THE PHARMACOLOGICAL ANALYSIS OF A LIPID FRACTION ISOLATED FROM NEURAL TISSUE

BY

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Abstract

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The biological actions of a lipid fraction (LF) isolated from neural tissue were analyzed using pharmacological methods. The original characterization of the LF, based on its actions on the guinea-pig ileum, was that it released acetylcholine (ACh) from nerve endings in a manner totally blocked by morphine. The LF was also blocked by the muscarinic antagonist, atropine, the choline uptake inhibitor, hemicholinium-3, and tissue manipulations which inhibit neural function such as anoxia or storage overnight at room temperature. The LF was not blocked by nicotinic, histaminic, or serotonin (5-HT) antagonists, and its action was unaffected by concentrations of cocaine that reduced the effect of 5-HT by two-thirds. Initial chemical characterization suggested that the LF was neither an acid nor an amide.

Based on the original findings, I decided to investigate two hypotheses concerning the action of the LF: first, that the LF was a universal cholinergic releasing agent which

did not require the presence of action potentials to produce its effect; and second, that the effect of the LF was necessarily linked to narcotic analgesic sensitivity of the stimulus evoked release of ACh.

The first hypothesis was tested by measuring the frequency and amplitude of miniature endplate potentials and showing that no consistent stimulation of mepp frequency or amplitude was demonstrable in the presence of the LF.

The second hypothesis was tested by exposing the rabbit ileum, a preparation known to be narcotic analgesic insensitive, to the LF. After a half minute lag, the spontaneous pendular motion of the ileum increased in amplitude reaching a maximum in one and a half minutes and then decaying with a half-life of three minutes. Morphine did not block the action of the LF or equiactive doses of ACh or acetyl-β-methylcholine (ABMeCh); neither did ganglionic and smooth muscle serotonergic, H_1 and H_2 histaminergic, and alpha and beta adrenergic antagonists in concentrations sufficient to block their respective agonists. Atropine caused an initial decrease in the response to the LF, arachidonic acid (20:4w6), and nicotine, but the response recovered with continued exposure (atropine-fast response). ACh and ABMeCh in previously equiactive doses were blocked without recovery. Tetrodotoxin (TTX), a potent local anaesthetic, at a concentration which blocked or reversed the effect of nicotine, decreased but did not totally block the effect of the LF, 20:4w6, prostaglandin E_2 (PGE_2), and $PGF_{2\alpha}$ in both normal and atropine-fast preparations. TTX also blocked the

effect of the LF and nicotine on the guinea-pig ileum.

Mefenamic acid, a reversible competitive inhibitor of the fatty acid cyclo-oxygenase (prostaglandin synthetase) blocked in a surmountable, reversible fashion the effect of the LF, 20:4w6, 8,11,14-eicosatrienoic acid (20:3w6), and 6, 9,12-octadecatrienoic acid (18:3w6). Indomethacin, a time-dependent competitive inhibitor of the cyclo-oxygenase, also blocked the action of the LF and 20:4w6. Comparison of the LF and 20:4w6 showed that they stimulate the rabbit ileum with the same maximal effect, while 20:3w6 and 18:3w6 caused progressively smaller maximum effects and 18:3w3 (9,12,15-octadecatrienoic acid) was essentially inactive.

Based on the linkage of the LF to the prostaglandin and thromboxane system, chemical characterization using TLC, GLC, IR, UV, NMR, and GC/CIMS demonstrated the LF to be a mixture of saturated fatty acids with sufficient amounts of cyclo-oxygenase substrates to account for the biological activity.

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This dissertation is dedicated to my princess, Sandra Joan Marshall: though I have known you all my life, I wish I had met you sooner.

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I. INTRODUCTION

A. A history of the active material (lipid fraction) isolated from neural tissue.

In 1963, while examining cholinergic substances present in rabbit sciatic nerve, Green, Carlini, and Robinson noted that acetone extracts of nerve caused a slow contraction of the guinea-pig ileum. Brain, similarly treated, yielded lipid extracts with the same effect, but intestine, kidney, liver and lung did not (Green, Carlini, & Robinson, 1963).

The active material (AM, a list of abbreviations may be found in Appendix D), was not affected by treatment with acetylcholinesterase (Green, Carlini, & Robinson, 1963). The yield was increased three-fold after heating at 100°C for five minutes at pH 10 (Green & Carlini, 1964). Refluxing in 1 N alcoholic KOH or HCl for one hour destroyed the activity (Robinson, Carlini, Green, Hargie, Weber, Hunter, & Schenck, 1967). When AM in petroleum ether was extracted with aqueous K_2CO_3, essentially all the activity remained in the petroleum ether; this was taken to indicate that the AM is not an acid (Robinson et al., 1967). The chemical properties of the AM will be discussed in greater detail in Section III.B.1.

All the published studies on AM described its contraction of the guinea-pig ileum (Green, Carlini, & Robinson, 1963; Green & Carlini, 1964; Robinson et al., 1967). Here a delay of 15 or more seconds in the onset of the response and one of at least 60 seconds for peak effect were observed. AM was completely blocked by the muscarinic antagonist, atropine, at concentrations of 2-4 x 10^{-8} M. Equiactive doses of acetylcholine (ACh) were also blocked by atropine at 2 x 10^{-8} M, a dose which did not inhibit bradykinin or substance P. On washout of 1 x 10^{-8} M atropine, the response to ACh recovered long before the response to AM (Green & Carlini, 1964). Hemicholinium-3 (HC-3), an inhibitor of choline uptake, at 2 x 10^{-5} M, slowly reduced the response to AM to about 10% of control in an hour without decreasing the response of ACh or bradykinin. When the bath fluid was collected from an ileum exposed to AM for 10 to 20 minutes, its content of ACh was approximately 50% higher than that collected in control periods. AM was without effect on ilea stored for 1 day at room temperature, 3 days at 4^{o}C or on ilea bathed in Tyrode's solution gassed with N_2. This was taken to indicate that AM required functioning nerves for it to have an effect. The ACh receptors and smooth muscle tissue were still functioning since, in both stored and anoxic ilea, ACh remained active. The ganglionic blocking agents hexamethonium and tetraethylammonium did not block the action of AM and at high doses appeared to slightly potentiate it. Cocaine,

6.6-13 x 10^{-6} M, did not alter the response to AM or ACh. The concentration of cocaine was thought to be sufficient for local anaesthesia as it reduced by two-thirds the response to 5-hydroxytryptamine (5-HT) at 2.8 x 10^{-6} M. The H_1 antihistamine, Mepyramine (pyrilamine), at 1.8 x 10^{-8} M or the 5-HT antagonist, lysergic acid diethylamide, at 1.5 x 10^{-6} M, did not alter on the response to AM. The narcotic analgesic, morphine, at 4.4-8.8 x 10^{-6} M, totally blocked the action of AM, without affecting the response to ACh (Green, Carlini, & Robinson, 1963; Green & Carlini, 1964).

This dissertation was conceived as an attempt to more completely characterize the pharmacological actions of the active material (AM). As will be demonstrated in Sections II and III, the AM is a mixture of substances which may be described more accurately as a lipid fraction (LF).

Pharmacologically active lipids (PALs) are not as much a part of the common experience of pharmacologists as are the bioactive amines such as acetylcholine, histamine, serotonin, or the catecholamines. The approaches and results of this dissertation may be easier to understand when they are compared with the results of other investigations of PALs. Therefore, the next subsection (I.B.) will be a review of the properties of pharmacologically active lipids and may provide an introduction to the complexities involved in their pharmacological analysis.

B. Review of pharmacologically active lipids.

1. Introduction

The purpose of this review is to survey the literature that has accumulated since Walther Vogt's 1958 review of "Naturally occurring lipid-soluble acids of pharmacological interest."

Where other pharmacologically active lipids (PALs), either synthetic or non acidic, provide additional insight into the mechanisms of the naturally occurring compounds, the other PALs will be included in the review.

The main purpose of this review is to provide a conceptual framework for understanding the interrelationships of the PALs by relating the known pharmacologically active lipids to the metabolic pathways involving the unsaturated fatty acids. Within this discussion, lipids which were originally named based on their pharmacological activity or source, such as Darmstoff, irin, vessiglandin, etc. will be classified as to their involvement with unsaturated fatty acid metabolism.

Emphasis will be primarily upon the actions of PALs on smooth muscle preparations, neural transmission, and platelets. A device that demonstrates the interrelationship of the PALs is to consider two broad categories of PALs: (1) those known to be either substrates or products of the enzymes involved in the metabolism of essential fatty acids, and (2) those whose relation to the metabolism of essential fatty acids remains unclear. For those PALs known to be associated

with the metabolism of the essential fatty acids, three main enzyme systems are involved: (a) the tissue acyl hydrolases and specifically phospholipase A_2; (b) the fatty acid cyclo-oxygenase and the enzymes which further metabolize its products; and (3) the lipoxygenases. For each enzyme system, consideration will be given to the general biochemistry of the enzymes, the substrates, the products, and pharmacological activation or inactivation.

2. General metabolic scheme for PALs related to unsaturated fatty acid metabolism.

The metabolic pathways for lipids have been reviewed (Gurr & James, 1975). Those pathways relevant to this review are outlined in fig. 1. As will be shown, many of the intermediates as well as the final products of the metabolism of essential fatty acids are pharmacologically active. An interesting feature of the metabolic pathways is their mutual interrelationship. For example, one of the products of the lipoxygenase, 15-hydroperoxy arachidonic acid, can act to shift the flow of the products of the fatty acid cyclo-oxygenase away from PGI toward TXs and the classical prostaglandins (Gryglewski, Bunting, Moncada, Flower, & Vane, 1976). Another example is the positive feedback system whereby lysolecithin, a product of phospholipase A_2 (PLA_2) increases the availability of PLA_2 substrates (Lankisch & Vogt, 1972). On the other hand, prostaglandins of the E series have been shown to inhibit the action of PLA_2, demonstrating classical negative feedback endproduct

inhibition (Feinstein, Becker & Fraser, 1976). These mechanisms will be discussed in more extensive detail in the appropriate sections of this review.

3. PALs related to phospholipase A_2.

a. General biochemistry.

Phospholipases are a group of acyl hydrolases which can liberate fatty acids from their stores in the various tissue lipids. Most studies of pharmacologically active lipids have concentrated on the actions and effects of enzymes with the biochemical properties of phospholipase A_2. Because PLA_2s appear to play a major role in the control of prostaglandin, thromboxane, as well as lipid peroxide synthesis, this portion of the review will concentrate on the properties of these phospholipases.

PLA_2s hydrolyze the 2-acyl groups in sn-3-phosphoglycerides (e.g., diacylphosphatidylcholine, lecithin) to yield a fatty acid (usually unsaturated) and a 2-lysophospholipid (e.g., lysolecithin). The pharmacology of the products of PLA_2 hydrolysis of phospholipids will be discussed subsequently.

The physical properties of the enzymes have been studied. PLA_2s (phosphatide-acyl hydrolase, E.C.3.1.1.4.), which have been isolated from snake venoms, are stable to boiling at acid pH (less than 5.9) for 15 min. while boiling at pH above 7 destroys their activity (Hughes, 1935; Magee, Gallai-Hatchard, Sanders, & Thompson, 1962). PLA_2 isolated from eastern diamondback rattlesnake (Crotalus adamanteus)

Fig. 1. General metabolic pathway for pharmacologically active lipids (PALs)

The major enzymes in the phospholipase/cyclo-oxygenase/lipoxygenase pathways are highlighted as they relate to their substrates and products. The structures for the abbreviations given in fig. 4. See text, Section I.B.2. for further details.

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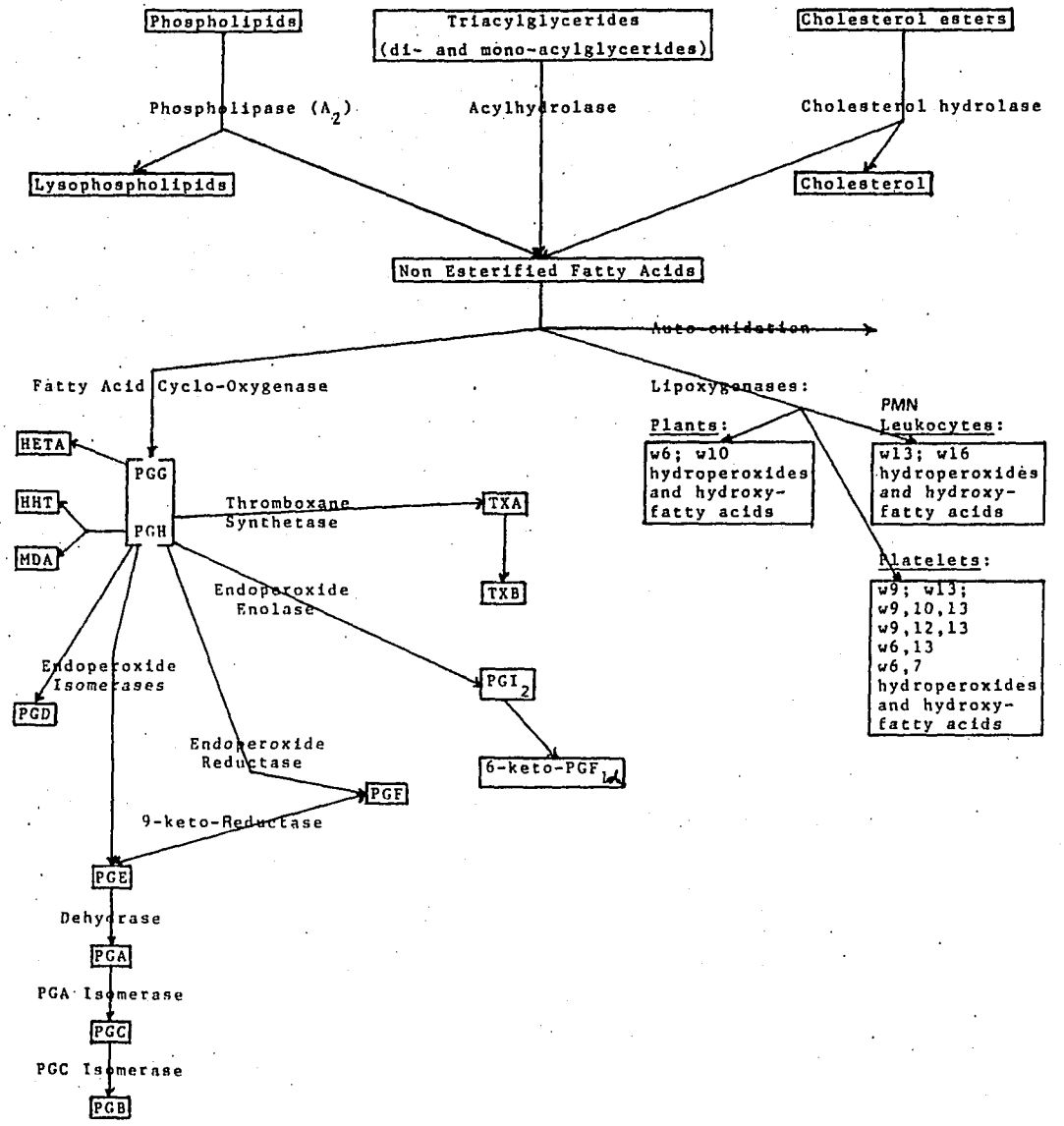
Phospholipids
Triacylglycerides
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Cholesterol esters
Phospholipase (A_2)
Acylhydrolase
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Lysophospholipids
Cholesterol
Non Esterified Fatty Acids
Auto-oxidation
Fatty Acid Cyclo-Oxygenase
Lipoxygenases:
Plants:
PMN Leukocytes:
w6; w10 hydroperoxides and hydroxy-fatty acids
w13; w16 hydroperoxides and hydroxy-fatty acids
Platelets:
w9; w13; w9,10,13 w9,12,13 w6,13 w6,7 hydroperoxides and hydroxy-fatty acids
HETA
PGG
HHT
PGH
MDA
Thromboxane Synthetase
TXA
TXB
Endoperoxide Enolase
Endoperoxide Isomerases
PGD
PGI_2
6-keto-$PGF_{1\alpha}$
Endoperoxide Reductase
PGF
9-keto-Reductase
PGE
Dehydrase
PGA
PGA Isomerase
PGC
PGC Isomerase
PGB

venom is a dimer whose subunits have a molecular weight of approximately 14,000 (Heinrikson, Krueger, & Keim, 1977). Indeed, the venom of Ringhals cobra (Hemachatus haemachetetes) contains two PLA_2s capable of passing through dialysis membrane (Bjork, 1961). The substrate specificity of the venom PLA_2s is such that phosphatidyl-choline, phosphatidyl-serine, phosphatidyl-inositol, and phosphatidyl-ethanol amine, phosphatidic acid, cardiolipin, and plasmenyl-choline (and possibly plasmenyl-serine and plasmenyl-ethanolamine) will act as substrates, but diphosphoinositide (1-phosphatidyl-inositol-3,4-biphosphate) and sphingomyelin will not (Meldrum, 1965). The fact that the preponderance of the fatty acids released from naturally occurring phosphatides are unsaturated has been explained (Meldrum, 1965) by the fact that the unsaturated fatty acids are usually esterified in the 2-position of the glycerol moiety rather than by an particular preference exhibited by the enzyme (Saito & Hanahan, 1962). However, recently evidence has been presented for specific release of arachidonic acid from lipid stores following heart tissue labelling with a mixture of ^{14}C-palmitate, -oleate, -linoleate, and -archidonate. It has been suggested that bradykinin stimulation activates either a specific lipase selective for arachidonate or a lipase in a separate compartment containing the arachidonate labelled lipids (Hsueh, Isakson, & Needleman, 1977). Some venom PLA_2s require an activator, such as Direct Lytic Factor (DLF), a peptide component of some snake venoms, for full action on membrane

bound substrates (Condrea, DeVries, & Mager, 1964; Lankisch & Vogt, 1972).

Mammalian PLA_2s occur in both membrane bound and soluble forms. The membrane bound form is activated by Ca^{2+} and has a pH optimum from 7-8. Mitochondrial PLA_2 is the classic example of the membrane bound PLA_2s (Lands & Rome, 1976). Other examples are the PLA_2 of plasma membranes (Victoria, Van Golde, Hostetler, Scherphof, & Van Deenen, 1971; Van Golde, Fleischer, & Fleischer, 1971), seminal plasma (Kunze, Nahas, & Wurl, 1974; Kunze, Nahas, Traynor & Wurl, 1976), and platelets (Schoene & Iacono, 1976); Vincent & Zijlastra, 1977; Feinstein, Becker, & Fraser, 1977). The soluble form of PLA_2 has an acid pH optimum (pH 4-6) and Ca^{2+} may act as an inhibitor (Lands & Rome, 1976). Soluble PLA_2 is released from lysosomes and has been demonstrated in liver (Franson, Waite, & La Via, 1971), spleen (Lloveras & Blazy, 1973), and polymorphonuclear leukocytes (Franson, Patricia, & Elsbach, 1974). The soluble PLA_2 activity from bovine seminal vesicular glands appears to be activated by Ca^{2+} (Kunze & Vogt, 1971). The interrelationship between the various forms of PLA_2 and how they affect the generation of PALs remains unclear.

PLA_2 itself acts on smooth muscle preparations. The original report of Feldberg, Holden, and Kellaway (1938) demonstrated that 4μg/ml of cobra venom causes a contraction with gradual decay of the isolated guinea-pig jejunum greater than the contraction caused by 20 ng/ml of histamine. A

repeated dose of the cobra venom failed to elicit the same effect, although there was possibly a slight slow contraction (Feldberg, Holden, & Kellaway, 1938). It seems likely that the action of PLA_2 on smooth muscle preparations is due to the enzymatic release by PLA_2 of PALs from the phospholipids in the cell membranes of the preparations.

b. The pharmacological actions of phospholipase substrates.

(1) Acidic phospholipids

(a) Phosphatidic acid (3-sn-phosphatidic acid): prepared from lecithin and tested on the rabbit duodenum by Vogt (1960a), 1 µg stimulated that isolated tissue to a level equivalent to 4 units of a standard preparation of Darmstoff (Vogt, 1953), which was originally described as dialysates of isolated frog intestine that caused a slow contraction of intestinal preparations (see Section II.B.4.d.(1).) Dimyristoyl phosphatidic acid, synthesized by Dr. E. Baer of Toronto, generated a response equivalent to 1.17 units/ µg. The purity of the phosphatidic acid preparations was not chromatographically verified (Vogt, 1960a).

(b) Cardiolipin: at concentrations ranging from 10 to 50 µg/ml, a preparation of cardiolipin, obtained from the State Serum Institute in Copenhagen, caused equivocal responses on the rabbit duodenum. The activity was assayed to be equivalent to 0.2 units of the previously mentioned Darmstoff preparation /µg (Vogt, 1960a).

(c) Phosphatidylserine (3-sn-phospha-

tidylserine): on the rabbit duodenum, distearyl-L-phosphatidylserine, prepared by Dr. Baer of Toronto, caused a contraction at a concentration of 2.5 µg/ml. In one experiment, the effect was reversed when more than 5 to 7 µg/ml were used. The effect was equivalent to 0.87 units of Darmstoff / µg (Vogt, 1960a). Other workers have found that cephalin (a crude mixture of phosphatidylserine and phosphatidylethanolamine) as well as the individual phospholipids, in concentrations from 10 µg to 10 mg/ml, potentiated the responses of the guinea-pig ileum to acetylcholine, histamine, 5-hydroxytryptamine (5-HT), tetramethylammonium, K^+, Ba^{2+}, and transmural stimulation. Phosphatidyl-L-serine was much less potent than phosphatidylethanolamine. Usually a slight inhibition preceded the potentiation, and, when concentrations greater than 250 µg/ml were used, the phospholipids also caused a direct contraction. When the Ca^{2+} concentration of the Tyrode's solution was increased up to 5 times normal, the potentiation of the responses to acetylcholine or transmural stimulation by cephalin was blocked. However, when the Ca^{2+} concentration of the Tyrode's solution was decreased down to 0.33 to 0.1 times normal, the potentiation by cephalin was increased (Brown, Poyser, & Telford, 1969). Previous studies in another laboratory had found no direct contractile effect of 100 µg/ml phosphatidylserine on the guinea-pig ileum (Green, Robinson, & Day, 1961).

(d) Monophosphoinositide (1-(3-sn-phosphatidyl) inositol): in concentrations up to 50 µg/ml, monophosphoinositide caused trace effects, at most. EDTA (ethyl-

enediaminetetraacetic acid) added 1:1, to remove any Ca^{2+} which may have been associated with the monophosphoinositide, did not increase the effect over the originally observed 0.045 units of Darmstoff/ µg (Vogt, 1960).

(e) Diphosphoinositide (1-phosphatidyl-inositol-3,4-biphosphate: at a concentration of 5 µg/ml, diphosphotidylinositol caused a stimulation equivalent to 0.36 units of Darmstoff /µg (Vogt, 1960).

(2) Neutral (zwitterionic) phospholipids

(a) Lecithin: diacylphosphatidylcholine prepared from animal sources (lecithin) contains a mixture of fatty acids, usually with saturated fatty acids esterified in the 3-position and unsaturated fatty acids in the 2-position (Sprecher, 1972). In the absence of added phospholipase or of base catalyzed hydrolysis, lecithin, obtained from egg yolks, had no stimulatory effect on the guinea-pig ileum (Vogt, 1957a). Lecithin inhibited the responses of the guinea-pig ileum to acetylcholine, histamine, 5-HT, tetramethylammonium, K^{+}, Ba^{2+}, and transmural stimulation. The inhibition was either increased or unaffected by increasing the Ca^{2+} concentration of the Tyrode's solution to 5 times normal, while the inhibition was prevented by decreasing the Ca^{2+} down to 0.33 to 0.1 times normal. The inhibition varied from batch to batch of crude extract made from egg yolk, and one batch produced different results. However, synthetic dipalmitoylphosphatidylcholine, which was chromatographically pure, produced solely inhibition (Brown, Poyser, & Telford, 1969). They

considered that the Ca^{2+} effect may have been due to differential binding effects of Ca^{2+} binding to lecithin as reported by Rojas and Tobias (1965). Another interpretation is that the difference might be due to activation of PLA_2 by Ca^{2+}. Also, crude batches might have had some arachidonate as a component fatty acid.

On the rabbit duodenum, at a concentration of 50 µg/ml, unpurified lecithin had an effect equivalent to 0.1 units of Darmstoff/ µg. However, lecithin that had been chromatographically purified lost its smooth muscle stimulating properties (Vogt, 1960).

Egg lecithin incubated with human plasma causes a slow contraction of the guinea-pig ileum. The effect is increased by incubation at pH 8 or 37°C and decreased by 25 mM Ca^{2+} or processing with kaolin. The effect was not antagonized by the antimuscarinic, atropine, the H_1-histamine receptor antagonist, antazolin, or incubation with the esterase-rich fraction of blood. This was not a general effect of all lipids because no slow contraction was generated after incubating the egg lecithin with a 5% saline solution of human serum albumin or after incubating emulsions of olive-oil, cottonseed oil, or butterfat with human plasma at pH 8 (Gabr, 1964).

(b) Sphingomyelin: in concentrations up to 50 µg/ml, it was without effect on the rabbit duodenum. Apart from one small enhancement of the pendular motion, the effect was less than 0.05 units of Darmstoff/ µg (Vogt, 1960).

(3) Glycolipids

(a) Ganglioside: the rabbit duodenum responded to 7.5 µg/ml of a preparation from brain with a strong excitation equivalent to 0.43 units of Darmstoff/ µg of the ganglioside preparation (Vogt, 1960). Gangliosides were identified in chloroform extracts of horse brain, and pharmacologically active substances were also detected in rabbit and guinea-pig brain (Kirschner & Vogt, 1961). The guinea-pig ileum was also thought to be stimulated by ganglioside preparations (Bogosch, Paasonen, & Trendelenburg, 1962). However, purification of crude ganglioside extracts from monkey brain led to the elimination of both a stimulatory and an inhibitory biological activity on both the rabbit duodenum and the guinea-pig ileum (Robinson, Carlini, & Green, 1963). The inhibitory component was identified as norepinephrine. The stimulatory component was not further characterized except to demonstrate profound tachiphylaxis to it on the guinea-pig ileum and its elimination after gel filtration with Sephadex G-50 following column chromatography with silic acid (Robinson, Carlini, & Green, 1963).

(b) Cerebroside: at a concentration of 10 µg/ml, the slow and small increase in tone was equivalent to less than 0.1 units of Darmstoff/ µg (Vogt, 1960). This slow contractile effect should be viewed with caution in view of the demonstrated binding by cerebroside of K+, norepinephrine, histamine, acetylcholine, and 5-HT (Green, Robinson, & Day, 1961).

(4) Summary of the actions of phospholipase substrates.

The actions of phospholipase substrates on smooth muscle preparations have not been studied either extensively or from the viewpoint that they provide substrates for the cyclo-oxygenase and lipoxygenase enzyme systems. As a result, the previous discussion may well seem fragmented and incomplete. Perhaps now that the relationship of PLA_2 substrates to other pharmacologically active lipids has become more obvious, the substrates will be subjected to a systematic reexamination.

c. The pharmacological actions of phospholipase products.

(1) Lysolecithin (2-lyso-3-sn-phosphatidylcholine).

The pharmacological actions of lysolecithin were originally described in terms of the hemolysis caused by the treatment of lecithin with cobra venom (Kyes, 1904). The actions of lysolecithin on smooth muscle preparations were first described by Feldberg and Kellaway (1938), but the stimulation of the guinea-pig ileum that they ascribed to "lysolecithin" is more likely to have been due to the release by tissue phospholipases of slow reacting substance-cobra venom (SRS-C, see Section I.B.4.d.(3).) Indeed, when Holden obtained a purer preparation of lysolecithin, it was shown to be free of stimulatory activity (Feldberg, Holden, & Kellaway, 1938). However, it was noticed that the purified

lysolecithin at concentrations greater than 0.33 mg/ml both produced a decreased excitability of the guinea-pig jejunum to histamine and directly contracted the guinea-pig uterus at concentrations greater than 0.33 mg/ml. Subsequent studies have shown that lysolecithin inhibits the response of the guinea-pig ileum to SRS-C, SRS-A, acetylcholine, K^+, histamine, 5-HT, and bradykinin (Kellaway & Trethewie, 1940; Rocha e Silva & Beraldo, 1948; Middleton & Phillips, 1963). The recovery of the excitability of the preparation was found not to be related to the number of attempted stimulations with the agonists but rather to both the concentration and the duration of exposure of the preparation to lysolecithin (Rocha e Silva, 1948; Middleton & Phillips, 1963). This suggests that the inhibition is due not to receptor antagonism but to direct depression of the smooth muscle preparation. It should be noted that the concentrations of lysolecithin used (4×10^{-5} to 1.2×10^{-4} M) approximate those (2.5×10^{-4} M) naturally occurring in human serum (Phillips, 1958).

In a recent report of PLA_2 (derived from the venom of Vipera russelli) inhibition of opiate receptor binding and the reversal of the inhibition by bovine serum albumin, bovine fatty acid poor albumin, porcine albumin, chicken albumin, rabbit albumin, human albumin, but not egg albumin (Lin & Simon, 1978), reference was made to unpublished work in which opiate receptor binding was inhibited by lysolecithin. This inhibition was not restored by bovine serum albumin (unpublished results cited in Lin &

Simon, 1978). The effect is intriguing in view of the postulated relationship between prostaglandins and the opiate receptor (Ehrenpreis, Greenberg, & Belman, 1973; see Section I.B.4.b & c).

The actions of lysolecithin on platelets are complex. At low concentrations (100-200 μM), lysolecithin stimulated reversible aggregation of human platelet rich plasma (PRP) induced by 5-HT (Besterman & Gillet, 1973). Other studies have shown a potentiation of aggregation of rabbit, human, and pig washed platelets resuspended in Tyrode's solution containing 0.35% albumin; the aggregation was induced by ADP, collagen, or thrombin. The potentiation, which was transient, was preceded by a period of inhibition (Joist, Dolezel, Cucuiana, Nishizawa, & Mustard, 1977). Higher concentrations of lysolecithin caused an instantaneous inhibition of aggregation of human, pig, or rabbit PRP induced by ADP, epinephrine (only with human PRP), collagen, or thrombin. At concentrations equivalent to the 250 μM found in normal plasma, lysolecithin may even induce aggregation (Kerr, Pirrie, MacAulay, & Bronte-Stewart, 1965).

The action of lysolecithin on platelets may be explained in two ways. The inhibition of aggregation may be due to damage to the platelet membrane. Lysolecithin is a detergent with well known hemolytic (Kyes, 1904; Reman, Demel, DeGrier, Van Deenen, Iebel, & Westphal, 1969; Weltzien, Arnold, & Reuther, 1977) and cell fusing properties (Lucy, 1970, 1971). The current hypothesis is that single molecules

(but not micelles) (Weltzien, Arnold, Blume, & Kalgoff, 1976) bind to the surface of cells and are incorporated into the membranes where they interact 1:1 with molecules of cholesterol to form a stable structure (Rand, Pangborn, Purdon, & Tinker, 1975). Once the amount of lysolecithin incorporated into the membrane exceeds a certain amount (perhaps roughly equal to the amount of cholesterol present), lysolecithin rich patches occur in the membrane (Weltzien et al., 1977) and a phase transition occurs in the membrane from ordered bimolecular lipid leaflet to a localized micellular structure (Lucy, 1970, 1971; Weltzien et al., 1977) leading to lysis. Thus at concentrations below those inducing lysis, lysolecithin may inhibit platelet function by membrane perturbation (Joist et al., 1977). On the other hand, the stimulation of aggregation might be due to lysolecithin induced degranulation of ADP (Vargaftig, 1977, p. 227).

A more specific action on platelet function has been proposed based on the rapid and selective metabolism of lysolecithin to glycerolphosphocholine and a fatty acid (Joist, Dolezel, Lloyd, & Mustard, 1976; Elsbach, Pettis, & Marcus, 1971). The fatty acid released is usually saturated, though some unsaturated fatty acids do occur (Joist et al., 1977) and saturated fatty acids such as palmitic or stearic have been shown to directly induce aggregation of washed platelets at a threshold concentration of 20-30 μM (Haslam, 1964) as well as potentiate the effects of other aggregating agents (Hoak, Spector, Fry, & Warner,

1970). However, the report that the inhibitory action of lysolecithin on platelet aggregation requires a high proportion of saturated fatty acids in the lysolecithin (Besterman & Gillett, 1972) is difficult to explain from this metabolic approach.

(2) Lysophosphatidic acid (monacyl glycerol phosphate)

When lysolecithin was subjected to brominolysis, the product (either chromatographically purified or containing 15% bromine) was extremely active on the rabbit duodenum. Stimulation of the isolated duodenum was caused by concentrations as low as 50 ng/ml and the addition of 300 ng/ml more lysophosphatidic acid increased the contractile response. The two preparations (pure and 15% bromine) had activities on the isolated rabbit duodenum equivalent to 49 and 30 units of Darmstoff/ µg, respectively (Vogt, 1960a, 1963).

Lysophosphatidic acid was identified as an active material isolated from horse brain (Kirschner & Vogt, 1961) although Vogt later described the activity as due to phosphatic acid (Vogt, 1963). Although lysophosphatidic acid is usually referred to as a product of phospholipase D, the monoacyl-glycerol phosphate is also a product of PLA_2. The confusion stems from the problem of trivial nomenclature, but from his papers on the chemical structure of Darmstoff, it seems clear that Vogt (1976) meant the monoacyl-glycerol phosphate.

(3) Lysophosphoinositide.

The biological activity of lipid extracts of intestine eluted from silicic acid chromatography columns with chloroform:methanol (3:2) was ascribed to a lysocephalin and lysophosphoinositide (Vogt, 1960b). The biological activity compared to other acidic phospholipids was reported for "lyso-monophosphoinositide" as 0.4 units of Darmstoff activity/ µg (Vogt, 1963).

(4) Fatty Acids.

The biological actions of the fatty acids libered by PLA_2s are described in Section I.B.4,5, & 6.

(5) Summary of the actions of phospholipase products.

The major pharmacologically active lipids derived from the actions of PLA_2 on its substrates are the fatty acids which are further metabolized by the enzymes of the cyclo-oxygenase and lipooxygenase systems. However, some of the PLA_2 products, such as lysolecithin, have interesting pharmacological properties of their own. For example, lysolecithin may act via positive feedback to stimulate the activity of PLA_2 (see Section I.B.3.d.(1).(c) on page 28 for futher discussions). The complexity of the interrelationships among the phospholipase/cyclo-oxygenase/lipooxygenase enzyme systems provide many examples of the intricacy of biological control.

d. Pharmacological manipulation of phospholipase activity.

(1) Activation.

(a) Ions and cyclic nucleotides.

The activation of the various PLA_2s by Ca^{2+} was discussed in Section I.A.3.a. The interrelation of Ca^{2+} and cyclic nucleotides is well established (Hardman & Sutherland, 1971; Goldberg, O'Dea, & Haddox, 1973). Since prostaglandins have a multiplicity of effects on cyclic nucleotides (Kuehl, Cirillo, & Oien, 1976; Nathanson, 1977), the possibility exists for both positive and negative feedback on PLA_2. For example, in washed human platelets stimulated with thrombin, a phospholipase is activated that releases arachidonate from the phospholipid stores. Addition of dibutyrl-cAMP or PGE_1 (which increased platelet cAMP levels (Miller & Gorman, 1976) prior to addition of thrombin blocks the release of arachidonate (Minkes, Stanford, Chi, Roth, Raz, Needleman, & Majerus, 1977; Gerrard, Peller, Krick, & White, 1977). In pig thyroid, thyroid stimulating hormone (TSH) activates a Ca^{2+} stimulated phospholipase that releases arachidonate from phosphatidylinositol. The prostaglandins formed serve as additional activators of the TSH stimulated adenyl cyclase that, in turn, activates a lipase that hydrolates triacylglycerides (triglycerides). The induced lipolysis frees more arachidonate which can be formed into additional prostaglandins which may cause additional stimulation of the adenyl cyclase (Haye, Champion, & Jacquemin, 1976).

Cyclic GMP production is stimulated by circumstances leading to platelet aggregation (Davies, Davidson, McClenaghan, Say, & Haslam, 1976) and by lipid

peroxides (Hidaka & Asano, 1977). The effects of cGMP on the PLA_2s in platelets have not been examined, but it is possible, in view of the "Yin-Yang" relationship between cAMP and cGMP (Goldberg et al., 1975), that PLA_2 activity may be stimulated by cGMP.

(b) Rabbit aorta contracting substance-releasing factor.

During an investigation of factors release from isolated guinea-pig lungs by in vitro anaphylaxis, Piper and Vane (1969) discovered a substance that caused a contraction of male rabbit aorta strips. The biological activity on the assay tissues of RCS (Rabbit-aorta Contracting Substance), which will be discussed in detail in Section I.B.4.e.(1)., was found to disappear after standing at room temperature for 20 min. However, if the perfusate, from which the RCS activity had disappeared, was passed through isolated unsensitized lungs, RCS activity was released. This RCS-releasing factor (RCS-RF) was originally reported to be non-dialzable and stable to 10 min. of boiling, standing at room temperature for 2 hr., or freezing at $-20^{\circ}C$ for 7 days (Piper & Vane, 1969). A significant observation was that the pharmacological action of RCS-RF was blocked by the non-steroidal anti-inflammatory (NSAI) drugs: aspirin, indomethacin, and mefenamate. RCS-RF, when continuously perfused through isolated guinea-pig lungs, induced only a transient release of RCS and prostaglandins while arachidonic acid (20:4w6) and dihomo- γ-linolenic acid (20:3w6) produced a

continuous release lasting throughout the perfusion. The release of RCS-RF as opposed to that of RCS was not blocked by pretreatment of the sensitized lungs with aspirin (Palmer, Piper, & Vane, 1973) nor by the following drugs in the perfusion fluid: aspirin, 200 µg/ml; indomethacin, 2 µg/ml; desamethasone, 2 µg/ml; disodium cromoglycate, 20 µg/ml; colchicine, 5 µg/ml; diethylcarbazine, 1 mg/ml; and mepacrine, 20 µg/ml. However, RCS-RF was not released from non-sensitized lungs by antigen challenge or by mechanical trauma (Nijkamp, Flower, Mondada, & Vane, 1976).

Partial purification of RCS-RF by adsorption onto Amberlite XAD-2 (BDH), elution with ethanol: water (80:20 v/v), rotary film evaporation of the eluant, washing 3 times with dry diethyl ether, and silica gel-impregnated paper chromatography (Whatman SG81) developed with ammonia:n-propanol:water (30:60:10 v/v) resulted in about 80% of the RCS-RF activity being associated with a single band with a Rf of 0.7.Fractions with Rfs of approximately 0.1, 0.2, and 0.8 accounted for the remaining RCS activity but were more effective (except for the fraction with Rf of 0.1) in stimulating the isolated guinea-pig ileum than the isolated rabbit aorta. The partially purified RCS-RF had no 'direct' effect on the rabbit aorta, rat stomach (fundic) strip, rat colon, or guinea-pig trachael chain all concurrently perfused with combined antagonists (Piper & Vane, 1969) with the unfortunate addition of indomethacin (Nijkamp et al.,1976). The partially purified RCS-RF did contract the

isolated guinea-pig ileum. A previously published preliminary communication mentioned that crude RCS-RF (a redissolved lyophilisate) in high doses (5 units: one unit causing the release of RCS activity equivalent to that of 1 μg of arachidonic acid) also contracted the rat stomach strip. Also the crude RCS-RF did not release RCS or prostaglandins from the perfused guinea-pig kidneys (which only convert arachidonic acid to PGE_2), was not active in the rat paw edema test for inflammation at a dose of 8 units/ rat (it was, however, hyperalgesic and this was 80% blocked by 10 mg/kg of indomethacin), and did not aggregate human platelets at a concentration of 10 units/ml (Flower, Harvey, Moncada, Nijkamp, & Vane, 1976).

A brief summary of the physicochemical properties of partially purified RCS-RF follows: it was insoluble in ether, chloroform, ethyl acetate, acetone, or ethanol, but it was about 70% as soluble in methanol as it was in water. Boiling for 10 min. decreased the activity by 75% but boiling for 1 min. had no effect (less than 10% decrease). Incubating for 1 hr. in 1 N hydrochloric acid reduced the RCS-RF activity by 100%, while a 1 hr. incubation with 1 N sodium hydroxide decreased the activity by 80%. In contrast to earlier reports (Piper & Vane, 1969), partially purified RCS-RF was found to pass through dialysis membranes suggesting a molecule of less than 5000 daltons. Incubation of RCS-RF with enzymes had the following effects on its biological activity: phospholipase A, C, or D, no effect;

trypsin, 15% reduction; carboxypeptidase B, 80-85% reduction; aminopeptidase M, 100% reduction; and arylsulfatase, 50-60% inactivation in 2 of 4 experiments (this is at odds with the report of the abstract in which it was stated "RCS-RF activity was often increased by incubation with arylsulphatase..." (Flower et al,,1976). Spraying of the freshly developed paper chromatogram with ninhydrin reagent resulted in several ninhydrin-positive areas of which the RCS-RF zone was one. Co-injection of the carboxypeptidase inhibitor BPP-5A caused a 3-5 fold potentiation of the action of RCS-RF. In summary they stated, "the limited physicochemical data obtained so far are compatible with RCS-RF being a peptide of less than 10 amino acids." (Nijkamp et al,,1976), although it is not clear how they arrived at such a small size for the molecule.

The isolated smooth muscle actions of RCS-RF (via the release of RCS) were blocked by aspirin (200 g/ml), indomethacin (2 μg/ml), mepacrine (20 μg/ml), and dexamethasone (2 μg/ml). In addition, only dexamethasone did not also block the action of arachidonic acid, implying that the others were acting at cyclo-oxygenase inhibiting concentrations. Both mepacrine and dexamethasone are usually thought to act via direct or indirect inhibition of PLA_2 action, respectively (see Section I.B.3.d.(2).), and the blockade by mepacrine of the action of arachidonic acid suggests that either a more specific PLA_2 inhibitor is needed (since mepacrine is known to inhibit the cyclo-oxygenase at concentrations that inhibit the PLA_2s (Flower & Blackwell,

1976)) or that exogenous arachidonic acid may act to stimulate the release or metabolism of endogenous arachidonate. The relative inhibitory potencies against RCS-RF of the anti-inflammatory steroids, dexamethasone, betamethasone, triamcinolone, fludrocortisone,prednisolone , prednisone, corticosterone, cortisone, and hydrocortisone paralleled their relative potencies as anti-inflammatory agents (Nijkamp et al., 1976). The anti-inflammatory steroid inhibition of the action of RCS-RF was slow in onset, reaching maximal effect after 10-20 min. of infusion, was reversible on washing for 30 min., and was readily surmountable by increasing the concentration of RCS-RF.

The parallels between RCS-RF and the previously mentioned DLF (Direct Lytic Factor, Damerau, Lege, Oldigs, & Vogt, 1975) are strengthened by the observation that the amount of hydrolysis of 2'-(1-^{14}C) oleoyl lecithin perfused through the isolated lung was doubled by 5 units of RCS-RF. Also, the basal rate of hydrolysis was decreased by dexamethasone or betamethasone (178.8 nmol/min). On the other hand, bradykinin, another peptide that released RCS activity from the lung (Vargaftig & Dao Hai, 1972) and whose action is also potentiated by BPP-5A, is not blocked by anti-inflammatory steroids (Nijkamp et al., 1976).

In summary, while it is fairly certain that the partially purified RCS-RF is contaminated with SRS-A-like activity, RCS-RF appears to act as an activator of PLA_2 activity, either directly or through a form of metabolic

control.

(c) Positive feedback.

The lysolecithin freed by the action of PLA_2 on diacylphosphatidylcholine acts as a membrane destablizer (as was discussed in Section I.A.3.c.(1).). It may act to facilitate the access of PLA_2 to membrane phospholipids whose cleavage is otherwise restricted (Condrea et al, 1964; Lankish & Vogt, 1972), perhaps by the asymetrical incorporation of different types of phospholipids into cell membranes (Zwaal, Roelofsen, & Colley, 1973; Vance et al., 1977).

(d) Induction of PLA_2 activity.

The regulation of the synthesis of PLA_2 provides another form of activation of phospholipase activity. The mineralocorticoids appear to regulate salt-water balance by a complex interaction between the kallikrein, kinin, and prostaglandin systems. In the kidney, aldosterone appears to act by stimulating the synthesis of PLA_2 (McGiff, Itskovitz, Terragno, & Wong, 1976). This hypothesis is supported by recent evidence demonstrating blockade of the action of aldosterone on the isolated frog skin by inhibitors of the cyclooxygenase, indomethacin and mefenamic acid (Yorio & Bentley, 1978). Indeed, the mediation of hormone-stimulated prostaglandin biosynthesis by activation of either phospholipase or acylhydrolase or both has been suggested as the universal mechanism for in vivo systems which are capable of hormone-stimulated prostaglandin biosynthesis (Zusman & Keiser, 1977).

(2) Inactivation.

(a) Agents acting directly on phospholipase A_2 activity.

In 1966, Vogt, Suzuki, and Babilli first proposed that PLA_2 might be of fundamental importance in the regulation of prostaglandin synthesis. It was subsequently demonstrated that the fatty acid cyclo-oxygenase does not act upon esterified fatty acids (Lands & Samuelsson, 1968; Vonkeman & Van Dorp, 1968), and the freeing of the fatty acid substrate is now considered to be the rate limiting step in prostaglandin biosynthesis (Kunze & Vogt, 1971; Flower & Blackwell, 1976). Local anaesthetics have been shown to inhibit the action of PLA_2 on both membrane bound (Seppala, Saris, & Gauffin, 1971) and free phospholipids (Scherphof, Scarpa, & Van Toorenbergen, 1972). The mechanism remains unclear (Kunze et al., 1976; Lands & Rome, 1976) but may involve both direct binding with the substrate and modifying its affinity for the enzyme (Scherphof et al., 1972). Some local anaesthetics also act as cyclo-oxygenase inhibitors (Kunze, Bohn, & Vogt, 1974): of a series of 9 agents, only tetracaine and possibly procaine acted specifically by inhibition of PLA_2. At low concentrations, some local anaesthetics, such as dibucaine, stimulate PLA_2 activity (Kunze, Nahas, Traynor, & Wurl, 1976). It is fairly clear that the local anaesthetics do not act as competitive inhibitors at the substrate site (Waite & Sisson, 1972) but may effect the adsorption or penetration of PLA_2 into the cell membrane phase (Hendrickson & Van Dam-Mieras, 1976).

Bromophenacyl bromide, by alkylating the imidazole at histidine 53, does appear to be an active site inhibitor of PLA_2 (Volwerk, Pieterson, & De Haas, 1974). However, at low concentrations (5-10 μM), it stimulates the release from platelets of smooth muscle contracting substances while it inhibits the release at higher concentrations (greater than 50 μM). It also appears to inhibit the synthesis of $PGF_{2\alpha}$ by guinea-pig uterine homogenates, from exogenous arachidonic acid (Mitchell, Poyser, & Wilson, 1977). Whether this is due to direct inhibition of the fatty acid cyclo-oxygenase or to inhibition of arachidonic acid stimulated activation of PLA_2 is not known.

Certain antimalarials, such as chloroquine and mapacrine, possess anti-inflammatory activity which may be due to direct inhibition of PLA_2 action (Lands & Rome, 1976; Vargaftig & Dao Hai, 1975). However, chloroquine has several nonspecific antagonist actions (Famaey, Fontaine, Reuse, 1977), and mepacrine appears to inhibit the cyclo-oxygenase at concentrations necessary to inhibit PLA_2 (Flower & Blackwell, 1976).

(b) Agents acting indirectly on PLA_2 activity.

Phenylmethylsulfonylfluoride, a serine protease inhibitor, has been demonstrated to block the activation of PLA_2 that occurs during platelet aggregation induced by thrombin and collagen (Feinstein, Becker, & Fraser, 1977; Vargaftig, 1977) but not the aggregation

induced by the Ca^{2+} ionophore A23187 (Feinstein et al, 1977).

The effects of corticosteroids on PLA_2 activity has recently been the subject of extensive discussion (Hong & Levine, 1976; Gryglewski, 1976; Floman & Zor, 1976). The anti-inflammatory steroids inhibit release of label from ^{3}H-arachidonate labelled cells with a relative potency roughly parallel to their anti-inflammatory potencies (Tam, Hong, & Levine, 1977). The effect is not due to direct action on the phospholipase (Tam, Hong, & Levine, 1977) but may be due to prevention of PLA_2 activation (Nijkamp et al., 1976) by a rapidly turning over protein (Pong et al., 1977), transport of the deacylated substrates to the cyclo-oxygenase (suggested by Tam et al., 1977), or inhibition of the extracellular release of the prostaglandins formed by the synthetase complex after the action of PLA_2 (Lewis & Piper, 1975, 1976; Chang, Lewis, & Piper, 1977) perhaps by a membrane stabilizing action (Weissman & Dingle, 1961). However, in view of the low concentrations necessary for 50% inhibition of the release of label from ^{3}H-arachidonate labelled cells (2.2×10^{-9} M) (Tam, Hong, & Levine, 1977) or of the release of lymphocytic thyroid stimulator (LTS), a mixture of prostaglandins of the E and F series, from cultured human lymphocytes (1×10^{-9} M) (Herman, Pillarisetty, & Rapoport, 1978), it seems more likely that the steroids are interacting with their receptors (O'Malley & Means, 1974; Jensen & DeSombre, 1973) and thereby modulating the activity of PLA_2.

Additional support for the contention

that the action of anti-inflammatory steroids is via inhibition of PLA_2 activity comes from the finding that glucocorticoids reduce the increased concentration of free arachidonate found in psoriatic tissue. The decrease came at a time consonant with the lag time necessary for drug penetration, activation of the glucocorticoid receptor, and transcription and translation of m-RNA but before any visible clinical improvement in the disease (Hammarstrom et al.,, 1977).

(c) Feedback.

Prostaglandin E_1, which might be formed from 20:3w6 released by the action of PLA_2, is known to inhibit the activation of platelet PLA_2, possibly by causing an increase in platelet cAMP and a diminution of free Ca^{2+} (Feinstein, et al, 1976). PGI_2 also inhibits the loss of label from arachidonate incorporated into platelet phospholipids following thrombin stimulation of platelets (Lapetina, Schmitges, Chandraboes, & Cuatrecasas, 1977). So, in a classic example of endproduct inhibition, the prostaglandins formed from the products of the action of PLA_2 can in turn act to inhibit PLA_2 activity.

4. Pharmacologically active lipids related to the fatty acid cyclo-oxygenase.

a. General biochemistry of the cyclo-oxygenase system.

Fatty acid cyclo-oxygenase (E.C.1.14.99.1, also named the prostaglandin endoperoxide synthetase (Miyamoto et al.,, 1976)), hereafter referred to as the cyclo-oxygenase, was

the name given to the enzyme involved in the initial step (see fig. 1 and fig. 2) in the synthesis of prostaglandins and thromboxanes (Hamberg, Svensson, Wakabayashi, & Samuelsson, 1974a), which will be further discussed in Section I.B.4.c. The cyclo-oxygenase is also a major site for the action of nonsteroidal anti-inflammatory (NSAI) agents, which will be discussed in more detail in Section I.B.4.a.(5).(b).

(1) Reactions catalyzed by the cyclo-oxygenase.

In the reaction catalyzed by the cyclo-oxygenase (see fig. 3), molecular oxygen and a free fatty acid are combined to generate a peroxy-cyclic endoperoxide (Lands & Rome, 1976; Lands & Samuelsson, 1968), PGG which is then converted to the hydroxy-cyclic endoperoxide, PGH (the structures of prototypical prostaglandins and thromboxanes are shown in fig. 4). The specificity of the cyclo-oxygenase is such that its activity is greatest with a substrate of 8,11,14-eicosatrienoic acid (Lands, LeTellier, Rome, & Vanderhoek, 1973) (dihomo-γ-linolenic acid) or 5,8,11,14-eicosatetraneoic acid (Beerthuis, Nugteren, Pabon, & Van Dorp, 1968; Beerthuis, Nugteren, Pabon, Steenhoek, & Van Dorp, 1971) (arachidonic acid) and much less with 5,8,11,14,17-eicosapentaenoic acid (Lands, LeTellier, Rome, & Vanderhoek, 1973). These three substrates give rise to the primary prostaglandins and thromboxanes of the 1, 2, and 3 series, respectively. Other fatty acids either have diminished activity as substrates (Beerthuis,

Nugteren, Pabon, & Van Dorp, 1968; Beerthuis, Nugteren, Pabon, Steenhoek, & Van Dorp, 1971; Lands, LeTellier, Rome, & Vanderhoek, 1973) or act as competitive inhibitors (Gryglewski, 1974; Hsia, Ziboh, & Snyder, 1974; Lands, LeTellier, Rome, & Vanderhoek, 1973; Nugteren, 1970; Pace-Asciak & Wolfe, 1968; Wallach & Daniels, 1971; Ziboh, 1973; Ziboh, Vanderhoek, & Lands, 1974). Some acetylenic fatty acids, such as 5,8,11, 14-eicosatetraynoic acid, are irreversible competitive inhibitors (Downing, Ahern, & Bachta, 1970; Downing, Barve, Gunstone, Jacobsberg, & Lie, Ken Jie, 1972; Vanderhoek & Lands, 1973a; Willis, Davison, & Ramwell, 1974).

The end product of the reaction, the cyclic endoperoxide, has the interesting property of stimulating its own production (Cook & Lands, 1975; Lands, LeTellier, Rome, & Vanderhoek, 1974; Smith & Lands, 1971; Smith & Lands, 1972). The proposed mechanism is shown in fig. 5. So far, not much is known about the interaction of the cyclic endoperoxide with the cyclo-oxygenase other than that it does act as an activator and that its action appears to be inhibited by certain NSAI agents (Lands, Cook, & Rome, 1976). Certain cyclic endoperoxide analogs inhibit the cyclo-oxygenase (Wlodawer, Samuelsson, Albonico, & Corey, 1971) as do some prostaglandin analogs (McDonald-Gibson, Frack, & Ramwell, 1973; Ohki, Ogino, Yamamoto, Hayaishi; Yamamoto, Miyake, Hayashi, 1977).

The self-catalyzed destructive property of the cyclo-oxygenase has also remained relatively unexplored (Smith, 1975; Smith & Lands, 1972). The reaction may involve

the production of singlet oxygen and/or superoxide anion which then attacks the enzyme and denatures it (Marnett, Wlodawer, & Sameulsson, 1974).

Previous studies of the cyclo-oxygenase have been performed on crude homogenates from a variety of tissues, such as bovine or sheep seminal vesicles, dog spleen, guinea-pig lung, or rabbit brain (Gryglewski, 1974; Lands, LeTellier, Rome, & Vanderhoek, 1973). Experiments on crude homogenates are complicated by the existence of a lipoxygenase in platelets, which are present in all blood-containing tissues (Nugteren, 1975). The lipoxygenase will be discussed in Section I.B.5. Cyclo-oxygenase activity has been measured by either substrate consumption or prostaglandin and thromboxane production (Bergström, Carlson, & Weeks, 1968; Flower, 1974; Lands & Rome, 1976). A problem with these approaches is that contaminating fatty acids, which may themselves be substrates, may act as inhibitors of more active primary substrates for the cyclo-oxygenase. If the synthesis of prostaglandins and thromboxanes is measured by their biological activity, the prostaglandins and thromboxane analogs formed from the less active fatty acids (Beerthuis, Nugteren, Pabon, & Van Dorp, 1968) may act as partial agonists (and therefore partial antagonists) (Ariëns, 1964) of the primary prostaglandins and thromboxanes.

(2) Physicochemical properties of the cyclo-oxygenase.

The cyclo-oxygenase has proved difficult to

Fig. 2. The central role of the fatty acid cyclo-oxygenase (E.C.1.14.99.1) in prostaglandin and thromboxane synthesis from the three main classes of substrates.

The importance of the cyclic endo-peroxide(s) as the "mother substance(s)" for the prostaglandin and thromboxane synthesis is demonstrated. The enzymes and compounds are further discussed in Section II.B.4.a. & c. The structures for the abbreviations are given in fig. 4.

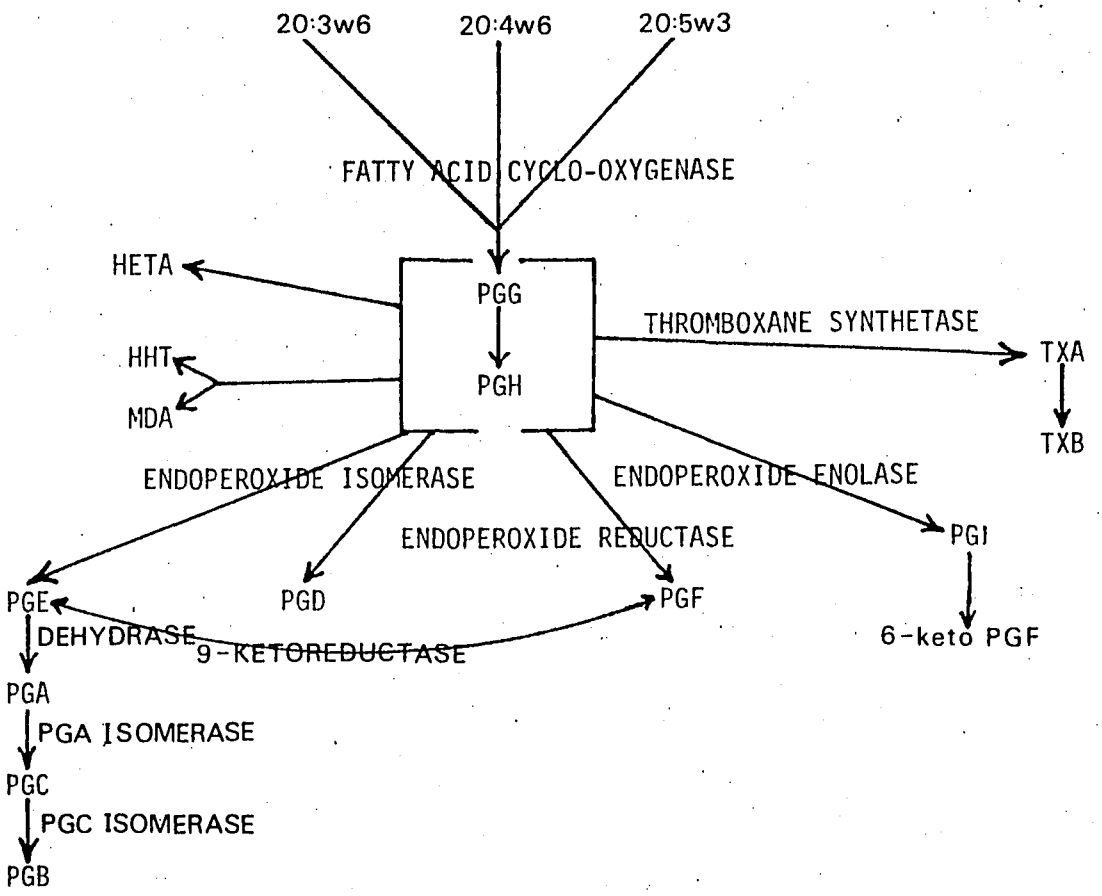

20:3w6
20:4w6
20:5w3
FATTY ACID CYCLO-OXYGENASE
HETA
PGG
THROMBOXANE SYNTHETASE
TXA
TXB
HHT
MDA
PGH
ENDOPEROXIDE ISOMERASE
ENDOPEROXIDE ENOLASE
ENDOPEROXIDE REDUCTASE
PGI
PGE
PGD
PGF
DEHYDRASE
9-KETOREDUCTASE
6-keto PGF
PGA
PGA ISOMERASE
PGC
PGC ISOMERASE
PGB

Fig. 3. Scheme for the reactions catalyzed by the cyclo-oxygenase using arachidonic acid, 20:4w6, as the substrate.

The proposed mechanism for the cyclo-oxygenase from Lands and Rome (1976) and from Lands and Samuellson (1968). Broken arrows indicate side reactions. The mechanism is further discussed in Section I.B.4.a.(1).

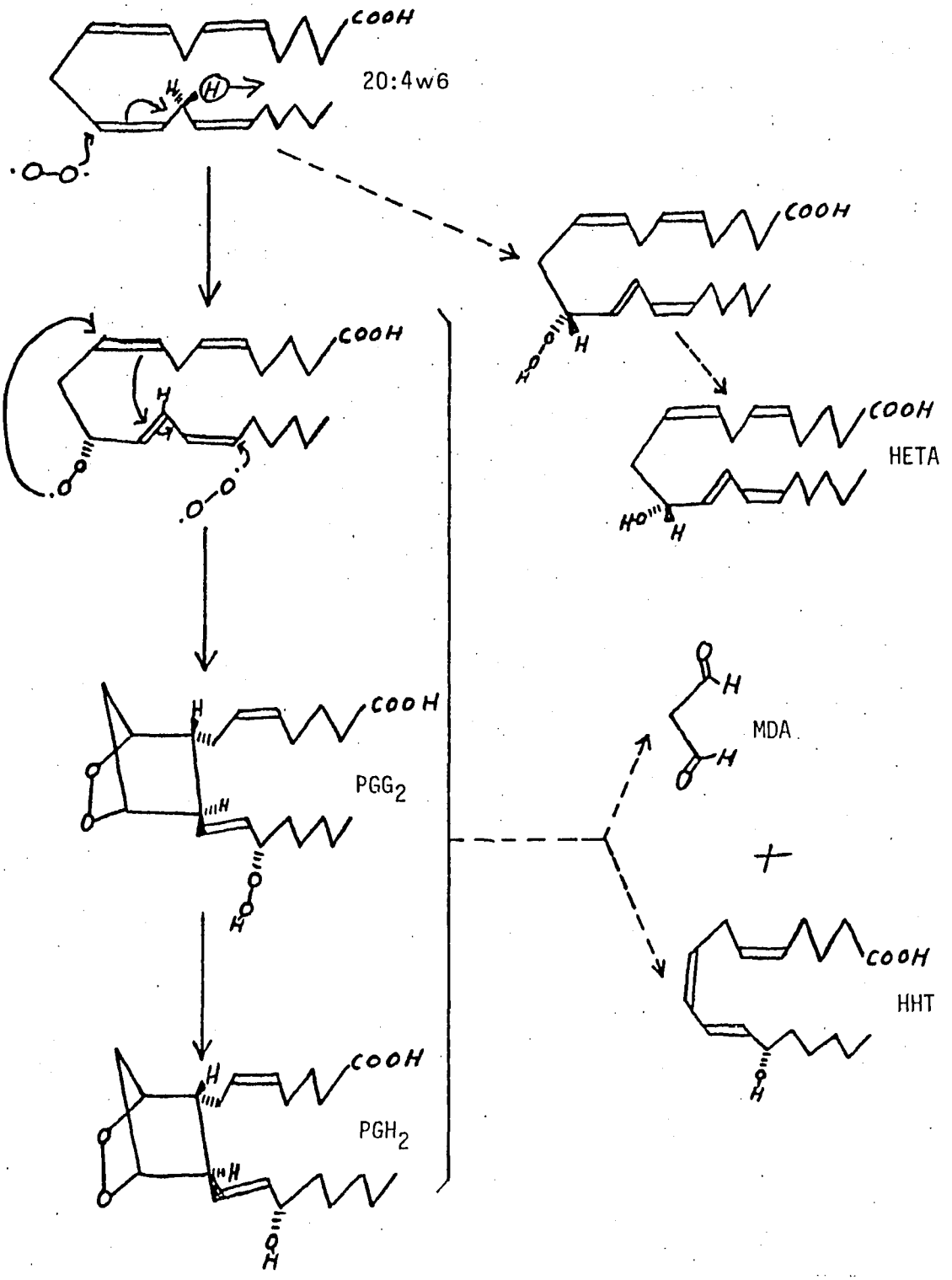
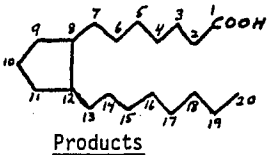

COOH
20:4w6
COOH
COOH
HETA
COOH
PGG2
MDA
+
COOH
HHT
COOH
PGH2

Fig. 4. Structures of prostaglandins and thromboxanes formed by the fatty acid cyclo-oxygenase and other enzymes in the prostaglandin synthetase complex.

The prototypical structure, prostanoic acid, and the classical substrates giving rise to the 1-, 2-, and 3- series prostaglandins are shown. Also shown are the structures of the precursor, arachidonic acid, (20:4w6), and the products formed from it.

Prototypical Structure: PROSTANOIC ACID



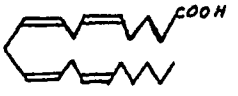
Substrates — Products

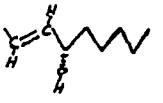
8,11,14-eicosatrienoic acid, 20:3w6 ⟶ prosta-13-enoic acids, PG_1 series

5,8,11,14-eicosatetraenoic acid, 20:4w6 ⟶ prosta-5,13-dienoic acids, PG_2 series

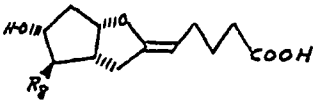
5,8,11,14,17-eicosapentaenoic acid, 20:5w3 ⟶ prosta-5,13,17-trienoic acids, PG_3 series

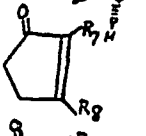
Prostaglandins and thromboxanes formed from arachidonic acid, 20:4w6:

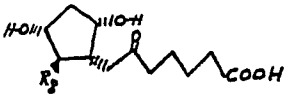
20:4w6 

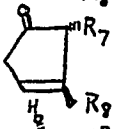
R_7  R_8 

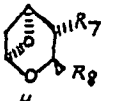
PGA_2 

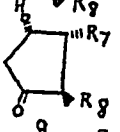
PGI_2 

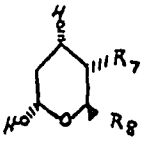
PGB_2 

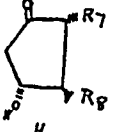
6-keto-$PGF_{1\alpha}$ 

PGC_2 

TXA_2 

PGD_2 

TXB_2 

PGE_2 

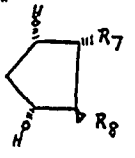
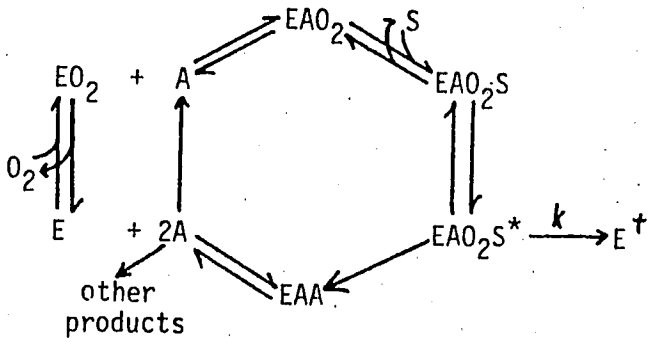
$PGF_{2\alpha}$ 

Fig. 5. The role of the activator, prostaglandin cyclic endoperoxide, in the proposed kinetic scheme for the fatty acid cyclo-oxygenase.

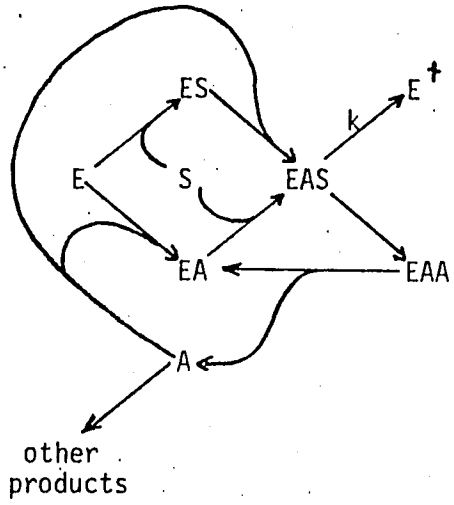
a. Scheme highlighting the central role of the activator (A) in the kinetic scheme involving the enzyme (E), oxygen (O_2), the substrate (S), and the inactivated enzyme (E+) (Lands, Cook, & Rome, 1976).

b. Scheme showing interrelationships between the enzyme (E), substrate (S), activator (A), and the inactivated enzyme (E+) as modified from Lands, Cook, and Rome (1976).

a.


EAO2
S
EO2
+
A
EAO2S
O2
E
+ 2A
EAO2S*
k
E†
other
products
EAA

b.


ES
E†
k
E
S
EAS
EA
EAA
A
other
products

purify (Miyamoto, Yamamoto, & Hayaishi, 1974; Raz, Kloog, Perel, & Kenig-Wakshal, 1975; Rome & Lands, 1975; Samuelsson, Granstrom, & Hamberg, 1967; Smith, 1975, Wallach & Daniels, 1971). Purification of bovine seminal vesicle gland microsomal "prostaglandin endoperoxide synthetase" yielded a single electrophoretic band in polyacrylamide gels containing 0.2% 'Tween 20'. Protein eluted from the gel had a molecular weight of between 3.0 and 3.5 x 10^5 and a specific activity of 2.4 units/mg protein at 24°C and pH 8.0 (90). Using sheep vesicular glands, the cyclo-oxygenase was purified to a single electrophoretic band in polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS). Protein eluted from the gel in this preparation had a molecular weight of 7 x 10^4 and a specific activity, expressed in the same terms as that above, of 24 units/mg protein at 30°C and pH 8.0 (56). The cyclo-oxygenase as purified by Hemler, Lands & Smith copurified with a low molecular weight compound that may be a heme cofactor, as the activity of the cyclo-oxygenase was stimulated by added hemin. In addition, non-heme iron also appeared to co-chromatograph with the purified enzyme. However, Van Der Ouderaa, Buytenhek, Nugteren, and Van Dorp (1977) have also purified a sheep vesicular prostaglandin endoperoxide synthetase (cyclo-oxygenase) using polyarcylamide gels containing either Tween-20 or SDS. The protein from the final isoelectric focusing step was found to have a molecular weight of 1.26 x 10^5 and bound about 12 molecules of mannose and 5 molecules of N-acetylglucosamine per molecule of apoprotein. The

apoprotein was composed of two polypeptide chains which, without sugars, weighed 6.9×10^4 daltons. The apoenzyme required added hemin, either as such or from added hemoglobin or myoglobin, and did not appear to contain any non heme iron. The best preparations had a specific activity of 8-11 units/mg protein at 25°C and pH 8.0. The difference between the findings of the Dutch and American groups with respect to iron may be due to the Dutch use of EDTA in the purification procedure which might eliminate non specific binding of iron by the cyclo-oxygenase. The difference in specific activity may be due to the differences in the temperatures at which they were calculated.

Of particular interest was the finding by Van Der Ouderaa et al (1977) that both hemin and a suitable reducing substance (hydroquinone, phenol, tryptophane, 5-HT, or epinephrine) were absolute requirements for reaction. The optimum fatty acid concentration was found to be 7×10^{-5} M; however, no mention was made as to which fatty acid was used. At the optimum fatty acid concentration and an enzyme concentration of 1.7×10^{-7} M, 1×10^{-6} M hemin, and $2.5\text{-}5.0 \times 10^{-4}$ M hydroquinone gave maximal initial oxygenation rates. The reducing agent, which is consumed in stoichiometric amounts, prevents the rapid inactivation of the enzyme by the hydroperoxy intermediate, PGG, or any fatty acid hydroperoxide derived from the action of the enzyme.

It appears that the cyclo-oxygenase catalyzes both the formation of the hydroperoxy-cyclic endo-

peroxide (PGG) and its reduction to the hydroxy-cyclic endoperoxide (PGH) Miyamoto, Yamamoto and Hayaishi (1974) were able to separate the cyclo-oxygenase activity from the endoperoxide isomerase activity but the oxygenase activity has not been separated from the peroxidase activity.

(3) Metabolism of the products of the cyclo-oxygenase.

As can be seen from fig. 2, the cyclo-oxygenase is the pivotal but only the initial step in the synthesis of the prostaglandins/thromboxanes. The reactions involved in the formation (fig. 2, clockwise from the top) of TXA, TXB, PGI, 6-keto-PGF, PGF, PGD, PGE, PGA, PGG, PGB, and the interconversion of PGE and PGF recently have been extensively discussed (Pong & Levine, 1977; Sun, Chapman, & McGuire, 1977). These reactions and the enzymes catalyzing them are not as well characterized as those of the cyclo-oxygenase. However, the control of the flow of the cyclic endoperoxides to these various enzymes and the biological effects of the pharmacological manipulation of that control (Needleman, Bryan, Wyche, Bronson, Eakins, Ferrendelli, & Minkes, 1977; Moncada, Needleman, Bunting, & Vane, 1976) provides a vast area for further research. Also, the regulation of the synthesis of the enzymes metabolizing the cyclic-endoperoxides remains open to further research.

(4) Catabolism of the products of the cyclo-oxygenase and their metabolites.

The catabolism of the prostaglandins and

thromboxanes is a major source of bio-inactivation of these compounds. The subject has been extensively reviewed (Samuelsson, 1972; Grandström, 1973; Wolfe, 1975, Pong & Levine, 1977; Ramwell, Leovey, & Sintetos, 1977). There are four main routes: 1) β-oxidation; 2) ω-oxidation; 3) Δ^{13}-hydrogenation; and 4) 15-hydroxydehydrogenation. All these routes allow endoperoxide metabolism to occur in almost any combination. The 15-hydroxydehydrogenation reaction has been the most extensively studied, since the enzyme, 15-hydroxy-prostaglandin dehydrogenase (PGDH), converts the prostaglandins/thromboxanes to compounds with considerably less biological activity (Hansen, 1976). Also, the enzyme lends itself to biochemical characterization since it is soluble (Pong & Levine, 1977). Two forms of the enzyme, which are respectively NAD^+ and $NADP^+$ dependent, have been isolated. The enzyme is itself metabolized with a half life of 1 hr. in rat kidneys (Blackwell, Flower, & Vane, 1975a). The inhibition of PGDH has been investigated and reviewed (Hansen, 1976). Two points of interest are that furosemide and ethacrynic acid are inhibitors and that steroids appear to be involved in the control of PGDH metabolism (Ramwell, Leovey, & Sintetos, 1977).

(5) Pharmacological manipulation of the cyclo-oxygenase.

(a) Activation.

The cyclo-oxygenase does not act on fatty acids when they are esterified either as their methyl esters (Struijk et al, 1966) or in phospholipids (Lands &

Samuelsson, 1968; Vonkeman & Van Dorp, 1968). The freeing of the fatty acid substrate for the cyclo-oxygenase is thought to be the rate limiting step in the synthesis of prostaglandins and thromboxanes (Kunze & Vogt, 1971). The actions of agents which stimulate the production of substrates for the cyclo-oxygenase was discussed in Section I.B.3.a. & d.(1).

The stimulation of the cyclo-oxygenase by its products, the cyclic-endoperoxides, was also previously discussed in Section I.B.4.a.(1)., as was the activation by hemin and reducing agents.

The synthesis of the cyclo-oxygenase is itself subject to activation, and, recently, the carcinogen benzo(a)pyrene has been shown to induce increased cyclo-oxygenase activity in cultured canine kidney cells. In addition, 7,8-benzoflavone, an agent that inhibits either the metabolic activation of the procarcinogen or the binding of the ultimate carcinogen (Kinoshita & Belboin, 1972), prevented the induction of increased prostaglandin synthesis by benzo(a)pyrene (Hassid & Levine, 1977). The interpretation of increased synthesis of the cyclo-oxygenase must be subject to the usual caveats of decreased inactivation, increased activation, etc. until an actual increase in the amount of cyclo-oxygenase protein has been demonstrated.

(b) Inactivation.

It is the cyclo-oxygenase on which many of the NSAI agents act. Though Jaques had demonstrated that the action of arachidonic acid (or perhaps arachidonic acid

peroxide (Dakhil & Vogt, 1962a & b) was blocked by acetylsalicylic acid (aspirin) as early as 1959 (Jaques, 1959), it was not until 1971 that Vane (1971), Ferreira, Moncada and Vane (1971) and Smith and Willis (1971) proposed that NSAI drugs acted by inhibiting prostaglandin synthesis. There is still some controversy about the exact mechanism of NSAI (Bonta, Bult, Vince,t, & Zijlstra, 1977; Smith, 1975), but the link to prostaglandin biosynthesis is very firmly established (Brune, Glatt, & Graf, 1976). Older studies on the mechanism of NSAI often used bioassay for quantitating the inhibition of prostaglandin synthesis (summarized by Flower, Robinson, & Vane, 1974; Tomlinson, Ringold, Qureshi, & Forchielli, 1972).

Some of the end products derived from the cyclic endoperoxide require enzymatic steps beyond the cyclo-oxygenase before they can be measured. These enzymes may also be inhibited by NSAI drugs (Flower, 1974). For example, Cushman and Cheung showed that benzydamine stimulated the production of PGE_2 from arachidonic acid by bovine seminal vesicle microsomes (Cushman & Cheung, 1976). At the same time, benzydamine inhibited the production of $PGF_{2\alpha}$ and PGD_2. These data may be explained by the work of Moncada, Needleman, Bunting, & Vane (1976), demonstrating that benzydamine blocks other enzymes in the prostaglandin synthetase complex at a lower concentration than that at which it blocks the cyclo-oxygenase.

Because of difficulties such as those

described in the previous paragraph, there are many conflicting data on the substrate specificity and inhibitor affinities of the "prostaglandin synthetase" (e.g., the published values for the I_{50} of aspirin and indomethacin on prostaglandin synthesis by bovine seminal vesicles vary from 30 μM (Flower, Cheung, & Cushman, 1973) to 15,000 μM (Tomlinson, Ringold, Qureshi, & Forchielli, 1972) and 0.1 μM (Gryglewski, 1974) to 10 μM (Flower, Cheung, & Cushman, 1973) respectively. The situation is further complicated by the almost universal use of the I_{50} by investigators to describe the various inhibitors (Flower, 1974; Gryglewski, 1974; Lands & Rome, 1976; Shen, Ham, Cirillo, & Zanetti, 1974).

The inhibition constant (K_i) is a measure of the dissociation constant of the enzyme inhibitor complex and is the reciprocal of the affinity of the enzyme for the inhibitor (Dixon & Webb, 1964). The I_{50} is the concentration of inhibitor which produces 50% inhibition of an enzymatic reaction under specified conditions (Tipton, 1973). The relationship between K_i and I_{50} is complex and, depending on the order and mechanism of the reaction, may be a function of both the concentration of the substrate(s) and the affinity of the enzyme for the substrate(s) (Cheng & Prusoff, 1973). For certain reaction mechanisms, simplifying assumptions may yield $I_{50}=K_i$, but this applies only to noncompetitive or uncompetitive inhibition (Cheng & Prusoff, 1973). Since most NSAI agents appear to act as competitive inhibitors of the cyclo-oxygenase (Flower, 1974; Gryglewski, 1974; Lands & Rome,

1976), it is very difficult to compare results produced in different laboratories under different experimental conditions (Lands & Rome, 1976).

An additional complicating factor is the existence of time-dependent inhibition of the cyclo-oxygenase by certain NSAI drugs. First reported by Smith and Lands in 1971, the apparent irreversible inhibition of the cyclo-oxygenase by NSAI agents such as aspirin and indomethacin (Lands & Rome, 1976) is a function of both the concentration of the inhibitor and the length of time that the enzyme is exposed to the inhibitor (Rome & Lands, 1975b). The kinetics of such a reaction have been described, and they involve an exponential decay in enzyme activity as an additional component in the rate equation (Laidler & Bunting, 1973, pp. 175-180; Lands & Rome, 1976). As a result of the existence of time-dependent inhibition, determination of K_i has physical meaning only when derived from extrapolation to zero exposure time to the time-dependent inhibitors. Otherwise, the apparent K_i will be a combination of K_i, k (the rate constant for the time-dependent inhibition), and the time over which the enzyme is exposed to the inhibitor. To demonstrate the effects of time-dependent inhibition, the following example was calculated using the K_i, the data in fig. 3.8, and equations 23 and 24 from Lands and Rome (1976).

Indomethacin is a time-dependent inhibitor with a proposed K_i (corrected to zero time of exposure) of 150 μM. If indomethacin is used at a concentration

equal to the K_i and if the substrate is used at a concentration equal to twice the K_m, then the expected initial velocity should be half the maximum velocity. However, if the enzyme is preincubated with the inhibitor for only one minute prior to the start of the reaction, then the initial velocity would fall to 5% of maximum velocity. If the incubation time is doubled, then the initial velocity would fall below one half of one percent of maximum velocity. From this example, it can be seen that, without taking into account the time-dependent inhibition caused by indomethacin, its affinity for the cyclo-oxygenase would be far over-estimated.

The inactivation of the cyclo-oxygenase is only one mechanism for the action of NSAI drugs. Another, equally important, action is the shifting of the flow of the cyclic-endoperoxides through their metabolic pathways, as was discussed in Section I.B.4.a.(3).

b. Pharmacologically active lipids related to cyclo-oxygenase substrates.

(1) Non esterified fatty acids.

(a) Eicosanoic acids.

((1)) Arachidonic acid (5,8,11,14-eicosatetraenoic acid,20:4w6).

Arachidonic acid is the predominant polyunsaturated fatty acid with pharmacological activity. In 1959, Jaques published his first paper on the actions of arachidonic acid on the isolated guinea-pig terminal ileum. In summary, he found that, after a latency of 10-15 sec.,

arachidonic acid produced a contraction of the ileum (which was recorded essentially isotonically in Mg^{2+} free Tyrode's solution) that peaked within 45-90 sec. and then gradually decayed to its original baseline within 3-5 min. Though he did not construct concentration-effect curves, he found that the threshold concentration varied from 3.3×10^{-8} to 2.0×10^{-7} M with a maximum response reached at 6.6×10^{-7} to 1.3×10^{-6} M; these latter concentrations were used as his test doses. Atropine, 2 to 4×10^{-7} M, inhibited the action of low concentrations of arachidonic acid but not the action of higher (3.3×10^{-6} to 1.6×10^{-5} M) concentrations. It should be noted that he found that atropine and mepyramine (pyrilamine, a H_1-antihistamine), at concentrations that blocked equieffective doses of acetylcholine or histamine, did not "appreciably" impair the effects of arachidonic acid. Morphine and other narcotic analgesics produced 90-100% inhibition of the action of arachidonic acid. The potency ratios for the blockade of arachidonic acid paralleled those for analgesia (Jaques, 1959, 1965). Nalorphine, a narcotic antagonist with partial agonist activity (Lewis, Bentley, & Cowan, 1971), was ineffective in blocking the action of arachidonic acid. The local anaesthetics, dibucaine and procaine, at concentrations of 2.6×10^{-5} M and 1.1×10^{-4} M respectively, caused 90-100% inhibition. The muscarinic antagonists, atropine and oxyphenonium bromide, at concentrations of approximately 2×10^{-7} M did block the action of low concentrations of arachidonic acid (6.6×10^{-7} M to 1.3×10^{-6} M),

but this action was considered to be nonspecific (i.e. physiological antagonism) since they also inhibited the actions of acetylcholine and 5-HT (actions that are now understandable in view of the stimulation of acetylcholine release by 5-HT (Brownlee & Johnson, 1963, 1965)) and since the stimulations by higher concentrations of arachidonic acid (3.3×10^{-6} M to 1.6×10^{-5}M) were not "appreciably" altered. Jaques noted that Vogt (1959) had made similar findings on the inhibition of Darmstoff by atropine and morphine. Isoproterenol, at a concentration of 5×10^{-7} M, inhibited the action of arachidonic acid in a manner that appeared similar to that of muscarinic antagonists. The effect of isoproterenol may now be explained by the direct relaxant effects of adrenergic agonists on intestinal smooth muscle (Kosterlitz, Lydon, and Watt, 1970). Paralyzing doses of nicotine (2×10^{-5} to 2×10^{-4} M) and the metabolic inhibitors cyanide (2×10^{-4} M) and 2,4-dinitrophenol (1×10^{-4} M), each caused 90-100% inhibition.

The H_1-antihistaminics, tripelennamine and mepyramine (pyrilamine) at concentrations as high as 4×10^{-6} M; the 5-HT antagonist, lysergic acid diethylamide (LSD), at 3×10^{-6} M; and azamethonium bromide, a ganglionic blocking agent at a concentration of 1×10^{-2} M, all had no effect on the response to arachidonic acid.

The metabolic inhibitor, F^- (2×10^{-3} M); the monoamine oxidase inhibitor, aminoguanidine (1×10^{-4} M); the soybean trypsin inhibitor (10^{-4} g/ml); and

the sulfhydryl agent, cystine (8×10^{-4} M) also had no effect on the arachidonic acid response. The ilea of guinea-pigs pretreated for 2 days with reserpine, 3 mg/kg s.c., "responded to arachidonic acid in a manner identical to that of untreated controls..." (Jaques, 1959).

Increasing the K^+ concentration of the Tyrode's solution to twice normal potentiated the response of the ileum to arachidonic acid, particularly at threshold doses. Tetraethylpyrophosphate, a cholinesterase inhibitor, at a concentration (3.4×10^{-6} M) that did not itself stimulate the ileum, also potentiated the response to arachidonic acid particularly at threshold doses. Additionally, pyridoxine, vitamin B_6, at a concentration of 2×10^{-5} M, had the same effect.

Perhaps the most significant finding, but one that has been completely overlooked, was that NSAI drugs such as aspirin (Jaques, 1959, 1965) phenylbutazone, aminopyrine, and methampyrone (Jaques, 1965) inhibited the response to arachidonic acid at concentrations (e.g., the D_{50} for aspirin was 1.5×10^{-4} M (Jaques, 1965)) that are effective for cyclo-oxygenase inhibition (Flower, 1974; Lands & Rome, 1976). Salicylic acid (7.4×10^{-4} M), an NSAI agent that is an order of magnitude less effective <u>in vitro</u> than aspirin (Vane, 1971), did not block the action of arachidonic acid. And prednisolone (3×10^{-5} M), a corticosteroid with anti-inflammatory activity whose action might be expected to fall into the category discussed in Section I.B.3.d.(2).(b).,

was also ineffective.

The action of arachidonic acid depended on its unsaturated nature, as incubation with dilute alcoholic iodine eliminated the stimulatory activity of a solution of arachidonic acid active at a concentration of 7×10^{-7} M. Also, hydrogenation by the platinum oxide method, eliminated the stimulatory activity of arachidonic acid at concentrations as high as 1.6×10^{-5} M.

In a comparative bioassay, arachidonic acid was found to stimulate the rabbit duodenum at concentrations greater than 3.3×10^{-6} M but to be inactive on both the rat colon at concentrations up to 3.3×10^{-4} M and the hamster colon at concentrations up to 1.6×10^{-4} M. The hamster colon did contract to ricinoleic acid at concentrations greater than 1×10^{-4} M but the guinea-pig ileum did not. Arachidonic acid, at doses greater than 3.3×10^{-7} moles/kg i.v., produced a brief fall in blood pressure in the anaesthetized cat; the fall was partially but never entirely blocked by atropine.

Oleic acid, elaidic acid (the trans isomer of oleic acid), linoleic, and linolenic acids were virtually inactive at concentrations up to 10^{-4} M on the guinea-pig ileum.

Jaques (1959) suggested that atropine blocked the action of arachidonic acid by inhibiting acetylcholine released by it and that the release was due to an action on the nerve fibers or their endings. In view of

the linkage of arachidonic acid to the PLA_2/cyclo-oxygenase/ lipoxygenase system, Jaques' speculation that "this fatty acid, or a substance with similar pharmacological characteristics, might be present in a pre-active form (bound to protein) in certain nerve structures - which are normally rich in lipids - and set free by some enzyme system, whereupon it would release the receptor-stimulating acetylcholine and among other things cause pain." (Jaques, 1959) seems remarkably prescient.

Unfortunately, three years later, Dakhil and Vogt (1962a & b) published an abstract and a paper demonstrating that freshly prepared solutions of linoleic, linolenic, and arachidonic acid had no stimulatory action on the guinea-pig or rabbit ileum. After standing for several days in contact with air, or treatment with hemoglobin, H_2O_2, or lipoxydase (Mann), the fatty acids acquired activity that paralleled the formation of peroxides (measured spectrophotometrically). However, when linoleic acid was incubated with alcoholic $KMnO_4$, the product, which was described as tetrahydroxystearic acid, had no biological activity up to 6×10^{-6} M. However, both linoleol and methyl arachidonate, which were ineffective on the guinea-pig ileum, became active when they were converted to their hydroperoxides. Treatment of the peroxidized fatty acids with $FeCl_2$ (one drop of a 0.22% solution in a 1 ml of 10^{-2} fatty acid), ascorbic acid (6×10^{-3} M), peroxidase, or triphenylphosphine entirely eliminated their stimulation of the guinea-pig ileum. In addition, ricinoleic acid and SRS-C were also inhibited by

triphenylphosphine, while phosphatidic acid and a preparation of 'prostaglandin' obtained from Dr. R. Eliasson in Sweden were not affected. The evidence was presented so forcefully that Jaques stated in his 1965 paper that the biological activity of arachidonic acid as reported earlier (Jaques, 1959) was due to the presence of arachidonic acid peroxide (Jaques, 1965).

At the time the evidence was presented, the link between arachidonic acid and prostaglandins via the cyclo-oxygenase had not been established (Bergström, Danielsson, & Samuelsson, 1964; Van Dorp, Beerthuis, Nugteren, & Vonkeman, 1964a & b). Additionally, the antioxidants used to destroy the hydroperoxides also act as cyclo-oxygenase inhibitors; e.g., ascorbic acid is a noncompetitive inhibitor with a K_i of 5×10^{-4} M (Vanderhoek & Lands, 1973b). Glutathione peroxidase, by destroying peroxides, prevents the activation of the cyclic endoperoxide and also perhaps by other lipid peroxides (Lands, Cook, & Rome, 1976).

If the action of arachidonic acid on the guinea-pig ileum is not via its formation into prostaglandins, the blockade by NSAI drugs must be explained in some other manner. A possibility suggested by Vane is that arachidonic acid peroxide (and other lipid peroxides) may act by shifting the flow of endoperoxide metabolism away from the formation of PGI toward the metabolism of the classical prostaglandins and the thromboxanes (Moncada, Needleman, Bunting, & Vane, 1976b). PGI_2 is a potent vasodilator that

contracts the rat stomach strip but relaxes the rat or hamster colon (Omini, Moncada, & Vane, 1977). If the longitudinal muscle of the intestinal preparation is under chronic PGI relaxation, then a shift away from its synthesis would tend to cause a contraction. Alternatively, the known activating properties of the hydroperoxy-fatty acids (Lands, Cook, & Rome, 1976) may stimulate ongoing prostaglandin synthesis which normally modulates longitudinal muscle tone (Bennett, Eley, & Stockley, 1975a & b; Eckenfels & Vane, 1972; Kadlec, Masek, & Seferna, 1974).

Certainly, not all effects of arachidonic acid on intestinal preparations are necessarily linked to the formation of its peroxides. Jager, comparing the effects of a series of unsaturated fatty acids on the guinea-pig ileum, found that only those fatty acids which could form prostaglandins or their homologs were stimulatory and summarized that "the results do not support the opinion that the stimulating effect of fatty acids on ileum contraction in vitro depends upon their contamination with unspecific autooxidation products." On the rabbit duodenum, Vargaftig and Dao (1970) demonstrated blockade by 3.6×10^{-6} M flufenamic acid of the response to 1.3×10^{-5} M arachidonic acid.

And on the rat fundus, arachidonic acid appeared to act through conversion to prostaglandins as freshly prepared solutions were used, the actions were blocked by anoxia (nitrogen replacing oxygen with pH

differences accounted for), indomethacin or 5,8,11,14-eicosatetraynoic acid (10^{-6} M), SC-19220, (a prostaglandin receptor antagonist (Sanner, 1969)) at concentrations up to 5×10^{-5} M (the slope of the Schild plot was the same for arachidonic acid and PGE_2 and $PGF_{2\alpha}$). At high concentrations giving responses approaching maximal, arachidonic acid or some compound formed from it appeared to have a direct effect as a partial agonist on prostaglandin receptors (Splawinski, Nies, Sweetman, & Oates, 1973). Splawinski et al.(1975) also analyzed the arachidonic acid for spontaneous conversion into prostaglandin like compounds (Nugteren, Vonkeman, & Van Dorp, 1967) and found less than 0.025% by mass spectrometric methods and 0.0006% by RSS bioassay. Unfortunately, they did not analyze for peroxides, but the precautions that they did take to avoid their formation (such as storage of the stock solutions in ethanol under nitrogen at $-20^{\circ}C$ and preparation of the working solution each day by conversion to the sodium salt, evaporation under nitrogen, and solvation in saline) probably prevented any contamination by auto-oxidation.

The effects of arachidonic acid on platelet aggregation have been widely examined (Blackwell, Duncombe, Flower, Parsons, & Vane, 1977; Chignard, Lefort, & Vargaftig, 1977a & b; Davies, Davidson, McClenaghan, Say, & Haslam, 1976; Feinstein, Becker, & Fraser, 1977; Vargaftig, 1977; Vargaftig & Zirinis, 1973) and no attempt will be made here to discuss any but a few relevant findings. Arachidonic acid can act to stimulate platelet aggregation via both the

cyclo-oxygenase and lipoxygenase metabolic pathways (see Vargaftig, 1977 for a more complete discussion). In addition, arachidonic acid is also released during platelet aggregation induced by other agents (Blackwell et al., 1977; Feinstein et al., 1977). Finally, arachidonic acid may induce platelet lysis by a detergent effect (Chignard et al., 1977a; Spector & Hoak, 1975). However, arachidonic acid is not a sine qua non for aggregation as it can be stimulated in the absence of endogenous precursors for the prostaglandin endoperoxides and the thromboxanes (Bult & Bonta, 1976).

In summary, the actions of arachidonic acid on isolated preparations appear to be due, in the main, to its metabolism to prostaglandins and thromboxanes (see Section I.B.4.c.) and to hydroperoxides by lipoxygenase and via auto oxidation (see Section I.B.5.).

((2)) Dihomo-γ-linolenic acid (8,11,14-eicosatrienoic acid, 20:3w6).

Dihomo-γ-linolenic acid acts as a precursor to prostaglandins and thromboxanes with only one double bond (see fig. 4 and Section I.B.4.c.). Because the sheep vesicular gland phospholipids contain much more 20:3w6 than they do 20:4w6, many of the initial experiments on the biosynthesis of prostaglandins were done using 20:3w6 as the substrate (Samuelsson, 1969).

The action of 20:3w6 on the guinea-pig ileum is a stimulation with a threshold concentration of 1.6×10^{-5} M; this was 100 times the threshold dose for 20:4w6

reported in the same system (Jager, 1970). When perfused through the isolated canine lung, 20:3w6 was approximately 2.5 to 5 times less potent than 20:4w6 in causing an increase in the perfusion pressure (Wicks, Ramwell, Rose, & Kot, 1977). When perfused through the isolated guinea-pig lung, 20:3w6 released both RCS and prostaglandin-like activity and this release, like that caused by the infusion of 20:4w6, lasted as long as the infusion continued. Aspirin (3×10^{-5} M in the perfusion fluid) blocked this action of both 20:3w6 and 20:4w6 (Palmer, Piper, & Vane, 1973).

The actions of 20:3w6 on platelets have been much more thoroughly investigated than its actions on isolated preparations. Whereas arachidonic acid is a potent proaggregatory compound with a threshold concentration of 2×10^{-5} to 5×10^{-4} M, depending on the species (Vargaftig & Zirinis, 1973; Silver, Smith, Ingerman, & Kocsis, 1973; Vargaftig, 1974), dihomo-γ-linolenic acid inhibits platelet aggregation induced by 20:4w6 (Silver et al,,1973), collagen, norepinephrine (Willis, Comai, Kuhn, & Paulsrud, 1974), and, in primates and man (Sim & McCraw, 1977) but not in rabbits (Oelz, Seyberth, Knapp, & Oates, 1976), adenosine diphosphate. Since 20:3w6 is incorporated into platelet phospholipids in very small quantities relative to 20:4w6 (Marcus, Ullman, & Safier, 1969), the normal action of PLA_2 is to release 20:4w6 which is then metabolized to the proaggregatory substances, PGG_2, PGH_2, and TXA_2 (Hamberg, Svensson, Wakabayashi, & Samuelsson, 1974; Hamberg & Samuelsson, 1974a & b; Hamberg,

Svensson, & Samuelsson, 1975). When the platelets are loaded with 20:3w6 (Willis et al., 1974: Sim & McCraw, 1977), or incubated with 20:3w6 (Silver et al., 1973), platelet aggregation is inhibited. The inhibition may either be at the level of competition with 20:4w6 for the enzymes involved in the latter's metabolic conversion to active substances or by conversion to substances such as PGE_1 which are potent inhibitors of platelet aggregation (Weiss, 1975). Since the cyclic endoperoxides formed from 20:3w6 induce aggregation (Svensson, Hamberg, & Samuelsson, unpublished; cited in Falardeau, Hamberg, & Samuelsson, 1976), the inhibition is not likely to be due to antagonism at the endoperoxide receptor level.

((3)) Other eicosanoic acids.

The precursor of prostaglandins and thromboxanes of the series containing three double bonds (e.g., PGE_3) (see fig. 4 and Section I.B.4.c.) is 5,8,11,14,17-eicosapentaenoic acid (Bergström, Danielsson, & Samuelsson, 1964; Van Dorp et al., 1964b). On the guinea-pig ileum, 20:5w3 caused a stimulation at a minimum effective concentration of 1.6×10^{-4} M (Jager, 1970). At a concentration of 5×10^{-4} M, 20:5w3 inhibited platelet aggregation induced by 20:4w6, collagen or ADP (Silver et al., 1973). The actions of the eicosenoic acids on isolated tissue preparations have not been examined to the extent that the prostaglandin precursors, 20:4w6 or 20:3w6, have been. Jager compared a series of eicosatrienoates on the guinea-pig ileum and found that 20:3w7 had a minimum effective concentration of

1.6×10^{-4} M while 20:3w9 may have exhibited slight activity at 3.2×10^{-4} M and 20:3w5 was inactive (Jager, 1970). This biological activity correlated with conversion of the fatty acids into prostaglandins by a sheep vesicular gland preparation (Struijk, Beerthuis, Pabon, & Van Dorp, 1966). Neither 20:3w3 nor 20:0 induced platelet aggregation and 20:3w3 inhibited the aggregation induced by 20:4w6 (Silver et al,,1973). Earlier reports showed an increase in the release of histamine from rabbit platelets by 20:0 at concentrations from 3×10^{-6} to 1×10^{-4} M (Shore & Alpers, 1963; Haslam, 1964), but, in view of the similar effects of other saturated fatty acids, an action by displacement of proaggregatory fatty acids (i.e., 20:4w6) from serum albumin must be considered an alternative mechanism (Birkett, Myers, & Sudlow, 1977; Sjodin, 1977; Wosilait & Soler-Argilaga, 1977).

(b) Other fatty acids.

((1)) Essential fatty acids.

The essential fatty acids (EFA) are those fatty acids which, when excluded from the diet, result in the development of the symptoms of essential fatty acid deficiency. The literature on the essential fatty acids is voluminous, and the reader is referred to recent extensive reviews of the subject for an in-depth discussion (Mead & Fulco, 1976; Alfin-Slater & Aftergood, 1976). The link between the prostaglandins and thromboxanes and the essential fatty acids is so strong that the Dutch group at Unilever was led to state, "We have found that biologically active

prostaglandins are formed only from unsaturated fatty acids that show appreciable EFA-activity. The correlation between the rate of prostaglandin formation and EFA-activity of the precursor is rather striking. It is highly attractive, therefore, to assume that the sole 'essential' function of the essential fatty acids is to act as precursors for the prostaglandins." (Beerthuis, Nugteren, Pabon, & Van Dorp, 1968). This hypothesis is still the subject of controversy (see Mead & Fulco, 1967, pp. 121ff). Although there is a certain amount of looseness in the specificity of the cyclo-oxygenase for its substrates (Van Dorp, 1966, 1971; Schlenck, 1972; Struijk, Beerthuis, Pabon, & Van Dorp, 1966; Beerthuis, Nugteren, Pabon, & Van Dorp, 1968; Beerthuis, Nugteren, Pabon, Steenhoek, & Van Dorp, 1971; Van Dorp & Christ, 1975), the basic structural requirements for a series of three methylene interrupted double bonds starting about eight carbons from a carboxylic acid function and ending about six carbons from a terminal methyl group.

Linoleic acid (18:3w6) is the essential fatty acid that serves as the basis of comparison for other EFA (Thomasson, 1962). By a series of reactions (Beerthuis et al., 1968; Sprecher, 1972), linoleic acid can be converted to the classical prostaglandin precursors, 20:3w6 and 20:4w6. In addition, activity on the guinea-pig ileum preparations has been reported (Gabr, 1956; Jager, 1970). However, other workers have not found any stimulatory action of 18:2w6 on the

guinea-pig ileum (Feldberg, Holden, & Kellaway, 1938; Jaques, 1959), and the possibility remains that the action was due to the formation of the hydroperoxides (Dakhil & Vogt, 1962a & b). Linoleic acid did not stimulate platelet aggregation (Silver et al, 1973) and has been shown to inhibit prostaglandin synthesis (Pace-Aciak & Wolfe, 1968; Downing, Ahern, & Bechta, 1970). Linoleic acid has also been shown to display anti-inflammatory activity in the rat adjuvant induced skin lesion test (Stuyvesant & Jolley, 1967). In unpublished work cited by Lin and Simon, linoleic acid inhibited opiate binding to a rat brain opiate receptor fraction (Lin & Simon, 1978).

γ-Linolenic acid (18:3w6) functions as a poor substrate for prostaglandin synthesis (Van Dorp, 1966; Struijk et al, 1966) and may stimulate the guinea-pig ileum (Jager, 1970). Although the authors claim that it does not do so, a close inspection of their data (table 3) reveals that 18:3w6 appears to cause a slight stimulation of the accumulation of cAMP in the rat myometrium ($p < 0.01$, 2-tailed Student's t-test) though to á much lower level than the classical prostaglandin precursors, 20:3 and 20:4w6 (Yesin, Do Khac, & Harbon, 1978). Linolenic acid (18:3w3) is an inhibitor of the prostaglandin synthesis (Pace-Aciak & Wolfe, 1968) and did not stimulate cAMP accumulation (Vesin et al,, 1978). However, it did appear to contract the guinea-pig ileum (Jager, 1970) perhaps due to either chain elongation and conversion to a prostaglandin precursor (Jager, 1970) or to auto-oxidation (Jager, 1970; Dakhil & Vogt, 1962a & b).

Linolenic has been shown not to stimulate platelet aggregation (Shore & Alpers, 1963; Silver et al, 1973).

((2)) Non essential fatty acids.

As mentioned in the previous section, the specificity of the cyclo-oxygenase is such that other fatty acids than those classically considered as "essential" can serve as substrates directly or be metabolized to substrates. Flower has summarized the actions of a number of cyclo-oxygenase substrate analogs that act as inhibitors of prostaglandin biosynthesis (Flower, 1974). Lands and Rome discussed the stimulation of the self-catalyzed destruction of the cyclo-oxygenase by a number of substrate analogs (Lands & Rome, 1976). Those fatty acids that are poor substrates for the cyclo-oxygenase act as partial antagonists of the better substrates (Struijk et al,,1966). Ziboh's group at the University of Miami School of Medicine has been active in the isolation of fatty acid inhibitors of the cyclo-oxygenase from skin (Ziboh, McElligott, & Hsia, 1973; Ziboh, 1973; Hsia, Ziboh, & Snyder, 1974; Ziboh, Vanderhoek, & Lands, 1974; Penneys, Lord, Ziboh, & Simon, 1977; Simon, Penneys, Lord, Ziboh, & Mandy, 1977). This work has been supported by other reports of similar activity (Hulan & Kramer, 1977). The saturated fatty acids from 10:0 to 16:0 have been shown to inhibit prostaglandin synthesis with the most active compound being myristic acid, 14:0 (Gryglewski, 1974), which has a K_i of 4×10^{-4} M as a non competitive inhibitor (Robak, Zmuda, & Gryglewski, 1976).

Synthetic inhibitors of the cyclo-oxygenase have also been summarized by Flower (1974) and by Lands and Rome (1976). The acetylenic substrate analog, 5,8,11,14-eicosatetraynoic acid was first reported by Ahern and Downing (1970), and the specificity of the inhibition with regard to the position of the triple bonds has been extensively examined (Vanderhoek & Lands, 1973a). Transition state analog inhibitors have also been examined (Nugteren, 1970), and the stereospecificity of the cyclo-oxygenase probed with substituted substrate analogs (Van Dorp & Christ, 1975; Do & Sprecher, 1976; Dawson, Reid, Hemler, & Lands, 1977). In addition, synthetic analogs of the cyclic-endoperoxides formed by the cyclo-oxygenase have also been demonstrated to inhibit its action (Ohki, Ogino, Yamamoto, Hayaishi, Yamomoto, Miyake, & Hayashi, 1977).

(2) Phospholipids and other lipids.

The actions of phospholipids as the storage sites for the cyclo-oxygenase substrates and the relationship between the phospholipids and PLA_2 were discussed in Section I.B.3. Those lipids with detergent action can also function to release fatty acids from their stores by disrupting cell membranes (Lucy, 1971; Weltzien et al,, 1977), allowing PLA_2 to have access to phospholipids which were previously restricted (Lankisch & Vogt, 1972), and increasing the availability of cyclo-oxygenase substrates.

c. Pharmacologically active lipids related to cyclo-oxygenase products.

The cyclic endoperoxides and their further metabolites, the prostaglandins (Von Euler, 1935b) and the thromboxanes (Hamberg, Svensson, Wakabayashi, & Samuelsson, 1974) are a group of fatty acids with a wide range of biological activities (Bergstrom, Carlson, & Weeks, 1968; Karim & Hillier, 1972, Nakano, 1973; Bennett, 1976). First described in 1930 by Kurzok and Lieb (1930), the biological action of human semen on smooth muscle was studied independently by Goldblatt (1933, 1935) and Von Euler (1934, 1935a). Although other workers had earlier studied the actions of tissue extracts that obviously contained prostaglandins (e.g., Battez & Boulet, 1913), Von Euler continued the investigation (1935b, 1937) and eventually suggested to Bergstrom the problem of the purification and identification of prostaglandins (as cited in Horton, 1972a, p. 1.) In the 1950s, Bergstrom and his associates separated the biological activity into its several component prostaglandins (Bergstrom, Eliasson, von Euler, & Sjovall, 1959; Bergstrom & Sjovall, 1957) and then determined their structures (Bergstrom, Ryhage, Samuelsson, & Sjovall, 1962, Nugteren, Van Dorp, Bergstrom, Hamberg, & Samuelsson, 1966). With that, the role of the essential fatty acids as precursors for the prostaglandins was hypothesized and then demonstrated (Bergstrom, Danielsson, Klenberg, & Samuelsson, 1964; Bergstrom, Danielsson, & Samuelsson, 1964; Van Dorp, Beerthuis, Nugteren, & Vonkeman, 1964a & b).

Since the early 1960s, a wealth of biological data has accumulated demonstrating the pharmacological

properties of the prostaglandins (Bergstrom, Carlson, & Weeks, 1968; Horton, 1972a & b, 1974, 1976; Kadowitz, Joiner, & Hyman, 1975; Karim, 1976; Karim & Hillier, 1973; Nakano, 1973), thromboxanes (Dawson, Boot, Cockerill, Mallen, & Osborne, 1976; Hamberg, Sevensson, & Samuelsson, 1975), and the precursors Dakhil & Vogt, 1962a & b; Ichikawa & Yamada, 1962; Jaques, 1959, 1965; Splawinski, Nies, Sweetman, & Oates, 1973; Vogt, 1958), intermediates (Hamberg & Samuelsson, 1966; Kuehl, Humes, Egan, Ham, Beveridge, & Van Arman, 1977; Balmsten, 1976), and side products of their synthetic reactions(Borgeat, Hamberg, & Samuelsson, 1976; Hamberg & Samuelsson, 1974b; Nugteren, 1975; Turner, Tainer, & Lynn, 1975). Prostaglandins seem to occur in almost all tissues (though in differing amounts) and in many phyla of the animal kingdom (Nomura & Ogata, 1976). Effects of this group of lipids have been observed on the reproductive (Karim, 1975), cardiovascular (Karim & Somers, 1972), respiratory (Cuthbert, 1973), nervous (Coceani, 1974; Wolfe, 1975, 1976), renal (Lee, 1974), gastrointestinal (Bennett, 1972, 1976), endocrine (Mashiter & Field, 1974), hematological (Mody, 1972; Needleman, Noncada, Bunting, Vane, Hamberg, & Samuelsson, 1976), ocular (Eakins, 1974, 1976), integumental (Hsia, Ziboh, & Snyder, 1974), and immunological systems (Bourne, 1974). Prostaglandins, thromboxanes, and their precursors have also been implicated in pathological states of all these organ systems including dysmenorrhea (Lindner, Zor, Baumminger, Tsafriri, Lamprecht, Koch, Antebi, & Schwartz, 1974; Pickles, Hall, Best, & Smith, 1965),

coronary ischemia (Needleman, Kulkarni, & Raz, 1977; Raz, Isakson, Minkes, & Needleman, 1977), asthma (Parker, 1973; Smith, 1972), pyrexia (Feldberg, 1974), hypertension (Armstrong, Blackwell, Flower, McGiff, Mullane, & Vane, 1976; Muirhead, 1973), ulcers (Wilson, 1973), and rheumatoid arthritis (Hart, 1974). For example, increased levels of prostaglandins have been demonstrated in the synovial fluids of patients with rheumatoid arthritis (Higgs, Vane, Hart, & Wojtulewski, 1974; Robinson & Levine, 1974). The role of prostaglandins has not been investigated extensively in non-mammalian species (Christ & Van Dorp, 1973; Nomura & Ogata, 1976).

Because the pharmacological actions of the prostaglandins and thromboxanes have been so extensively reviewed (Bergström, Carlson, & Weeks, 1968; Horton, 1972a & b; Karim, 1976; Vargaftig, 1974; Wolfe, 1975, 1976) this review will not concern itself with the actions of the products of the cyclo-oxygenase except in cursory detail. The structures of the products of the cyclo-oxygenase are shown in figs. 2, 3, and 4.

(1) Cyclic endoperoxides.

The cyclic endoperoxides, PGG and PGH, were initially postulated by Hamberg and Samuelsson (1966), who then proceeded to detect them, isolate them (Hamberg & Samuelsson, 1973), determine their structures (Hamberg, Svensson, Wakabayashi, & Samuelsson, 1974), and finally their functions (Hamberg, Svensson, & Samuelsson, 1974). On all

smooth muscles on which they have been tested, PGG_2 and PGH_2 have been shown to be stimulatory with actions similar to the classical prostaglandins (Hamberg, Hedqvist, Strandberg, Svensson, & Samuelsson, 1975a). The actions of the cyclic-endoperoxides on gerbil colon and the rat stomach strip were stimulatory, but only about equal in potency to $PGF_{2\alpha}$ and less potent than PGE_2 (Hamber et al, 1975a). Because of the short (about 5 min.) half life of the endoperoxides in aqueous solutions (Hamberg, Svensson, Wakabayashi, & Samuelsson, 1974), synthetic stable analogs of PGH have been synthesized (Corey, Nicolaou, Machida, Malmsten, & Samuelsson, 1975; Bundy, 1975; Portoghese, Larson, Abatjoglou, Dunham, Gerrard, & White, 1977; Okuma, Yoshimoto, & Yamomoto, 1977) and tested on various smooth muscle preparations (Malmsten, 1976; Corey et al, 1975; Chijimatsu, Van Nguyen, & Said, 1977).

The potency of the analogs on intestinal preparations (rat stomach strip, rat colon, chick rectum, guinea-pig gallbladder, guinea-pig ileum) was of the same order of magnitude as that of the classical prostaglandins, except on the rat colon and guinea-pig ileum where they were much less potent. On vascular and lung tissue, and on platelets, the analogs were much more potent than those prostaglandins that were normally stimulatory.

(2) Metabolic products of the cyclic endoperoxides.

(a) Classical prostaglandins.

((1)) PGF.

Of particular interest is the antagonism by $PGF_{2\alpha}$ of the increase in vascular permeability caused by PGE_1 or PGE_2 (Crunkhorn & Willis, 1971) demonstrating that the prostaglandins can act either as physiological or pharmacological antagonists of each other. Various isolated tissues exhibit a range of sensitivities to the different prostaglandins. For example, the rabbit jejunum is more than an order of magnitude more sensitive to the stimulation produced by $PGF_{2\alpha}$ than it is to that produced by PGE_1 (Horton & Main, 1965) while in the guinea-pig ileum the relative potencies are reversed (Horton & Main, 1963, 1965). The antagonism of the actions of $PGF_{2\alpha}$ by the fenamates is fairly well established (Collier & Sweatman, 1968; Levy & Lindner, 1971; Panczenko, Grodzinska & Gryglewski, 1975), although whether the action is due to receptor antagonism or to blockade of $PGF_{2\alpha}$ induced stimulation of the synthesis of other prostaglandins is a matter of debate (Collier & Sweatman, 1968; Panczenko, Grodzinska, & Gryglewski, 1975).

((2)) PGE.

The relation between the prostaglandins and narcotic analgesics is still a matter of controversy. On the guinea-pig intestine, morphine and other narcotic analgesics antagonize the actions of prostaglandins at low doses but not at high ones (Jaques, 1969; Sanner, 1971). An interesting feature of the antagonism is that the narcotic analgesics are more effective against the stimulation due to PGE_1 than PGE_2 (Sanner, 1971; Ruegg & Jaques,

1973). The actions of narcotic analgesics on PGF compounds apparently have not been studied. The effects of narcotic analgesics on blocking the electrical stimulated contractions of the guinea-pig ileum and their reversal by PGE_1 or PGE_2 are an interesting side topic (Ehrenpreis, Greenberg, & Belman, 1973; Ehrenpreis, Greenberg, & Comaty, 1976) and also the subject of some controversy (Gintzler & Musacchio, 1974; Hazra, 1975a) which may be due to seasonal variation in the stimulus evoked release of acetylcholine (Hazra, 1975b).

((3)) PGD.

The action of PGD on intestinal preparations has not been the subject of much examination; however, what has been reported provides another example of the variation between tissues in their sensitivities to the prostaglandins. For example, on the rat stomach strip, PGD_2 is an order of magnitude more potent than $PGF_{2\alpha}$ and almost two orders of magnitude more potent than PGE_2; while on the rabbit jejunum, PGD_2 was a bit less than an order of magnitude more potent than $PGF_{2\alpha}$ (Horton & Jones, 1974). However, results from a different laboratory suggest that PGD_2 is only slightly more potent than $PGF_{2\alpha}$ and considerably less potent than PGE_2 on the rat stomach strip; this same series of experiments demonstrated that both PGD_2 and $PGF_{2\alpha}$ were less potent that PGE_2 on the gerbil colon preparation (Hamberg, Hedqvist, Strandberg, Svensson, & Samuelsson, 1975a). In the sheep and horse, PGD_2 is 50% as potent as PGI_2 for inhibiting ADP induced platelet aggregation (Moncada, Vane,& Whittle, (1977).

((4)) PGA.

Prostaglandins of the A family are virtually without effect on smooth muscle preparations (Bergström, Carlson, & Weeks, 1968) though they appear to relax the rabbit stomach strip and coeliac artery preparations (Moncada, Mugridge, & Whittle, 1977).

((5)) PGC.

The actions of this prostaglandin have been studied on vascular smooth muscle where it relaxes the tissue rather more potently than its immediate precursor, PGA (Horton, 1972).

((6)) PGB.

Prostaglandins of the B family are between 2 and 3 orders of magnitude less potent on smooth muscle preparations than PGE_2 (Salmon & Karim, 1976).

(b) Thromboxanes.

((1)) TXA.

The isolation and identification of TXA_2 as the predominant biologically active compound in RCS (Hamberg, Svensson, & Samuelsson, 1975) led to the concept that the classical prostaglandins might not have been the biologically active compounds that various investigators thought they were examining (Horton, 1976). TXA_2 contracts all smooth muscle preparations on which it has been examined (Nijkamp, Moncada, White, & Vane, 1977; Moncada, Mugridge, & Whittle, 1977), and it is a potent aggregating agent of platelets (Hamberg, Svensson, & Samuelsson, 1975b).

Thromboxane synthase has been identified in platelet microsomes (Needleman, Moncada, Bunting, Vane, Hamberg, & Samuelsson, 1976) and resolved from the cyclooxygenase (Hammarström & Falardeau, 1977), and the enzyme is selectively inhibited by benzydamine (Moncada, Needleman, Bunting, & Vane, 1976 and by L-8027 (2-isopropyl-3-nicotinyl indole, Gryglewski, Zmuda, Korbut, Krecioch, & Bieron, 1977). Other inhibitors such as imidazole, N-0164 (sodium p-benzyl-4-(1-oxo-2-(4-chlorobenzyl)-3-phenyl propyl) phenyl phosphonate), and U-51605 (9,11-azoprosta-5,13-dienoic acid) are much less specific (Needleman, Bryan, Wyche, Bronson, Eakins, Ferrendelli, & Minkes, 1977), and their use to control the flow of the metabolism of the endoperoxides is limited.

((2)) TXB.

Originally, the breakdown product of TXA_2 was thought to be inactive; however, TXB_2 has recently been shown to weakly stimulate the guinea-pig trachea smooth muscle with a potency from one to three orders of magnitude less than that of $PGF_{2\alpha}$ (Wasserman & Griffin, 1977; Dawson, Boot, Cockerill, Mallen, & Osborne, 1976). In addition, TXB_2 has been shown to demonstrate leukocyte chemotactic activity (Boot, Dawson, & Kitchen, 1976) and to stimulate the release of slow reacting substance-anaphylaxis (SRS-A); Boot, Dawson, & Osborne, 1976). SRS-A will be further discussed in Section II.B.4.d.(4).

(c) Prostacyclin.

((1)) PGI.

Because the pharmacological actions of PGI have not been extensively reviewed, I will include more detail in this section. The discovery that the intima of arteries was able to synthesize a compound from prostaglandin endoperoxides that inhibited platelet aggregation (Moncada, Gryglewski, Bunting, & Vane, 1976a) led to the characterization of PGI_2 (prostacyclin; Johnson, Morton, Kinner, Gorman, McGuire, Sun, Whittaker, Bunting, Salmon, Moncada, & Vane, 1976) a compound whose structure had been suggested half a dozen years earlier by Pace-Asciak and Wolfe (1970) but whose significance had not been recognized. The enzyme 6(9)oxy-cyclase that forms PGI_2 from the cyclic endoperoxides is inhibited by lipid peroxides (Gryglewski, Bunting, Moncada, & Vane, 1976).

PGI_2 relaxed all vascular tissue except the rabbit aorta, caused either relaxation or a slight contraction of the rat and hamster colons, stimulated the rat stomach strip with a potency 0.5 that of $PGF_{2\alpha}$ and 0.2-0.1 that of PGE_2, stimulated the hamster stomach strip and the chick rectum with a potency 0.2-0.1 that of PGE_2, contracted the guinea-pig tracheal strips, stimulated the rat uterus with a potency 0.04 that of $PGF_{2\alpha}$ and 0.5-0.25 that of PGE_2, and was inactive on the cat terminal ileum (Omini, Moncada, & Vane, 1977).

It has been proposed that PGI_2 and PGE_1 act on the same or similar sites on platelets and that these sites differ from the sites for PGD_2 (Moncada, Vane, & Whittle, 1977). By inspection of the molecular structures,

it is easy to see that prostaglandins with only one trans double bond at the 13,14-position can assume the conformation of PGI_2 more readily than prostaglandins with additional cis-double bond at the 5-or 5,17-positions. It should be noted that of the prostaglandins tested, PGI_2 was more potent in inhibiting platelet aggregation (Moncada, Vane, & Whittle, 1977).

((2)) 6-keto $PGF_{1\alpha}$.

Prostacyclin (PGI_2) spontaneously decomposes in aqueous solutions with a half life of about 10 min. to 6-keto $PGF_{1\alpha}$ (Johnson et al, 1976). This novel prostaglandin (6-keto $PGF_{1\alpha}$) had been discovered earlier in homogenates of rat stomach incubated with 20:4w6 (Pace-Asciak & Wolfe, 1970), but, as previously mentioned, its significance had not been appreciated. The biological activity of 6-keto $PGF_{1\alpha}$ is much different from that of PGI_2; 6-keto $PGF_{1\alpha}$ does not inhibit platelet aggregation, is ineffective on the vascular or the gastrointestinal smooth muscle preparations, but stimulates the rat uterus to the same extent that PGI_2 does (Omini, Moncada, & Vane, 1977).

(d) Hydroperoxy- and hydroxy-fatty acids.

((1)) 11-hydroperoxy and 11-hydroxy derivatives of 8,12,14-eicosatrienoic acid and 5,8,12,14-eicosatetraenoic acid.

The mechanism of the fatty acid cyclo-oxygenase is such that the first molecule of oxygen is

inserted at the 11-position of the eicosapolyenoic acid substrates; it is possible for this peroxide to dissociate from the enzyme and be degraded to the hydroxide (Hamberg & Samuelsson, 1976b & c). The biological activities of these fatty acids and peroxides on smooth muscle preparations have not been examined.

((2)) Hydroperoxy- and hydroxy-heptadecatrienoic acid and malondialdehyde.

Once the cyclic-endoperoxide has formed, it is possible for it to decompose into malondialdehyde (MDA) and a Hydroperoxy- or Hydroxy-Hepta-deca-Di- or Trienoic acid (depending on whether 20:3w6 or 20:4w6 was the substrate), that is MDA and HHD or HHT (Hamberg & Samuelsson, 1967b & c; 1974b). No studies have been reported of the biological activities of these fatty acids and peroxides on smooth muscle preparations.

(3) Pharmacological manipulation of the metabolic pathways for the cyclic endoperoxides.

(a) Activation.

The various metabolic pathways open to the cyclic endoperoxides appear, in part, to be regulated by the availability of substrate for the enzymes involved. For example, rabbit lung microsomes produce mainly PGI_2 at a PGH_2 concentration of 2×10^{-7} M while at a PGH_2 concentration of 2×10^{-5} M they produce mainly TXA_2 (Sun, Chapman, & McGuire, 1977). The enzymes that metabolize the endoperoxides are subject to control by the presence or absence of cofactors

such as the shift from the production of PGE_2 to the production of PGD_2 caused by gluthathione-S-transferase (Christ-Hazelhof, Nugteren, & Van Dorp, 1976). In addition, the synthesis of the enzyme or the provision of substrate for it may be subject to activation such as the stimulation of endothelial cell PGI_2 synthesis by a plasma factor similar to RCS-RF (MacIntyre, Pearson, & Gordon, 1978).

(b) Inactivation.

The action of 15-hydroperoxy arachidonic acid (15-HPAA) on the synthesis of PGI_2 has already been mentioned (Sections I.B.4.a.(3). and c.(2).(c).((1)).) The 15-hydroperoxy arachidonic acid apparently combines with the enzyme irreversibly and thereby inactivates it (Moncada, Gryglewski, Bunting, & Vane, 1976a). The effects of inhibitors of thromboxane synthase were discussed in Section I.B.4.c.(2).(b). The bicyclo (2.2.1.) heptene analog of prostanoic acid has been demonstrated to inhibit the endoperoxide isomerase that forms PGEs (Wlodawer, Samuelsson, Albonico, & Corey, 1971). The manipulation of the cyclic endoperoxide metabolism by pharmacological agents will become a major therapeutic field.

d. Naturally occurring pharmacologically active lipids related to cyclo-oxygenase substrates.

(1) Darmstoff.

Dialysates of the isolated frog intestine cause a slow contraction of intestinal preprations (Vogt, 1949, 1959, 1953, 1955, 1957b, 1958, 1959); the dialysates

were named "Darmstoff" by Vogt who spent the next 15 years pharmacologically characterizing and chemically identifying them. In summary, the pharmacological actions of Darmstoff are stimulation of the longitudinal muscle of the dog, cat, rat, mouse, guinea-pig and rabbit intestine, uterus of the rat, guinea-pig, and rabbit, and the urinary bladder of the rat, rabbit, and frog. The effect of low doses of Darmstoff on guinea-pig but not rabbit intestine were completely antagonized by atropine and morphine, but, when the concentration of Darmstoff was increased, the blockade was surmounted in such a fashion that a hundred fold increase in the concentrations of the antagonists caused no further antagonism. Botulinum toxin either reduced, abolished or reversed the response of the rabbit ileum to Darmstoff (Ambache, 1954a); when 4-8 times the original concentration was used, there was still no response. The action of Darmstoff was potentiated by acetylcholinesterase inhibitors and was unaffected by antihistaminics, antiadrenergics, ganglionic blocking doses of hexamethonium, and cocaine. Methamphetamine antagonized the action of Darmstoff, which, in turn, itself antagonized the action of methamphetamine. Darmstoff did not stimulate chilled rabbit ileum, which still responded to acetylcholine. And, as long as 10 min. after a single dose of Darmstoff, the actions of histamine and acetylcholine were potentiated (Vogt, 1958).

Darmstoff was isolated two ways: either by diffusion from an isolated intestinal preparation or by

extraction of horse intestine with boiling 0.2 N NaOH. In 1957, Vogt published that the Darmstoff that had been extracted from horse intestine was a mixture of acidic phosphatides, and it was assumed that the structure of the Darmstoff from intestinal dialysates and perfusates was the same. Almost a decade later, Suzuki and Vogt further characterized frog Darmstoff as containing a mixture of prostaglandins and hydroxy fatty acids (Suzuki & Vogt, 1965). A minor biologically active constituent that was not further investigated may have been the unsaturated fatty acids which serve as the substrates for the cyclo-oxygenase.

(2) Brain lipids.

Aqueous, acetone (Ambache, Reynolds, & Whiting, 1963), and chloroform-methanol (Kirschner & Vogt, 1961; Toh, 1963) extracts of brain contain smooth muscle stimulating lipids. The extracts could be separated into different fractions; one of which stimulated the rabbit intestine in the presence of atropine and LSD or of hexamethonium but was blocked by dibucaine and stimulated guinea-pig ileum only in the absence of atropine, LSD, and mepyramine (pyrilamine). Another fraction stimulated the rabbit intestine in the presence of dibucaine or hexamethonium and the guinea-pig ileum in the presence of atropine, LSD and mepyramine (Toh, 1963). Since the biological activity of these lipids was blocked by substances that destroy double bonds, such as $KMnO_4$, I_2, or IBr, the presence of cyclo-oxygenase substrates in these extracts may reasonably be suspected. The presence

of other biologically active lipids is also assured since the inactivation of hydroxyl groups also decreased their activity (Ambache et al, 1963). These lipids will be further discussed in Section I.B.4.c.(6).

(3) Slow reacting substance-C (SRS-C).

The interaction of venom phospholipases with isolated tissues has been previously discussed in Section I.B.3. Feldberg and Kellaway (1938) initially studied the actions of cobra venom on isolated preparations and on lecithin, and, indeed, the designation SRS-C stands for "cobra venom". The biological activity of the SRS-C was distinguished from the histamine, which is liberated from isolated lungs perfused with venom, by the slowness of its onset and the marked tachyphylaxis to its action (Feldberg & Kellaway, 1938; Feldberg, Holden, & Kellaway, 1938). The actions of a large variety of snake and bee venoms, containing phospholipase activity on egg lecithin, have been studied and all release SRS-C (summarized by Vargaftig, 1974). Crude extracts of the products of incubation of PLA_2 with lecithin contain equimolar amounts of lysolecithin (see Section I.B.3.c.(1).). The fatty acids, which were released, produced a slow contraction of the guinea-pig ileum and sensitized the preparation to subsequent doses of histamine (Vogt, 1957a). The actions of SRS-C on the rabbit intestine and the rat stomach strip were blocked by NSAI agents (Vargaftig & Dao, 1970; Ferreira & Vargaftig, 1974). The rat colon, which has very little prostaglandin synthetase activity, was not affected by SRS-C that had not been previously incubated with preparations containing cyclo-

oxygenase or lipoxygenase (Vargaftig, 1974; Ferreira & Vargaftig, 1974).

SRS-C activity on isolated tissues depends upon its conversion to more active molecules, and this depends on the presence of endogenous cyclo-oxygenase and/or lipo-oxygenase activity in the isolated tissue (Vargaftig, 1974). The actions of SRS-C on platelets are those that would be expected of a mixture of unsaturated fatty acids containing 5% arachidonic acid (personal communication of Bruneau cited in Vargaftig, 1974).

(4) Slow reacting substance-anaphylaxis (SRS-A).

In 1960, Brocklehurst introduced the term "slow reacting substance of anaphylaxis" to describe the characteristic pharmacological activity on smooth muscle preparations of an immunologically generated material (Brocklehurst, 1960). First reported by Kellaway and Trethewie (1940), SRS-A is only one of a number of agents released by tissues upon immunologic challenge (for reviews see Austen, Wasserman, & Goetzl, 1976; Orange & Austen, 1969; Piper, 1977; Stechschulte, 1974). SRS-A produces a slow and prolonged contraction of the guinea-pig terminal ileum, rabbit intestine, chick rectum, and human and guinea-pig bronchiole smooth muscle preparations; it is inactive on the gerbil colon and the estrous rat uterus (Stechschulte, 1974).

SRS-A is a water soluble, lipid-like substance with a molecular weight of about 300. Acid incubation

and aryl sulfatase B (E.C.3.1.6.1.) inactivate it, and it is a competitive inhibitor of the aryl sulfatase B. (Austen, Wasserman, & Goetzl). Recently, it has been suggested that SRS-A is a family of compounds, only one of whose activity is destroyed by aryl sulfatase B (Takahashi, Webster, & Newball, 1976). It has previously been suggested that SRS-A was a mixture of gangliosides (Smith, 1962, 1966). Tritiated arachidonic acid was incorporated into SRS-A, and the production of SRS-A was inhibited by NSAI, by 9,11-azoprosta-5,13-dienoic acid (an agent that inhibits thromboxane synthase), and by hydrocortisone, in a manner that could be partly overridden by addition of either 20:4w6 or, to a lesser extent, 20:3w6 (Bach, Brashler, & Gorman, 1977).

The actions of SRS-A are antagonized by a series of chromone-2-carboxylic acids (Augstein, Farmer, Lee, Sheard, & Tattersall, 1973; Appleton, Bantick, Chamberlain, Hardern, Lee, & Pratt, 1977), but the slope of the Schild plot is 0.57 which indicates that the antagonism may not be purely competitive (Waud & Parker, 1971). Atropine is ineffective as an antagonist, and the standard assay for SRS-A activity is on the atropinized guinea-pig terminal ileum. The contraction of guinea-pig and human isolated trachial and bronchial smooth muscle by SRS-A is antagonized by NSAI agents (Berry & Collier, 1964; Sweatman & Collier, 1968) at high concentrations.

Although it has long been thought that SRS-A was not stored in tissues but was synthesized and released solely on stimulation (Austen, Wasserman, & Goetzl, 1976;

Piper, 1977), there are reports that it is a normal constituent of human lung (Turnbull, Jones, & Kay, 1976).

(5) G acid.

A slow contracting substance first isolated from the G.2 fraction of human plasma in 1956, G acid was originally identified as 3-octadecenoic acid (Gabr, 1956). A similar activity was liberated from egg lecithin upon incubation with human plasma (Gabr & Amin, 1967). In view of the presence in plasma of the enzymes necessary to metabolize lecithin to prostaglandins/thromboxanes or lipoperoxides, the biological activity of G acid is more likely to be due to its ability to function as a substrate for this system with the subsequent generation of biologically active compound than to its direct action on the atropinized guinea-pig ileum in the presence of antihistamine (Gabr, 1964).

(6) Muscle-stimulating fatty acid isolated from placenta.

Acetone extracts of human placenta produced a slow contraction of the rabbit and guinea-pig intestine and uterus that was not blocked by atropine, or pentamethonium but was antagonized by papaverine (Ichikawa, 1960). Chemical identification revealed 3 fractions with the bulk of the biological activity associated with a tetraenoic acid with an neutralization number of 180.3; less active fractions appeared to be hexaenoic and dienoic acids. The biologically most active fraction was tentatively identified as arachidonic acid (Ichikawa, 1960), and further studies found that both the

guinea-pig and rabbit intestine responded to concentrations as low as 1 x 10^{-8} M while the rat intestine was two orders of magnitude less sensitive (Ichikawa & Yamada, 1962). Both free and albumin bound arachidonic acid exhibited the same smooth muscle stimulatory activity, and both caused a precipitous fall in the rabbit but not the dog blood pressure (Ichikawa & Yamada, 1962). The sensitivity of the smooth muscle preparations suggests that the fatty acids may have been converted to their peroxides; however, though the partially purified mixture was described as "brown", the purified fatty acids were described as "clear transparent" and ultraviolet measurements were made which should have revealed the presence of any peroxides (Ichikawa, 1960).

e. Naturally occurring pharmacologically active lipids related to cyclo-oxygenase products.

(1) Rabbit aorta contracting substance (RCS).

Isolated guinea-pig lungs from animals sensitized to ovalbumin release histamine, SRS-A, prostaglandins, and a substance (RCS) that contracts the isolated male rabbit aorta upon challenge with ovalbumin in the perfusion fluid (Piper & Vane, 1969). The pharmacology of RCS has been extensively investigated; its formation but not its action is blocked by both steroid and non-steroidal anti-inflammatory (NSAI) agents (Piper & Vane, 1969), it is unstable in aqueous solution with a half-life of under 2 min. (Palmer, Piper, & Vane, 1973), and the cyclo-oxygenase substrates, 20:3w6 and 20:4w6, stimulate its release as long as

they are maintained in the perfusion fluid (Vargaftig & Dao, 1971; Palmer, Piper, & Vane, 1973).

The chemical identification of RCS followed the suggestion that it was a prostaglandin endoperoxide (Gryglewski & Vane, 1972a & b). Once the isolation and structural analysis of the cyclic endoperoxides, PGG_2 and PGH_2 had been completed (Hamberg, Svensson, Wakabayashi, & Samuelsson, 1974), they were characterized as having RCS activity which decayed with a half-life of about 5 min. This pharmacological characterization was followed by the isolation and identification of TXA_2 as an unstable (decaying with a half-life of 0.5 min.) product of the cyclic endoperoxide. It was then shown that TXA_2 was the major biologically active substance in RCS with the cyclic endoperoxides themselves representing the rest of the biological activity (Hamberg, Svensson, & Samuelsson, 1975, Sevensson, Hamberg, & Samuelsson, 1975).

(2) Labile aggregation-stimulating substance (LASS).

Incubation of 20:4w6 with sheep vesicular microsomes generated a labile aggregation-stimulating substance whose formation was inhibited by aspirin (Willis & Kuhn, 1973). The aggregating substance reaches a maximum within a minute of the start of the incubation and then decays with what appears to be a half-life of about a minute (see fig. 2 of Willis, 1974). The LASS appears to be a precursor to RCS activity, which, in turn, decays with a half-life of less than 5 minutes. Though Willis suggests that

LASS has properties that cannot be distinguished from the cyclic endoperoxides (Willis, 1974), it seems likely that a major portion of the LASS activity is due to the presence of TXA_2 (Samuelsson, Hambert, Malmsten, & Svensson, 1976).

(3) Irin.

In the middle 1950s, Ambache described a substance obtained from the iris that was soluble in ether or acetone and that stimulated atropinzed preparations of the rat and hamster colon, the guinea-pig and rabbit ileum, and the guinea-pig and rat uterus (Ambache, 1957, 1959, 1963). The biological activity of these iris extracts has been identified as due to the presence of PGE_2 and $PGF_{2\alpha}$ (Anggard & Samuellson, 1964; Waitzman, Bailey, & Kirby, 1967; Eakins, Whitelocke, Perkins, Bennett, & Unger, 1972a; Eakins, 1976).

(4) Medullin.

Low molecular weight lipids with antihypertensive properties have been isolated from the renal medulla, have been named "medullin" and have been identified as a mixture of prostaglandins, PGE_2, $PGF_{2\alpha}$, and PGA_2 (Daniels, Himman, Leach, & Muirhead, 1967; Lee, Crowshaw, Takman, Attrep, & Gougoutas, 1967). However, in view of the properties of PGI_2 on the kidney, the possibility that PGI_2 may play a major role in the functions of the kidney must be considered (Bolger, Eisner, Ramwell, Slotkoff, & Corey, 1978).

(5) Darmstoff.

As previously mentioned in Section I.B.4.d.(1)., Darmstoff was originally described as a smooth muscle

stimulating principle which diffused from isolated intestinal preparations. Although the biological activity of the extracts of intestine was attributed to the presence of acidic phosphatides and fatty acids (Vogt, 1975b), the biological activity of the diffusates has been attributed to prostaglandins; PGE_1 and $PGF_{1\alpha}$ in the case of the isolated frog intestine (Suzuki & Vogt, 1965; Vogt, Suzuki, & Babilli, 1966) and PGE_2 and $PGF_{2\alpha}$ in the case of the rabbit intestine (Ferreira, Herman, & Vane, 1972, 1976). Other isolated smooth muscle preparations, such as the rat stomach (Bennett, Freidmann, & Vane, 1967; Coceani, Pace-Asciak, Volta, & Wolfe, 1967) and uterus (Vane & Williams, 1973), have been demonstrated to release prostaglandins into the bathing solution.

The release of prostaglandins by isolated smooth muscle preparation can be pharmacologically manipulated. Paralyzing doses of cocaine cause an initial increase in PG release and then a sustained decrease (Vogt & Distelkötter, 1967) from the isolated frog intestine. NSAI agents and anoxia decreased the release from the isolated rabbit intestine; however, no change in prostaglandin release was demonstrated on contraction with acetylcholine, physostigmine, or arachidonic acid or on relaxation with epinephrine, hyoscine, papaverine, dinitrophenol, or calcium-free Krebs solution (Ferreira, Herman, & Vane, 1976). The response to 20:4w6 was described as "quite instantaneous" (Ferreira, Herman, & Vane, 1976) and very likely may have been due to the peroxides rather than the free acid, as the response of

the rabbit intestine to 20:4w6 is a slow increase in the pendular motion and tone following a lag that is inversely related to the dose used (e.g., this dissertation, Section II.B.1. and Vargaftig & Dao, 1970). The lack of response to cholinergic agents is at variance with the report of increased release of prostaglandins from the perfused frog intestine by acetylcholine or dimethyl-phenyl-piperazinium (Bartels, Kunze, Vogt, & Willis, 1970).

(6) Brain lipids.

The occurrence of prostaglandins in brain was first unequivocally demonstrated by Samuelsson, who, in 1964, identified $PGF_{2\alpha}$ in bovine brain extracts by gas chromotographic/mass spectrometric means (Samuelsson, 1964). Other prostaglandins, such as PGE_1, PGE_2, and $PGF_{1\alpha}$, have also been identified in some species. But the finding that 20:3w6 is only present in minute quantities or is undetectable in rat and mouse brain lipids (Lunt & Rowe, 1968; Bazan, 1970; Baker & Thompson, 1972) coupled with finding that 6-keto $PGF_{1\alpha}$, the breakdown product of PGI_2, cochromatographs with PGE_2 in several commonly used solvent systems (Cottee, Flower, Moncada, Salmon, & Vane, 1977) suggests that the identification of the various prostaglandins in brain be viewed with caution (for an extensive review, see Wolfe, 1975).

(7) Slow reacting substance-C.

The composition of SRS-C depends upon the manner in which it is isolated; incubation of purified lecithin with PLA_2 generates a mixture of fatty acids

(including 5% 20:4w6) and lysolecithin (Vargaftig, 1974), while perfusion of PLA_2 through isolated guinea-pig lungs generates prostaglandins and lipid peroxides (Babilli & Vogt, 1965; Vogt, Suzuki, & Babilli, 1966; Vogt, Meyer, Kunze, Lufft, & Babilli, 1969), which probably include RCS (Vargaftic & Dao, 1971).

(8) Menstrual fluid stimulants.

Lipid soluble smooth muscle stimulants were first reported to be present in menstrual fluid in 1957 (Pickles, 1957). The stimulants, which were extracted with acetone from menstrual fluid or dressings, were separated into three fractions (Clithroe & Pickles, 1961), two of which were subsequently identified as PGE_2 and $PGF_{2\alpha}$ (Eglington, Raphael, Smith, Hall, & Pickles, 1963). The role of the balance of PGE_2 to $PGF_{2\alpha}$ in the production of dysmenorrhea (Pickles, Hall, Best, & Smith, 1965; Hall, 1966) needs to be re-examined in light of recent findings that PGI_2 and 6-keto-$PGF_{1\alpha}$ stimulate the rat uterus with potencies 1/25th that of $PGF_{2\alpha}$ and 1/2-1/4 that of PGE_2 (Omini, Moncada, & Vane, 1977) and that, as mentioned in Section I.B.4.e.(6) above, the breakdown product of PGI_2, 6-keto-$PGF_{1\alpha}$, co-chromatographs with PGE_2 in several commonly used solvent systems (Cottee, Flower, Moncada, Salmon, & Vane, 1977).

(9) Lipid soluble substance from nasal mucosa (N* acid).

Extraction of sheep or dog nasal mucosa with acid, phosphate buffer, or acetone resulted in a lipid

soluble acid that contracted the atropinized tortoise jejunum and the atropinized rat colon in the presence of Brom-LSD (Toh & Mohiuddin, 1958). Further characterization revealed stimulation of the atropinized hamster colon but no effect at high concentrations on the guinea-pig ileum in the presence of atropine and mepyramine (Ambach, Reynolds, & Toh, 1958 unpublished but cited in Ambache, 1963). These further studies also served to distinguish the activity of N*from irin. However, the finding that human nasal polyps synthesize prostaglandins (bioassayed as PGE_2 equivalents; Szczeklik, Gryglewski, Olszewski, Dembinska-Kiec, & Czerniawska-Mysik, 1977) suggests that the activity of N* is probably due to prostaglandins.

(10) Intestinal stimulant acidic lipids.

Dialysates of rabbit and puppy intestine contained lipid soluble acids that stimulated the isolated rabbit or puppy intestine in the presence of atropine (Gray, 1958, 1962, 1964). Though no attempt was made to characterize the acidic lipids chemically, the pharmacological characteristics suggest that the biological activity is due to the presence of prostaglandin-like substances.

(11) Unidentified smooth-muscle-contracting agent (UVS).

Ethyl acetate extracts of subcutaneous perfusates of ultraviolet irradiated or allergic contact eczematic skin contain a fatty acid with pharmacological properties identical to those of PGE_1 on the isolated rat uterus. (Sondergaard & Graves, 1970).

5. Pharmacologically active lipids related to lipoxygenases.

a. General biochemistry of lipoxygenases.

The vast majority of studies of lipoxygenases have been conducted on enzymes of plant origin (Hamberg, Samuelsson, Bjorkhem, & Danielsson, 1974). The fatty acid cyclo-oxygenase, whose actions were discussed in Section I.A.4., functions as a lipoxygenase during the formation of the 11-hydroperoxy and 11-hydroxy derivatives of 20:3w6 and 20:4w6 (Hamberg & Samuelsson, 1967a & b). A lipoxygenase present in blood platelets that catalyzes the formation of w9 (i.e., n-8 hydroperoxy-fatty acids has been described (Hamberg & Samuelsson, 1974b) and characterized (Nugteren, 1975). It is apparent that a substantial proportion of 20:4w6 added to platelets is metabolized by the lipoxygenase (Hamberg & Samuelsson, 1974b), and that the lipoxygenase pathway is, therefore, a major metabolic route for the fatty acid products of PLA_2's action (Section II.A.3.). The actions of plant and platelet lipoxygenase on 20:4w6 and 20:3w6 are shown in fig. 6 and fig. 7.

Soybean lipoxygenase (E.C. 1.13.1.13) has been isolated and purified to the extent that it appears to be a dimer with subunits weighing 54,000 daltons (Stevens, Brown, & Smith, 1970). The enzyme catalyzes the insertion of molecular oxygen at the w6 and/or w10 position with the major product at w6. Other requirements are for a pair of methylene interrupted double bonds located between w6 and w10 (Hamberg & Samuelsson, 1967a), although a substantial activity occurs with the w5, w2 substrate,

Fig. 6. Products of the action of plant, platelet, and polymorphonuclear (PMN) leukocyte lipoxygenase on arachidonic acid (20:4w6).

The reactions catalyzed by the lipoxygenases of plants, platelets, and PMN leukocytes have different specificities which are discussed in Section I.B.5.a.

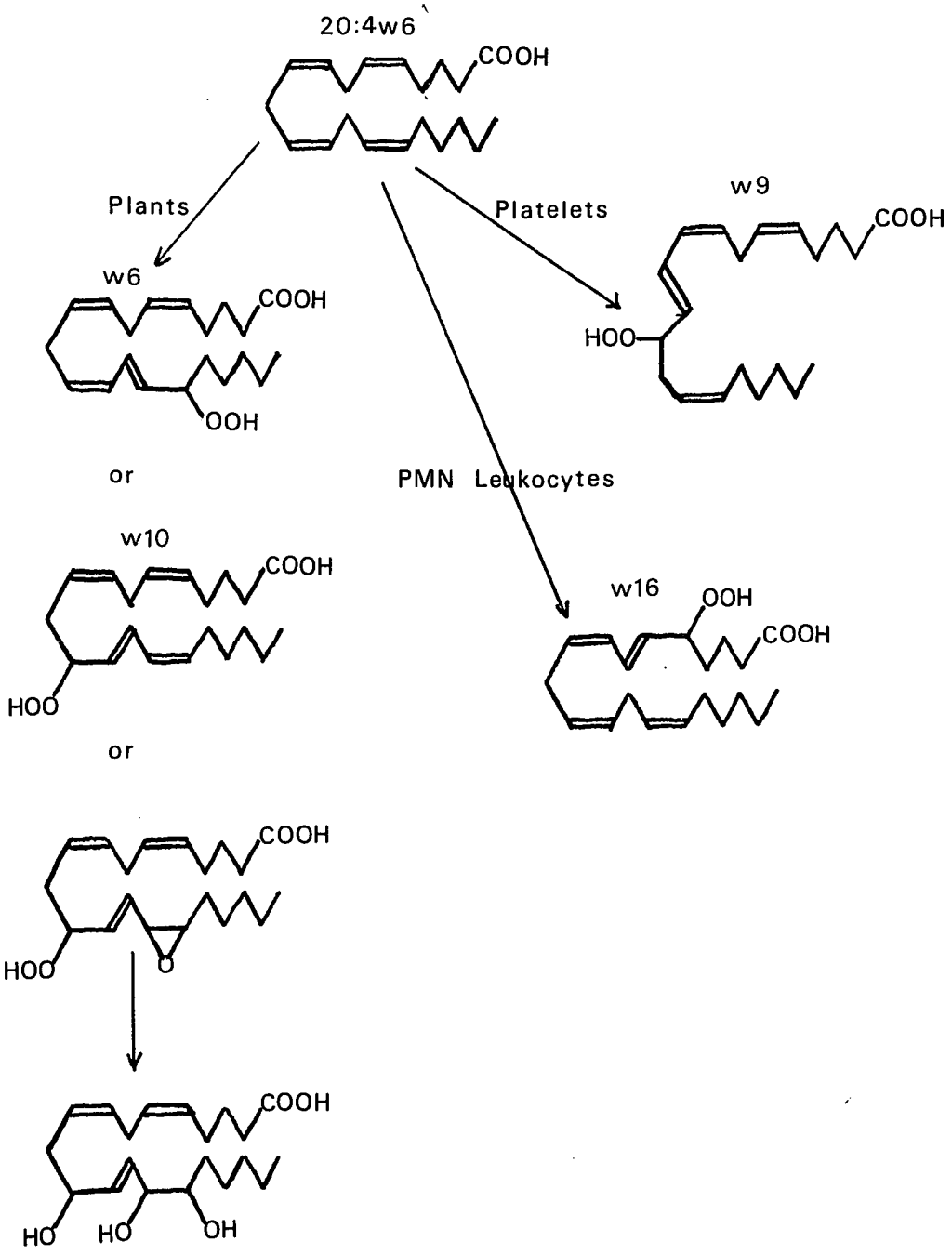
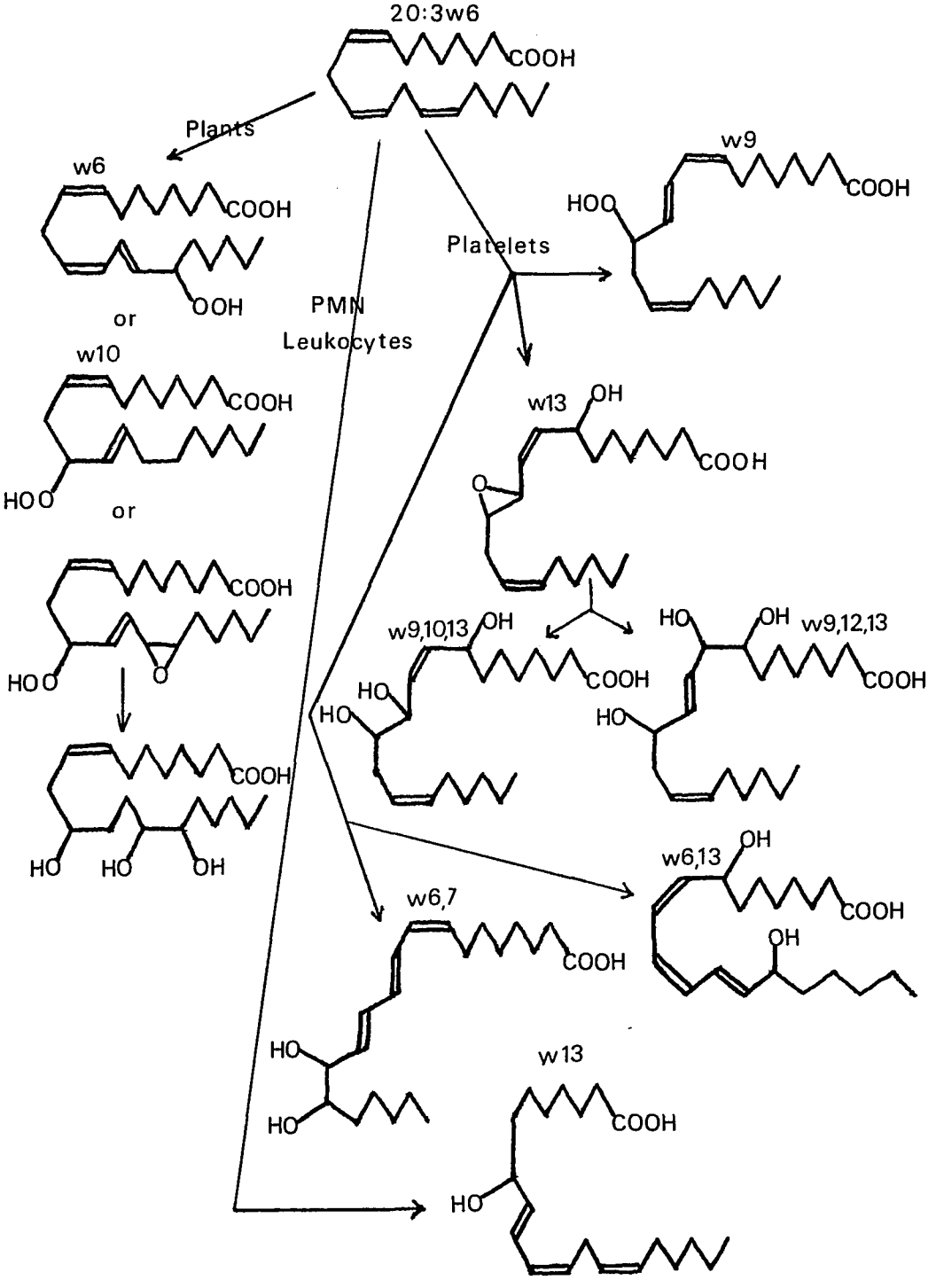

20:4w6
COOH
Plants
Platelets
w9
COOH
HOO
w6
COOH
OOH
or
PMN Leukocytes
w10
COOH
HOO
w16
OOH
COOH
or
COOH
HOO
O
COOH
HO
HO
OH

Fig. 7. Products of the action of plant, platelet, and polymorphonuclear (PMN) leukocyte lipoxygenases on 8,11,14-eicosatrienoic acid (20:3w6).

The reactions catalyzed by the lipoxygenases of plant, platelets, and PMN leukocytes which are discussed in Section II.A.5.a.


20:3w6
COOH
Plants
w6
COOH
OOH
or
w10
COOH
HOO
or
COOH
HOO
O
COOH
HO
HO
OH
PMN
Leukocytes
Platelets
w9
HOO
COOH
w13
OH
COOH
O
w9,10,13
OH
HO
HO
COOH
HO
OH
w9,12,13
COOH
HO
w6,13
OH
COOH
OH
w6,7
COOH
HO
HO
w13
COOH
HO

13,16-octadecdienoic acid (Holman, Egwim, & Christie, 1969).

The platelet lipoxygenase inserts molecular oxygen (Hamberg & Samuelsson, unpublished, cited in Hamberg & Samuelsson, 1974b) into the pro-L 12-position of 20:4w6, followed by 20:3w9, 81%; 20:3w6 and 20:5w3, 60%; 20:2w9, 26%; 22:6w3, and 18:3w6, 14%; and 20:2w6, 18:2w6, and 18:3w3, all less than 5%. This suggests that the primary requirement is for two methylene interrupted double bonds at the w9 and w12 positions (Nugteren, 1975). It appears that the platelet lipoxygenase, like the cyclo-oxygenase (Lands & Samuelsson, 1968), requires a free carboxyl function, as the methyl ester of 20:4w6 was not a substrate (Nugteren, 1975).

Platelet lipoxygenase can also catalyze the formation of 8,11,12-trihydroxy-9,14-eicosadienoic acid, 8,9,12-trihydroxy-10,14-eicosadienoic acid, 8,15-dihydroxy-9,11,13-eicosatrienoic acid, and 14,15-dihydroxy=8,10,12-eicosatrienoic acid from 20:3w6 in addition to 12-hydroxy-8,10,14-eicosatrienoic acid (Falardeau, Hamberg, & Samuelsson, 1976). These fatty acids are of interest in light of the findings that 20:3w6 does not stimulate platelet aggregation but in fact inhibits it (Silver, Smith, Ingerman, & Kocsis, 1973; Willis, Comai, Kuhn, & Paulsrud, 1974).

Rabbit polymorphonuclear leukocytes contain lipoxygenase activity that forms 5-D-hydroxy-6,8,11,14-eicosatetraenoic acid from 20:4w6 and 8-D-hydroxy-9,11,14-eicosatrienoic acid from 20:3w6; whether the lipoxygenase activity is due to one enzyme with a higher specificity for the

20:4w6 structure or to two separate enzymes with different activities is unknown (Borgeat, Hamberg, & Samuelsson, 1976).

The lipoxygenases from soybeans and platelets are inhibited by 5,8,11,14-eicosatetraynoic acid (Ahern & Downing, 1970; Hamberg & Samuelsson, 1974b; Hammarström, Hamberg, Samuelsson, Duell, Stawiski, & Voorhees, 1975). For a series of linoleic acid (18:2w6) analogs, only 9,12-cotadecadiynoic acid significantly inhibited soybean lipoxygenase. Recently, Hammarström has shown that 5,8,11-eicosatriynoic acid inhibits platelet lipoxygenase (Hammarström, 1977). Interestingly, the rabbit PMN leukocyte lipoxygenase activity is not inhibited by 5,8,11,14-eicosatetraynoic acid (Borgeat, Hamberg, & Samuelsson, 1976). The lipoxygenases are not inhibited by NSAI drugs (Hamberg & Samuelsson, 1974b; Falardeau et al., 1976; Borgeat et al., 1976) such as aspirin or indomethacin.

Antioxidants, such as α-naphthol, DL-α-tocopherol, or 2,6-di-tert-butyl-4-hydroxytoluene (BHT) are potent inhibitors of soybean lipoxygenase (Vanderhoek & Lands, 1973b; Panganamala, Milier, Gwebu, Sharma, & Cornwell, 1977). The actions of antioxidants on the other lipoxygenases have not yet been examined.

Soybean lipoxygenase is activated by a lipid peroxide (Smith & Lands, 1972b), and the stimulation of lipid peroxide production by the formation of free radicals (Tappel, 1973) may explain the stimulation of platelet aggregation by ultraviolet light (Dickson, Doery, & Lewis, 1971). Arguing against such an interpretation is the finding that

incubates of 20:4w6 with soybean lipoxygenase failed to stimulate platelet aggregation (Vargaftig & Zirinis, 1973).

b. Pharmacologically active lipids related to lipoxygenase substrates.

As mentioned in the previous section, the specificity of the lipoxygenases is less rigid than that of the cyclo-oxygenase. Therefore, any of the unsaturated fatty acids that come in contact with a tissue lipoxygenase are likely to be converted to their respective hydroperoxides. Nugteren (1975) found that the platelets contained most of the tissue lipoxygenase. However, exogenously added substrates such as linoleic or stearic acids appear not to gain ready access to the platelet lipoxygenase, as incubation of linoleic or linolenic acid with platelet rich plasma does not generate RCS activity (Vargaftig & Zirinis, 1973). The pharmacology of hydroperoxides of the various fatty acids was discussed in relation to their action as cyclo-oxygenase substrates in Section I.B.4.b.

c. Pharmacologically active lipids related to lipoxygenase products.

Ambache divided the receptors for fatty acids into those receptors which responded to unsaturated fatty acids without hydroxyl groups such as are found in the guinea-pig and rabbit ileum, and those receptors that respond to unsaturated hydroxy-fatty acids, such as are found in the hamster and rat colon (Ambache, 1963). While this heuristic device has been superceded by the concept of tissues with high

and low levels of "prostaglandin synthetase" activity, the specificity of the different isolated tissues in the perfusion cascade allows for the determination of the 'pharmacological fingerprint' which has been the basis for unravelling the prostaglandin/thromboxane/lipoperoxide system (Vane, 1964).

Fatty acids incubated with lipoxygenases develop rabbit aorta contracting activity; e.g., 20:4w6 incubated with soybean lipoxygenase contracted the rabbit aorta, rat stomach strip and rat colon without inducing bronchoconstriction in the guinea-pig or aggregation of human or rabbit platelet rich plasma (Vargaftig & Zirinis, 1973). Soybean lipoxygenase, superfused over the isolated rabbit aorta and rat stomach strip, "had little or no effect", but, when it was infused into superfusion medium already containing 20:4w6, contractions of both tissues occurred. The RCS activity formed by the action of lipoxygenase on 20:4w6 did not disappear within 3 min., while RCS activity generated by the cyclo-oxygenase did. In addition, there was no increase in the amount of prostaglandin-like activity following the decay of lipoxygenase formed RCS activity (Gryglewski & Vane, 1972b).

Superfusion of the rabbit aorta, rat stomach strip and rat colon with soybean lipoxygenase caused no stimulation, but when 18:2w6, 18:3w3, 20:3w6, 20:4w6, or alcoholic extracts of SRS-C were infused along with lipoxygenase and allowed to incubate for 2 min. in a coil of tubing before cascading over the tissues, RCS activity was generated. Only 20:4w6 had any appreciable effect on the assay

tissues when superfused without the lipoxygenase, and, in combination with the lipoxygenase, the effect of 20:4w6 was always greater than when it was superfused by itself (Ferreira & Vargaftig, 1974).

15-Hydroperoxy arachidonic acid (15-hydroperoxy-5,8,11,13-eicosatetraenoic acid: 15:HPAA) can be quantitatively synthesized by incubation of 20:4w6 with soybean lipoxygenase (Funk, Isaac, & Porter, 1976). The actions of 15-HPAA on prostacyclin synthetase were discussed in Section II.A.c.(2).(c).((1)). In addition, incubation of 18:3w6 with the same preparation of soybean lipoxygenase yielded both the 9- and 13-hydroperoxy-acids (w10 and w6). The reaction conditions are critical. Using conditions reported in the literature and commercially prepared lipoxygenase, Funk et al., 1976 were able to prepare hydroperoxides of 18:3w6 with a yield of no more than 5%. These authors also found that extended incubation led to the formation of non-peroxide polar by products. This last finding may be the explanation of the discrepancy between the previously mentioned failure of incubates of soybean lipoxygenase and 20:4w6 to aggregate platelets (Vargaftig & Zirinis, 1973) and the recent reports of lipid peroxide stimulation of platelet guanylate cyclase activity (Hidaka & Asano, 1977). These authors found that unsaturated fatty acids stimulated guanylate cyclase activity in proportion to the amount of peroxides formed. While the relationship between increased cGMP levels and platelet aggregation is still uncertain (Davies, Davidson, McClenaghan, Say &

Haslam, 1976), the role of increased levels of cAMP in inhibiting platelet aggregation is well established (Salzman, 1972; Vincent, Zijlstra, & Bonta, 1976. It may be that different fatty acid peroxides have different effects upon the purine nucleotide cyclases. As an aside, in essential fatty acid deficient rats, the amount of 20:3w9 present in erythrocyte total fat increases by two orders of magnitude (Bult & Bonta, 1976). If the same effect occurs in platelet phospholipids, then there would be a selective enrichment of the second best substrate (after 20:4w6) for the platelet lipoxygenase (Nugteren, 1975). Perhaps the aggregation of platelets from essential fatty acid deficient rats involves the production of w9 lipid peroxides as opposed to the normal reaction to w6 and/or w10 lipid peroxides.

The end product of the action of platelet lipoxygenase on 20:4w6 (HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid) has been demonstrated to be chemotactic for neutrophils (Turner, Tainer, & Lynn, 1975) and eosinophils without effecting mononuclear leukocytes (Goetzl, Woods, & Gorman, 1977). At minimally chemotactic concentrations, HETE also enhanced the random migration of human polymorphonuclear leukocytes (Goetzl, Woods, & Gorman, 1977). The actions of the hydroperoxide (HPETE) were not studied, but it seems likely that it also may have actions on the reticuloendothelial system. Unlike 15-HPAA, 12-hydroperoxy arachidonic acid did not inhibit PGI_2 formation at concentrations up to 3×10^{-4} M (Moncada, Gryglewski, Bunting, & Vane, 1976b).

The actions of the multihydroxy fatty acids, whose synthesis was discussed in Section I.B.5.a., have not been studied on isolated smooth muscle preparations.

6. Pharmacologically active lipids of unknown relation to the cyclo-oxygenase/lipoxygenase system.

a. Short chain fatty acids.

In a series of short chain fatty acids at concentrations of 1×10^{-3} M, acetic, proprionic, lactic, and butyric acids all stimulated the isolated rat colon; the stomach was depressed by proprionic and butyric acids, which, on the other hand, stimulated the rectum; the duodenum was stimulated by both lactic and acetic acid; and the jejunum and ileum were stimulated only by lactic acid (Yokokura, Yajima, & Hashimoto, 1977). Acetate esters including aspirin, aspirin methyl ester, and resorcinol monoacetate contracted the guinea-pig ileum in a manner that was blocked by atropine and cholinesterase inhibitors, potentiated by choline, and not affected by morphine. Hemicholinium slightly reduced the responses (Moritoki & Ishida, 1977). In view of the actions of aspirin (Vane, 1971), interpretation of these results is complex, though hydrolysis of the acetate ester followed by incorporation into newly synthesized acetylcholine would seem to be a possibility. On the other hand, fatty acids have been shown to inhibit the acetylcholine receptor at low concentrations (Brisson, Devaux, & Changeaux, 1975).

b. Long chain fatty acids.

Palmic acid has been shown to be arrhythmogenic

in the isolated perfused guinea-pig heart (Wasilewska-Dziubinska, 1975). Since prostaglandins and their precursors are antiarrhythmic (Mest, Blass, & Forster, 1977), it is possible that the long chain fatty acids exert their actions by inhibition of myocardial adenine nucleotide translocase, the enzyme responsible for the transfer of energy out of mitochondria (for a review, see Shrago, 1978). Alternatively, the action could be by inhibition of myocardial Na^+K^+-ATPase in analogy to the inhibition of brain Na^+K^+-ATPase by fatty acids (Dahl, 1968).

c. Hydroxy-fatty acids.

Ambache's classification of fatty acid receptors had a category for unsaturated hydroxy-fatty acids (Ambache, 1963). The rat and hamster colons are the prototypical preparations containing this type of receptor and have the advantage of being relatively insensitive to hydroperoxides (Ambache, 1966). Ricinoleic acid, 12-hydroxy-cis-9-octadecenoic acid, is the protypical agonist of this series and stimulates the hamster colon at a concentration of 3×10^{-6} M, as does its trans isomer ricinelaidic acid and the triple-bonded analog, ricinstearolic acid (Ambache, 1959). The saturated analog, 12-hydroxy-octadecanoic acid, was active at a concentration of 2×10^{-5} M. The actions of ricinoleic acid on other intestinal preparations are inhibitory, producing a concentration related depression (starting at 1×10^{-5} M) of the coaxially stimulated guinea-pig ileum, the rabbit jejunum, and the guinea-pig taenia coli and rat

colon which had been depolarized with 9 x 10^{-2} M K^+ (Stewart, Gaginella, & Bass, 1975; Stewart, 1975). Other fatty acids, such as oleic, elaidic, linoleic, 12-hydroxystearic 10(9)-hydroxystearic, or ricinelaidic, and methyl ricinoleate did not produce the same action at matching concentrations, but the effect was matched by surface active agents, such as bile acids, sodium dodecyl sulfate, dioctyl sodium sulfosuccinate, and polysorbate 80, at similar concentrations (Gaginella, Stewart, Gullison, Olsen, & Bass, 1975; Stewart, 1975). It is therefore likely that the depressant action is due to membrane stabilizing effects (Mead & Fulco, 1976, pp. 122-226).

The stimulatory action of ricinoleic acid was originally suggested to be due to its structural similarity to prostaglandins (Ambache, 1966). An alternative might be an action by modulating the PLA_2/cylco-oxygenase/lipoxygenase system; specifically, by shifting the metabolic flow of the precursor fatty acids.

d. Platelet activating factor (PAF).

A lipid soluble factor that stimulated the aggregation of platelets has been isolated from rabbit and human mixed leukocytes (Benveniste, 1974). PAF has a molecular weight of 300-500 daltons, copurifies with SRS-A, and is activated by phospholipase D, but not by PLA or PLB, arylsulfatase B, pronase, or subtilisn (Kater, Goetzl, & Austen, 1976). As with SRS-A, PAF may be a family of compounds since PAF from lung does not crossdesensitize with PAF from basophils (Kravis & Henson, 1977). Although PAF

stimulates the release of cyclo-oxygenase products, its action is not blocked by NSAI drugs, aspirin and indomethacin (Shaw, Printz, Stewart, & Henson, 1977).

e. Spontaneous lipid peroxides.

The actions of auto-oxidized fatty acids on smooth muscle preparations were discussed in Sections I.B.4.b. and 5.b. & c., An extensive discussion of the biochemical and physiological aspects of autoxidation can be found in Mead and Fulco (1976, pp. 153-174).

7. Summary.

a. Interrelationships among the pharmacologically active lipids.

The actions of PALs appear to be due mainly to their involvement in the metabolism of essential fatty acids. Other PALs, not directly involved in the phospholipase/cyclo-oxygenase/lipoxygenase system, have a variety of actions, perhaps the most important of which are on the transport of energy out of the mitochondria (Shrago, 1978) and on the structure and function of biological membranes (Mead & Fulco, 1976, pp. 122-139). As can be seen from a casual observation of any of the maps of cellular metabolism (e.g., Biochemical Pathways by Michal, 1974), the processes involved in lipid metabolism are extensively interdigitated.

As an example, the release of SRS-A is subject to extensive modulation by positive and negative feedback. SRS-A stimulates the production of the cyclic endoperoxides which are further metabolized to prostaglandins and thromox-

anes (Piper & Vane, 1969; Hamberg, Svensson, Hedqvist, Strandberg, & Samuelsson, 1976; Mathe, Strandberg, & Yen, 1977). $PGF_{2\alpha}$ and TXB_2, released by immunological challenge themselves, increased the release of SRS-A (Boot, Dawson & Osborne, 1976; Boot, Dawson, Cockerill, Mallen, & Osborne, 1977). A more complex interaction was shown by the inhibition of IgE stimulated SRS-A release by PGE_1 and PGE_2 (Koopman, Orange, & Austen, 1971) while IgG_a stimulated release of SRS-A was inhibited by PGI_2 (Burka & Garland, 1976).

NSAI drugs can potentiate the anaphylactic response by inhibiting the release of the negative feedback modulators (Miller & Robson, 1976; Walker, 1972; Engineer, Piper, & Sirois, 1976), but the effect is biphasic, since at low doses aspirin (Mielens & Rosenberg, 1976), indomethacin, ibuprofen, and flurbiprofen (Bach, Brashler, & Gorman, 1977), and 5,8,11,14-eicosatetraynoic acid (Jakschik & Parker, 1976) inhibit the action of SRS-A.

b. Pharmacological manipulation.

A theme throughout this review has been the control of the actions of PALs by the modulation of their metabolic pathways or the pathways that they impinge upon. While the dissection of these pathways has just begun, the use of pharmacological agents as probes of PAL structure and function provides a fruitful area for futher research.

C. Purpose.

The purpose of this dissertation was to determine, by a pharmacological analysis, the nature of the active material.

The strategy used in the pharmacological analysis was to combine the use of isolated tissue preparations and qualitative organic analysis. These two approaches were conducted in parallel so that information derived from each of the different experimental techniques could be employed to guide further experiments.

1. Hypotheses.

Based on the original findings reported in the literature (see Section I.A.), I decided to investigate two main hypotheses concerning the action of the active material: First, that the active material was a universal cholinergic releasing agent which did not require the presence of action potentials to produce its effect; and, second, that the effect of the active material was necessarily linked to narcotic analgesic sensitivity of the stimulus evoked release of acetylcholine.

The first hypothesis was tested by measuring the frequency and amplitude of miniature endplate potentials at the frog sciatic sartorius neuromuscular junction. The methods are described in Section II.A.1.c. and the results are described and discussed in Section II.B.3.

The second hypothesis was tested by exposing a preparation in which the stimulus evoked release of acetylcholine was known to be insensitive to narcotic analgesics, i.e., the rabbit ileum (Schaumann, 1956), to the active material. The methods employed are discussed in Section II.A.1.a. and the results, which comprise the bulk of the isolated

tissue preparation experiments, are described and discussed in Section II.B.1.

2. Additional research goals.

When the two hypotheses concerning the pharmacological mechanism of action were not supported by the results, additional pharmacological experiments were performed to attempt to elucidate the mechanism of action of the active material. Once a conceptual link had been formed to the prostaglandin and thromboxane system, the pharmacological and qualitative organic analyses proceeded in parallel in a mutually reinforcing effort. Specific research goals included the identification and quantitation of cyclo-oxygenase substrates in the active material, determination and comparison of the biological activity of the pure cyclo-oxygenase substrates with the active material, and comparison of the inhibition by nonsteroidal anti-inflammatory drugs of active material and the pure cyclo-oxygenase substrates.

II. PHARMACOLOGICAL ANALYSIS OF THE ACTIONS OF THE LIPID FRACTION ON ISOLATED TISSUE PREPARATIONS

A. Methods.

1. Isolated tissue preparations.

The definition of the active material in the lipid fraction derived from its actions on the guinea-pig ileum (Green, Carlini, & Robinson, 1963; Green & Carlini, 1964). The analysis of the actions of biological unknowns involves the use of several isolated tissue preparations to determine the pharmacological 'fingerprint' (Bennett, 1973). In this dissertation, attempts were made to test two experimental hypotheses formulated from the previously published findings: first, that the lipid fraction was a universal acetylcholine releasing agent which did not require the presence of action potentials to produce its effect; and second, that the effect of the lipid fraction was necessarily linked to narcotic analgesic sensitivity. When both experimental hypotheses were rejected based on the findings described in Section III.B., the previously published findings on the guinea-pig ileum and the responses obtained on the rabbit ileum were explored in the attempt to uncover the mechanism of action of the lipid fraction. Through the kindness of Drs. Peter Bentley and Thomas Yorio and Dr. L. Allen Barker, additional screening experiments were performed on the isolated frog skin and

rectus abdominus preparations.

a. Rabbit ileum preparation

The rabbit ileum was chosen to test the hypothesis that the effect of the lipid fraction was necessarily linked to narcotic analgesic sensitivity. The rabbit ileum has long been known to release acetylcholine in a manner that is unaffected by narcotic analgesics (Schaumann, 1957). Although rabbits and guinea-pigs both belong to the cohort Glires, the former belongs to the order Lagomorpha while the latter belongs to the order Rodentia. Since some rabbits have a serum atropine acyl-hydrolase (E.C.3.1.1.10) which has been demonstrated to be controlled by a semi-dominant gene (As) linked to the gene (E) that codes for black in the animal's coat (Glick, 1949; Glick & Glaubach, 1941; Sawin & Glick, 1943; Margolis & Feigelson, 1964), rabbits whose coats contained black pigmentation, were excluded from the study.

Male Dutch-Belted rabbits (Rudolf Vrana's Rabbit Farm) were killed by air embolism or decapitation so as to eliminate drug interactions with anaesthetics and so that tissues could be made available to other investigators.

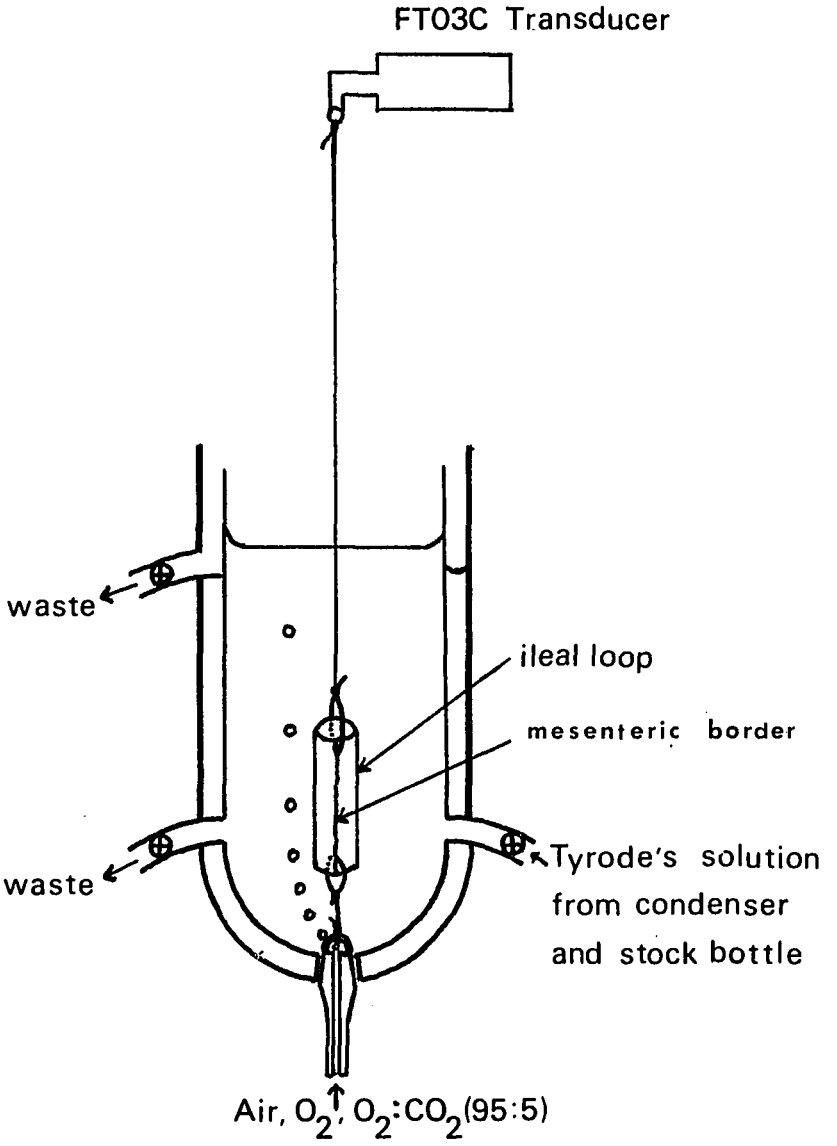
The intestines were exposed, and the peritoneal cavity was filled with warm Tyrode's solution to prevent the ileum from drying out. The ileo-caecal junction was located by the presence of the vermiform appendix at the start of the large bowel. In the rabbit, the jejunum can only be distinguished macroscopically (Richardson, 1958) from the ileum by the slightly lighter color of the former (Bensley, 1920,

p. 82, 190). It was found that reproducible results could be obtained from approximately 1.5 cm long sections of ileum cut from the intestine just orad to the termination of the mesentery which connects the ileum, the caecum, and the appendix (i.e., by following the ileum orad from the ileo-caecal junction to just beyond the tip of the appendix).

The ileal sections (subsequently referred to as "loops") were obtained by making transverse sections of the ileum approximately 0.75 cm on either side of the insertion of the bundle consisting of a mesenteric artery, vein, and nerve. If more than 1.5 to 2 cm of ileum were used, waves of contraction were sometimes visible along the ileal loop, as the waves of spontaneous depolarization and relaxation spread from one intestinal segment to the following (aborad) segment (Daniel, 1973). After placing the ileal loop in a petri dish filled with warm Tyrode's solution, the chyme was extruded, using light pressure and a rolling motion of the finger from the oral to the aboral opening of the loop.

The ileal loops were suspended (see fig. 8) in a 10 ml isolated organ bath (K.C. & N.Y. Krebs) which was maintained at 37^{o}C by means of a constant temperature bath (Precision Scientific, Lo-Temptrol 154). Skin suture silk (Deknatel, #4-0) was used to tie the loop to the gas bubbler of the organ bath and to the transducer. In order to allow the mucus which continuously formed in the lumen of the ileal loop to be extruded by the rhythmic contractile motion (pendular motion) of the rabbit ileum and so that only the

Fig. 8. Diagram of rabbit ileum "loop" suspended in an isolated tissue bath (gut bath).


FT03C Transducer
waste
waste
ileal loop
mesenteric border
Tyrode's solution
from condenser
and stock bottle
Air, O_2, O_2:CO_2(95:5)

contractions of the longitudinal muscle coat would be recorded, the sutures were threaded across the lumen of the intestinal loop from the mesenteric border to the antimesenteric border and were knotted on a bight at both the oral and aboral ends of the loop. Care was taken to ensure that the entrance and exit of the thread at the oral end of the loop were at the same sites relative to those at the aboral end. Otherwise, the loop would be twisted, and this would allow the circular muscles to contribute a force vector in the direction in which the longitudinal muscle contraction was recorded.

The suture at the oral end of the loop was attached to a force-displacement transducer (Grass, FT03C), and the tension was recorded isometrically, or as nearly isometrically as possible (Daniel, 1973). The transducer output was amplified and recorded on a linear displacement chartrecorder (Clevite Brush, Mark 200 or 250). The loop was suspended with approximately 0.5 g of tension on it. As the tissue stretched, slack was taken up by moving the transducer which was attached (as was the organ bath) to a vibration damped frame. Care was taken not to overstretch the tissue as this would prevent the formation of tone; that is, the spontaneous resting tension of the preparation (Lum, Kermani, & Heilman, 1966).

The recorder gain was adjusted to give maximum sensitivity without going off scale at the top of dose-effect curves. The recorder sensitivity was calibrated by suspending weights (0-15 g) from the transducer. The effects of

drugs on the isolated ileal preparation were recorded in arbitrary standardized chart divisions equivalent to 1.82 grams/division. Since the rabbit ileum is a spontaneously motile preparation with a regular pendular motion, the responses were taken as the increase above the baseline average peak height.

The ileal loops were bathed in Tyrode'e solution (Tyrode, 1910) prepared according to the procedure outlined in Pharmacological Experiments in Isolated Preparations, Second Ed. (Staff of the Department of Pharmacology of the University of Edinburgh, 1970, pp. 152-153). The Tyrode's solution had the following composition in mM: NaCl, 136.9; KCl, 2.7; $MgCl_2$, 1.0; $CaCl_2$, 1.8; NaH_2PO_4, 0.4; $NaHCO_3$, 11.9; and D-glucose, 5.6. According to the manual, the Tyrode's solution could be gassed with air, O_2-CO_2 (95:5), or O_2. However, gassing the Tyrode's solution with air or O_2-CO_2 (95:5) resulted in a bath pH of 7.6 at 37°C. On the other hand, gassing the Tyrode's solution with O_2 resulted in a bath pH of 8.6. Since the degree of ionization of some of the drugs tested depends on the pH, differences due to the pH of the medium, which were noted, are mentioned in Section III.B.

b. Guinea-pig ileum preparation.

The actions of the lipid fraction were originally defined on the guinea-pig ileum (Green, Carlini, & Robinson, 1963; Green & Carlini, 1964; Robinson et al, 1967). Therefore, findings on the mechanism of action of the lipid fraction, made using the rabbit ileum, were confirmed on the guinea-pig ileum.

Male albino guinea-pigs (Perfection Breeders)

were killed by stunning and decapitation. A 15 to 20 cm section of ileum was obtained, which ended approximately 5 cm orad to the terminal Peyer's patch at the ileo-caecal junction. The mesentery was completely stripped off the section of ileum. The intestinal contents were cleaned from the section by inserting the tip of a pipet filled with warm Tyrode's solution into the oral end of the section of ileum and allowing the Tyrode's solution to flow through the ileal section under a 1 to 2 cm head of pressure. A 1.5 to 2 cm loop of ileum was sectioned from the aboral portion of the section of ileum. The loop of guinea-pig ileum was suspended in the organ bath in the same manner as was the loop of rabbit ileum. However, it was found that, if 5 g of tension were placed on the loop for 1 hr prior to the adding of any drugs, when the loop was relaxed to 1 g of tension, the responses to drugs added subsequently were more reproducible. All other aspects of the guinea-pig ileum preparation were comparable to those of the rabbit ileum preparation.

c. Frog sciatic-sartorius neuromuscular junction preparation.

Intracellular recording depends upon the use of micro-electrodes, the basic techniques of which were developed by Graham and Gerard (1946) and Ling and Gerard (1949). The frog sciatic-sartorius neuromuscular preparation has been classically described by Fatt and Katz (1951). The dissection was done according to a procedure developed by Alan Fein of the Marine Biological Laboratory at Woods Hole,

Massachusetts. Male 'winter' frogs (Rana pipiens) were obtained from West Jersey Biological Supply Farm and kept at $4^{o}C$ until used. The dissected sartorius muscle and attached sciatic nerve were pinned in a tissue bath and bathed in Ringers solution. The Ringers solution used was Amphibian Ringer - Glades and had the following composition: (mM): NaCl, 111.23; KCl, 1.88; $CaCl_2$, 1.08; $NaHCO_3$, 2.38; Na_2HPO_4, 0.08; and glucose, 11.1. The solution was aerated to maintain pH at 7.6 (Cavanaugh, 1964, p. 70).

Glass microelectrodes were pulled on a Kopf Vertical pipette puller (700C) from thick walled Mertex capillary tubes (150 x 1.0 mm I.D. obtained from the Mercer Glassworks). A strand from a glass fiber optics light guide #2516 (Edmund Scientific Co.) was inserted before pulling to aid in the filling of the microelectrode (Tsaki, Tsukakava, Ito, Wayner, & Yu, 1968). The microelectrodes were back filled with 3 M sodium acetate under 100-400 X magnification to allow continuous checking for bubble formation. Microelectrodes of approximately 20 Mohms were found to have the best combination of low noise and muscle fiber penetration.

The microelectrode was placed in a Pfeiffer micromanipulator, model PBL-5, mounted on a rigid vibration damped frame. Signals were amplified by a Mentor N-950 intracellular probe system, and the amplified signals were displayed on Tektronix D11 single beam storage oscilloscope. The stored traces were recorded on Polaroid type 107 film with a Tektronix C5A oscilloscope camera. Alternatively, the

signals were displayed on a Tektronix type RM 565 dual beam oscilloscope, and photographed with a Grass kymograph camera, model C4R. Endplate potentials were obtained by stimulating the sciatic nerve with bipolar platinum electrodes. Stimuli were square waves of 0.05 sec. duration and were isolated from ground. The sciatic nerve was isolated from the sartorius muscle by a barrier of petroleum jelly, or the stimulating electrode with the nerve draped across it was raised above the surface of the bathing solution immediately prior to the stimulation.

d. Frog skin preparation.

The frog skin preparation was made by the method of Yorio and Bentley (1976) which is summarized below. 'Winter' leopard frogs (Rana pipiens, Lake Champlain Frog Farm) were stored at room temperature, 21°C, in tap water with access to a raft. The frogs were double pithed, and the ventral skins removed and placed in Ussing-type chambers made of Lucite. A ring of 'parafilm' was placed on either side of the chamber in order to minimize damage to the edges of the preparation. Each side of the skin preparation was bathed with 10 ml of Ringer's solution and the total surface area of each side exposed to the Ringer's solution was 3 cm^2. The potential difference (pd) across the frog skin preparation was measured by Ringer-agar bridges which were connected to a potentiometric recorder by means of calomel cells. The short-circuiting current (scc) was passed through a similar pair of bridges and Ag-AgCl cells. An automatic voltage

clamp was used to maintain the potential difference at zero, and the scc was continually recorded.

The frog Ringer's solution used for bathing the preparation had the following composition (mM): NaCl, 111; KCl, 3.35; $CaCl_2$, 2.54; $NaHCO_3$, 4; and D-glucose, 5. The solution was maintained at about pH 8.0 by means of aeration.

e. Frog rectus abdominus preparation.

Male 'winter' frogs (Rana pipiens, Lake Champlain Frog Farm) were pithed and the recti abdomini were dissected by the method of Whittaker and Barker (1972). A single rectus abdominus was mounted in a 5 ml isolated organ bath (K.C. & N.Y. Krebs) which was maintained at 25°C. One end of the rectus was attached to the bubbler of the organ bath, the other end to a force displacement transducer (Grass, FT03C). Signals were amplified and recorded by a curvilinear chart recorder (Grass, model 7 Polygraph).

A load of 1 g was placed on the tissue, and the preparation was allowed to relax for 30 min. in frog Ringer's solution. The preparation was then sensitized by switching to eserinized frog Ringer's solution and waiting for 30 min. After exposure to drugs which caused a contraction of the preparation, it was relaxed by gentle stretching to its control length. The frog Ringer's solution used for bathing the preparation had the following composition: (mM): NaCl, 107.8; KCl, 3.9; $CaCl_2$, 1.5; $NaHCO_3$, 4.2; D-glucose, 3.9. The eserinized frog Ringer's solution had the same composition plus 4.8×10^{-6} M physostigmine salicylate (Whittaker &

Barker, 1972).

2. Drugs.

The drugs used in the pharmacological analysis are expressed in molar concentration and are listed in appendix B. The drugs were dissolved in Tyrode's solution. Mefenamic acid and indomethacin, which were not freely soluble in Tyrode's solution, were first dissolved in the smallest amount of 0.1 N sodium hydroxide in which they were soluble and then diluted with Tyrode's solution. In no case did this procedure affect the pH of the Tyrode's solution in the organ bath.

Storage stock solutions (0.1 M) of the fatty acids and the prostaglandins were prepared in N_2 gassed ethanol and kept at $-40^{\circ}C$ under nitrogen. Working stock solutions for injection into the organ bath were prepared by drying an appropriate amount of the storage stock solution under a stream of nitrogen and, in the case of the prostaglandins, adding Tyrode's solution to yield the working stock solution. The fatty acids were not freely soluble as prepared and formed white suspensions only after they were agitated (Vortex) in a test tube in which a glass stirring rod was held. The concentrations of the working stock solutions were critical. For example, when working stock solutions of arachidonic acid were prepared, if the dilution was made from 10^{-2} to 10^{-3} M, the arachidonic acid was found to coalesce into a single fatty blob after a few stirrings. However, when the working stock solutions were injected into the organ bath using 10, 50, or 100 ul microsyringes (Hamilton), the arachidonic acid remained

in fine suspension during the course of the response.

Stock solutions of norepinephrine, epinephrine, and isoproterenol were prepared daily at acid pH and stored at $4^{o}C$ until immediately prior to injection into the organ bath. These precautions were found to be necessary to prevent the formation of adrenochrome.

Working stock solutions for all other drugs were prepared daily from storage stock solutions maintained under the conditions described in appendix II of Pharmacological Experiments in Isolated Preparations, Second Ed. (Staff of the Department of Pharmacology of the University of Edinburgh, 1970).

Antagonists were added to the organ bath by switching the infusion Tyrode's solution to Tyrode's solution containing the antagonist. The isolated tissue was allowed to equilibrate for a minimum of 6 min. before any agonists were tested.

3. Data analysis.

Statistical analysis (Snedecor & Cochran, 1967) of the data was performed with the aid of the PROPHET system (a specialized computer resource for the study of chemical/biological interrelationships funded by the Chemical/Biological Information-Handling Program of the National Institute of Health). Analysis of dose-effect relationships was performed using the LOGISTIC and ANTAGONIST programs develped by Dr. Carl L. Johnson as public procedures for the PROPHET system (Wood, 1977). The procedures are

based on the method of Parker and Waud (1971) and Waud and Parker (1971). Analysis of the half-life of the response to the lipid fraction was performed using the public EXPFIT program developed by Johnson, Sher, Risley, and Wood (Wood, 1977). Other statistical analyses compared single does effects before and after treatment and were analyzed by paired t-test or the signed rank test as appropriate.

B. Results and discussion.

1. Rabbit ileum preparation.

a. Effect of the active material.

The rabbit ileum is a spontaneously motile preparation which develops a "pendular motion" as can be seen from the control period in fig. 9. Active material added to the bath produces a slow increase in the pendular motion with a lag period that is inversely related to the amount of active material (AM) in the lipid fraction (LF). Fig. 10 shows the average effect on six different rabbit ileum preparations of a concentration of AM causing an approximately half-maximal effect. The lag period (0.35 min.) was determined by calculating the x-intercept for a weighted least-squares fit of the rising phase of the effect of AM shown in fig. 11. The time to the peak effect was 2.40 $\pm$ 0.30 min. (the average $\pm$ the standard error of the mean, n=6, length of time for the individual preparations to reach their peak effect). Fig. 12 shows the weighted least-squares exponential fit of the average decay from the peak effect of a single dose of AM. The half-life of the decay, calculated by the formula:

$T_{/2} = \frac{0.693}{k}$, was 2.80 min.

In view of the stimulation by the lipid fraction of the rabbit ileum, a preliminary pharmacological screen was employed to ascertain the mechanism of action of the lipid fraction on the rabbit ileum. It was considered that the comparative pharmacology of the lipid fraction on the guinea-pig and rabbit ilea would provide insights into the general mechanism of the action of the lipid fraction on isolated tissues.

b. The effect of morphine on the response of the rabbit ileum to the lipid fraction and to acetylcholine.

The rabbit intestine is known to be insensitive to narcotic analgesics. As early as 1955, Schaumann demonstrated that, while 8.8×10^{-8} M morphine blocked the nicotine stimulation of the guinea-pig ileum, morphine at concentrations up to 5.9×10^{-5} M had no effect (or possibly slightly enhanced) nicotine stimulations of the rabbit jejunum (Schaumann, 1955). This lack of effect was confirmed by Goldenberg using morphine at a concentration of 2×10^{-6} M on the ileum from either normal or vagotomized rabbits (Goldenberg, 1968). Morphine depressed the stimulus evoked release of acetylcholine from the guinea-pig ileum (Paton, 1957; Schaumann, 1957) but not from the rabbit ileum (Greenberg, Kosterlitz, & Waterfield, 1970; Lees, Kosterlitz, & Waterfield, 1973).

However, because the lipid fraction caused a stimulation of the rabbit ileum with a latency and a time to peak effect similar to those demonstrated on the guinea-

Fig. 9. Effect of atropine (1×10^{-6} M) on the response of the rabbit ileum to approximately half-maximal concentrations of active material.

a. ACh, 7×10^{-8} M. b. AM, 2µl/ml - a concentration producing approximately half the maximal response to AM in this particular "loop". c. atropine, 1×10^{-6} M in the Tyrode's solution. w. wash, approximately 5 exchanges of the volume of the tissue bath with Tyrode's solution. numerals. time in minutes from the replacement of plain Tyrode's solution with Tyrode's solution containing 1×10^{-6} M atropine.

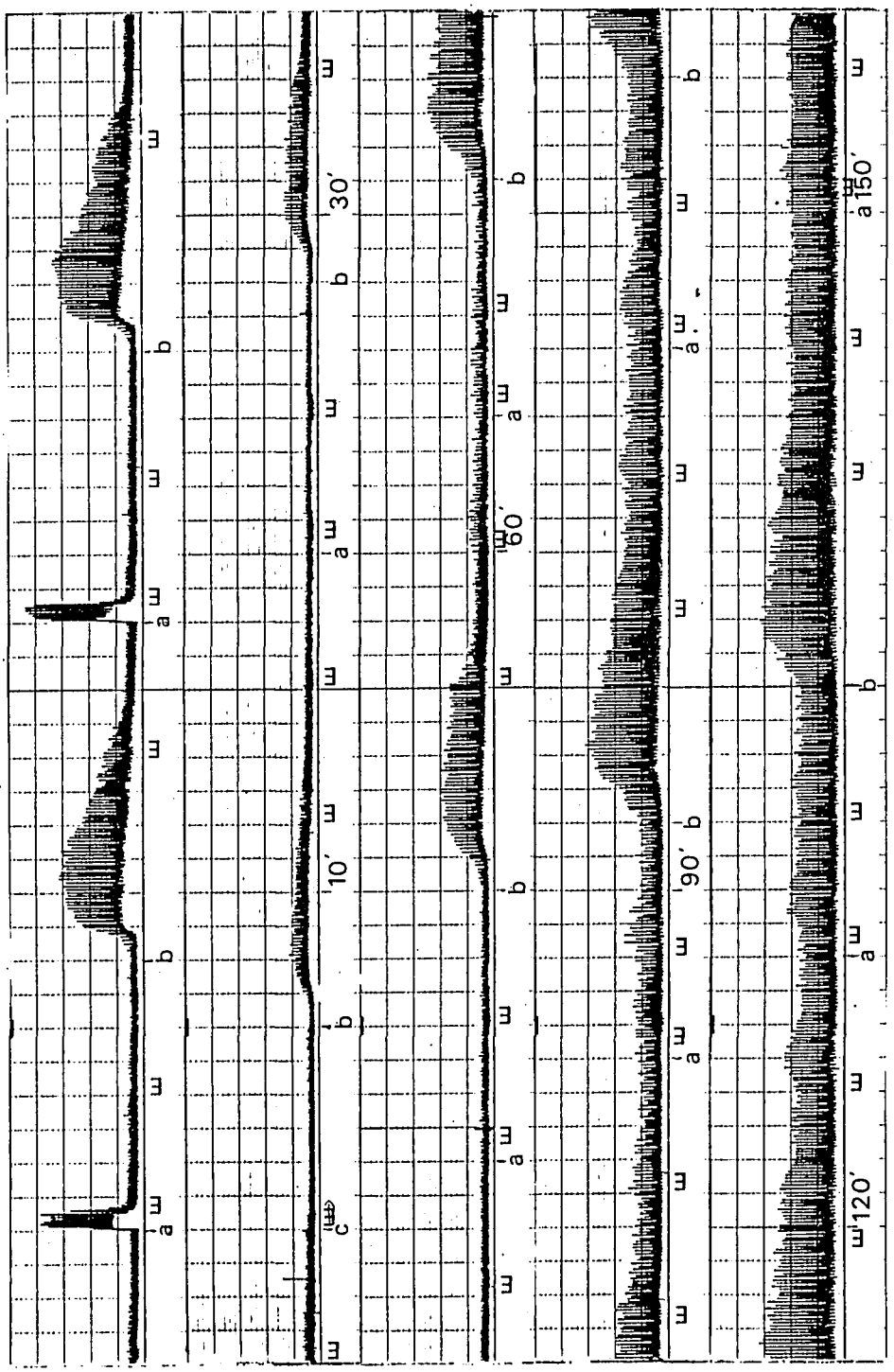

a
b
c
10'
30'
60'
90'
120'
150'

Fig. 10. Average effect of a single dose of active material (AM).

The average effect of approximately half-maximal single doses of AM on six different rabbit ileum preparations is shown. To eliminate the variation between preparations, the effect is shown as a percent of the peak effect of that dose for each individual preparation.

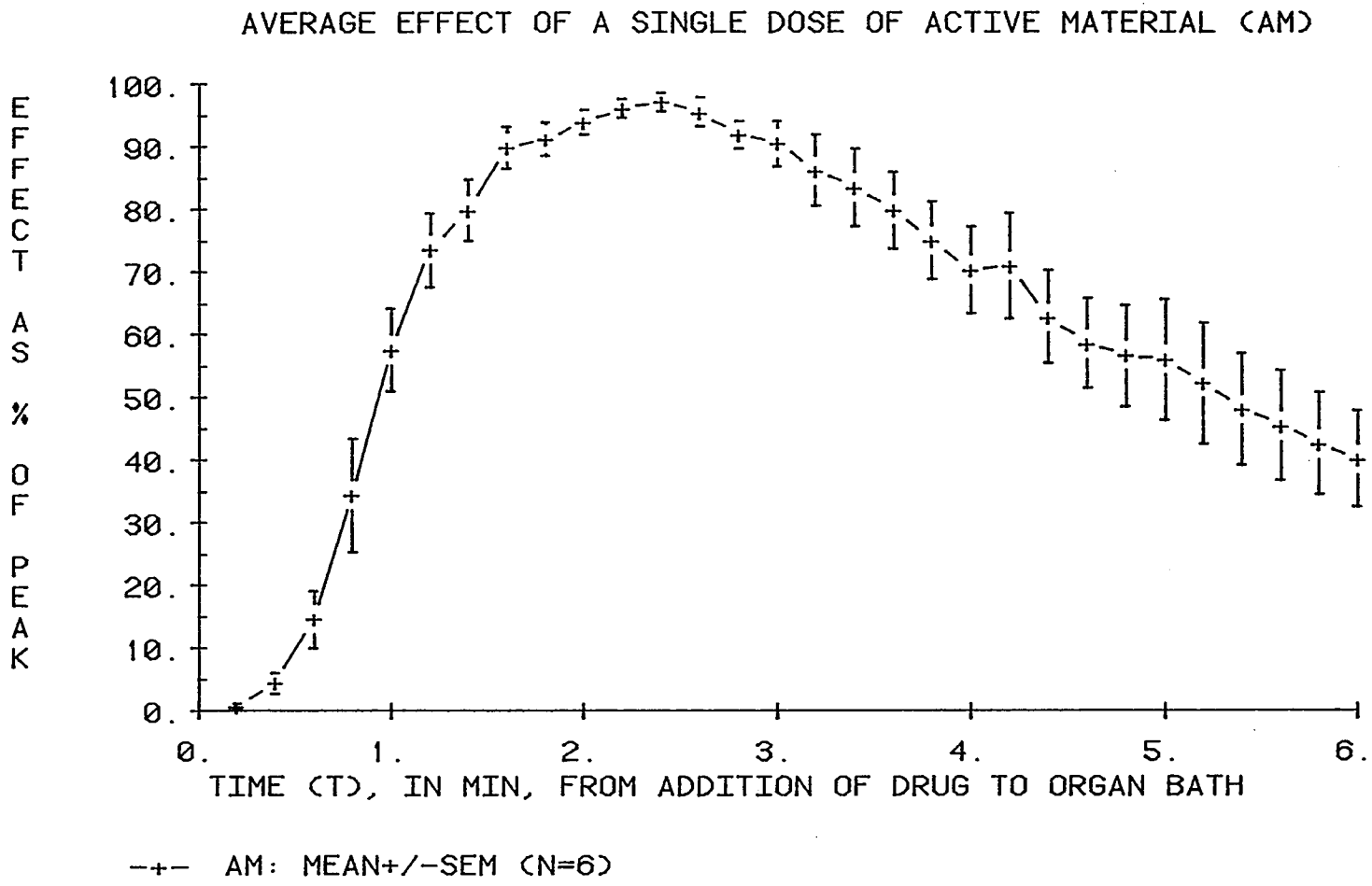

AVERAGE EFFECT OF A SINGLE DOSE OF ACTIVE MATERIAL (AM)
EFFECT AS % OF PEAK
100.
90.
80.
70.
60.
50.
40.
30.
20.
10.
0.
0.
1.
2.
3.
4.
5.
6.
TIME (T), IN MIN, FROM ADDITION OF DRUG TO ORGAN BATH
-+- AM: MEAN+/-SEM (N=6)

Fig. 11. Latency of the effect of a single half-maximal dose of active material (AM): weighted least-squares plot.

The latency of the effect of the half-maximal doses of AM shown in fig. 10.

The points were taken from the rising phase of fig. 10, and a regression line fitted using the least-squares method weighted by the inverse of the variance. The latency was taken as the x- intercept: $y = 85.1 x + (-30.2)$ ($y = m X + b$); for $y = 0$, $x = 0.35$ min.

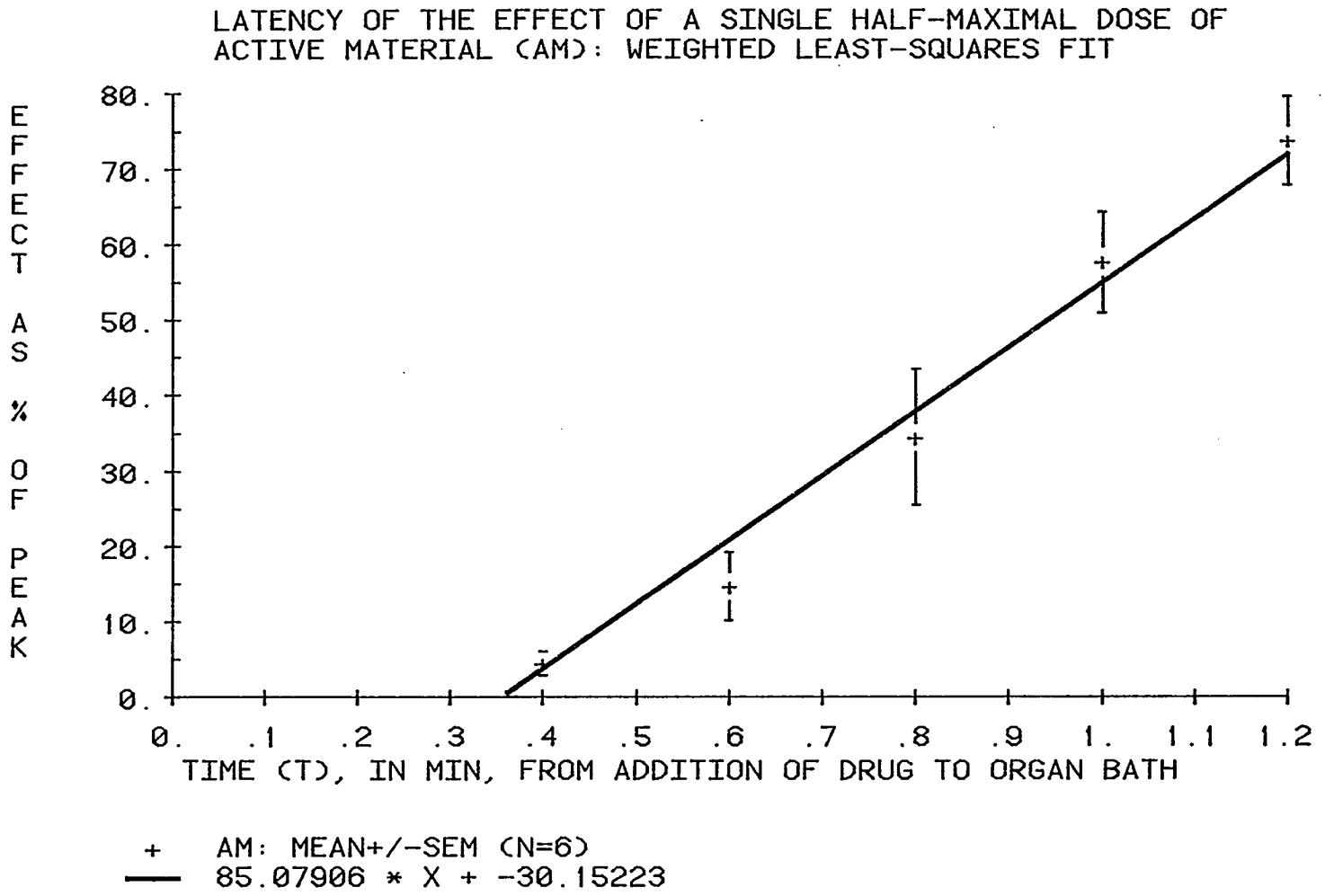
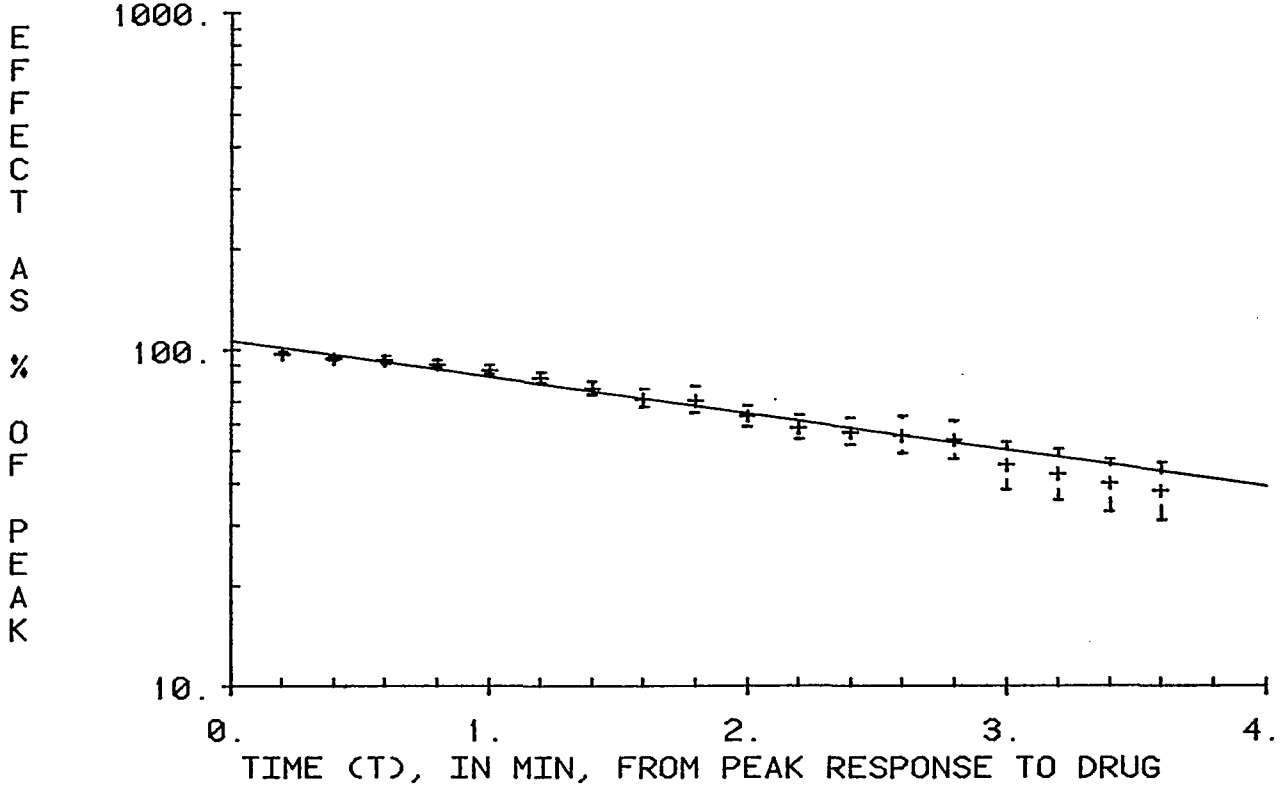

LATENCY OF THE EFFECT OF A SINGLE HALF-MAXIMAL DOSE OF ACTIVE MATERIAL (AM): WEIGHTED LEAST-SQUARES FIT
EFFECT AS % OF PEAK
80.
70.
60.
50.
40.
30.
20.
10.
0.
0. .1 .2 .3 .4 .5 .6 .7 .8 .9 1. 1.1 1.2
TIME (T), IN MIN, FROM ADDITION OF DRUG TO ORGAN BATH
+ AM: MEAN+/-SEM (N=6)
85.07906 * X + -30.15223

Fig. 12. Weighted least-squares exponential fit of the decay of the average effect of a single dose of active material (AM).

The data shown in fig. 10 were used to calculate the decay of the effect of AM from its peak effect. The peak effect of each individual experiment was determined, and the curve shown was the best fit of an exponential function to the average decay of the effect.

WEIGHTED LEAST-SQUARES EXPONENTIAL FIT OF THE DECAY OF THE AVERAGE EFFECT OF A SINGLE DOSE OF ACTIVE MATERIAL (AM)



pig ileum and because blockade by narcotic analgesics was a defining property of the lipid fraction on the guinea-pig ileum, the action of morphine on the effect of single doses of the lipid fraction was examined. After obtaining control responses using doses of the lipid fraction that caused approximately a half-maximal effect, the tissue was exposed to 1×10^{-6} M morphine for six minutes. At that time, the lipid fraction was added to the bath and the effect measured. The average effect of the lipid fraction, pre(control) and post morphine, for each ileal preparation were calculated, and then the groups of preparations compared using a paired t-test (Snedecor & Cochran, 1967). No significant difference was found (pre = 2.7, post = 2.3, $\Delta = -0.4 \pm 0.3$; $n = 4; p > 0.2$) in the effects produced by active material, nor were there any differences in the effects of ACh. The results with acetylcholine are consistent with those of Goldenberg (1968).

In view of the previously reported lack of effect of narcotic analgesics on the stimulus evoked release of acetylcholine by the rabbit intestine (Schaumann, 1955; Goldenberg 1968; Greenberg, Kosterlitz, & Waterfield, 1970; Lees, Kosterlitz, & Waterfield, 1973), it is not surprising that the actions of the lipid fraction on the rabbit ileum were not blocked by morphine. The experimental evidence that the lipid fraction does stimulate the rabbit ileum and that the actions of the lipid fraction on the rabbit ileum are not blocked by narcotic analgesics refutes the hypothesis that the actions of the lipid fraction are necessarily

linked to cellular components sensitive to narcotic analgesics.

c. The effects of muscarinic antagonists on the response of the rabbit ileum to the lipid fraction.

Atropine blocks the action of acetylcholine at muscarinic receptors (Ambache, 1955). In previous experiments using the guinea-pig ileum, the action of AM was blocked by 2.1×10^{-8} M atropine (Green & Carlini, 1964). The rabbit ileum is known to be relatively sensitive to atropine (Ellis & Rasmussen, 1951; Goldenberg, 1968). For example, transmural stimulation of the guinea-pig ileum causes a contraction that is blocked by atropine (Paton, 1955) while transmural stimulation of the rabbit ileum causes a stimulation (increase in the size of the pendular motion) and then a depression (decrease in the pendular motion). In an ileum responding to transmural stimulation by excitation and slight depression, hyoscine (scopolamine) at 3.3×10^{-6} M converted the response to a large depression followed by a decreased excitation (Day & Warren, 1968). Hyoscine was used because atropine at concentrations greater than 3.4×10^{-7} M frequently inhibited the spontaneous activity of the rabbit ileum (Day & Warren, 1968; Holman & Hughes, 1965). Atropine in similar concentrations was capable of blocking exogenous acetylcholine causing similar stimulations (Ellis & Rasmussen, 1951).

Because the inhibition of the action of AM on the guinea-pig ileum by atropine had been described as 100%

(Green, Carlini & Robinsin, 1963), screening experiments on the rabbit ileum were conducted using 1×10^{-9} to 1×10^{-5} M atropine; no consistent effects on the action of AM could be determined.

When the rabbit ileum was bathed in Tyrode's solution containing 1×10^{-6} M atropine, the pendular motion usually decreased to a minimum and then slowly recovered toward the baseline level. Fig. 9 shows the effect of 1×10^{-6} M atropine on the actions of previously equiactive doses of acetylcholine (ACh) and AM. The action of AM was initially decreased by 50% but the action appeared to recover to about 80% of the control (pre-atropine) levels within an hour. The effect of ACh or acetyl-β -methylcholine (ABMeCh), in equiactive doses to those of AM, was always completely blocked. Nicotine also stimulated the rabbit ileum, and, when previously equiactive doses of nicotine and AM were exposed to 1×10^{-6} M atropine, the action of nicotine was decreased and then recovered in parallel with the actions of AM. This action of nicotine in the presence of atropine was described by Ellis & Rasmussen (1951) as "the atropine-fast nicotine stimulation of the rabbit's intestine". Ellis and Rasmussen (1951) also suggested that Darmstoff had much to recommend it as the putative neurotransmitter involved. This atropine-fast nicotine stimulation of the rabbit intestine is not due to the presence of atropinesterase in the intestinal preparations, as Ambache has reported the occurrence of atropine-

fast contractions due to nicotine in intestinal preparations obtained from rabbits whose serum lacked atropinesterase (1955). In a series of three experiments done in atropinized Tyrode's solution, the actions of AM, (n=1), the fatty acids extracted from the lipid fraction (NLE#3 FFA1-3), see Section III.B.2.d.(2), n=2), and arachidonic acid (20:4w6, n=2) were not affected by 5×10^{-5} hexamethonium chloride, while the action of previously equiactive doses of nicotine was blocked. This result may be interpreted to mean that the lipids were not releasing acetylcholine which was then interacting with nicotinic cholinergic receptors, subsequently causing a stimulation of the preparation.

The action of atropine on the effect of AM is more readily understood by comparing the maximum effect (EMAX) and the concentration producing half the maximum effect (ED_{50}) of concentration-effect curves obtained after exposure to 1×10^{-6} M atropine with paired controls: table 1 lists the EMAXs and ED_{50}s of AM, arachidonic acid, and of the average response (AVE CH) of the rabbit ileum to ACh and/or ABMeCh. The reason for using AVE CH was to allow the pooling of data from two series of experiments. The rationale for the pooling was that there proved to be no statistical differences between the EMAXs and ED_{50} of ACh (6.06 ± 0.42, $1.41 \pm 0.57 \times 10^{-7}$ M, n=12) and of ABMeCh (6.21 ± 0.29, $8.48 \pm 0.98 \times 10^{-8}$ M, n=54). While the AVE CH EMAX is unaffected by atropine, the EMAXs of arachidonic acid and AM are decreased, the latter significantly

$p < 0.001$. The ED_{50}s are all shifted to the right, although only the ED_{50}s of AVE CH and AM are shifted significantly. The effect of atropine on the action of arachidonic acid is in the same direction as the effect of atropine on the action of AM.

Because the K_b is dependent on the receptor and not the agonist, no significant differences were found in affinity of the muscarinic receptor of the rabbit ileum for atropine when either ACh or ABMeCh was used as the agonist (table 2). In addition, no differences were noted in the calculated K_b of atropine when either O_2, O_2/CO_2:95/5%, or air were used to oxygenate the Tyrode's solution. The values of the K_b of atropine determined on the rabbit ileum are not much different from those determined by others on the guinea-pig ileum (Barlow et al., 1973; Yavetz, 1976). Also, as the concentration of atropine used for testing its action on the effects of the various agonists was 3 orders of magniture greater than the K_b, a Schild plot (Arunlakshana & Schild, 1959; fig. 13) was used to demonstrate that the action of atropine as an antagonist of muscarinic agonists had not changed in character from that of a competitive antagonist to that of a local anaesthetic (i.e., the slope of the Schild plot remained 1.0 over the range of concentraetions tested).

In one experiment, 1×10^{-6} M scopolamine reduced the action of single doses of AM by 67% and shifted the concentration-effect curve of ACh approximately two log

units to the right.

d. The effects of fatty acids.

During the initial screen of pharmacological antagonists, the observation was made that a fatty acid cyclo-oxygenase inhibitor, mefenamic acid, inhibited the action of AM. This finding provided the conceptual link between the action of the AM and the actions of the fatty acids related to cyclo-oxygenase substrates. Based on this initial clue, it was decided to obtain the pharmacological evidence presented below and the qualitative organic analysis described in Section III.B. These experiments led to the subsequent identification of the lipid fraction as a mixture of saturated and unsaturated fatty acids with sufficient cyclo-oxygenase substrates to yield the prostaglandins and/or thromboxanes which account for the biological activity of the AM.

(1) Actions as agonists.

The effects of several different preparations of the lipid fraction (AM, NLE#3, and the products of the serial extraction procedure discussed in Section III.B.2.d.(2); the different preparations of the lipid fraction are described in appendix A) were compared with the cholinergic agonists, acetylcholine (ACh), acetyl-β-methylcholine (ABMβCh), the average cholinergic response (AVE CH), several fatty acids; arachidonic (20:4w6), 8,11,14-eicosatrienoic (20:3w6), γ-linolenic (18:3w6), and linolenic (18:3w3), and the prostaglandins, PGE_2 (PGE2) and

TABLE 1

ACTION OF 1 x 10^{-6} M ATROPINE ON PAIRED CONCENTRATION-EFFECT CURVES OF AVE CH, 20:4w6, AND AM

Agonist	n	$EMAX_{pre}$	$EMAX_{post}$	P	ED_{50} pre	ED_{50} post	P
AVE CH	13	6.0	6.5	>0.33	5.9 x 10^{-8} M	6.1 x 10^{-5} M	<0.002
20:4w6	4	5.2	3.4	<0.17	2.5 x 10^{-6} M	3.2 x 10^{-5} M	<0.07
AM	6	5.5	2.6	<0.001	2.9 µl	12.2 µl	<0.04

TABLE 2

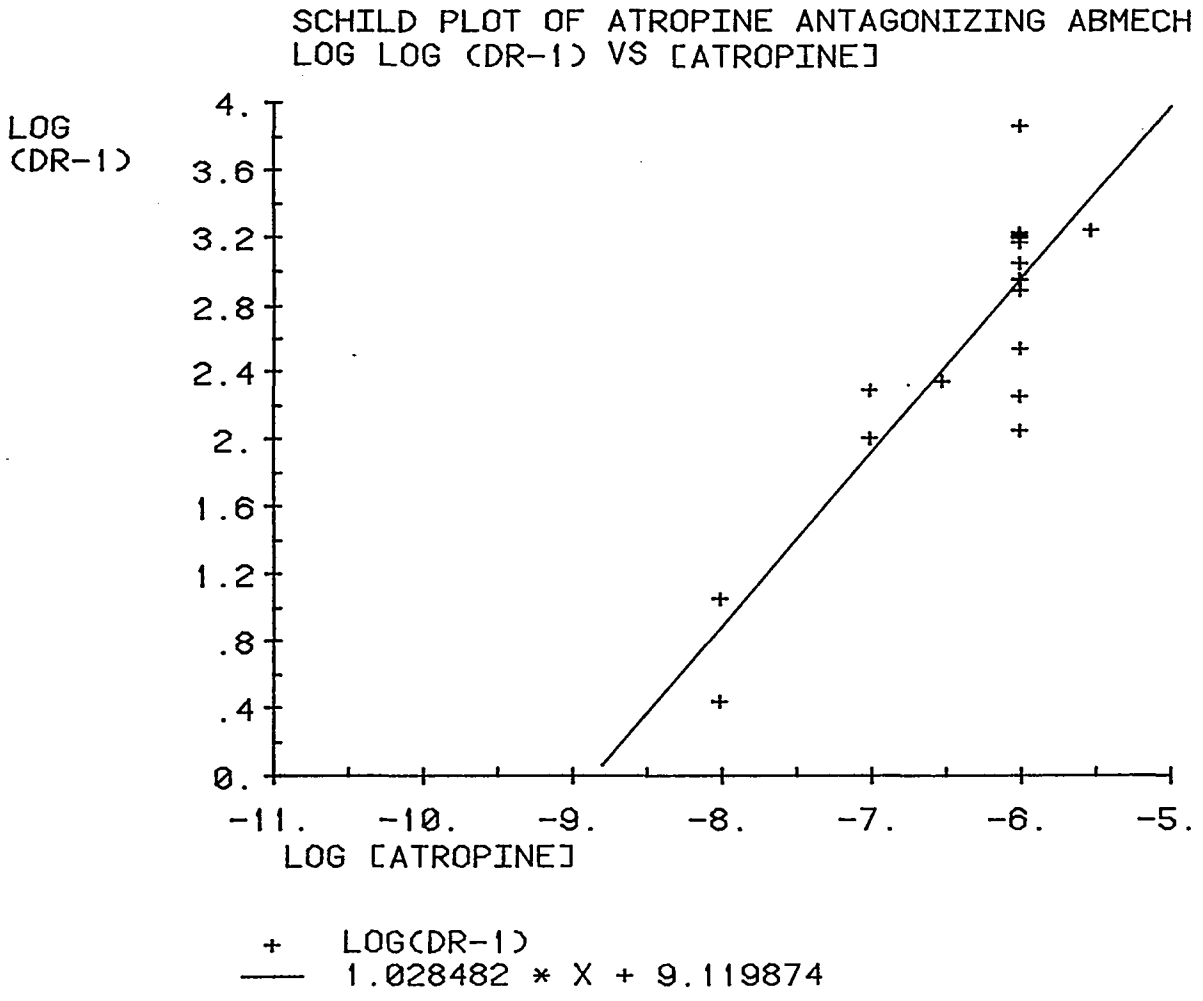
K_b s DETERMINED FOR ALL CONCENTRATIONS OF ATROPINE AS AN ANTAGONIST OF THE ACTIONS OF ACh AND ABMeCh

Agonist	Tyrode's Solution Gassed with	$K_b \pm$ SEM M*	n
ACh	air	$3.4 \pm 2.1 \times 10^{-9}$	6
ABMeCh	O_2/CO_2:95/5%	$6.4 \pm 1.7 \times 10^{-10}$	7
ABMeCh	O_2:100%	$1.9 \pm 0.6 \times 10^{-9}$	16
ABMeCh	combined	$1.5 \pm 0.4 \times 10^{-9}$	23

*Comparison of K_b s revealed no significant differences between agonists or gases.

Fig. 13. Schild plot of atropine antagonizing ABMeCh: log (DR-1) vs log atropine.

The Schild plot (Arunlakshana & Schild, 1959; Waud & Parker, 1971) of atropine antagonizing the actions of acetyl-β-methylcholine (ABMeCh) was a straight line with a slope not different than 1.0. The K_b, calculated from the coefficients of the regression equation: $y = 1.03 (X) + 9.12$ at $y = 0$ and $X = -8.87$, was 1.3×10^{-9} M.


SCHILD PLOT OF ATROPINE ANTAGONIZING ABMECH
LOG LOG (DR-1) VS [ATROPINE]
LOG
(DR-1)
4.
3.6
3.2
2.8
2.4
2.
1.6
1.2
.8
.4
0.
-11.
-10.
-9.
-8.
-7.
-6.
-5.
LOG [ATROPINE]
+ LOG(DR-1)
1.028482 * X + 9.119874

$PGF_{2\alpha}$ (PGF2A). The results of this comparison are shown in table 3.

In order to ascertain if there was a relationship between the effects of the different agonists, regression analyses were performed comparing the maximum effects (EMAXs) of the various agonists with the paired maximum effects of AVE CH or arachidonic acid. Table 4 shows the regression of the EMAXs of the fatty acids on the average cholinergic EMAXx (AVE CH EMAX). Both the slopes and the intercepts of the regressions of arachidonic acid and of AM on AVE CH are significant; the variation in the AVE CH EMAX explaining 38% and 35% of the variation in their respective EMAXs. The positive values of the intercepts may be explained by experimental bias introduced by discarding ileal preparations with small responses to ACh or ABMeCh. The lack of a significant correlation between the EMAXs of NLE#3 and AVE CH EMAX may be explained by the lack of correlation between the EMAXs of arachidonic acid and AVE CH in those preparations in which both NLE#3 and arachidonic acid could be compared with AVE CH. Comparison of the concentration of agonists producing half-maximal effects (ED_{50}s) revealed no correlation between the ED_{50} of AM or arachidonic acid and the ED_{50} of the AVE CH: the correlation coefficients were 0.021 and 0.118. Ichikawa and Yamada (1962) stated that "organs that exhibited high sensitivity to ACh were more sensitive to the acid (arachidonic) than the others." If they meant an increased EMAX

TABLE 3

COMPARISON OF AGONISTS ON THE RABBIT ILEUM

Agonist	EMAX	SEM	n	EMAX as % of AVE Ch EMAX Paired	SEM	n	EMAX as % of 20:4w6 EMAX Paired	SEM	n	ED_{50}	SEM	M	n
ACh	6.06	0.42	12							1.41	.57	10^{-7}	12
ABMeCh	6.21	0.29	54							8.48	.98	10^{-8}	54
AVE Ch	6.24	0.25	65	100	0	65	127	8	29	9.46	1.34	10^{-8}	65
PGE_2	7.50	1.26	6	104	11	4	122	7	6	6.27	1.47	10^{-8}	6
$PGF_{2\alpha}$	6.32	1.05	6	84	11	4	102	6	6	2.00	0.57	10^{-9}	6
20:4w6	5.13	0.27	34	86	5	29	100	0	34	2.97	0.51	10^{-6}	34
20:3w6	3.09	0.48	4	58	13	4	68	11	4	2.58	1.03	10^{-5}	4
18:3w6	2.14	0.69	4	41	12	4	50	14	4	2.30	1.53	10^{-4}	4
18:3w3	.5	-	2	12	-	2	11	-	2	5.5	-	10^{-3}	2
AM	5.09	0.26	38	84	4	36	92	13	6	7.19	1.37	in µl	38
NLE#3	3.62	.35	13	66	8	13	87	9	11	5.09	111	in µl	13
NLE#3 FFA	4.25	0.23	5	86	11	5	88	9	5	249	249	in µl	5

TABLE 4

REGRESSION OF FATTY ACID EMAXs ON AVE Ch EMAX

Agonist	Slope	P≤	Intercept	P≤	r	P	n
AM	0.45*	0.001	2.22	0.003	0.59	<0.01	37
20:4w6	0.41*	0.001	2.45	0.001	0.62	<0.01	29
20:3w6	-0.13	0.74	3.86	0.2	-0.26	>0.1	4
18:3w6	1.07	0.28	-3.17	0.48	0.72	>0.1	4
NLE#3	-0.01	0.97	3.72	0.02	-0.01	>0.1	12
NLE#3FFA	0.05	0.85	4.53	0.05	-0.12	>0.1	5
PGE_2	1.13	0.04	-0.52	0.77	0.97	<0.05	4
$PGF_{2\alpha}$	0.74	0.09	0.56	0.78	0.91	<0.1	4

*The values of the slopes of AM vs AVE Ch and 20:4w6 vs AVE Ch are the same with p=0.78.

by "high sensitivity", then the results reported in this dissertation are in accord with their report.

In addition, regression analyses were performed on the agonist EMAX and ED_{50} values of arachidonic acid. The only significant correlation was between the AM ED_{50} and the arachidonic acid ED_{50}: slope = 2.71×10^{6}, $p<0.04$; intercept = 2.56, $p>0.60$; correlation coefficient = 0.839.

(2) Effects of glutathione.

Glutathione (GSH) has been described as an activator of prostaglandin synthesis (Splawinski et al., 1973), though more recent evidence suggests that it merely serves to shift the flow of metabolism of the cyclic-endoperoxide (Sun, Chapman, & McGuire, 1977). At a concentration of 5×10^{-4} M, the concentration used by Splawinski et al., (1973), GSH had no effect on the EMAX or ED_{50} of arachidonic acid (n=4), dihomo-γ-linolenic acid (n=2), or AVE CH (n=5).

(3) Inhibition.

(a) Anoxia.

In one experiment, baseline responses to AM and ACh were established with Tyrode's solution bubbled with air. Switching from air to pure N_2 was followed by an initial increase and then a subsequent decrease in the pendular motion and tone of the rabbit ileum. Over the course of 4 min., the pendular motion was replaced by sporadic contractions of diminished frequency. These

results are similar to those reported previously for the anoxic rabbit ileum (West, Hadden, & Farah, 1951).

The ACh EMAX was decreased to 81% of the control value (duplicate samples), and the ED_{50} was shifted slightly to the right. The effect of single doses of AM was decreased to less than the limit of error of the preparation; i.e., about 10%. On restoration of the air, the ACh and AM responses returned to their control values.

The results for the anoxic ileum of the rabbit are similar to those found for the anoxic guinea-pig ileum (Green, Carlini, & Robinson, 1963; Green & Carlini, 1964).

(b) Cyclo-oxygenase inhibitors.

((1)) Mefenamic acid.

From its original description in 1963 by Green, Carlini, and Robinson, the AM was known to be lipoidal in nature. In 1972, Ferreira, Herman, and Vane published an abstract stating that prostaglandin generation maintained the tone of the rabbit isolated jejunum. Since, at the time of the original biological characterization of AM, Vane had not yet demonstrated that non-narcotic analgesics (non-steroidal anti-inflammatories, NSAI) inhibit prostaglandin synthesis (1971), it seemed appropriate to determine if AM was involved in the prostaglandin system or any of the other compounds for which the cyclo-oxygenase is a metabolic step.

Ferreira, Herman, and Vane (1972, 1976) used indomethacin (10 ug/ml = 2.8 x 10^{-5} M) as their cyclo-oxygenase inhibitor. However, for an initial pharmacological screen, indomethacin presented a number of disadvantages: it was not particularly soluble in aqueous solutions (The Merck Index, 8th Ed.); it had a fairly high K_i (Rome & Lands, 1975b) of 1 x 10^{-4} M; and it was a time dependent inhibitor of the cyclo-oxygenase (Smith & Lands, 1971; see Lands & Rome, 1976 for a complete discussion). Therefore, when Rome and Lands (1975b) demonstrated that mefenamic acid combined the advantages of relatively high aqueous solubility (The Merck Index Index, 8th Ed.), low K_i (1 x 10^{-6} M), and simple competitive inhibition kinetics, mefenamic acid was chosen as the initial cyclo-oxygenase inhibitor for the pharmacological screen.

Since claims have been made that mefenamic acid also functions as a prostaglandin antagonist (although whether the effect is due to receptor antagonism, Collier & Sweatman, 1968; Panczenko, Grodzinski, & Gryglewski, 1975) or to blockade of $PGF_{2\alpha}$ induced stimulation of the synthesis of other prostaglandins is a matter of interpretation), the effects of mefenamic acid on the actions of a variety of agonists were tested. These are summarized in table 5. As can be seen by an inspection of part a. of the table, the action effects of AM were antagonized to a greater extent than either nicotine or acetyl-β-

TABLE 5

EFFECTS OF MEFENAMIC ACID (1×10^{-5} M)
ON THE ACTIONS OF SEVERAL AGONISTS

a. Action of mefenamic acid (MFA) on the effects of equiactive doses of active material (AM), nicotine (Nic), and acetyl-β-methylcholine (ABMeCh) in 2 different rabbit ileum preparations (average of duplicates).

Agonist	Control	Post MFA	Recovery post Wash
AM	100%	5, 19%	Up to 19%
Nic	100%	83, 52%	Up to 147%
ABMeCh	100%	106, 59%	Up to 178%

b. Mefenamic acid: K_b and effect on EMAX.

Agonist	$K_b \pm$ SEM in M	$EMAX_{pre}$	$EMAX_{post}$	n	P≥
ABMeCh	*	4.9	5.2	7	0.4
PGE	*	6.3	4.1	1	-
$PGF_{2\alpha}$	6.2×10^{-6}	5.6	3.4	1	-
20:4w6	$5.6 \pm 2.1 \times 10^{-7}$	4.7	4.3	8	0.3
20:3w6	2.3×10^{-6}, 1.0×10^{-7}	2.3	2.3	2	-
18:3w6	2.2×10^{-7}	0.5	0.5	1	-
AM	$1.0 \pm 0.5 \times 10^{-5}$	5.5	5.1	3	0.6
NLE#3	1.7×10^{-6}, 2.3×10^{-7}	3.8	3.8	2	-
NLE#3FFA	$4.3 \pm 1.9 \times 10^{-6}$	4.7	4.3	3	0.3

*Apparent potentiation.

methylcholine. While the average response to AM was only 12% of the control response, the average response to nicotine and acetyl-β-methylcholine were 68% and 83% of their respective control responses. A possible explanation for the decrease seen with the nicotinic and muscarinic agonists may be that mefenamic acid prevents the synthesis of endogenous prostaglandin-like substances which normally enhance cholinergic agonists (Clegg, Hall, & Pickles, 1966). On the other hand, the decrease in the effect of ABMeCh seemed to be more apparent than real as part b. of table 5 shows that, when complete concentration-effect curves were obtained, mefenamic acid appeared to slightly potentiate the action of ABMeCh and of PGE_2.

Inspection of part b. of the table will demonstrate the mefenamic acid had no consistent action on the EMAX of the various agonists, (although the 37% decrease of EMAX for PGE_2 and $PGF_{2\alpha}$ in one experiment remains unexplained), and therefore it appears to have acted as a purely competitive antagonist. The K_bs against the various agonists range over an order of magnitude, but the mean value was $2.9 \pm 1.1 \times 10^{-6}$ M ($\bar{x} \pm$SEM, n=20). This value approximates that (1 μM) reported by Rome and Lands for the sheep seminal vesicle preparation (1975b). The K_bs show considerable variation from ileal preparation to preparation, and this variation may reflect the level of endogenous cyclooxygenase substrates or inhibitory fatty acids (Pace-Asciak

& Wolfe, 1968) which may compete with mefenamic acid for the substrate site (Lands & Rome, 1976). Indeed, the competing fatty acids present in the AM, NLE#3, and NLE#3 FFA may explain the order of magnitude difference in the average K_b for all the extracts of $5.6 \pm 2.3\ 10^{-6}$ M ($\bar{X} \pm$SEM, n=8) compared to the average K_b for all pure fatty acids of $5.6 \pm 2.4 \times 10^{-7}$ M.

Since the average K_b for all the extracts was so similar to that of the apparent K_b of mefenamic acid against $PGF_{2\alpha}$ (6.2×10^{-6} M), it was considered necessary to test another cyclo-oxygenase inhibitor, indomethacin, whose actions have been claimed not to involve antagonism of prostaglandin receptors (Panczenko, Grodzinska, & Gryglewski, 1975).

((2)) Indomethacin.

Despite the disadvantages discussed in Section II.B.1.d.(3).(b)., indomethacin was chosen as the confirmatory cyclo-oxygenase inhibitor because it was the standard inhibitor used in isolated smooth muscle preparations (Flower, 1974) and had been reported to inhibit the actions of arachidonic acid on the rabbit jejunum (Ferreira, Herman, & Vane, 1976). The effects of indomethacin (5×10^{-5} M) on the actions of ABMeCh, arachidonic acid, AM, and NLE#3 FFA are shown in table 6. While no significant effects were observed on the EMAXs of the various agonists, there was a trend toward a decreased EMAX, suggesting that the inhibition may not be purely competive.

TABLE 6

EFFECTS OF INDOMETHACIN (5×10^{-5} M) ON THE ACTIONS OF ABMeCh, 20:4w6, AM, AND NLE#3 FFA

Agonist	$K_b \pm$ SEM in M	$EMAX_{pre}$	$EMAX_{post}$	n	P≥
ABMeCh	*	5.4	2.7	3	.10
20:4w6	$6.2 \pm 2.9 \times 10^{-6}$	5.4	3.9	3	.30
AM	3.4×10^{-6}	6.1	1.1	1	-
NLE#3 FFA	2.3×10^{-5}, 7.7×10^{-6}	4.5, 4.1	4.4, 1.2	2	-

*Apparent potentiation

Indomethacin is known to inhibit phosphodiesterase at concentrations approximately equal to the one chosen for this study (Flower, 1974), and, since both phosphodiesterase inhibitors and cAMP have been demonstrated to depress the pendular motion of the rabbit intestine (Bowman & Hall, 1970), it is possible that the depression of EMAXs may be due to an increase in the intestinal cAMP. On the other hand, the reason such a high concentration of indomethacin was chosen in the first place stemmed from the report that 2.8×10^{-5} M indomethacin only blocked the action of arachidonic acid after between 0.5 and 2 hours of exposure of the preparation (Ferreira, Herman, & Vane, 1976). In any case, a non-specific decrease in the excitability of the tissue would not necessarily be expected to change the ED_{50} of an agonist.

It should be noted that the apparent K_bs of indomethacin as an antagonist of the actions of arachidonic acid, AM, and NLE#3 FFA are all within the same order of magnitude. An explanation may be that since indomethacin is a time dependent inhibitor, its effects may not be so sensitive to competition from non-cyclo-oxygenase substrate fatty acids. The K_b against arachidonic acid is similar to that reported for the K_i in the sheep seminal vesicle preparation, without correction for the time dependent inhibition (Ku & Wasvary, 1973).

(c) Tetrodotoxin.

Tetrodotoxin is known to inhibit Na^+ dependent action potentials (Kao, 1966; Dettbarn, 1971;

Cuthbert, 1976) and has been used to inhibit neuronal firing in the rabbit ileum as a method of chemical denervation (Gershon, 1967b). Bearing in mind the existence of Ca^{2+} dependent action potentials in neurons of intestinal preparations (North, 1973), tetrodotoxin (TTX) may be used to determine if pharmacological agents require nerve firing for their actions; or, more specifically, Na^{+} dependent actions potentials. Therefore, TTX may be considered to be a more generally acting ganglionic transmission depressant than any of the transmitter specific ganglionic inhibitors (e.g., hexamethonium). Inhibition by TTX of the effect of AM would be evidence that neuronal firing is required for the expression of AM's effect. One interpretation would be that AM acts prior to the initiation of the action potential; in other words, at the cell soma or other action potential initiation site. Another interpretation might be that AM works at the synaptic bouton but requires action potentials for its effect.

When matched doses of ACh or ABMeCh, nicotine, and AM were exposed to 3.1×10^{-7} M TTX, there was no effect on the action of ACh or ABMeCh, the action of nicotine was blocked or reversed (i.e., converted from a stimulation to an inhibition), and the action of AM was decreased by 60% ($p<0.005$, $n=5$) while the nature of the action was converted from a slow stimulation to a rapid stimulation more like the action of PGE (Horton & Main, 1965). Increasing the concentration of TTX beyond 3×10^{-7} M

had no greater inhibitory action. In the atropinized rabbit ileum, TTX had the same action on the atropine-fast effects of AM and nicotine (n=3). In 2 experiments, comparing the action of TTX on the effects of PGE_2, $PGE_{2\alpha}$, and arachidonic acid with ABMeCh or AM, the effects of PGE_2 were decreased 76% and 37%; $PGF_{2\alpha}$, 68% and 79%; arachidonic acid, 91% and 50%; ABMeCh, not tested and -2%; and AM, 91% and not tested.

When cumulative concentration-effect curves were determined before and after exposure of the rabbit ileal preparations to Tyrode's solution containing 3.1×10^{-7} M TTX, no significant decrease in the EMAX or increase in the ED_{50} of any agonist occurred. However, inspection of table 7 shows a trend toward a decrease in the EMAXs of PGE_2, $PGF_{2\alpha}$, arachidonic acid, and AM and an increase in the Ed_{50}s of PGE_2 and $PGF_{2\alpha}$. On the other hand, there is a trend toward an increase in the EMAX and a decrease in the ED_{50} of the AVE CH. These data are suggestive of a difference in the effect of TTX on the actions of cholinergic agonists compared with the actions of prostaglandins or prostaglandin/thromboxane precursors.

e. Pharmacological screen.

As was mentioned in Sections II.B.1.a. and d., a preliminary pharmacological screen was employed to attempt to determine the mechanism of action of the lipid fraction on the rabbit ileum. The results of the pharmacological screen are of interest as they confirm the similarity of

TABLE 7

EFFECT OF 3 x 10^{-7} M TETRODOTOXIN ON THE EMAXs AND ED_{50}s OF AVE CH, PGE_2, $PGF_{2\alpha}$, 20:4w6, AND AM

Agonist	n	$EMAX_{pre}$	$EMAX_{post}$	p	ED_{50pre}	ED_{50post}	p
AVE CH	6	5.8	7.8	>0.89	7.5 x 10^{-8} M	6.6 x 10^{-8} M	>0.36
PGE_2	4	7.4	4.0	<0.09	6.6 x 10^{-8} M	2.2 x 10^{-7} M	<0.09
$PGF_{2\alpha}$	4	5.8	3.1	<0.09	1.6 x 10^{-9} M	5.5 x 10^{-9} M	<0.09
20:4w6	5	6.3	3.5	<0.08	3.0 x 10^{-6} M	3.5 x 10^{-6} M	≥0.35
AM	3	6.4	1.6	<0.07	15.1 µl	23.1 µl	>0.37

the actions of the lipid fraction and of arachidonic acid and they provide some insight into the mechanism of action of fatty acids on the isolated rabbit ileum.

(1) Cholinergics.

Hemicholinium-3 inhibits the actions of cholinergic nerves by blocking the uptake of choline by the synaptic bouton and thereby depleting the store of ACh available for release upon stimulation (Gardiner, 1961; Birks & MacIntosh, 1961). In the guinea-pig ileum, hemicholinium-3 (HC-3) caused a progressive decline in the effect of AM reaching about 85% inhibition after an hour; previously equiactive doses of ACh or bradykinin were unaffected (Green, Carlini, & Robinson, 1963). In a pair of experiments on the rabbit ileum, the action of AM was decreased by only 20% after an hour of exposure to Tyrode's solution containing 3×10^{-5} M HC-3 (Bentley, 1962). Previously equiactive doses of ACh were not decreased. In a subsequent experiment, the atropine-fast stimulation of the rabbit ileum by matching doses of NLE#3, arachidonic acid, and nicotine did not appear to be affected by the addition of 3×10^{-5} M HC-3. This latter result implies that the atropine-fast nicotine (and fatty acid) stimulation of the rabbit ileum is not due to the release of ACh which subsequently interacts with a class of ACh receptor insensitive to muscarinic antagonists.

In two experiments, rabbit ileum preparations stored overnight responded to ACh while AM was without effect.

(2) Tryptaminergics.

Rabbit intestine is known to release 5-hydroxytryptamine (5-HT, serotonin) from the serosal side of the tissue (Gwee & Yeoh, 1968), and, by analogy to the guinea-pig ileum, it may contain 5-HT interneurons (Robinson & Gershon, 1971). In view of this, it was considered possible that AM might act directly by stimulating the 5-HT interneurons causing the release of 5-HT or by releasing another transmitter which in turn might stimulate the 5-HT interneuron.

The primary action of 5-HT on the rabbit intestine appears to be excitatory and via neuronal stimulation, as TTX reduces the response to exogenously added 5-HT to a small remnant which is blocked by 3×10^{-7} M methysergide (Gershon, 1967b). Because of the apparent neural component of 5-HT's action, phenylbiguanide, which has been described as a neuronal 5-HT receptor antagonist (Fastier, McDowall, & Waal, 1959; Drakontides & Gershon, 1968) was used in addition to the smooth muscle 5-HT receptor antagonist, methysergide. The response to 5-HT is complex, as the contraction of the rabbit intestine is often preceded by a relaxation, which is completely blockable by TTX (Gershon, 1967b).

Seven experiments using desensitizing concentrations of 5-HT (1×10^{-4} M) demonstrated no consistent effect on the action of AM. Methysergide, at a concentration of 1×10^{-6} M, potentiated the effect of AM

100 $\pm$ 10% (n=3, $p < 0.02$) while previously equiactive doses of 5-HT and ABMeCh were decreased 80$\pm$15% (n=3, $p < 0.05$) and increased 80$\pm$25% (n=3, $p < 0.1$), respectively. Phenylbiguanide alone, at concentrations ranging from 3×10^{-6} to 1×10^{-4} M, did not appear to affect the actions of AM, ABMeCh, or 5-HT in the one experiment in which it was tested. When methysergide, 1×10^{-6} M, and phenylbiguanide, 1×10^{-4} M, were used in combination, the subsequent increases in the actions of AM and ABMeCh of 90 and 70%, respectively, were not significant compared with the increases caused by methysergide alone. However, it should be noted that phenylbiguanide itself increased the baseline pendular motion of the rabbit ileum by 100$\pm$10% (n=4, $p < 0.005$), either when added after methysergide (which itself had no effect, n=3) or in the one experiment in which it was tested without prior exposure of the preparation to methysergide.

(3) Histaminergics.

Although the preparation is not very sensitive to it, histamine (HA) contracts the rabbit ileum (Gershon, 1967b). For this reason, the effects of HA, at desensitizing concentrations, the H_1 antagonist, pyrilamine (mepyramine), and H_2 antagonist, metiamide were tested on the actions of AM, ABMeCh, and HA. At a desensitizing concentration of 3×10^{-5} M, HA had no effect on the action of AM or matching doses of ABMeCh, while previously equiactive doses of HA were blocked. Pyrilamine, 1×10^{-6} M, had no significant effect on the action of AM or ABMeCh while

previously equiactive doses of HA were decreased $84\pm10\%$ n=5, $p < 0.005$). When 3×10^{-5} M metiamide was added to the Tyrode's solution containing 1×10^{-6} M pyrilamine, there was no further effect on the action of AM.

(4) Adrenergics.

Finkleman demonstrated in 1930 that stimulation of the splachnic nerve fibers (perivascular nerves) running to the isolated rabbit gut caused a decrease in the amplitude of the pendular motion and a relaxation of the "tone" of the preparation. Gershon (1967a) concluded that the effect was due to direct action of the released norepinephrine on the intestinal smooth muscle. Although there is evidence to suggest that, in the guinea-pig ileum, the primary action of adrenergics interacting on alpha receptors is via inhibition of ACh release (Paton & Vizi, 1969; Kosterlitz, Lydon, & Watt, 1970), the beta receptors do appear to be directly on the rabbit intestinal smooth muscle (Bowman & Hall, 1970). In view of the stimulatory nature of the action of AM, it was not expected that adrenergic antagonists would inhibit its effect. However, the possibility existed that AM might act by decreasing the release or effect of norepinephrine within the rabbit ileum.

In one experiment, epinephrine at a concentration of 6.6×10^{-5} M completely blocked the pendular motion of the rabbit ileum and decreased its tone. When AM was added during the blockade of the pendular motion, there was no apparent effect. On the other hand, when

5×10^{-9} M epinephrine was added at the peak effect of AM, the pendular motion and tone immediately were decreased by the same amount as when the epinephrine was added in the absence of AM (n=2). Propranolol, a beta adrenergic antagonist with local anaesthetic properties, at a concentration of 3×10^{-6} M, decreased not only the inhibition of the pendular motion caused by isoproterenol but also the actions of matching doses of AM and ACh and the pendular motion itself (n=3). These results suggest that the inhibition of AM and ACh was due to the local anaesthetic effect of the propranolol. Sotalol, a beta adrenergic antagonist without local anaesthetic properties, at concentration ranging from 1×10^{-6} M to 3×10^{-4} M, appeared to slightly potentiate the actions of AM and ABMeCh at concentrations above 5×10^{-6} M; however, the increases of $24 \pm 10\%$ (n=4, $p < 0.1$) and $19 \pm 13\%$ (n=4, $p > 0.2$) were not significant. The effect of isoproterenol was blocked with a K_b of 1×10^{-7} M, which approaches the literature value of 2×10^{-7} M (Salimi, 1975).

Phentolamine, an alpha adrenergic antagonist, at a concentration of 2×10^{-6} M, blocked the action of half maximal doses of phenylephrine, had no effect on the action of AM, and increased the action of matching doses of ABMeCh $23 \pm 3\%$ (n=3, $p < 0.01$).

In four experiments, when a combination of phentolamine (2×10^{-6} M) and sotalol (3×10^{-5} M) were used, the actions of phenylephrine and isoproterenol were blocked while the actions of AM were increased $55 \pm 15\%$ (n=4,

$p < 0.05$) and the actions of ABMeCh were unaffected (increased $6 \pm 8\%$ n=4, $p > 0.52$). The non significant increase in the action of AM by sotalol alone and the barely significant increase by the combination of phentolamine and sotalol are interesting in view of the recent report that sotalol potentiates the actions of $PGF_{2\alpha}$ on the isolated rat uterus (Narendranth & Sharma, 1977). However the potentiation is caused by (+)-sotalol, whereas (-)-sotalol, which is the stereoisomer with beta adrenergic receptor activity, was without effect. The sotalol used in the experiments reported in this dissertation was the racemic mixture.

2. Guinea-pig ileum preparation.

Since the pharmacological properties of AM were initially defined on the guinea-pig ileum, findings, made with the rabbit ileum which appeared to be at variance with the reported properties (Green, Carlini, & Robinson, 1963; Green & Carlini, 1964; Robinson et al., 1967), were checked on the guinea-pig ileum.

a. Agonists.

All the previous studies on the actions of AM had employed single doses which were matched by other agonists. Single doses of AM, NLE#3, or arachidonic acid, which were on the same order of magnitude as those causing maximal response of the rabbit ileum, did stimulate the guinea-pig ileum. However, it proved impossible to obtain cumulative concentration-effect curves, such as could be

obtained using the rabbit ileum, due to an apparent profound tachyphylaxis.

b. Antagonists.

(1) Mefenamic acid.

In a single experiment, 1×10^{-5} M mefenamic acid decreased the action of previously approximately equiactive repeated doses of AM and ABMeCh by 95 and 100% and by 73 and 80%, respectively. The EMAX and ED_{50} for ABMeCh were 1.09 and 5.8×10^{-8} M for the control periods and 0.93 and 6.6×10^{-8} M during exposure to Tyrode's solution containing 1×10^{-5} M mefenamic acid; the apparent K_b for mefenamic acid as an antagonist of ABMeCh was 7.3×10^{-5} M. The EMAX and ED_{50} for AM were 0.21 and 0.7 µl for the control period and 0.21 and 20.7 µl during exposure to Tyrode's solution containing 1×10^{-5} M mefenamic acid; the apparent K_b for mefenamic acid as an antagonist of AM was 3.7×10^{-7} M. The results suggest that the effect of AM on the guinea-pig ileum is antagonized by mefenamic acid in a manner consistent with mefenamic acid's actions as a cyclo-oxygenase inhibitor, while the effects of single low doses of ABMeCh may have been diminished due to a change in the length-tension relationship for the preparation (Bozler, 1976; Paton, 1975). The antagonism of ABMeCh by mefenamic acid is probably physiological since the apparent K_b of 7×10^{-5} M is more than two log units greater than the K_b against AM and 73 times that for inhibition of the sheep seminal vesicle cyclo-oxygenase (Rome & Lands, 1975b).

(2) Tetrodotoxin.

In two experiments, the effect of single doses of AM was completely blocked by 3×10^{-7} M TTX; in one experiment, increasing the dose of AM 100 fold did not restore any activity. In the other experiment, matching single doses of ABMeCh, nicotine, and AM were decreased 65%, 100%, and 100%, respectively. The inhibitory effect of TTX on the action of ABMeCh was more apparent than real. When complete concentration-effect curves for ABMeCh were obtained, the EMAXs were 1.16 and 1.24 for the controls and 1.02 and 0.96 for the curves obtained in the presence of TTX while the ED_{50}s were 1.0, 1.4, and 1.1, 1.5×10^{-7} M, respectively. The results of the experiments with TTX on the guinea-pig ileum are consistent with those on the rabbit ileum, indicating that the effect of AM requires the presence of Na^{+} dependent action potentials for its expression.

3. Frog neuromuscular junction preparation.

The release of neurotransmitter substances at chemical synapses has been the subject of intensive research since Loewi's demonstration of neurohumoral transmission from the vagus nerve to the frog heart (Loewi, 1921). To summarize the elegant description by Sir Bernard Katz, the action potential wave invades the nerve terminal and the subsequent depolarization causes an increase in the probability that the intracellular vesicles containing the neurotransmitter substance will interact successfully with release sites on the inside of the synaptic bouton. When the contents of a

vesicle are released into the synaptic cleft, the transmitter substance diffuses across the synaptic gap and interacts with receptors on the postsynaptic membrane. The transmitter-receptor interaction may stimulate the flow of ions across the postsynaptic membrane generating a postsynaptic potential. At the neuromuscular junction, the postsynaptic potential is called the "end-plate potential". The end-plate potential is caused by the electrical summation of the postsynaptic potential generated by the "simulataneous" release of many quanta from the presynaptic terminal (Katz, 1969).

In the absence of nerve impulses, there remains a diminished probability that a vesicle will successfully interact with a release site. Should the vesicle do so, it will cause a postsynaptic event comparable to the end-plate potential but of much smaller magnitude. This postsynaptic event is called a "miniature end-plate potential" (Fatt & Katz, 1950, 1952).

Miniature end-plate potentials (mepps) vary in both their amplitude and frequency of occurrence. When recording mepps using an intracellular electrode inserted in a single muscle fiber at the frog neuromuscular junction, the mean mepp amplitudes vary directly with the fiber resistance and inversely to the 3/2 power with the fiber diameter (Katz & Thesleff, 1957). The distribution of mepp amplitudes is normal with a coefficient of variation ($o/\bar{X}$) of approximately 0.25. The recorded amplitude may vary due

to changes in the presynaptic terminal or postsynaptic membrane. If the supply of neurotransmitter is interrupted as by decreasing choline uptake with hemicholinium-3 (Gardiner, 1961), the amplitude of the mepps decreases with time (Elmqvist & Quastel, 1965). Modifications of the postsynaptic membrance or receptor may also change the amplitude of the mepps. Anything that decreases the postsynaptic membrane resistance, such as increasing the pH of the bathing solution, will decrease mepp amplitude (Del Castillo, Nelson, & Sanchez, 1962). Pharmacological blockade of the neurotransmitter receptors will also decrease mepp amplitude. However, pharmacological manipulations, such as inhibiting the metabolism of the neurotransmitter, may also increase the mepp amplitude.

The frequency of mepps increases with temperature (Boyd & Martin, 1956), osmotic pressure (Fatt & Katz, 1952; Hubbard, Jones, & Landau, 1968), and presynaptic depolarization (Del Castillo & Katz, 1954; Liley, 1956). Botulinum toxin, which interferes with transmitter release by cholinergic nerves, decreases mepp frequency to zero (Brooks, 1956).

While there are many interrelations between pre- and post synaptic conditions,

"A useful generalization is that, except for metabolic effects such as that produced by hemicholinium, the frequency of miniature potentials appears to be controlled by the state of the presynaptic terminal, whereas

the amplitude of these potentials is determined by the state of the postsynaptic membrane." (Auerbach, 1972).

With these concepts in mind, the results of the experiments on the effects of the lipid fraction on the frog sciatic-sartorius neuromuscular junction are presented below.

The frequency of miniature end-plate potentials was unaffected by exposure to the lipid fraction suspended in Ringers solution. Table 8 summarizes the results of four separate experiments and shows that there is no significant difference between the mepp frequencies before and after exposure to the lipid fraction. The forty-fold increase in mepp frequency after exposure to the lipid fraction during experiment #1 could not be repeated. However, it should be noted that the lipid fraction was stored in chloroform, and that the laboratory conditions at the Marine Biological Laboratory, where experiment #1 was conducted, prevented the evaporation of the lipid fraction to dryness under a stream of N_2. Since the lipid fraction was evaporated at room temperature in open air in a beaker, it is possible that some chloroform remained in the beaker. When 0.1 ml of chloroform was added to a muscle preparation, the resultant tracings (fig. 14a) were very similar to those of the initial experiment (fig. 14b).

In no experiment was the amplitude distribution of the mepps affected. The results of experiment #4 are shown in fig. 15. The mean mepp amplitude was approximately

TABLE 8

THE EFFECT OF THE LIPID FRACTION (AM) ON MEPP FREQUENCY

Experiment #	Mepp Frequency (Hz) Pre AM	Post AM	Δ = Post - Pre
1	0.83	35.3	34.5
2	5.68	1.23	-4.45
3	2.01	2.12	0.11
4	0.35	0.24	-0.11
mean	2.22	9.72	7.51*
SEM	1.21	8.53	9.05

*Probability of no significant difference is p = 0.468

Fig. 14. The effect of air dried lipid fraction or of 0.1 chloroform on the frequency of mepps at the frog sciatic sartorius neuromuscular function.

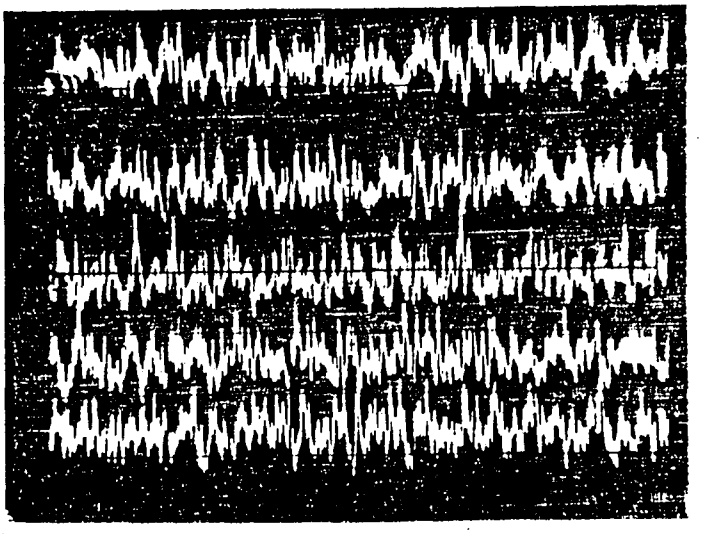
a. The effect of the lipid fraction (active material) in experiment #1 on the frequency of mepps.

The tracing was made approximately 1 hr. after flooding the lipid fraction (which had been prepared by allowing the chloroform to evaporate in air and then suspending the residue in MBL Frog Ringer's solution) over the preparation. One vertical division is equivalent to 2. mV, and one horizontal division is equivalent to 1 sec.

b. The effect, in a different experiment, of flooding 0.1 ml of chloroform over the preparation.

One vertical division is equivalent to 0.5 mV, and one horizontal division is equivalent to 20 m sec.

a.



b.

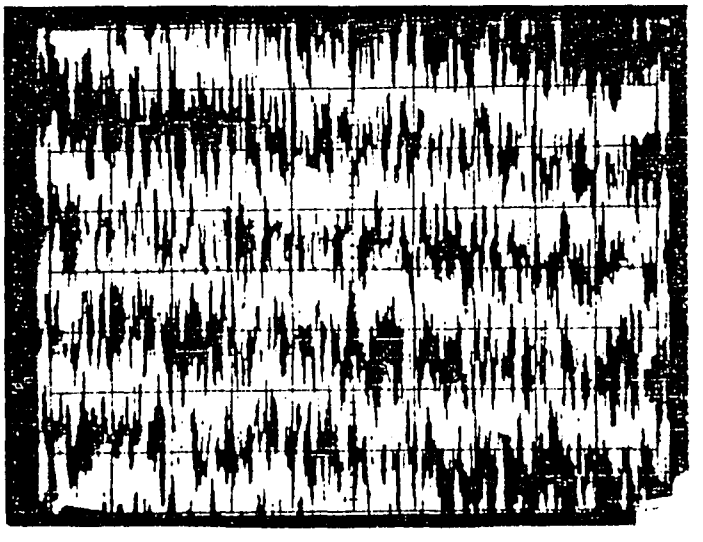
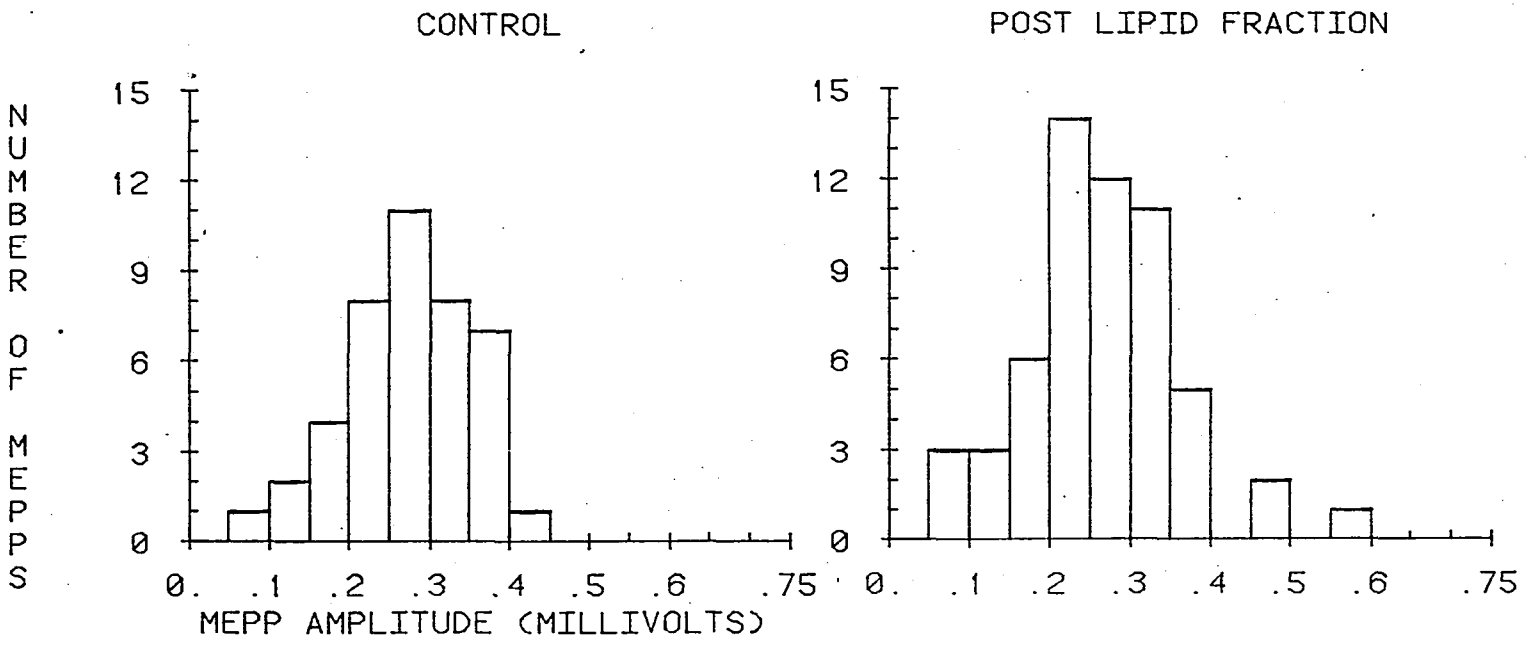


Fig. 15. Histograms of the miniature end-plate potential (mepp) amplitude distributions pre- and post-exposure to the lipid fraction.

Mepp amplitudes were obtained by comparison with calibration voltages. The lipid fraction added to the muscle bath had a biological activity, as measured on the guinea-pig ileum, equivalent to 8×10^{-5} M ACh.

a. Control: mean mepp amplitude of 0.27 ± 0.01 mV (mean $\pm$ SEM, n = 42).

b. Post exposure to the lipid fraction: mean mepp amplitude of 0.26 ± 0.01 mV (n = 52).


CONTROL
POST LIPID FRACTION
NUMBER OF MEPPS
15
12
9
6
3
0
0.
.1
.2
.3
.4
.5
.6
.75
MEPP AMPLITUDE (MILLIVOLTS)

0.3 mV and the coefficient of variation was about 0.3.

In an experiment to examine the effects of the lipid fraction on stimulus evoked end-plate potentials, no change in the amplitude or duration of the end-plate potential was apparent after exposure to the lipid fraction.

During experiment #1 fasiculations were evident in the muscle. As with the increased mepp frequency, only the addition of 0.1 ml chloroform produced a similar result.

The conclusion to be drawn from the electrophysiological analysis of the lipid fraction is that the lipid fraction does not function as a universal cholinergic releasing agent that does not require the presence of action potentials to produce its effect. In light of the linkage of the lipid fraction to the prostaglandin/thromboxane system, the lack of effect on mepp frequency or amplitude agrees with the findings of Gripenberg, Jansson, Heinänen, Heinonen, Hyvärinen, and Tolppanen (1976). Using a cut rat diaphragm, they showed that 1×10^{-7} M PGE_1 had no effect on either mepp frequency or mepp amplitude. While a lack of response to one dose of a single prostaglandin in a different species does not preclude an effect of prostaglandins and/or thromboxanes on mepp frequency or amplitude, these findings are consistent with the results reported in this dissertation.

Gripenberg et al., (1976) also reported that PGE_1 did cause intermittent failures in the generation of the end-plate potential in response to repeated indirect

stimulation. Though the current study reports no effect of the lipid fraction on the end-plate potential, Gripenberg et al. stated that "the effect occurred only at 36-38°C when the nerve was stimulated at 30-80 Hz." (Gripenberg et al., 1976). Since the conditions of temperature and frequency used in the current study did not fall within the specified limited range, it is not surprising that this study did not uncover the effect.

Stimulation of the rat phrenic nerve-diaphragm preparation has been to release prostaglandins (Ramwell, Shaw, & Kucharski, 1965), but the report by Gripenberg et al. (1976) is the first report of prostaglandin effects on the release of acetylcholine by the neuromuscular junction. Indeed, in his 1976 review, Wolfe wrote the following, "There is no evidence that prostaglandins have any action on the release of acetylcholine or on the effector responses at nicotinic cholinoreceptors in sympathetic ganglia or at the neuromuscular junction." (Wolfe, 1976).

4. Frog skin preparation.

Prostaglandins have been implicated in the maintenance of the permeability and sodium transporting capability of the isolated frog skin (Fassina, Carpenedo, & Santi, 1969; Ramwell & Shaw, 1970; Barry, Hall, & Martin, 1975; Hall, O'Donoghue, O'Regan, & Penny, 1976; Haylor & Lote, 1976; Hall, O'Regan, & Quigley, 1977). Frog skin has been demonstrated to synthesize prostaglandins (Ramwell & Shaw, 1970, Hashikawa, Sakurai, & Susuki, 1971; Hall et al., 1976).

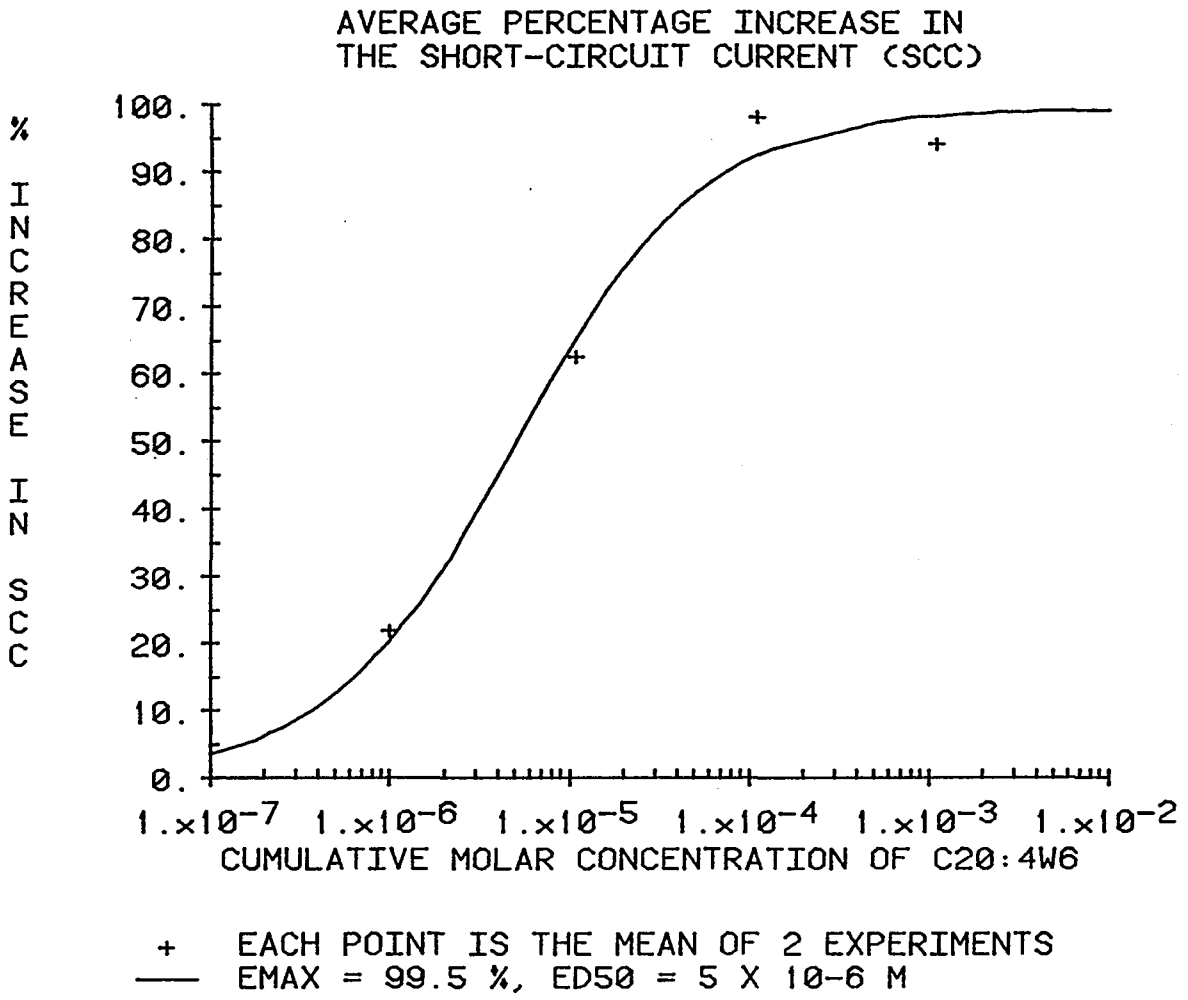
Recently, the mechanism of aldosterone on the frog skin has been linked to the prostaglandin/thromboxane system (Yorio & Bentley, 1978).

When a dose, which had a half-maximal effect on the rabbit ileum, of the lipid fraction was added to the blood side of the frog skin preparation, it caused a slow increase in the short circuit current (scc) from 22 $\mu A/cm^2$ to 35 $\mu A/cm^2$. The effect reached a peak at approximately 55 min., by which time the open circuit potential difference (pd) had increased from 13 mV to 25 mV. Repetition of the original dose of the lipid fraction at the peak of its effect had no further effect.

Following the linkage of the lipid fraction to the prostaglandin/thromboxane system, several experiments were performed testing the effects of arachidonic acid on the isolated frog skin preparation. Arachidonic acid was found to increase both the scc and the pd in a dose-related manner. The threshold dose was approximately 1×10^{-6} M, and the maximum effect occurred at a concentration of approximately 1×10^{-4} M. The concentration-effect curve for arachidonic acid (20:4w6) on the scc is shown in fig. 16. The effect was blocked by 1×10^{-5} M mefenamic acid; this concentration is approximately ten times the K_i of mefenamic acid as a competitive inhibitor of bovine seminal vesicle fatty acid cyclo-oxygenase (Rome & Lands, 1975b). Amiloride, 1×10^{-5} M which is a concentration known to inhibit active sodium transport in amphibian skin (Bentley, 1968; Ehrlich

Fig. 16. Average percentage increase in the short-circuit current (SCC).

Cumulative concentration-effect curve for arachidonic acid (C20:4w6) stimulation of the SCC. Each point is the mean of two experiments.


AVERAGE PERCENTAGE INCREASE IN
THE SHORT-CIRCUIT CURRENT (SCC)
% INCREASE IN SCC
100.
90.
80.
70.
60.
50.
40.
30.
20.
10.
0.
1.x10-7 1.x10-6 1.x10-5 1.x10-4 1.x10-3 1.x10-2
CUMULATIVE MOLAR CONCENTRATION OF C20:4W6
+ EACH POINT IS THE MEAN OF 2 EXPERIMENTS
—— EMAX = 99.5 %, ED50 = 5 X 10-6 M

& Crabbé, 1968; Crabbé & Ehrlich, 1968), added to the external surface of the preparation at the peak effect of arachidonic acid, dropped the scc to zero (Yorio, Bentley, & Bergamini, unpublished).

These results are consistent with the findings of Hall (1973) and Hall et al. (1976) that arachidonic acid increased the scc across the frog skin with a maximum effect of about a 50% increase and with an ED_{50} of approximately 3×10^{-5} (estimated from fig. 2 of Hall et al., 1976). The pd was affected in a similar manner. Paracetamol (4-acetamidophenol), a fatty acid cyclo-oxygenase inhibitor (Flower & Vane, 1972) which may function at the activator site (Lands, Cook, & Rome, 1976), inhibited the arachidonic acid induced increase in the scc with a K_i of approximately 8×10^{-5} M (calculated from fig. 2 of Hall et al., 1976).

In summary, the effects of the lipid fraction on the isolated frog skin are not inconsistent with the effects that would be expected from a fatty acid mixture that contained a considerable proportion of arachidonic acid.

5. Frog rectus abdominus preparation.

The frog rectus abdominum preparation has been classically described as responding to nicotinic agonists. Though the actions of the lipid fraction on the guinea-pig ileum were not blocked by the ganglionic cholinergic antagonists, hexamethonium (9.9×10^{-5} M) or tetraethylammonium (3.1×10^{-4} M), the initial results from the rabbit ileum

preparation suggested that the lipid fraction might be acting in an entirely different manner than had been postulated for the guinea-pig ileum (Green, Carlini, & Robinson, 1963; Green & Carlini, 1964). Therefore, with the assistance of Dr. L Allen Barker, a frog rectus abdominus preparation was used as part of the pharmacological screen.

A dose of the lipid fraction, which caused an increase in the amplitude of the pendular motion of the rabbit ileum equivalent to the increase caused by 5.5×10^{-7} M ACh, was added to the organ bath containing the rectus. Since the rectus organ bath had half the volume of the rabbit ileum organ both, the concentration of the lipid fraction was doubled. However, as can be seen in fig. 17a there was no discernible response of the rectus after an exposure period of 6 min. An additional dose of the lipid fraction ten times larger than the previous dose was added, and no response was observed over an additional exposure period of 6 min. (fig. 17c). At the end of this period, a single dose of ACh was added to the preparation. The effect of this dose of ACh was equivalent to that of previous doses of ACh. The results of this experiment are shown in fig. 17, which was redrawn from the original polygraph recording. The dose of ACh (2×10^{-7} M) used was slightly less than that (2.7×10^{-7} M) having a half maximal effect (fig. 18).

The results from the frog rectus abdominus preparation were taken to indicate that the lipid fraction did not act via neuromuscular nicotinic acetylcholine receptors.

Fig. 17. The effect of the lipid fraction and ACh on the frog rectus abominus preparation.

The figure is redrawn from the original polygraph recording. The vertical bar is equal to one chart division (200 mg); the horizontal bar is equal to 1 min: a) Control period of 6 min; b) A dose of the lipid fraction having a slightly greater than half maximal effect for the lipid fraction on the rabbit ileum. This dose had an effect on the rabbit ileum equivalent to that of 5.5×10^{-7} M ACh. The ED_{50} for ACh on this particular rabbit ileum was 7.3×10^{-7} M; c) A dose of the lipid fraction ten times larger than the dose in part a; d) A dose of acetylcholine, 2×10^{-7} M, slightly less than the ED_{50}, 2×10^{-7} M, of ACh on this rectus.

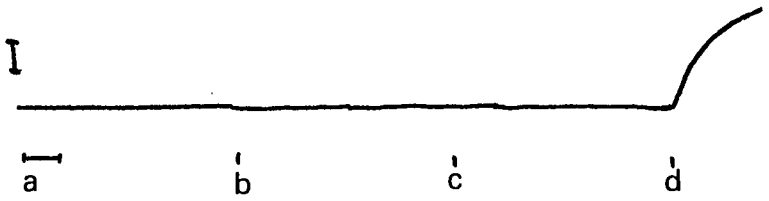
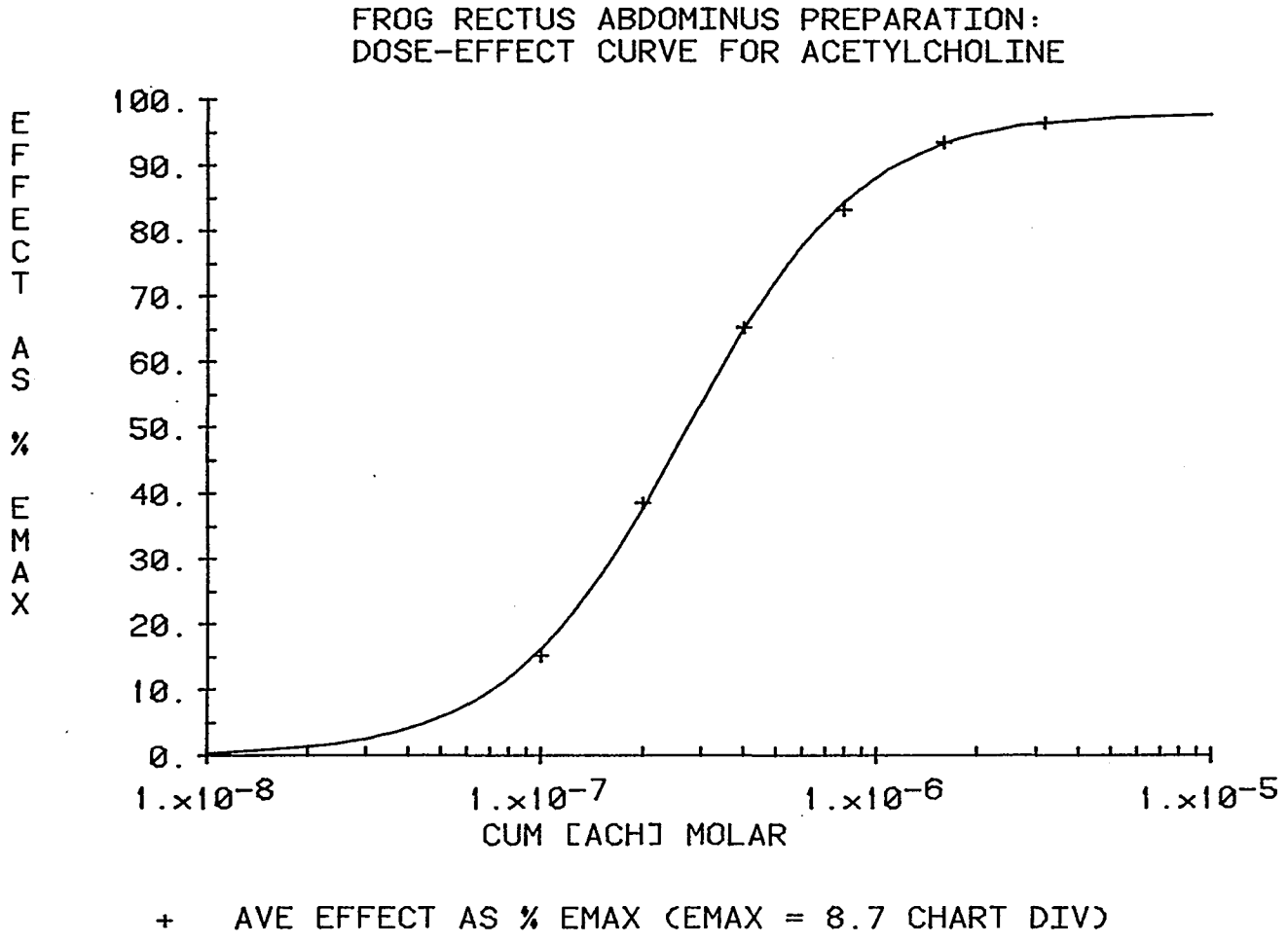

a
b
c
d

Fig. 18. The concentration effect curve of ACh on the same frog rectus abdominus preparation shown in fig. 17.

The curve is the average of two determinations in the same preparation. The ordinate is the effect as a percentage of the maximum effect (EMAX). The EMAX was equivalent to 8.7 chart divisions in fig. 17. The abscissa is the molar concentration of ACh. The ED_{50} was 2.7×10^{-7} M.


FROG RECTUS ABDOMINUS PREPARATION:
DOSE-EFFECT CURVE FOR ACETYLCHOLINE
EFFECT AS % EMAX
100.
90.
80.
70.
60.
50.
40.
30.
20.
10.
0.
1.×10⁻⁸
1.×10⁻⁷
1.×10⁻⁶
1.×10⁻⁵
CUM [ACH] MOLAR
+ AVE EFFECT AS % EMAX (EMAX = 8.7 CHART DIV)

III. STRUCTURAL ANALYSIS AND CHARACTERIZATION OF THE ACTIVE MATERIAL IN THE LIPID FRACTION

The purpose of the pharmacological analysis of an unknown compound(s) is not only to learn its biological 'fingerprint' and, where possible, its mechanism of action, but also to assist in the determination of the chemical structure.

Therefore, in addition to the pharmacological experiments on isolated tissues, qualitative organic analytical experiments were also performed.

A. Methods.

1. Chemical methods of analysis.

a. Chlorine-tolidine test for compounds convertible to chloramines.

A previously prepared 0.5 mm silica gel HR (E. Merck) 20x20 cm thin layer chromatographic (TLC) plate was heated for 1/2 hour at 125°C to activate the silica gel. The plate was removed and allowed to cool under a stream of N_2 in a TLC plate spotting box. Within 1 hr. of removal from the drying oven, samples of the lipid fraction, partially methylated lipid fraction, hydrogenated lipid fraction, nicotinamide, succinamide, succinimide, sphingomyelin, urea, and procainamide were spotted on the

TLC plate using a Hamilton 50 μ l syringe. The spot size was kept below 1 cm, and the plate was continually flushed with N_2 during the spotting. The spotted TLC plate was chromatographed using a mixture of petroleum ether: diethyl ether: acetic acid (90:10:1) until the solvent front was within 1 cm of the top of the plate. The TLC plate was air dried until the odor of acetic acid disappeared. The dried TLC plate was chlorinated by exposing it to a chlorine atmosphere for 15 to 20 min. The chlorine atmosphere was generated by adding concentrated hydrochloric acid to a 1.5% aqueous solution of potassium permanganate. The chlorinated chromatographed TLC plate was allowed to stand for 5 min. in air to remove excess chlorine. A corner of the chromatogram was sprayed with a solution made by dissolving 0.16 g o-tolidine in 30 ml glacial acetic acid diluted to 500 ml with distilled water and to which 1 g of potassium iodide had been added. If no color appeared, the entire plate was sprayed with a mist of the o-tolidine solution and observed for the development of a positive reaction. (#61 in Dyeing Agents for thin layer and paper chromatography (E. Merck Laboratories, 500 Executive Boulevard, Elmsford, New York, undated); Pataki, 1963).

b. Attempted reduction of the lipid fraction with sodium borohydride.

Two 0.5 ml aliquots of the lipid fraction dissolved in chloroform served as experimental and control. The aliquots were taken to dryness under a stream of

nitrogen, redissolved in 1.0 ml of methanol, and chilled in ice. Thirty mg of sodium borohydride was dissolved in 3.5 ml of methanol and chilled on ice. The lipid fraction and the sodium borohydride (experimental) or the lipid fraction and blank methanol (control) were combined and chilled at 4°C for 20 min. in a hood. The pH of the control was adjusted to rough equivalency to the experimental with sodium hydroxide. The reaction mixtures were incubated at room temperature for 1 hr. in a hood. Five ml of water were added to the reaction mixtures, and the solution was flash evaporated at room temperature. Both experimental and control were acidified with 1 N hydrochloric acid to pH 3.0, and the aqueous solutions were extracted three times with 10 ml diethyl ether. The ether extracts were combined, washed with 5.0 ml of distilled water, and brought to dryness at room temperature under a stream of nitrogen. The experimental and control were each redissolved in 0.5 ml of chloroform, and 10 ml aliquots of experimental, control, and untreated lipid fraction were thin layer chromatographed on a 0.5 mm silica gel HR 5 x 20 cm TLC plate using a solvent system of petroleum ether: diethyl ether (90:10) and stained with iodine vapor. When the results of the thin layer chromatography and infrared spectroscopy showed no apparent differences between the starting material, the control, and the experimental, the experimental was re-chromatogrammed to remove sodium borohydride on a previously prepared 0.5 mm silica gel HR 20x20 cm TLC plate, using

petroleum ether: diethyl ether (90:10). A one cm edge strip was stained with iodine vapor. The rest of the lipid fraction band was scraped off and extracted three times with chloroform. The chloroform extract was brought to dryness under a stream of nitrogen. The control was also brought to dryness under a stream of nitrogen. Both were dissolved in 1.0 ml aliquots of ethanol and chilled on ice. One hundred fifty mg of sodium borohydride was dissolved in 19 ml of ethanol and added to each of the experimental and control samples at room temperature. The control and experimental reaction mixtures were refluxed at 100^{o}C for 4 hrs. Distilled water (20 ml) was added to the reaction mixtures, and they were flash evaporated until the ethanol started to boil. The samples were then acidified with 1 N hydrochloric acid to approximately pH 3 and extracted thrice with 25 ml portions of diethyl ether. The diethyl ether phase was washed once with 25 ml of distilled water, decanted, and taken to dryness with a stream of nitrogen. The residue was dissolved in approximately 0.5 ml of chloroform, and both analytical and preparative TLC were performed. The TLC band was scraped off the preparative plate and extracted three times with 15 ml aliquots of chloroform through an ultrafine fritted glass filter funnel. The chloroform was concentrated under a stream of nitrogen, and infrared spectroscopic analysis was performed (Jones & Wise, 1962; Fieser & Fieser, 1967, p. 1050).

c. Determination of the effects of suspension in Tyrode's solution on the thin layer chromatographic properties of the lipid fraction.

Twenty µl aliquots of lipid fraction from the chloroform stock solution or the equivalent amount of lipid fraction suspended in Tyrode's solution were placed in 13 ml centrifuge tubes. All samples were taken to dryness under a stream of nitrogen. To one of the aliquots taken from the chloroform stock solution, 40 microliters of Tyrode's solution was added, and the sample was again taken to dryness under a stream of nitrogen. Five ml of chloroform:methanol (2:1) was added to each tube, and the samples stored overnight. One ml of distilled deionized water was added to each tube, and the samples were Vortex'd three times for ten seconds each. Samples were centrifuged at 600 x g for 10 min., and the upper phase removed with a Pasteur pipette. The separated phases were taken to dryness under a stream of nitrogen. Fifty µl of chloroform was added to each lower (chloroform) phase sample, and the entire sample was spotted on a 0.5 mm previously prepared silica gel HR TLC plate. Fifty µl of ethanol was added to each upper (methanol/water) phase sample, and the entire sample spotted on a TLC plate. The TLC plates were developed in petroleum ether: diethyl ether: acetic acid (90:10:1), air dried until the odor of acetic acid disappeared, and stained with iodine vapor.

d. Alcoholic potassium carbonate extraction of the lipid fraction from organic solvent.

(1) Qualitative.

Six aliquots each of roughly equivalent amounts, as determined by iodine staining, of organic material were taken from ethanol or chloroform stock solutions of arachidonic acid and lipid fraction. One aliquot of each was combined, and the eleven samples taken to dryness under a stream of nitrogen. Hexane, 0.1 ml, was added to one of the lipid fraction samples, one of the arachidonic acid samples, and to the combined sample. Each of these was spotted separately on a previously prepared 0.5 mm silica gel HR TLC plate (20x20 cm). Each of the remaining samples of lipid fraction was treated differently. To one was added only 1 ml hexane; the second received the hexane plus 1 ml ethanol: water (1:1); the third, hexane plus 1 ml water, and the fourth, hexane plus 1 ml of 1.4 N K_2CO_3. The four arachidonic acid samples were treated similarly. The mixtures were Vortex'd, and the hexane (upper) phase was removed by Pasteur pipette, taken to dryness under a stream of nitrogen, dissolved in 0.1 ml of hexane and spotted on the TLC plate. The spotted TLC plate was developed in hexane:diethyl ether:acetic acid (90:10:1), air dried, and stained with iodine vapor. A subsequent experiment repeated the above with the addition of samples extracted with ethanol:2.8 N (20%) aqueous potassium carbonate (1:1), 1 N (6.9%) aqueous potassium carbonate

and 0.1 N (0.69%) aqueous potassium carbonate.

(2) Quantitative.

A 200 ml aliquot was withdrawn from the NLE#3 sample of the lipid fraction; the remainder was stored for comparison testing by bioassay. The 200 ml aliquot was concentrated by flash evaporation to yield 2.11 g of a yellow grease. This residue was dissolved in 200 ml of hexane, and the hexane solution was extracted three times with 150 ml of 10% (1.4 N) K_2CO_3 (aqueous): methanol (1:1). The organic phase was flash evaporated yielding 1.97 g of material. The methanolic K_2CO_3 phases containing the free fatty acids (FFA) were combined, chilled in an ice bath, and brought to approximately pH 4 with 1.0 N HCl. It was noted that white solid material floated to the surface as the solution was acidified. The acidified methanolic K_2CO_3 was extracted three times with 300 ml portions of hexane. These hexane extracts, containing the FFA, were combined and washed three times with 200 ml of water. The washed hexane (FFA) extracts were dried over Na_2SO_4 and then flash evaporated to dryness. The residue was weighed and then taken up in 300 µl of hexane for quantitation. The organic phase from the original aqueous K_2CO_3/methanol extraction was twice resubjected to the same procedure with five extractions with aqueous K_2CO_3/methanol per procedure instead of three.

The fatty acids extracted from the NLE#3 were either bioassayed or converted to their methyl

esters for quantitation by GLC. An aliquot of the hexane fatty acid solution was taken to dryness under a stream of nitrogen and dissolved in benzene. Three hundred μl of BF_3-methanol solution (Supelco) was added to the benzene solution. The mixture was refluxed for 1 hour, and the organic phase was washed with 50 ml of 5% aqueous K_2CO_3 and then three times with 50 ml of water. The organic phase was dried over Na_2SO_4, flash evaporated, and taken up in 300 μl of hexane for chromatographic quantitation.

Bioassay was performed on aliquots from the original NLE#3 sample and from the organic phases remaining after each of the three extractions of the FFA. The aliquots were taken to dryness under a stream of nitrogen and suspended in Tyrode's solution in a test tube by means of a Vortex mixer and glass stirring rod.

2. Physical method of analysis.

a. Chromatographic.

(1) Thin layer chromatography of lipid fraction and fatty acid methyl esters.

Thin layer chromatographic plates were prepared as in Section III.A.1.a. except that silver nitrate, 6% w/w, was mixed with the silica gel HR. Care was taken to avoid exposure of the impregnated TLC plates to ultraviolet light (Cubero & Mangold, 1965).

(2) Gas-liquid chromatography of lipid fraction and fatty acid methyl esters.

Gas-liquid chromatography (GLC) of the

methyl esters of the lipid fraction and the hydrogenated lipid fraction was performed on a Fischer Victoreen gas chromatograph, model 4000-4M, equipped with a flame-ionization detector (O_2-750 ml/min, H_2 - 50 ml/min). The separation was performed using a 9 ft x 0.125 in (I.D.) coiled aluminum column packed with 3% Silar 10C coated 100/120 mesh Gas Chrom Q (Applied Science Laboratories, Inc.) and treated with trifluoroacetyl acetone and Silyl-8 (Pierce Chemical Co.) to inactivate any aluminum filings and silylate any exposed surfaces (Heckers, Dittmar, Melcher, & Kalinowski, 1977; Myher, Marzi, & Kukis, 1974; Takagi & Itabashi, 1977). The carrier gas was helium (40 ml/min). The temperatures of the injection port, detector, and column were 232°C, 242°C, and 178°C, respectively. Fatty acid methyl ester mixtures of known composition (GLC-60, GLC-100, PUFA-1, and PUFA-2, purchased from Supelco were chromatographed under identical conditions in order to provide standards for the comparison of retention times (Holman & Rahm, 1966 to 1970; Takagi, Sakai, Itabashi, & Hayashi, 1977).

b. Spectroscopic.

(1) Ultraviolet and visible spectroscopy of the lipid fraction.

The lipid fraction and the products of the hydrogenation and methanolic HCl reactions with the lipid fraction were dissolved in Fisher "spectro grade" n-heptane and placed in 1 cm x 3 mm quartz microcuvettes

(0.3 ml capacity). Difference spectra against a solvent reference were generated with Beckman "Acta II" or "Acta III" spectrophotometers.

(2) Infrared spectroscopy of the lipid fraction and fatty acids.

The lipid fraction, the products of the hydrogenation and methanolic hydrochloric acid reactions, and fatty acid standards were dissolved in Fisher spectro-grade chloroform or carbon tetrachloride and placed in a Perkin-Elmer 0.125 mm sodium chloride liquid sample cell. Difference spectra against pure solvent in a similar cell as a reference were run on a Perkin-Elmer grating infrared spectrophotometer, model 457.

(3) Nuclear magnetic resonance spectroscopy of the lipid fraction, its methyl esters, fatty acids, and their methyl esters.

Sample of the lipid fraction, methyl esters of the lipid fraction, hydrogenated lipid fraction, or known fatty acids and fatty acid methyl esters were taken to dryness under a stream of nitrogen and redissolved in chloroform. This process was repeated three times. The samples were then taken to dryness with nitrogen and dissolved in deuterated chloroform twice. Finally the samples were filtered through a glass wool plug in a Pasteur pipette into standard NMR sample tubes flushed with nitrogen.

Two hundred seventy MHz NMR spectra were

obtained with the Bruker HX270 superconducting NMR spectrometer at the Southern New England High Field NMR Facility (Yale University, Department of Chemistry) using tetramethylsilane as a standard.

c. Spectrometric.

(1) Gas chromatography/chemical ionization mass spectrometry of lipid fraction and fatty acid methyl esters.

Chemical ionization mass spectra were obtained on a Hewlett Packard 5930A dodecapole mass spectrometer. The Hewlett Packard 5710A gas chromatographic inlet system was equipped with a 67.1 m x 0.77 mm I.D. coiled glass capillary column, coated with Silar 10CP. The chromatographic conditions were: column temperature, 140°C; injector port temperature, 250°C, carrier gas, 25 ml/min (1 torr) of helium. The reagent gas was isobutane (0.35 torr). The ion source temperature was maintained at 165°C and the mass filter at 90°C. The source envelope pressure was 2×10^{-4} torr. The ionizing potential was 170 eV, and the ionizing current was 0.15 mA. Spectra from 100 - 650 amu were recorded and stored, and the background substracted, using a Hewlett Packard 5933A data system. Fatty acid identification was confirmed by the presence of the $(m+1)^+$ ion at a retention time comparable to standards. Unresolved total ion current (TIC) peaks and peaks of very low intensity were tentatively identified by comparing the TIC chromatogram with mass-fragmento-

grams of the various $(m+1)^+$ ions (Araki, Ariga, & Murata, 1976; Ariga, Araki, & Murata, 1977; Dommes, Wirtz-Peitz, & Kunau, 1976; Phillipou, Bigham, & Seamark, 1975).

B. Results and discussion.

1. Summary of previously known chemical properties of the lipid fraction.

The lipid fraction was originally isolated as an acetone extract of rabbit sciatic nerve (Green, Carlini, & Robinson, 1963). Acetone extracts of pig and bovine brain yielded similar biological activity (Green, Carlini, & Robinson, 1963; Green & Carlini, 1964; Robinson et al., 1967). The biological activity was increased three fold after heating the lipid fraction in a boiling water bath for 5 minutes at pH 10 (Green & Carlini, 1964). On the other hand, refluxing the lipid fraction in 1 N alcoholic KOH or HCL for one hour destroyed the biological activity (Robinson et al., 1967).

Silicic acid column chromatography of the lipid fraction revealed no correlation between the biological activity of the elutions and their content of "cholesterol, long chain aldehydes, and glycerol both after saponification (representing the fatty acid glycerides) and after subsequent hydrolysis (representing the alkenyl analogs of the glycerides)." (Robinson et al., 1967). Based upon the pattern of elution from the silicic acid column, the lipid fraction was classified as a nonpolar neutral lipid, specifically eliminating phospho- and sphingo-lipids.

Paper chromatography of the products of the hydrolysis with 1 N alcoholic KOH or HCl demonstrated no spots stainable by ninhydrin; this supported the contention that the lipid fraction does not include an amine or an amide (Robinson et al., 1967).

And, "finally, when either the crude lipid extract or the active eluate resuspended in petroleum ether was extracted with aqueous K_2CO_3, essentially all the activity remained in the petroleum ether, indicating that the active material was not an acid." (Robinson et al., 1967).

2. Chemical Methods.

a. Chlorine-tolidine test for compounds convertible to chloramines.

Although the original chemical analysis of the lipid fraction by Robinson et al. (1967) had revealed no ninhydrin-positive material, which might indicate that the lipid fraction was not a fatty acid amide of an organic amine, the results of the infra red spectroscopic analysis (cited in Section III.B.4.a.(2).) suggested the presence of either an amide or a carboxylic acid proton. Since there was evidence for the absence of a carboxyl function (Robinson, et al., 1967) a more specific test for the presence of functional nitrogen, the Chlorine-tolidine test for compounds convertible to chloramines (Pataki, 1963), was attempted.

Neither the lipid fraction, the partially methylated lipid fraction, nor the hydrogenated lipid

fraction gave any reaction to the test while nicotinamide, succinamide, succinimide, sphingomyelin, urea, and procainamide all gave positive reactions.

The results of the chlorine-tolidine test for compounds convertible to chloramines are not consistent with either an amide or imide nitrogen in either the lipid fraction, partially methylated lipid fraction, or hydrogenated lipid fraction.

b. Attempted reduction of the lipid fraction with sodium borohydride.

The results of the infrared spectroscopic analysis of the lipid fraction (cited in Section III.B.4.a.(2).) suggested the presence of a carbonyl group. The shoulder at 1750 cm^{-1} combined with the absence of detectable nitrogen in the chlorine-tolidine test and the previously published data on the lack of a carboxyl function led to the hypothesis that the carbonyl group might be a ketone. In order to test this hypothesis, a sodium borohydride reduction was attempted under conditions known to reduce aliphatic ketones (Jones & Wise, 1962; Fieser & Fieser, 1967, p. 1050).

The results of the attempted sodium borohydride's reduction of the lipid fraction are not consistent with the presence of a ketone group in the lipid fraction as no hydroxyl absorption bands appeared in the infrared spectra of the reaction products whose IR spectrum is listed in table 13.

In view of the previously published report indicating the lipid fraction was not an acid (Robinson et al., 1967) and of the results of the chlorine-tolidine test for compounds convertible to chloramines, the infrared spectra of the lipid fraction, with its absorption band at 1710 cm^{-1} and shoulder at 1745 cm^{-1}, was difficult to rationalize. Since sodium borohydride would not be expected to reduce an acid (Fieser & Fieser, 1967, p. 1050), the failure of the sodium borohydride reaction to produce an alcohol under conditions in which aliphatic ketones are reduced (Jones & Wise, 1967) is consistent with the subsequently determined acidic nature of the lipid fraction.

c. Determination of the effects of suspension in Tyrode's solution on the thin layer chromatographic properties of the lipid fraction.

A possibility existed that the suspension of the lipid fraction in Tyrode's solution was chemically altering the lipid fraction so as to increase its biological activity. Since unsaturated lipids are known to oxidize fairly readily to their hydroperoxides (Holman, 1966 to 1970; Mead & Fulco, 1976), it was considered possible that the method of suspending the lipid fraction was forming hydroperoxides which were having the smooth-muscle stimulating activity. Should such an event occur, the chromatographic properties of the lipid fraction should change dramatically as the hydroperoxides are much more polar, and, therefore, the amount of organic material near the

origin should increase.

The thin layer chromatogram of the lower phase (chloroform layer) showed no increase in the amount of organic material staining with iodine vapor close to the origin. There was no apparent qualitative difference between the samples of lipid fraction to which Tyrode's solution had been added or which had suspended in Tyrode's solution and the control samples. However, the thin layer chromatogram of the upper phase (methanol:water) showed an appreciable amount of staining with a Rf. equivalent to that of the lipid fraction.

While suspension in Tyrode's solution did not appear to change the chemical properties of the lipid fraction, the appearance of iodine vapor stainable material with the same Rf. as that of the lipid fraction in the methanol: water phase suggested behavior similar to that expected of an organic acid.

d. Alcoholic potassium carbonate extraction of the lipid fraction from organic solvent.

(1) Qualitative.

Since the only known substrates for the fatty-acid cyclo-oxygenase are free fatty acids (Lands & Samuelson, 1968; Vonkeman & van Dorp, 1968), the finding that the action of the lipid fraction was inhibited by the fatty acid cyclo-oxygenase inhibitor, mefenamic acid (cited in Section II.B.1.d.(3).(b).((1)).) strongly suggested that the biological activity of the lipid fraction might be due

to the presence of fatty acid substrates for the cyclo-oxygenase. In addition, the finding that material with the same Rf and iodine vapor stainability as the lipid fraction was extractable (cited in Section III.B.2.c.) from a chloroform:methanol solution of the lipid fraction with Tyrode's solution, which is weakly basic, also suggested that the lipid fraction was behaving as an acid. So, in spite of the published finding that the lipid fraction was not an acid (Robinson et al., 1967), a concerted effort was made to determine if the lipid fraction behaved as an acid.

The lipid fraction and arachidonic acid cochromatographed on silica gel HR when developed with hexane:diethyl ether:acetic acid (90:10:1). Taking the samples to dryness under a stream of nitrogen and then redissolving them in hexane had no effect on the chromatographic properties of the lipid fraction or arachidonic acid. There was no apparent decrease in the amount of iodine vapor stainable material in the hexane phase when the samples were extracted with either water or ethanol:water (1:1). However, when the hexane phase was extracted with ethanol:2.8 N (20%) aqueous potassium carbonate (1:1) or with 1.4 N (10%), 1.0N (6.9%), or 0.1 N (0.69%) aqueous potassium carbonate, essentially all the iodine vapor stainable material disappeared from the hexane phase in both the arachidonic acid and the lipid fraction samples. There was a slight staining of the hexane phase at the 0.1 N aqueous potassium carbonate concentration in the lipid fraction

sample, which appeared to be due to contamination with the aqueous phase. The ethanol: 2.8 N (20%) aqueous potassium carbonate samples formed slight emulsions at the hexane/water interface, and, therefore, the staining may also be due to contamination. The results of these experiments are summarized in table 9.

The behavior of the lipid fraction parallels that of arachidonic acid in its extraction by weak base from organic solvent and is consistent with the interpretation that the lipid fraction is composed of fatty acid(s).

(2) Quantitative.

Aliquots of the original NLE#3, of the organic phases remaining after each of the three extractions, and of the fatty acids extracted from the NLE#3 were bioassayed on the rabbit ileum preparation. Additional aliquots of the fatty acids extracted from the NLE#3 were converted to their methyl esters for identification and quantitation by GLC and GC/CIMS.

A 200 ml aliquot of the NLE#3 contained 2.11 g of solids. The first extraction procedure yielded 80 mg of fatty acids. The remaining solids in the organic phase weighed 1.97 g. The second extraction procedure yielded 32 mg of fatty acids. The third extraction procedure yielded only an additional 15 mg.

The total amount of fatty acids present in the NLE#3 was roughly estimated to be less than 140 mg,

TABLE 9

EXTRACTION OF THE LIPID FRACTION OR ARACHIDONIC ACID FROM HEXANE

Sample Manipulation	Intensity of I_2 Staining of Hexane Phase	
	Lipid Fraction	Arachidonic Acid
Spotted directly from stock solution	++++	++++
Blown to dryness with N_2, redissolved in hexane, and spotted	++++	++++
Lipid fraction and arachidonic acid combined	++++	
Hexane phase extracted with: H_2 only	++++	++++
Hexane phase extracted with: ethanol:H_2O (1:1)	++++	++++
Hexane phase extracted with: ethanol:2.8 N K_2CO_3 (1:1)	+	++
Hexane phase extracted with: 1.4 N K_2CO_3	-	-
Hexane phase extracted with: 1.0 N K_2CO_3	-	-
Hexane phase extracted with: 0.1 N K_2CO_3	+	-

and the partition coefficient was estimated to be about 0.25. Least squares analysis of the data minimizing the mass error in mg for each extraction procedure was performed. This analysis estimated a total of 126 mg of fatty acids with a partition coefficient of 0.29. The estimated yields of fatty acids were 81 mg for the first extraction procedure, 40 mg for the second extraction procedure, and 7 mg for the third extraction procedure. Since the estimated yield for the third extraction procedure was less than 50% of the experimental yield, a least squares analysis of the extraction data was performed minimizing the percentage mass error for each extraction. The analysis minimizing the percentage error estimated a total of 119 mg of fatty acid present in the NLE#3 with a partition coefficient of 0.21. The estimated yields of fatty acid were 60 mg for the first extraction procedure, 41 mg for the second extraction procedure, and 13 mg for the third extraction procedure.

Both least squares analyses underestimated the total amount of fatty acids which were experimentally determined to be present in NLE#3. This underestimation may be due either to improved experimental proficiency in the second and third extraction procedures or to increased extraction of non-fatty acid substances along with the fatty acids as the amount of fatty acid remaining in the NLE#3 declined.

The fatty acid composition of the

methylated products of each extraction procedure is listed in table 12.

Aliquots from the unextracted NLE#3, the fatty acids extracted from NLE#3, and the organic phase remaining after the fatty acid extraction were taken to dryness under a stream of N_2 and suspended in Tryrode's solution for bioassay. Cumulative dose-effect (Ariëns, 1964) curves were obtained for the aliquots, and the results are summarized in table 10. The ED_{50}s are expressed in terms of the comparable initial volume of the NLE#3. The percent of biological activity is expressed as the ratio, $\frac{\text{NLE\#3 } ED_{50}}{\text{sample } ED_{50}} \times 100$.

As can be seen from an inspection of table 10, the maximum response (EMAX) of the rabbit ileum to the NLE#3, the fatty acids extracted from the NLE#3, and the organic phase remaining after the fatty acid extractions is roughly the same. It should be noted that, as the fatty acids are extracted from the NLE#3, the increase in biological activity in the fatty acid fraction is matched by the decrease in the biological activity of the organic phase remaining after the fatty acids are extracted. The increase of 104% above the biological activity of the NLE#3 displayed by the combined fatty acids of extractions one to three may be due to the separation of the prostaglandin/thromboxane (and their homologs) precursors from other compounds with which they may have formed micelles, prevent-

TABLE 10

BIOLOGICAL ACTIVITY OF NLE#3, THE FATTY ACIDS EXTRACTED, AND REMAINING EXTRACTED ORGANIC PHASES

Sample[1]	n	EMAX[2]	ED_{50}[3]	% Biological Activity of NLE#3[4]
NLE#3	13	3.6 ± 0.4[5]	51 ± 11 (537 ± 116 µg/ml)	100
Fatty Acids:				
Extraction 1	2	4.2 ± 1.5	268 ± 232	19
Extractions 2+3	1	5.0	58	88
Combined Extractions 1,2+3	5	4.3 ± 0.2	25 ± 3	204
Organic Phases:				
Extraction 1	1	3.0	107	48
Extraction 2	1	2.6	200	25
Extraction 3	2	2.1 ± 0.1	990 ± 10	5

1. Samples described in III.B.2.d.(2).
2. EMAX expressed in chart divisions corrected to constant scale. One corrected chart division equals 1.82 grams.
3. To avoid possible misinterpretation of what is, essentially, a purification procedure for fatty acids, the ED_{50}s of the fatty acids extracted and the extracted organic phases were expressed in terms of equivalent volumes (in µl) of the original NLE#3 per ml Tyrode's solution in the gut bath.
4. Calculation by the formula: $\frac{ED_{50}\ NLE\#3}{ED_{50}\ Sample} \times 100$.
5. Mean ± standard error of the mean.

ing the prostaglandins/thromboxanes and their homologs from stimulating the intestinal preparation.

An alternative explanation for the two fold increase in biological activity of the extracted fatty acids above the biological activity of the NLE#3 may be that the fatty acid cyclo-oxygenase is known to require an activator, thought to be the cyclic endoperoxide (Lands, Cook, & Rome, 1976), for maximal function. Many of the fatty acids known to be present in the NLE#3 (see Section III.B.3.a.(2)) act as inhibitors of the cyclo-oxygenase (by acting as substrates with lower turnover numbers, as pure competitive inhibitors, and as stimulators of the irreversible inactivation reaction (Pace-Aciak, & Wolfe, 1968; Lands, Lee, & Smith, 1971; Lands, LeTellier, Rome, & Vanderhöek, 1973; Ziboh, Vanderhöek, & Lands, 1974)). It is possible that the extraction of the fatty acids from the NLE#3 may have enriched the fatty acid fraction in active substrates, while decreasing the relative proportions of inhibitory compounds. This contention is supported by the decrease in arachidonic acid in the third and final extraction suggesting that most of the arachidonic acid was extracted in the initial two procedures.

Finally, the separation of other lipophilic compounds in the NLE#3 from the polyunsaturated fatty acids may have decreased the competition for non-specific binding sites near the substrate or activator site of the fatty acid cyclo-oxygenase.

The biological activity of the NLE#3 may be compared with that of pure arachidonic acid in the following manner: (1) The lower limit of the estimated total amount of fatty acids in the NLE#3 is approximately 120 mg/ 200 ml aliquot of NLE#3 = 0.6 mg fatty acids/ ml NLE#3; (2) From table 11, arachidonic acid makes up roughly 15% of the total fatty acids or (0.6 mg fatty acids/ml NLE#3) x (0.15 arachidonic acid/fatty acids) = 0.09 mg arachidonic acid/ ml NLE#3 = 0.09 µg arachidonic acid / µl NLE#3; (3) From table 10, the ED_{50} of the NLE#3 is 51 µl NLE#3 / ml organ bath or (0.09 µg arachidonic acid / µl NLE#3 x (51 µl NLE#3 / ml organ bath = 4.6 mg arachidonic acid / 1 organ bath; (4) The molecular weight of arachidonic acid is 304.4; therefore, ((4.6 x 10^{-3} g arachidonic acid / 1 organ bath) / (304.4 g arachidonic acid / 1 organ bath) x 1.0 M = 1.5 x 10^{-5} M. This ED_{50} of 1.5 x 10^{-5} M is more than five times the ED_{50} of the arachidonic acid alone, but it is not unexpected if the other fatty acids present in the NLE#3 can act as inhibitors of the fatty acid cyclooxygenase (see above, Pace-Aciak & Wolfe, 1968).

3. Physical methods of analysis by separation.

a. Chromatographic.

(1) Thin layer chromatography of lipid fraction and fatty acid methyl esters.

Silver nitrate impregnated TLC plates provide a simple and convenient method for the separation of fatty acid methyl esters based on the number of

TABLE 11

FATTY ACID COMPOSITION OF THE LIPID FRACTION AS DETERMINED BY GLC

Assigned Structure[1]	% Total Area under Chromatogram Peaks[2] Samples[3] NLE#3	AM High Activity	AM Low Activity	H_2 AM	D_2 AM	% Total Fatty Acids Rat Brain[4]
16:0	9.7 ± 0.1[5]	40.1	18.9	25	12.1	17.4
16:1	3.4 ± 0.6					0.4
18:0	17.5 ± 1.2	8.0	26.0	48	39.4	24.6
18:1	19.6 ± 1.4	29.2	18.2			22.3
18:2	1.2 ± 0.1	1.6	30.2			0.5
18:3	2.8 ± 0.2	8.1	1.2			
20:0				17	19.6	0.6
20:3	1.4 ± 0.2	0.1				0.2
20:4	15.4 ± 1.6	6.2	2.6			10.5
20:5	1.2 ± 0.4	0.3				0.3
22:0				6	20.3	
22:5w6	2.6 ± 0.7					1.9
22:5w3	1.6 ± 0.2					
22:6	14.0 ± 1.2					14.7
Total % of Assigned Structures	90.5 ± 2.1	93.6	97.1	96	91.4	93.4[6]

1. Structures assigned on the basis of retention times.
2. % total area under chromatogram peaks determined by weighing peaks cut from chromatogram.
3. Samples described in III.B.3.a.(2).
4. Data from Shimasaki, Phillips, & Privett (1977).
5. Mean ± standard error of the mean (n = 3)
6. Excluding fatty acids not assigned by the method in Note 1.

unsaturations and the carbon chain length (William E. M. Lands, personal communication; Cubero & Mangold, 1965; Holman & Rahm, 1966 to 1970). A double bond causes a fatty acid to migrate as though it were a saturated fatty acid with two fewer carbon atoms (Holman, 1951). While the lipid fraction (either AM or hydrogenated AM) migrated on silica gel HR as a single spot with a Rf equivalent to arachidonic acid, γ-linolenic acid, or linolenic acid (Rf = 0.12), the methyl esters of AM migrated as at least four separate spots with Rf's equivalent to 18:0, 18:1, 18:2, and 18:3. This finding confirmed the results of the NMR spectroscopic analysis (see Section III.B.3.b.(3).) that the lipid fraction was not homogenous but a mixture.

(2) Gas liquid chromatography of lipid fraction and fatty acid methyl esters.

GLC of the methyl esters of the lipid fraction revealed peaks with rentention times similar to those of fatty acid methyl ester standards. Therefore, methylated NLE#3, two batches of AM with differing amounts of biological activity, hydrogenated AM (H_2AM), and deuterated AM (D_2AM) were chromatographed under identical conditions with fatty acid methyl ester standard mixtures of known composition. The proposed fatty acid percentage compositions of the samples are listed in table 11. As can be seen from the last column of the table, the proposed fatty acid compositions are similar to that previously reported for lipid extracts of neural tissue (Shimasaki,

Phillips, & Privett, 1977; Pullarkat & Reha, 1976).

The hydrogenaged and deuterated AM, which were taken from different batches of AM than were the high and low activity AM samples, are composed almost entirely of fatty acids with retention times equivalent to 16:0, 18:0, 20:0, and 22:0. This composition is what would be expected from the saturation of the unsaturated fatty acids present in the lipid fraction.

The fatty acid percentage compositions of the methylated products of the three serial extraction procedures described in Section III.B.2.d.(2) are listed in table 12. The percentage compositions of the extracted fatty acids remained relatively constant except for a decrease in arachidonic acid and 22:6w3 and an increase in 18:0 and 18:1 in the third and final extraction. This difference might result if the partition coefficients of the fatty acids present in the lipid fraction varied widely and the first two extraction procedures were enriched in the polyunsaturated fatty acids.

b. Spectroscopic.

(1) Ultraviolet and visible spectroscopic analysis of the lipid fraction.

When samples of the lipid fraction were taken to dryness under a stream of nitrogen, it was noted that often, just before the sample dried, the fluid appeared to take on a light bluish green (aqua) hue. Therefore, it was felt that full visible and near UV spectra might be

TABLE 12

COMPOSITION OF THE FATTY ACID MIXTURE
EXTRACTED FROM NLE#3 DISSOLVED IN HEXANE

Assigned Structure[1]	% Total Area under Chromatogram Peaks[2] Extraction[3] #1	Extraction #2	Extraction #3
16:0	9.1	9.9	9.7
16:1	4.3	3.9	4.2
18:0	19.8	21.0	32.3
18:1	20.0	18.0	25.0
18:2	0.9	1.3	0.1
18:3	2.7	1.8	2.9
20:3	1.3	2.3	1.4
20:4	14.9	15.3	4.9
20:5	1.4	1.2	2.6
22:5	3.6	1.2	3.2
22:5	1.2	0.8	2.1
22:6	11.8	11.8	4.2
Total % of Assigned Structures	91.5	88.6	92.6

1. Structures assigned on the basis of retention times.
2. % total area under chromatogram peaks determined by weighing peaks cut from chromatogram.
3. Extraction procedures described in IIIA.1.d.(2).

useful in the qualitative analysis of the lipid fraction.

The spectrum of the lipid fraction from 200 to 800 nm showed a single absorption at 205 to 215 nm with a shoulder from 225 to 245 nm and a slight shoulder from 255 to 295 nm. The hydrogenated lipid fraction showed a single peak at 205 to 215 nm with a shoulder from 220 to 240 nm and a slight shoulder from 265 to 290 nm. The methyl esters of the lipid fraction show a single peak at 205 to 215 nm with a shoulder from 225 to 245 nm and slight shoulders from 255 to 270 nm and from 270 to 295 nm.

The absence of absorption in the visible region is puzzling in view of the initial observation of the slight bluish green tinge to the samples as they were being taken to dryness. A possible explanation is that inorganic ions may be present in very low concentrations in the lipid fraction. The near ultraviolet spectrum is not inconsistent with a mixture of fatty acids and is suggestive of the absence of ketones, aldehydes, or hydroperoxides (Silverstein & Bassler, 1967; Holman & Rahm, 1966 to 1970).

(2) Infrared spectrometric analysis of the lipid fraction.

With the availability of sufficient quantities of the lipid fraction, infrared spectra were run on it and on the products of various qualitative chemical procedures to assist in the determination of the structure of the lipid fraction.

The infrared spectra of the lipid fraction

the 'products' of the attempted reactions with sodium borohydride and ethanolic potassium hydroxide, the products of the reactions with ethanolic and methanolic hydrochloric acid and with hydrogen over a palladium on carbon catalyst, and of authentic samples of oleic and arachidonic acid are summarized in table 13.

Inspection of the table indicates that the spectra of the lipid fraction and the 'products' of the attempted reactions with sodium borohydride and with ethanolic potassium hydroxide are essentially identical. These spectra may be rationalized as follows (Holman & Rahm, 1966 to 1970; Jones, 1962a & b; Jones, McKay & Sinclair, 1952; Parker, 1971; Silverstein & Bassler, 1967; Sinclair, McKay, & Jones, 1952; Sinclair, McKay, Myers, & Jones, 1952; and Stennagen, 1966): the weak and broad absorption at 3530 cm^{-1} is due to O-H stretching, the medium and broad absorption shoulder rising in intensity from 3400 to 3100 cm^{-1} is due to stretching of the O-H...O hydrogen bonded acid dimer, the medium absorption at 3025 cm^{-1} is due to C-H olefinic stretching, the strong absorption at 2940 cm^{-1} is due to a combination of C-H asymmetrical methyl and methylene stretching, the strong abosrption at 2860 cm^{-1} is due to symmetrical methyl and methylene C-H stretching, the weak and broad absorption at 2680 cm^{-1} is a C-H and bonded O-H...O combination band, the medium shoulder increasing from 1745 cm^{-1} is due to saturated carboxylic monomer C=O stretching, the weak and broad shoulder decreasing from 1650 cm^{-1} is due to

TABLE 13

INFRARED ABSORPTION BANDS OF THE LIPID FRACTION AND FATTY ACIDS

Wave # (CM^{-1})	AM	$NaBH_4$ Attempted Reduction #1	$NaBH_4$ Attempted Reduction #2	ETOH KOH of AM	Hydrogenated AM	ETOH HCl of AM	MEOH HCl of AM	18:2w6	20:4w6
3530	w,b	w,b	w,b	w,b	w,b	-	-	w,b	w,b
3400-3100	m,b,shd	m,b,shd	m,b,shd	m,b,shd	w,b,shd	-	-	m,b,shd	m,b,shd
3025	m	m	m	m	-	m	m	-	-
3005	-	-	-	-	-	-	-	m	S
2955	-	-	-	-	-	-	-	m,shd	S
2940	S	S	S	S	S	S	S	-	-
2930	-	-	-	-	-	-	-	S	S
2870	-	-	-	-	-	-	-	-	S
2860	S	S	S	S	S	S	S	S	
2850	-	-	-	-	-	-	-	-	S
2680	w,b	w,b	w,b	w,b	w,b	-	-	w,b	w,b
1745	m,shd	m,shd	m,shd	m,shd	m,shd	-	-	m,shd	m,shd
1730	-	-	-	-	-	S	S	-	-
1710	S	S	S	S	S	-	-	S	S
1650	w,b,shd	w,b,shd	w	w,shd	-	w	w	w,shd	w,b
1470	m }d	m }d	m }d	m }d	m }d	m }d	m }d	m }d	-
1460	m	m	m	m	m	m	m	m	m }d
1450	-	-	-	-	-	-	-	m	m
1430	-	-	-	-	w,b	w	w,b	m,shd	m,shd
1415	m	w	w	w,b	w	-	-	m	m
1380	w	w	w	w,b	w	-	-	w,shd	w
1375	-	-	-	-	-	m	m	-	-
1350	-	-	-	-	-	w,b	w,b	-	-
1300	-	-	-	-	-	w,b	w,b	-	-
1290	m	m,b	m,b	m,b	m,b	-	-	m,b	-
1265	-	-	-	-	-	w,b	w,b	-	m,b
1230	-	-	-	-	m	-	-	-	-
1190	w	-	-	-	-	m	m,b	-	w
1130	w	w	m,b	-	w	-	-	w,b	w
1120	-	-	-	w	-	w	m	-	-
1100	-	-	-	-	-	w	w	-	-
1020	-	-	-	-	-	w	m	-	-
940	w,b	w,b	m,b	w,b	-	-	-	m	m,b
930	-	- }d	-	- }d	m,b	-	-	-	- }d
920	w,b	w,b	m,b	w,b	-	-	-	m	m,b
865	w,b					w,b	w,b		

1. b = broad
2. }d = doublet
3. m = medium
4. S = strong
5. shd = shoulder
6. w = weak

stretching of a cis C=C double bond, the medium absorption doublet at 1470 and 1460 cm^{-1} is due to $-CH_2-$ methylene scissoring and CH_3 asymmetrical methyl bending, the weak to medium absorption at 1415 cm^{-1} is due to O-H and C-O-H bending, the weak absorption at 1380 cm^{-1} is due to O-H and C-O-H bending and CH_3 symmetrical methyl bending, the weak to medium absorption at 1290 cm^{-1} is due to C-O carboxylic acid dimer stretching, and the weak and broad doublet at 940 and 920 cm^{-1} is due to O-H...O hydrogen bonded carboxylic acid out of plane bending. Not all the absorption bands have been rationalized, and some may have been obscured by interference from the chloroform solvent or by contamination of the sample cells.

The infrared spectra of the products of the hydrogenation of the lipid fraction are identical with those of the lipid fraction itself with the following exceptions: the C-H olefinic stretching absorption at 3025 cm^{-1} and the cis C=C double bond stretching absorption at 1650 cm^{-1} are absent, there is a medium absorption at 1230 cm^{-1} due to C-O monomer stretching, and the weak and broad absorption due to O-H...O hydrogen bonded carboxylic acid dimer out of plane bending doublet at 940 and 920 cm^{-1} has been replaced by a medium and broad absorption at 930 cm^{-1}.

The infrared spectra of the products of the reactions of ethanolic and methanolic hydrochloric acid with the lipid fraction differed from those of the starting material by the absence of the O-H stretching band at 3530 cm^{-1},

the O-H...O dimer stretching band from 3400 to 3100 cm^{-1}, the C-H and bonded O-H...O combination band at 2630 cm^{-1}, C=O saturated carboxylic acid dimer stretching band at 1710 cm^{-1}, the O-H and C-O-H bending band at 1415 cm^{-1}, and O-H and C-O-H bending and CH_3 symmetrical methyl bending band at 1380 cm^{-1}, the C-O carboxylic acid dimer stretching band at 1290 cm^{-1}, and the O-H...O bonded carboxylic acid dimer out of plane bending doublet at 940 and 920 cm^{-1} and by the presence of a C=O saturated ester carbonyl stretching band at 1730 cm^{-1}, a CH_3 symmetrical methyl bending band at 1375 cm^{-1}, and C-O ester stretching bands at 1265 and 1190 cm^{-1}.

The infrared spectra of oleic and arachidonic acids differ from those of the lipid fraction only by small shifts in the absorption frequencies of several functional groups and not by major differences in the absorption intensity. The only exception is the strong absorption of arachidonic acid at 3005 cm^{-1} showing a greatly increased intensity of the C-H olefinic stretching band, consistent with the presence of four methylene interrupted C=C double bonds.

The infrared spectra of the lipid fraction and the products of the reactions and attempted reactions are consistent with a mixture of long chain saturated and unsaturated fatty acids. The spectra are inconsistent with the presence of ketones, aldehydes, acid halides, anhydrides, amides, and lactams (Silverstein and Bassler (1967)). The disappearance of the C-H olefinic stretching band at 3025 cm^{-1} and the C=C cis double bond stretching band at 1650 cm^{-1} in the hydrogenated lipid fraction confirms that the lipid

fraction contains cis double bonds and that the reaction was essentially complete. The failure of the attempted reduction of the lipid fraction with sodium borohydride, as indicated by the lack of alteration in the infrared spectrum, suggests the absence of a keto-carbonyl. The failure of the attempted ethanolic potassium hydroxide hydrolysis of the lipid fraction suggests the absence of an amide or ester function in the lipid fraction. The disappearance of the O-H stretching, O-H...O dimer stretching, C-H and bonded O-H...O combination, C=O saturated carboxylic acid monomer and dimer stretching, O-H and C-O-H bending, C-O dimer stretching, and O-H...O bonded dimer out of plane bending bands and the appearance of the C=O saturated ester carbonyl stretching and C-O ester stretching bands following the reaction of the lipid fraction with ethanolic or methanolic hydrochloric acid suggest that the ethyl and methyl esters were formed.

(3) NMR spectroscopy of the lipid fraction, methyl esters of the lipid fraction, hydrogenated lipid fraction, and known fatty acids and fatty acid methyl esters.

Both the pharmacological and chemical analysis suggested that the lipid fraction was a long chain hydrocarbon with unsaturation and a carbonyl group. Nuclear magnetic resonance (NMR) spectrometry is a useful tool "... for the characterization of organic compounds and for the elucidation of molecular structure and stereochemistry." (Jackman & Sternhell, 1966). Accordingly, the samples listed

in table 14 were run to provide a basis for analysis of the lipid fraction and its comparison with known fatty acids. The results of the NMR spectroscopic analysis listed in table 14 may be interpreted as follows (Holman & Rahm, 1966 to 1970; Jackman & Sternhell, 1969; Silverstein & Bassler, 1967; Stenhagen, 1966): The first three resonances at 0.9, 1.3, and 1.7 ppm (δ from tetramethylsilane (TMS)) are due to terminal $-CH_3$, $-CH_2-$ in an alkyl chain and $-CH_2-$ β to carbonyl protons, respectively, and are present in the spectra of all the samples. The resonance at 2.1 ppm is due to $-CH_2$ protons α to a double bond and is present in the AM sample and in the unsaturated reference samples, 20:4w6, 18:2w6, and methyl 18:2w6. In the crude methyl esters of AM, the intensity is reduced to the point of uncertainty as to its presence or absence. The resonance at 2.4 ppm is due to $-CH_2-$ protons α to carbonyl and is present in all samples. The resonance at 2.8 ppm is due to the $-CH_2-$ protons between two double bonds and is present in the AM sample as well as the unsaturated reference samples of 20:4w6, 18:2w6, and the methyl ester of 18:2w6. In the crude methyl esters of AM the absorption is very faint. The resonance at a δ of 3.7 ppm is due to $-CH_3$ protons of methyl esters and is present in the methyl esters of AM and in methyl linoeate and methyl stearate. Trace resonances, present in the spectra of AM and palmitic acid, suggest that the samples may be partly esterified. The resonance at 5.4 ppm is due to olefinic protons and is present in AM, 20:4w6,

TABLE 14

CHEMICAL SHIFTS[1] OF THE LIPID FRACTION, HYDROGENATED LIPID FRACTION METHYL ESTERS OF THE LIPID FRACTION, AND KNOWN FATTY ACIDS AND FATTY ACID METHYL ESTERS

Chemical Shift (in ppm)[1]	Proton Assignments[2]	Sample AM	Methyl Esters of AM	Hydrogenated AM	20:4w6	18:2w6	Methyl 18:2w6	Methyl 18:0	16:0	14:0
0.9	A	+	+	+	+	+	+	+	+	+
1.3	B	+	+	+	+	+	+	+	+	+
1.7	C	+	+	+	+	+	+	+	+	+
2.1	D	+	+	-	+	+	+	-	-	-
2.4	E	+	+	+	+	+	+	+	+	+
2.8	F	+	+	-	+	+	+	-	-	-
3.7	G	-	+	-	-	-	+	+	-	-
5.4	H	+	+	-	+	+	+	-	-	-

1. Chemical shifts are expressed as σ, in ppm, from tetramethylsilane (TMS).

2. Prototypical fatty acid

(and methyl ester): $CH_3-(CH_2)_n-CH_2-CH=CH-(CH_2-CH=CH)_n-CH_2-(CH_2)_n-CH_2-CH_2-CO-O-H$ (CH_3)

Proton type assignments: A B D H H F H H D B C E G

18:2w6, and methyl 18:2w6. A slight resonance is present in the methyl esters of AM. The ratio of olefinic proton absorption in the AM sample to that of the terminal alkyl methyl proton absorption is less than 2:3.

The conclusion to be drawn from the NMR spectroscopic analysis is that the lipid fraction displays the same characteristics as polyunsaturated fatty acids. The products of the hydrogenation and esterification reactions yield similar spectra to those of saturated fatty acids and unsaturated methyl esters. The products of the methyl esterification reaction were not purified, and the ambiguity as to the presence or absence of the unsaturations may be due to interference from side products of the reaction. Perhaps the most significant finding is the small ratio of olefinic protons to that of the terminal alkyl methyl group protons. As there is no resonance at 1.1 ppm due to methyl substitutions on the alkyl chain (Jackman & Sternhell, 1969, p. 166), this result, combined with the presence of the protons of $-CH_2-$ between two double bonds, indicate that the AM is a mixture of fatty acids of varying degree of unsaturation.

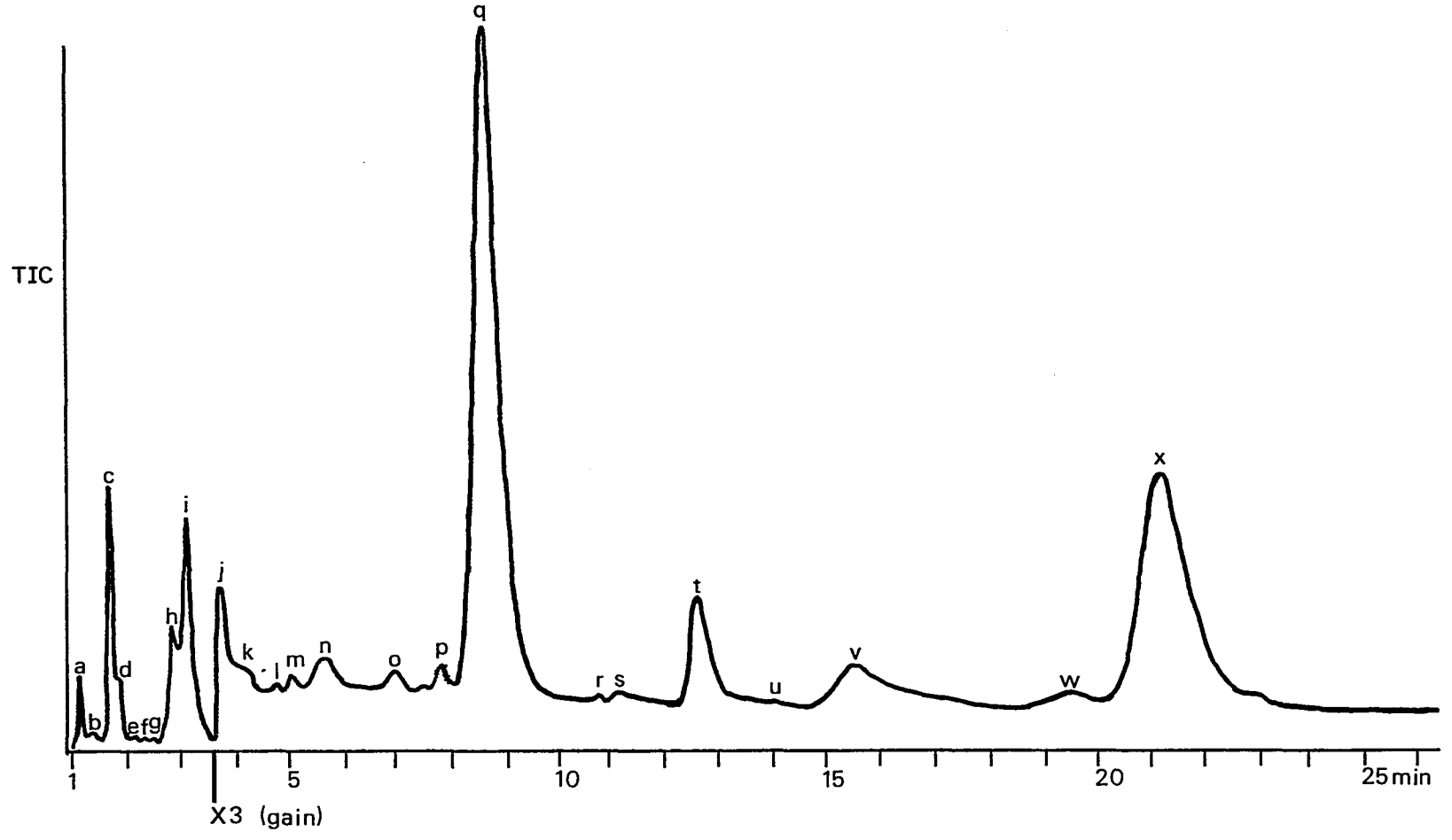
b. Spectrometric.

(1) Mass spectrometry of the lipid fraction and fatty acid methyl esters.

The assignments of the molecular ions to and the rationalization for the fragmentation patterns (McLafferty, 1973) of the various total ion current chromatographic peaks, shown in fig. 19, are consistent with the

Fig. 19. Redrawn total ion current (TIC) chromatogram of the methyl esters of NLE#3.

Chromatograph and mass spectrometer conditions were as described in Section IV.A., 3.b.(1). The ordinate is the TIC expressed in arbitrary units (note the 3 fold gain increase at 3.6 min). The abscissa is the time, in minutes, after the sample was injected into the chromatograph. The TIC chromatographic peaks have the following: label - m/e of the $(M+1)^+$ ion, and the assigned structure; a - 243.4, 14:0; b - 257.4, 15:0; c - 271.4, 16:0; d - 269.4, 16:1; e - 285.4, 17:0; f - 283.4, 17:1; g - 267.4, 16:2; h - 299.4, 18:0; i - 297.4, 18:1; j - 295.4, 18:2; k - 293.4, 18:3w6; l - 293.4, 18:3w3; m - 223.3, unknown; n - 325.4, 20:1; o - 321.4, 20:3w6; p - 321.4, 20:3w3; q - 319.4, 20:4; r - 317.4, 20:5; s - 352.4, 22:1; t - 279.3, unknown; u - 349.4, 22:3; v - 347.4, 22:4; w - 345.5, 22:5; x - 343.5, 22:6.


TIC
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v
w
x
1
5
10
15
20
25 min
X3 (gain)

fatty acid composition reported in table 15. The presence of arachidonic acid (20:4w6) is confirmed by the mass spectra shown in figures 20 and 21. These figures show the correspondence between the chemical ionization fragmentation patterns of authentic methyl arachidonate and of the GLC peak with equivalent retention time to methyl arachidonate in the methyl esters of the lipid fraction. The four largest fragments in figure 20 may be assigned the following structures (McLafferty, 1973; Holman & Rahm, 1966 to 1970; Ryhage & Stenhagen, 1960; Stenhagen, 1966): m/e=319.4, $(M+1)^+$; m/e= 320.4, ((M+1)+1+; m/e=137.2, $(CH_3-(CH_2)_3-(CH_2-CH=CH)_2)+$; m/e=287.4, $((M+1\_-CH_3OH)+$. The determined relative abundance of the assigned ((M+1)+1+ ion is 22.3% while the theoretical relative abundance is 23% (McLafferty, 1973).

The other ions in the mass spectrum follow typical fragmentation patterns for polyunsaturated fatty acids: $CH_3-(CH_2)_n-(CH=CH)_2+$, n = 3 to 7, m/e = 109,123,137, 151, & 165; $CH_3-(CH_2)_n-(CH=CH)_3+$, n = 1 to 8, m/e = 107, 121, 135, 149, 163, 177, 191, & 205; $CH_3-(CH_2)_n-(CH=CH)_4+$, n = 3 to 7, 9, m/e = 161, 175, 189, 203, 217, & 245; $CH_3-)-CO-(CH_2)_n-CH=CH+$, n = 4, 6, 7, 8, m/e = 167, 181, 209, & 223; $CH_3-O-CO-(CH_2)_n-(CH=CH)_3+$, n = 5 to 8, m/e = 207, 221, 235, & 249.

The largest four fragments in the mass spectrum of the GLC peak with a retention time equivalent to methyl 20:4w6 in the methyl esters of NLE#3 (see fig. 21),

TABLE 15

FATTY ACIDS IDENTIFIED IN THE METHYLATED PRODUCTS OF THE LIPID FRACTION BY GC/CIMS

Assigned Structure[1]	Samples[2] NLE#3	AM: High Activity	AM: Low Activity	H_2 AM	D_2 AM
14:0	+	+	+	+	+
15:0	+	+	+	+	
16:0	+	+	+	+	+
16:1	+	+			+
16:2	+				
17:0	+	+	+	+	+
17:1	+	+			
18:0	+	+	+	+	+
18:1	+	+	+		+
18:2	+	+	+		+
18:3	+		+		+
19:0				+	
20:0				+	
20:1	+	+	+		
20:3	+	+			+
20:4	+	+	+		+
20:5	+	+	+		+
22:0				+	
22:1	+				
22:3	+				
22:4	+	+			
22:5	+	+			
22:6	+	+			

1. Structures assigned on the basis of $(M + 1)^+$ ion, retention time, and characteristics of the mass spectrum.
2. Samples described in Section III.B.3.a.(2).

Fig. 20. Redrawn chemical ionization mass spectrum of the methyl ester of arachidonic acid (20:4w6).

Chromatograph and mass spectrometer conditions were as described in Section III. A.3.b.(1). The ordinate is the relative abundance expressed as percentage of the base peak. The abscissa is the m/e of the fragment ions expressed in atomic mass units (amu). The rationalization of the spectrum is presented in Section III.B.4.b.(1).

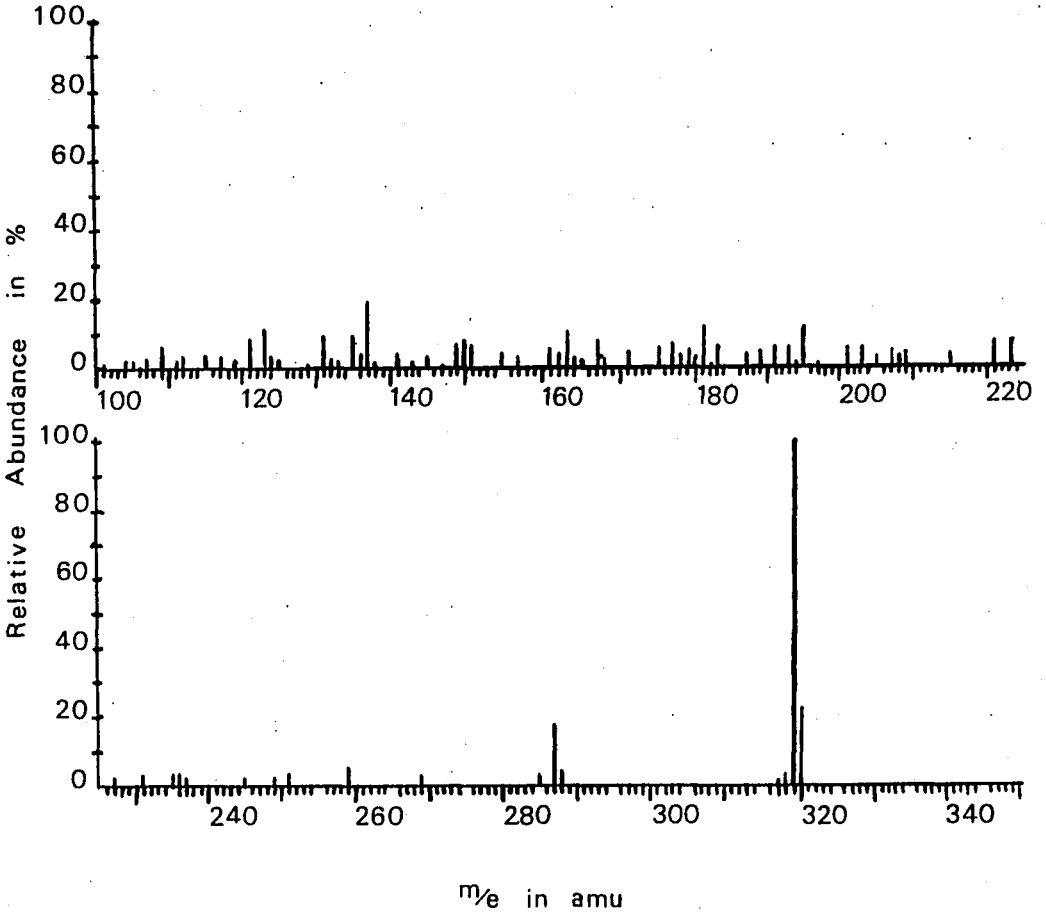
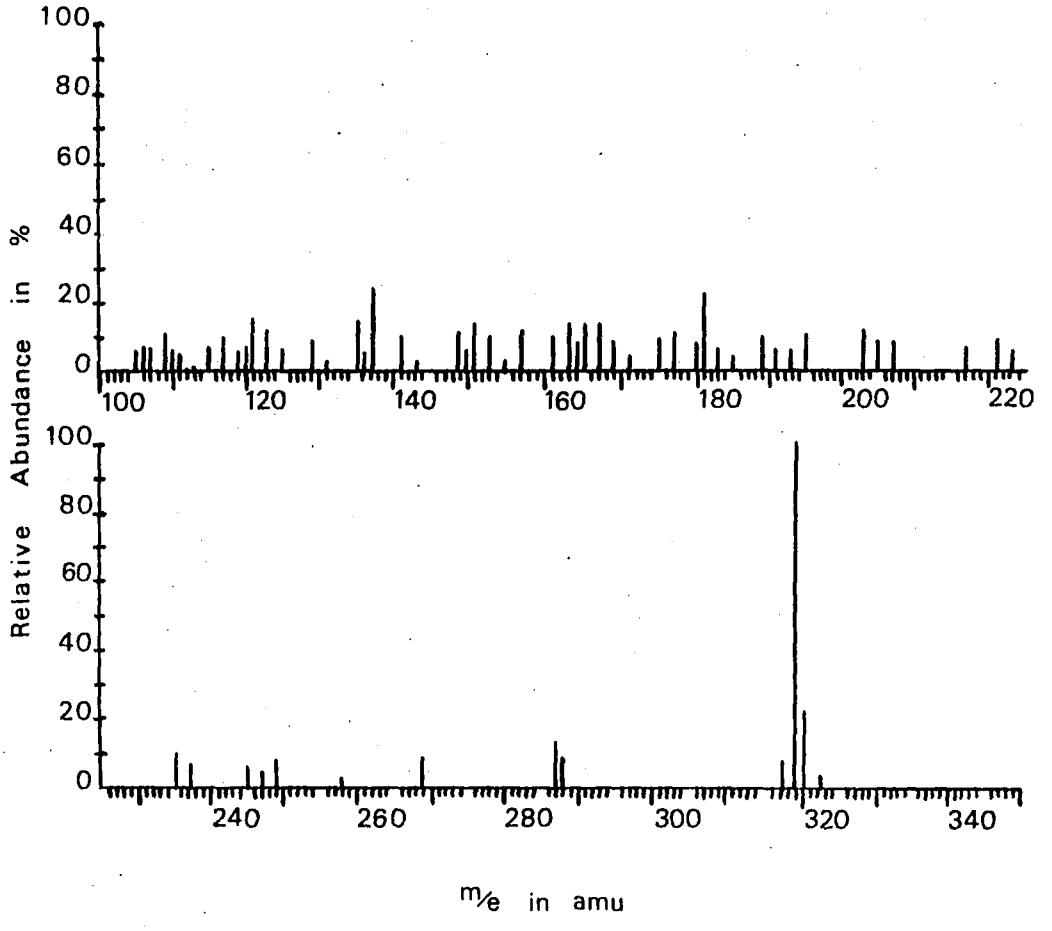

100
80
60
40
20
0
Relative Abundance in %
100
120
140
160
180
200
220
100
80
60
40
20
0
240
260
280
300
320
340
m/e in amu

Fig. 21. Redrawn chemical ionization mass spectrum of the capillary column TIC chromatographic peak in the methyl esters of NLE#3 with a retention time equivalent to that of methyl arachidonate.

Chromatograph and mass spectrometer conditions were as described in Section IV.B. 3.b.(1). The ordinate is the relative abundance expressed as percentage of the base peak. The abscissa is the m/e of the fragment ions expressed in atomic mass units (amu). The rationalization of the spectrum is presented in Section IV.B. 4.b.(1).


Relative Abundance in %
100
80
60
40
20
0
100
120
140
160
180
200
220
240
260
280
300
320
340
m/e in amu

may be assigned the following structures (Holman & Rahm, 1966 to 1970; McLafferty, 1973; Ryhage & Stenhagen, 1960; Stenhagen, 1966): m/e = 319.4, (M+1); m/e = 137.2, $(CH_3-(CH_2)_3-(CH_2-CH=CH_2)+$; m/e = 181.2, $(CH_3-O-CO-(CH_2)_3-(CH=CH-CH_2)_2)+$; and m/e = 320.4, ((M+1)+1)+. The determined relative abundance of the assigned ((M+1)+1)+ ion is 22.2% while the theoretical relative abundance is 23% (McLafferty, 1973). The other ions in the spectrum follow the same pattern as that for authentic methyl arachidonate as described in the preceding paragraph.

In addition to the methyl esters of NLE#3, the other methyl ester samples, which were subjected to the GLC analysis described in Sections III.A.2.a.(2) and III.B.3.a.(2), were also analyzed by capillary column GC/CIMS. The results of this analysis are also displayed in table 16. The assignments of chemical structure were made on the basis of the chromatographic retention time, the m/e of the $(M+1)^+$ ion, and the characteristics of each TIC peak's mass spectrum. For those fatty acids which were present in very low quantities, mass fragmentograms were constructed using the computer to search for the hypothesized $(M+1)^+$ ion in the TIC chromatogram. The mass spectrum was then taken at the top of the mass fragmentographic peak, and the characteristics of the mass spectrum were either compared with the mass spectrum of an authentic sample of the hypothesized fatty acid from the standards or rationalized using standard techniques for the interpretation of mass

TABLE 16

COMPARISON OF THE FATTY ACIDS IN THE METHYLATED PRODUCTS OF THE LIPID FRACTION IDENTIFIED BY EITHER GLC (*) OR GC/CIMS (+)

Assigned Structure[1]	Samples:[2] NLE#3	AM: High Activity	AM: Low Activity	H_2 AM	D_2 AM
14:0	+	+	+	+	+
15:0	+	+	+	+	
16:0	* +	* +	* +	* +	* +
16:1	* +	+			+
16:2	+				+
17:0	+	+	+	+	+
17:1	+	+			
18:0	* +	* +	* +	* +	* +
18:1	* +	* +	* +		+
18:2	* +	* +	* +		+
18:3	* +	*	* +		+
19:0				+	
20:0				* +	*
20:1	+	+	+		
20:3	* +	* +			+
20:4	* +	* +	+		+
20:5	* +	* +	+		+
22:0				* +	*
22:1	+				
22:3	+				
22:4	* +	+			
22:5	* +	+			
22:6	* +	+			

1. Structures assigned on the basis of the m/e of the $(M + 1)^+$ ion, characteristics of the mass spectra, and/or the chromatographic retention time.
2. Samples described in Section IV.B.3.a.(2).

spectra (McLafferty, 1973).

The assignments of the structures for the components of the D_2AM were made from the presence of multiple $(M+1)^+$ peaks in the mass spectrum of each TIC chromatographic peak. Due to deuterium scrambling (Stenhagen, 1966) and the ^{13}C isotopic abundance contribution for long alkyl chains (McLafferty, 1973), there was not always a clear difference of 2 amu between the $(M+1)^+$ peaks of the assigned structures. However, by combining the information from the D_2AM mass spectra with the chemical structures assigned to the high and low activity AM and the H_2AM samples, it was possible to assign the structures indicated in column six of table 15.

The chemical structures assigned by comparison of the relative retention times of the samples and standards on the GLC closely matched those assigned using capillary column GC/CIMS. Table 16 contains a comparison of the assigned structures using the two methods. The differences may be explained as follows: The capillary column GC/CIMS allows structure assignment in the absence of standard. Those fatty acids not assigned using GLC, which were assigned using GC/CIMS, may have been present in the GLC chromatograms, but no standard was available for comparison. Additionally, the technique of mass fragmentography allows structural assignments for those fatty acids present in such small quantities that the GLC peak may not have been distinguishable from baseline. On the other hand, those fatty acids which were not assigned by GC/CIMS but

were originally assigned using GLC retention times are likely to have been incorrectly assigned by the GLC method. This incorrect assignment may have been due to the presence of substances which chromatographed with the same retention time as the standard. Since the GC/CIMS scans were done using a lower m/e cutoff at 100 amu, any low molecular weight compounds were eliminated from the TIC chromatogram. Also, for those TIC chromatographic peaks whose $(M+1)^+$ ion did not match that of any known fatty acid, chemical structures were not assigned. It is possible these unassigned TIC chromatographic peaks may be due to side products of the methylation reaction, as the fractional m/e of 0.3 amu is not consistent with a structure composed solely of C,H, and O.

The mass spectrometric analysis provides confirmatory evidence that the lipid fraction is a mixture of unsaturated and saturated fatty acids. Additionally, the presence in the lipid fraction of arachidonic acid (20:4w6) and other prostaglandin/thromboxane precursors and their homologs is also confirmed.

IV. GENERAL DISCUSSION

A. Review of the problem.

At the time that this dissertation was initiated, all that was known of the pharmacological properties of the lipid fraction was what had been summarized in Section I.A. The preparation of the lipid fraction, active material (AM), isolated by the procedure discussed in Appendix A had been brought by thin layer chromatographic techniques to a single spot by Dr. Edward J. Ronan. He had confirmed the pharmacological properties of blockade by atropine or morphine of the effect on the guinea-pig ileum of the AM eluted from the TLC spot. The elucidation of the pharmacological mechanism of action of the lipid fraction was of interest in view of the speculation that "Among substances present in mammalian nerve, the active compound may be unique in its property of effecting release of the cholinergic transmitter, an action that morphine totally blocks.", and, "The active substance may be a component in the presynaptic event leading to the release of the cholinergic transmitter after nerve stimulation." (Green & Carlini, 1964).

Based on the published properties of the lipid fraction and the speculations mentioned above, the two major hypotheses described in Section I.C. were formulated and tested: that the lipid fraction was a universal cholinergic

releasing agent which did not require the presence of action potentials to produce its effect and that the effect of the lipid fraction was necessarily linked to narcotic analgesic sensitivity of the stimulus evoked release of acetylcholine.

B. Approaches to the problem.

In order to determine the nature of the lipid fraction, it was decided to combine the use of a pharmacological screen with qualitative organic analysis. By doing so, it was possible to make maximum use of the research resources available and of the different and often complementary information derived from the two techniques. Once the two major hypotheses were disproved, the pharmacological screen was initiated to determine if the lipid fraction functioned on the rabbit ileum in a similar manner to its actions on the guinea-pig ileum. The first major finding was the atropine-fast stimulation of the rabbit ileum. This was followed by the blockade of AM by tetrodotoxin, and this finding was confirmed on the guinea-pig ileum. However, though these findings suggested the involvement of non-cholinergic excitatory neurones, the real breakthrough was the demonstration of the reversible, surmountable blockade of the effect of AM by the cyclo-oxygenase inhibitor, mefenamic acid.

Once the lipid fraction was conceptually linked to the metabolic products of the cyclo-oxygenase, the acidic nature of the lipid fraction was established and then its long chain unsaturated fatty acid character. Next, it was

identified as a mixture of saturated and unsaturated fatty acids with sufficient cyclo-oxygenase substrates to account for the biological activity. Following the chemical characterization of the lipid fraction, other fatty acids obtained from commercial sources were examined for their biological activity and compared and contrasted with the lipid fraction. Finally, GC/CIMS provided positive identification of the component fatty acids and confirmatory evidence for the presence of arachidonic acid and other cyclo-oxygenase substrates as well as inhibitors. For example, table 11 shows the difference in the fatty acid composition between the high activity AM and the low activity AM. The high activity AM contains roughly 2.5 times the amount of classical cyclo-oxygenase substrates that the low activity sample has, while the low activity AM contains almost 20 times more than the high activity AM of 18:2w9, a compound known to inhibit prostaglandin synthesis (Pace-Asciak & Wolfe, 1968; Lands, Lee, & Smith, 1971; Lands, LeTellier, Rome, & Vanderhoek, 1973; Ziboh, Vanderhoek, & Lands, 1974; Lands & Rome, 1976).

C. Integration of the results.

1. Comparison of the results with previously published findings.

Comparing the properties of the AM (Sections I.A. and III.B.1.) with the properties of the pharmacologically active lipids described in Section I.B., it is

apparent that there are similarities between the lipid fraction and the PALs related to the cyclo-oxygenase system (section I.B.4.). Four main properties of, or rather, conclusions about, the lipid fraction mitigated against a conceptual linkage of it with the PALs related to the cyclo-oxygenase system: First, the total blockade of the action of AM by morphine. From the experiments of Jaques (1959) with 20:4w6, Vogt (1959) with Darmstoff, and Jaques (1969) and Sanner (1971) with PGEs, it was known that only part of the action of cyclo-oxygenase substrates or products was blockable by morphine. Arachidonic acid, in low doses, is totally blocked by narcotic analgesics (Jaques-1959). As the dose of arachidonic acid is increased, the blockade is surmounted, and increasing concentrations of narcotic analgesics to not re-establish the blockade. Splawinski et al. (1973) have demonstrated that, on the rat fundus preparation, arachidonic acid has indirect actions via formation of prostaglandins as well as direct actions as a partial agonist on prostaglandin receptors. Therefore, increasing concentrations of arachidonic acid may have direct action on the smooth muscle prostaglandin receptors of the ileum as a partial agonist as well as via prostaglandin formation. At lower doses, arachidonic acid stimulates the ganglionic prostaglandin receptors both directly as a partial agonist and indirectly via prostaglandin formation.

An alternative explanation of the narcotic analgesic resistant stimulation by high concentrations of arachidonic acid is that narcotic analgesics only inhibit the stimulus evoked release of acetylcholine at low levels of stimulation (Paton, 1957; Schauman, 1957; Cowie, Kosterlitz, & Watt, 1968; Lees, Kosterlitz, & Waterfield, 1973). If a high concentration of arachidonic acid produces a high level of stimulation at the ganglionic prostaglandin receptors, either via the synthesis of prostaglandins or via arachidonic acid's own partial agonist activity, then the inhibition by narcotic analgesics of the stimulus evoked release of acetylcholine may be indetectably slight.

Second, the total blockade by atropine. Again, Jaques (1959) had demonstrated that atropine totally blocked only the lower doses of 20:4w6. Vogt (1959) had shown the same for Darmstoff, and Bennett, Eley, and Scholes (1968), Harry (1968), and Sanner (1971) demonstrated blockade of only low doses of PGEs by atropine.

Third, the fact that the AM was only found in neural tissue and not in extracts of intestine, kidney, liver, or lung which were known to contain cyclo-oxygenase precursors and/or products (e.g., Darmstoff, Medullin, 20:4w6, RCS and SRS-A, respectively. (See Section I.B.4.b. through e.).

And fourth, the failure to demonstrate extraction from petroleum ether with aqueous K_2CO_3 was taken to indicate that the AM was not an acid, whereas all of the aforementioned PALs were known to have acidic properties.

These four conclusions are readily explainable in hindsight. Because so little of the AM was available for pharmacological analysis, it was not possible for earlier investigators to obtain complete concentration-effect curves for the AM. Had they been able to do so, they might have uncovered the resistance of the effects of high doses of AM to morphine or atropine. The third and fourth conclusions both can be explained by the partition coefficient of AM, which has been estimated to be between 0.29 and 0.21, (See Section III.B.2.d.(2).). As can be seen from column 5 of table 10, the initial extraction procedure (which itself was a triple extraction) contained only 19% of the biological activity. Considering the wide range of sensitivities demonstrated between individual ileal preparations, it is certainly conceivable that "essentially all of the activity remained in the petroleum ether, indicating that the active material was not an acid." (Robinson et al., 1967).

The ability to demonstrate biological activity in acetone extracts of nerve or brain, while "similar activity could not be detected in kidney, intestine, lung,, or liver" (Green, Carlini, & Robinson, 1963), might have

been due either to the partition coefficient or to relatively higher content of unsaturated long chain fatty acids in neural tissue (Mead & Fulco, 1976, p. 11).

From the foregoing discussion, it is possible to see how the apparent inconsistencies between the original published properties and the results of this dissertation can be resolved. The total blockade of the action of AM by morphine and atropine is likely to have been due to the inability to test high doses of AM. The failure to find AM in organs other than nervous tissue and to demonstrate its acidic nature is likely to have been due to its small partition coefficient and its isolation by a procedure designed to eliminate fatty acids. One inconsistency has so far remained unexplained.

"Since cocaine did not prevent its action, the material (lipid fraction) cannot be acting through stimulation of the nerve fibers of the ileum." (Green, Carlini, & Robinson, 1963). This statement is difficult to reconcile with the evidence presented in this thesis (see Sections II.B.1.d.(3).(c) and II.B.2.b.(2) demonstrating that Tetrodotoxin, at a concentration of 3.1×10^{-7} M, is a nonsurmountable physiological antagonist of the lipid fraction, arachidonic acid, and the prostaglandins, PGE_2 and $PGF_{2\alpha}$. A possible explanation of the apparent inconsistency may be found in the multiple actions of cocaine on intestinal preparations.

Cocaine has been classically described as inhibiting gastrointestinal preparations by blocking the postganglionic nerve fibers in the gut. Reference is usually made to the classical papers of Feldberg and Lin, 1949, Rocha e Silva, Valle, and Picarelli, 1953, Blair and Clark, 1956, and Gaddum and Picarelli, 1957. Cocaine is known to be a local anaesthetic (Ritchie, 1971), and has been shown to block the ganglionic stimulation due to nicotine at doses that did not inhibit the direct smooth muscle stimulation due to acetylcholine or histamine (Feldberg & Lin, 1949). When it was shown that cocaine blocked the action of both 5-hydroxytryptamine (5-HT) and nicotine (Gaddum & Picarelli, 1957), it was assumed that the mechanism of the blockade by cocaine was the same for both agonists, i.e., blockade of postganglionic nerve fibers. However, evidence did exist that the mechanisms of action were not necessarily identical. Rocha e Silva et al., 1953, found that the effect of 5-HT could be blocked by cocaine concentrations as low as 2.2×10^{-6} M. Gaddum and Picarelli, 1957, showed that 3.3×10^{-5} M cocaine generated a dose ratio for 5-HT which was, on the average, five and a half times the dose ratio for nicotine. Indeed, the tone and movements of small intestinal muscle had long been known to be increased in most animals by small doses of cocaine and decreased by large doses (Bayliss & Starling, 1899; Langley & Magnus, 1905; Trendelenberg, 1917; Feldberg & Lin, 1947).

Recently, it has been reported that cocaine is an antagonist of 5-HT. In the rabbit heart, 5-HT stimulates the release of norepinephrine from sympathetic nerves (Fozard & Mobarok Ali, 1976). This action of 5-HT is blocked by both (+)- and (-)-cocaine with pA_2s of 6.9 and 6.2, respectively. Dimethylphenylpiperizinium (DMPP), a ganglionic nicotinic agonist, also stimulates the release of norepinephrine from sympathetic nerves. DMPP is also antagonized by (+)- and (-) cocaine, but the antagonism is not stereospecific and occurrs at a higher concentration with a pA_2 of 5.0 (Fozard, Mobarok Ali, & Muscholl, 1977). While there was no indication that the antagonism of the actions of 5-HT by cocaine is competitive, there was a clear distinction between the antagonism of 5-HT and the antagonism of DMPP. Other drugs, classically described as local anaesthetics also acted as 5-HT antagonists. Metaclopromide was the most potent with a pA_2 of 7.3 against 5-HT and of 4.5 against DMPP. Neopsicaine (pseudococaine), with pA_2s against 5-HT and DMPP of 6.4 and 5.1, respectively; tropacocaine, pA_2s of 6.8 and 4.8; and procaine, pA_2s of 5.6 and 4.1, were also selective 5-HT antagonists. Lidocaine, with pA_2s against 5-HT and DMPP of 3.9 and 4.2, respectively; tropine, pA_2s of 4.2 and 4.3; and atropine, pA_2s of 4.8 and 4.6, were all non-selective 5-HT antagonists.

Metaclopramide has been shown to antagonize, at low concentrations, the actions of 5-HT on the guinea-pig ileum (Fontaine & Reuse, 1973; Birtley & Baines, 1973)

and colon (Bianchi, Beani, & Crema, 1970). Metaclopramide also potentiates the effect of acetylcholine on human intestinal preparations (Eisner, 1968), on guinea-pig colon (Beani, Bianchi, & Crema, 1970), and on guinea-pig ileum (Birtley & Baines, 1973; Bury & Mashford, 1976). In the guinea-pig ileum preparation, the potentiation by metaclopramide of the effect of acetylcholine, substance P, histamine, and barium chloride was completely eliminated by the presence of 3.1×10^{-7}M TTX. Atropine, 1.4×10^{-7} M, reduced the potentiation of the effect of substance P,histamine, and barium chloride, but the reduction was not complete. The potentiation of the effect of exogenously added acetylcholine was completely abolished by atropine (Bury & Mashford, 1976).

The actions of 5-HT on intestinal preparations are complex. Drakontides and Gershon (1968) applied the findings of Fastier, McDowell, and Waal (1959), that phenylbiguanide interacts with a particular class of 5-HT receptors, to determine that 5-HT can activate receptors at three different sites in the mouse duodenum. In addition to the excitatory receptor directly on the smooth muscle, 5-HT stimulates receptors on both excitatory and inhibitory neurones (Drakontides & Gershon, 1968). The inhibitory neurone on which 5-HT acts may be the non-adrenergic inhibitory neurone (Bulbring & Gershon, 1967; Burnstock, 1972). The excitatory neurone may be the classical cholinergic excitatory neurone (Roche e Silva et al., 1953;

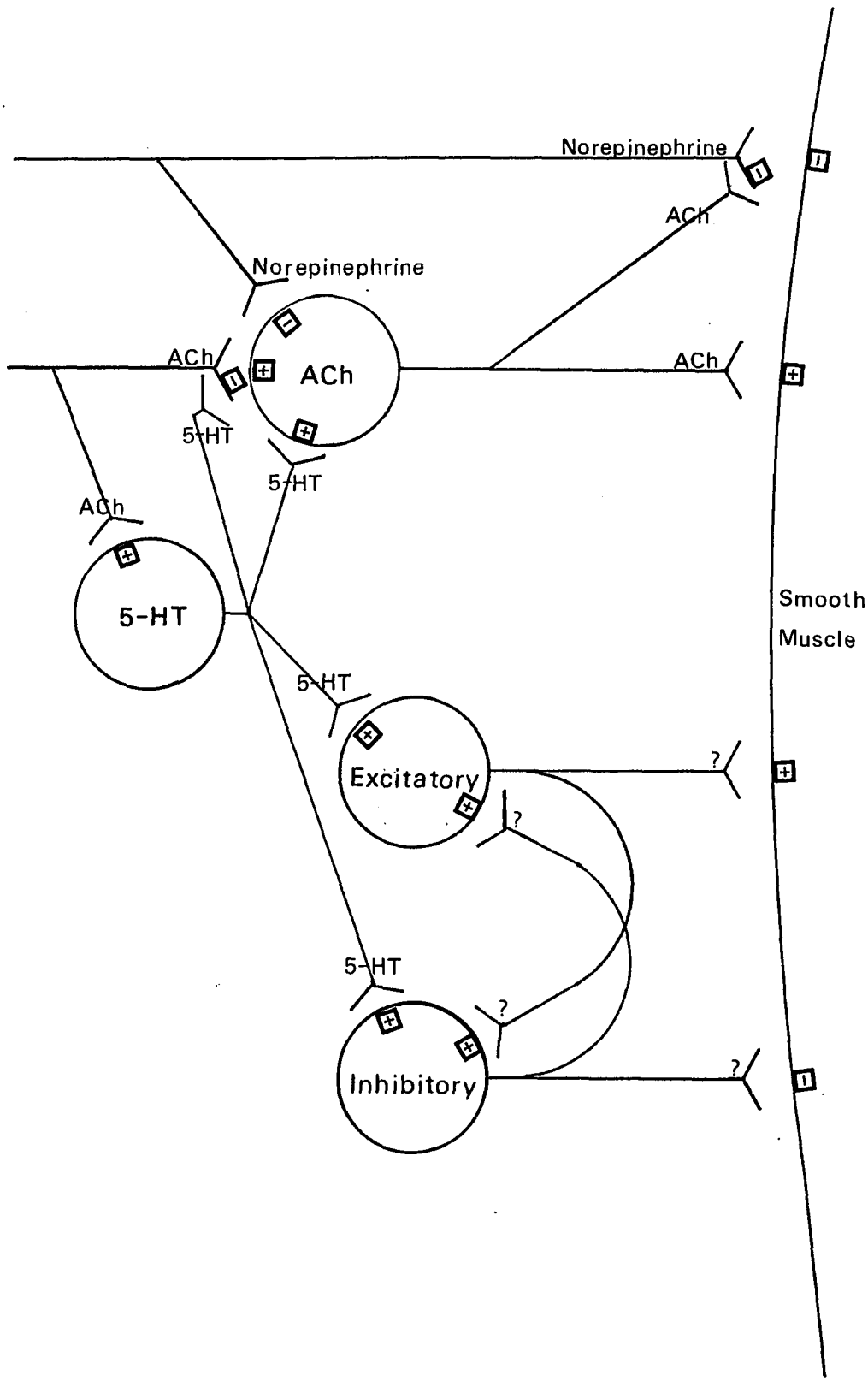
Gaddum & Picarelli, 1957) or a noncholinergic neurone whose transmitter has not been characterized (Ambache & Zar, 1970; Furness & Costa, 1973). Indeed, the situation in the guinea-pig ileum is still further complicated by the presence of 5-HT receptors, blockable by methysergide, which reduce the release of ACh from most presynaptic terminals (Henderson & North, 1975). Finally, there is evidence that 5-HT and norepinephrine can interact on the same class of receptors to cause a stimulation of the preparation (Innes & Kohli, 1969).

Sorting out the effects of 5-HT antagonists becomes a problem of recognizing which class of receptors on which type of effector cells are being inhibited by which range of concentrations. For example, the potentiation of nicotine by metaclopramide might be due to the inhibition of the action of 5-HT (released by nicotinic stimulation) at inhibitory neurones or by blockade of the action of endogenous levels of 5-HT which act presynaptically to inhibit the relase of acetylcholine. The potentiation of acetylcholine might be due to a simple removal of endogenous inhibitory tone. The diagram of the proposed sites of interaction of 5-HT and nicotine (fig. 22 redrawn from fig. 8 of Bianchi et al., 1970) may be of use in the interpretation of the actions of 5-HT on intestinal preparations.

In view of the above discussion on the 5-HT antagonizing activities of drugs whose actions have been considered to be primarily as local anaesthetics, it is

Fig. 22. A possible neuronal "hook-up" for the rabbit ileum.

A possible neuronal "hook up" for the rabbit ileum. ACh released by parasympathetic stimulation interacts with nicotinic ganglionic ACh receptors to stimulate ⊞ neuronal firing. 5-HT released by nicotinic ACh stimulation interacts with preganglionic parasympathetic nerve fibers to inhibit ⊟ ACh release and with postganglionic parasympathetic neurones, excitatory neurones, and inhibitory neurones to stimulate their firing.


Norepinephrine
ACh
Norepinephrine
ACh
ACh
ACh
5-HT
5-HT
ACh
5-HT
Smooth
Muscle
5-HT
Excitatory
?
?
5-HT
?
Inhibitory
?

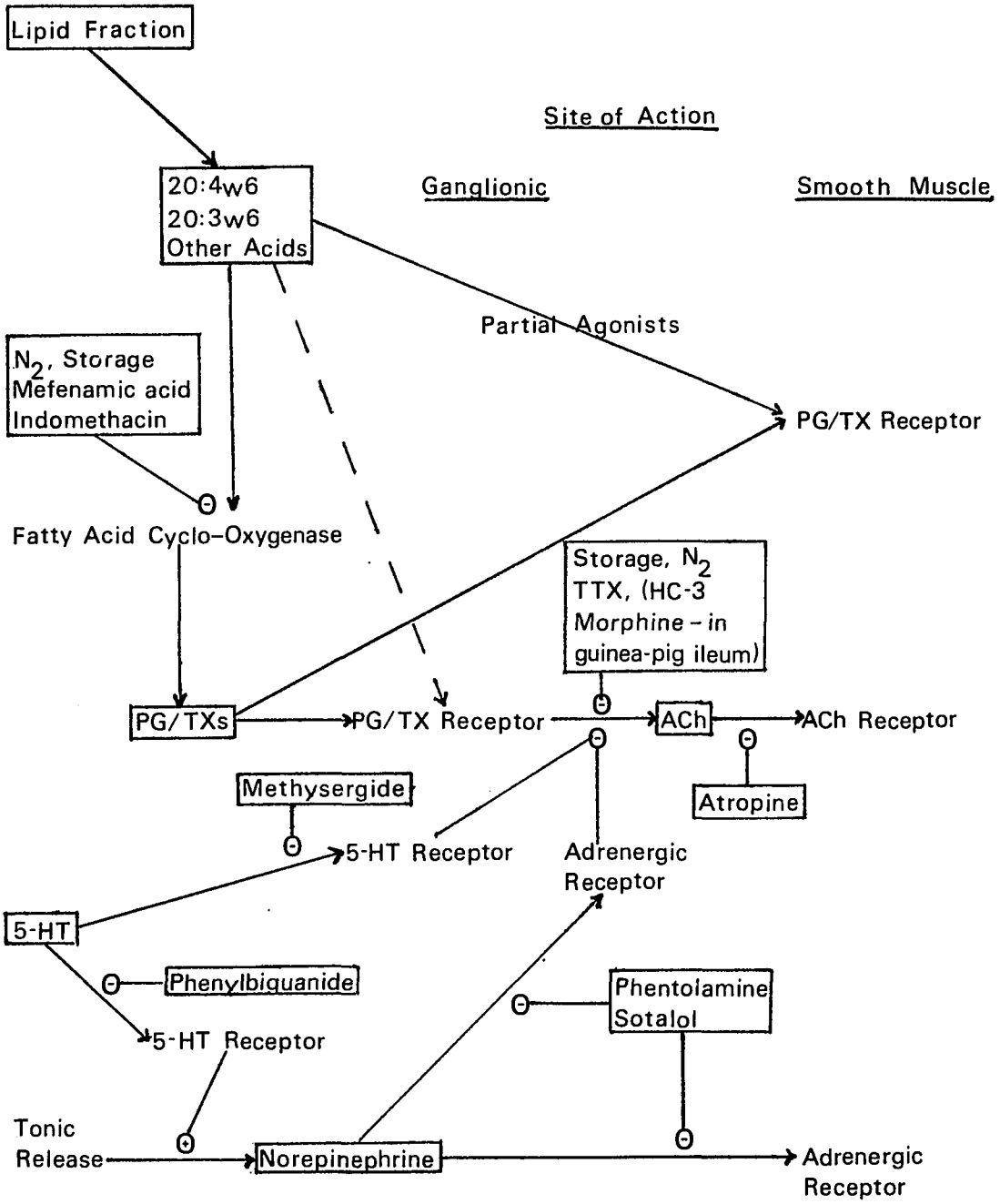
possible that the reported failure to block the action of AM with concentrations of cocaine that decreased the effect of added 5-HT by two-thirds (Green, Carlini, & Robinson, 1963; Green & Carlini, 1964) may have been due to a failure to establish local anaesthesia, even though the concentrations used (5.9×10^{-6} and 1.2×10^{-5} M) were closer to the pA_2 for local anaesthesia (5.0) than to the pA_2 for 5-HT antagonism (about 6.5 for racemic cocaine) in the rabbit heart (Fozard, Mobarok Ali, & Muscholl, 1977).

2. Proposed mechanism of action of the lipid fraction on intestinal preparations.

A possible mechanism of action of the lipid fraction is shown in fig. 23. The mechanism provides for the lipid fraction to either serve as cyclo-oxygenase substrates (in the rabbit ileum and perhaps the guinea-pig ileum) or to shift the metabolic flow of endogenous cyclo-oxygenase products (perhaps in the guinea-pig ileum). The result is the production of cyclo-oxygenase products (the endoperoxides, prostaglandins, and/or thromboxanes) which stimulate the release of ACh and, perhaps, inhibit the release of norepinephrine. The cyclo-oxygenase is blocked by anoxia, mefenamic acid, indomethacin, and possibly storage of the tissue overnight. The products of the cyclo-oxygenase stimulate the release of ACh from neuronal sites, and this ACh release is blocked by storage, HC-3, TTX, morphine, (in the guinea-pig ileum), and adrenergic stimulation.

Fig. 23. A possible scheme to explain the mechanism of action of the lipid fraction.

A possible scheme to explain the mechanism of action of the lipid fraction. ⊕ denotes apparent potentiation, ⊖ denotes inhibition. For a detailed explanation see text, Section IV.C.2.


Lipid Fraction
Site of Action
Ganglionic
Smooth Muscle
20:4w6
20:3w6
Other Acids
Partial Agonists
N_2, Storage
Mefenamic acid
Indomethacin
PG/TX Receptor
Fatty Acid Cyclo-Oxygenase
Storage, N_2
TTX, (HC-3
Morphine - in
guinea-pig ileum)
PG/TXs
PG/TX Receptor
ACh
ACh Receptor
Atropine
Methysergide
5-HT Receptor
Adrenergic
Receptor
5-HT
Phenylbiguanide
5-HT Receptor
Phentolamine
Sotalol
Tonic
Release
Norepinephrine
Adrenergic
Receptor

The released ACh interacts with muscarinic receptors, and this interaction is blocked by atropine.

The rabbit ileum (and, perhaps, the guinea-pig ileum) is under tonic adrenergic inhibition which is itself blocked by sotalol, phentolamine, and prostaglandins of the E series. The potentiation of the action of AM by methysergide may be partly explained by the finding that 5-HT reduces the release of ACh from presynaptic terminals within the myenteric plexus and that this reduction of release was prevented by methysergide (Henderson & North, 1975). It is possible that the lipid fraction acts to stimulate the same cholinergic nerve that 5-HT inhibits. On the other hand, phenylbiguanide increases the baseline pendular motion without much effect on the action of the lipid fraction. This may be explained by 5-HT acting to stimulate the tonic adrenergic inhibition of the pendular motion.

In addition, the products of the cyclo-oxygenase can directly stimulate the smooth muscle. Finally, it is possible that the component fatty acids can themselves act as partial agonists of the prostaglandin/thromboxane receptors (e.g., Splawinski et al., 1973).

V. SUMMARY AND CONCLUSIONS

A. Pharmacological analysis.

1. The lipid fraction is not a universal cholinergic releasing agent which does not require the presence of action potentials to produce its effect.

2. The effect of the lipid fraction is not necessarily linked to narcotic analgesic sensitivity of the stimulus evoked release of acetylcholine by isolated smooth muscle preparations.

3. The action of the lipid fraction is blocked by inhibitors of the fatty acid cyclo-oxygenase.

4. Fatty acids which are cyclo-oxygenase substrates stimulate the rabbit ileum in a manner similar to the lipid fraction.

5. In the rabbit ileum, the lipid fraction may function as a substrate for the cyclo-oxygenase and, possibly, as a partial agonist at cyclo-oxygenase metabolite receptors. In the guinea-pig ileum, the lipid fraction may either function as in the rabbit ileum or act to shift the flow of the metabolic products of the cyclo-oxygenase.

6. The effect of the lipid fraction is not limited to the release of acetylcholine. In the rabbit ileum the effect of the lipid fraction involves the release of excitatory transmitter(s) other than and in addition to acetylcholine. In the absence of action potentials, the lipid fraction causes a direct stimulation of the rabbit ileum.

7. The lipid fraction stimulates the frog skin preparation in a manner similar to the stimulation caused by arachidonic acid.

8. The lipid fraction does not act via nicotinic neuromuscular acetylcholine receptors in the frog rectus abdominus preparation.

B. Chemical analysis.

1. The lipid fraction behaves as a long chain fatty acid with a partition coefficient from hexane: alcoholic K_2CO_3 of approximately 0.25. Serial extraction of the fatty acids from the organic phase results in a decrease in the biological activity of the organic phase that is proportional to the increase in the biological activity of the aqueous phase.

2. Tests for nitrogen and for ketone groups failed to reveal either.

3. Argentation thin layer chromatography of the methyl esters of the lipid fraction reveals it to be composed of a mixture of saturated and unsaturated fatty acids.

4. Gas liquid chromatography of the methyl esters of the lipid fraction shows it to be a mixture of fatty acids similar in composition to the fatty acids extracted from neural tissue as reported in the literature. The biological activity of the lipid fraction seems to parallel its content of arachidonic acid.

5. Ultraviolet, infrared, and nuclear magnetic resonance spectroscopic analyses of the lipid fraction suggest that it contains unsaturated fatty acids and eliminates the possible presence of ketones, aldehydes, acid halides, anhydrides, amides, lactones, and hydroperoxides. These analyses further suggest that the lipid fraction is a mixture of saturated and unsaturated fatty acids.

6. Mass spectrometric analysis confirms the structures of the fatty acids present in the lipid fraction, provides the identification of fatty acids for which standards were not available, and also confirms the presence of arachidonic acid and other substrates for the fatty acid cyclo-oxygenase.

C. Overall Conclusions.

1. While the structures and properties of many of the compounds related to the phospholipase/cyclo-oxygenase/lipoxygenase system remain to be elucidated, it is likely that any newly discovered pharmacologically active lipid material will be related to this system.

2. The pharmacological activities and chemical properties of the lipid fraction are accounted for by its characterization as a mixture of fatty acids containing arachidonic acid and other cyclo-oxygenase substrates.

APPENDIX A: Preparation of the Lipid Fraction (LF)

I. Active Material (AM)

The preparation of active material used in this dissertation was developed by Dr. Edward J. Ronan with the assistance of Ms. Barbara Craddock as a modification of the original procedure developed by Robinson et al. (1967). Frozen bovine brain was placed in acetone (1:10 w/v), blended, and stirred at 4^{o} C for one hour. The homogenate was then strained through cheesecloth and centrifuged at low speeds (600 x g) to remove the remaining large tissue particles. The supernatant was flash evaporated, and the residue was dissolved/suspended in the minimum volume of chloroform:methanol (1:1 v/v). Distilled water, equal to 1/5 the volume of the chloroform:methanol solution/suspension, was added, and the mixture was centrifuged at low speed (600 x g) to separate the phases. The resulting Folsch-Pi extraction yielded neutral lipids in the lower (chloroform: water) phase and polar lipids in the upper (methanol: water) phase. The lower neutral lipid phase was flash evaporated and dissolved in hexane. The hexane solution was chromatographed on silicic acid columns (1 cm diameter x 25 cm length) first with hexane, then 5% diethyl ether in hexane, and the active material was found to be eluted with 12% diethyl ether in hexane. The active material fraction was layered onto a 1 cm diameter x 25 cm length LH-20 lipid Sephadex

chromatographic column and eluted with chloroform: hexane (1:1 v/v). The active material fraction eluted just before the bright yellow band of cholesterol. The eluant was flash evaporated and taken up in the minimum volume of chloroform. The active material fraction was streaked onto a 0.5 mm layer of silica gel HA (non calcium binder) on 20 cm x 20 cm glass thin layer chromatographic plates. The thin layer chromatogram was developed with hexane:diethyl ether (90:10 v/v), and the band containing the active material (Rf. = 0.1-0.2) was scraped off. The silica gel with the active material adsorbed was placed in a funnel with a fritted glass bottom, and the active material was eluted with chloroform. The chloroform was flash evaporated, and the active material was dissolved in nitrogen flushed chloroform to yield a chloroform stock solution with a final concentration of approximately 1 mg/ml. The active material was then stored under nitrogen at -40° C until the day before it was to be used. A 1 ml aliquot of the active material in chloroform was removed and evaporated under a stream of nitrogen. When the chloroform had completely evaporated, Tyrode's solution was added in 100 µl portions, and the mixture was suspended in a test tube by means of a Vortex mixer and a glass stirring rod. Sufficient additional Tyrode's solution was added to bring the suspension to the original volume of the chloroform aliquot. The suspension of active material

in Tyrode's solution was then flushed with nitrogen and frozen overnight at -40° C. On the morning of the experiment, the active material suspended in Tyrode's solution was thawed at room temperature and kept under nitrogen until ready for use.

II. Neutral Lipid Extract (NLE)

The preparation of neutral lipid extract used in this dissertation was developed by Drs. L. Allen Barker and Edward J. Ronan. Frozen bovine brain (100 g) was added to 300 ml of chloroform:methanol (1:2 v/v) and blended for 2 min. at maximum speed. An additional 100 ml of chloroform was added to bring the mixture to chloroform:methanol (1:1 v/v), and the mixture was blended again for 30 sec. Distilled water (100 ml) was added to the mixture to bring the composition to chloroform:methanol:water (1:1:0.9 v/v/v), assuming that the brain tissue was 80% water. The mixture was then filtered through paper, and the filtrate allowed to separate into two phases overnight at 4° C. The lower neutral lipid phase of chloroform:water was poured through a silicic acid column, and everything that passed through the column was flash evaporated. The residue from the flash evaporation was dissolved in chloroform:methanol (1:1 v/v), and distilled water equal to 1/5 the volume was added. The resulting mixture was centrifuged at low speed (600 x g) to separate the phases. The lower neutral lipid phase was flash evaporated and dissolved

in 200 ml of hexane. This neutral lipid extract was stored under nitrogen at -40° C until the day prior to use. A 5 ml aliquot of the neutral lipid extract in hexane was removed and evaporated under a stream of nitrogen. When the hexane had completely evaporated, Tyrode's solution was added in 0.25 ml portions, and the mixture was suspended in a test tube by means of a Vortex mixer and a glass stirring rod. Sufficient additional Tyrode's solution was added to bring the suspension to 1/5 of the original volume of the hexane aliquot. The suspension of neutral lipid extract in Tyrode's solution was then flushed with nitrogen and frozen overnight at -40° C. On the morning of the experiment, the neutral lipid extract suspended in Tyrode's solution was thawed at room temperature and kept under nitrogen until ready for use. The results from experiments done with the neutral lipid extract were always expressed in terms of the volume of the original hexane aliquot rather than the volume of the suspension in Tyrode's solution that was added to the isolated tissue bath.

APPENDIX B: Drugs used in the course of this dissertation.

acetylcholine chloride: Sigma

acetyl-β- methylcholine chloride: Sigma

amiloride: Merck and Company

arachidonic acid: Supelco

atropine sulfate: ICN

dihoma-γ -linolenic acid: Supelco

d,l-epinephrine hydrochloride: K & K Labs

eserine (physostigmine) salicylate: Sigma

gluthathione, reduced: Sigma

hemicholinium-3: Aldrich

hexamethonium chloride: Nutritional Biochemicals

histamine dihydrochloride: Sigma

5-hydroxytryptamine (serotonin) creatinine sulfate: Sigma

indomethacin: Sigma

l-isoproterenol hydrochloride: Pfaltz and Baur, Inc.

linolenic acid: Supelco

γ-linolenic acid: Supelco

mefenamic acid: gift of Parke-Davis; the kindness of Dr. Alexander M. Moore is gratefully acknowledged.

methysergide maleate: gift of Sandoz, Inc.; the kindness of Kathleen D. Roskaz is gratefully acknowledged.

metiamide: Smith, Kline, and French

morphine sulfate: Mallinckrodt

nicotine bitartrate: City Chemical

phentolamine hydrochloride: gift of Ciba Pharmaceutical Company, the kindness of Charles A. Brownley, Jr. is gratefully acknowledged.

phenylbiguanide: ICN

phenylephrine: California Corporation for Biochemical Research

propranolol: Sigma

prostaglandin E_2: gift of The Upjohn Company; the kindness of Dr. John E. Pike is gratefully acknowledged.

prostaglandin $F_{2\alpha}$: gift of The Upjohn Company; the kindness of Dr. John E. Pike is gratefully acknowledged.

pyrilamine (mepyramine) maleate: gift of Sandoz, Inc.; the kindness of Kathleen D. Roskaz is gratefully acknowledged.

(-)-scopolamine (hyoscine) hydrochloride: Sigma.

sotalol hydrochloride: gift of Regis Chemical Company, the kindness of Charles Feit is gratefully acknowledged.

tetrodotoxin: Sigma

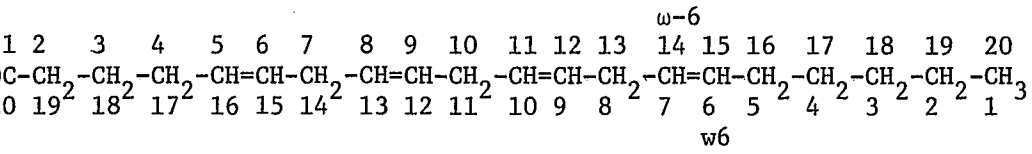
APPENDIX C: Fatty Acid Nomenclature

The nomenclature of fatty acids varies considerably from author to author. Holman (1966-1970) discussed the abbreviated notations that have been used and concluded that few offered any distinct advantages. The IUPAC nomenclature, although unambiguous, is exceedingly cumbersome for repeated references to structurally similar fatty acids. And the trivial names, such as γ-linolenic and arachidonic acids, do not imply their metabolic interrelationship. Because the specificity of the enzymes involved in the metabolism of polyunsaturated fatty acids appears in many cases to depend more on the unsaturated alkenyl tail structure than on the position of the double bonds relative to the carboxyl function, I have made use of a modification of the ω-n numbering system.

In the ω-n numbering system, arachidonic acid (5,8,11,14-eicosatetraenoic acid) is abbreviated 20:4ω-6; where 20 is the number of carbons in the alkyl chain counting the carboxyl group, : stands for methylene interrupted double bonds, 4 is the number of double bonds, and ω-6 stands for the first carbon of the double bond closest to the terminal methyl. However, this carbon is actually the 7th carbon from the end of the alkenyl chain, since ω-1 stands for the 19th carbon of a 20 carbon chain. Thus for 5,8,11,14-eicosatetraenoic acid, the double bond furthest from the carboxyl group is ω-6. At first glance, this system

seems to make sense since 20-6=14. But, if one views the fatty acid as, in anthropomorphic terms, the enzymes that metabolize it view it, one is interested in the start of the double bond counting backwards from the terminal methyl group.

In this dissertation, I have modified the ω-n numbering system to a wn system where, for example, w6 stands for the 6th carbon from the terminal methyl group. This wn system avoids the ambiguity of the ω-n numbering system as can be seen in the following example for arachidonic acid:



While the difference between the carboxyl and the terminal methyl side of a double bond is not necessarily of major importance, for ricinoleic acid (12-hydroxy-octadeca-9-enoic acid) the hydroxyl group is at ω-8 but at w9. The lipoxygenase that inserts the hydroxyl function does so at the 9th carbon from the end of the chain, and the w9 nomenclature specifies that carbon unambiguously.

APPENDIX D: Abbreviations

5-HT	5-hydroxytryptamine, serotonin
15-HPAA	15-hydroperoxy arachidonic acid
18:3w3	9,12,15-octadecatrienoic acid, linolenic acid
18:3w6	6,9,12-octadecatrienoic acid, γ-linolenic acid
20:3w6	8,11,14-eicosatrienoic acid, dihomo-γ-linolenic acid
20:4w6	5,8,11,14-eicosatetraenoic acid, arachidonic acid
ABMeCh	acetyl-β-methylcholine
ACh	acetylcholine
ADP	adenosine diphosphate
AM	active material - see Appendix A
amu	atomic mass unit
AVE CH	the average response of the rabbit ileum to ACh and/or ABMeCh
BHT	butylated hydroxytoluene, 2,6,-di-tert-butyl-4-hydroxytoluene
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
DLF	direct lytic factor
DMPP	dimethylphenylpiperizinium
EDTA	ethylenediaminetetraacetic acid
EFA	essential fatty acid
ED_{50}	the concentration of an agonist that produces half its maximum effect
EMAX	maximum effect of an agonist
FFA	free fatty acid
GC/CIMS	gas chromatography/chemical ionization mass spectrometry

GLC	gas liquid chromatography
GSH	glutathione
HC-3	hemicholinium-3
HETE	12-hydroxy-5,8,11,14-eicosatetraenoic acid
HPETE	12-hydroperoxy-5,8,10,14-eicosatetraenoic acid
I_{50}	concentration of an inhibitor that produces 50% inhibition
IR	infrared
k	rate constant for time-dependent inhibition
K_b	apparent affinity of a receptor for an antagonist
K_i	inhibition constant; dissociation constant of the enzyme-inhibitor complex
LASS	labile aggregation-stimulating substance
LF	lipid fraction - see Appendix A
LSD	lysergic acid diethyl amide
LTS	lymphocitic thyroid stimulator
(M+1)+	molecular ion that has picked up a hydrogen from the reagent gas
((M+1)+1)+	(M+1)+ ion with one ^{13}C isotope replacing a ^{12}C
m/e	mass to charge ratio
mepp	minature end-plate potential
NAD+	oxidized nicotinamide-adenine dinacleotide
NADP+	oxidized nicotinamide-adenine dinucleotide phosphate
NLE#3	the 3rd batch of neutral lipid extract, see Appendix A
NLE#3 FFA1-3	the free fatty acids obtained from the serial extractions of NLE#3, see section III.B.2.d.(2).
NMR	nuclear magnetic resonance
NSAI	non-steroidal anti-inflammatory

pA_2	negative logarithm to the base 10 of the concentration of an antagonist that requires a doubling of the dose of the agonist to produce the same effect
PAF	platelet activating factor
PAL	pharmacologically active lipid
pd	potential difference
PG___	prostaglandin___ (A-I), see figure 4.
PGDH	15 hydroxy-prostaglandin dehydrogenase
PLA_2	phospholipase A_2
PLB	phospholipase B
PRP	platelet rich plasma
RCS	rabbit aorta contracting substance
RCS-RF	RCS - releasing factor
Rf	ratio of the distance a solute travels from the origin relative to the solvent front
RSS	rat stomach strip
SCC	short circuit current
SDS	sodium dodecylsulfate
SRS-A	slow reacting substance - anaphylaxis
SRS-C	slow reacting substance - cobra venom
TIC	total ion current
TLC	thin layer chromatography
TMS	tetramethylsilane
TSH	thyroid stimulating hormone
TTX	tetrodotoxin
TX___	thromboxane___ (A-B), see figure 4.
UV	ultraviolet

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