DEVELOPMENT OF A TECHNIQUE FOR *IN SITU* PERFUSION OF THE RAT ADRENAL GLAND: EFFECTS OF ACTH AND PROSTAGLANDINS ON CORTICOSTERONE OUTPUT

by

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ABSTRACT

A technique is described for in situ perfusion of a single rat adrenal gland through its arterial circulation permitting continuous collection of secretory products from the single vein that drains the gland. Male hooded Long Evans rats (300 - 450 gms) were anesthetized with urethane and the arterial and venous circulations of the left gland isolated. Oxygenated Krebs Ringer bicarbonate glucose (KRBG) at 38° C was perfused within 60 seconds after interruption of the circulation. X-ray studies of glands perfused with Thorotrast or $BaSO_4$ confirmed that the perfusate passed through the adrenal and was present in the venous effluent. Flow rate was maintained at 1.8 to 6.0 ml/hr with a peristaltic pump and remained constant for a given experiment. Thirty minute samples of venous effluent were collected and analyzed for corticosterone (B) by acid-fluorescence. Both B output and flow of effluent fell to immeasurable levels when the adrenal vein was ligated during a perfusion. This confirmed the adrenal origin of the venous effluent.

Perfusion with unsupplemented KRBG (controls) of adrenals from intact rats showed 2 different patterns of response over a 6 hour period; (1) a high initial output of B during the first 30 minutes of perfusion which decreased with time (Pattern I) and, (2) a high initial output which decreased during the first hour, followed by spontaneous transient increases which then decreased with time (Pattern II). Regardless of the pattern of response, mean outputs of 1.8 ($\pm$ 0.25 S.E.) µg B during the first 30 minutes declined to 1.0 ($\pm$ 0.25 S.E.) after 3 hours and 0.62 ($\pm$ 0.20 S.E.)

μg B after 6 hours of perfusion.

Perfusion of adrenals from rats 2 to 3 hours after hypophysectomy showed low initial outputs of B which gradually increased with time. Mean outputs of 0.12 (± 0.06 S.E.) μg B during the first 30 minutes increased (spontaneously) to 0.66 (± 0.28 S.E.) μg after 5 to 6 hours of perfusion.

Most adrenals from intact rats continuously perfused with ACTH (100 mU/ml) maintained relatively high outputs of B for 3 to 4 hours or longer before showing any decline. Mean outputs of 1.8 (± 0.21 S.E.) μg B during the first 30 minutes increased to 3.2 (± 0.80 S.E.) μg after 3 hours and declined to 1.1 (± 0.18 S.E.) after 6 hours of perfusion. Response to ACTH in most perfusions was immediate showing little or no lag period. The mean total 3 hour B output from KRBG-perfused adrenals was 7.2 (± 1.4 S.E.) μg B; this increased to 12.87 (± 1.4 S.E.) under the influence of ACTH ($P < 0.05$). No relationship was observed between flow rate and B output from any control or ACTH-perfused adrenals.

Attempts were made to find an explanation for the transient increases in output observed from some KRBG-perfused adrenals. They did not appear to be due to an effect of ACTH or nembutal, nor were they directly related to the time of day. The increases were eliminated or diminished by perfusion with dextran and with indomethacin, an inhibitor of prostaglandin synthetase. Addition of 5 μg/ml prostaglandin E_2 (PGE_2) to the indomethacin-containing medium resulted in a transient increase in B output suggesting that the transient increases may represent a response to increased endogenous adrenal prostaglandin synthesis. Other possible explanations for the

transient increases are discussed.

Perfusion with PGE_1, PGE_2 and $PGF_{1\alpha}$ of adrenals from intact rats increased B output. Variation in the response of different glands to a particular prostaglandin was observed. PGE_1 and PGE_2 did not increase B output from in vitro-incubated adrenal sections. The mean total 3 hour B output from adrenals perfused only with indomethacin was 6.1o ($\pm$ 0.69 S.E.) μg; this was increased to 16.83 ($\pm$ 2.5 S.E.) when PGE_2 (5 μg/ml) was added to the indomethacin-containing medium. This stimulatory effect was highly significant ($P < 0.001$) and not accompanied by an increase in flow rate. PGE_1, PGE_2 and $PGF_{1\alpha}$ at levels of 10 μg/ml increased adrenal flow rate as well as B output.

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DEDICATION

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INTRODUCTION

This thesis describes the development of a technique for *in situ* perfusion of the rat adrenal gland. The reason for pursuing this approach was the desire to study the adrenal cortex under more physiological conditions than one encounters using conventional *in vitro* procedures - and at the same time, to eliminate or control some of the variables present *in vivo*.

The objectives of this study were:

(1) Development of the surgical techniques necessary for perfusion of a single rat adrenal gland through its arterial circulation. Perfusion at a constant rate with a physiological solution would then permit continuous collection of the secretory products from the single vein that drains the gland.

(2) Establishment of well-defined experimental conditions under which the perfusion system could be used to study steroidogenesis.

(3) Modification of experimental conditions through addition of adrenocorticotropic hormone (ACTH), prostaglandins and other agents to the perfusing medium in order to study qualitative and quantitative changes in adrenal secretory activity in response to substances known to influence corticosteroidogenesis.

(4) Comparison of the secretory activity of the perfused adrenal with that of single glands incubated *in vitro* using a classical *in vitro* incubation system.

I. The adrenocortical secretion

A. Historical background

Most students of endocrine history agree that our knowledge concerning the adrenal cortex appears to have begun in 1563. In that year Bartolomeo Eustacchio first described the adrenal glands, although it took another 150 years before his anatomical drawings were actually published (Castiglioni, 1941; Jones, 1957). The function of the adrenals appears to have remained completely unknown for nearly 3 centuries following Eustacchio's description. In 1855 Thomas Addison published a classical work in which he described the effects of diseases of the adrenals. His description of the metabolic disturbances leading to death in cases of adrenal insufficiency established the physiologic importance of the adrenal glands. In the following year Brown-Sequard showed that bilateral adrenalectomies in various experimental animals proved to be fatal, although animals subjected to sham operation survived. These experiments established that the adrenals are essential for life.

Houssay and Lewis (1923) showed that dogs that underwent bilateral adrenalectomy died whereas those having one adrenal removed and the medulla from the other gland destroyed remained healthy. This was convincing evidence that the cortex and not the medulla was the element essential for life.

During the 1890's and early 1900's many attempts were made to prepare potent adrenal extracts for the clinical treatment of Addison's disease. Osler (1896), using a glycerin extract of hog adrenals, was able to treat the symptoms of adrenal cortical insufficiency in man. Several groups of workers succeeded in preparing more potent extracts

which were able to maintain the lives of adrenalectomized animals. However, the extracts prepared by different laboratories differed in biological activity (Hartman et al, 1928; Rogoff and Stewart, 1928; Hartman, 1937). At that time, the cortical secretory product was viewed as a single hormone designated as "cortin" (Hartman et al, 1928).

The observation by Swingle and Pfiffner (1931) that the biological activity was concentrated in the lipid fractions of adrenal extracts led to the successful isolation and characterization of most of the active principles. This was accomplished in the period of 1935 - 1940 by groups of chemists associated with Reichstein at Basel, Kendall at the Mayo Clinic and Wintersteiner and Pfiffner at Columbia. The results of early studies created a confusing nomenclature, but led to identification of some 21 different types of steroidal substances. By the late 1940's, 28 different, but chemically related steroid hormones were known (Hechter and Pincus, 1954). The first to be isolated and shown to possess biological activity was designated corticosterone (Reichstein and Shoppee, 1943). The other active compounds were shown to be derived from this substance. It was not known at this time whether one, several or all of these substances were actually secreted into the bloodstream. As the procedures for assaying adrenocortical activity improved it became apparent that the cortical secretion contained at least 3 different types of steroid hormones: an 11-oxygenated carbohydrate-active steroid i.e., a glucocorticoid, an electrolyte-regulating steroid, i.e., a mineralocorticoid and an adrenal androgen (Hechter and Pincus, 1954).

In 1943, Vogt developed a technique for cannulating the adrenal

vein in dogs and other species. By collecting and analyzing the venous blood leaving the gland, she was able to show that there was far more adreno-cortical activity (as measured biologically) in the effluent, than could be extracted from the glands themselves. Subsequently, Vogt (1947) demonstrated that ACTH added to the blood perfusing the isolated dog adrenal increased the output of cortical hormones. During the same year, Long (1947) showed that injections of ACTH into intact rats stimulated adrenocortical secretion and caused a rapid depletion of cholesterol from the inner two zones of the cortex. Thus, by the early 1950's, there appeard to be agreement in at least 3 areas:

(1) That the adrenal cortex synthesizes and secretes hormonal products which are steroid in nature. (2) That ACTH stimulates the synthesis and release of these hormones and (3) that adrenal cholesterol is the most probable precursor of the corticosteroids.

It is important to point out that further biochemical developments such as identification of specific steroids and the elucidation of biosynthetic pathways would not have been possible without improved techniques for the extraction, isolation, fractionation and estimation of microquantities of adrenal steroids from biological fluids. Using these newly-developed methods, Bush (1952,1953b) studied the qualitative and quantitative aspects of the adrenal secretory products in a variety of species. The principal glucocorticoid secreted by the rat and rabbit is corticosterone, whereas the cat, dog and monkey secretes predominantly cortisol.

Hechter and associates (Hechter, 1949; Hechter et al, 1949, 1951) perfused isolated cow adrenals with steroid hormone precursors

under controlled conditions and determined the sequence of biochemical reactions involved in corticosteroidogenesis. They were also able to demonstrate where in this biosynthetic pathway the adrenocorticotropic hormone exerted its action (Stone and Hechter, 1954).

B. Pathways of adrenocortical hormone biosynthesis

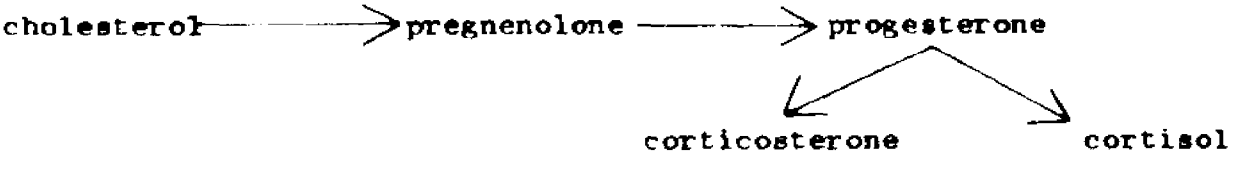
The rapid depletion of adrenal cholesterol in response to injections of ACTH (Long, 1947) plus the obvious structural similarity of the steroid hormones to the first 21 carbons of cholesterol led to the suggestion that cholesterol is the most obvious precursor of the adrenocortical hormones. Zaffaroni and co-workers (1951) found that perfusion of the isolated cow adrenal with ^{14}C-labeled cholesterol led to the formation of radioactive cortisol and corticosterone. Radioactive acetate was also converted into corticosteroids although cholesterol was the more efficient precursor. The question of whether the conversion of acetate to corticosteroids via an alternative pathway not involving cholesterol has been considered but remains essentially unresolved (Stone and Hechter, 1954; Hechter and Pincus, 1954; Kowal, 1969a).

A stoichiometric relationship between the disappearance of cholesterol and the appearance of corticoids in rat adrenals has been demonstrated (Peron and Robidoux, 1964). Studies such as this leave little doubt that cholesterol is an efficient precursor of adrenal steroids and may be the main one in many species. The possibility that the esters of cholesterol are the significant corticoid precursors has been previously suggested (Goodman, 1965; Grant, 1968; Gidez and Feller, 1969). This point will be discussed below in more detail in reference to prostaglandin involvement in adrenal steroid bio-

synthesis.

Another unresolved problem is that of the origin of adrenal cholesterol *in vivo*, i.e., is adrenal cholesterol derived from circulating cholesterol or is it synthetized *in situ*? Perhaps both sources are utilized in corticosteroid biosynthesis. In rats and dogs, it appears that adrenal cholesterol is derived entirely from circulating cholesterol, while in the guinea pig and rabbit, 30 - 40% is synthesized *in situ*. In man, 80% of the adrenal cortisol has been shown to be derived from circulating cholesterol (Vinson and Whitehouse, 1970).

The major biochemical reactions in corticosteroidogenesis involve a series of hydroxylation reactions. Hechter and co-workers (1949) first demonstrated that the adrenal gland has the capacity to introduce hydroxyl groups at various positions in the steroid molecule. Perfusion of 11-deoxycorticosterone (DOC) through the adrenal followed by isolation of corticosterone (compound B) from the venous effluent indicated that the gland has the enzymatic capacity to introduce a hydroxyl group at carbon-11 of the steroid molecule. From studies with pregnenolone, progesterone and 17-α-OH progesterone they demonstrated that the gland also has the capacity to introduce hydroxyl functions into the C-17 and C-21 positions. The conversion of pregnenolone into progesterone and into corticosterone indicated that the gland can transform a 5-ene-3β hydroxyl function into an α - β unsaturated ketone. Hechter and associates suggested that corticosteroidogenesis involves the following pathway:



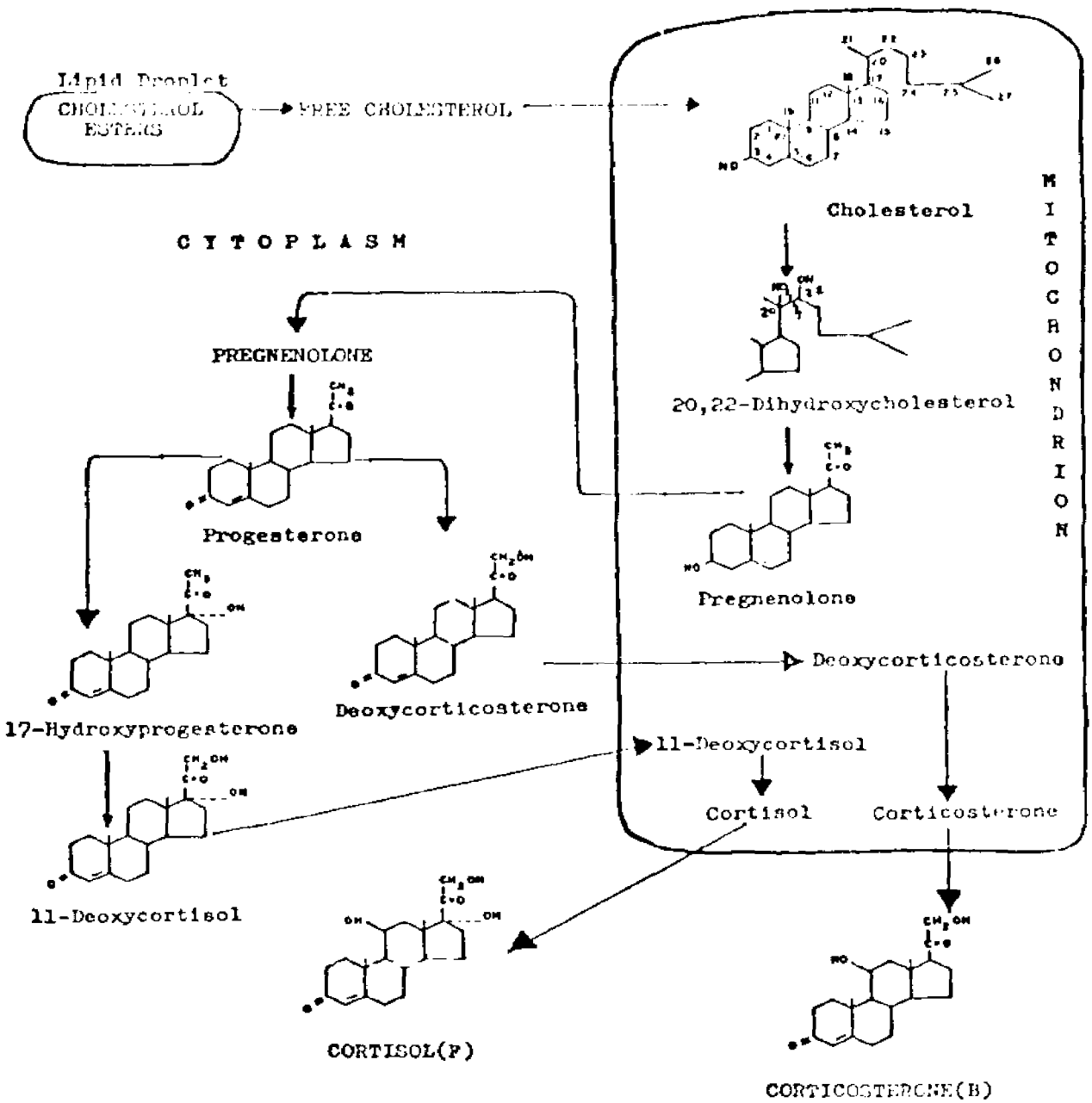
Cortisol and corticosterone were believed to be the end products in the pathway and progesterone their common intermediate (Hechter et al, 1951).

Studies which followed showed that all the above transformations could take place in adrenal homogenates except that cholesterol was always found to be transformed into progesterone rather than pregnenolone. To explain this apparent anomaly, it was postulated that the enzyme involved in the conversion of pregnenolone to progesterone (3β-dehydrogenase) was very close to the site of pregnenolone formation (in these preparations). Hence, all the pregnenolone formed from cholesterol was believed to be immediately converted into progesterone (Hayano et al, 1956).

The studies with the perfused bovine adrenal and subsequent work with adrenal homogenates and slices (Hayano et al, 1956) laid the basis for formulation of the biosynthetic pathway leading to the synthesis of glucocorticoids in the adrenal cortex (figure 1). A great deal of evidence has accumulated to support the hypothesis and it has changed little with time.

The synthesis of glucocorticoids from cholesterol is accomplished through a series of hydroxylation reactions which take place in different subcellular compartments. Enzymic mechanisms for hydroxylation and side-chain cleavage of cholesterol to give pregnenolone, and for hydroxylation at position 11, are localized within the mitochondria of the adrenal gland (Halkerston et al, 1961; Sweat, 1951). Those for hydroxylating at positions 17 and 21 seem to be restricted to the microsomal fraction (Hechter et al, 1951; Levy et al, 1954; Ryan and Engel, 1957; Estabrook et al, 1963). A cholesterol molecule

FIGURE 1: PATHWAYS OF GLUCOCORTICOID BIOSYNTHESIS



in the course of its metabolism, say to cortisol (cholesterol side-chain cleavage + C-21 hydroxylation + 11 hydroxylation) has to shuttle back and forth between mitochondria and endoplasmic reticulum. One imagines that each trip involves dissociation of the steroid molecule from an enzyme, passage through a membrane, and binding to another enzyme. The reason(s) for the compartmentalization of reactions is not known but must somehow be related to control of hormone synthesis.

Cholesterol, the initial substrate, (Zaffaroni et al, 1951) enters the mitochondrion where it is transformed into pregnenolone by cleavage of its side-chain (Saba et al, 1954). Large amounts of esterified cholesterol are "stored" within cytoplasmic lipid droplets; free cholesterol which results from the hydrolysis of these esters is believed to be the immediate source of the substrate (Garren, 1968). The availability of extra-adrenal (circulating) cholesterol to the steroidogenic enzyme is not clear. The first step in the metabolism of cholesterol to steroid hormones is cleavage of the cholesterol side-chain. This is believed to be catalyzed by an enzyme complex which converts cholesterol into hydroxylated derivatives and finally to pregnenolone. Hydroxylation of the side-chain is a prerequisite for the cleavage reaction (Constantopoulos and Tchen, 1961; Constantopoulos et al, 1966). Difficulties have been encountered in attempting to isolate the intermediates in the reaction (Solomon et al, 1956; Tchen, 1968), hence the exact reaction sequence is still a matter of controversy. More recently, Burstein and Gut (1969) studied the reaction sequences from cholesterol to pregnenolone; several possible pathways were described and all the intermediates were shown to be hydroxylated derivatives of cholesterol. The most substantial portion of

pregnenolone formation from cholesterol could be accounted for by assuming an enzymatic concerted attack of oxygen on cholesterol to form 20α, 22 R dihydroxycholesterol which is then oxidatively cleaved to pregnenolone. It has also been suggested that the intermediates may be steroidal free radicals rather than side-chain hydroxylated steroids (Hochberg et al, 1971).

Pregnenolone, once formed, leaves the mitochondrion and is transformed into progesterone by the action of a DPN-dependent 3β - hydroxysteroid dehydrogenase which resides in the microsomal fraction (Beyer and Samuels, 1956). Progesterone can then be converted to 17-αhydroxyprogesterone by a cytoplasmic 17-hydroxylase. The occurrence of this reaction appears to vary from species to species (Bush, 1953a; Hechter and Pincus, 1954). Cortisol producers (cat, dog, monkey and man), hydroxylate progesterone at carbon 17 whereas rats, mice and rabbits under normal conditions appear not to. Progesterone and/or 17-α-hydroxyprogesterone are next converted to deoxycorticosterone (DOC) and to 17-α hydroxydeoxycorticosterone respectively by the insertion of a hydroxyl group at carbon 21 (Hechter et al, 1951). The enzymatic system concerned with 21 hydroxylation has been shown to reside in the microsomal fraction (Ryan and Engel, 1957). The intermediates, DOC and 17-hydroxydeoxycorticosterone (11-deoxycortisol) now return to the mitochondrion where they are hydroxylated at position 11 to give the final glucocorticoid products, cortisol and corticosterone (Hayano and Dorfman, 1953).

The oxygenases which catalyze these hydroxylations have all been shown to require NADPH and molecular oxygen (Hayano and Dorfman, 1953; Ryan and Engel, 1957; Tchen, 1968). Recent studies show that the

oxygenases catalyzing cholesterol conversion to pregnenolone, and the 11-hydroxylation of DOC in addition to requiring oxygen and NADPH also require, a flavoprotein specific for NADPH, a non-heme iron-containing protein, and a particulate fraction containing the hemoprotein, cytochrome P-450 (Simpson et al, 1969). Cytochrome P-450 is present in both the mitochondrial and microsomal fractions of the adrenal cortex; it is believed to be the oxygen-activating enzyme which participates in the hydroxylations of cholesterol, progesterone, and DOC (Tchen, 1968; Cooper et al, 1968; Harding et al, 1968). The sequence of events in the hydroxylation of a steroid is initiated when NADPH donates an electron to the flavoprotein which then mediates the reduction of the non-heme iron protein. The non-heme iron protein is probably the intermediate in the reduction of cytochrome P-450. The P-450 is presumed to act as the oxygen-activating component resulting in the conversion of the steroid into its hydroxylated derivative (Cammer et al, 1968; Harding et al, 1968). This electron transport pathway appears to be well established for cholesterol side-chain cleavage and for 11-hydroxylation but it is not known whether the flavoprotein and non-heme iron protein are components of the 21 hydroxylase system. The carrier(s) between NADPH and cytochrome P-450 remain to be identified for the C-21 microsomal system (Bryson and Sweat, 1968).

II. The adrenocorticotropic hormone (ACTH)

A. Chemical structure and biological activity

A voluminous amount of evidence has accumulated to support the current belief that both growth of the adrenal cortex and hormone biosynthesis are controlled by the pituitary polypeptide, adrenocorticotropic hormone (ACTH) (Garren, 1968).

The complete amino acid sequences of pig, beef, sheep and human ACTH have been determined (see Li and Oelofsen, 1967). The ACTH molecule from all species studied contains 39 amino acids. The sequence of the first 24 and the last 7 amino acids are identical in the hormones from pig, beef, sheep and man. The structure required for biological activity was shown to be contained in the N-terminal 24 amino acid residues (Bell et al, 1956; Cole et al, 1956; Li, 1962). In 1963, Schwyzer and Sieber completed the formidable task of synthesizing the procine ACTH molecule. Synthetic ACTH preparations are at least as active as the hormones isolated from natural sources (Barthe et al, 1964; Bradlow et al, 1963).

B. The steroidogenic effect of ACTH

Pituitary ACTH stimulates both synthesis and release of adrenocortical hormones. Addition of ACTH to blood perfusing the isolated dog adrenal caused an immediate increase in the rate of secretion of cortical hormones (Vogt, 1951). ACTH increased corticosteroid output 10 -20 fold in perfused cow adrenals (Hechter et al, 1951). Microunit amounts of ACTH increased steroid output from cat adrenals; the quantity of steroid released was shown to depend on the ACTH concentration as well as the duration of exposure to ACTH (Jaanus et al, 1970).

Cannulation of the adrenal vein in several species has permitted direct measurement of corticosteroids in the adrenal venous effluent. Such measurements have been made following injections of ACTH. The results have varied from one laboratory to another. Bush (1953a, 1953b) was unable to find a consistent rise in adrenal steroid output after ACTH injections to normal rats and rabbits but observed an increase in cats, dogs and sheep. Kass et al (1954) were also unable to detect an ACTH effect on the rate of steroid produced in rabbits, and only small changes in corticosterone output were detected in rats after intravenous infusions of ACTH (Holzbauer and Vogt, 1957). Holzbauer and Vogt (1957), believe that the anterior pituitary of rats subjected to the stress of vein cannulation releases enough ACTH to ensure that the adrenal cortex is maximally stimulated, and, under these conditions very little further stimulation can be elicited by exogenous ACTH. Other workers have shown that corticosteroid output, as determined by vein cannulation in stressed rats, can be increased by as much as five times the normal value (Henkin and Knigge, as reported by Schindler (1959)). An increase in corticosteroid secretion in the adrenal venous blood of normal dogs after ACTH administration has also been reported by Hume and Nelson (1954).

The stimulatory effect of ACTH on cortical hormone production can also be observed in various in vitro systems using adrenal slices from different animals, including the rat (Saffran et al, 1952; Hofmann and Davison, 1953) and the cow (Haynes et al, 1952). Superfused adrenal glands as well as isolated rat adrenal cell preparations respond to ACTH by increasing corticosteroid production (Saffran et al, 1967; Tait et al, 1970; Swallow and Sayers, 1969). It is

apparent from such studies that ACTH stimulates corticosteroid output both *in vivo* and *in vitro*. The response of perfused adrenals and of *in vitro* adrenal preparations to ACTH demonstrates that the effect of ACTH upon the gland is direct.

There appear to be differences in sensitivity to ACTH when one compares responses to ACTH *in vivo*, by perfused adrenals and isolated adrenal cells, on the one hand and superfused or *in vitro*-incubated adrenal slices on the other. In general, *in vivo* preparations, perfused glands and isolated cells respond to microunits of ACTH, whereas superfused adrenals and *in vitro*-incubated adrenal sections require milliunit quantities of the hormone (Lipscomb and Nelson, 1962; Jaanus et al, 1970; Swallow and Sayers, 1969; Saffran et al, 1953, 1967). No thoroughly adequate explanation has been found to explain the differences. Although the inference made is that the perfused adrenal and the isolated adrenal cell are the more physiologic preparations.

Attempts to demonstrate stimulatory effects of ACTH on adrenal homogenates have not been successful (Macchi and Hechter, 1954; Reich and Lehninger, 1955). Cell damage resulting from freezing and thawing of adrenal tissue seems to abolish the response to ACTH (Haynes et al, 1954; Koritz and Peron, 1958). One concludes from these studies that an intact adrenal cell is essential for demonstration of ACTH action.

The adrenal response to exogenous ACTH is rapid. In the isolated cow adrenal, an increase in corticosteroid output was detected within 2 to 10 minutes (Hechter et al, 1951). The maximum response in perfused cat adrenals is reached as early as 20 minutes after administration of ACTH (Jaanus et al, 1970). Collection of adrenal

venous blood from hypophysectomized rats following aortic injections of microunit quantities of ACTH, showed that corticosterone secretion reached maximum levels 5 to 9 minutes after the injection of ACTH, with return to basal levels within 20 minutes (Girard and Vance, 1962). Similar results have been reported after intravenous administration of ACTH (Lipscomb and Nelson, 1962). In this latter study, the concentration of corticosterone in adrenal venous blood began to rise as early as 2 minutes after injection of ACTH into the jugular vein. These studies indicate, that at least in the hypophysectomized animal, the adrenal is extremely sensitive to and responds rapidly to small amounts of ACTH.

The release of cortical hormones either by unstimulated or ACTH-stimulated adrenal glands results from synthesis and secretion rather than from release of stored hormone. Several investigators, working with different animal species, have observed that the quantity of hormone released by the gland was greater than the amount present in the cortex at any one time under any set of experimental conditions (Vogt, 1943; Holzbauer, 1957; Holzbauer and Newport, 1969; Jaanus et al, 1970). Perfusion of cow adrenals with ACTH elicited an increase in corticosteroid output in the venous effluent, which greatly exceeded the difference in corticosteroid content between the perfused gland and its contralateral unperfused control. It was concluded that the increased output was the result of biosynthesis of corticosteroids by the perfused gland (Hechter et al, 1951). Holzbauer (1957), studied the relationship between corticosteroid content and rate of secretion in rats. She found that the amount of corticosterone stored in a single rat adrenal gland would represent the amount secreted in about

3 minutes under the stressful conditions of adrenal vein cannulation. Hence, "the acute effect of ACTH on steroid secretion can therefore not be due to the activation of a mechanism which enables the release of preformed steroids from a storage site in the tissue, but must be due to an increase in the velocity at which corticosterone is formed" (Holzbauer and Newport, 1967). Perfusion of isolated cat adrenals with ACTH elicited a four fold increase in adrenal corticoid content (Jaanus et al, 1970). One concludes from all of these studies that the adrenals of most species do not contain enough stored secretory product to account for their rates of secretion. High secretory rates elicited by ACTH stimulation must be due to de novo synthesis followed by secretion.

Elimination of the endogenous source of ACTH by hypophysectomy leads to diminished adrenocortical secretion rates for cortisol and corticosterone, to levels only 20% of those found in intact animals. With time, the secretory rates fall even lower (Farrell et al, 1955; Singer and Stack-Dunne, 1955; Sweat and Farrell, 1954). Within half an hour after hypophysectomy in rats, the corticosterone secretion falls 12 fold, and by 4 hours the output has dropped to almost zero (Harding and Nelson, 1964). There is some corticosteroid secretion after hypophysectomy, but the levels are very low. This residual secretion has been observed in superfused rat adrenals taken from hypophysectomized rats (Tait et al, 1970).

Treatment of hypophysectomized animals with ACTH has been shown to increase corticosteroid secretion rates toward the normal level (Farrell et al, 1955; Porter and Klaiber, 1964). It appears, that in the absence of ACTH there is a low residual level of steroid

output, but that "normal" adrenal steroid release is almost completely dependent upon a supply of ACTH to the gland. When rats are hypophysectomized there is a gradual decrease in the steroidogenic response of the adrenal to ACTH (Dear and Guillemin, 1960; Liddle et al, 1962; Grahame-Smith et al, 1967). An exponential decrease in adrenal sensitivity to ACTH was observed in rats as a function of time. Two hours after hypophysectomy, the adrenal was extremely sensitive to an injection of one milliunit of ACTH. Ten days later, the adrenal no longer responded to this same dose of ACTH (Dear and Guillemin, 1960). Liddle and co-workers (1962) found that at 24 hours after hypophysectomy they needed about four times as much ACTH to produce a given response, as was required 2 hours after hypophysectomy. Grahame-Smith et al (1967) confirmed these observations by showing that the amount of corticosterone secreted in response to two milliunits of ACTH had fallen by 70% within 24 hours after hypophysectomy, and after 6 days there was no longer any response to ACTH. Thus it appears that the response of the adrenal to hypophysectomy involves a rapid decrease in steroid output which reaches low residual levels as well as an actual decrease in the sensitivity of the adrenal gland to ACTH stimulation, the maximum sensitivity being observed at two hours after hypophysectomy and decreasing thereafter.

C. Effect on adrenal blood flow

A close relationship exists between the functional activity of a tissue and its blood supply. The relationship is of obvious importance in the case of an endocrine gland whose metabolic processes are controlled and regulated by a trophic hormone. The secretory activity of the adrenal cortex is dependent on ACTH being delivered

to the gland. The amount of ACTH arriving at the adrenal in a given time period is dependent on both the concentration of ACTH in the blood and, the flow of blood through the adrenal (Porter and Klaiber, 1965).

One problem is whether or not steroid secretion can be influenced by blood flow in adrenals not maximally stimulated by ACTH. This question has been studied by experimentally varying blood flow to adrenals exposed to different concentrations of ACTH. Urquhart (1965) found that the rate of cortisol secretion from the perfused canine adrenal was markedly influenced by adrenal blood flow only when blood ACTH concentration was low. When the ACTH concentration was high, blood flow had no effect on cortisol secretion. In the intact dog, cortisol secretion was also shown to be dependent on adrenal blood flow only when the adrenal was exposed to submaximal stimulatory concentrations of ACTH (L'Age et al, 1970). Porter and Klaiber (1965) have shown that corticosterone secretion by the rat adrenal under conditions of constant intravenous infusion of low concentrations of ACTH increased with increasing flow of blood through the adrenal. These studies indicate that adrenal blood flow, under conditions of submaximal ACTH stimulation, may be an important factor in the regulation of adrenal cortical secretory function.

It is possible that the rapid steroidogenic action of ACTH could be due at least in part to a temporary increase in adrenal blood flow. Several factors may be involved in the regulation of flow through the adrenal. Infusion of catecholamines in the conscious rat caused an increase in net blood flow to the adrenal glands (Goldman, 1966). Symington (1962) believes that the longitudinal muscle bundles present

in the central adrenal vein in man serve as valves which regulate blood flow through the cortex. Carotid artery ligation in the rat, increased blood flow to both the cortex and medulla (Kramer and Sapirstein, 1967). Carotid ligation provokes sympathetic discharge and epinephrine secretion. The epinephrine released was believed to exert part of its effect on adrenal blood flow by eliciting release of ACTH. ACTH would then act directly on the adrenal to increase its blood flow.

Many workers have sought to determine whether ACTH exerts a direct effect on the blood supply of the adrenal cortex and if it does, to determine whether this effect is related to the steroidogenic function of the hormone. Table I summarizes some studies which have examined these questions. Species differences as well as the different techniques that were used to measure adrenal blood flow vary from one study to another and hence make exact comparisons difficult. In spite of these differences, some comments can be made.

In the rat, it would appear, under the conditions of these studies, that ACTH increases adrenal blood flow. The dose of ACTH listed in Table I for each study was the lowest dose used which elicited an effect on adrenal blood flow. Most studies which reported an effect of ACTH on blood flow had been using unit or milliunit quantities of ACTH. Physiologic levels of ACTH in resting animals are usually measured in microunit amounts. For example, rat plasma ACTH concentrations vary from 2 to 6 microunits per ml in intact resting animals (Rees et al, 1971). This suggests that the reported ACTH effects on blood flow may be pharmacologic. Porter and Klaiber (1964) used microunit quantities of ACTH in their study on the rat and detected no

TABLE I. EFFECT OF ACTH ON BLOOD FLOW THROUGH THE ADRENAL GLAND

ANIMAL	EXPERIMENTAL PROCEDURE	EFFECT OF ACTH ON BLOOD FLOW	REFERENCE
rat(intact)	Adrenal vein cannulation ACTH infusion (250 mU)	increased about 50%	Holzbauer & Vogt (1957)
rat(intact)	2 techniques:(1) cardiac output fractionation using rubidium ^{86}, (2) adrenal vein cannulation i.v. injection of ACTH (100 mU)	approximately doubled	Sapirstein & Goldman (1959)
rat (hypophyx)	adrenal vein cannulation ACTH infusion 26-104 micro-units/min	no change	Porter & Klaiber (1964)
rat (hypophyx)	adrenal vein cannulation i.v. injection of ACTH (300 mU/kg)	doubled	Staehelin et al (1965)
dog hamster rat(intact)	(1) heated thermocouples embedded into adrenals (dogs) (2) fractionation method using rubidium ^{86} i.v. injection of ACTH (2U/kg)	approximately doubled in the 3 species	Stark & Varga (1968)
dog	cannulation of lumboadrenal vein ACTH dosage not specified	increased	Hartman et al (1955)
dog	blood-perfused adrenal, micro-units of ACTH	no change	Urquhart (1965)

TABLE I. EFFECT OF ACTH ON BLOOD FLOW THROUGH THE ADRENAL GLAND

ANIMAL	EXPERIMENTAL PROCEDURE	EFFECT OF ACTH ON BLOOD FLOW	REFERENCE
dog	indwelling cannula in lumbo-adrenal vein	increased only with high doses (4U/dog)	L'Age et al (1970)
sheep	autotransplanted adrenal i.v. infusion of ACTH (5-6U/hr)	no change	McDonald & Reich (1959)
sheep	autotransplanted adrenal-intra-arterial (1-60 mU/hr) and i.v. (1-24 U/hr) admin.of ACTH	increased only with unit amts. of ACTH	Wright (1963)
sheep	autotransplanted adrenal i.v. infusion of ACTH (microunits)	increased only at high ACTH infusion rates	Espiner et al (1972)
bovine	isolated blood-perfused adrenal	no change	Hechter et al (1951)
calf (8-40 days old)	collection of blood leaving adrenal vein-i.v. infusion of "small amounts" of ACTH	temporary increase	Balfour (1953)

Hypophx = hypophysectomized

change in adrenal blood flow (see Table I).

In the other species studied, (Table I) it also appears that an effect on blood flow was elicited only when high doses of ACTH were used i.e., unit or milliunit amounts. There was no correlation found between steroid secretion and adrenal blood flow in any of the studies summarized in Table I. Two important points stand out: (1) high levels of ACTH are necessary to stimulate adrenal blood flow and (2) steroid secretion appears to be independent of adrenal flow rate.

Cycloheximide will block the steroidogenic effect in vivo but does not affect the increase in blood flow lending support to the concept that the two effects of ACTH are separate (Maier and Staehelin, 1968). The effect of ACTH on blood flow, could either be due to its direct action on the adrenal vasculature or its stimulation of the production of adrenal metabolites which could cause local vasolidation (Lever, 1955).

D. Mechanism of action

(1) The steroidogenic effect

The purpose of this section is to briefly review what is currently known about the mechanism of ACTH stimulation of steroidogenesis, i.e., how the actions of ACTH at the cellular level lead to the stimulation of steroidogenesis that one observes in vivo.

The stimulatory effect of ACTH on steroidogenesis appears to involve the following events; (1) the binding of a specific portion of the ACTH molecule to a receptor located on the exterior of cell membranes of adrenocortical cells. (2) Activation of the enzyme adenyl cyclase believed to be located on the interior face of the cell membrane. This leads to increased intracellular production of

adenosine 3':5' cyclic monophosphate (cyclic AMP). (3) The binding of cyclic AMP to a cytoplasmic receptor protein kinase resulting in activation of the kinase. (4) Phosphorylation of cellular protein(s) and/or synthesis of labile proteins implicated in the conversion of cholesterol to pregnenolone i.e., the proposed rate-limiting step, (Gill, 1972).

Several studies have indicated that ACTH can exert its effect without entering the adrenal cortical cells. ACTH, when covalently-linked to cellulose, or agarose, or when diazotized to polyacrylamide beads, retained its ability to stimulate steroidogenenesis in adrenocortical cells (Schimmer et al, 1968; Selinger and Given, 1971; Richardson and Schulster, 1972). When bound to these macromolecules, the ACTH-complex is presumed, by virtue of its size, to be prevented from entering the cell. There have also been suggestions that ACTH may penetrate these cells and accumulate intracellurlarly (Muller and Scriba, 1969).

The initial event which sets the steroidogenesis into motion appears to be the binding of ACTH to the membrane of adrenal cortical cells. This results in the activation of the enzyme, adenyl cyclase (Taunton et al, 1967; Lefkowitz et al, 1970; Finn et al, 1972). Adenyl cyclase is believed to reside in the cell membrane and to be oriented toward the interior of the cell. The receptor for ACTH is thought to be on the exterior of the cell (Robinson, Butcher and Sutherland, 1971).

Haynes (1958) was the first to show that ACTH increased the content of cyclic AMP within adrenal cortical slices and that cyclic AMP stimulated steroidogenesis (Haynes et al, 1959). ACTH has been

shown to increase cyclic AMP levels in adrenal quarters in vitro, in intact adrenals in vivo as well as in adrenal homogenates. It appears that activation of adenyl cyclase results in an increased conversion of ATP to cyclic AMP within the adrenal cell and that this increase in the nucleotide occurs prior to the increase in steroidogenesis. This supports the concept of cyclic AMP as the intra-cellular mediator of ACTH action (Grahame-Smith et al, 1967).

The next step is the binding of cyclic AMP to a receptor protein which has been localized in the cytosol and endoplasmic reticulum fractions of the cell (Walton et al, 1971). The receptor protein is specific for cyclic AMP; its major function appears to be the regulation of a cyclic AMP dependent protein kinase (Gill, 1972; Gill and Garren, 1970).

Gill and Garren (1970, 1971) have purified and studied an adrenal cortical cyclic AMP dependent protein kinase and found it to consist of two subunits; a regulatory receptor unit and a catalytic kinase unit. The receptor functions to inhibit the kinase; the binding of cyclic AMP to the receptor results in dissociation of the two subunits, thereby freeing the kinase. The free kinase is the activated form of the enzyme.

The conversion of cholesterol to pregnenolone is the ACTH-sensitive rate-limiting step in the steroidogenic pathway. Stone and Hechter (1954) demonstrated that ACTH stimulates the conversion of labeled cholesterol to cortisol and corticosterone but the conversion of labeled progesterone to these end products was not affected. This finding has been confirmed by others (Karaboyas and Koritz, 1965; Hall and Young, 1968; Kortiz and Kumar, 1970; Farese, 1971). Cyclic

AMP also exerts its control at this rate-limiting step providing further evidence that it is the intra-cellular mediator of ACTH action (Karaboyas and Koritz, 1965; Tchen, 1968; Burstein and Gut, 1971). A more exact identification of the regulatory site has not been made.

Ferguson (1963) was the first to show that protein synthesis is associated with stimulation of steroidogenesis by ACTH. He observed that puromycin, an inhibitor of protein synthesis at the translational level, blocked both ACTH and cyclic AMP induced steroidogenesis in vitro. Garren and co-workers (1965) showed that cycloheximide as well as puromycin, in doses that block adrenal protein synthesis, inhibited the increase in corticosterone output that follows ACTH administration in vivo. Administration of cycloheximide to maximally-stimulated adrenals was shown to cause a rapid decrease in corticosterone production even in the presence of amounts of ACTH sufficient to maintain high levels of corticosterone output. They concluded that ACTH stimulates the production of a short-lived regulator protein. They estimated the half-life of the protein to be about ten minutes. They went on to show that the site of cycloheximide inhibition was the proposed rate-limiting step (cholesterol to pregnenolone) which had previously been shown to be controlled by ACTH and cyclic AMP. Actinomycin D, an inhibitor of RNA synthesis, failed to prevent the action of ACTH in vitro (Ferguson and Morita, 1964) as well as in vivo (Garren et al, 1965; Ney et al, 1966). This indicates that the ACTH effect on protein synthesis does not require new RNA synthesis and probably involves relatively stable mRNA's.

Based on these studies, it is reasonable to suggest that ACTH

is involved in the synthesis of a labile protein at the translational level. It appears that the protein exerts its effect on a rate-limiting step in the steroidogenic pathway and is required for both the initiation and maintenance of the steroidogenic effect.

Although the involvement of protein synthesis in the steroidogenic effect of ACTH appears to be well-accepted, there have been reports which suggest that stimulation of new protein synthesis by ACTH is not required for its steroidogenic action (Koritz et al, 1957; Hechter and Halkerston, 1964; McKerns, 1968). These reports taken together suggest that ACTH does not increase steroid synthesis by stimulating the synthesis of a labile protein nor by increasing the level of this protein. Koritz (personal communication) has suggested that neither initiation of synthesis nor control of the level of the labile protein is regulated by ACTH, but that the phosphorylation and hence activation of an already existing rapidly-turning over protein is what is being regulated by ACTH.

The chain of events set into motion by ACTH results in the activation of a protein kinase and the synthesis of specific regulator protein(s), or the activation of pre-existing protein(s). It remains to be seen how the catalytic activity of the kinase, i.e., how phosphorylation leads to the synthesis or activation of specific proteins which control the steroidogenic pathway. Localization of the cyclic AMP receptor-protein kinase complex in the endoplasmic reticulum makes it easy to visualize its interaction with the protein-synthesizing machinery of the cell (Walton et al, 1971).

(2) Involvement of calcium

Calcium has been shown to play an important role at various

sites within cells of the adrenal cortex. The absence of calcium from the incubation medium abolishes the steroidogenic response of adrenal quarters to ACTH and cyclic AMP (Birmingham et al, 1953; 1960; Triller and Birmingham, 1965). Corticosterone production in response to ACTH is reduced when isolated adrenal cells are incubated in a calcium-free medium (Sayers et al, 1972). Other workers have demonstrated a calcium requirement in the activation of adenyl cyclase by ACTH *in vitro* (Bar and Hechter, 1969; Lefkowitz et al, 1970). It has also been shown that calcium inhibits adenyl cyclase activity and the suggestion has been advanced that ACTH may activate adenyl cyclase by releasing this calcium inhibition (Bar and Hechter, 1969; Kelly and Koritz, 1971; Carchman et al, 1971).

The ability of the perfused adrenal to secrete corticosteroids in response to low concentrations of ACTH is dependent upon calcium. This stimulatory effect of ACTH has been shown to be associated with a redistribution of intra-cellular calcium (Jaanus et al, 1970; Jaanus and Rubin, 1971).

Several investigators have demonstrated a site of action for calcium beyond the step of cyclic AMP production. Peron and McCarthy (1968) have suggested that a site of action for calcium may be within the mitochondrion. Farese (1971) has shown that a significant correlation exists between incorporation of amino acid into adrenal protein and the steroidogenic effect of ACTH and cyclic AMP in incubated rat adrenal sections. Both processes appear to require calcium.

It thus appears obvious that calcium is intimately concerned with the functioning of adrenal cortical cells. Rubin (1970) envisions calcium as having at least two effects on the cortical cells; one

concerned with synthesis of corticosteroids and the other with their release.

The role(s) of calcium and cyclic AMP in the action of ACTH has been extensively studied using the isolated perfused cat adrenal gland (Jaanus et al, 1970; 1972; Jaanus and Rubin, 1971; Carchman et al, 1971; Rubin et al, 1969; 1972). These studies have led Rubin and co-workers (1972) to propose a model which attempts to explain the role of calcium and cyclic AMP in the action of ACTH upon the cortical cells.

The model proposes that a calcium fraction associated with adenyl cyclase keeps the activity of this enzyme at a low level. Interaction of ACTH with the cortical cell leads to dissociation of this calcium fraction resulting in activation (release of inhibition) of adenyl cyclase and production of cyclic AMP. Cyclic AMP activation of a protein kinase leads to an increase in steroidogenesis. The liberated calcium fraction is believed to move into the cell, where it triggers the release of newly formed steroid. Elimination of calcium from incubation and perfusion media has been shown to interfere with steroid production (Birmingham et al, 1953; 1960; Jaanus et al, 1972; Rubin et al, 1972) as well as with adrenal protein synthesis which might be required for steroidogenesis (Farese, 1971). Hence, the model suggests that the translocated calcium stimulates steroidogenesis and protein synthesis as well as playing a role in steroid release (Rubin et al, 1972).

III. Prostaglandins

A. Occurrence and release from tissues

The prostaglandins are a group of substances structurally related to C-20 essential fatty acids and found to be present in most mammalian tissues and cells examined so far (Ramwell and Shaw, 1970; 1971).

Their biological effects are numerous and include lowering and elevation of blood pressure, stimulation of many smooth-muscle organs as well as effects on most mammalian organ systems. They transiently affect physiological processes, such as blood pressure, lipolysis, gastric secretion, steroidogenesis and the functioning of platlets, to cite only a few of their influences. The specific effect is dependent on the precise structure of the prostaglandin in question. They are of high biological potency; nanomolar concentrations produce well-marked responses (Weeks, 1972; Horton, 1972).

The ability to produce prostaglandins appears to be widespread throughout the animal kingdom. Most mammalian tissues release prostaglandins in response to stimuli. Lungs, adrenals, stomach and intestine release more prostaglandins than can be extracted from unstimulated tissue (Ramwell and Shaw, 1970). The dog spleen contains less than one microgram of PGE_2 but is able to release up to 10 micrograms during the first minute of stimulation (Gilmore et al, 1968). Thus, increased efflux is believed to reflect increased biosynthesis followed by release, rather than release from a stored form (Piper and Vane, 1971; Ramwell and Shaw, 1970).

Neural, hormonal, and mechanical stimulation can provoke prosta-

glandin synthesis and release. Both catecholamines and nerve stimulation cause prostaglandin release from the dog spleen (Ferreira and Vane, 1967). The perfused guinea pig lung releases prostaglandins in response to histamine and serotonin, to anaphylaxis, and to many different forms of mechanical stimulation such as: perfusion of particles, distension, and gentle massage. Gentle stirring of chopped guinea pig lung also caused release of prostaglandins (Piper and Vane, 1971). The various stimuli evoking prostaglandin release and the tissue types responding have recently been summarized (Ramwell and Shaw, 1970; Piper and Vane, 1971). There appears to be little in common between one type of stimulus and another, except perhaps a generalized disturbance of the cell. As Piper and Vane (1971) have stated, "mammalian cells seem to disgorge prostaglandins at the slightest provocation".

B. Chemical structure

Prostanoic acid is the trivial name given to the C-20 unsubstituted carboxylic acid taken as the parent compound of the family of compounds referred to as prostaglandins (see figure 2). There are two series of prostaglandins, based on the degree of unsaturation: the subscript 1 denotes the monenoic series, while two double bonds (subscript 2) gives the dienoic series. There is also a trienoic series but it is encountered only rarely in nature. The basic structure of all naturally occurring prostaglandins include a carbon-13 double bond, a hydroxyl group at carbon-15 and carbons 8 to 12 are joined to form a 5-membered ring (see figure 2). All PGE and PGF compounds possess a fully saturated 5-membered ring and a hydroxyl group at carbon-11. The PGE compounds have a ketone at carbon 9 while

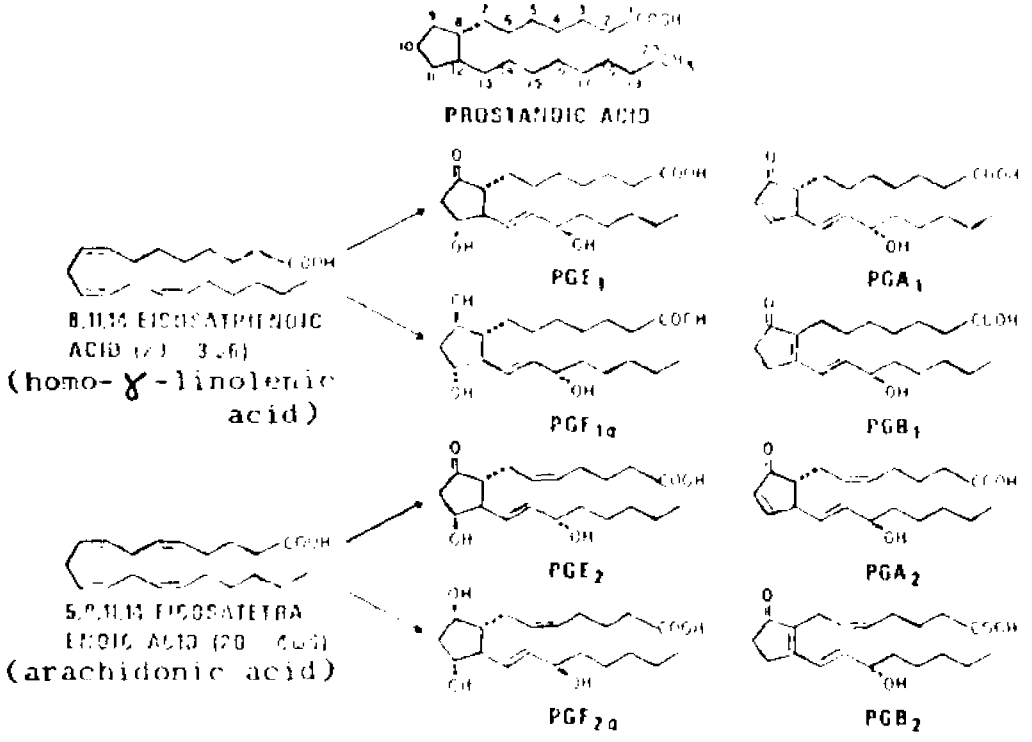


Figure 2. Structures and precursors of the prostaglandins.
(modified from Hinman, 1972)

the PGFs have a hydroxyl at this position. The PGAs are produced by loss of water from the 5-membered ring of the PGEs. PGB compounds result from isomerization of the ring double bond of PGAs (Hinman, 1972; Bergström, 1967).

C. Biosynthesis and metabolism

The essential fatty acids have been shown to be the precursors of prostaglandins in various organs of the sheep, bull, rat and guinea pig (Samuelsson, 1972). Incubation of labeled arachidonic acid (5,8,11,14-eicosatetraenoic acid) with different tissue preparations gave rise to PGE_2 and $PGF_{2\alpha}$ (Bergström et al, 1964b, Van Dorp et al, 1964a; Anggård and Samuelsson, 1965), whereas homo- γ-linolenic acid (8,11,14-eicosatrienoic acid) was converted into PGE_1 and $PGF_{1\alpha}$ (Bergström et al, 1964a; Van Dorp et al, 1964b; Hamberg and Samuelsson, 1966).

The conversion of these fatty acid precursors to prostaglandins is catalyzed by a multi-enzyme system (prostaglandin synthetase) which resides in the microsomal fraction of the cell (Samuelsson, 1967; Van Dorp, 1967; Flack et al, 1971). The precursors undergo cyclization by a complex reaction sequence to form prostaglandins (see figure 2). The reaction sequence has been well studied but has not been completely defined; it is known to involve the incorporation of molecular oxygen (Samuelsson, 1972). The end products formed by the synthetase system depend upon many enzymes and co-factors; of which most are unknown (Hinman, 1972). The ratio of PG_1 to PG_2 compounds depends on the relative amounts of homo- γ-linolenic and arachidonic acids available to the enzyme system.

Biosynthesis of prostaglandins initially depends on the tissue

concentration of the free essential fatty acids (Lands and Samuelsson, 1968). Most of these acids appear to be incorporated into membrane phospholipids and other esterified forms from which they would have to be hydrolyzed before prostaglandin synthetase can act (Horton, P.59, 1972).

Kunze and Vogt (1971) have suggested that phosphilipase A, which cleaves fatty acids from phospholipids, may play a role in supplying substrate for conversion to prostaglandins. These studies have indicated that the prostaglandin synthetase exists in a highly active state within intact cells and that its synthetic ability is limited by the availability of fatty acid substrate. Enzyme-catalyzed release of polyunsaturated fatty acids from cellular storage sites appears to be an important means of control of endogenous prostaglandin biosynthesis (Hinman, 1972; Samuelsson, 1969).

Metabolism of endogenous prostaglandins to less active products occurs rapidly at or near their sites of production as well as in the general circulation. Lungs and liver can inactivate at least 90% of circulating prostaglandins during a single passage (Dawson et al, 1968; Ferreira and Vane, 1967). These two organs appear to be responsible for inactivation of any circulating prostaglandins.

The rapid inactivation of prostaglandins at or near their sites of synthesis as well as inactivation by the lungs and liver could explain the short duration of their effects after injection into the general circulation. This point taken together with the failure to detect significant amounts of circulating prostaglandins suggests that the biological effects of endogenous prostaglandins are restricted to their tissues of origin (Flack et al, 1971). This could

also explain the transient nature of the biological effects.

D. Role in hormone action

The available evidence rules against consideration of the prostaglandins as classical circulating hormones; their transient activity, rapid inactivation and low circulating levels support the concept that they are local humoral substances (Flack et al, 1971). Neither the true physiological role nor the molecular mode of action is understood for any of the many biological effects of the prostaglandins.

A close relationship appears to exist between prostaglandins and both hormone-secreting and hormone-sensitive tissues. Hormonal stimulation ofmany target tissues elicits prostaglandin formation and release. In some tissues added prostaglandin mimics the hormonal response while in other tissues the response is antagonized. A major problem has been to relate the effects of added prostaglandins (pharmacological effects) and of endogenous prostaglandins.

Rapidly accumulating evidence suggests a role for prostaglandins in modulating the adenyl cyclase system in hormone-sensitive tissues. As a broad generalization, it appears that where prostaglandins inhibit the hormonal response, that they do so by inhibiting the accumulation of cyclic AMP; where they mimic the hormone, they increase the intracellular levels of cyclic AMP (Ramwell and Shaw, 1970).

Several workers have suggested a role for prostaglandins in local control of blood flow. Vasodilator prostaglandins, produced in response to hormonal or other stimulation, could act to increase blood flow through the organ in question. The funtional hyperemia observed upon activation of adipose tissue has been attributed to prostaglandins

(Lewis and Matthews, 1969). The increases in blood flow observed by some workers in adrenal glands after stimulation with ACTH have been attributed to prostaglandins (Grant et al, 1968; Maier and Staehelin, 1968). A vasodilatory mechanism could also explain the effects of prostaglandins on other tissues (Hinman, 1972).

Prostaglandins are intimately related to hormone action and any attempt to study the so called "classical hormone" without considering the role of prostaglandins would be incomplete. At the present time, their role in those tissues where they are synthesized upon humoral stimulation, appears to be one of an intra-cellular regulator of the hormone's action. This role may be effected by increasing or decreasing the accompanying cyclic AMP response (Flack et al, 1971). A single concept which explains all the effects of prostaglandins has not been successfully formulated. Differences in dosage, prostaglandin type and type of biological preparation used (homogenate vs tissue slices) are factors which present difficulties in trying to make valid comparisons between one experiment and another. The suggestion of more than one prostaglandin-sensitive adenyl cyclase in a single cell type (Lipson et al, 1971), the presence of multiple cell types and concentration dependent effects, further complicates the picture.

E. Involvement in adrenocortical function

(1) Prostaglandins and steroid-producing organs

The presence of prostaglandins in the ovary, testis and adrenal may be related to a role for these substances in steroid-producing organs. Although prostaglandin effects on steroidogenesis are well-documented, their physiological role(s) in these tissues remains unknown.

Prostaglandins have been shown to both inhibit and to stimulate

ovarian steroidogenesis. Several prostaglandins (PGE_1, $PGF_{1\alpha}$ and $PGF_{2\alpha}$) when infused directly into the artery of the autotransplanted sheep ovary, are capable of inhibiting ovarian progesterone secretion (Aldridge et al, 1970; McCracken et al, 1970). When administered in vivo prostaglandins depress ovarian steroidogenesis in rats, rabbits, hamsters, guinea pigs and in the rhesus monkey (Pharriss, 1970). Exposure of either rat or bovine ovaries to prostaglandin enhances the production of progesterone in vitro, (Pharriss et al, 1968; Speroff and Ramwell, 1970). A recent study by Chasalow and Pharriss (1972) has shown that ovarian homogenates from pregnant rats are able to incorporate ^{3}H-arachidonic acid into "physiologically significant" amounts of PGE_2. Agents known to inhibit progesterone synthesis were shown to suppress incorporation of arachidonic acid, suggesting a role for progesterone or one of its metabolites in the control of ovarian prostaglandin synthetase. These studies and others taken from an enormous literature suggest a role for prostaglandins in ovarian steroidogenesis (see, Flack et al, 1971).

A much less extensive literature indicates that a relationship exists between prostaglandins and testicular steroid biosynthesis. PGE compounds stimulate the secretion of testosterone in the perfused dog testis (Eik-Nes, 1971). Testicular tissue has been shown to contain prostaglandins (Carpenter and Wiseman, 1970) and to possess the capacity to convert labeled precursors into prostaglandins (Carpenter et al, 1971). Prostaglandin synthetase activity and androgen synthesis were both diminished by hypophysectomy, while the addition of LH to these "rat testicular preparations" showed increases in both these parameters. This suggests a close relationship between testicular

prostaglandin synthesis and steroidogenesis (Ellis, 1972).

Prostaglandins stimulate adrenal steroidogenesis and are present in adrenal glands (Flack et al, 1971). The relationship between prostaglandins, their release upon adrenal stimulation and their possible role in corticosteroidogenesis will be discussed below.

(2) The presence of prostaglandin precursors in rat adrenal glands

It has been known for some time that cholesterol esters and triglycerides are the predominant lipids found in rat adrenal glands (Goodman, 1965; Rudman and Garcia, 1966). The adrenal glands of rats contain more esterified cholesterol in proportion to their weight than any other tissue in the body. Four to six percent of the wet weight of the rat adrenal can be accounted for by cholesteryl esters (Carroll, 1962). These lipids contain large amounts of polyunsaturated fatty acids. High proportions of arachidonic and lesser amounts of eicosatrienoic acids (prostaglandin precursors) have been found esterified with cholesterol in the rat adrenal. Cholesteryl arachidonate is a major adrenal cholesteryl ester in the rat (Goodman, 1965).

Adrenal glands contain the enzymes capable of catalyzing the hydrolysis of cholesteryl esters and of triglycerides. ACTH stimulation of adrenal steroidogenesis causes a rapid depletion of adrenal lipids. This appears to involve the selective activation of adrenal lipase activities (Rudman and Garcia, 1966; Macho and Palkovic, 1970) and of cholesterol esterase (Behrman and Greep, 1972). A substantial and rapid depletion of the cholesterol ester fraction upon adrenal stimulation has been observed by many investigators (Glick and Ochs, 1955; Goodman, 1965; Gidez and Feller, 1969). The consensus of

opinion is that the cholesterol ester fraction represents a reservoir of cholesterol molecules which are the immediate precursors for steroid hormone biosynthesis (Goodman, 1965; Gill, 1972).

It also appears that the cholesterol molecules destined to become steroid hormones may arise from the preferential hydrolysis of particular cholesteryl esters. Gidez and Feller (1969) have shown that cholesteryl arachidonate, a major adrenal cholesterol ester is selectively hydrolyzed during steroidogenesis in the rat adrenal. A preferential decrease in adrenal cholesteryl arachidonate in response to ACTH injection has also been reported (Muraoka, 1965). Grant et al (1968) had observed that stress decreased the cholesterol ester concentration in human adrenals, and that this decrease was selective for the more highly unsaturated fatty acid esters, especially for arachidonic acid. Thus adrenal stimulation results in hydrolysis of adrenal triglycerides and cholesterol esters. Certain cholesterol esters are preferentially hydrolyzed leading to the accumulation of free cholesterol for steroid hormone biosynthesis and long chain polyunsaturated fatty acids.

The fate of the liberated fatty acids is essentially unknown. Their unusual composition (high in polyunsaturates) led Goodman (1965) to suggest that they might play some special role in the adrenal. Other workers have suggested possible functions for the adrenal fatty acids, but no evidence has been presented in what remains a relatively unexplored area. It has been suggested that the fatty acids liberated upon adrenal stimulation, could serve as substrates for energy metabolism, for hormone biosynthesis, for the synthesis of other adrenal lipids (Walker and Carney, 1971; Macho and Saffran, 1967). Maier and

Staehlin (1968) and Grant (1968) have suggested that the polyunsaturated fatty acids resulting from cleavage of cholesterol esters are precursors of the prostaglandins and their liberation leads to adrenal prostaglandin biosynthesis. This speculation appears reasonable based on the following observations:

(1) Polyunsaturated fatty acids are prostaglandin precursors (Samuelsson, 1972).

(2) The biosynthesis of prostaglandins has been shown to depend on the tissue concentration of free essential fatty acids (Lands and Samuelsson, 1968).

(3) The tissue concentration of these precursor molecules depends on their release from esterified forms (Lands et al, 1971).

Thus, stimulation of the adrenal cortex enhances steroidogenesis and appears to increase the availability of substrates which could be used for prostaglandin biosynthesis.

(3) <u>The presence and release of prostaglandins from adrenal glands</u>

Prostaglandins can be released from adrenal glands upon stimulation in amounts which greatly exceed their endogenous concentration (Ramwell and Shaw, 1970). This indicates that the enzymatic machinery for producing prostaglandins is present in adrenal glands and that increased release reflects increased biosynthesis.

$PGF_{1\alpha}$ appeared in the venous effluent when catecholamine secretion was evoked by acetylcholine in perfused cat adrenals. When the glands were assayed, $PGF_{1\alpha}$ was found to be present in both the cortex and medulla. The amount released upon stimulation greatly

exceeded the amount present in the gland (Ramwell et al, 1966). A number of prostaglandins (PGE_1, PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, and PGA_1) were formed by incubating homogenates of rat adrenal glands. The formation of several of these was increased by both ACTH and acetylcholine. An 18-fold increase in both PGE_2 and $PGF_{2\alpha}$ was elicited by ACTH, while $PGF_{1\alpha}$ formation was increased about 4-fold (Shaw and Ramwell, 1967). It should be recalled, that arachidonic acid is the precursor of the PG_2 compounds (Samuelsson, 1972) and that adrenal cholesteryl arachidonate appears to be selectively hydrolyzed upon adrenal stimulation (Gidez and Feller, 1969). Carney and co-workers (1972) also incubated rat adrenal homogenates with ACTH and observed stimulation of the production of prostaglandins E_2, $F_{2\alpha}$ and to a lesser degree, $F_{1\alpha}$.

Superfused rat adrenals have been shown to release PGE and PGF compounds. The release decreased with time of superfusion. ACTH stimulation resulted in a further decrease. Administration of ACTH in vivo caused a decrease in adrenal prostaglandin content (Ramwell and Shaw, 1970). Hence, both the efflux and adrenal content of prostaglandins appear to decrease as a result of ACTH stimulation (Ramwell and Shaw, 1970). It is difficult to resolve these data with those showing ACTH stimulation of prostaglandin synthesis in adrenal homogenates (Shaw and Ramwell, 1967; Carney et al, 1972). It is possible that these differences stem from the different preparations used i.e., intact cells versus homogenates. It has been suggested that the decreasing prostaglandin efflux, observed upon superfusion with ACTH could be due to increased utilization or decreased biosynthesis of endogenous prostaglandins (Ramwell and Shaw, 1970). It is possible that ACTH is stimulating prostaglandin synthesis in both

preparations but that an intact adrenal cell is required for utilization or metabolism of the prostaglandin. Hence, in adrenal homogenates, prostaglandins would be formed in response to ACTH, but they would not be metabolized. The studies discussed in this section leave little doubt that prostaglandins are synthsized and released from adrenal glands.

(4) Stimulation of adrenal steroidogenesis by prostaglandins

(a) In vivo studies

The wide diversity of biological effects and rapid metabolism of the prostaglandins makes in vivo studies difficult to carry out and to interpret. Nevertheless, several investigators have attempted to study the effects of the prostaglandins on adrenocortical function in vivo. Peng and co-workers (1970) showed that PGE_1 (0.125 - 4.0 μg), but not PGA_1 or $PGF_{2\alpha}$, when injected intravenously into rats, increased plasma corticosterone concentration, and decreased both adrenal ascorbic acid and cholesterol concentrations. These effects were not observed in hypophysectomized animals nor in rats treated with morphine to block "the hypothalamus or a higher nervous center". They concluded that PGE_1 was acting at some higher center in the brain to elicit ACTH release and not directly on the pituitary or adrenal glands. Flack and Ramwell (1972) have indicated that this effect of PGE_1 was probably due to cardiovascular stress and not to a direct ACTH releasing effect.

Using several assay systems for measuring corticotropic activity (i.e., rats in which the nonspecific release of ACTH was blocked with various drugs) de Wied et al (1969) found that only the prostaglandins of the E type were able to stimulate ACTH discharge. However, these

prostaglandins were unable to release ACTH in all the assay systems employed. Flack et al (1971) have pointed out again that this effect on ACTH release may be due to peripheral effects of the injected prostaglandins. The dose of prostaglandin used (10 μg/rat) appeared to exert effects on vascular and alimentary smooth muscle, which can modify blood pressure and cause diarrhea (Flack et al, 1971). This same laboratory (Flack et al, 1969) had reported the stimulation of plasma and adrenal concentrations of corticosterone after i.p. injection of PGE_2 into hypophysectomized rats. The dose employed in this study was more than 100 μg/rat. In this instance the steroidogenic effect was not believed to be due to any peripheral effect of the prostaglandin (hypotension or diarrhea) since it could be elicited by the prostaglandin in vitro (Flack et al, 1969).

In a recent study, several prostaglandins were stereotaxically injected into the anterior pituitary and hypothalamus (median eminence) of dexamethasone pretreated rats. Plasma corticosterone levels were used as an index of ACTH secretion. PGE_1, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ were all shown to stimulate ACTH secretion by an indirect effect exerted at the level of the median eminence (Hedge, 1972). This study together with that of de Wied et al (1969) prompted Hedge (1972) to suggest that the prostaglandins stimulate ACTH secretion via corticotropin-releasing factor.

Wilson and co-workers (1971), found that infusions of PGA_1 into man had no effect on cortisol secretion. This study conflicts with that of Fichman et al (1972) who found that infusions of PGA_1 in man caused a rise in plasma cortisol which could be prevented by administration of dexamethasone. This suggested to the authors that the

prostaglandin influenced cortisol production by a hypothalamic effect on ACTH release rather than by a direct action on the adrenal gland (Fichman et al, 1972). These studies indicate that, at least pharmacologically, the prostaglandins may elicit ACTH secretion and they might be exerting this effect at the level of the brain. The physiological significance of these observations remains obscure.

Infusion of relatively low levels of PGE_1 (2 or 20 µg/hour) directly into the autotransplanted adrenal gland of the sodium deficient sheep showed variability in the steroidogenic response. Some animals showed a marked decrease, while others only slight variation in aldosterone secretion rate. Cortisol and corticosterone output were slightly increased but not significantly. All animals showed a sustained increase in adrenal blood flow. The variability observed in this study suggests the interesting possibility that differences in biosynthetic capabilities might exist from individual to individual. This variability doesn't permit one to postulate a mechanism which would explain the action of PGE_1 upon adrenal steroidogenesis. The highest dosage used in this study (20 µg/hour) gave an adrenal arterial blood concentration of 20 nanograms/ml, which is lower than any dose previously used in other *in vivo* studies. Under these more physiological conditions, it would appear that PGE_1 may increase adrenal blood flow without an effect on steroidogenesis (Blair-West et al, 1971). This study is important because it is the first demonstration of prostaglandin effects on the adrenal using near physiological conditions. In this same study, ACTH secretion was not stimulated when low levels of PGE_1 (20 µg/hour) were infused into the systemic circulation. This casts doubt on the belief held by some workers (Peng et al, 1970;

de Wied et al, 1969; Hedge, 1972) that prostaglandin may be a factor in the physiological release of ACTH.

(b) In vitro studies

Direct stimulatory effects of the prostaglandins on adrenal steroidogenesis have been reported in several in vitro systems. Decapsulated adrenal glands from acutely hypophysectomized rats (3 - 4 hours post-hypophysectomy) superfused in vitro, increased their output of corticosterone in response to added prostaglandins (Flack et al, 1969). PGE_2 was the most potent prostaglandin and caused a doubling of corticosterone secretion. The effect of PGE_2 was dose dependent, the lower limit of response being 2.8 µM PGE_2. The order of potency for the prostaglandins used was, $PGE_2 > PGF_{2\alpha} > PGA_2 > PGE_1$. The adrenals were exposed to the prostaglandins after being superfused with buffer for 90 minutes. PGE_2 caused a rapid and highly significant increase in steroidogenesis which was maximal one hour after its addition to the medium. The stimulation was not sustained; 3 hours after superfusion with PGE_2, corticosterone output had returned to control levels. The response to PGE_2 was insignificant 12 hours after hypophysectomy and could not be elicited with adrenals from intact rats - it was also inhibited by cycloheximide (Flack et al, 1969).

The steroidogenic response to PGE_2 in this same superfusion system was compared to ACTH, cyclic AMP and dibutyryl cyclic AMP. The initial rate of corticosterone output was the same for both PGE_2 and ACTH; both reached a maximum level 60 minutes after their addition to the medium. Cyclic AMP and dibutyryl cyclic AMP induced maximal corticosterone formation at 90 and 150 minutes respectively. The prostaglandin response decayed rapidly while those for ACTH and the

nucleotides decayed at a slower rate. These data indicate that the steroidogenic response to PGE_2 is different from the response to ACTH. PGE_2 causes a transient stimulation which decays rapidly, while ACTH and cyclic AMP maintain steroid secretion for several hours. The transient nature of the PGE_2 response led these authors to believe that perhaps PGE_2 was releasing preformed corticosterone. It was subsequently shown that PGE_2 increased the concentration of corticosterone both in the adrenal and in the medium, and that this effect was inhibited by cycloheximide. Hence, PGE_2 was acting directly on the adrenal to stimulate synthesis of corticosterone (Flack and Ramwell, 1972). Doses of PGE_2 and ACTH which resulted in submaximal stimulation of corticosterone formation were additive when superfused simultaneously, while there was no potentiation of the effect when PGE_2 was added to adrenal glands maximally stimulated with ACTH. This suggests that PGE_2 and ACTH may be stimulating steroidogenesis by similar mechanisms (Flack et al, 1971; Flack and Ramwell, 1972).

Other investigators have also shown direct prostaglandin effects on corticosteroidogenesis. In a classical *in vitro* system, PGE_2 (3 μM) was shown to significantly increase both corticosterone and aldosterone production in adrenals from dexamethasone-treated rats (Sprott et al, 1971). PGE_1 and PGE_2 (100 μg/gm tissue) significantly stimulated the production of aldosterone, corticosterone and to a lesser extent, cortisol from beef adrenal slices incubated *in vitro*, while PGA and the F prostaglandins were without effect (Saruta and Kaplan, 1972). The steroidogenic effect of PGE_1 was shown to share certain similarities with that of ACTH in that calcium was required, puromycin but not actinomycin D inhibited the effect, cyclic AMP levels were increased,

and it did not produce an additive effect with exogenous cyclic AMP. Additive effects were not observed when PGE_1 was used with submaximal or maximal amounts of ACTH. Based on these data, the authors suggest that PGE_1 and ACTH share the same receptor site on the cell membrane. PGE_1 would then exert its steroidogenic effect via stimulation of adenyl cyclase and generation of cyclic AMP (Saruta and Kaplan, 1972).

(5) Possible mechanisms for prostaglandin effect on the adrenal cortex

Currently two hypotheses have been proposed to explain the mechanism by which prostaglandins may modulate adrenal steroidogenesis. One is by an effect on intra-cellular cyclic AMP levels and the other by controlling adrenal blood flow (Flack et al, 1969; Flack et al, 1972). Each hypothesis is compatible with some of the available evidence but neither is comfortable with all of it.

(a) Regulation of cyclic AMP levels

Zor and co-workers (1969) using concentrations of PGE_1 and PGE_2 which stimulated steroidogenesis in vitro (Flack et al, 1969), were not able to demonstrate increases in rat adrenal cyclic AMP concentrations. PGE_1 was also shown to have no effect on an ACTH-sensitive adenyl cyclase preparation, partially purified from a functional steroid producing mouse adrenal tumor (Taunton et al, 1969). Flack and Ramwell (1972) have reported no PGE_2-induced increase in cyclic AMP concentration although corticosteroid synthesis was increased in superfused rat adrenals at the same time. These results argue against a role for cyclic AMP in the stimulation of corticosteroidogenesis by prostaglandins. The difference in steroid output patterns in response to PGE_2 on one hand, and ACTH and cyclic AMP on

the other, also argues against cyclic AMP as the prostaglandin mediator of steroidogenesis. PGE_2 transiently stimulated steroidogenesis, while ACTH and cyclic AMP maintained steroid secretion for several hours (Flack and Ramwell, 1972).

In contrast to the above studies, there are several reports which suggest that prostaglandins might increase cyclic AMP levels. PGE_1 was found to stimulate steroidogenesis and to increase cyclic AMP levels in beef adrenal slices incubated in vitro (Saruta and Kaplan, 1972). Wilson and Kitabchi (1971), have reported that prostaglandins increased cyclic AMP formation in isolated adrenal cells without any concomitant increase in steroidogenesis. ACTH stimulated adenyl cyclase and steroidogenesis in this preparation. The absence of a steroidogenic effect for the prostaglandins in this system is not clear. The data suggest that there might be two adenyl cyclase systems, one sensitive to ACTH and the other to the prostaglandins, or that the procedure used to isolate adrenal cells may destroy a site of action for the prostaglandins.

(b) Effect on blood flow

It has been suggested that the increase in adrenal blood flow sometimes observed after administration of ACTH may be due to prostaglandins present in the adrenal gland (Grant et al, 1968; Maier and Staehelin, 1968). Cleavage of cholesterol esters resulting from ACTH stimulation, may liberate unsaturated fatty acids which could undergo cyclization to form vasodilator prostaglandins. Maier and Staehelin (1968) cannulated the adrenal vein in rats and measured flow rate and steroid production after the administration of ACTH. ACTH was shown to increase steroidogenesis as well as adrenal blood flow. Adminis-

tration of cycloheximide blocked the steroidogenic response but not the increase in blood flow. Cycloheximide is known not to prevent the cleavage of cholesterol esters but to inhibit the subsequent conversions of the resulting free cholesterol (Davis and Garren, 1966). This type of information led Maier and Staehelin (1968) to hypothesize that fatty acids liberated from cholesterol esters might be converted into vasodilator prostaglandins which then act by increasing adrenal blood flow. As a result of the increased flow, the oxygen supply to the adrenal would be augmented, thereby activating adrenal energy metabolism which might potentiate the effect of cyclic AMP on steroidogenesis. This could account for the very rapid steroidogenic response to ACTH observed in vivo. There is little evidence to support this hypothesis.

Infusion of several doses of PGE_1 into autotransplanted sheep adrenal glands showed a consistent increase in adrenal blood flow which was independent of steroid secretion (Funder et al, 1969; Blair-West et al, 1971). These experiments question the hypothesis that the steroidogenic effect of prostaglandin results from an increase in blood flow.

If prostaglandins are the mediators of the vasodilation observed after ACTH, then one would expect their efflux and their adrenal content to increase upon stimulation with ACTH. A contradictory observation, i.e., a consistent decrease in prostaglandin release and content has been observed after in vivo or in vitro stimulation of corticosterone production by ACTH (Flack et al, 1971). One possible explanation for this decrease in prostaglandin release and content may be that the prostaglandins formed in response to ACTH are rapidly degraded after exerting their effect. When this possibility was tested no increase in

prostaglandin metabolites was observed when labeled PGE_1 was incubated with adrenal slices or homogenates in the presence of ACTH (Flack et al, 1971). Another possible explanation for this decrease was that the steroid itself was inhibiting the further synthesis of adrenal prostaglandins. This possibility was tested by adding corticosterone to an *in vitro* prostaglandin-synthesizing system which consisted of beef seminal vesicle microsomes plus labeled arachidonic acid. No decrease in the rate of prostaglandin formation was observed (Flack et al, 1971). Hence, the ACTH-induced decrease in prostaglandin release and content does not appear to be due to either decreased adrenal prostaglandin synthesis nor to increased prostaglandin metabolism.

The reason(s) for the presence of prostaglandins in adrenal tissue as well as the significance of their stimulatory effects on adrenal blood flow and steroidogenesis is not clear. The isolated perfused rat adrenal appeared to be an ideal preparation for studying these effects of prostaglandins on the adrenal gland. It was hoped, that with this preparation, one could determine whether prostaglandins were stimulating steroidogenesis through an effect on adrenal blood flow or via another mechanism.

IV. Methods of approach to the study of adrenal steroidogenesis

An ideal method for study of adrenal steroidogenesis would permit direct measurement of steroid production rate while at the same time preserving the adrenal gland in an undisturbed unstressed, "normal" state. Recognition that this goal has been and is presently unachievable has led to the development of *in vivo* and *in vitro* approaches to the problem. The purpose of this section is to briefly assess some of these methods.

A. In vivo

The sampling of adrenal venous blood by cannulation of the adrenal vein first introduced by Vogt (1943) represents a direct approach to the study of adrenal secretion. This approach has been used extensively in rats where the collection of adrenal venous blood can be effected through either adrenal (Munson and Toepel, 1958; Cade and Perenich, 1965) or renal vein cannulation (Bush, 1953a;Holzbauer and Vogt, 1957).

The surgical procedures involved in adrenal vein cannulation cannot be carried out without stressing the animal. Corticosteroid outputs under these conditions of ACTH stimulation may be near maximal, as suggested by the lack of a significant steroidogenic effect after administration of exogenous ACTH to rats undergoing adrenal vein cannulation (Holzbauer and Vogt, 1957). This would explain the high secretory rates in rats of 10 to 60 µg of corticosterone per adrenal per hour observed *in vivo* compared to 1.4 to 3.3 µg *in vitro* (Tait et al, 1970; Cade and Perenich, 1965). The technique gives information about maximal secretory rates under conditions of stress but tells us nothing about the *in vivo* resting output. Release of endogenous ACTH

can be eliminated by using hypophysectomized rats but this approach also eliminates other pituitary hormones which may influence adrenal secretion.

The injection of stimulatory or inhibitory agents can be studied by injecting them into a peripheral vein before collection of adrenal venous blood. This is not a completely satisfactory technique because the injected material circulates throughout the body where it may influence the function of other glands or organs and in this way indirectly alter adrenal secretion.

B. In vitro incubation

The method generally used for in vitro studies of adrenal glands (whole glands, bisects, quarters, or slices) has involved incubation in closed or aerated flasks in a shaking water bath for fixed periods of time. Most investigators have incubated adrenal tissue for periods of 30 to 60 minutes (preincubation period) in buffer alone before incubation for one and a half to three hours (incubation period) in fresh buffer containing the stimulatory or inhibitory agent, e.g., ACTH (Saffran and Schally, 1955; Bakker and deWied, 1961; Birmingham et al, 1968).

Although the reasons for preincubation have not been clearly established, it appears to be important for increasing the sensitivity of rat adrenals to added ACTH. In the presence of "small" amounts of ACTH (110 mU/100 mg adrenal) only preincubated glands produced significant amounts of corticoids (Saffran and Bayliss, 1953). The following suggestions have been advanced to explain the effect of preincubation on the sensitivity of in vitro-incubated adrenals to ACTH; (1) the adrenal tissue needs time to recover from any previous stimulation by

ACTH *in vivo* (Saffran and Bayliss, 1953), (2) preincubation is necessary to remove preformed corticoids (McKerns and Nordstrand, 1955). (3) Corticoids present in the preincubation medium can inhibit steroid formation rate *in vitro* (Birmingham and Kurlents, 1958). (4) A substance that inhibits or inactivates ACTH accumulates in the preincubation medium (Bakker and deWied, 1961).

Two general approaches have been used to assess adrenal cortical activity after incubation of the tissue for fixed periods of time: (1) the direct quantitative measurement of endogenous steroid hormone products. With this approach information has been obtained concerning the physiological status of the gland and its response to *in vitro* and *in vivo* stimulation (Saffran and Schally, 1955; Bakker and deWied, 1961; Birmingham et al, 1968). (2) Isolation of steroid hormone products and isotope determination after incubation with labelled precursors (Lucis et al, 1965; Vinson, 1966; Laplante and Stachenko, 1966; Whitehouse and Vinson, 1971). This approach has been of great value in determining the biosynthetic pathways by which the steroid hormone products are elaborated.

Although the *in vitro* incubation approach has serious limitations (to be discussed below), it possesses several positive characteristics which have led to its widespread use.

Sectioned adrenals maintain the ability to respond to their trophic hormone *in vitro* (Saffran et al, 1953). Unstimulated steroid output can be maintained *in vitro* for many hours. Birmingham and coworkers (1968) have shown that sectioned rat adrenals can continue to put out steroids for 70 hours at which time they still retain responsiveness to ACTH. The *in vitro*-incubated adrenal gland also possesses the

ability to maintain a steady rate of conversion of added progesterone and deoxycorticosterone to 18-hydroxydeoxycorticosterone and corticosterone for periods of at least 8 hours (Birmingham et al, 1968). Hence, adrenal enzyme activity appears to persist for many hours *in vitro*.

The *in vitro* method has been useful for obtaining information on factors affecting secretion of the unstimulated gland and on the requirements for the ACTH response. For example, both calcium and glucose were shown to be needed for optimal ACTH response *in vitro*; in the absence of ACTH both substances were ineffective for promoting endogenous steroid output (Birmingham et al, 1953; Schonbaum et al, 1956).

The *in vitro* approach possesses the obvious advantage of studies on isolated systems, i.e., ability to study the behavior of adrenal tissue in an environment not subjected to the many unknown and uncontrollable circumstances which prevail in the intact organism.

Although *in vitro* incubation studies have yielded much meaningful information concerning adrenal cortical physiology and biochemistry, the technique is beset with serious limitations and disadvantages.

(1) Tissue damage *in vitro*

The sectioning of the adrenal in preparation for its incubation results in some maceration of the tissue, although this probably occurs to a greater extent as a result of the agitation provided by the shaking water bath. The cellular debris which is likely to accumulate may have biosynthetic capabilities and a response to various agents different from those of the tissue being incubated. Enzymes, co-factors and biosynthetic precursors may leak out of the incubating tissue.

Several different enzyme activities have been demonstrated in the incubation media from adrenal tissue incubations. The preincubation medium from rat adrenal quarters but not from whole adrenals has the capacity to metabolize labelled progesterone; the 2 major conversion products were corticosterone and 11- β -hydroxyprogesterone (Tsang and Carballeira, 1966). Bovine cortical preincubation media has the capacity to effect side-chain cleavage of labelled cholesterol and its further conversion into corticosterone and cortisol (Carballeira and Durnhoffer, 1967). In demonstrating the above enzyme activities present in preincubation media, NADPH-generating systems were added. These studies demonstrated that enzymes are leached out of incubating adrenal sections.

Tsang and Stachenko (1970) observed that glucose-6-phosphate + NADP stimulated corticosterone production from incubated rat adrenal quarters but elicited no effect on whole adrenal glands in vitro. ACTH stimulated corticosterone production by both the quartered and whole glands. This study suggested that the steroidogenic effect of glucose-6-phosphate and NADP on sectioned adrenal glands involves activation of enzymes in the incubation medium and the stimulation of enzyme systems in damaged cells. The steroidogenic effect of ACTH was believed to be exerted only on intact cells. The possible existence of two types of cells in rat adrenal sections incubated in vitro had previously been suggested by Halkerston and co-workers (1968). On type (intact cells) was believed to respond to ACTH and cyclic AMP while the others contained damaged cells with altered permeability and enzymatic properties. The damaged cells were envisioned as being responsive to glucose-6-phosphate plus NADP but not to ACTH and cyclic AMP.

The sectioning of adrenal tissue and its subsequent incubation *in vitro* would thus appear to damage at least some of the adrenal tissue thereby altering its biosynthetic capacilities and response to various agents.

(2) Limitations arising from the static nature of the incubation

Certain limitations of the *in vitro* incubation technique arise from its static nature, i.e., the incubation of tissue without change of medium for fixed periods of time. In such a closed system, nutrients utilized by the tissue are not replaced and the products of biosynthetic pathways are not removed and remain in the medium. In this respect, the most obvious disadvantages are: (1) by remaining in the medium, the product(s) of biosynthetic pathway(s) being studies can alter reaction rates; inhibition of biosynthetic reactions may occur. (2) Biosynthetic products, which would normally be removed from the tissue *in vivo* accumulate in the incubation medium and could be further metabolized by the tissue, thereby giving rise to "abnormal" end products, i.e., products that would not have been synthetized *in vivo* (Orti et al, 1965).

A serious disadvantage of *in vitro* incubation techniques is that they are restricted to measuring steroid output for fixed or definite time intervals; temporal aspects of hormone output are difficult to study. Several approaches can and have been used in an attempt to more fully appreciate the temporal aspects *in vitro*. These approaches and their limitations have been discussed (Saffran et al, 1967). One can sample the incubation medium from the same flask at many time intervals. The obvious disadvantage here is that each sample

removed will decrease the volume of the incubation medium. One can also incubate several flasks, each for a different period of time. In this case, hormone output for each point in time is determined by a different tissue sample. Birmingham and co-workers (1968) have overcome these objections by completely changing the incubation medium every 2 hours. A technique has also been described in which adrenal glands are transferred to fresh medium every 3 minutes. The transfer reportedly takes 10 to 15 seconds (Margoulies et al, 1970). Periodic changes of the entire incubation medium satisfies the objections inherent in frequent sampling of the medium and in using different tissue samples for each point in time, but the tissue is still exposed to an increasing accumulation of tissue products.

The technique of adrenal superfusion described by several laboratories (Orti et al, 1965; Tait et al, 1967; Saffran et al, 1967; Schulster et al, 1970; Huibregtse and Ungar, 1970) overcomes most of the difficulties inherent in using a closed (static) system in vitro. In superfusion systems, small organs or tissue sections are incubated in vessels through which nutrient medium is allowed to flow in and out. The tissues are believed to be thin enough to exchange respiratory gases and nutrients with the incubating medium which is continuously being replaced. The tissue can be exposed to stimulatory and inhibitory agents continuously, or for fixed periods of time. The volume of the incubation medium remains unchanged for the duration of a superfusion experiment; the same tissue sample is used for measurements taken at each point in time and tissue products are removed from the medium at a constant rate. Hence it would appear that the superfusion technique eliminates most of the disadvantages which

stem (arise) from the static nature of the *in vitro* technique.

(3) Sensitivity to ACTH in vitro

Rat adrenal sections incubated *in vitro* retain the ability to respond to ACTH but require amounts of the trophic hormone which far exceed amounts known to be effective *in vivo*. The rat adrenal responds to as little as 0.06 mU of ACTH when injected into the whole animal (Liddle et al, 1962), while rat adrenal slices *in vitro* require at least 1 mU ACTH/ml for a response (Saffran and Schally, 1955). Superfused rat adrenal bisects required 0.6 mU ACTH/ml at a flow rate of about 45 ml medium/hour (i.e. the adrenal tissue is exposed to 27 mU ACTH over a one hour period) in order to stimulate steroidogenesis (Schulster et al, 1970).

The steroid production rate *in vitro* in response to ACTH is usually 5 to 10 times less than that observed *in vivo*; 10 to 60 µg corticosterone/adrenal/hour *in vivo* compared to 1.4 to 3.3 µg when adrenal bisects are superfused *in vitro* (Cade and Perenich, 1965; Harding and Nelson, 1964; Tait et al, 1970). The reason(s) for this relative insensitivity to ACTH *in vitro* is not established but has been discussed in detail (Kloppenberg et al, 1968; Richardson and Schulster, 1972).

The devlopment of techniques for isolating rat adrenal cells and the subsequent finding that they are extremely sensitive to ACTH has suggested possible explanations for the relative insensitivity of adrenal sections to ACTH (Kloppenberg et al, 1968; Sayers et al, 1971; Richardson and Schulster, 1972). For example, Sayers and co-workers (1971) prepare suspensions of isolated rat adrenal cells by means of tryptic digestion and mechanical agitation. When these isolated cells

are incubated *in vitro* they can respond to one-tenth of a microunit (one picogram) of ACTH (minimum effective dose), by increasing their production of corticosterone.

It has been suggested that the insensitivity of adrenal sections to ACTH may be due to the fact that relatively few of its cells are in contact with the incubation medium, whereas in cell suspensions most of the cells are in contact with the medium. The ACTH molecules may be able to gain access to only a small percentage of their target sites (Kloppenberg et al, 1968; Richardson and Schulster, 1972).

Adrenal sections *in vitro* appear to be unable to eliminate corticosterone after stimulation with ACTH. High concentrations of corticosterone accumulate in the gland and can inhibit protein synthesis (Clayman et al, 1970). Hence, the comparative insensitivity of the adrenal section to ACTH could be due to an inability of the tissue to clear itself of biosynthetic products (Richardson and Schulster, 1972).

Another possible explanation for the difference in sensitivity to ACTH between adrenal cell suspensions and sections might have to do with the removal of the endogenous ACTH stimulus i.e., the ACTH bound to the gland as a result of secretion *in vivo*. Preparation of isolated adrenal cells involves the use of proteolytic enzymes which could destroy any ACTH bound to the gland. This would not be the case with adrenal sections which might still be under the influence of the endogenous ACTH. This possible difference between the two preparations has been suggested by Richardson and Schulster (1972). They have also pointed out that the maximum levels of stimulation are similar for cell suspensions and for superfused adrenal bisects. Maximum levels of

corticosterone are 1.0 to 3.5 µg/adrenal/hour for cell suspensions and 1.4 to 3.3 µg for the superfused tissue. The difference in sensitivity to ACTH between the two preparations arises from the fact that basal levels of steroid production are much lower in the isolated cell suspension. This leads to a stimulation in corticosterone output of 100 fold or more upon addition of ACTH. The lower basal levels of production in the isolated cell system may be due to the removal of endogenous ACTH by proteolytic digestion during preparation of the dispersed cells. It is also possible that the proteolytic enzyme alters the cell membrane in such a way as to make the ACTH target site more accessible to the trophic hormone.

The relative insensitivity of *in vitro* incubated adrenal sections to ACTH could also be due to lack of some factor normally present *in vivo* but absent in the incubation medium.

C. Adrenal perfusion

Techniques have been described which permit the "in situ" perfusion with either blood or physiological salt solutions, of the adrenal gland of the dog (Vogt, 1951; Hilton et al, 1958; Urquhart, 1965), of the cat (Feldberg, 1940; Douglas and Rubin, 1961) and of the rat (Cession-Fossion, 1964a). Methods have also been developed for perfusing the adrenal glands from slaughtered cattle (Hechter et al, 1953; Hechter and Pincus, 1954).

In most of the investigations, the goal has been to closely approximate the *in vivo* conditions. Vogt (1951) perfused dog adrenals with heparinized blood derived from the same animal; the natural circulation to the gland was maintained until the start of the perfusion and in general, the experimental conditions could be

described as "close to physiologic". The rate of corticoid output from perfused glands compared well with Vogt's values *in vivo* (adrenal vein cannulation). The technique, in spite of some problems inherent in all adrenal perfusions (to be discussed below), would appear to permit one to study the adrenal cortex under "near physiological conditions".

Adrenal perfusion has also been undertaken with a different objective. The perfusion of beef adrenals by Hechter and co-workers (Hechter et al, 1951; 1953; Hechter and Pincus, 1954) had as its main goal the elucidation of the enzymatic conversions involved in the production of corticosteroids, and led to the first biochemical reaction sequence of corticosteroid biosynthesis. The conditions for perfusion, i.e. perfusion pressures, perfusion media, whether to perfuse through an artery or vein and other factors were chosen with one consideration in mind; that of "the influence of these variables upon the rate of the *in vitro* reaction under investigation" (Hechter et al, 1951). The question of whether the perfusion method was "physiological" or not was of little consequence. For example, Hechter and co-workers (1953) found that in their hands, perfusion of lacerated beef adrenals by means of gravity flow through the adrenal vein (retrograde perfusion) were the best conditions for studying the bioconversion of deoxycorticosterone to corticosterone.

Implicit in this approach was the clear realization that data obtained under such abnormal conditions could only reveal reactions which may occur *in vivo* but makes no statement as to their importance under physiological conditions. They (Hechter et al, 1951) felt that even the best perfusion conditions would be unable to exactly mimic

physiological conditions and hence used adrenal perfusion as a tool "to study steroid metabolism, wherein substrates in perfusion media are presented through vascular channels to surviving enzyme systems present in cells" (Hechter et al, 1951). No matter what ones primary objective is in studying a particular organ, the ultimate goal is of course to gain greater understanding of events which occur in vivo. Regardless of what the purpose is for undertaking adrenal perfusion, the technique possesses important attributes as well as certain problems inherent in organ perfusion.

The most obvious advantage of perfusion techniques is that vascular channels remain relatively intact thereby enabling one to provide oxygen and nutrients (e.g., glucose) to the adrenal gland in a manner similar to that which exists in vivo. The effects of stimulatory and inhibitory agents can also be studied by presenting them to the gland via its circulation. The perfusion technique also retains one of the most important advantages of classical in vitro adrenal (slices or bisects) incubation techniques, that of being able to isolate the gland and thereby eliminate the complicated interactions that occur between organs in vivo. Elimination of the many factors which undoubtedly influence adrenal function in vivo could also be disadvantageous since it may create a functional state which differs markedly from that existing in vivo.

In classical in vitro studies, (adrenal slices, bisects etc), only adrenal production for a given time period can be measured. With adrenal perfusion, sampling takes place without interruption of the incubation and for this reason is ideally suited to studies of the dynamic aspects of stimulation and response, i.e., one can deter-

mine the duration of stimulatory or inhibitory effects.

The need to compare the behavior of two adrenal glands (or two groups of glands) to particular stimulatory or inhibitory agents can be avoided with adrenal perfusion. The technique often permits one to use a single adrenal gland as its own control in a stimulation experiment. One can also test the effect of two or more doses of a particular agent on a single adrenal gland.

Like other approaches to the study of endocrine tissues, adrenal perfusion is beset with experimental difficulties. One problem arises from exposure of the gland to the stimulatory effects of unknown amounts of endogenous ACTH. Perfusion with ACTH-free solutions or with blood drawn from hypophysectomized rather than intact animals would provide an ACTH-free medium to which known amounts of ACTH could be added. It has previously been pointed out (Hechter and Pincus, 1954) that even when perfused with an ACTH-free medium, an adrenal gland from an intact animal could still be under the influence of ACTH. The surgical preparation of an adrenal for perfusion probably stimulates the release of unknown amounts of ACTH from the animal's pituitary. It is possible that this ACTH is fixed or retained by the gland or that it triggers stimulatory effects which last long after the ACTH is no longer present. Saffran and Bayliss (1953) have shown that the ACTH effect persists in rat adrenal tissue incubated in vitro following withdrawal of the ACTH. Hence, adrenal glands from intact animals even when perfused with ACTH-free media would still be subjected to the stimulatory effects of unknown quantities of endogenous ACTH; the influence of which would be difficult to assess.

In an introductory lecture to a symposium entitled, "In vitro versus In vivo", Bush (1968) criticized certain aspects of perfusion studies and discussed some of the experimental difficulties encountered when using this technique. The following is a summary of the more important points made in this lecture: (1) Heparin is usually used in perfusion studies to prevent clotting and or release of vasoactive substances. The functions of heparin are poorly understood in mammals and it is possible that it may exert unexpected or adverse effects in perfusion studies. (2) In most published perfusion studies no reported attempts are made to preserve a normal flow of lymph. The tying of arterial and venous cannulae in place plus ligatures used to prevent leakage usually result in blockage of most of the lymphatics which normally lie in these areas. Interference with lymphatic drainage leads to edema and can cause a decrease or complete block of perfusate flow. (3) The surgical procedure and the placing of ligatures can cause injury to sympathetic nerves supplying the perfused organ. Injury to these nerves can affect cellular function directly or indirectly via release of catacholamines, with concomitant vasoconstriction. (4) The anesthetic used during the surgical procedure can stimulate the sympathetic nervous system (volatile anesthetics) or depress it (barbiturates).

It is apparent that the performance of an isolated perfused organ is subject to deficiencies when compared with its counterpart in the conscious animal. Whether it is technically possible to overcome the experimental difficulties discussed above is an open question. Hence, when describing the performance of a perfused organ, the terms "physiologic" and "normal" should be used with caution. In spite of

the experimental difficulties involved, much valuable information concerning the physiology and biochemistry of the adrenal cortex has been obtained using the technique of adrenal perfusion (see Vogt, 1951; Hechter and Pincus, 1954; Urquhart, 1965; Urquhart and Li, 1968; Rubin et al, 1972).

PRELIMINARY STUDIES

I. General anatomy

The anatomy of the area in question was thoroughly studied before any attempt was made to surgically isolate the circulation to a single adrenal gland. Figure 3 illustrates the significant anatomical relationships of the kidneys, the adrenal glands and the major blood vessels. The right adrenal gland is situated anterior to and slightly medial to the right kidney and lies close to the vena cava. All the aforementioned structures are dorsal to and covered by the liver. A single short vein drains the gland and empties directly into the vena cava. The location of the right adrenal gland under the liver, plus its close proximity to the vena cava makes surgical manipulation in the area extremely difficult. For these reasons it was decided early in this study to perfuse the left adrenal.

The left adrenal is located 4 - 7 mm anterior to, and slightly medial to the left kidney. It is enveloped by the perirenal fat. A single adrenal vein (8 - 10 mm in length) leaves the inferomedial surface of the gland and empties into the left renal vein. The renal vein is approximately 10 - 16 mm in length and 1.5 - 2.5 mm in diameter. The point of entry of the adrenal vein into the renal vein is usually about 3 - 6 mm from the entry of the latter into the vena cava (figure 4A). Occasionally the adrenal vein emptied into the renal vein just at its point of junction with the vena cava (figure 4B). When this situation occurred, the surgical procedure was modified as described below under surgical technique. The adrenal vein is joined by one or two slightly smaller veins which are branches of

Figure 3. Ventral view of the kidneys and adrenal region showing the relations of the major vessels.

Key

R.A.G.	Right Adrenal Gland
S.A.A.	Superior Adrenal Arteries
I.A.A.	Inferior Adrenal Artery
I.P.V.	Branches of Inferior Phrenic Vein
L.R.A.	Left Renal Artery
L.R.V.	Left Renal Vein
S.A.V.	Spermatic Arteries and Veins
I.L.V.	Left Iliolumbar Artery and Vein
L.A.V.	Left Adrenal Vein
C.A.	Coeliac Artery
S.M.A.	Superior Mesenteric Artery
(x)	numerous small veins which drain the fat pad and empty into left adrenal vein

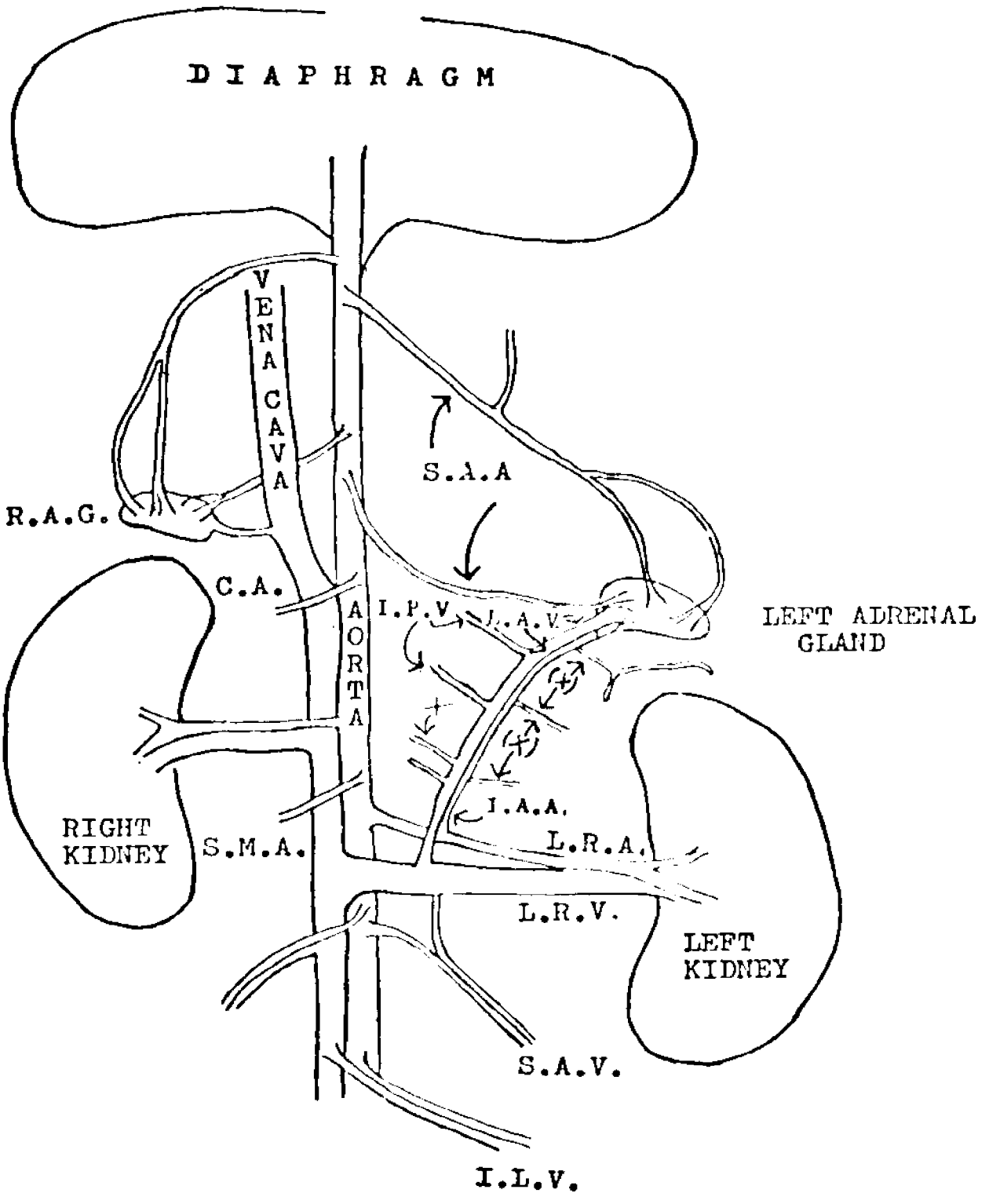

DIAPHRAGM
VENA CAVA
S.A.A
R.A.G.
C.A.
AORTA
I.P.V
L.A.V
LEFT ADRENAL GLAND
I.A.A.
RIGHT KIDNEY
S.M.A.
L.R.A.
L.R.V.
LEFT KIDNEY
S.A.V.
I.L.V.

FIGURE 3

Figure 4A. Point of entry of the adrenal vein into the left renal vein as observed in most animals studied.

Figure 4B. Point of entry of the adrenal vein into the left renal vein at the renal vein's point of junction with the vena cava (an infrequent variation).

L.A.V. Left Adrenal Vein

L.R.V. Left Renal Vein

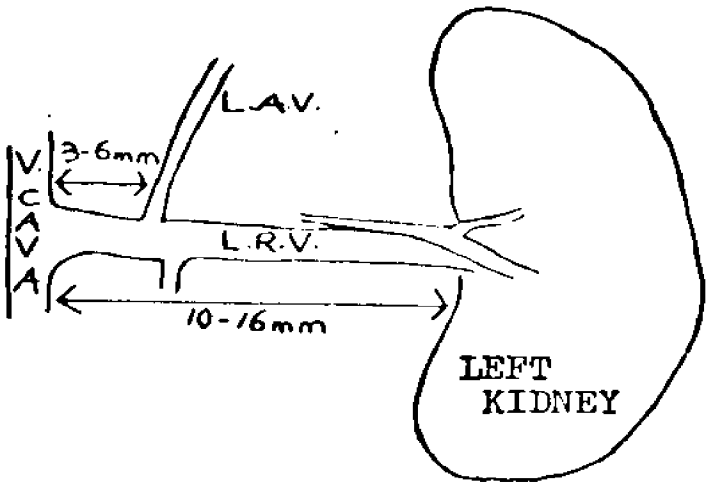


FIGURE 4A

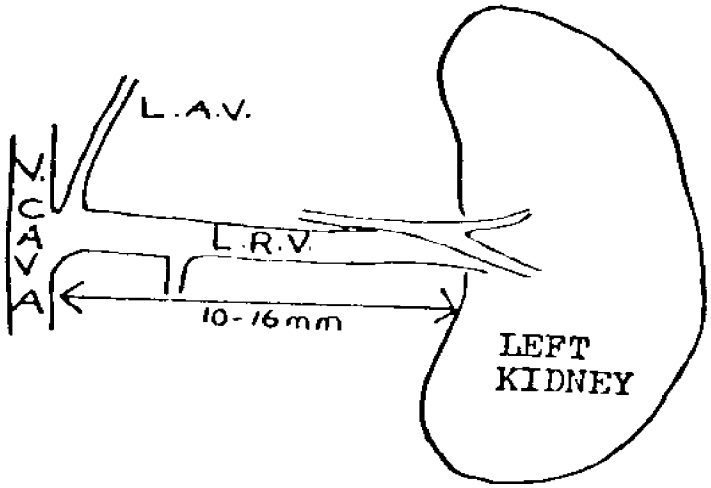


FIGURE 4B

the left inferior phrenic vein. Many extremely small veins coming from the perirenal fat also join the adrenal vein (see figure 3, I.P.V.and (x)).

The aorta gives rise to a right and left spermatic artery just below the left renal vein. The right spermatic vein empties into the vena cava while the left spermatic vein enters the left renal vein approximately 3 - 5 mm from the junction of the latter with the vena cava.

II. The arterial blood supply to adrenal glands in the rat.

A. Previous descriptions

The arterial circulation to the adrenal glands of the Eutheria has been extensively studied. The adrenal blood supply has been described in the dog (Flint, 1900), in the cat (Bennett and Kilham, 1940), in rats, rabbits and cats (Harrison, 1951) and in man (Anson et al, 1947). A detailed description of the gross anatomy of the adrenal circulation in these species was summarized and published as a monograph in 1960 (Harrison and Hoey, 1960). A uniform feature of the adrenal vascularization of all mammals studied is a multiplicity of arteries whereas there is usually one vein, (or at the most three) draining each adrenal (Harrison and Hoey, 1960). The arterial blood supply to the adrenals of the albino rat, according to the published description, is derived from a number of arteries which arborize extensively near the glands. The arteries are of very fine calibre; many cannot be seen with the naked eye. Two main arterial branches arise from the aorta and supply the left adrenal gland (figure 3). The more superior branch supplies the cephalic aspect of the gland while the other branch vascularized the more medial aspect. The left

renal artery provides an inconstant adrenal artery to the inferior pole of the gland. A similar arterial supply is provided to the right adrenal gland.

A somewhat different description of the arterial circulation can be found in Greene's treatise on the anatomy of the rat (1968). In this work, the left inferior phrenic artery (a branch of the aorta) is described as giving rise to a single suprarenal branch which presumably supplies the cephalic and medial aspects of the gland. A branch of the renal artery gives a branch to the inferior aspect of the gland. These descriptions of the gland's arterial supply left several unanswered questions: (1) How many arterial branches actually supply the adrenal and what is their distribution? (2) Does the left renal artery contribute to the vascularization of the adrenal? (3) Are there any strain differences in the adrenal circulation between the albino rat from which the above descriptions come and the Long Evans rat used in the present study? Since this thesis deals with the surgical isolation of the circulation to a single adrenal gland, and its subsequent perfusion, it was deemed necessary to study the blood supply in more detail.

B. Perfusion of blood vessels for visualization.

Twelve male Long Evans hooded rats, bred in our colony and weighing 325 to 500 grams were used in this part of the study. Anesthesia was achieved by intraperitoneal administration of sodium pentobarbital (Diabutal, Diamond Labs., Des Moines, Iowa) at a dose of 6 mg per 100 grams of body eight. In order to remove the gastrointestinal tract from the general arterial circulation, the coeliac axis and the inferior and superior mesenteric arteries were ligated.

The circulation to the right adrenal gland was ligated as were the spermatic arteries and veins of both sides. Next the right and left renal arteries and veins were ligated at the hilus and 3000 units of heparin (Liquaemin Sodium "50", Organon, Inc., West Orange, N.J.) were injected into a superficial leg vein. Sufficient time (5 minutes) was allowed for the heparin to circulate throughout the animal. The thoracic cavity was then opened and the arterial cannula (Intramedic Polyethylene Catheters, PE 190, Clay Adams) attached to a peristaltic pump was inserted into the aorta, advanced downward to a point just above the level of the diaphragm and tied into place at this point. Oxygenated Krebs Ringer bicarbonate glucose (KRBG) at 38°C flowed through the cannula during its insertion into the thoracic aorta. Surgical threads that had been previously placed around the aorta below the renal arteries and around the left renal vein at its entry into the vena cava were tied immediately after the start of the perfusion. Drainage was accomplished by cutting the left renal vein right after the start of the perfusion. The pump was set to give a flow rate of about 6.0 ml/hour. Perfusion with KRBG was continued for twenty minutes followed by perfusion with a 25% suspension of barium sulfate made up in Krebs Ringer bicarbonate glucose buffer (Ultrapaque A, barium sulfate U.S.P., Bell-Craig, Inc., N.Y.C.). Perfusion with $BaSO_4$ was continued until the arteries could be clearly visualized as white strands. The arterial cannula was removed and the aorta ligated to prevent leakage of the $BaSO_4$. The animal was then completely immersed in 10% formalin and stored this way for a period of at least 12 hours. After this treatment the vessels can be dissected more easily, since the $BaSO_4$ hardens and does not diffuse

out of the vessels. Dissection of the adrenal arteries was perfomed using a binocular dissecting microscope.

The above procedure with minor variations is essentially the same as the one previously described by Harrison and Hoey (1960) for the examination of gross adrenal arterial blood supply in rats, rabbits, cats and monkeys. According to these workers, when $BaSO_4$ is injected or perfused into a major artery, it penetrates the finest branches of the arterial system but because of its particle size does not penetrate capillaries. The present study is in agreement with this observation. In the 12 rats studied, the $BaSO_4$ was never observed in the venous system.

C. The blood supply of the left adrenal gland of the Long Evans rat

The left adrenal gland receives two main arterial branches from the ventral aspect of the aorta. These are illustrated in figure 3 and referred to as the superior adrenal arteries. The more cranial of the arteries sends a branch to the diaphragm - it then splits into two branches, supplying the lateral and the medial portion of the cephalic aspect of the gland. The second arterial branch arising from the aorta supplies the medial aspect of the gland. The superior adrenal arteries were present in all animals studied although their points of origin from the aorta varied from animal to animal. In four out of the twelve animals studied, the left renal artery gave rise to a branch, the inferior adrenal artery, which supplied the inferior aspect of the gland. The venous drainage was the same in all animals studied; a single major vein left the inferior surface of the

gland and emptied into the left renal vein. When an inferior adrenal artery was present, it adhered to and traveled with the adrenal vein.

MATERIALS AND METHODS

I. Perfusion of the adrenal gland

A. Surgical technique

Male hooded Long Evans rats purchased from a commercial source (Carworth, New City, N.Y. and Blue Spruce Farms, Altamont, N.Y.) or bred in our animal facilities were used in all of these studies. The strain of rat was chosen because it is believed to be physiologically more robust and healthier than the albino rat (Weisbroth, 1969). In my hands the Long Evans rat was better able to survive the surgical procedure than albino rats.

Animals weighing 300 to 450 grams were anesthetized by intraperitoneal injection of sodium pentobarbital (6 mg/100 gm. body weight) or urethane (200 mg/100 gm.). When the surgical procedure was carried out under urethane anesthesia, the animals' respiratory rate was more regular than under pentobarbital. All urethane-anesthetized animals survived the surgery but pento-barbital-anesthetized rats often did not. Most of the studies were performed on urethane-anesthetized animals.

Most of the surgery was performed with the aid of a 2.5 times magnifying loupe (Binoc-Loupe, Clay Adams). An adjustable loupe (Lempert-Storz Loupe, Storz Instrument Co., St. Louis, Mo.) having magnification from 3 to 6 power was employed to check for damage and for some of the more delicate surgery. Most ligatures and all surgical loops were made with silk suturing material (Ethicon Surgical Silk, Ethicon Inc., Somerville, N.J.). Size 3 - 0 was used for most ligatures but 4 - 0 was required for smaller blood vessels such as the branches of

the inferior phrenic vein and other small veins which empty into the adrenal vein. Small surgical clips (Hemoclips Edw. Weck & Co., Long Island City, N.Y.) were used to ligate blood vessels that could not be manipulated with suturing material.

(1) Standard procedure (protocol)

In order to facilitate breathing, a tracheotomy was performed by inserting a polyethylene tube (Adams Intramedic PE 260, I.D., .070", O.D., .110") into the trachea. A midline incision was made through the ventral body wall and peritoneum. Lateral incisions were made on each side at the level of the kidneys. The gastrointestinal tract was reflected to the right in order to view the left adrenal gland and its associated blood vessels. The viscera were kept moist with warm saline and manipulated with moist gauze pads. An incandescent lamp was used to keep the animal warm.

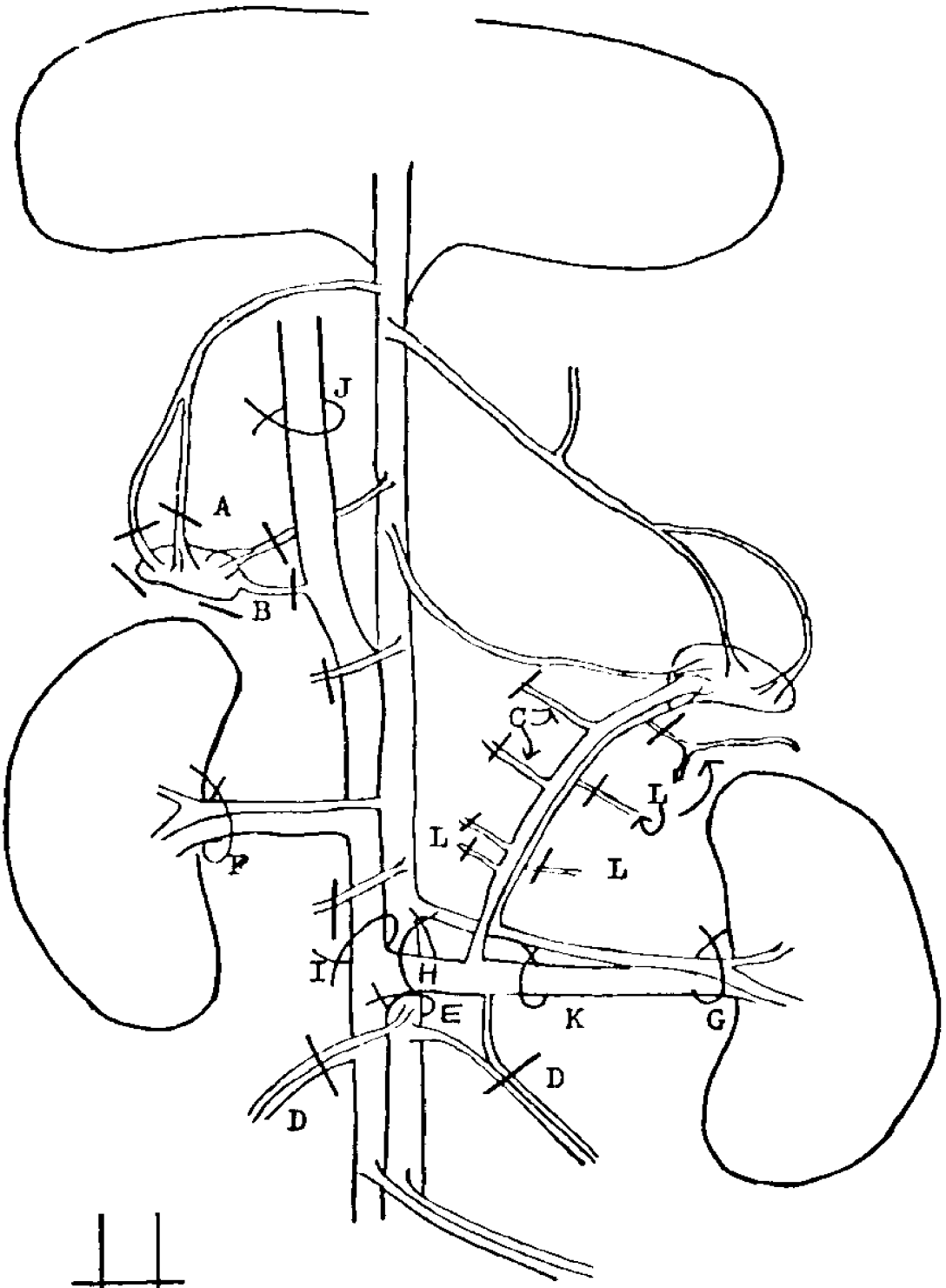
With the aid of the Storz loupe, the area of the left adrenal gland, its blood vessels and the left renal vein was carefully examined in order to (1) assure that the anatomy of the area conformed with the expected pattern and (2) to look for possible superficial damage to the gland. Occasionally the gland had one or more blemishes or darkened spots which were visible on its surface. These were interpreted as being small areas of vasoconstriction which were present in vivo before the start of the surgery, or as having resulted from the stress involved in administering the anesthetic. The blemishes did not always persist but when they did, the animal was discarded. Manipulation of the area cephalad to the left kidney was kept at a minimum in order to avoid trauma or damage to the adrenal and its associated blood vessels.

The arteries supplying the right adrenal gland and the single vein that drains it were ligated next. These arteries arborize extensively in the fat pad just anterior to the gland. Ligation was accomplished by surrounding the gland with sutures or hemoclips as shown in figure 5A. The right adrenal vein was then tied at its exit from the gland (figure 5B). The gland when deprived of its blood supply darkens immediately. It is important to completely cut off the blood supply to the right adrenal in order to avoid its perfusion. In some studies, the right adrenal was then surgically removed and incubated in vitro.

Next, the animal was eviscerated by ligating and cutting the following structures in sequential order: the inferior mesenteric artery, the rectum, the superior mesenteric and coeliac arteries, the upper end of the esophagus and the portal vein and bile duct. Removal of the entire gastrointestinal tract provided easy access to the left adrenal and its associated blood vessels. The surgical manipulations which followed evisceration involved the careful dissection and separation of certain blood vessels from their surrounding structures (i.e., connective tissue and other blood vessels), the placing of loose ties around some of these, and the immediate ligation of others. The aim was to surgically isolate the arterial and venous circulation to the left adrenal. When this was accomplished, the aorta and left renal vein were cannulated to serve as perfusate inflow and outflow channels respectively. Placement of loose ties around blood vessels and the designation of blood vessels to be ligated are shown in figure 5A. A detailed description of these manipulations follows.

Several branches of the inferior phrenic vein (figure 5C) which empty into the left adrenal vein were carefully ligated to avoid

Figure 5. Ventral view of adrenal region showing placement of loose ties and blood vessels to be ligated.



loose tie

ligated vessel

FIGURE 5

disturbing the arterial circulation to the gland. Failure to securely ligate these veins can result in inadequate adrenal perfusion as well as extra-adrenal flow into the venous effluent to be collected. The importance of ligating these veins and others which empty into the adrenal vein will be discussed below. The spermatic artery and vein of each side (figure 5D) were tied off next. Loose ties were then placed around the following blood vessels:

(1) The aorta just below the point where it gives rise to the left renal artery (figure 5E).

(2) The right renal artery and vein at the hilus (figure 5F).

(3) The left renal artery and vein at the hilus (figure 5G).

(4) The left renal vein at its entry into the vena cava (figure 5H).

(5) The vena cava above the point of entry of the left renal vein (figure 5I).

(6) The vena cava just below the point where it enters the liver (figure 5J).

(7) The left renal vein about halfway between its exit from the kidney and its entry into the vena cava (figure 5K). This tie is later used to hold the venous cannula in place, and must go only around the renal vein, after separation from the accompanying renal artery.

In some animals, as mentioned above, the renal artery sends a branch to the inferior aspect of the left adrenal gland. Hence, occlusion of the renal artery at this point could prevent adequate perfusion of the inferior aspect of the gland. Ties 1 through 6 (above) are ligated later in the procedure.

Fine sutures or surgical clips were then used to ligate the numerous small veins which empty, both medially and laterally, into the adrenal vein (figure 5L). The renal arteries and veins of each side (ties 2 & 3 above, figure 5F & G) were tied off at the hilus. The animal was then heparinized by slow injection into the left iliolumbar vein of 3000 units of heparin (Sodium heparin injection, Ivenex Pharmaceuticals, San Fransisco, Calif.) dissolved in 1.0 ml of physiological saline. The heparin reaches the general circulation via the vena cava. Sufficient time (5 minutes) was allowed for the heparin to circulate throughout the animal.

Next, the thoracic cavity was opened and the arterial cannula (Adams Intramedic Catheter, PE 190, I.D.,.047", O.D., .067") with perfusate flowing through it (Oxygenated Krebs Ringer bicarbonate glucose at 38°C) and attached to a peristaltic pump (Sigmamotor AL-4E, Sigmamotor Inc., Middleport, N.Y.) was inserted into the thoracic aorta and guided downward to a point 1 - 2 cm below the diaphragm's attachment to the dorsal body wall. When the gland was observed to completely whiten, the cannula was tied into place at this point. This whitening usually occurred within 60 seconds after interruption of the arterial circulation and apparently represents a complete washout of blood remaining in the gland. Flow rate was set at a level which effected washout within 60 seconds after insertion of the cannula. The pump flow rate was then decreased by about 25% and the aorta ligated below the left renal artery (tie 1 above, figure 5E). The left renal vein was then tied off at its entry into the vena cava (tie 4 above, figure 5H). Adrenal venous effluent now accumulated in the renal vein which could no longer empty its contents into the vena

cava. As a result of this the renal vein becomes distended. It was then cut and the venous cannula inserted (PE 160, I.D., .045", O.D., .062" to PE 240, I.D., .066", O.D., .095", depending on the diameter of the renal vein). The bevel of the cannula was always positioned so that it faced towards the entrance of the adrenal vein (figure 6A). The cannula was tied into place within the renal vein (tie 7 above, figure 5K), when adequate perfusion was attained. Sometimes it was necessary to increase the pump flow rate at this point.

Occasionally the adrenal vein emptied into the renal vein at its point of junction with the vena cava (figure 6B). When this occurred it was not possible to ligate the renal vein at its entry into the vena cava without obstructing the flow within the adrenal vein. The surgical procedure was then adapted to the situation in the following way: loose ties (ties H and I, figure 6B), were previously placed around the vena cava above (tie I) and below (tie H) the entry point of the renal vein. Another tie (tie X, figure 6B) was placed between ties H and I, dorsal to the vena cava and fixed securely in place to prevent flow from any veins (such as the lumbars) which might empty dorsally into the vena cava at this point. Veins emptying into the vena cava at this point and remaining patent would result in contamination of the adrenal venous effluent. The vena cava was then ligated, first with tie H and then with tie I. The renal vein was cut, the venous cannula inserted, positioned and tied in place so that its bevel faced toward the entrance of the adrenal vein as shown in figure 6B.

After cannulation of the renal vein, the vena cava was ligated at two points with ties J and I (figure 5). This must be done in

Figure 6. Position of venous cannulae and arrangement of sutures for the two possible points of entry of the adrenal vein into the left renal vein.

Figure 6A. Arrangement as observed in most animals studied.

Figure 6B. Arrangement used when adrenal vein empties into the renal vein at its point of junction with the vena cava.

L.A.V. Left Adrenal Vein

L.R.V. Left Renal Vein

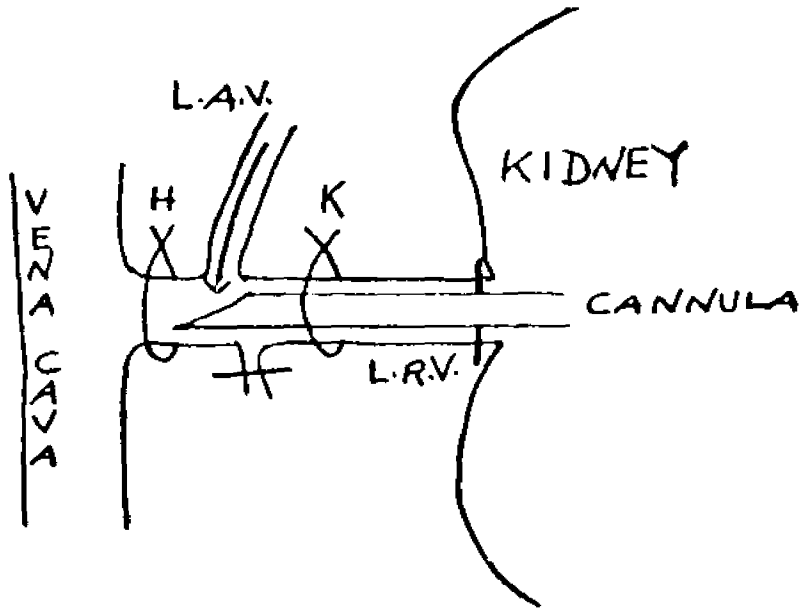


FIGURE 6A

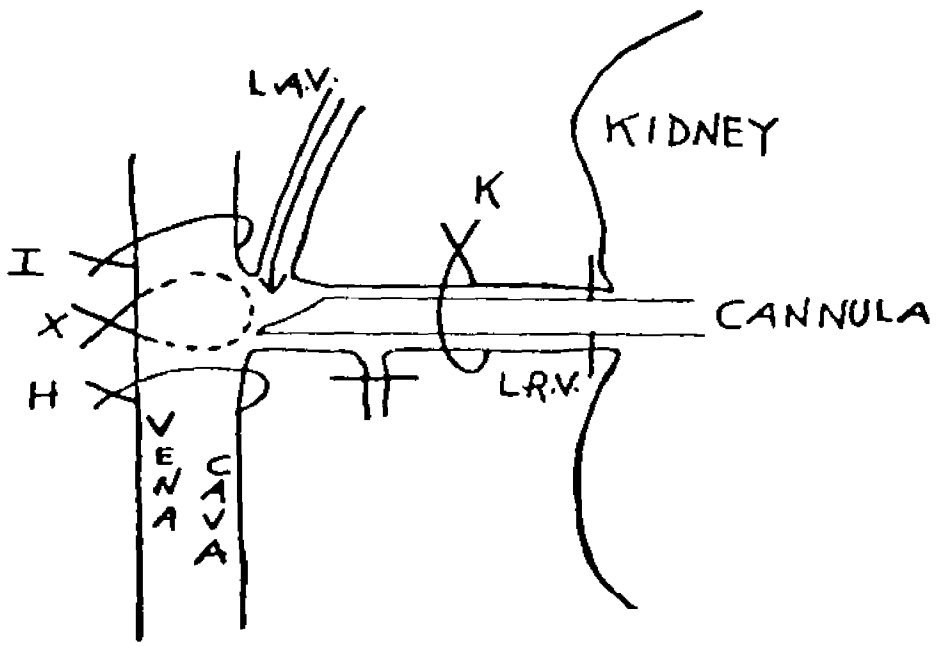


FIGURE 6B

order to prevent flow of perfusate within the vena cava. In the early studies it was observed that perfusate flowed into the vena cava via lumbar veins which drain the dorsal musculature. Perfusate gains access to the dorsal musculature via lumbar arteries which leave the aorta from its dorsal aspect. I have never been able to ligate the lumbar arteries without inflicting damage to the adrenal and its circulation. It is thus not possible to prevent perfusate from leaking into the dorsal musculature and from there into the lumbar veins which empty into the vena cava at several points between ties J and I. If the vena cava is not ligated at these two points then perfusate will flow throughout its length, and can gain access to the liver via backflow through hepatic veins which empty into the vena cava within the liver. Ligation of the vena cava creates a dead space which rapidly fills with perfusate. Once filled with perfusate, the dead space is believed to act as a counter pressure to incoming perfusate and in this way prevents further flow of perfusate into this area or at least keeps this flow at bare minimum.

Perfusate flowing into the abdominal aorta gains access to the adrenal through the adrenal arteries. The venous effluent passes from the adrenal vein into the cannula in the renal vein. Flow of perfusate is shown in figure 7.

The gland was then covered with a gauze pad which was periodically moistened with saline and warmed by an incandescent bulb. The animal was placed on a slanted board to facilitate the flow of venous effluent. The entire procedure (see Summary of Surgical Techniques, pages 88 and 89), from intraperitoneal administration of the anesthetic to collection of the first sample, is completed within

Figure 7. Ventral view of adrenal region showing ligated blood vessels and flow of perfusate.

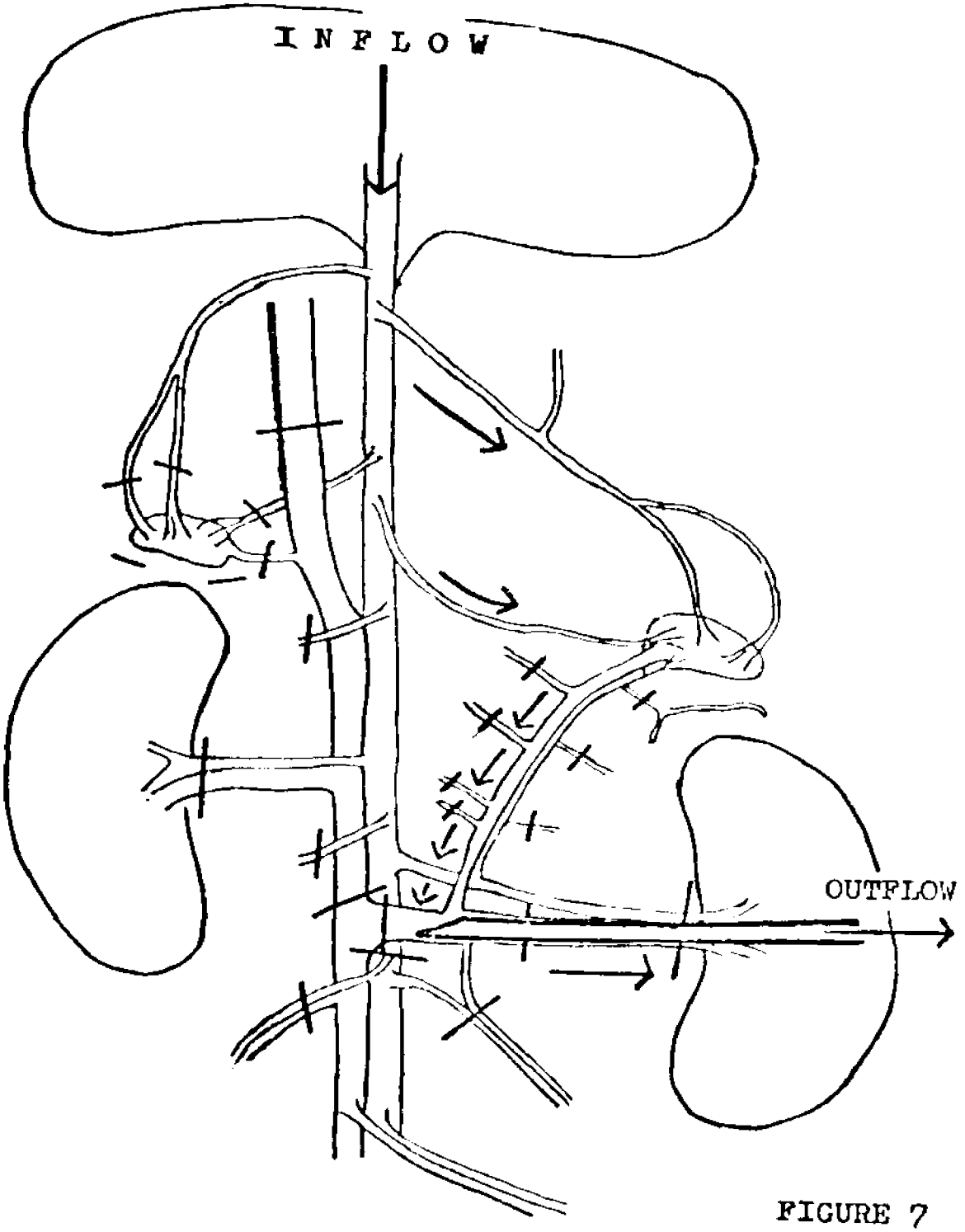


FIGURE 7

50 to 80 minutes.

(2) Summary of surgical technique

A. Intraperitoneal injection of anesthesia.

B. Tracheotomy.

C. Ligation of right adrenal arteries and vein.

D. Evisceration.

E. Ligation of inferior phrenic vein branches which empty into left adrenal vein.

F. Ligation of spermatic blood vessels.

G. Placing loose ties around:

(1) Aorta just below the left renal artery.

(2) Right renal artery and vein at hilus.

(3) Left renal artery and vein at hilus.

(4) Left renal vein at entry into vena cava.

(5) Vena cava just above left renal vein.

(6) Vena cava just below point of entry into liver.

(7) Left renal vein between kidney and vena cava.

(1) through (6) to be ligated later in procedure.

(7) will be used to hold venous cannula in place.

H. Ligation of small veins emptying into adrenal vein.

I. Ligation of ties (2) and (3) above.

J. Injection of 3000 units of heparin into left iliolumbar vein.

K. Insertion of arterial cannula

(1) Opening of thoracic cavity.

(2) Insertion of cannula into thoracic aorta.

(3) Guiding of cannula to a point below diaphragm's attachment to dorsal body wall.

(4) Tying cannula in place when adrenal completely whitens.

L. Lowering flow rate by 25% followed by ligation of tie (1) above.

M. Ligation of tie (4) above, followed by insertion of cannula into the renal vein. Tying cannula into place when perfusate flows through it.

N. Ligation of ties (5) and (6) above.

O. Placing animal on slanted board.

P. Covering adrenal with moistened gauze pad warmed by incandescent bulb.

Q. Collection of first sample started.

(3) Adrenal vein cannulation

Collection of adrenal venous effluent from the perfused adrenal can also be accomplished by direct cannulation of the adrenal vein. This is feasible only if the left renal artery and vein are far enough apart or can be separated so that a loose tie can be placed around the adrenal vein just at its junction with the left renal vein (tie X, figure 8). If this can be done, then a piece of polyethylene tubing (PE 10, I.D., .011", O.D., .024" or PE 20, I.D., .015", O.D., .043" depending on the diameter of the adrenal vein) with a beveled edge can be inserted into the adrenal vein. The procedure followed was the same as that described above under standard procedure, except that once perfusate flowed down the aorta, the adrenal vein rather than the renal vein was cannulated. First tie K (figure 8) was ligated

Figure 8. Position of venous cannula and arrangement of sutures for direct cannulation of the adrenal vein.

L.R.V. Left Renal Vein

L.R.A. Left Renal Artery

I.A.A. Inferior Adrenal Artery

L.A.V. Left Adrenal Vein

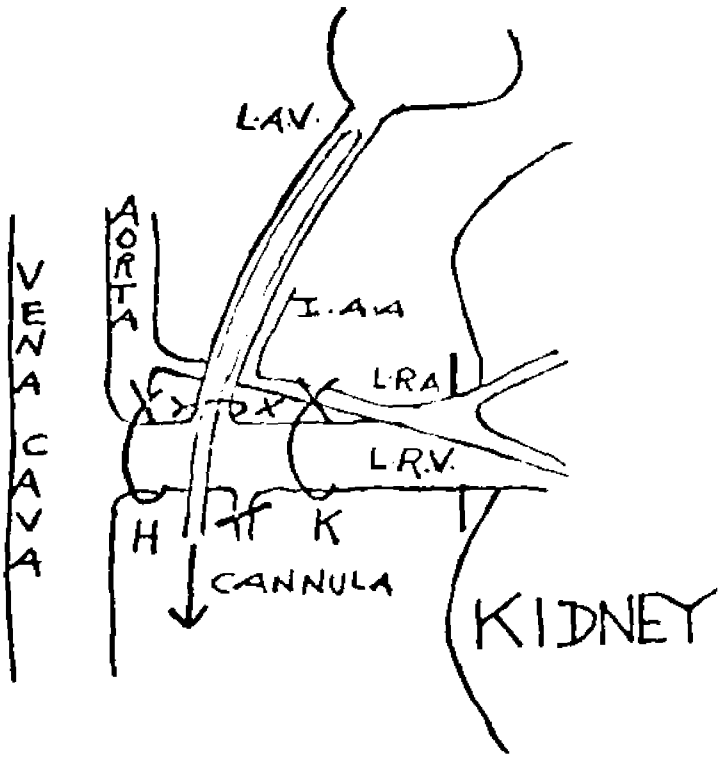


FIGURE 8

followed by tie H and the renal vein cut just below the entry of the adrenal vein. The venous cannula (PE 10 or 20) was inserted into the adrenal vein through the cut in the renal vein and advanced upward to a point several millimeters below the adrenal gland. The cannula was tied in place with tie X when perfusate was observed to flow within it. After ligation of the vena cava (ties I and J, figure 5), the animal was placed on a slanted board so that flow of perfusate down the venous cannula was not opposed by the force of gravity.

B. Experimental procedure

(1) Preparation of the perfusion medium

Adrenals were perfused at room temperature with bicarbonate-buffered Krebs Ringer solution containing 200 mg % glucose (KRBG). The solution was freshly prepared and filtered through a fine sintered glass funnel to remove any particles. It has the following composition (mM concentration): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.1; $NaHCO_3$, 25; glucose 11.1. The perfusate was continuously oxygenated with a gas mixture of 95% oxygen and 5% carbon dioxide and had a pH of 7.2 to 7.4.

(2) Flow rate and perfusion pressure

A Sigmamotor peristaltic pump (Model AL-4E) was equipped with tygon tubing (I.D., 1/32", O.D., 1/16") which provided flow rates of 0.035 to 1.2 ml per minute (2.0 to 72 ml per hour). Perfusion flow rates were expressed as milliliters of effluent collected per 30 minutes. The flow rate was maintained constant for each experiment but was observed to vary from animal to animal within a range of 1.0 to 5.0 ml per hour. This is in accord with the great variability reported for rates of blood flow through the rat adrenal as determined

experimentally using isotopic methods (Sapirstein and Goldman, 1959; Goldman, 1961; Kramer and Sapirstein, 1967) as well as from adrenal vein cannulation studies (Bush, 1953a; Porter and Klaiber, 1964; Holzbauer et al, 1969). In all of these experiments the pump was adjusted so that the rate of collection of adrenal effluent fell within the range (1.8 - 6.0 ml/hr) reported in the literature for adrenal blood flow rates. Perfusion pressure was found in several experiments to lie between 35 and 60 mm Hg.

(3) Collection and handling of samples

Samples of adrenal venous effluent were collected every 30 minutes in ice-cooled 15 ml graduated centrifuge tubes. In some earlier studies, 10 minute samples were collected. The volume was recorded, the samples centrifuged at 4°C and the supernatant frozen for future analysis. The samples were analyzed for corticosterone (B) by acid-fluorescence within one week after completion of an experiment. Outputs were expressed as micrograms of corticosterone per 30 minutes. Most perfusions were started between 10:30 and 11:30 A.M. and were run for periods of 5 to 6 hours.

C. Additions to the perfusion medium

(1) Adrenocorticotropic hormone

Synthetic ACTH (β 1 - 24 Synachten), generously supplied by Dr. J.J. Chart of the Ciba-Geigy Corporation was used. Stock solutions were prepared in 0.5% bovine serum albumin at pH 3.0 and stored in siliconized glass tubes at -4°C. The solutions, which had a concentration of 10 units ACTH per ml (10,000 milliunits/ml), were thawed out just before being added to the standard perfusate (KRBG) to give final concentrations of 10, 100 and 1000 milliunits of ACTH

per ml KRBG. Glass tubes used to hold ACTH solutions, pipettes, tubing and cannulae, all of which came in contact with the hormone, were provided with a silicone coating (Siliclad, Clay Adams Inc., N.Y.) to prevent absorption of the polypeptide to glass or other material. Adrenals were usually exposed to a given concentration of ACTH for the entire duration of the perfusion (5 - 6 hours). In some experiments, a 90 minute washout period with unsupplemented KRBG was followed by exposure to ACTH for 270 minutes.

(2) Dextran

In an attempt to reduce the edema that one frequently encounters in these perfusions, a series of experiments was carried out in which dextran (Clinical Grade, M.W. 60 - 90,000, Nutritional Biochemical Corp., Cleveland, Ohio) was added to the perfusion medium. Five grams of dextran was dissolved in a total volume of 100 ml of previously warmed (38°C) KRBG. The resulting clear, viscous solution (5% dextran in KRBG) was then filtered through a fine sintered glass funnel.

(3) Aminoglutethimide

Aminoglutethimide, an inhibitor of steroidogenesis (Dexter et al, 1967) and freely soluble in aqueous solutions, was dissolved in KRBG to give a final concentration of 100 µg per ml KRBG. Aminoglutethimide phosphate (Elipten Phosphate) was generously supplied by Dr. J.J. Chart of the Ciba-Geigy Corporation.

(4) Indomethacin

Indomethacin, an inhibitor of prostaglandin biosynthesis (Vane, 1971; Ferreira et al, 1971; Smith and Lands, 1971) was generously supplied by Dr. Carl R. Stevenson of Merck Sharp and Dohme Research

Laboratories, West Point, Penna. Ten mg of indomethacin was dissolved in 5 ml of 95% ethanol to produce a concentration of 2 mg per ml. A volume of 0.25 ml of this solution was added to 100 ml of KRBG to produce a final concentration of 5 μg indomethacin per ml KRBG. This solution was then filtered through a fine sintered glass funnel.

(5) Prostaglandins

The prostaglandins used in this study (PGE_1, PGE_2 and $PGF_{1\alpha}$) were generously supplied by Dr. J.E. Pike of the Upjohn Company, Kalamazoo, Michigan. Required amount of these substances were carefully weighed out and dissolved in small volumes (0.3 - 0.5 ml) of 95% ethanol. The prostaglandins were then added to indomethacin-containing KRBG solutions (5 μg indomethacin/ml KRBG) to produce final concentrations of 5 or 10 μg of prostaglandin plus 5 μg indomethacin per ml KRBG. Indomethacin was used in an attempt to prevent possible endogenous prostaglandin biosynthesis. Adrenals were exposed to the prostaglandin-indomethacin solutions after a 90 minute washout with unsupplemented KRBG, or for the entire duration of a perfusion (5 - 6 hours).

II. Hypophysectomy

In some of the perfusion studies performed on hypophysectomized rats, surgically prepared animals were purchased from a commercial source (Carworth, New City, N.Y.). Hypophysectomized male Long Evans rats arrived at this laboratory 22 - 24 hours after surgery and were immediately set up for adrenal perfusion. Adrenals were perfused 24 - 26 hours after the animal had undergone hypophysectomy. In other studies, hypophysectomies were performed in this laboratory and perfusions were carried out 2 - 3 hours after removal of the pituitary.

Male Long Evans rats weighing 250 - 350 gm. were anesthetized intraperitoneally with urethane and an intratracheal cannula was inserted to facilitate breathing, especially during manipulation of the trachea. The animals were hypophysectomized by the standard parapharyngeal approach originally devised by Smith (1930). The operation took 15 minutes to complete. Preparation was begun for adrenal perfusion approximately 45 minutes after removal of the pituitary. Perfusion was started between 2 and 3 hours after completion of the hypophysectomy. The success of the operation was determined at the end of the perfusion by removing the calvarium from the skull, lifting the brain to expose the pituitary area and looking for possible remnants, using a loupe if necessary.

III. Fluorometric assay of corticosterone

A. Assay of adrenal venous effluent

Samples of adrenal venous effluent were assayed for corticosterone (B) by a fluorometric determination which differs only slightly from the method furnished by Dr. J.W. Kendall, Jr. (Personal Communication, 1970). Samples of effluent (0.5 ml) were extracted in 7.5 ml of methylene chloride, and centrifuged for 3 to 5 minutes at 1500 - 2000 rpm. The aqueous layer was removed by aspiration and discarded. A 5 ml aliquot of the remaining methylene chloride extract was transferred to a 12 ml glass-stoppered centrifuge tube containing 1.0 ml of the fluorescent reagent (sulfuric acid: ethanol 3:1 v/v). The tubes were shaken vigorously for 30 seconds and then allowed to stand for 5 minutes. The acid layer was transferred to pyrex tubes and the fluorescence intensity read in an Aminco-Bowman spectrofluorometer with activation wavelength at 470 mμ and

emission wavelength at 525 mμ. The resulting fluorescence reached a maximum intensity within 15 minutes and was observed to remain stable for 60 minutes. Thus, all samples were read after 15 minutes and before 60 minutes. Corticosterone standards (Steraloids Inc., Pawling, N.Y.) of 0.10, 0.25 and 0.50 μg were run through the procedure with each assay. The aqueous working standard solution was freshly prepared on the day of the assay, from an ethanolic standard stock solution of corticosterone stored at 4°C. Since the major corticosteroid secreted by the rat adrenal is corticosterone (Bush, 1953a; the output of steroid was expressed as ug corticosterone per 30 minutes.

The fluorometric method is an extremely sensitive assay but is subject to interference from fluorescent contaminants often found in reagents and in detergents normally employed to wash glassware. For this reason, the following precautions were taken:

(1) It was ascertained that the reagents listed below were free of substances which interferred with the assay and hence substitutes were never used. The reagents were used as supplied by the manufacturer.

(A) Methylene Chloride
Baker-Instra-Analyzed
GC-Spectrophotometric quality solvent

(B) Concentrated sulfuric acid
Mallinckrodt analytic reagent

(C) Ethyl alcohol
Pharmaco
Publicker Industries Inc.

(2) Glassware was washed thoroughly in an acid bath (HNO_3: HCl 1:1) about once every 2 - 3 months.

After use, glassware was washed with Prell (Palmolive Peet & Co.), which is known not to fluoresce under the conditions of the assay.

B. Precision and accuracy of the assay method

When duplicate derminations of both corticosterone standards and unknown samples of perfusion effluent were assayed simultaneously and on different days they gave similar readings. The lower limit of corticosterone concentration which could be realiably detected was 0.02 µg/ml. All samples of perfusate (KRBG) exhibited almost the same blank values. Only when the fluorometer readings of unknowns were at least 50% or higher than those of the blanks were they considered to be an accurate measure of the corticosterone content of the sample. When corticosterone standards ranging from 0.1 to 2.0 micrograms were assayed by the procedure, a linear relationship was observed between fluorescence intensity and concentration of steroid. The time course to reach maximum fluorescent intensity was the same for both standards and unknowns.

Corticosterone recovery was determined by assaying samples of pooled venous effluent to which known amounts of the steroid had been added. The values obtained were compared with aliquots of the same effluent containing no added steroid. The recovery was found to be between 95 and 103%. Sample blanks were determined by adding ethanol to methylene dichloride extracts of perfusion effluents and determining their fluorescent intensity. Under these conditions fluorescence never developed.

The measurement of fluorescence at 520 mµ under the conditions of the assay appears to be an accurate measure of corticosterone

concentration. Moncloa and co-workers (1959) showed that values for corticosterone content of rat adrenals assayed with the fluorometric method agreed with those values obtained by a procedure involving paper chromatographic separation followed by assay with blue tetrazolium. In addition, the corticosterone concentrations obtained in the present study are similar to those obtained from superfused rat adrenals when assayed by competitive protein binding or soda fluorescence (Schulster et al, 1970). Hence, it would appear that the direct fluorometric analysis of perfusion effluents without prior chromatographic isolation of steroids is an accurate method for measuring changes in adrenal corticosterone output which may arise as a result of altering the experimental conditions.

C. Endogenous rat adrenal steroid output and specificity of the method

In addition to corticosterone, a number of other steroids are produced endogenously by the rat adrenal gland. The following steroids have been shown to be present either in rat adrenal vein blood and/or in incubation media from adrenals incubated in vitro;

Corticosterone 18-hydroxydeoxycorticosterone deoxycorticosterone 11-dehydrocorticosterone	Bush, 1953a; Ward and Birmingham, 1960; Cortés et al, 1963; Birmingham et al, 1968.
Aldosterone	Singer and Stack-Dunne, 1955; Giroud et al, 1962.
18-hydroxycorticosterone	Péron, 1961
Progesterone	Holzbauer et al, 1969

11-βhydroxyprogesterone (tentatively identified)	Traikov and Birmingham, 1966.

Both corticosterone and 18-hydroxydeoxycorticosterone are the major secretory products of the rat adrenal. Cortés et al (1963), have shown that large amounts of these steroids are secreted into the blood stream; corticosterone levels were approximately two times higher than those found for 18-hydroxydeoxycorticosterone.

The specificity of the fluorometric technique has been extensively investigated by other workers who have determined the fluorescence of many steroid hormones under conditions similar to those used in the present assay (Zenker and Bernstein, 1958; Silber et al, 1958; Cortés et al, 1963; Uete et al, 1970; Monder and Kendall, Unpublished Observations, 1972). Of all the steroid hormones studied, corticosterone always showed the greatest fluorescent intensity. None of the other steroids listed above (i.e., those isolated and identified from rat adrenal glands) manifested enough fluorescence to interfere with the assay.

In the present study some preliminary work was done to confirm that corticosterone was the substance responsible for the fluorescence measured in venous effluents of perfused rat adrenals. Venous effluents from several control (KRBG) perfusions were pooled, extracted with methylene dichloride and evaporated to dryness. The extracts were redissolved in ethanol and chromatographed on thin layer plates (Silica Gel 60 F-254 Brinkman Instruments, Westbury, N.Y.) along with a corticosterone standard in chloroform: methanol (95:6). The resulting spots were removed from the plates, extracted from the silica gel with methylene dichloride and their fluorescence deter-

mined. Several components separated on the thin layer plates, but only the one migrating with standard corticosterone manifested any fluorescence. This together with the investigations concerning the specificity of the fluorometric method (cited above) suggested that corticosterone accounts for the fluorescence in the methylene dichloride extracts of venous effluents from perfused rat adrenals.

The fluorometric technique has been extensively used to measure plasma corticosterone levels in rats (Guillemin et al, 1959; Moncloa et al, 1959). A major interfering factor in this assay is an unidentified fluorescing substance present in the plasma of rats following adrenalectomy and hypophysectomy. Hence, the source of this "residual fluorescence" cannot be of adrenal origin. In the present study, venous effluent being collected is primarily of adrenal origin (see Appendix); non-adrenal contaminants would not be present in the samples collected. This eliminates the major source of error reported in the literature for the fluorometric method.

IV. In vitro studies

Whenever possible, comparisons were made between the behavior of the in situ perfused adrenal and that of single adrenal glands incubated in vitro. Attempts were made to expose the in vitro-incubated adrenals to experimental conditions closely approximating those existing during the perfusion.

Adrenals were incubated in vitro by varying the conditions of the classical technique of Saffran and Schally (1955). After intraperitoneal administration of the anesthetic, adrenals were surgically removed from male Long Evans rats. In some experiments, only the right adrenal was removed and immediately incubated in vitro while the

left gland was prepared for perfusion. Adrenals, once removed from the animal, were placed in ice cold KRBG, and the adhering fat was removed with a pair of fine scissors. The glands were then blotted on a piece of filter paper and weighed on a torsion balance. They were then placed in a petri dish lined with moistened filter paper, and cut into quarters with a razor blade. The quarters from one single adrenal gland were placed in a flask containing 1.5 ml of KRBG and were preincubated in a shaking water bath for 30 minutes at 38°C and in an atmosphere of 95% O_2:5% CO_2. At the end of the preincubation period, the medium was removed and replaced by 1.5 ml of fresh medium. When stimulatory or inhibitory agents were used, they were added at this point in volumes of 0.05 or 0.10 ml to 1.45 or 1.40 ml respectively of fresh medium resulting in a final volume of 1.5 ml. The flasks were then returned to the waterbath and incubated for two hours at 38°C (incubation period). At the end of the incubation period the medium was removed and immediately frozen for future analysis. Aliquots of 0.1 ml of incubation medium were analyzed fluorometrically for corticosterone as described above. Steroid output was expressed as µg corticosterone per adrenal gland or as µg corticosterone per 100 mg. of adrenal tissue per two hours. Small fragments of liver, skeletal muscle, kidney, aorta, pituitary gland and fat pad, approximately the size of an adrenal gland, were incubated *in vitro* according to the above procedure. The fluorescence in the medium from these incubations was equivalent to that in the KRBG blank.

RESULTS AND DISCUSSION OF RESULTS

I. X-ray studies: perfusion with radioopaque substances

Perfusion of adrenal glands with radioopaque materials such as $BaSO_4$ or Thorotrast (a 25% colloidal solution of thorium dioxide) followed by x-rays of the area or, photography of the area after perfusion with a dye, confirmed that the perfusate passed through the adrenal and was present in the venous effluent.

Figure 9 is a photograph of an x-ray taken after perfusion with Thorotrast. The perfusion was continued until the Thorotrast was observed in the adrenal and renal veins. The preparation was then x-rayed and a photograph taken of the x-ray. The gland appears to be completely perfused and the adrenal and renal veins are clearly visible. A branch of the inferior phrenic vein can also be seen emptying into the adrenal vein. (see insert, figure 9).

Visualization of the area with a dissecting microscope after perfusion with various dyes further confirmed that the perfusate gained access to the adrenal via the adrenal arteries and that the venous effluent passed from the adrenal vein into the renal vein.

Early studies which demonstrated the importance of ligating branches of the inferior phrenic vein and other small branches which empty into the adrenal vein (see I.P.V. and (x) figure 3) are described in the appendix.

II. Patterns of response to unsupplemented perfusate (KRBG) in the intact rat

Control perfusions with Krebs Ringer bicarbonate glucose (KRBG), of adrenal glands from intact rats elicited responses which

Figure 9. Photograph of an x-ray taken after adrenal perfusion with Thorotrast.

Perfusion was continued until the Thorotrast could be seen within the adrenal and renal veins. The animal was then x-rayed and a photograph taken of the x-ray plate.

Insert:

A. Left Adrenal Gland

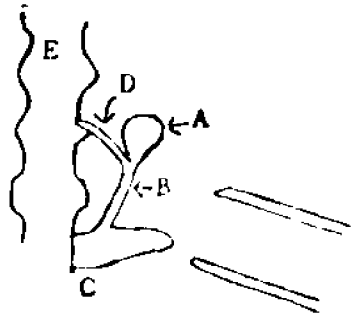
B. Adrenal Vein

C. Renal Vein

D. Branch of Inferior Phrenic Vein

E. Vertebral Column

INSERT

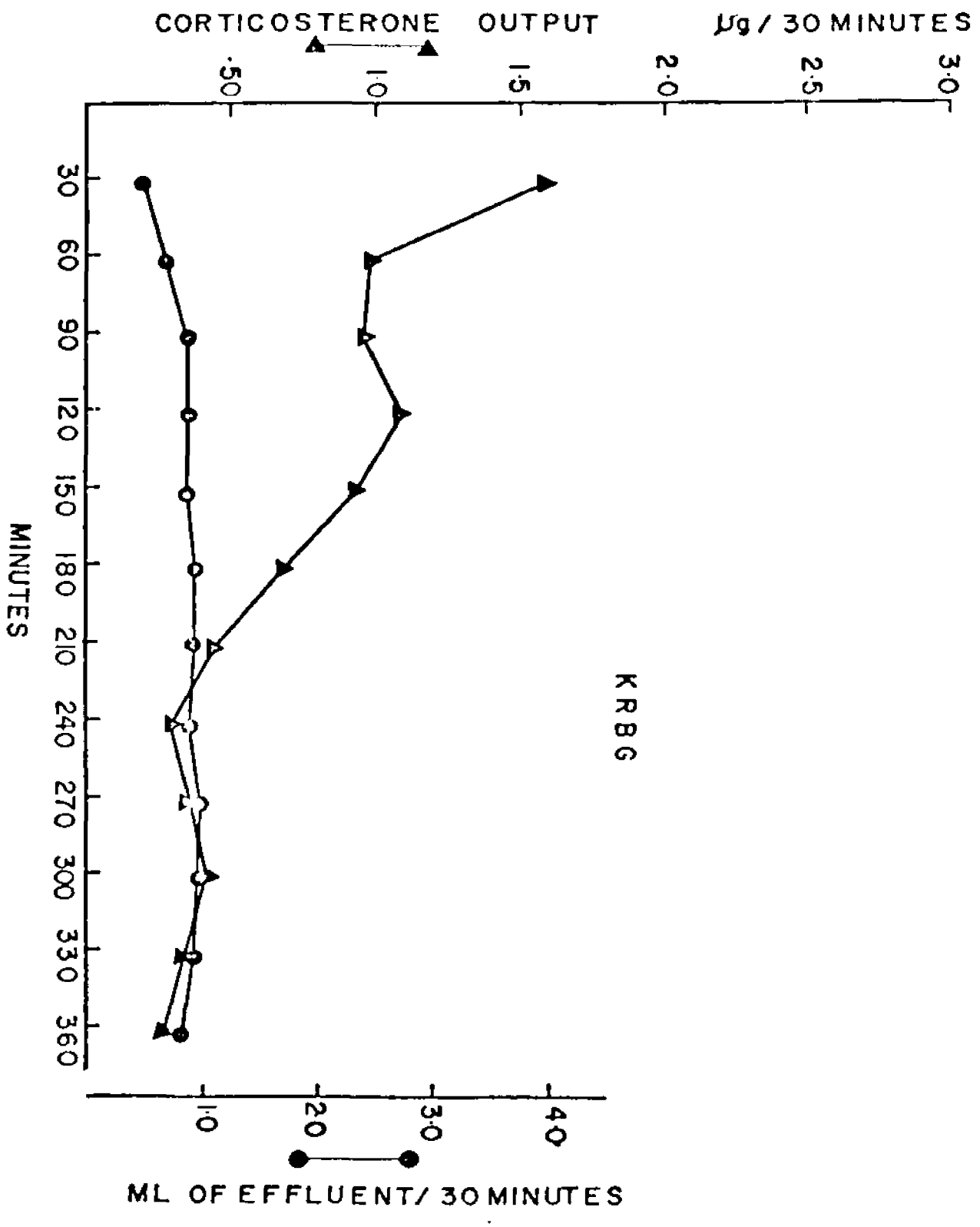


fall into two patterns over a 6 hour period. Nine out of seventeen control perfusions showed high initial outputs of corticosterone (B) which decreased with time (Pattern I). In the remaining eight perfusions an overall decrease in output was also observed, but was accompanied by transient increases in output (Pattern II). These increases in B output occurred at different time intervals over the 6 hour period. In these and all subsequent perfusions steroid output was expressed as micrograms of corticosterone per 30 minutes (μg B/30 minutes).

A. The initial output of corticosterone decreases with time (Pattern I)

Figure 10 shows a typical control experiment representative of the pattern I type of response. The gland was perfused for 6 hours with KRBG. Corticosterone output (triangles, upper curve) was seen to be high at the start of the perfusion and to reach low levels at the end of 6 hours. Regardless of the pattern of response, mean outputs of 1.8 ($\pm$ 0.25 S.E.) of corticosterone during the first 30 minutes declined to 0.62($\pm$ 0.20) after 6 hours of perfusion in control experiments. This is similar to what others have observed with *in vitro* incubation (Birmingham et al, 1968; Margoulies et al, 1970) as well as with superfusion systems (Tait et al, 1970; Saffran et al, 1971). High initial outputs were obtained in almost all perfusions with intact rats. This probably reflects the response of the adrenal to ACTH stimulation elicited by the stress of the surgical procedure. The high initial outputs of corticosterone were not observed in adrenal perfusions from hypophysectomized rats. The flow rate (circles, lower curve, figure 10) remained constant over the 6 hour period. In general,

Figure 10. Time courses of corticosterone output and flow rate for a control (KRBG) adrenal perfusion (experiment 2 C) showing pattern I type of response.


CORTICOSTERONE OUTPUT µg / 30 MINUTES
·50
1·0
1·5
2·0
2·5
3·0
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES
KRBG
1·0
2·0
3·0
4·0
ML OF EFFLUENT/ 30 MINUTES

no relationship appeared to exist between variations in flow rate from one experiment to another and steroid output in any control perfusions.

Figures 11 and 12 are control perfusions showing the same type of response to KRBG, i.e. high initial outputs which decline with time. Although corticosterone output declined with time of perfusion, qualitative differences in the time course of the decline were observed from one experiment to another. For example, figure 12 shows a generally steady time-dependent decline in output. In contrast to this, the perfusions represented by figure 10 and 11 showed that the declining output can be interrupted by time sequences during which fairly steady outputs of corticosterone can be maintained.

The data for the 9 perfusions showing this type of response (Pattern I) are presented in Table II. The mean corticosterone outputs for each 30 minute collection period and their standard errors are included in the table.

B. Transient increase in corticosterone output (Pattern II)

Frequently, in perfusions with unsupplemented KRBG, a pattern of response (Pattern II) characterized by transient spontaneous rises in corticosterone (B) output was observed. These occurred at different time intervals over the 6 hour period, were superimposed upon the overall decrease in output, were not accompanied by changes in adrenal flow rate, and were not related to the time of day.

Figure 13 illustrates this type of response. The high initial output of B (triangles, figure 13) decreased rapidly during the first hour of perfusion and was then followed by an increase in output which surpassed the initial value. The maximal B output was at 120 minutes

Figure 11. Time course of corticosterone output for a control (KRBG) perfusion (experiment 3 C) showing pattern I type of response.

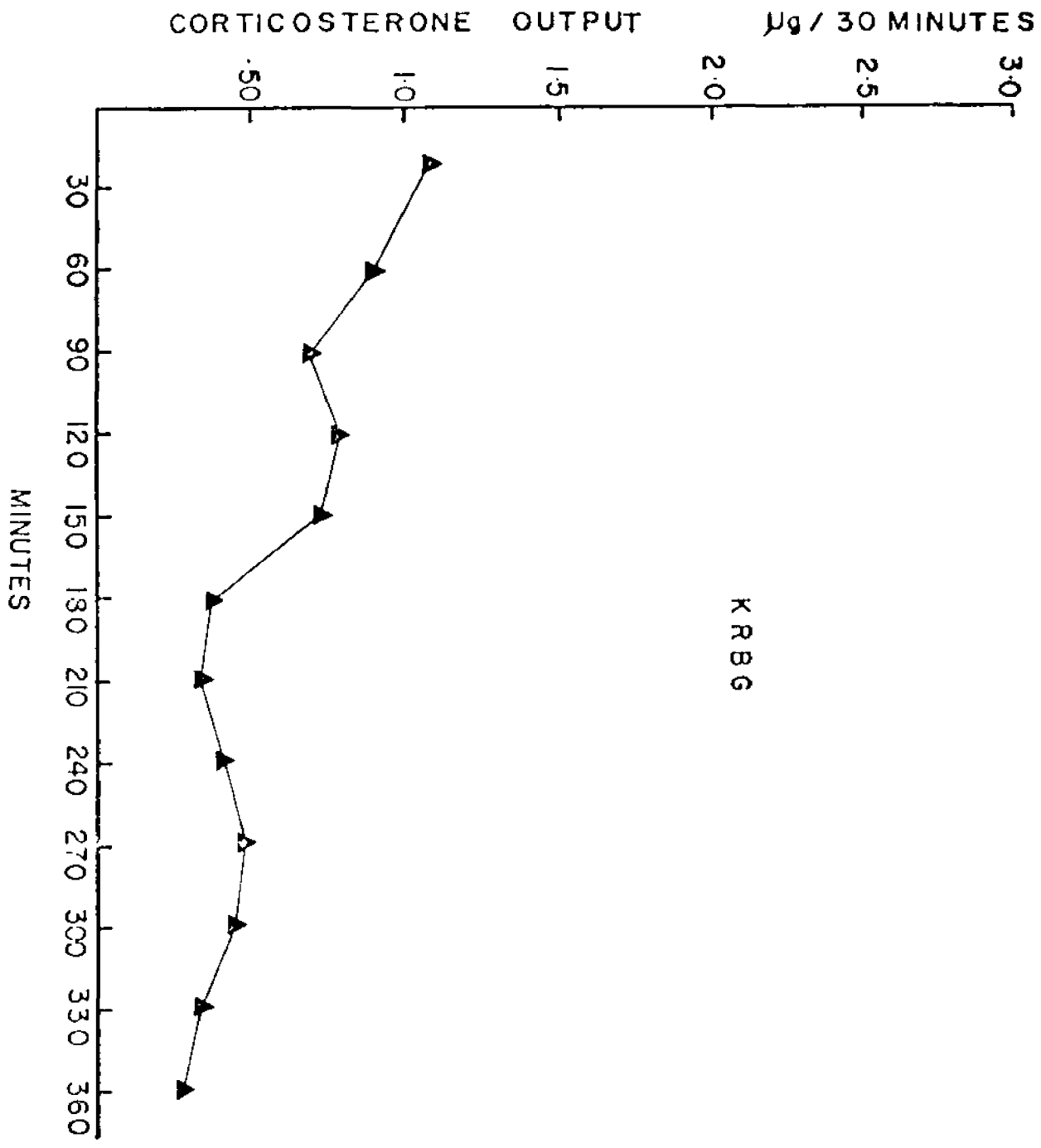

CORTICOSTERONE OUTPUT µg / 30 MINUTES
·50
1·0
1·5
2·0
2·5
3·0
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES
KRBG

Figure 12. Time course of corticosterone output for a control (KRBG) perfusion (experiment 4 C) showing pattern I type of response.

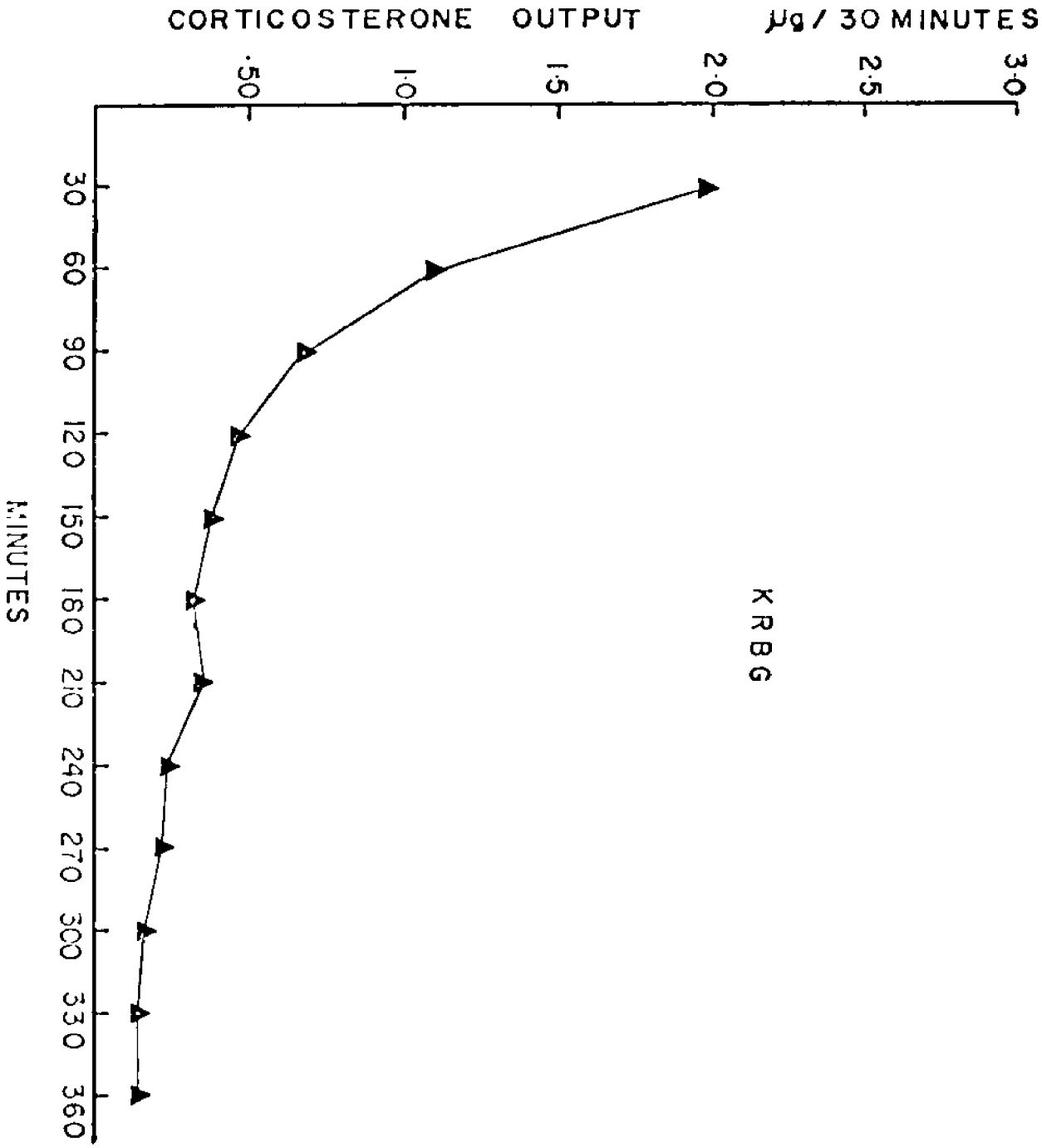

CORTICOSTERONE OUTPUT µg / 30 MINUTES
·50
1·0
1·5
2·0
2·5
3·0
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES
KRBG

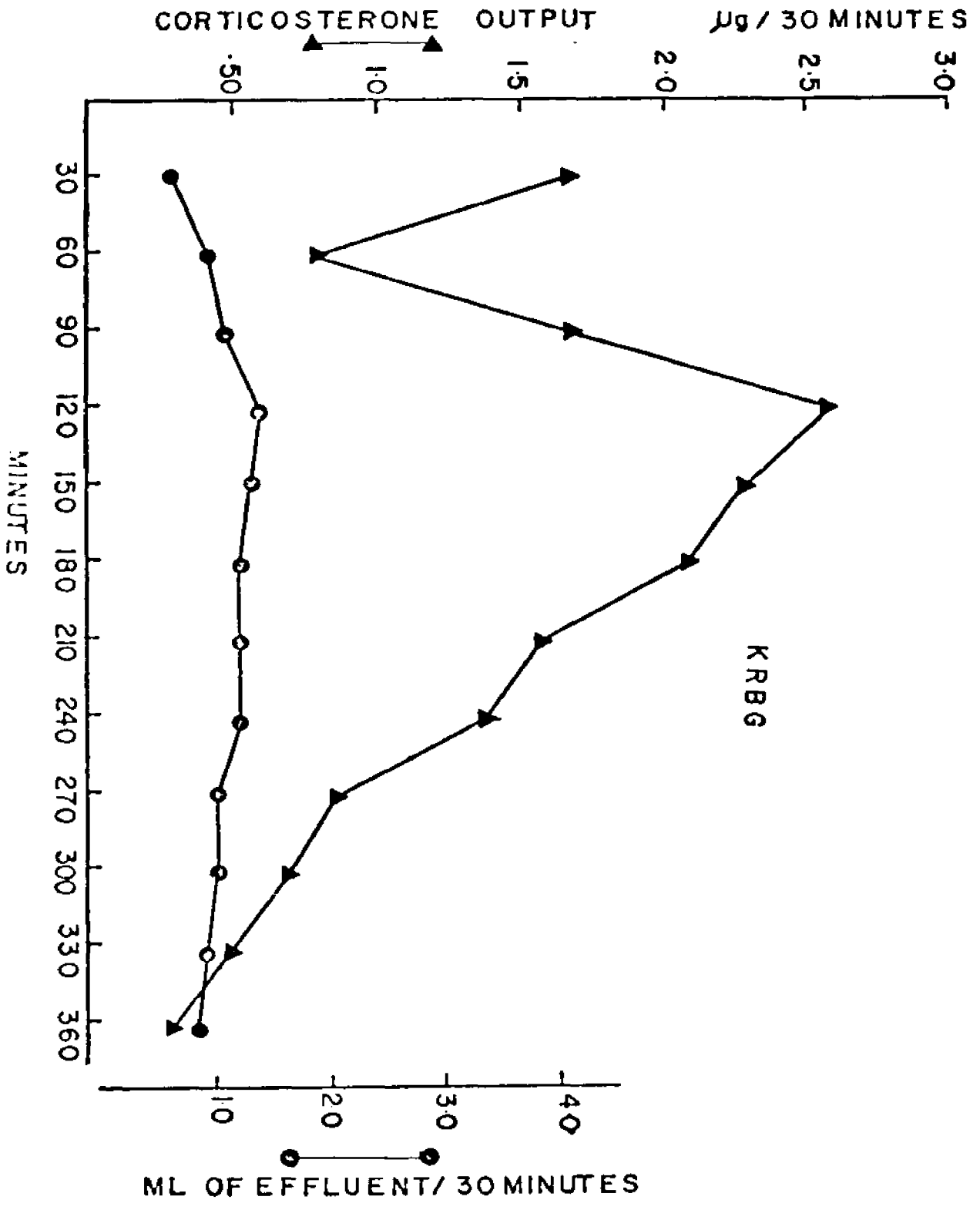
TABLE II. CORTICOSTERONE OUTPUT FROM RAT ADRENAL GLANDS PERFUSED WITH KRBG SHOWING PATTERN I TYPE RESPONSE.

µg CORTICOSTERONE/30 MINUTE SAMPLE

Minutes after start of perfusion	0 - 30	30 - 60	60 - 90	90 - 120	120 - 150	150 - 180	180 - 210	210 - 240	240 - 270	270 - 300	300 - 330	330 - 360
Experiment												
1C	2.0	0.31	0.22	0.24	0.20	0.39	0.52	0.53	0.40	0.27	0.18	0.14
2C	1.6	0.99	0.97	1.1	0.95	0.69	0.45	0.39	0.37	0.42	0.34	0.27
3C	1.1	0.91	0.75	0.79	0.73	0.37	0.33	0.40	0.48	0.44	0.33	0.27
4C	2.0	1.1	0.67	0.45	0.37	0.31	0.34	0.22	0.20	0.14	0.12	0.12
5C	1.8	0.88	0.75	0.63	0.50	0.36	0.46	0.45	0.30	0.21	0.16	0.08
6C	0.59	0.40	0.59	0.61	0.48	0.48	0.45	0.33	0.44	0.35	0.21	0.15
7C	1.1	0.21	0.16	0.21	0.20	0.14	0.10	0.12	0.08	0.07	0.07	0.09
8C	1.7	1.0	0.62	0.53	0.58	0.30	0.46	0.24	0.14	0.07	0.04	0.04
9C	2.0	1.6	1.1	0.71	0.77	0.55	0.47	0.45	0.39	0.43	-	-
MEAN	1.5	0.82	0.65	0.59	0.53	0.39	0.39	0.35	0.31	0.27	0.18	0.14
± S.E.	0.16	0.15	0.10	0.09	0.08	0.05	0.04	0.04	0.05	0.05	0.04	0.02

S.E. = standard error C= control (KRBG-perfused)

Figure 13. Time courses of corticosterone outout and flow rate for a control (KRBG) adrenal perfusion (experiment 13 C) showing pattern II type of response.


CORTICOSTERONE OUTPUT µg / 30 MINUTES
·50
1·0
1·5
2·0
2·5
3·0
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES
KRBG
10
20
30
40
ML OF EFFLUENT/ 30 MINUTES

after which there was a slow decrease with time, reaching low levels of B at the end of 6 hours of perfusion. The flow rate (circles, figure 13) remained constant for the duration of the perfusion.

The spontaneous increase in B output has been observed to occur at different time intervals over the 6 hour period. In experiment 14C (Table III) the maximal increase occurred late (at 240 minutes) in the perfusion. Here the maximal output did not surpass the high initial value and was followed by a gradual decline. Experiment 10C (Table III) showed a spontaneous increase which occurred early in the perfusion (at 90 minutes) and another increase of lesser magnitude later in the experiment (at 270 minutes) also followed by a decline. The low initial output observed in this perfusion was a rare occurrence. In most perfusions of adrenals from intact rats, high initial outputs of B were observed.

The data for the 8 perfusions which showed transient increases in B output (Pattern II) are summarized in Table III. Large individual differences in B output from one adrenal to another were found within this series of experiments, e.g., in experiment 11C, the gland had a mean output over a 6 hour period of 0.44 µg B per 30 minutes compared to 3.3 µg for the adrenal in experiment 17.

Spontaneous transient increases in B output from adrenal preparations have been observed by others (Huibregtse and Ungar, 1970; Flack and Ramwell, 1972). This phenomenon is difficult to explain especially in light of the fact that it occurred in the absence of any obvious stimulatory agents.

It was thought, at first, Nembutal (sodium pentobarbital) anesthesia used in some of the early perfusion studies might be involved

TABLE III. CORTICOSTERONE OUTPUT FROM RAT ADRENAL GLANDS PERFUSED WITH KRBG SHOWING TRANSIENT INCREASE IN OUTPUT: PATTERN II TYPE OF RESPONSE

Minutes after start of perfusion	μg CORTICOSTERONE/30 MINUTE SAMPLE											
	0 - 30	30 - 60	60 - 90	90 - 120	120 - 150	150 - 180	180 - 210	210 - 240	240 - 270	270 - 300	300 - 330	330 - 360
Experiment												
10C	0.59	0.51	1.4	1.2	0.75	0.60	0.51	0.65	0.80	0.60	0.51	0.37
11C	1.0	0.43	0.26	0.40	0.62	0.66	0.42	0.38	0.30	0.33	0.25	0.25
12C	1.9	2.3	3.7	4.3	4.7	4.1	3.0	2.3	1.9	2.1	1.8	1.6
13C	1.6	0.71	1.6	2.5	2.2	2.0	1.5	1.3	0.78	0.61	0.40	0.20
14C	2.1	0.69	0.32	0.54	0.81	0.98	1.1	1.6	1.2	0.79	0.75	0.61
15C	1.9	1.2	1.1	1.1	1.0	1.4	1.6	1.8	2.0	1.8	1.8	1.7
16C	2.0	0.77	0.68	0.80	0.97	1.3	1.5	1.8	1.9	1.8	1.7	1.4
17C	5.3	2.6	2.6	5.4	3.8	2.9	3.1	2.9	3.2	2.8	2.2	2.7

C = control (KRBG - perfused)

Underlined samples refer to point where maximal increase in output occurred.

in the spontaneous increases in B output. Biegelman and co-workers (1956) had compared the effects of anesthetics (ether vs nembutal) on adrenal B output in rats that had undergone adrenal vein cannulation. This study showed that under nembutal anesthesia, B secretion was low at the start of the collection period but increased some three fold by the end of a two hour period. In contrast, ether anesthesia resulted in raised early levels of B output, followed by a decrease over the 2 hour period. They suggested that the B output under nembutal anesthesia reflected the basal or non-stressed output of the rat adrenal or a partial inhibition of the adrenal response to the stress of laparaotomy and vein cannulation. Although they did not consider that nembutal was acting directly on the adrenal gland, this possibility seemed reasonable in the current studies.

Sodium pentobarbital is a short-acting barbiturate which is metabolized by enzymes of the liver. Any factor such as liver damage which adversely influences its metabolism may result in a longer biological half-life for the anesthetic (Ben et al, 1969; Strobel and Wollman, 1969). This would certainly be true in the eviscerated animal.

Rough calculations were made to estimate the amount of nembutal which might be circulating in an eviscerated animal. Based on the total blood volume for the rat (about 5 ml/100 gm. body weight), an arbitrary assumption was made that about half of the injected dose of nembutal would reach the circulation and be present in an unmetabolized form by the time the evisceration had been completed. The adrenal would then be exposed to concentrations of 0.5 - 1.0 mg nembutal per ml KRBG. Perfusions were then carried out in which these concentrations of

nembutal were added to the medium perfusing the gland in urethane-anesthetized animals. Under these conditions, nembutal was observed to inhibit rather than stimulate corticosterone output. Nembutal concentrations of 0.5 - 1.0 mg per ml KRBG were also shown to inhibit corticosterone output from adrenal glands incubated in vitro. Hence, these experiments indicated that under these conditions, the spontaneous increases in B output from the perfused adrenal gland could not be attributed to an effect of nembutal.

The possibility that the spontaneous increase(s) in B output seen over a 6 hour period might be related to diurnal variation in output from the perfused adrenal was also explored. Diurnal variations (rhythms) in B output have been shown to occur in vivo (Guillemin et al, 1959; Halberg et al, 1959; Krieger, 1972) as well as in vitro (Ungar and Halberg, 1962). Hence, there appears to be an intrinsic mechanism within the adrenal which controls secretory rhythmicity.

In the rat, steroid levels are low in the early morning (0700 to 1100); they then start to increase at about 11:00 A.M.; maximum levels are reached between 3:00 and 7:00 P.M. (Guillemin et al, 1959; Critchlow et al, 1963). In the present study, most adrenal perfusions were started between 10:30 and 11:30 A.M. and were run for periods of 5 to 6 hours, i.e., a time of day when adrenal output is increasing. This suggested that the spontaneous increase(s) were perhaps due to the fact that perfusions were carried out during a time period when the adrenal output is increasing. To test this possibility, several perfusions were started between 3:00 and 4:00 P.M. (a period of near maximum steroid output level) and continued for 5 to 6 hours until 9:00 to 10:00 P.M., when steroid output would be expected to be on the

decline. Transient spontaneous increase(s) in B output were still observed to occur and at different time intervals, even when the adrenals were perfused later in the day. Thus, the spontaneous increase(s) in B output do not appear to be directly related to a mechanism within the adrenal which controls diurnal secretory rhythmicity.

III. Response to unsupplemented perfusate (KRBG) in the hypophysectomized rat

Control perfusions with Krebs Ringer bicarbonate glucose (KRBG) of adrenal glands from hypophysectomized rats were started two to three hours after removal of the pituitary gland. The decision to start the perfusion of an adrenal gland at this time was based on reports that the adrenal gland shows maximum sensitivity to ACTH two to three hours after hypophysectomy and decreases thereafter (Dear and Guillemin, 1960; Liddle et al, 1962; Grahame-Smith et al, 1967). The original intention was to study the effects of ACTH in the hypophysectomized rat.

In general, adrenal perfusions in hypophysectomized rats were difficult to perform. Frequently, animals would succumb while being surgically prepared for perfusion. To get around this problem, three animals were hypophysectomized on each day of an experiment and hence were available for perfusion if needed.

Another difficulty encountered with hypophysectomized animals was that of controlling adrenal flow rate. It was difficult to maintain a constant flow rate and at times impossible to obtain flow through the gland at all. Perfusions which resulted in wide fluctuations in flow rates and those with flow rates less than 1.0 and

greater than 5.0 milliliters per hour were discarded.

Output of B during the first 30 minutes of perfusion was always low. The small initial outputs gradually increased with time of perfusion. Figure 14 (experiment 1Hx, Table IV) depicts a typical experiment. Two hours after hypophysectomy, the adrenal was perfused with KRBG for a period of six hours. The initial output of B was low during the first hour of perfusion and gradually increased to maximal values by the end of two and a half hours. The maximal output was maintained for about one hour and then output slowly declined reaching lower levels during the last hour of perfusion.

Figure 15 (experiment 4Hx, Table IV) shows a somewhat different pattern of response to perfusion in a hypophysectomized rat. The initial output of B was low; this was followed by a gradual increase in output which continued throughout the duration of the perfusion. No decrease in B output was observed.

Five successful control (KRBG) perfusions were carried out on adrenals from hypophysectomized rats. Flow rate was observed to remain constant over the 6 hour period in all of these experiments. Low initial mean outputs of 0.12 ($\pm$ 0.06 S.E.) µg of B during the first 30 minutes gradually increased (spontaneously) to 0.66 ($\pm$ 0.28 S.E.) µg after five to six hours of perfusion. The low initial outputs were observed to slowly increase to some maximal level. With the exception of the experiment depicted in figure 15, a decrease from the maximal level attained was always observed, although B output during the last hour never declined to the low initial level. The data for the 5 control perfusions from hypophysectomized rats are summarized in Table IV.

Figure 14. Time courses of corticosterone output and flow rate for an adrenal gland (experiment 1 Hx) perfused with KRBG 2 hours after hypophysectomy.

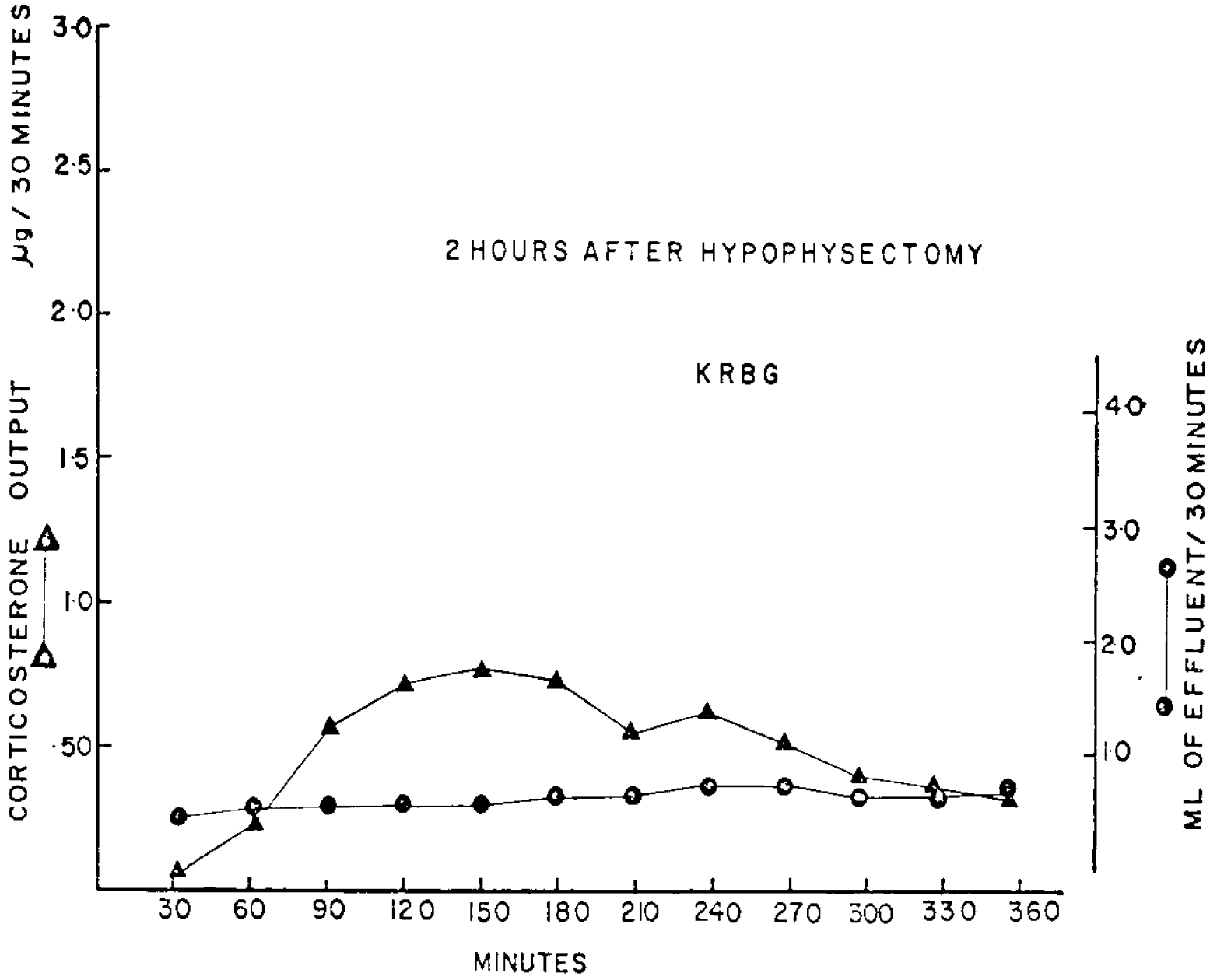

2 HOURS AFTER HYPOPHYSECTOMY
KRBG
CORTICOSTERONE OUTPUT µg / 30 MINUTES
ML OF EFFLUENT / 30 MINUTES
3·0
2·5
2·0
1·5
1·0
·50
40
30
20
10
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES

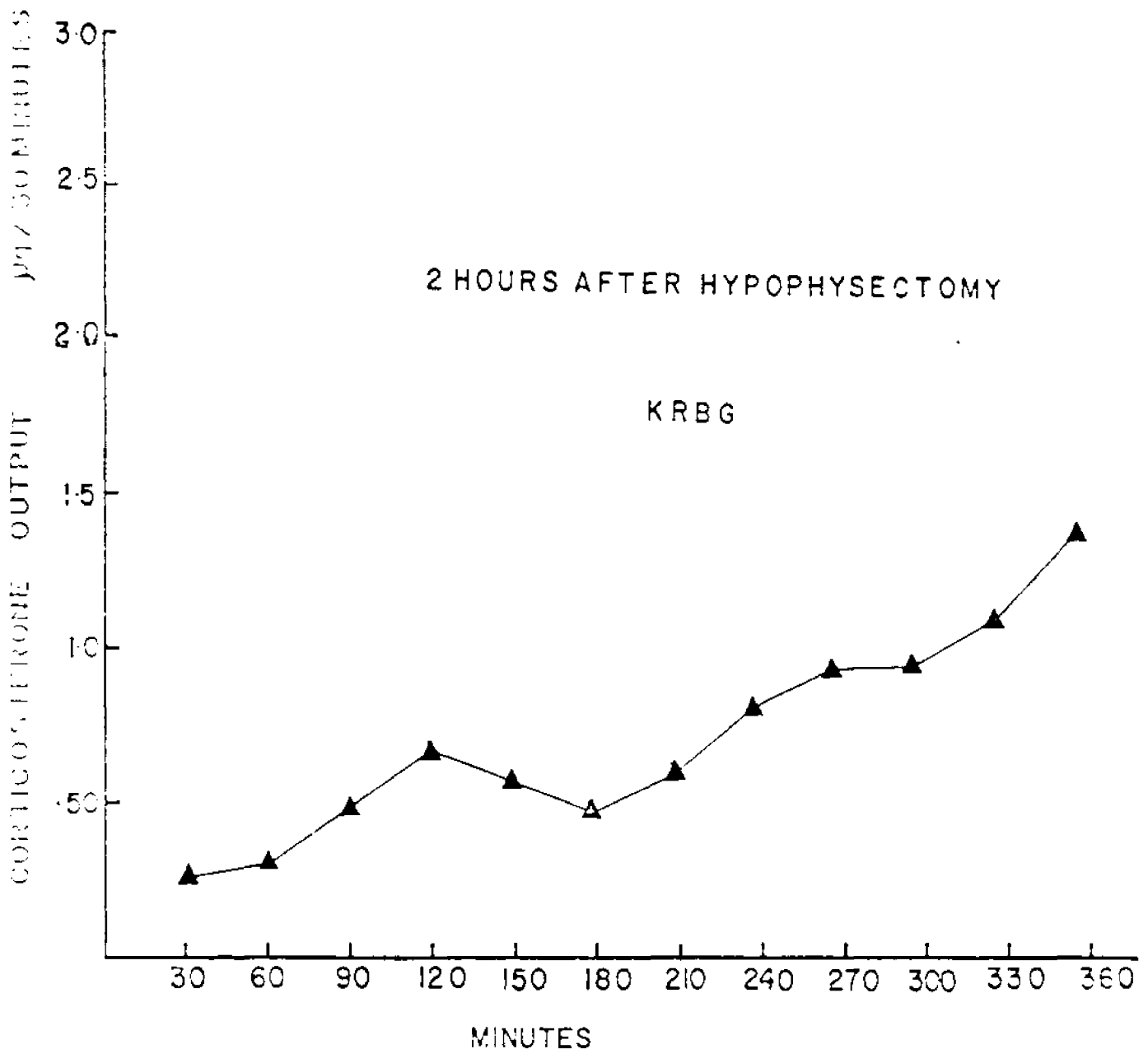
TABLE IV. CORTICOSTERONE OUTPUT FROM RAT ADRENAL GLANDS PERFUSED WITH KRBG 2 TO 3 HOURS AFTER HYPOPHYSECTOMY.

μg CORTICOSTERONE/30 MINUTE SAMPLE

Minutes after start of perfusion	0 - 30	30 - 60	60 - 90	90 - 120	120 - 150	150 - 180	180 - 210	210 - 240	240 - 270	270 - 300	300 - 330	330 - 360
Experiment												
1Hx	0.04	0.21	0.57	0.72	0.78	0.73	0.54	0.62	0.51	0.39	0.35	0.30
2Hx	0.02	0.07	0.13	0.24	0.41	0.52	0.46	0.54	0.56	0.61	0.26	-
3Hx	0.04	0.16	0.26	0.20	0.11	0.11	0.19	0.21	0.23	0.17	0.18	0.07
4Hx	0.24	0.28	0.47	0.66	0.56	0.45	0.58	0.80	0.93	0.94	1.1	1.4
5Hx	0.30	0.32	0.38	0.49	0.80	0.89	0.63	1.6	1.9	1.5	1.3	-
MEAN	0.13	0.21	0.36	0.46	0.53	0.54	0.48	0.75	0.83	0.72	0.64	0.59
± S.E.	0.05	0.04	0.07	0.11	0.13	0.13	0.08	0.23	0.29	0.23	0.23	0.41

Hx = hypophysectomized

Figure 15. Time course of corticosterone output for an adrenal gland (experiment 4 Hx) perfused with KRBG 2 hours after hypophysectomy.


3·0
2·5
2·0
1·5
1·0
·50
2 HOURS AFTER HYPOPHYSECTOMY
KRBG
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES

The finding of low initial outputs of B in perfusions from hypophysectomized rats suggests that the high initial outputs observed in adrenal perfusions from intact rats may be due to ACTH stimulation. These high initial values, which were never observed in hypophysectomized rats, may represent the response of the adrenal to ACTH stimulation elicited by the stress of the surgical procedure. The rapid rate of disappearance of endogenous ACTH following hypophysectomy supports this belief. Both bioactive (Sydnor and Sayers, 1953) and immunoreactive ACTH (Cook et al, 1972; Matsuyama et al, 1972) have been shown to disappear rapidly (half-life less than 5 minutes) from the plasma of rats following hypophysectomy. The biological life of the ACTH molecule as manifested by its ability to stimulate steroidogenesis appears to be somewhat longer. It takes 30 minutes for B secretion to fall 12-fold following hypophysectomy in rats (Harding and Nelson, 1964).

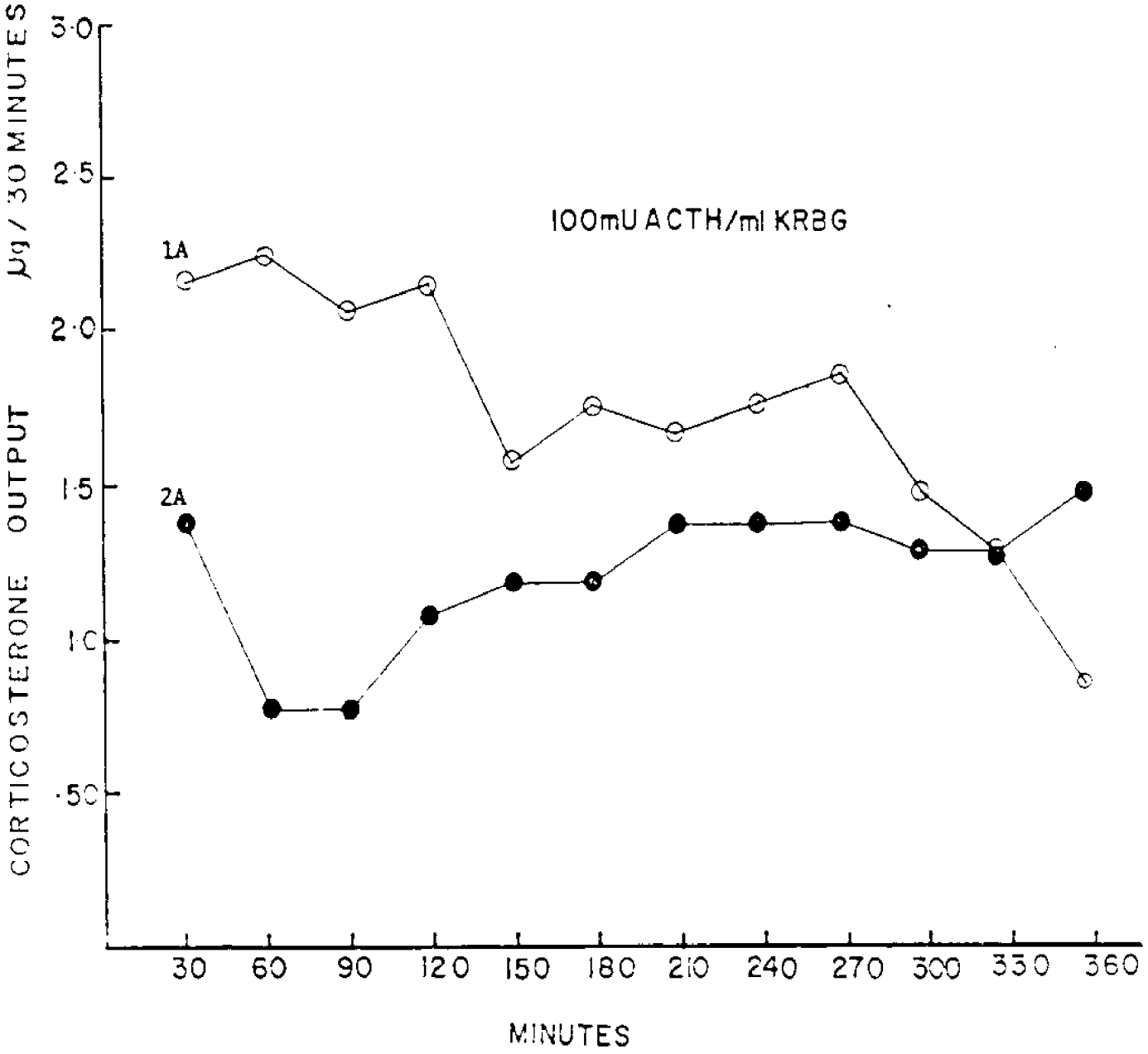
IV. Response to ACTH

A. Continuous perfusion with ACTH 100 mU ACTH/ml KRBG

In these studies adrenal glands were continuously perfused with ACTH (100 mU/ml KRBG) from the start of perfusion until its completion, i.e. for periods of 3 and 6 hours. The response to ACTH was variable, yet in most of the experiments the high initial corticosterone (B) output observed in control perfusions was maintained for several hours under the influence of ACTH. Some adrenal glands exhibited an initial decline in output followed by a rise to a higher level which was then maintained for several hours. A rise to some peak level followed by a decline was also observed in response to ACTH.

Figure 16 shows the response to continuous perfusion with ACTH

Figure 16. Time courses for corticosterone output for 2 adrenal glands (experiments 1 A and 2 A) perfused with ACTH (100 mU/ml KRBG) for 6 hours.


CORTICOSTERONE OUTPUT µg / 30 MINUTES
3·0
2·5
2·0
1·5
1·0
·50
100mU ACTH/ml KRBG
1A
2A
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES

over a 6 hour period in two separate experiments. In experiment 1A (open circles) the high initial output of B was maintained for two hours and declined slightly during the third hour. The slightly lower output was maintained for another two hours followed by a decline. In this experiment, ACTH was able to maintain an output of 3 - 4 µg B/ hour (see Table V, experiment 1A) for four and a half hours before starting to decline.

In experiment 2A (closed circles, figure 16) the high initial output of B declined to about half the initial value during the first 90 minutes of perfusion. The decline was followed by a slow increase which after another 30 minutes attained an output of between 2 and 3 µg B/hour. This level of output was maintained for the duration of the perfusion (another 4 hours) and no decline was observed. The adrenal glands of both animals (experiments 1A and 2A) perfused with ACTH maintained a relatively high output of B when compared with control perfusions.

The responses to ACTH were similar in both experiments; however qualitative and quantitative differences were observed. There was an initial drop in B output in experiment 2A but not in 1A. The adrenal perfused in 2A maintained a high output without showing any decline while in 1A a decline could be observed after four and a half hours of perfusion with ACTH. The rate of B output/adrenal/hour was greater for 1A than for 2A (see Table V). This may have been related to a difference in size of the two adrenal glands. The wet weight of the adrenal gland in 1A after 6 hours of perfusion was 32.0 mg while the gland in 2A weighed 22.0 mg. In general, no definite correlation could be made between B output of a perfused adrenal gland and its wet weight

TABLE V. CORTICOSTERONE OUTPUT FROM RAT ADRENAL GLANDS PERFUSED WITH ACTH (100 mU/ml KRBG) FOR 3 AND 6 HOURS.

µg CORTICOSTERONE/30 MINUTE SAMPLE

Minutes after start of perfusion	0 - 30	30 - 60	60 - 90	90 - 120	120 - 150	150 - 180	180 - 210	210 - 240	240 - 270	270 - 300	300 - 330	330 - 360
Experiment												
1A	2.2	2.3	2.1	2.2	1.6	1.8	1.7	1.8	1.9	1.5	1.3	0.85
2A	1.4	0.78	0.77	1.1	1.2	1.2	1.4	1.4	1.4	1.3	1.3	1.5
3A	1.3	1.4	2.0	1.9	3.5	3.1	2.2	1.8	1.3	0.63	0.43	0.40
4A	2.7	0.71	1.2	1.9	2.5	2.9	2.9	2.7	2.3	2.2	1.8	1.2
5A	1.2	0.85	1.2	2.3	4.6	7.7	3.3	3.0	2.6	1.7	0.95	1.2
6A	2.2	3.1	3.1	2.9	2.7	2.9						
7A	1.7	1.8	1.7	1.7	2.3	2.5						
MEAN	1.8	1.6	1.7	2.0	2.6	3.2	2.3	2.1	1.9	1.5	1.2	1.0
± S.E.	0.21	0.34	0.29	0.21	0.43	0.79	0.36	0.30	0.25	0.26	0.23	0.18

A = ACTH

Underlined values refer to point where maximal increase in output occurred.

after perfusion.

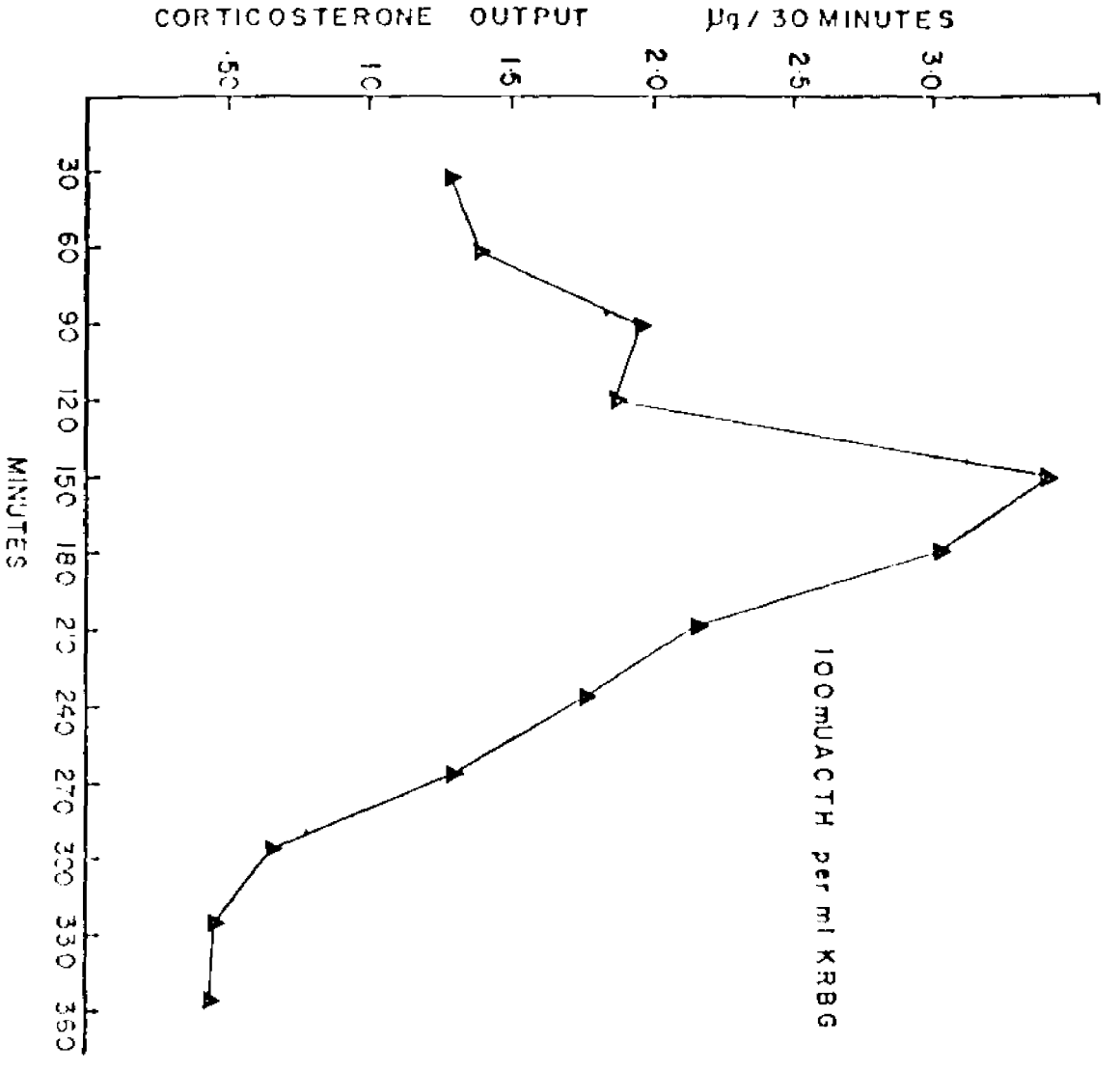
Figure 17 (experiment 3A) illustrates another type of response to continuous perfusion with ACTH. In this experiment a high initial output of B (2.7 µg B during the first hour) increased to a maximal value (6.6 µg/hour) during the third hour of perfusion. These high values declined during the next 3 hours reaching low levels at the end of 6 hours of perfusion.

The data showing the response to continuous perfusion with ACTH (100 mU/ml KRBG) of 7 adrenal glands are summarized in Table V. With the exception of experiments 6A and 7A (3 hour perfusions with ACTH) all the glands were exposed to ACTH for periods of 6 hours. When these experiments are taken together, mean outputs of 1.8 ($\pm$ 0.21 S.E.) µg B during the first 30 minutes increases to 3.2 ($\pm$ 0.80 S.E.) µg after 3 hours of perfusion with ACTH. By the end of 6 hours of perfusion these values have declined to 1.1 ($\pm$ 0.18 S.E.) µg B. Control (KRBG-perfused) adrenals (Pattern I plus II) had mean outputs of 1.8 ($\pm$ 0.25 S.E.) µg B during the first 30 minutes; this declined to 1.0 ($\pm$ 0.25 S.E.) µg B after 3 hours and to 0.62 ($\pm$ 0.20 S.E.) µg after 6 hours of perfusion.

A decline in B output from adrenal glands continuously exposed to ACTH has been observed by other workers. Rat adrenals continuously superfused with ACTH showed a decline in B output during the later time periods of superfusion (Schulster et al, 1970). In vivo perfused dog adrenals showed a maximum rate of cortisol secretion followed by a subsequent decline in response to continuous ACTH perfusion (Urquhart and Li, 1968).

ACTH (100 mU/ml KRBG) had no obvious effect on the flow rate

Figure 17. Time course for corticosterone output for an adrenal gland (experiment 3 A) perfused with ACTH (100 mU/ml KRBG) for 6 hours.


CORTICOSTERONE OUTPUT µg / 30 MINUTES
·50
1·0
1·5
2·0
2·5
3·0
30
60
90
120
150
180
210
240
270
300
330
350
MINUTES
100mUACTH per ml KRBG

which remained constant in all of these experiments. There was no difference in the magnitude of flow between adrenals perfused with ACTH and those perfused with KRBG.

Comparisons of total B output for the first 3 hours of perfusion were made between KRBG-perfused adrenals from intact and hypophysectomized rats and ACTH-perfused adrenals from intact rats (Table VI). The mean B output of adrenals from intact rats was observed to be 7.2 ($\pm$ 1.4 S.E.) µg B/3 hours when perfused with KRBG, this increased to 12.87 ($\pm$ 1.4 S.E.) when ACTH (100 mU/ml KRBG) was added to the perfusing medium ($p = < 0.05$). The output was decreased as a result of hypophysectomy to 2.23 ($\pm$ 0.46 S.E.) µg B/3 hours. These data are summarized and compared in Table VI and in figure 18.

B. Continuous perfusion with ACTH (100 mU/ml KRBG) after a 120 minute washout with KRBG

Adrenal glands were also exposed to a constant amount of ACTH (100 mU/ml KRBG) after being perfused for 120 minutes with KRBG. It was hoped that exposure of the gland to unsupplemented KRBG for 120 minutes would "washout" any residual ACTH or any ACTH effect which might have persisted from in vivo exposure to the trophic hormone.

Figures 19 and 20 illustrate this type of perfusion experiment, i.e. perfusion with KRBG for 2 hours followed by exposure to a constant amount of ACTH (100 mU/ml KRBG) for the remaining 4 hours of perfusion. In experiment 8A (figure 19) a transient increase in B output was observed after 30 minutes of perfusion with KRBG. Difficulty had been encountered in inserting the venous cannula into the renal vein in this particular experiment. This probably resulted in the gland being inadequately perfused during collection of the first

TABLE VI. TOTAL 3 HOUR CORTICOSTERONE OUTPUT FROM PERFUSED RAT ADRENAL GLANDS: A COMPARISON BETWEEN KRBG-PERFUSED ADRENALS FROM INTACT AND HYPOPHYSECTOMIZED RATS AND ACTH-PERFUSED ADRENALS FROM INTACT RATS.

TOTAL 3 HOUR OUTPUT

μg CORTICOSTERONE/3 HOURS

EXPT.		EXPT.		EXPT.	
1C	3.35	1Hx	3.05	1A	12.20
2C	6.30	2Hx	1.39	2A	6.45
3C	3.64	3Hx	0.88	3A	13.20
4C	4.88	4Hx	2.66	4A	11.90
5C	4.96	5Hx	3.18	5A	17.80
6C	3.15			6A	16.90
7C	2.02			7A	11.70
8C	4.73				
9C	6.73				
10C	4.85				
11C	3.37				
12C	21.00				
13C	10.60				
14C	5.48				
15C	7.70				
16C	7.60				
17C	22.60				
MEAN OUTPUT (±S.E.)	7.23(±1.4)		2.23(±0.46)		12.87(±1.4)*

C = control (KRBG - perfused)
Hx = hypophysectomized (KRBG-perfused)
A = ACTH (100 mU/ml KRBG)

* significantly different from KRBG-controls ($P < 0.05$)

Figure 18. Data from Table VI depicted as a scattergram. Total 3 hour corticosterone output from perfused rat adrenal glands: A comparison between KRBG-perfused adrenals from intact and hypophysectomized rats and ACTH-perfused adrenals from intact rats.

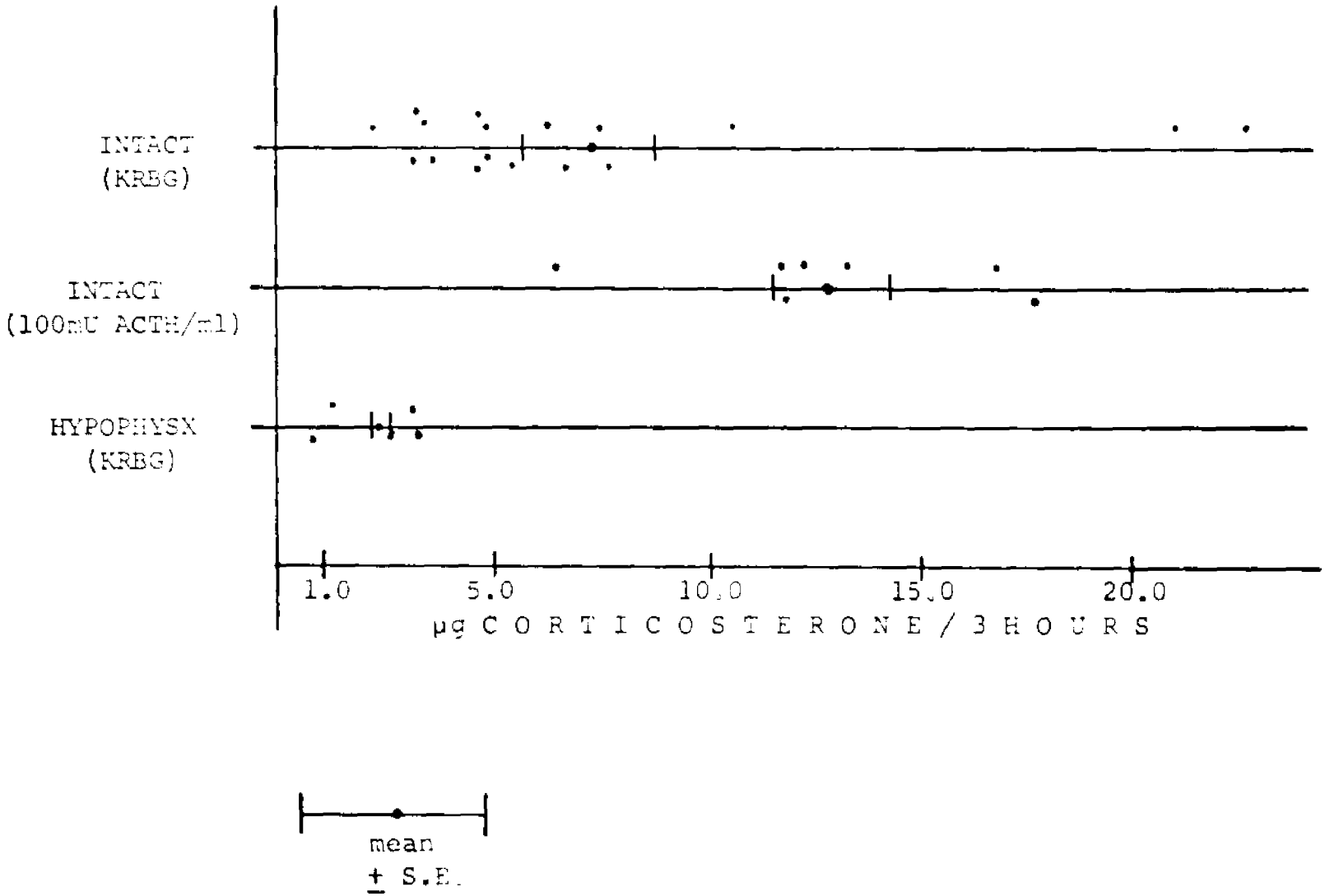

INTACT
(KRBG)
INTACT
(100mU ACTH/ml)
HYPOPHYSX
(KRBG)
1.0
5.0
10.0
15.0
20.0
µg C O R T I C O S T E R O N E / 3 H O U R S
mean
± S.E.

Figure 19. Time courses of corticosterone output and flow rate for an adrenal gland (experiment 8 A) perfused for 2 hours with KRBG followed by 4 hours with ACTH (100 mU/ml KRBG).

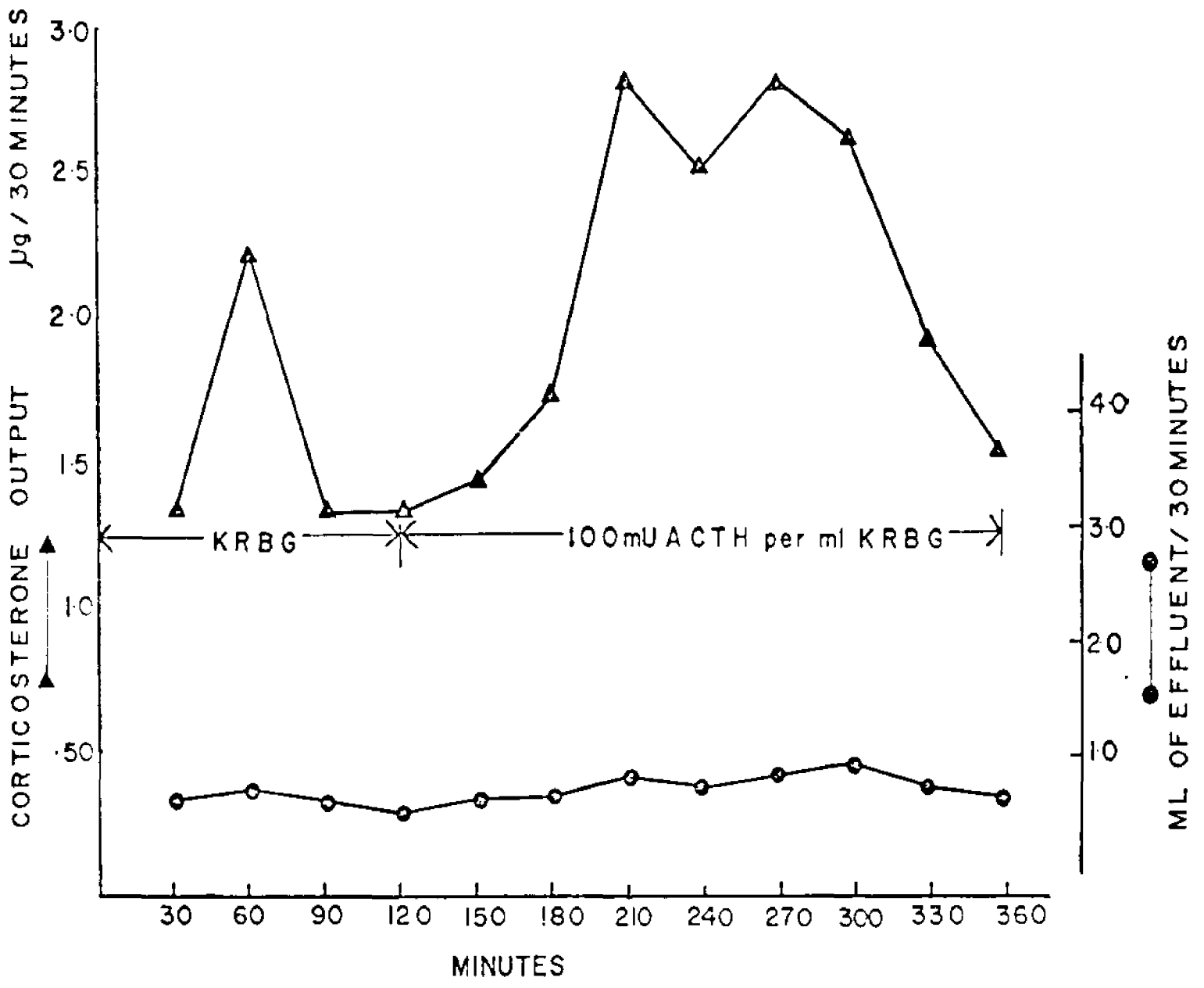
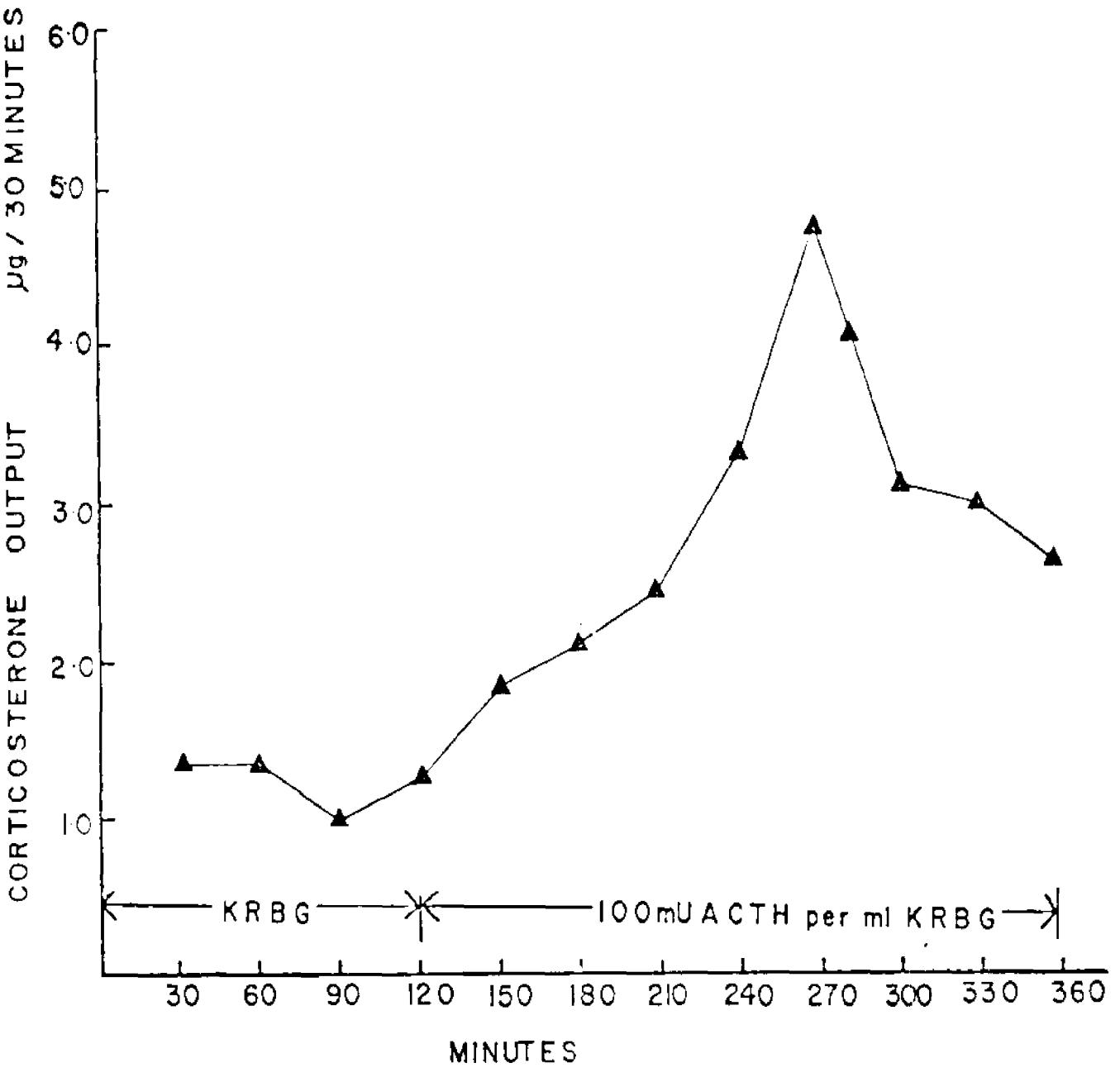

CORTICOSTERONE OUTPUT µg / 30 MINUTES
3·0
2·5
2·0
1·5
1·0
·50
KRBG
100mU ACTH per ml KRBG
ML OF EFFLUENT/ 30 MINUTES
40
30
20
10
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES

Figure 20. Time course of corticosterone output for an adrenal (experiment 9 A) perfused for 2 hours with KRBG followed by 4 hours with ACTH (100 mU/ml KRBG).


CORTICOSTERONE OUTPUT µg / 30 MINUTES
6·0
5·0
4·0
3·0
2·0
1·0
KRBG
100mU ACTH per ml KRBG
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES

30 minute sample of venous effluent. Hence, the second 30 minute sample would be more representative of the initial level of B output in this perfusion. In any case, the gland responded to the addition of ACTH with an almost immediate increase in B output. The maximal response was reached 90 minutes after addition of ACTH and was maintained at this level for another 90 minutes before starting to decline.

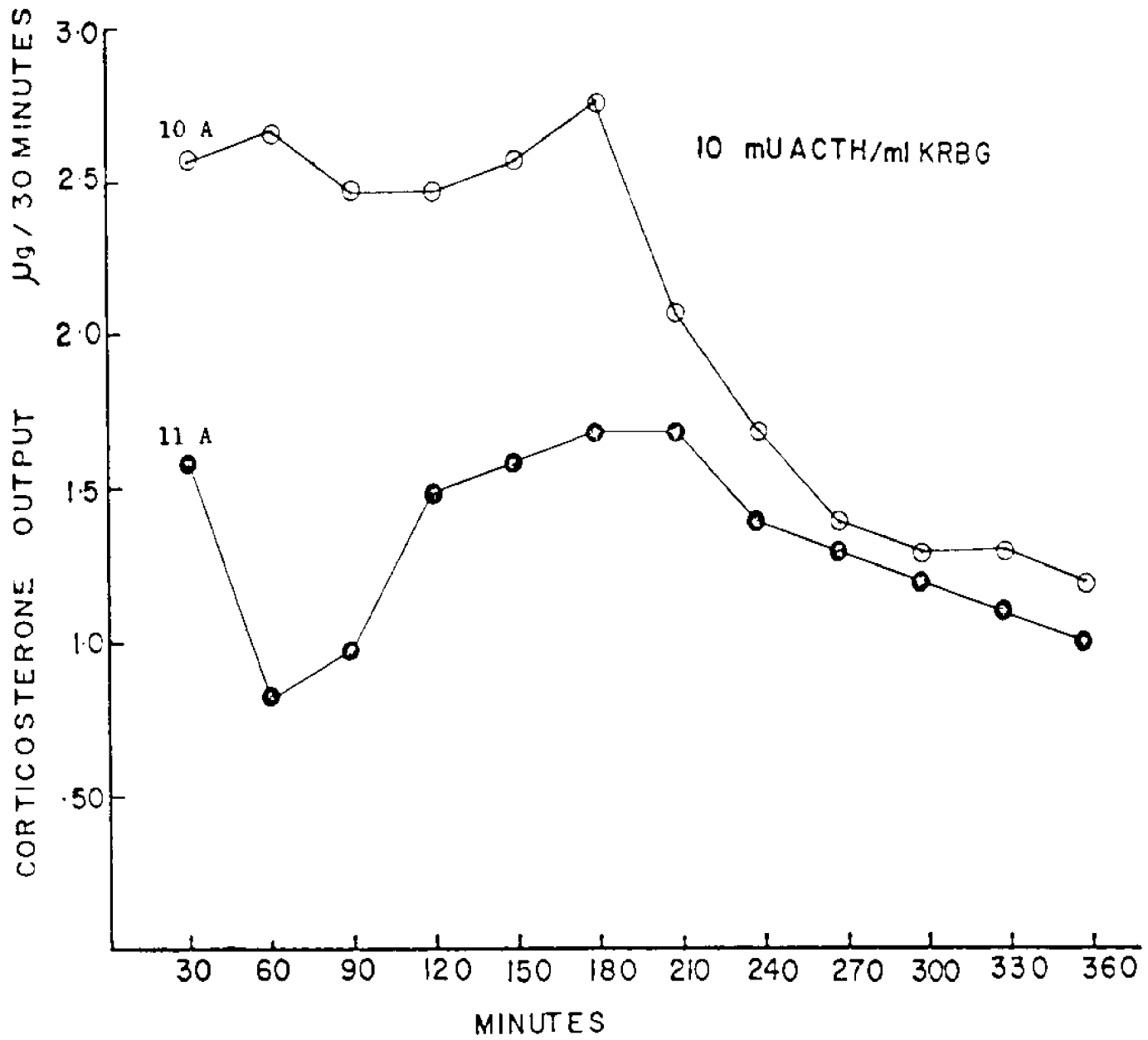
Figure 20 (experiment 9A) illustrates the same type of experiment but shows a different response to ACTH. This adrenal also responded immediately to ACTH with an increase in B output but here it took 150 minutes to reach a maximal level after the addition of ACTH. This level once reached was not maintained but began to decline immediately.

C. Continuous perfusion with ACTH: 10 mU ACTH / ml KRBG

In order to determine whether the isolated perfused adrenal would respond to levels of ACTH lower than 100 mU, perfusions were carried out with 10 mU ACTH/ml KRBG. Adrenal glands from six animals were continuously perfused for 6 hours with this concentration of ACTH (10 mU/ml KRBG). Only two responded to ACTH. The remaining 4 adrenals showed levels of B output which declined with time and reached low levels at the end of 6 hours of perfusion. The pattern of response of these 4 adrenals was the same as the pattern I response to unsupplemented buffer as described in Section IIA.

Figure 21 illustrates the time course of B output for the two adrenal glands that did respond to continuous perfusion with 10 mU ACTH. Both adrenals maintained a high level of B output. In experiment 10A a high initial level of B was maintained for 3 hours followed

Figure 21. Time courses for corticosterone output for adrenal glands of 2 rats (experiments 10A and 11A) perfused with ACTH (10 mU/ml KRBG) for 6 hours.


CORTICOSTERONE OUTPUT µg / 30 MINUTES
3·0
2·5
2·0
1·5
1·0
·50
10 A
11 A
10 mU ACTH/ml KRBG
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES

by a decline. In 11A, an initial decline during the first hour was followed by an increase to a higher level of output. Relatively high levels were then maintained for about 2 hours followed by a decline. The response of the 2 adrenals to 10 mU of ACTH was similar to the pattern of response observed in adrenals exposed to 100 mU ACTH (see figure 16).

D. Continuous perfusion with ACTH (100 mU/ml KRBG) plus amino-glutethimide (100 µg/ml KRBG)

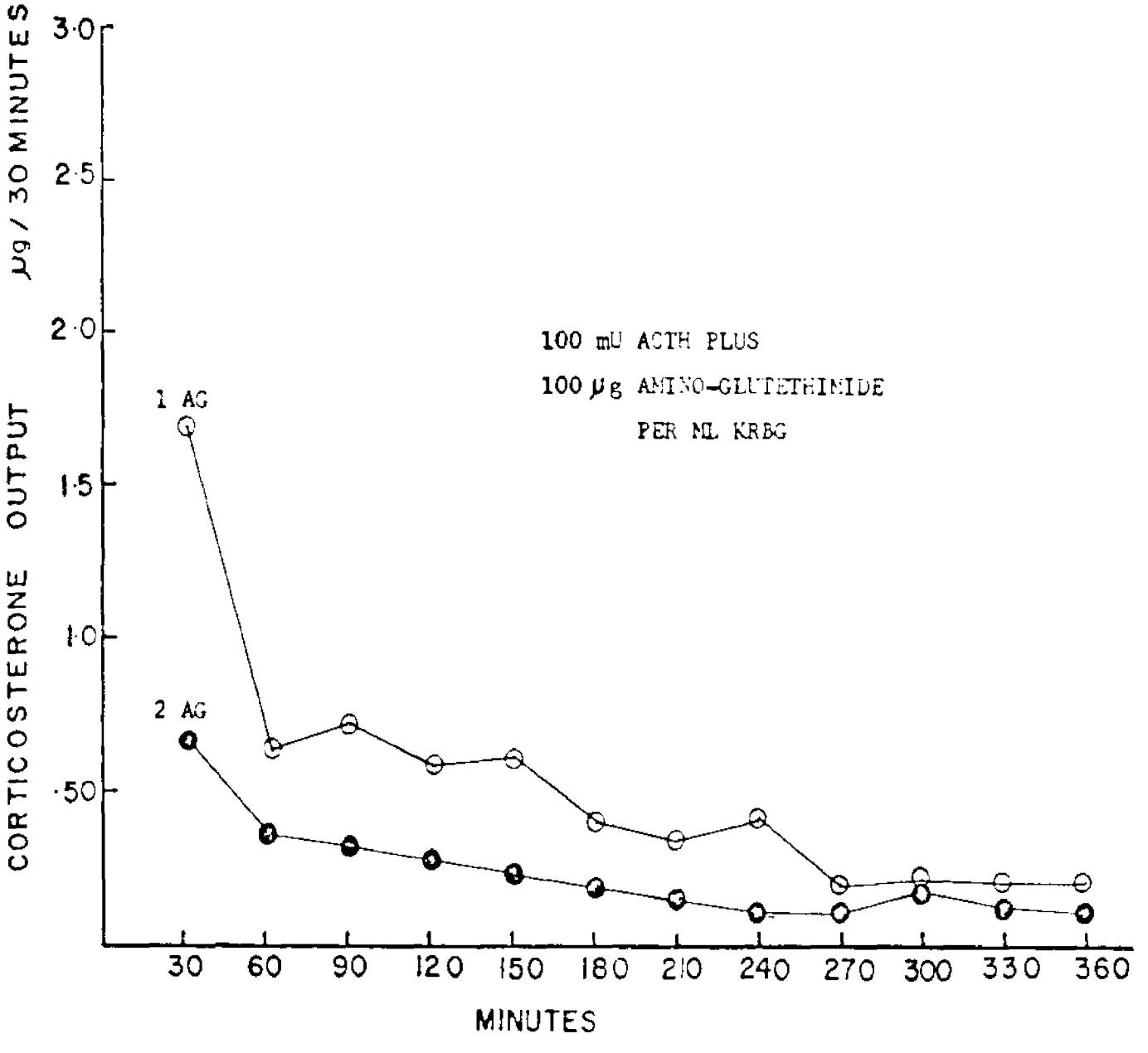
Amino-glutethimide (α-ethyl-α-p-amino-phenyl-glutarimide) is a compound originally used clinically as an anticonvulsant and later shown to inhibit adrenal corticosteroid biosynthesis both in vivo and in vitro (Dexter et al, 1967). Amino-glutethimide (AG) has been shown to inhibit adrenal steroid biosynthesis in several different types of biological preparations: (1) in rats, both in vivo and in incubated adrenal quarters (Dexter et al, 1967); (2) in dogs, in vivo after a single intravenous injection of AG (Wilroy et al, 1968) and (3) in functional monolayer cultures of adrenal cortex tumors exposed to AG (Kowal, 1969b). Dexter and co-workers have shown that the inhibitor does not affect the conversion of exogenous pregnenolone, progesterone or deoxycorticosterone to corticosterone in rat adrenal secretions incubated in vitro. This suggests that the inhibitory effect of AG on adrenal corticosterone biosynthesis occurs prior to the formation of pregnenolone. The data of Wilroy et al (1968) and those of Kowal (1969b) agree with the view that AG interferes with the conversion of cholesterol to pregnenolone. The response of intravenous injection of AG has been shown to be rapid. In dogs, a rapid decrease in corticosteroid secretion has been observed 20 minutes after injection

of AG (Wilroy et al, 1968).

The effect of AG on the ACTH-stimulated perfused rat adrenal was studied in two experiments. The adrenals were perfused for 6 hours with KRBG containing 100 mU ACTH plus 100 μg AG per ml. The two experiments are illustrated in figure 22. AG abolished the response to constant perfusion with ACTH. Neither maintenance of a high level of B output nor any effect which could be attributed to ACTH was observed. In experiment 1AG (figure 22, open circles) a relatively high initial level of B was observed to decline rapidly during the first hour of perfusion, followed by a slower rate of decline over the next 3 hours. A basal level of B output was reached at the end of 270 minutes of perfusion and was maintained until the experiment was terminated. This result of perfusion with ACTH + AG is similar to the pattern I type of response observed in control (KRBG) perfusions (see Results, Section IIA). In experiment 1AG total B output during the first 3 hours of perfusion was 4.58 μg B, which compares well with total 3 hour outputs observed in the pattern I type of response (see Table VI, experiments 1C to 9C).

The response of the adrenal gland in experiment 2AG (figure 22, closed circles) was similar to that of the adrenal in 1AG, except that the initial and overall outputs of B were lower. Although, in both of the perfusions (experiments 1AG and 2AG) the flow rate (measured in ml effluent/30 minutes) remained constant for the 6 hour period, it was approximately 25 to 50% higher than that observed in control or ACTH-stimulated adrenals. This suggests that AG or AG in combination with ACTH may exert a vasodilatory effect.

Figure 22. Time courses for corticosterone output for 2 adrenal glands (experiments 1 AG and 2 AG) perfused with ACTH (100 mU/ml KRBG) plus aminoglutethimide (100 µg/ml KRBG).


CORTICOSTERONE OUTPUT µg / 30 MINUTES
3·0
2·5
2·0
1·5
1·0
·50
100 mU ACTH PLUS
100 µg AMINO-GLUTETHIMIDE
PER ML KRBG
1 AG
2 AG
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES

V. The effect of adding dextran to the perfusion medium (5% dextran in KRBG)

A problem frequently encountered when one attempts to perfuse an isolated organ either with blood or with isotonic salt solutions is the formation of edema fluid (lymph) in the perfused organ. The problem becomes more acute when the perfusate consists of only an isotonic salt solution which lacks the cellular and protein components of blood. This creates an osmotic imbalance between the intercellular fluids and the perfusate flowing though the organ. Under such conditions, and as a result of the osmotic imbalance, fluid that would normally leak out of capillaries and into the tissue spaces (lymph) is not able to return to the circulation and hence accumulates in the tissue spaces. Accumulation of fluid in the tissue spaces can cause an increase in interstitial tissue pressure leading to vasoconstriction, blockage of flow and deterioration of the physiological condition of the organ. A perfused organ which becomes edematous cannot be expected to function "normally".

The problem of edema formation was encountered in the isolated KRBG-perfused rat adrenal gland. Edema, or swelling of a single adrenal gland, could not be observed during the course of a perfusion experiment, because of the small size of the gland. As the experiment progressed tissue fluids (edema) accumulated within the perirenal fat surrounding the gland. The edema was first evident after about 2 hours of perfusion; it became progressively worse with increasing time of perfusion. This observation, plus the fact that adrenal glands weighed more after being perfused for 6 hours than adrenals removed from rats of the same size immediately after sacrifice, indicated that perfusate

was also accumulating within the gland and perhaps influencing its function.

An attempt was made to reduce the edema by adding dextran (M.W. 60 - 90,000) to the perfusing medium. The dextran was used as an osmotic substitute for the protein components normally found in blood. Adrenal glands of 4 rats were perfused with a 5% solution of dextran in KRBG (control experiments).

Figure 23 illustrates a typical perfusion experiment carried out for 6 hours with KRBG-containing 5% dextran. Corticosterone output was observed to be high at the start of the perfusion and to reach low levels at the end of 6 hours. This response has been previously described as the pattern I type of response observed in control (KRBG) perfusions (see Results and Discussion of Results, Section IIA) i.e., the initial output of B decreases with time. The data for the 4 adrenals perfused with KRBG-containing 5% dextran are summarized in Table VII.

Perfusion with dextran appeared to eliminate or diminish the spontaneous transient increases in B output that had previously been described as the pattern II type of response in control (KRBG) perfusions (see Results and Discussion of Results, Section IIB). Addition of dextran to the perfusing medium had no effect on flow rate.

VI. Perfusion with indomethacin

A. Rationale for studying the effects of indomethacin on the perfused rat adrenal gland

A major problem which prevailed throughout both the development and utilization of the perfusion technique was that of finding an explanation for the transient spontaneous increases in corticosterone

Figure 23. Time course of corticosterone output for an adrenal gland (experiment 1 D) perfused with KRBG-containing 5% dextran.

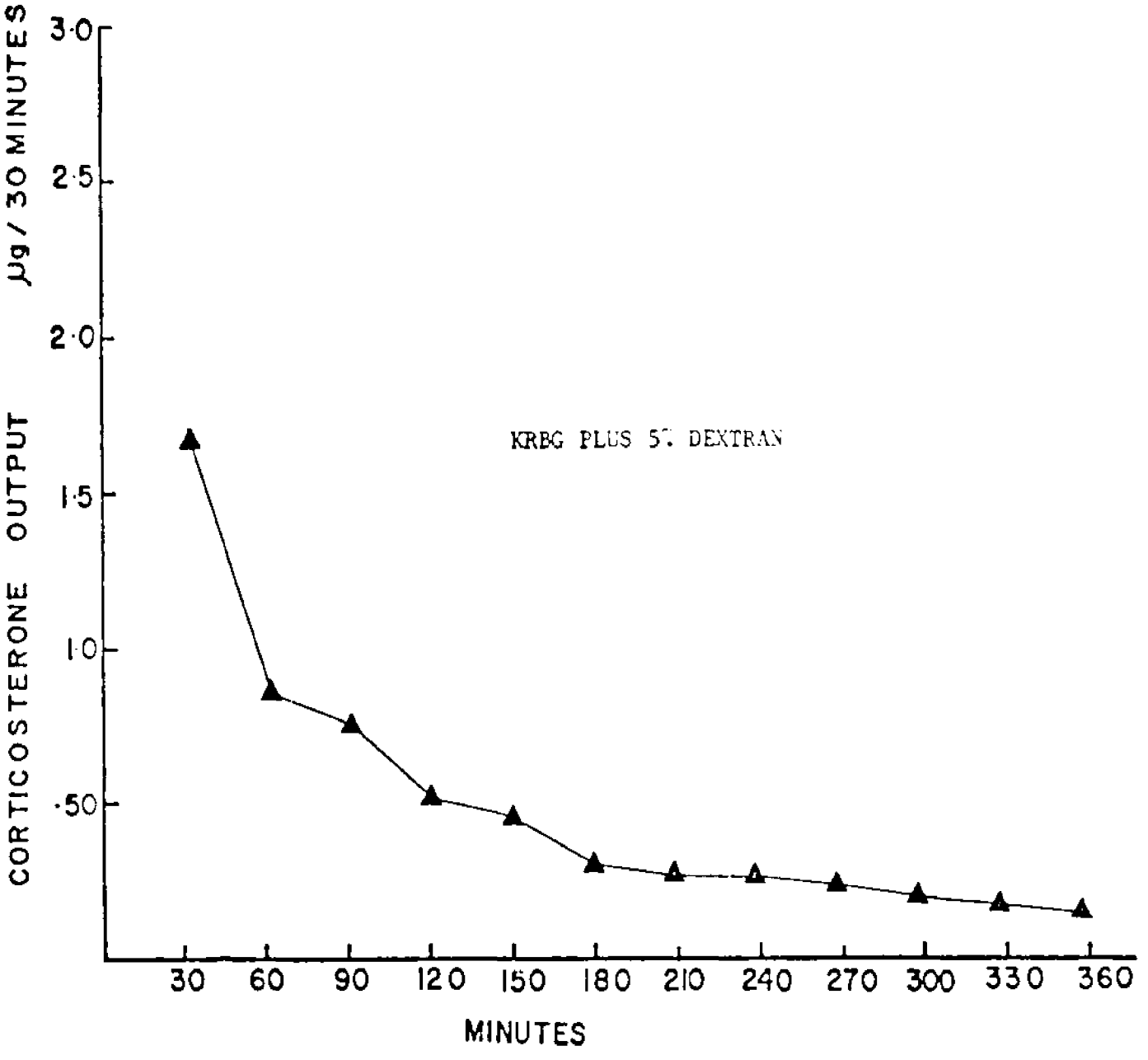

CORTICOSTERONE OUTPUT µg / 30 MINUTES
3·0
2·5
2·0
1·5
1·0
·50
KREBG PLUS 5% DEXTRAN
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES

TABLE VII. CORTICOSTERONE OUTPUT FROM RAT ADRENAL GLANDS PERFUSED WITH KRBG-CONTAINING 5% DEXTRAN.

Minutes after start of perfusion	μg CORTICOSTERONE/30 MINUTE SAMPLE											
	0 - 30	30 - 60	60 - 90	90 - 120	120 - 150	150 - 180	180 - 210	210 - 240	240 - 270	270 - 300	300 - 330	330 - 360
Experiment												
1D	1.7	0.85	0.75	0.50	0.44	0.28	0.24	0.24	0.21	0.17	0.15	0.11
2D	1.4	1.2	0.50	0.59	0.40	0.36	0.35	0.14	0.25	0.18	0.18	0.14
3D	1.2	0.74	0.48	0.40	0.29	0.54	0.63	0.37	0.22	0.11	0.08	0.06
4D	1.8	1.4	1.0	0.82	0.60	0.62	0.71	1.2	0.91	0.57	0.65	0.49
MEAN	1.5	1.05	0.68	0.58	0.43	0.45	0.48	0.49	0.39	0.26	0.26	0.20
± S.E.	0.13	0.15	0.12	0.08	0.06	0.07	0.11	0.24	0.17	0.10	0.13	0.09

(B) output (Pattern II type of response) observed in control perfusions (see Results and Discussion of Results, IIB).

Several studies by Flack and co-workers led to the suggestion that the transient increase in B output might represent a steroidogenic response of the adrenal to endogenous prostaglandin biosynthesis. These workers have reported (1) the presence of several prostaglandins in rat adrenal glands (Shaw and Ramwell, 1967; Ramwell and Shaw, 1970), (2) the release of prostaglandins from rat adrenals upon stimulation (Ramwell and Shaw, 1970), (3) the stimulation by prostaglandins of corticosterone synthesis in superfused rat adrenal glands (Flack et al, 1969) and (4) that prostaglandin E_2 causes a transient stimulation of corticosterone synthesis in superfused rat adrenals, while ACTH maintains steroid secretion for several hours (Flack and Ramwell, 1972). As pointed out before (Results and Discussion of Results, Section IIA and C), ACTH maintains a high level of B output for several hours in the perfused rat adrenal. These observations, together with reports that some organs can release prostaglandins in response to mechanical stimuli such as perfusion and gentle massage (Piper and Vane, 1971), led to the hypothesis that mechanical stimulation resulting from surgical manipilation and/or the perfusion itself was promoting endogenous adrenal prostaglandin biosynthesis, and transiently stimulating steroidogenesis.

B. Indomethacin inhibits prostaglandin biosynthesis in several biological preparations

Indomethacin (1-(p-chlorobenzoyl)5-methoxy-2-methyl-indole-3-acetic acid), a potent anti-inflammatory drug, has been shown to inhibit the biosynthesis of prostaglandins in several biological

preparations. Vane (1971) reported that the synthesis of prostaglandins E_2 and $F_{2\alpha}$ from arachidonic acid by cell-free homogenates of guinea pig lung can be blocked by indomethacin. The adrenaline-induced synthesis of prostaglandins E_2 and $F_{2\alpha}$ in the isolated perfused dog spleen was inhibited by infusion of indomethacin (Ferreira et al, 1971).

Smith and Willis (1971) have shown that a suspension of human platelets will produce prostaglandins when incubated with thrombin. The platelets were unable to produce prostaglandins in response to thrombin when indomethacin was added to the incubating medium, or when the platelets were withdrawn from volunteers who had ingested indomethacin one hour before (Smith and Willis, 1971).

Smith and Lands (1971) have studied in detail a prostaglandin-synthesizing system isolated from sheep vesicular glands. They reported that the enzymic system is inhibited by indomethacin in a time-dependent, concentration-dependent manner. The drug irreversibly blocked the full activity of the synthetase system. These studies taken together leave little doubt that indomethacin can block prostaglandin synthesis both in vivo and in vitro.

C. The effect of adding indomethacin (5 µg/ml KRBG) to the perfusion medium

In these studies adrenal glands were continuously perfused with 5 µg indomethacin/ml KRBG for periods of 6 hours. The decision to use this concentration of indomethacin was based on the following considerations: (1) to inhibit prostaglandin release from the isolated perfused dog spleen, Ferreira et al (1971) used concentrations of 0.37 to 4 µg indomethacin per ml of perfusate. Perfusion with 4 µg

indomethacin/ml for 15 minutes completely abolished prostaglandin output. (2) In man, therapeutic doses of indomethacin produce plasma concentrations of between 2 and 10 μg/ml (Personal Communication from Dr. C.R. Stevenson, Merck Sharp and Dohme).

Figure 24 illustrates two separate perfusion experiments in which the adrenals were exposed to indomethacin (5 μg/ml KRBG) for 6 hours. In experiment 1I (closed circles) a high initial output of B was observed to decay with time (Pattern I type of control response). No transient increase in B output was observed. The adrenal in experiment 2I (open circles) showed essentially the same picture except that slight increases in B output were observed during the second and third hours of perfusion, the output then decayed with time.

Table VIII summarizes the B output data for 9 adrenal glands perfused with indomethacin (5 μg/ml KRBG) for periods of 6 hours. Four of the adrenals (experiments 1I, 4I, 5I and 6I) showed no transient increase in B output, only a decay with time. Three adrenals (experiments 2I, 3I and 7I) showed slight increases in B output but the overall 6 hour picture was one of decreasing output. The two remaining adrenals (experiments 8I and 9I) showed definite spontaneous increases in B output which were comparable to the transient increases observed in the pattern II type of control response. Perfusions with higher concentrations of indomethacin were not carried out. Hence, it is not known whether higher levels of the drug might have completely eliminated the transient increases.

The mean corticosterone outputs for each 30 minute collection period for the 9 perfusions with indomethacin are included in Table VIII. The mean outputs for the KRBG control perfusions

Figure 24. Time courses for corticosterone output for 2 adrenal glands (experiments 1 I and 2 I) perfused with indomethacin (5 µg/ml KRBG) for 6 hours.

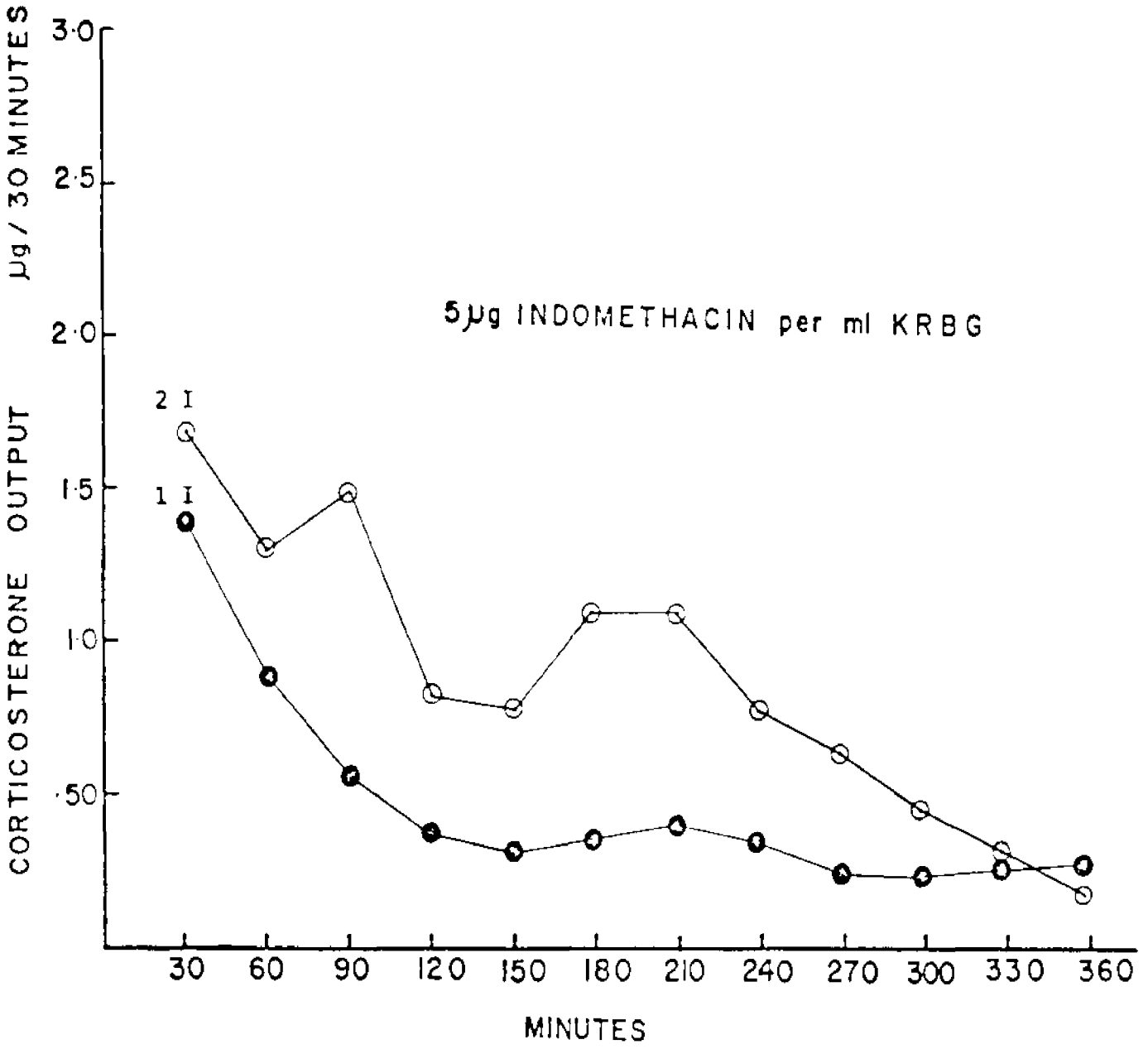

CORTICOSTERONE OUTPUT µg / 30 MINUTES
3·0
2·5
2·0
1·5
1·0
·50
5µg INDOMETHACIN per ml KRBG
2 I
1 I
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES

TABLE VIII. CORTICOSTERONE OUTPUT FROM RAT ADRENAL GLANDS PERFUSED WITH INDOMETHACIN (5 µg/ml KRBG).

µg CORTICOSTERONE/30 MINUTE SAMPLE

Minutes after start of perfusion	0 - 30	30 - 60	60 - 90	90 - 120	120 - 150	150 - 180	180 - 210	210 - 240	240 - 270	270 - 300	300 - 330	330 - 360
Experiment												
1 I	1.4	0.88	0.55	0.36	0.30	0.34	0.39	0.33	0.23	0.22	0.30	0.26
2 I	1.7	1.3	1.5	0.82	0.77	1.1	1.1	0.77	0.63	0.44	0.24	0.16
3 I	1.6	1.9	1.4	1.5	1.4	1.3	1.1	0.97	0.66	0.33	0.25	0.14
4 I	2.4	2.1	0.99	0.62	0.33	0.26	0.39	0.32	0.23	0.20	0.18	0.11
5 I	1.8	0.49	0.35	0.38	0.48	0.34	0.25	0.17	0.15	0.11	0.08	0.09
6 I	2.0	1.8	1.0	0.62	0.53	0.49	0.35	0.34	0.32	0.27	0.17	0.17
7 I	0.90	0.61	0.72	0.72	0.68	0.51	0.48	0.70	0.63	0.41	0.28	0.25
8 I	2.1	1.3	0.82	1.1	1.8	2.1	1.8	1.3	0.74	0.55	0.38	0.20
9 I	0.56	0.50	0.86	0.90	0.82	1.1	0.54	0.22	0.11	0.12	0.04	0.03
MEAN	1.6	1.2	0.91	0.78	0.79	0.84	0.71	0.57	0.41	0.29	0.21	0.16
± S.E.	0.19	0.21	0.12	0.12	0.17	0.20	0.17	0.13	0.08	0.05	0.03	0.02
MEAN (I)	1.5	0.82	0.65	0.59	0.53	0.39	0.39	0.35	0.31	0.27	0.18	0.14
MEAN (II)	2.0	1.6	1.5	2.0	1.9	1.7	1.6	1.6	1.5	1.4	1.2	0.89

(Patterns I and II) are also included in the table for comparison purposes. When the 9 perfusions with indomethacin taken together are compared to control (KRBG) perfusions, their response appears to be similar to the pattern I type of response, i.e., the initial output of corticosterone decreases with time.

In general, addition of indomethacin to the perfusing medium at the dose level employed (5 μg/ml KRBG) eliminated or greatly diminished the transient increase in B output. Indomethacin had no effect on the flow rate which remained constant and within an acceptable range in all these perfusions.

VII. Response to prostaglandins

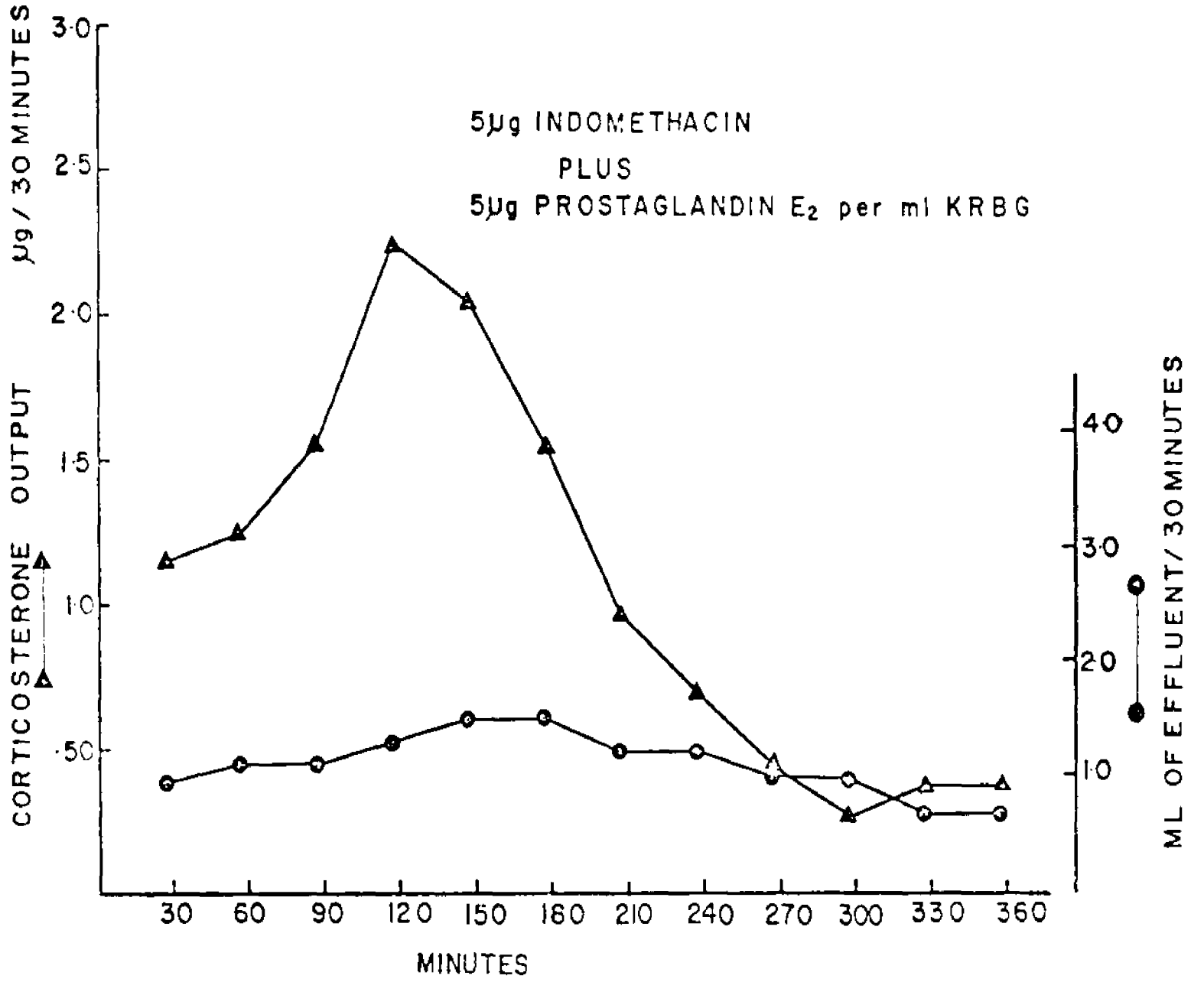
A. Prostaglandin E_2

(1) Continuous perfusion with 5 μg prostaglandin E_2 plus 5 μg indomethacin per ml KRBG

The effects of several prostaglandins on corticosterone (B) output were studied by perfusing adrenal glands with indomethacin-containing KRBG solutions (5 μg indomethacin/ml KRBG) to which the prostaglandins were added.

The steroidogenic response to prostaglandin E_2 (PGE_2) was studied in 5 adrenal glands which were continuously perfused with 5 μg PGE_2 plus 5 μg indomethacin per ml KRBG for periods of 6 hours. Figure 25 (experiment II) illustrates such a perfusion. The initial output of B increased to a maximal level after 2 hours of perfusion. A fairly rapid decline occurred over the next 3 hours, reaching a low level at the end of 5 hours of perfusion. There appeared to be no time lag in response of the adrenal gland to PGE_2, i.e. the increase in B output was immediate. Adrenal flow rate remained constant

Figure 25. Time courses for corticosterone output and flow rate for an adrenal gland (experiment 1 P) continuously perfused with 5 μg prostaglandin E_2 plus 5 μg indomethacin per ml KRBG.


5µg INDOMETHACIN
PLUS
5µg PROSTAGLANDIN E2 per ml KRBG
CORTICOSTERONE OUTPUT µg / 30 MINUTES
3·0
2·5
2·0
1·5
1·0
·50
ML OF EFFLUENT/ 30 MINUTES
40
30
20
10
30 60 90 120 150 180 210 240 270 300 330 360
MINUTES

during the 6 hours of perfusion and had no obvious relationship to B output.

Figure 26 (experiment 4P) shows a somewhat different response to continuous perfusion with PGE_2. Corticosterone output rose immediately and reached a maximal level after 90 minutes of perfusion. This level was maintained for one hour after which it slowly declined with time. In this perfusion (experiment 4P) higher levels of B output were observed than in experiment 1P. High levels of B were also maintained for a longer period of time in this perfusion than in experiment 1P (figure 25). No relationship between the flow rate, which remained constant, and B output was observed (figure 26, experiment 4P).

The data showing B output per 30 minute sample for the 5 adrenal glands continuously exposed to PGE_2 are summarized in Table IX. Three of the adrenals responded to PGE_2 with a transient rise in output followed by a fairly rapid decline (experiments 1P, 2P and to a lesser extent 5P). The other 2 adrenals responded to PGE_2 with an initial stimulation of B output; the resulting higher level of B was maintained for a short period of time followed by a slow decline (experiments 3P and 4P).

Although neither qualitative nor quantitative responses to perfusion with PGE_2 were exactly the same for each adrenal gland, some general observations can be made concerning the influences of PGE_2. (1) Relatively high levels of B output were observed in response to PGE_2. Mean outputs of 2.0 ($\pm$ 0.45 S.E.) µg B during the first 30 minutes increased to 3.2 ($\pm$ 1.9 S.E.) µg after 3 hours of perfusion with PGE_2. By the end of 6 hours of perfusion, B output

Figure 26. Time courses for corticosterone output and flow rate for an adrenal gland (experiment 4 P) continuously perfused with 5 µg prostaglandin E_2 plus 5 µg indomethacin per ml KRBG.

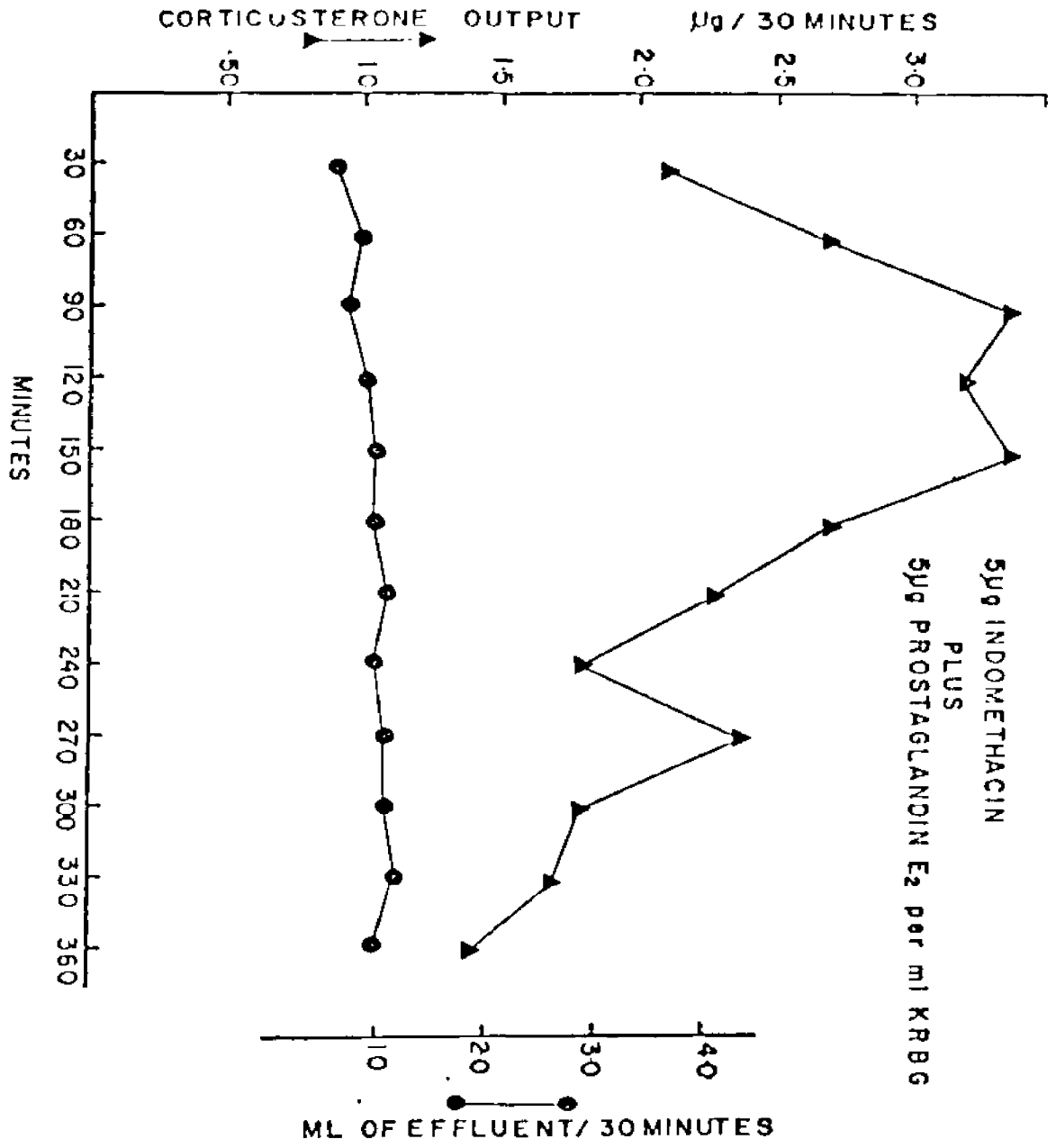

CORTICOSTERONE OUTPUT
µg / 30 MINUTES
·50
1·0
1·5
2·0
2·5
3·0
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES
5µg INDOMETHACIN
PLUS
5µg PROSTAGLANDIN E2 per ml KRBG
10
20
30
40
ML OF EFFLUENT/ 30 MINUTES

TABLE IX. CORTICOSTERONE OUTPUT FROM RAT ADRENAL GLANDS PERFUSED WITH PROSTAGLANDIN E_2 (5 µg/ml KRBG) PLUS INDOMETHACIN (5 µg/ml KRBG) FOR 6 HOURS.

µg CORTICOSTERONE/30 MINUTE SAMPLE

Minutes after start of perfusion	0 - 30	30 - 60	60 - 90	90 - 120	120 - 150	150 - 180	180 - 210	210 - 240	240 - 270	270 - 300	300 - 330	330 - 360
Experiment												
1 P	1.1	1.2	1.5	2.2	2.0	1.5	0.92	0.65	0.38	0.22	0.31	0.31
2 P	0.93	0.92	2.4	4.1	5.0	6.1	3.4	2.7	1.8	1.4	0.98	0.68
3 P	3.3	3.9	4.8	4.8	3.4	4.1	3.2	3.2	2.9	2.4	2.0	2.4
4 P	2.1	2.7	3.4	3.2	3.4	2.7	2.3	1.8	2.4	1.8	1.7	1.4
5 P	2.6	2.0	2.4	2.7	2.1	1.6	1.3	0.60	0.34	0.27	0.17	0.15

underlined values refer to point where maximal increase in output occurred.

declined to 0.98 (± 0.41 S.E.) µg/30 minutes. Control(indomethacin-perfused) adrenals had mean outputs of 1.6 (± 0.19 S.E.) µg B during the first 30 minutes; this declined to 0.84 (±0.20 S.E.) µg after 3 hours and to 0.16 (± 0.02 S.E.)µg B after 6 hours of perfusion. Comparisons of total B output for the first 3 hours of perfusion were made between indomethacin-perfused adrenals and those perfused with PGE_2 plus indomethacin (Table X). The mean B output of adrenals exposed only to indomethacin was 6.16 (± 0.69 S.E.) µg B/3 hours. Addition of PGE_2 to the indomethacin-containing medium increased the output to 16.83 (± 2.5 S.E.) µg for the 3 hour period. This stimulatory effect of PGE_2 was highly significant ($P < 0.001$) and was found to be even greater than that of ACTH over a 3 hour period (see Table X). Hence, the conclusion that PGE_2 elicits a relatively high rate of B output appears to be justified. (2) The response of the perfused adrenal to PGE_2 appeared to be immediate although in 2 perfusions (experiments 2P and 5P, Table IX) there was a short time lag.(3) When PGE_2 was present in the perfusing medium the high initial output of B (the first 30 minute sample) was always surpassed early in the perfusion, i.e., peak B outputs were always higher than the initial outputs (see Table IX). (4) At the concentration used (5 µg/ml KRBG), PGE_2 stimulation of output occurred with no concomitant change in flow rate, hence there appeared to be no relationship between adrenal flow rate and steroid output. (5) The response to PGE_2 always declined with time of perfusion.

TABLE X. TOTAL 3 HOUR CORTICOSTERONE OUTPUT FROM PERFUSED RAT ADRENAL GLANDS: A COMPARISON BETWEEN PERFUSION WITH KRBG, ACTH, INDOMETHACIN AND INDOMETHACIN PLUS PROSTAGLANDIN E_2

Perfusion Medium	Number of experiments	Total 3 hour output (µg B/3 hours)
KRBG	17	7.23 ($\pm$ 1.4)
ACTH (100 mU/ml)	7	12.87 ($\pm$ 1.4)
INDOMETHACIN (5 µg/ml)	9	6.16 ($\pm$ 0.69)
INDOMETHACIN (5 µg/ml) plus PGE_2 (5 µg/ml)	5	16.83 ($\pm$ 2.5)x

All values represent means ($\pm$ S.E.)

x $p < 0.001$ when compared with perfusion with indomethacin alone.

(2) Continuous perfusion with 10 µg prostaglandin E_2 plus 5 µg indomethacin per ml KRBG

The effect(s) of increasing the concentration of prostaglandin E_2 (PGE_2) to 10 µg/ml of the indomethacin-containing medium was studied in 4 adrenal perfusion experiments. The adrenal response to doubling of the prostaglandin concentration was different in each experiment; no clear pattern of response was evident. This is illustrated by the B output data which are summarized in Table XI. After a 60 minute time lag, the adrenal gland in experiment 6P appeared to respond to PGE_2 by maintaining a high level of B output for the duration of the experiment. In 7P (a four hour perfusion), a 2 hour time lag was followed by a transient increase in output which started to decline after it reached a peak level about one hour later. In experiments 8P and 9P high levels of B output were maintained for the first 90 minutes of perfusion followed by levels which declined for the durations of the experiments.

When the four adrenal glands perfused with 10 µg PGE_2/ml were taken together, a relatively high output of B was observed to occur during the first 3 hours of perfusion. The mean output for the first 30 minutes of perfusion was 2.2 ($\pm$ 0.56 S.E.) µg B. This declined only slightly to 1.7 ($\pm$ 0.36 S.E.) µg/30 minutes at the end of 3 hours of perfusion.

The mean total 3 hour B output for the 4 adrenals perfused with 10 µg PGE_2 plus 5 ug indomethacin/ml KRBG was 10.83 ($\pm$ 1.9 S.E.) µg B. This is less than the mean total 3 hour output of adrenals

TABLE XI. CORTICOSTERONE OUTPUT FROM RAT ADRENAL GLANDS PERFUSED WITH 10 µg PROSTAGLANDIN E_2 PLUS 5 µg INDOMETHACIN PER ml KRBG.

Minutes after start of perfusion	µg CORTICOSTERONE/30 MINUTE SAMPLE											
	0 - 30	30 - 60	60 - 90	90 - 120	120 - 150	150 - 180	180 - 210	210 - 240	240 - 270	270 - 300	300 - 330	330 - 360
Experiment												
6 P	2.8	1.5	2.1	2.1	1.4	1.4	1.6	1.7	1.5	1.4	1.3	1.3
7 P	0.55	0.59	0.62	0.53	0.69	2.6	2.6	1.9	-	-	-	-
8 P	3.0	2.9	3.3	2.1	1.9	1.8	1.4	1.1	0.89	0.53	0.51	0.57
9 P	2.5	2.4	2.3	1.9	1.5	0.87	0.87	0.48	0.35	0.24	0.20	0.23
MEAN	2.2	1.9	2.1	1.7	1.4	1.7	1.6	1.3	0.91	0.72	0.67	0.70
± S.E.	0.56	0.51	0.55	0.38	0.25	0.36	0.36	0.32	0.33	0.35	0.33	0.31

underlined values refer to point where maximal increase in output occurred.

perfused with 5 µg PGE_2 plus 5 µg indomethacin (16.83 ($\pm$ 2.5 S.E.) ug B/3 hours), but still significantly different ($P < 0.02$) from 6.16 ($\pm$ 0.69 S.E.) µg B which was the mean total 3 hour output from adrenals perfused with indomethacin only (see Table X).

Perfusion with 10 µg PGE_2 resulted in irregular (inconstant) adrenal flow rates (measured as ml of effluent collected/30 minutes) which were higher than those observed in most adrenal perfusions. Often a relationship appeared to exist between flow rate and B output i.e., flow rate increased with time of perfusion and was accompanied either by increasing or high levels of B output. This was observed in most adrenal perfusions when the concentration of the prostaglandin used was 10 µg/ml (see data below for PGE_1 and $PGF_{1\alpha}$). In all other perfusions, whenever the adrenal flow rate was observed to increase, a concomitant decrease in B output occurred. This has been interpreted to mean that extra-adrenal areas were being perfused and drained via small veins which empty into the adrenal or renal veins; the net effect was a partially perfused adrenal. This would account for the decrease in B output in face of an increase in flow rate (see Results and Discussion of Results, Section IB). Thus, perfusions which showed either high, irregular or increasing flow rates were accompanied by diminishing levels of B output and for the above reason were discarded. The prostaglandins (PGE_2, PGE_1 and $PGF_{1\alpha}$) at a concentration of 10 µg/ml KRBG were the only agents used in this study which showed stimulation of steroid output which was accompanied by an increase in flow rate.

Three adrenal glands (experiments 6P, 7P and 8P) out of the 4 continuously perfused with 10 µg PGE_2 showed increases in flow rate

with concomitant increases in B output. Figure 27 shows the time courses of B output and flow rate for one of the adrenals (experiment 6P) perfused with 10 μg PGE_2. The flow rate slowly increased from less than 3.0 ml/hour during the second hour of perfusion to over 6.0 ml/hour during the fourth hour. From 60 to 120 minutes after the start of perfusion, the increasing flow rate was accompanied by stimulation of B output. Thereafter, from 120 minutes until the end of the perfusion a high level of B output was maintained.

Two adrenal glands were perfused for 90 minutes with KRBG containing only indomethacin. PGE_2 (10 μg/ml KRBG) was added to the indomethacin-containing medium and the perfusion continued for another 270 minutes. Both adrenals responded to the addition of 10 μg PGE_2 after a short time lag with increases in B output. The stimulation of steroid output in each case was accompanied by an increasing flow rate.

Perfusion of adrenal glands with PGE_2 at a concentration of 10 μg/ml KRBG appears to influence flow rate as well as steroid output.

B. <u>Prostaglandin E_1: continuous perfusion with 10 μg prostaglandin E_1 plus 5 μg indomethacin per ml KRBG after 90 minutes of perfusion with indomethacin (5 μg/ml KRBG)</u>

Three adrenal glands were perfused for 90 minutes with KRBG containing only indomethacin. PGE_1 (10 μg/ml KRBG) was added to the indomethacin-containing medium and the perfusion continued for another 210 minutes. Corticosterone output data for the 3 perfusions are summarized in Table XII. Two adrenals (experiments 10P and 11P)

Figure 27. Time courses for corticosterone output and flow rate for an adrenal gland (experiment 6P) continuously perfused with 10 µg prostaglandin E_2 plus 5 µg indomethacin per ml KRBG.

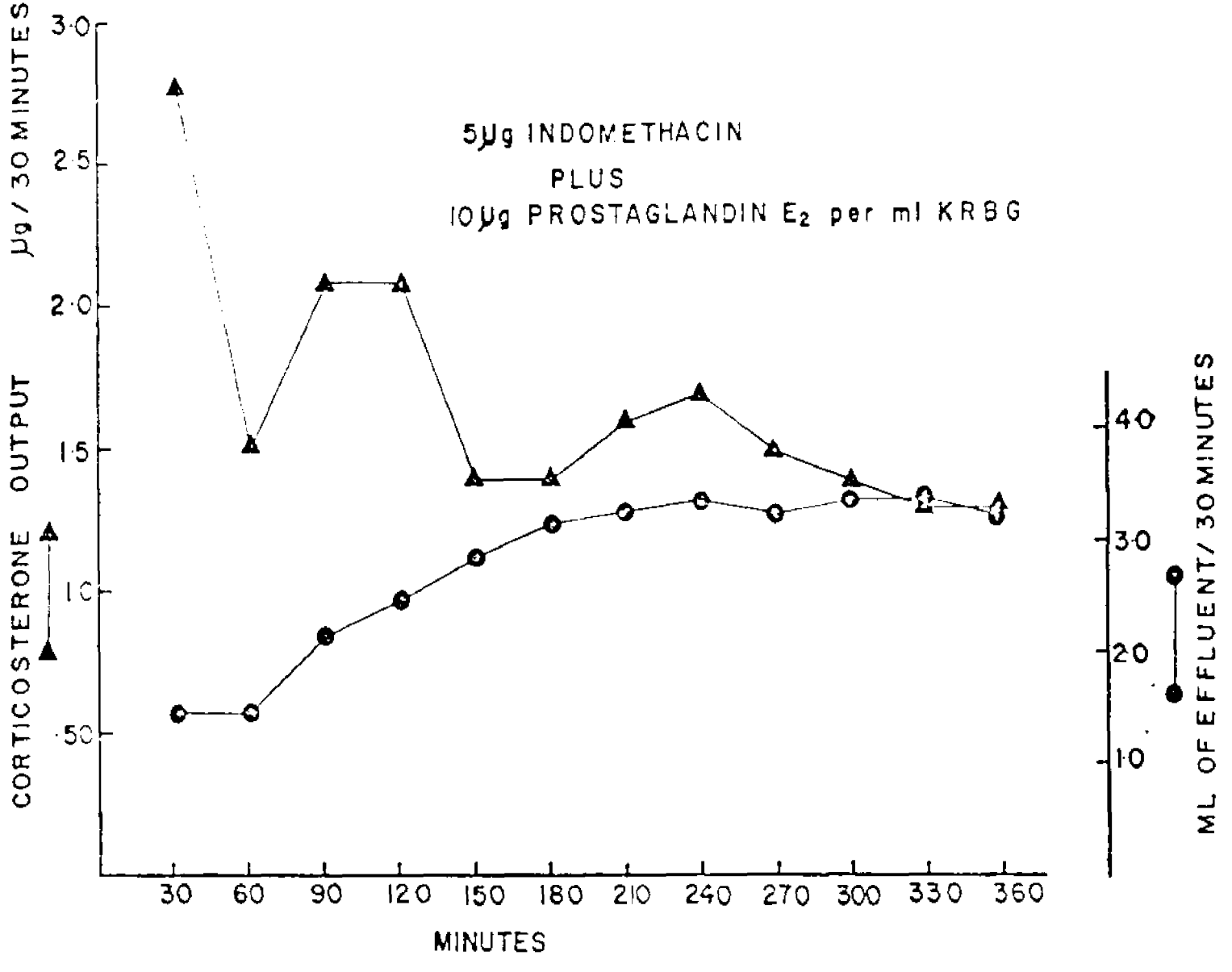

5µg INDOMETHACIN
PLUS
10µg PROSTAGLANDIN E2 per ml KRBG
CORTICOSTERONE OUTPUT µg/30 MINUTES
3·0
2·5
2·0
1·5
1·0
·50
ML OF EFFLUENT/30 MINUTES
40
30
20
10
30 60 90 120 150 180 210 240 270 300 330 360
MINUTES

TABLE XII. CORTICOSTERONE OUTPUT FROM RAT ADRENAL GLANDS PERFUSED WITH 10 μg PROSTAGLANDIN E_1 PLUS 5 μg INDOMETHACIN/ml KRBG AFTER PERFUSION FOR 90 MINUTES WITH INDOMETHACIN (5 μg/ml KRBG).

Minutes after start of perfusion	μg CORTICOSTERONE/30 MINUTE SAMPLE									
	0 - 30	30 - 60	60 - 90	90 - 120	120 - 150	150 - 180	180 - 210	210 - 240	240 - 270	270 - 300
	5 μg Indomethacin per ml KRBG			10 μg PGE_1 plus 5 μg Indomethacin/ml KRBG						
Experiment										
10 P	1.6	1.4	0.68	5.5	5.1	3.1	3.0	3.1	2.2	1.5
11 P	0.88	0.56	0.53	0.68	0.61	0.77	1.1	1.2	1.5	2.0
12 P	0.74	0.50	0.32	0.42	0.37	0.49	0.63	0.86	0.48	0.33

responded immediately (no apparent lag period) to the addition of 10 μg PGE_1/ml with increases in B output. Only a slight increase in B output was observed for the remaining adrenal (experiment 12P) although the flow rate in this experiment increased after addition of the prostaglandin. In experiments 10P and 11P the stimulation of B output was accompanied by increases in flow rate.

Figure 28 illustrates one of these perfusions with PGE_1. A transient increase in output reached a maximal level within 30 minutes after the addition of PGE_1. The actual levels of B output remained high but declined with time of perfusion. The flow rate also appeared to respond immediately to PGE_1, reaching high levels and remaining there for the duration of the perfusion (see figure 28).

The perfusions described in this section suggest that PGE_1 (10 μg/ml KRBG) exerts an influence on adrenal flow rate and B output.

C. <u>Prostaglandin $F_{1\alpha}$: continuous perfusion with 10 μg prostaglandin $F_{1\alpha}$ plus 5 μg indomethacin per ml KRBG after 90 minutes of perfusion with indomethacin (5 μg/ml KRBG)</u>

Three adrenal glands were perfused for 90 minutes with KRBG-containing only indomethacin. $PGF_{1\alpha}$ (10 μg/ml KRBG) was then added to the indomethacin-containing medium and the perfusion continued for 270 minutes (experiments 13P and 14P) and 210 minutes (experiment 15P). The corticosterone output data for the 3 perfusions are summarized in Table XIII. All 3 adrenals responded with increases in B output; high levels of B were maintained for several hours under the influence of $PGF_{1\alpha}$. Increases in flow rate which appeared related to increased steroid levels were observed in experiments 13P and 15P. However,

Figure 28. Time courses of corticosterone output and flow rate for an adrenal gland (experiment 10P) perfused for 90 minutes with indomethacin (5 μg/ml KRBG) followed by continuous perfusion (210 minutes) with 10 μg prostaglandin E_1 plus 5 μg indomethacin per ml KRBG.

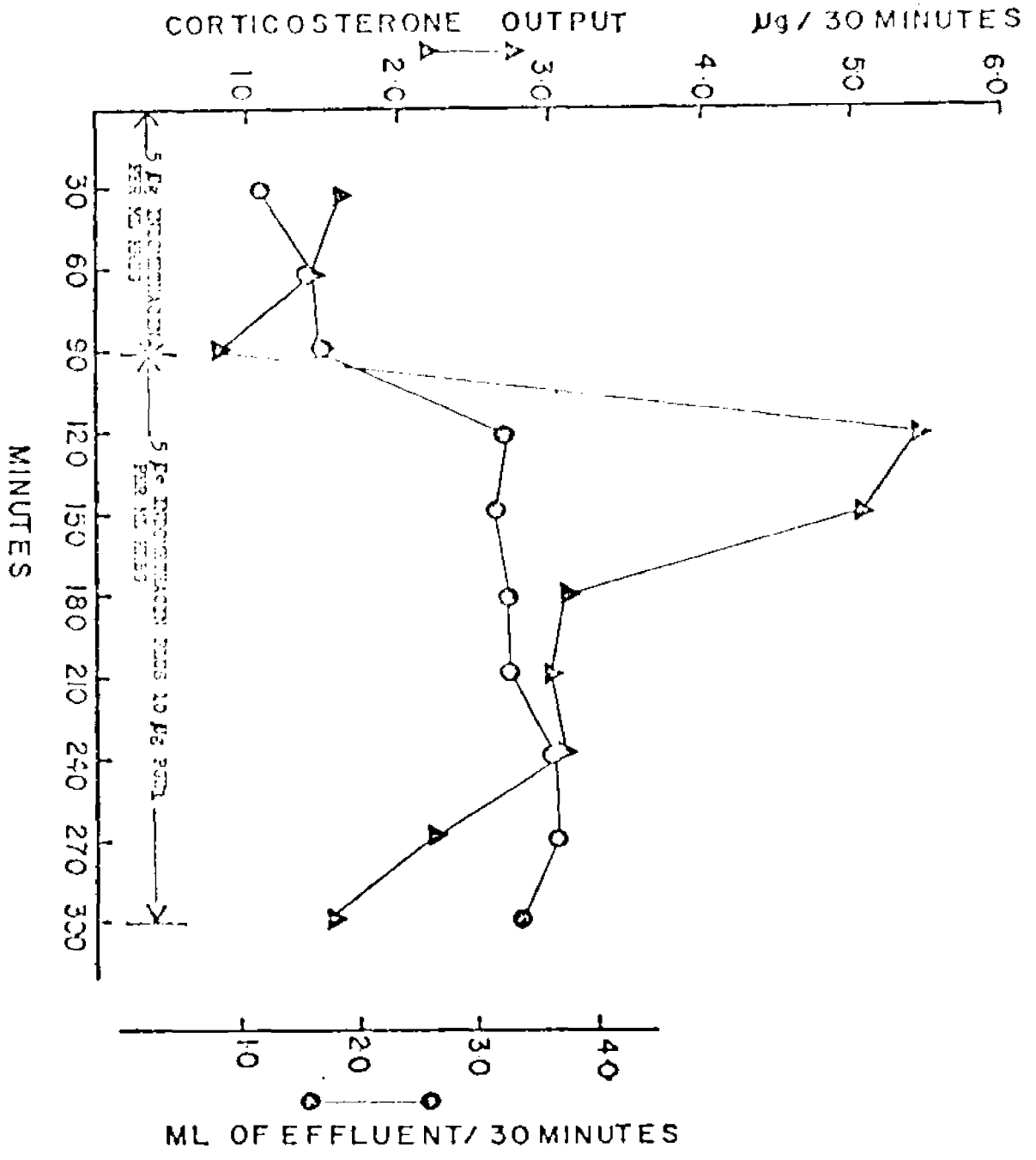

CORTICOSTERONE OUTPUT µg / 30 MINUTES
1·0
2·0
3·0
4·0
5·0
6·0
30
60
90
120
150
180
210
240
270
300
MINUTES
10
20
30
40
ML OF EFFLUENT / 30 MINUTES

TABLE XIII. CORTICOSTERONE OUTPUT FROM RAT ADRENAL GLANDS PERFUSED WITH 10 µg PROSTAGLANDIN $F_{1\alpha}$ PLUS 5 µg INDOMETHACIN/ml KRBG AFTER PERFUSION FOR 90 MINUTES WITH INDOMETHACIN (5 µg/ml KRBG).

µg CORTICOSTERONE/30 MINUTE SAMPLE

Minutes after start of perfusion	0 - 30	30 - 60	60 - 90	90 - 120	120 - 150	150 - 180	180 - 210	210 - 240	240 - 270	270 - 300	300 - 330	330 - 360
	5 µg Indomethacin per ml KRBG			10 µg Prostaglandin $F_{1\alpha}$ plus 5 µg indomethacin/ml KRBG								
Experiment												
13 P	1.2	1.5	2.0	5.3	4.5	5.1	2.7	2.0	1.9	0.92	0.64	0.48
14 P	2.3	3.1	1.6	1.5	1.9	1.7	1.4	1.5	1.0	0.90	0.57	0.49
15 P	1.0	0.99	1.2	1.5	1.2	1.3	1.3	0.95	0.71	0.45	-	-

in experiment 14P the flow rate did not change during the 6 hours of perfusion.

Figure 29 illustrates the time courses of B output and flow rate for experiment 13P. Steroid output and flow rate were seen to increase after addition of $PGF_{1\alpha}$.

The perfusions described in this section suggest that $PGF_{1\alpha}$ (10 µg/ml KRBG) may also exert an influence on steroid output and flow rate.

VIII. Perfusion with direct cannulation of the adrenal vein

Adrenal venous effluent can also be collected by direct cannulation of the adrenal vein. This variation of the standard procedure, which simply replaces cannulation of the renal vein (see Surgical Techniques under Materials and Methods), was developed in an attempt to improve the technique of adrenal perfusion.

Using this variation, six successful studies were carried out. Three adrenals were perfused with KRBG (control) and the other three continuously with 100 mU ACTH/ml KRBG.

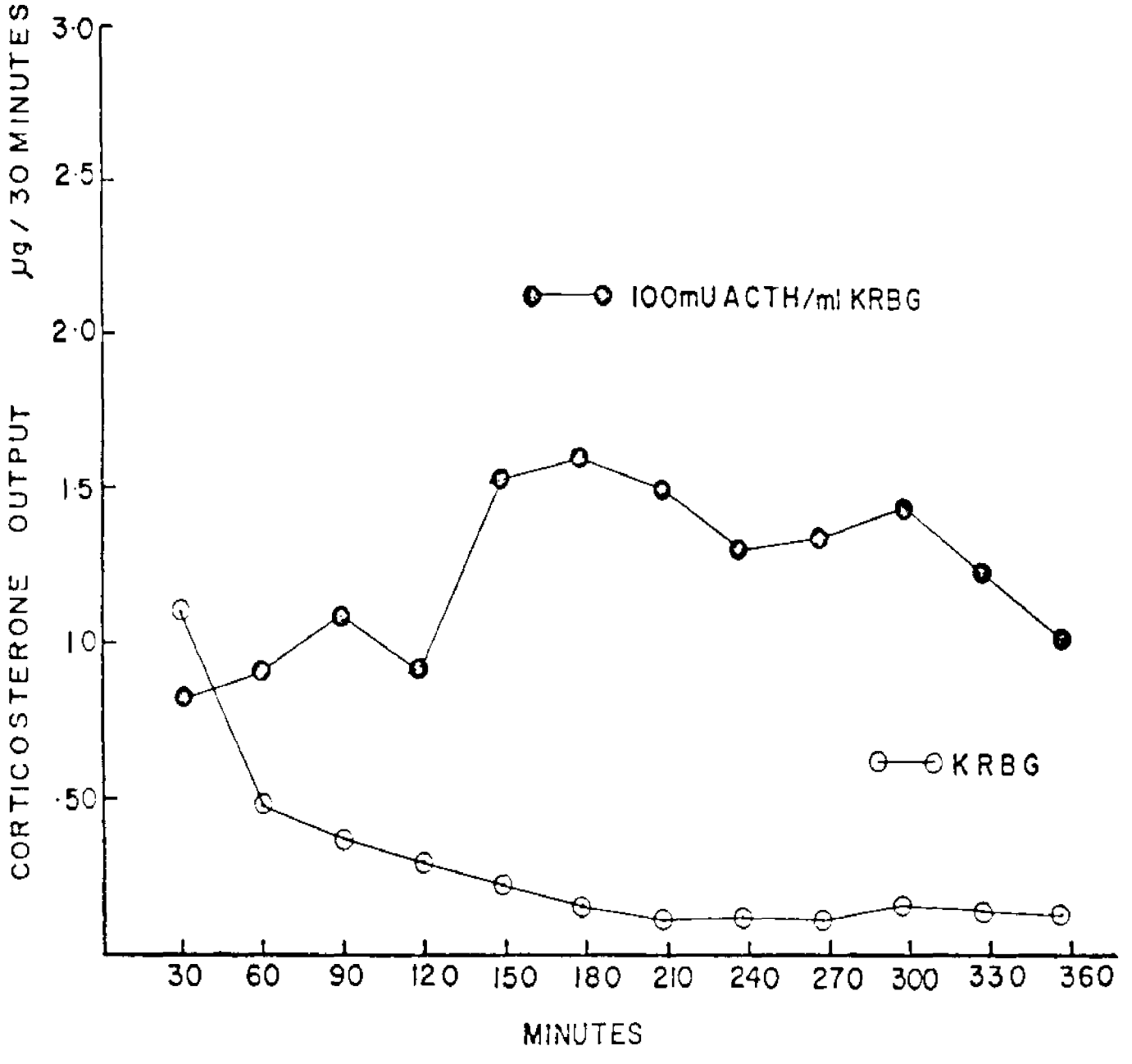
Figure 30 depicts the time courses of corticosterone (B) output for two typical experiments. One adrenal was perfused with KRBG (open circles) and the other with ACTH (closed circles). The adrenal venous effluents were collected by means of cannulae inserted in the adrenal veins. The output of B from the control experiment showed a decline with time. Corticosterone output reached a low basal level at the end of three hours of perfusion. The low basal level persisted until the experiment was terminated at the end of six hours.

A spontaneous increase in B output was observed in only one out of three control perfusions. The magnitude of the increase was less

Figure 29. Time courses of corticosterone output and flow rate for an adrenal gland (experiment 13P) perfused for 90 minutes with indomethacin (5 µg/ml KRBG) followed by continuous perfusion (270 minutes) with 10 µg prostaglandin $F_{1\alpha}$ plus 5 µg indomethacin per ml KRBG.

CORTICOSTERONE OUTPUT µg / 30 MINUTES
1·0
2·0
3·0
4·0
5·0
6·0
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES
10
20
30
40
ML OF EFFLUENT/ 30 MINUTES

Figure 30. Time courses of corticosterone output for two adrenal glands with the venous effluent being collected via cannulae inserted in the adrenal veins.
Open circles: KRBG-control.
Closed circles: continuous perfusion with 100 mU ACTH/ml KRBG.


CORTICOSTERONE OUTPUT µg / 30 MINUTES
3·0
2·5
2·0
1·5
1·0
·50
100mU ACTH/ml KRBG
KRBG
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES

than that which had been previously observed in control experiments when the renal vein was cannulated.

A higher level of B output was maintained when adrenals were continuously perfused with ACTH (100 mU/ml KRBG) and the venous effluent collected via the cannulated adrenal vein. In the ACTH-perfused adrenal illustrated in figure 30 (closed circles), the initial output (output during the first 30 minutes) was maintained for the first two hours of perfusion. During the third hour, the output increased above the initial value and was maintained at this higher level until the end of five hours of perfusion, at which time it started to decline.

When the cannula was in the adrenal vein, B output was less from both control and ACTH-perfused adrenals (see Table V) than when it was in the renal vein. When the adrenal vein was cannulated the flow rates remained constant in both types of perfusions (control and ACTH-stimulated) but were 1.0 to 2.0 ml/hour, which was lower than those encountered when the procedure involved renal vein cannulation. The presence of the polyethylene cannula within the very narrow adrenal vein probably interfered with the flow of adrenal venous effluent.

For the reasons just mentioned, and because cannulation of the adrenal vein was difficult and often time consuming, it was decided that no advantage would be gained by pursuing this variation of the technique.

IX. In vitro incubation studies on quartered rat adrenal glands

The reasons for carrying out in vitro studies were (1) to determine the rate of corticosterone (B) output in vitro of adrenal

glands from Long Evans rats and, (2) to use this information to compare the behavior of the in situ perfused adrenal and that of single adrenal glands incubated in vitro. Attempts were made to expose the in vitro-incubated adrenals to experimental conditions closely approximating those existing during the perfusion (see Materials and Methods).

A. In vitro response to ACTH

Adrenal quarters were first incubated for 30 minutes (pre-incubation period) in medium (KRBG) alone before incubation for two hours (incubation period) in fresh medium containing ACTH. The reasons for pre-incubation have not been clearly established, but appear to be important for increasing the sensitivity of rat adrenals to added ACTH (Saffran and Bayliss, 1953).

The ability of ACTH to stimulate corticosterone output from adrenal quarters in vitro was determined at 3 different concencentrations; 10, 100 and 1000 mU ACTH/ml KRBG. The data for these experiments are summarized in Table XIV. ACTH was observed to elicit a 3 - 4 fold increase in B output over the two hour incubation period. Corticosterone output from ACTH-stimulated adrenals at all three doses used was significantly different ($P < 0.001$) from the KRBG controls. Increasing the ACTH concentration from 10 to 100 mU/ml KRBG resulted in a small increase in B output per adrenal gland ($P < 0.10$). Corticosterone output of adrenals exposed to 100 mU ACTH and those exposed to 1000 mU was not significantly different. Thus, 100 mU ACTH/ml KRBG appears to elicit a maximal response in this in vitro system.

TABLE XIV. CORTICOSTERONE OUTPUT FROM SINGLE QUARTERED RAT ADRENAL GLANDS INCUBATED FOR 2 HOURS IN VITRO[a].

INCUBATION MEDIUM	NUMBER OF ADRENALS	TOTAL 2 HOUR OUTPUT (Incubation Period)	
		µg B/gland/2 Hrs.	µg B/100 mg Adrenal/2 Hrs.
KRBG	30	2.6 (± 0.16)	11.1 (± 0.77)
ACTH mU/ml KRBG			
10	7	7.2 (± 0.91)*	28.6 (± 3.6)*
100	10	9.5 (± 0.80)*	39.8 (± 2.7)*
1000	5	10.9 (± 3.1)*	48.3 (± 12.7)*

All values represent means (± S.E.).

[a] Adrenals were pre-incubated in KRBG for 30 minutes. The medium was then replaced with fresh KRBG with or without (controls) the addition of ACTH, and incubated for 2 hours (incubation period).

* Significantly different from KRBG controls ($P < 0.001$)

B. Comparisons between in situ perfused and in vitro incubated adrenal glands

Comparisons between corticosterone (B) output from perfused adrenals and from single adrenal glands incubated in vitro were made for equivalent time periods. This data is summarized in Table XV. The mean B output during the first 30 minutes of incubation (pre-incubation period) was found to be 2.99 ($\pm$ 0.12 S.E.) µg/gland while the first 30 minutes of perfusion with KRBG produced a somewhat lower level; 1.78 ($\pm$ 0.25 S.E.) µg/gland ($P < 0.001$). Thus it appears that the in vitro - incubated adrenal puts out more B during the first 30 minutes of incubation than does the perfused gland. This difference could be attributed to greater release of preformed B from the in vitro- incubated glands. Adrenal glands destined to be incubated in vitro were immediately removed from animals after they were anesthetized; they were thus exposed for only a short time (several minutes) to in vivo ACTH stimulation which could cause release of some preformed steroid. Perfused adrenals were exposed to in vivo ACTH stimulation for about 1 hour, the time required to surgically prepare them for perfusion. This relatively long exposure to ACTH might have caused depletion of all or most of their preformed steroid and hence could account for the lower B output observed during the first 30 minutes of perfusion. In other words, less preformed steroid would be present in perfused adrenals during the first 30 minutes of perfusion than would be present in incubated adrenals during the pre-incubation period.

When the total B output from the pre-incubation plus the incubation period of in vitro-incubated glands was compared with the

TABLE XV. A COMPARISON BETWEEN CORTICOSTERONE OUTPUT FROM SINGLE ADRENAL GLANDS INCUBATED IN VITRO AND FROM IN SITU PERFUSED ADRENALS.

	IN VITRO		PERFUSION
MEDIUM			
	Pre-incubation period		0-30 minutes of perfusion
	µg B/adrenal/30 minutes		
KRBG	2.99 (± 0.12) (n = 51)	P < 0.001	1.78 (± 0.25) (n = 17)
	Pre-incubation + incubation periods		0-150 minutes of perfusion
	µg B/adrenal/2 ½ hours		
KRBG	5.40 (± 0.28) (n = 29)		6.20 (± 1.1) (n = 17)
	Incubation Period		30-150 minutes of perfusion
	µg B/adrenal/2 hours		
ACTH (100 mU/ml KRBG)	9.5 (± 0.80) (n = 10)		8.2 (± 0.99) (n = 7

All values represent means (± S.E.)

first two and a half hours from control perfusions, the outputs were not significantly different. Adrenals incubated *in vitro* had a mean output of 5.40 (± 0.28 S.E.) µg B/gland/two and a half hours, while perfusion for two and half hours resulted in 6.20 (± 1.1 S.E.) µg/gland. Thus it appears that B output during the first two and half hours of perfusion with KRBG is about the same as that produced by a single quartered adrenal gland incubated *in vitro*. Making the assumption that the *in situ* perfused adrenal is the more physiologic of the two preparations, one would intuitively expect a higher rate of output from the perfused adrenal, i.e. closer to that which has been observed *in vivo*. Under conditions which probably elicit maximum ACTH stimulation (adrenal vein cannulation), a single rat adrenal gland has an output of from 10 to 60 µg B/hour (Tait et al, 1970). These differences will be discussed below (see General Considerations and Conclusions).

More meaningful comparisons between *in vitro* incubations and the perfusion system require that the incubations be carried out for longer time periods and with more frequent changes of incubation media.

Adrenal perfusions with ACTH were not designed so that they could be easily compared to adrenal glands incubated *in vitro* in an ACTH-containing medium. It was previously mentioned that in order to demonstrate a significant effect of ACTH *in vitro*, the glands had to be pre-incubated before exposure to ACTH. In most perfusions with ACTH, the adrenals were continuously exposed to the trophic hormone from the start of the perfusion and hence there was no time period comparable to the 30 minute pre-incubation carried out *in vitro*.

In order to be able to make a comparison between the response of the *in vitro*-incubated and the perfused adrenal to ACTH, the first 30 minutes of perfusion with ACTH was assumed to be equivalent to the pre-incubation period *in vitro*. It is clear that these two time periods and their effects on the adrenals are probably not equivalent. The mean total output of B for the incubation period *in vitro* with 100 mU ACTH was compared to 30 to 150 minutes of continuous perfusion with 100 mU ACTH/ml KRBG (see Table XV). The mean B output was 9.5 ($\pm$ 0.80 S.E.) µg B per/gland over a two hour period of incubation with ACTH. This was not significantly different from 8.2 ($\pm$ 0.99 S.E.) µg which was the mean two hour B output from adrenals perfused with 100 mU ACTH. Thus, for two hour periods which are not exactly equivalent, the mean B output from *in vitro*-incubated and *in situ*-perfused adrenals exposed to 100 mU ACTH was about the same.

In vitro incubations were also carried out with indomethacin, PGE_1 and PGE_2 in order to determine whether the effects of these agents were similar to the effects observed when they were added to the perfusion medium. Quartered adrenal glands were incubated for two hours with five different concentrations of indomethacin (5, 25, 50, 100 and 200 µg indomethacin per ml KRBG). Indomethacin had no effect on B output at any of the dose levels used. Total B output was equivalent to that of KRBG-incubated controls (see Table XIV). PGE_1 and PGE_2, at two different concentrations (5 and 10 µg/ml KRBG) alone and in combination with indomethacin (5 µg/ml KRBG) had no effect on control levels of B output (see Table XIV) over a two hour incubation period. This suggests that differences exist between the response of the perfused adrenal and the adrenal incubated *in vitro*.

GENERAL CONSIDERATIONS AND CONCLUSIONS

I. The Technique of adrenal perfusion

To this writer's knowledge, only one other technique for *in situ* perfusion of rat adrenal glands has been described in the literature (Cession-Fossion, 1964a, 1964b). The method was developed to study adrenal catecholamine secretion and involved perfusion of both the kidneys and the adrenal glands via a cannula placed in the aorta. The vessels supplying the gastrointestinal tract and the genital organs were ligated and the venous effluent was collected from the cannulated vena cava. Edema formation which led to a rapid decrease in flow rate, limited the perfusions to periods of no more than 30 minutes. Large amounts of perfusate were required to irrigate this preparation and were probably an important factor leading to the edema formation. Cession-Fossion (1964b; personal communication, 1969), believed that the exclusion of the kidneys from the perfusion circuit by ligation of their blood vessels would cut off the blood supply to the inferior poles of the adrenal glands. The present study has demonstrated that the blood vessels at the hilus of a kidney can be carefully ligated without damaging the circulation to the adrenal gland. This manupulation makes it possible to perfuse the rat adrenal gland without having to include the kidney in the perfusion circuit.

Of the several surgical manipulations required, two are crucial to the success of a perfusion experiment; (a) the left renal artery and vein must be carefully separated so that the tie for holding the venous cannula in place can be put only around the renal vein

(see figure 3). Failure to do this could result in damage to the renal artery, occlusion or constriction of the inferior adrenal artery (if one is present) and inadequate perfusion of the gland. (b) The small venous tributaries which empty into the adrenal vein must be ligated. Failure to do this may lead to an adrenal which is only partially perfused (see Results and Discussion of Results, Section IB).

Perfusion of the adrenal with Thorotrast followed by x-rays of the area established that the gland was being perfused (see Results and Discussion of Results, Section 1A). Perfusate penetrated most of the adrenal capillaries; if the intracortical vessels were not filled with radioopaque medium, the gland would not be visible radiographically (Harrison and Hoey, 1960). This type of experiment indicates that flow is occurring in most parts of the gland but it cannot adequately assess the microcirculation. It is possible that contrast medium enters one segment of the circulation yet the flow to that segment may be extremely slow and the function quite disturbed.

Several parameters were routinely utilized in an attempt to estimate the overall well-being of the gland and hence the success of a perfusion experiment. These were, flow rate, visual observation of the isolated gland, vascular leakage and edema formation within the perirenal fat surrounding the gland.

Most important of these parameters was the flow rate which usually remained constant and within an acceptable range (see Materials and Methods, IB (2)) for the duration of most experiments. Increases or decreases in flow rate (inconstant flow rates) usually signaled failure of the perfusion. Blotches or blemishes on the

surface of the gland were visible signs of vasoconstriction or gross damage to small areas of the adrenal gland and when they persisted, the experiment was discarded. The surface of the successfully perfused adrenal appeared clear, moist and glistening. Extensive vascular leakage of perfusate occurred infrequently and could be detected by periodic observations of the preparation with a loupe. When leakage did occur it was usually accompanied by a decrease in flow rate.

It was not possible to assess the effect of edema on the functional status of the perfused adrenal, and hence only some suggestions can be offered. As previously mentioned (Results and Discussion of Results, Section V), edema fluid could be observed to accumulate within the perirenal fat. Most of the time progressive accumulation of fluid was observed with no concomitant decrease in flow rate. This suggested that the increased tissue pressure which usually accompanies edema formation was not severe enough to affect adrenal flow and that probably tissue damage was kept at a minimum. A possible effect of increased tissue pressure on prostaglandin biosynthesis will be discussed below. Edema accompanied by increased tissue pressure, vasoconstriction and decreased flow is probably the most common cause of failure in most organ perfusion studies. In the present study, because edema formation had little effect on flow rate, it rarely resulted in the failure of a perfusion.

No attempts were made in the present study to experimentally determine the oxygen requirements of the rat adrenal nor could they be found in the literature. However, it is known that adrenal glands from most species studied have very rich blood supplies (Lever, 1955;

Jones, 1957; Rhodin, 1971) suggesting that their oxygen requirement is relatively high. In preparing an adrenal for perfusion, the gland is deprived of a circulation for no longer than 60 seconds, a relatively short period of time. It is doubtful whether this short period of hypoxia results in permanent damage to the adrenal. The question that does arise is whether the oxygenated perfusate (KRBG), after some loss of oxygen in transit to the gland, is able to supply the gland with sufficient oxygen to maintain it in a functional state for fairly long periods of time. On the other hand studies both in this laboratory and in another (Liemann, 1971) have shown that adrenal glands can be deprived of oxygen (i.e. cut off from their blood supply) for varying periods of time (60 to 120 minutes) and still produce corticosterone as well as respond to ACTH in vitro. Although, the perfused adrenal is undoubtedly receiving less oxygen that it would in vivo, it can still put out measurable amounts of corticosterone for periods of at least 6 hours and can respond to various stimuli.

II. Control (KRBG) perfusions

Most adrenal glands perfused with KRBG (controls) showed high initial outputs of corticosterone which declined with time of perfusion. Regardless of whether the decline was accompanied by transient rises in output or not, it was a highly reproducible phenomena. This has been observed by others studying superfused rat adrenals (Saffran and Rowell, 1969; Tait et al, 1970; Huibregtse and Ungar, 1970; Schulster et al, 1970). The high initial output is not believed to be due to a washout of preformed steroid from the gland. Tait and coworkers (1970) have shown that the amount of corticosterone "stored" in an adrenal gland would be completely depleted after ten minutes of

superfusion. Thus, the observed initial decline in output would appear to be due to a decline in de novo synthesis rather than a release of preformed steroid.

Figure 31 illustrates the time courses of mean corticosterone (B) output for each 30 minute collection period both for the pattern I (open circles) and pattern II (closed circles) types of control responses; these are plotted separately and together (I plus II, triangles). The pattern I type of response has been previously described as a decrease in B output with time of perfusion, while pattern II involves an overall decrease in output accompanied by transient increases (see Results and Discussion of Results, Sections IIA and B). Also included in figure 31 are data recalculated from superfusion studies of Schulster and co-workers (1970) and expressed as micrograms of corticosterone per adrenal gland per hour. This curve (solid squares) shows B output as a function of time of superfusion.

Valid comparisons can be made between adrenal perfusion and superfusion, although one must realize that the experimental conditions differ greatly from one technique to the other. In the superfusion study (Schulster et al, 1970), decapsulated (zona fasiculata and reticularis plus the medulla) adrenals from 12 intact female rats (24 adrenal glands, i.e. 350 -450 mg of adrenal tissue) were used for each 5 hour incubation. The curve for superfusion in figure 31 (solid squares) represents the mean for 6 superfusion experiments, i.e., the data obtained from 144 adrenal glands or over 2.0 grams of tissue. The adrenals were superfused at a flow rate of about 45 ml KRBG/hour; which is at least ten fold greater than that used in adrenal perfusion.

Figure 31. Time courses of mean corticosterone output for adrenal glands superfused and perfused with KRBG for 5 and 6 hours respectively.

The mean B outputs for each 30 minute sample for control perfusions (Pattern I, II and I + II) were calculated from the data of Tables II and III.

The mean outputs for each 60 minute sample for superfused adrenals were recalculated from the data of Schulster et al (1970) which was reported as μg B/rat/hour.

Perfusion:

Pattern I	open circles
Pattern II	closed circles
Patterns I + II	triangles

Superfusion: solid squares

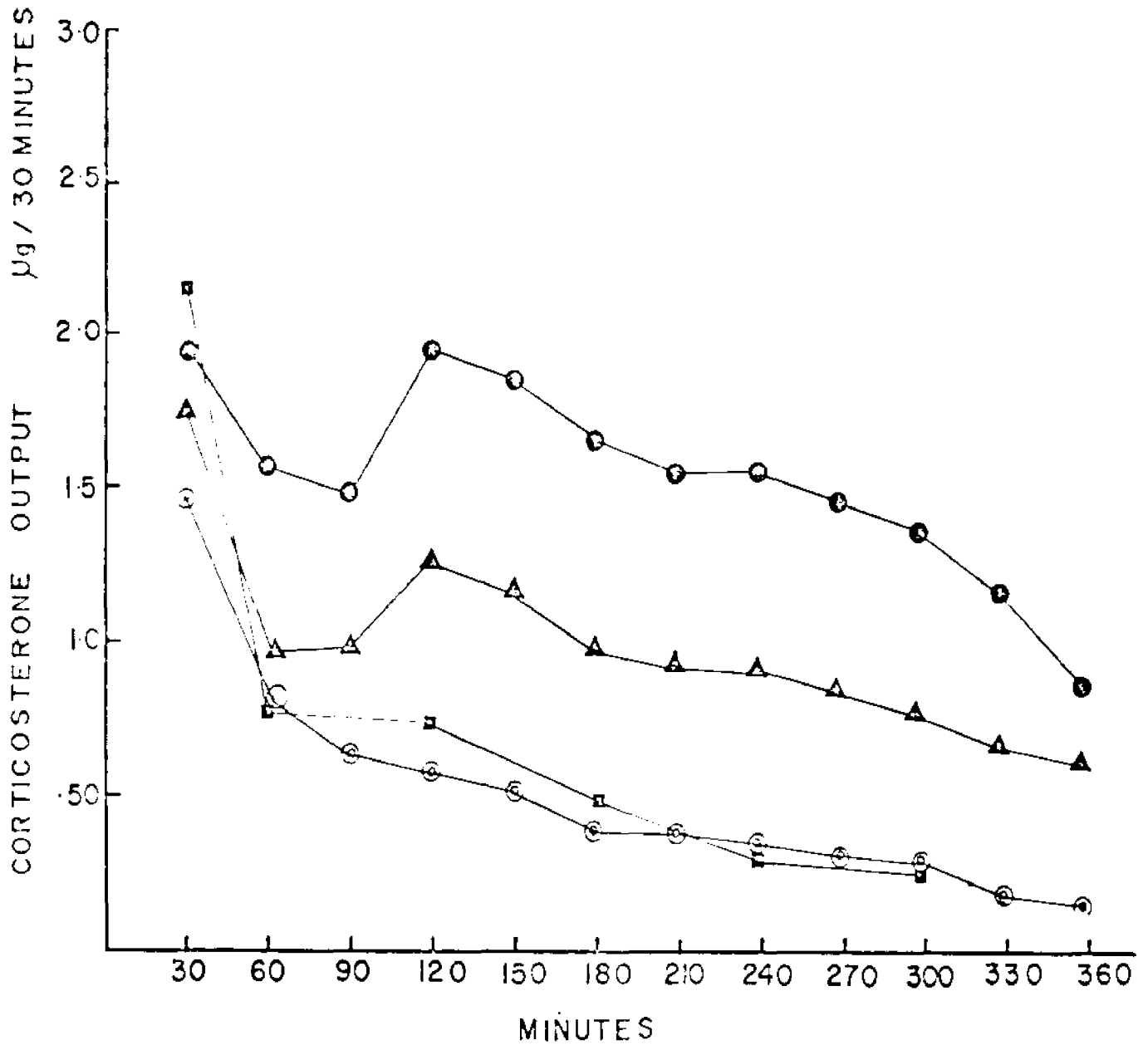

CORTICOSTERONE OUTPUT µg / 30 MINUTES
3·0
2·5
2·0
1·5
1·0
·50
30
60
90
120
150
180
210
240
270
300
330
360
MINUTES

Figure 31 shows that high initial levels of B decline with time from both perfused and superfused adrenals. Corticosterone output was lower from superfused adrenals when compared with the outputs for pattern II and for pattern I plus II responses for perfused adrenals. Spontaneous increases in B output were not observed in this superfusion study. The superfusion curve represents the mean output for 144 adrenal glands and any increases in B output by some glands would be averaged out. This possibility illustrates an important contribution of adrenal perfusion; that of providing a technique which allows one to study individual differences between isolated adrenal glands.

The output from superfused adrenals was qualitatively and quantitatively similar to the pattern I type of response for perfused adrenals. Table XVI expresses the data from figure 31 in terms of hourly outputs of B. The corticosterone output/adrenal/hour can be seen to be as much as 3 to 10 times higher from perfused adrenals than from superfused adrenals. The relatively high levels of B output from intact perfused adrenals suggests that perfusion may be more representative of what occurs *in vivo* than superfusion.

A decline with time of B output was observed from most KRBG-perfused adrenal glands. Baniukiewicz et al (1968) have suggested that this decline at least in the case of superfused adrenals is due to the decay *in vitro* of the *in vivo* effect of the trophic hormone ("in vivo decay hypothesis"). They feel that the decline would result from inactivation of secreted ACTH still bound to the tissue and to a decay of the effects initiated *in vivo*. This explanation is not completely satisfactory because the decline in output was also

TABLE XVI. MEAN CORTICOSTERONE OUTPUT PER ADRENAL PER HOUR: A COMPARISON BETWEEN PERFUSED (KRBG) RAT ADRENAL GLANDS AND SUPERFUSED (KRBG) DECAPSULATED ADRENAL GLANDS.[x]

	μg CORTICOSTERONE/ADRENAL/HOUR			
	PERFUSION			SUPERFUSION
MINUTES	I	II	I + II	
0 - 60	2.3	3.6	2.8	2.2
60 -120	1.2	3.5	2.3	0.75
120-180	0.92	3.6	2.2	0.50
180-240	0.74	3.2	1.9	0.30
240-300	0.58	2.9	1.7	0.25
300-360	0.32	2.0	1.3	
	(n=9)	(n=8)	n=17)	(n=144)

x superfusion data modified from Schulster et al (1970).

observed when adrenals were perfused or superfused with ACTH. Regardless of the approach used, perfusion or superfusion etc., knowledge of *in vivo* cell function is not advanced enough to permit an adequate reproduction of the *in vivo* conditions in any of these isolated systems. For this reason, one would intuitively expect the steroid output to decline with time; many *in vivo* requirements for optimal function must be lacking.

III. Transient increase in corticosterone output from KRBG-perfused adrenals

As previously described (see Results and Discussion of Results, IIB), transient increases in corticosterone (B) output occurred frequently in control (KRBG) perfusions and were especially difficult to explain because they occurred in the absence of any obvious stimulatory agents. One of my primary goals was to find a reasonable explanation for this phenomenon.

The transient increase may have been the result of stimulation by ACTH. The finding of low initial outputs of B in perfusions from hypophysectomized rats compared to high initial outputs from intact rats suggests that the low outputs might represent the response of an adrenal which is no longer under the influence of ACTH. Endogenous ACTH and its steroidogenic effect have been shown to disappear rapidly from the plasma of rats following hypophysectomy (Harding and Nelson, 1964; Cook et al, 1972; Matsuyama et al, 1972). This together with the observation that the low initial outputs in perfusions from hypophysectomized rats increased with time of perfusion, would suggest that the increases in B output are not due to an effect of ACTH.

Studies have been described (see Results and Discussion of

Results, IIB) which have indicated that the spontaneous increases in B output from the perfused adrenal could not be attributed to an effect of nembutal. Neither do they appear to be directly related to time of day. In this respect one could suggest (a speculation) that adrenal glands might possess intrinsic mechanisms unrelated to environmental cues or to diurnal variations, which would control their secretory rhythmicity. Secretory rhythms might be different for adrenals from different animals; rhythmicity would be controlled by an intra-adrenal mechanism. Spontaneous increases in B output would occur when an adrenal was being perfused at a time when its secretory output was increasing.

The possibity that prostaglandins of adrenal origin synthesized in response to mechanical stimulation (surgical manipulation and/or the perfusion itself) were stimulating steroid output was also considered. It has been reported that some organs can release prostaglandins in response to mechanical stimuli (Piper and Vane, 1971), that prostaglandins were present in and released by rat adrenal glands (Shaw and Ramwell, 1967; Ramwell and Shaw, 1970) and that prostaglandins could stimulate corticosteroidogenesis (Flack et al, 1969).

The increased tissue pressure which usually accompanies edema formation could result in mechanical stimulation of a perfused adrenal leading to prostaglandin biosynthesis and stimulation of B output. No spontaneous increases in corticosterone output were observed when adrenals were perfused with dextran-supplemented KRBG (see Table VII). The experiments could be interpreted to mean that the dextran-perfused adrenal was subjected to less mechanical stimulation (less edema) and

to a decrease or elimination of endogenous prostaglandin biosynthesis.

The perfusions carried out with indomethacin, an inhibitor of prostaglandin synthesis (Vane, 1971), suggest that prostaglandins may be involved in the transient increases in output. When the mean B outputs from adrenals perfused with indomethacin were compared with mean outputs from control (KRBG) perfusions (see Table VIII), the indomethacin appeared to eliminate or greatly diminish the transient increases in corticosterone output. Several adrenal glands perfused with indomethacin still showed increases in corticosterone output (see Table VIII). If one assumes that these increases in output are due to prostaglandin stimulation of steroidogenesis, then their occurrence in perfusions carried out with indomethacin raises two questions: (1) Is indomethacin really inhibiting prostaglandin synthesis in the perfused adrenal? and, (2) if indomethacin is inhibiting prostaglandin synthesis then why doesn't it do so in all adrenals perfused with the inhibitor? The first question can only be asnwered by further experimentation. This would require the identification of prostaglandins in the venous effluent from control (KRBG) perfusions. One would then have to show that perfusion with indomethacin eliminates or decreases the concentration of prostaglandins previously found in the venous effluent. Several possible explanations can be given to explain why indomethacin did not eliminate or diminish the increases in output from all of the adrenals perfused with the inhibitor (question 2). It is not clear from the present study whether indomethacin can easily penetrate the adrenocortical cells. It is possible that not enough indomethacin gained access to the prostaglandin-

synthesizing machinery in those perfusions which manifested increases in output even in the presence of the inhibitor. In addition, some adrenal glands might require concentrations of indomethacin greater than the 5 μg/ml used in the present study. The activity and/or the amount of prostaglandin synthetase from one adrenal to another might not be the same. Some of the adrenals perfused with 5 μg of indomethacin might have required more of the inhibitor to completely inhibit prostaglandin biosynthesis.

Perfusion of adrenals with a medium containing 5 μg prostaglandin E_2 plus 5 μg indomethacin resulted in transient increases in B output which resembled those observed in control (KRBG) perfusions (see figure 25 and 26 and Table IX). The date are consistent with the suggestion that the spontaneous increase in B output may represent a (steroidogenic) response of the perfused adrenal to endogenous prostaglandin.

Additional suggestions can be advanced to explain the transient increase in output. Deterioration of some adrenal tissue during the course of a perfusion could result in leakage of potassium from damaged cells thereby increasing the intra-adrenal potassium concentration and enhancing steroid production. Exposure of adrenal glands to excess potassium has been shown to stimulate steroidogenesis. Addition of potassium to the blood perfusing the isolated dog adrenal caused a rise in cortical hormone production (Vogt, 1951). More recently, Matthews and Saffran (1972) have shown that excess potassium enhanced corticosterone production in superfused rabbit adrenals.

A transient increase in B output could occur as a result of redistribution of perfusate within the adrenal. For example, vaso-

constriction could occur in a relatively inactive area of the gland (i.e. an area producing little or no corticosterone) thereby decreasing flow to the inactive part with subsequent diversion of perfusate (increase of flow) to a more active area (i.e. a major corticosterone-producing area). If such a redistribution of intra-adrenal flow occurred during a perfusion experiment, it might not be detectable as a change in the volume of venous effluent collected per unit time (i.e. flow rate). The intra-glandular distribution of arteries in the rat adrenal suggests that such a redistribution of flow is possible. This circulation consists of two types of blood vessels which arise from a plexus in the capsule. The first type, the arteriae medullae pass through the cortex to supply the medulla without giving off any branches to the cortex. These empty into medullary sinusoids. The other type of blood vessel consists of capillary networks arising from the capsular plexus and irrigating the various zones of the cortex as well as the medulla (Gersh and Grollman, 1941). Harrison and Hoey (1960), have proposed that changes in blood flow to the cortex may be due to constriction of the arteriae medullae with subsequent diversion of blood flow from the medulla to the cortex. Other investigators do not agree with this hypothesis (Kramer and Sapirstein, 1967). Nevertheless, redistribution of flow, if it were to occur during adrenal perfusion would appear to be a possible mechanism which could explain spontaneous increase in output.

Some component of the perfusate (KRBG) could be responsible for the observed enhancement of steroidogenesis. This could be easily tested by removing one ionic constituent of the buffer at a time, adding enough sucrose to maintain tonicity and observing the steroid-

ogenic effect of this modified buffer on the perfused adrenal. However, additional assumptions would have to be introduced in order to explain why enhanced output would occur in some control perfusions and not in others.

As previously mentioned, spontaneous increases in steroid output have been observed in the data of others (Huibregtse and Ungar, 1970; Flack and Ramwell, 1972) but it does not appear that any serious attempts have been made to explain their occurrence. Huibregtse and Ungar (1970) have developed a system in which the adrenals from a single rat (about 30 mg of tissue) can be superfused. Figure 32 was modified from their study and shows corticosterone output from two pairs of adrenal glands as a function of time of superfusion. Both pairs of adrenals were taken from intact rats, one was handled ("stressed") prior to sacrifice (triangles) while the other was killed rapidly (minimum stress) by cervical dislocation (circles). When the adrenals were superfused with KRBG (no ACTH in the medium), a "spontaneous rise in corticosterone secretion was noted at 3 hours" but could not be consistently reproduced. No possible explanation was offered for this enhancement of corticosterone output.

A "transient increase in corticosterone formation" was also observed from superfused decapsulated adrenals taken from rats 3 to 6 hours after hypophysectomy (Flack and Ramwell, 1972). In each control experiment the adrenals from at least ten rats (about 400 mg of tissue) were superfused for six hours with KRBG. The transient rise occurred during the first 90 minutes of superfusion. These workers suggest that the increase may result from transfer of the glands from 4° C to 37° C but apparently haven't experimentally tested this

Figure 32. Time courses for corticosterone output for KRBG-superfused adrenal glands from intact rats.

Circles: rat killed by rapid cervical dislocation.

Triangles: rat handled prior to sacrifice ("stressed").

Data modified from Guilbreatse and Unwar (1970).

µg CORTICOSTERONE/100 mg ADRENAL/30 MINUTES
.20
.40
.60
.80
4.0
4.2
1
3
5
7
HOURS

possibility. They also indicate that the rise in output may be due to "recovery from a short period of ACTH deprivation" (Flack and Ramwell, 1972).

Spontaneous increases in corticosterone output have been observed in some adrenal superfusion studies (Huibregtse and Ungar, 1970; Flack and Ramwell, 1972) but not in others (Tait et al, 1970; Schulster et al, 1970). When single adrenals are perfused with KRBG the phenomenon occurs frequently and is often of a greater magnitude than that observed from superfused adrenals. The absence of transient increases in output, or their occurrence but at a lesser magnitude from superfused adrenals is understandable. Superfusion systems usually involve the incubation of many adrenal glands and give information concerning the average behavior of the incubated tissue; this tends to diminish the importance of individual differences. In the present study, where the behavior of single adrenal glands were investigated, spontaneous rises in steroid output were more apparent and the importance of individual differences could be more fully appreciated.

IV. Response to ACTH

Continuous perfusion of adrenal glands with ACTH (100 mU/ml KRBG) for periods of up to six hours has resulted in high levels of corticosterone (B) output for relatively long periods of time. The adrenal perfused with ACTH has the ability to maintain high outputs for 3 to 4 hours and longer before showing any decline (see Table V). In most of these perfusions the response to ACTH was immediate with practically no lag period, although several adrenals did require a short interval before they responded.

Huibregtse and Ungar (1970) have shown that adrenal sections superfused with 100 mU ACTH/ml can maintain stimulated levels of B for 3 to 4 hours before starting to decline. However, their levels of B output are at least ten fold lower than outputs detected in the present study as well as in other adrenal superfusion studies (Tait et al, 1970; Schulster et al, 1970). Schulster and co-workers (1970) superfused decapsulated adrenal glands with ACTH (64 mU/ml) for periods of 5 hours. This data is expressed in Table XVII as hourly outputs of B and compared with the data from 7 adrenal glands (see Table V) continuously perfused with ACTH (100 mU/ml). The superfusion data were obtained from incubations involving 72 adrenal glands (over 1.0 gram of tissue). The ACTH-stimulated levels, when compared in this matter can be seen to be similar for both perfused and superfused adrenals. The major difference between the two systems appears to be the point in time where the stimulated levels begin to decline. Perfused adrenals maintain the high levels for a longer period of time. This difference could be due to the different levels of ACTH used, 100 mU/ml in the perfusion experiments and 64 mU/ml in the superfusion studies. Both levels of ACTH probably result in maximal stimulation of steroidogenesis, although these concentrations of ACTH are certainly much higher than found under physiological conditions. Physiologic levels of ACTH measured in resting intact rats has been shown to vary from 2 to 6 microunits/ml of plasma (Rees et al, 1971). However, the means by which the ACTH gains access to the adrenocortical cells is certainly different in perfusion and superfusion from that which occurs in vivo. Maybe only a very small amount that is present actually gets to its site of action.

TABLE XVII. MEAN CORTICOSTERONE OUTPUT PER ADRENAL PER HOUR: A COMPARISON BETWEEN RAT ADRENALS CONTINUOUSLY PERFUSED WITH ACTH (100 mU/ml) AND DECAPSULATED ADRENAL GLANDS CONTINUOUSLY SUPERFUSED WITH ACTH (64 mU/ml).x

μg CORTICOSTERONE/ADRENAL/HOUR

MINUTES	PERFUSION (100 mU/ml)	SUPERFUSION (64 mU/ml)
0 - 60	3.4	5.2
60 -120	3.7	3.4
120-180	5.8	3.0
180-240	4.4	1.9
240-300	3.4	1.4
300-360	2.2	

x superfusion data modified from Schulster et al, 1970.

As a result of the great difference in flow rates between superfused (45 ml/hour) and perfused adrenals (1.8 to 6.0 ml/hour), the superfused adrenals were exposed to a greater total amount of ACTH for a given time period than were the perfused adrenals. The superfused adrenals were exposed to a concentration of 64 mU ACTH/ml of medium which was completely replaced every two and half minutes. The superfused adrenals were thus exposed to over 2 units of ACTH/ hour. The perfused adrenals were exposed to a concentration of 100 mU ACTH/ml of medium. At this concentration of ACTH and at the highest acceptable flow rate, i.e., 6.0 ml/hour; a perfused adrenal gland would be exposed to a maximum dose of 600 mU ACTH/hour. This type of comparison points out one of the difficulties encountered in trying to compare the perfused rat adrenal with other approaches previously used to study rat adrenal glands.

Corticosterone output from adrenal glands continuously perfused with ACTH (100 mU/ml) declines during the later time periods of perfusion. A decline in output even in the presence of ACTH has been observed from blood-perfused dog adrenals (Urquhart and Li,1968), from superfused rat adrenals (Saffran and Rowell, 1969; Huibregtse and Ungar, 1970; Schulster et al, 1970) and from rat adrenal sections incubated in vitro (Birmingham et al, 1968). The question of why the response to ACTH falls off when the gland is continually supplied with its trophic hormone has been discussed by others (Schulster et al, 1970; Birmingham et al, 1968).

One possible explanation is that as the incubation or perfusion precedes the tissue deteriorates and irreversible changes ensue. This explanation has been discounted in the superfusion

studies. ACTH given 5 to 7 hours after the start of superfusion gave maximum responses which were similar to those observed when ACTH was given at the start of the incubation (Schulster et al, 1970; Saffran et al, 1971). Thus it would appear that adrenal tissue is still fully responsive to ACTH for at least 5 to 7 hours. In the present study the perfused adrenal responded to ACTH after a 120 minute washout period with KRBG. Longer periods of perfusion with KRBG before stimulation with ACTH were not carried out. As previously mentioned, some adrenals continuously perfused with ACTH (100 mU/ml) maintained high outputs for longer than 4 hours. Hence, perfused adrenals also respond to ACTH for relatively long periods of time.

Another possible explanation for the declining output in the presence of ACTH could be the depletion of a steroid hormone precursor or of some other important component of the steroid biosynthetic pathway. A likely candidate would be adrenal esterified cholesterol which is known to greatly decrease as a result of ACTH administration (Goodman, 1965). Gidez and Feller (1969) have measured cholesterol ester concentration in adrenal glands which were surgically removed from etherized ("stressed") rats. They found 44 micrograms of esterified cholesterol per milligram of adrenal tissue. This would be more than enough precursor to account for the 2.0 to 5.8 micrograms of corticosterone put out by adrenals perfused with ACTH (see Table V). , although one does not know how much of the cholesterol would be available for hormone biosynthesis. Birmingham and co-workers (1968) believe that the fall off in steroid output from rat adrenal sections exposed to continuous ACTH is due to depletion of a steroid hormone precursor. In this system the decline

always occurs after approximately 100 micrograms of ultra-violet-absorbing steroids have been produced. When decapsulated adrenals from hypophysectomized rats were continuously superfused with increasing concentrations of ACTH (0.61 to 680 mU/ml) a decline in corticosterone output was always observed (Schulster et al, 1970). At any time during the 5 hour superfusion the total amount of B produced could be increased by a higher concentration of ACTH. Hence, in this study it appears unlikely that the decline observed with low concentrations of ACTH was due to depletion of an essential precursor or cofactor.

The factor(s) which contribute to the decline in steroid output from adrenal glands continuously supplied with ACTH are not known. The decline in output observed in vitro and in perfusion experiments may be a result of removal of the adrenal from the environment which exists in the intact living animal. However, adrenal glands in vivo under "normal physiologic conditions" also respond to continuous ACTH stimulation with an eventual decline in steroid output.

Detailed investigations to determine the sensitivity of the perfused adrenal to ACTH were not carried out in the present study. Two out of six adrenals responded to 10 mU of ACTH in a way that was similar to that observed with 100 mU of ACTH. This may be interpreted as a relative insensitivity of the perfused rat adrenal gland to ACTH, although other interpretations are possible. The sensitivity of a perfused adrenal to ACTH is probably related to the degree of pituitary stimulation that the gland was subjected to during its surgical preparation for perfusion. Different adrenals might be exposed to the stimulatory effects of differing amounts of endogenous

ACTH. In a recent study on the ultrastructure of the rat adrenal cortex, adrenals were perfused with a fixative for electron microscopy (Rhodin, 1971). These "perfused" adrenals from "normal" rats showed variations in the number of lipid droplets present in the zona fasiculata. This was believed to reflect a difference in the degree of stress under which the animal was killed; yet all the animals were killed in the same manner (Rhodin, 1971). The sensitivity to ACTH might differ from one adrenal to another depending on the degree of stress and of ACTH exposure that the animal was subjected to before death. Adrenals subjected to intense endogenous ACTH stimulation during the surgical preparations might be less responsive to addition of ACTH to the perfusion medium. This interpretation could explain the relative insensitivity of some adrenals to ACTH as well as explain why some adrenals take more time to respond maximally to ACTH than do others (compare figures 16 and 17). Insensitivity to ACTH could also result from vasoconstriction with concomitant collapse of part of the microcirculation thereby preventing ACTH from reaching all parts of the gland.

V. Response to prostaglandins

The results of the present study show that prostaglandin E_2 (PGE_2) stimulates corticosterone output by a direct action on perfused adrenal glands from intact rats. The data confirm previous work which demonstrated a direct stimulatory effect of prostaglandins on superfused decapsulated adrenal glands (Flack et al, 1969; Flack and Ramwell, 1972).

The perfused adrenal illustrates certain aspects of the prostaglandin response which were not observed in the superfusion

system. In the present study, three prostaglandins (PGE_2, PGE_1 and $PGF_{1\alpha}$) enhanced steroid output in adrenals from intact rats. In the superfusion system, prostaglandin stimulation was not observed with adrenals from intact rats. In addition, the response to PGE_2 was no longer significant 12 hours after hypophysectomy (Flack et al, 1971). It is not at all clear, why adrenals from acutely hypophysectomized rats are required in order to elicit a prostaglandin response during superfusion. The differences in response to be discussed below might be due to the different types of preparations used, i.e., intact versus hypophysectomized. Adrenals superfused with PGE_2 (10 μg/ml) showed a rapid doubling of corticosterone (B) output (Flack and Ramwell, 1972). One hour after the addition of the prostaglandin, the response was maximal; 3 hours later it had decreased to control levels (i.e. a transient response). In comparison, when adrenals were continuously perfused with half the above dose of PGE_2 (5 μg PGE2 + 5 μg indomethacin/ml KRBG) individual differences in response were observed. Increases in B output were followed by declines which were not as rapid as those from superfused adrenals (see Table IX). Peak responses were observed anywhere from 90 to 180 minutes. When compared to superfusion with PGE_2 or to control (5 μg indomethacin/ml KRBG) perfusions, relatively high levels of B were maintained by PGE_2 (see Table X). In most PGE_2-perfused adrenals, the maximal response was less than a doubling in output except in one experiment (2P, Table IX) where there was a 6 fold increase. In general, the superfused adrenal responds to PGE_2 with a rapid increase (doubling) in output which it appears unable to maintain while the perfused adrenal does not increase its output as rapidly

but can maintain a high level for a longer period of time.

With superfused adrenals the response to ACTH decays slowly; this is clearly different from the rapid decay observed with PGE_2. In general, continuous perfusion with ACTH (see Table V) usually results in maintenance of the high initial output for several hours. Perfusion with PGE_2 elicits an increase in output which rises to some peak level (not usually seen with ACTH) followed by a decay which is more rapid than that observed with ACTH.

The flow rates for the adrenals perfused with 5 µg PGE_2 remained constant and within an acceptable rangefor the duration of the experiments. This suggests that PGE_2 (5 µg/ml) was not stimulating B output through an increase in flow rate. It has been suggested that unsaturated fatty acids resulting from the hydrolysis of adrenal cholesterol esters could be converted into vasodilator prostaglandins which could then stimulate steroidogenesis via a mechanism involving an increase in blood flow (Grant et al, 1968; Maier and Staehelin, 1968). The data of the present study do not support this hypothesis.

Perfusions carried out with a higher concentration of PGE_2 (10 µg/ml), with PGE_1 (10 µg/ml) and with $PGF_{1\alpha}$ (10 µg/ml) also showed stimulation of B output as well as individual variation in response (see Tables XI, XII and XIII). In some of the studies, adrenals were perfused with indomethacin (5 µg/ml KRBG) for 90 minutes followed by addition of the prostaglandin to the medium and the perfusion continued for varying amounts of time. Most of the adrenals responded within 60 minutes of the addition of the prostaglandin (see TablesXII and XIII). The washout period with the indomethacin-containing KRBG appeared to have shortened the lag period.

It may be that before the prostaglandin can act some interfering substance (perhaps bound ACTH) must be washed from the gland. This might be the reason why adrenals from hypophysectomized rats when superfused with PGE_2 showed an almost immediate response (Flack and Ramwell, 1972).

Prostaglandins E_1, E_2 and A have been shown to stimulate steroidogenesis in beef adrenal slices incubated in vitro (Saruta and Kaplan, 1972), while $PGF_{1\alpha}$ decreased both aldosterone and corticosterone synthesis. There appear to be no other reports pertaining to possible effects of $PGF_{1\alpha}$ on adrenal steroid synthesis. The present study is probably the first report of a stimulatory effect of $PGF_{1\alpha}$ on adrenal steroid output. This difference in effect of $PGF_{1\alpha}$ on beef adrenal slices and in the perfused rat adrenal may be related to a species difference or to the different methodology used.

PGE_1 and PGE_2 at two different concentrations (5 and 10 µg/ml) had no effect on control levels of corticosterone output from adrenal quarters incubated in vitro. This shows that there can be difference(s) between exposing an adrenal gland to a stimulatory substance in a flask and presenting the adrenal with the same substance via its vascular channels.

Most adrenals perfused with 10 µg of a prostaglandin (either PGE_1, PGE_2 or $PGF_{1\alpha}$) showed increases in adrenal flow rate which were accompanied by stimulation of B output (see figures 27, 28 and 29). The prostaglandins at the higher concentration used (10 µg/ml) were the only agents in the present study which led to an increase in flow which was directly related to increased B output. Prostaglandins of the E series are known to be vasodilators but the vascular effects

of different prostaglandins depend upon the species and the tissue type being studied (Horton, 1969). The effects of prostaglandins on adrenal vasculature in the rat are not known. PGE_2 at 5 μg/ml stimulated B output from perfused adrenals. An increase in concentration to 10 μg/ml didn't increase the magnitude of the steroidogenic response but caused an increase in adrenal flow rate. This suggests that at least for PGE_2, the only prostaglandin studied at two different doses (5 and 10 μg/ml) that the effect on flow rate may be a pharmacologic one.

The prostaglandin effects on adrenal corticosterone output described in this study can be summarized as follows;

(1) PGE_1, PGE_2 and $PGF_{1\alpha}$ can increase corticosterone output in perfused adrenal glands from intact rats.

(2) Considerable individual variation exists in the response of different adrenal glands to a particular prostaglandin.

(3) PGE_2 can increase corticosterone output in perfused adrenal glands from intact rats without concomitant increase in flow rate.

(4) PGE_1, PGE_2 and $PGF_{1\alpha}$ at levels of 10 μg/ml increase adrenal flow rates which are accompanied by increases in corticosterone output.

(5) PGE_1 and PGE_2 do not increase corticosterone output from in vitro-incubated adrenal sections.

APPENDIX

The importance of ligating venous branches which empty into the adrenal vein; early studies

The importance of ligating branches of the inferior phrenic vein and other small veins which empty into the adrenal vein (see I.P.V. and (x) figure 3) was not fully appreciated during the early stages in the development of this perfusion technique. Hence, the early studies were carried out with these veins remaining patent.

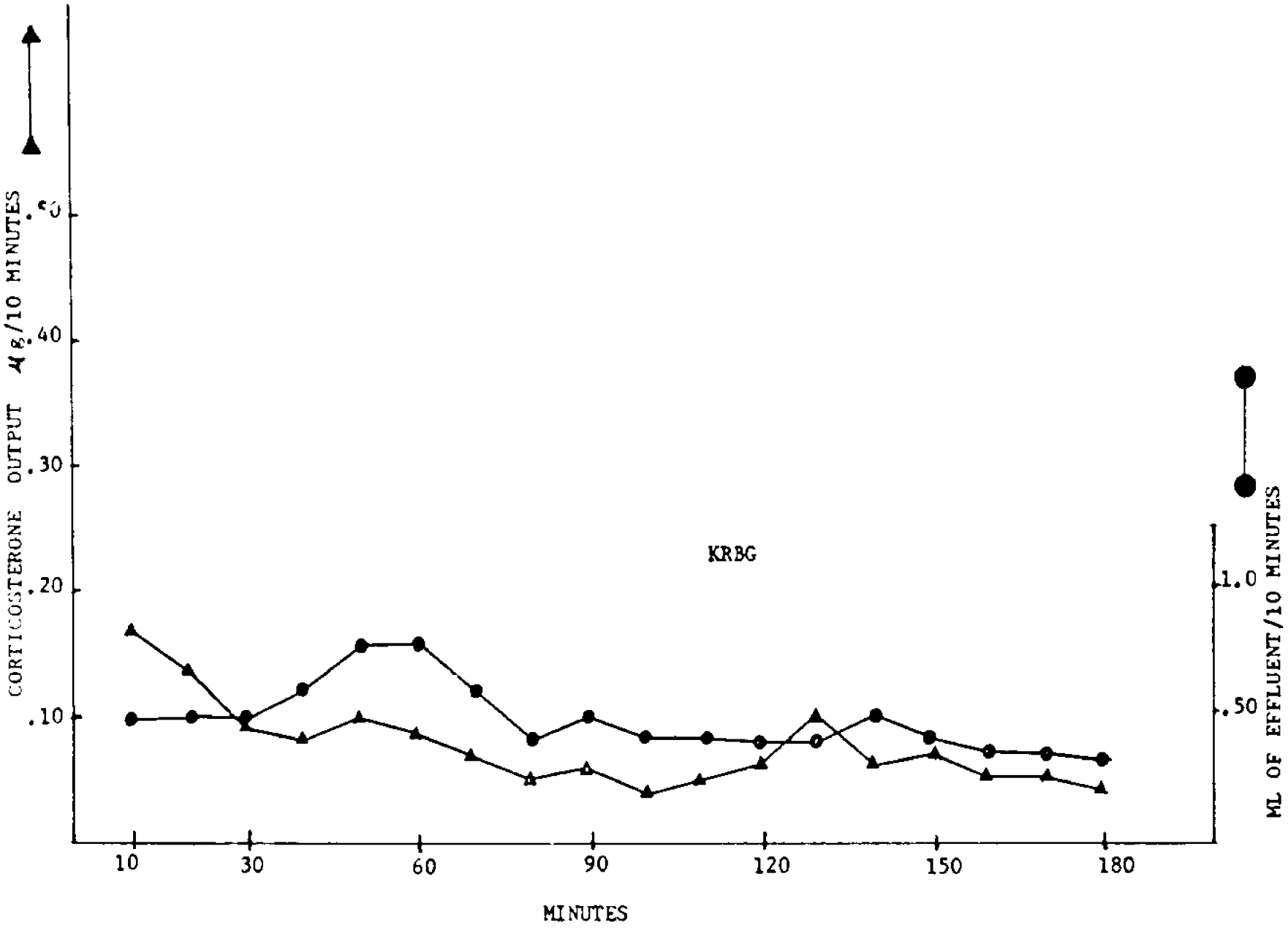
Perfusions were first carried out using unsupplemented Krebs Ringer bicarbonate glucose (KRBG). The objective of the early studies was to determine the rate of corticosterone output. Ten minute samples of venous effluent were collected and analyzed for corticosterone. In later experiments 30 minute samples were collected.

Difficulty was encountered in attempting to obtain an adequate output of corticosterone. The values ranged from less than 0.25 µg to 0.30 µg corticosterone per 10 minutes. This was 5 to 10 fold lower than levels previously reported by other workers using conventional *in vitro* incubation and superfusion methods (van der Vies, 1960; Birmingham et al, 1968; Tait et al, 1970).

Figure 33 shows a typical experiment of this type. Corticosterone (B) output is shown as a function of time of perfusion. Corticosterone output is slightly higher at the start of the perfusion (figure 33) but after 30 minutes drops to values, most of which are less than 0.10 µg B/10 minutes. In experiments of this type, the output often fell below the sensitivity of the fluorometric assay method (i.e. below 0.02 µg B/ml).

Figure 33. Time courses of corticosterone output and flow rate for an adrenal gland perfused for 3 hours with KRBG, venous tributaries emptying into the adrenal vein were not ligated.

Ordinate on left depicts B output expressed in micrograms/10 minutes.
Ordinate on right depicts flow rate expressed as milliliters of effluent collected per 10 minutes.
Abscissa is time in minutes.
Triangles represent total output of B in a 10 minute sample.
Circles represent total volume of a 10 minute sample (flow rate).


CORTICOSTERONE OUTPUT µg/10 MINUTES
.50
.40
.30
.20
.10
KRBG
1.0
.50
ML OF EFFLUENT/10 MINUTES
10
30
60
90
120
150
180
MINUTES

After addition of ACTH to the perfusing medium, no stimulation of B output was observed in most experiments. In some perfusions a high level of ACTH (1000 mU/ml KRBG) appeared to slightly increase the output of B and to maintain this increase for 60 to 90 minutes, after which the response decayed with time. Such an experiment is shown in figure 34. Perfusion for 45 minutes with KRBG was followed by exposing the gland to ACTH (1000 mU/ml KRBG) for another 45 minutes. The ACTH was then removed and the perfusion continued for 90 minutes with KRBG. ACTH appeared to slightly increase B output and to maintain this slight increase for 60 to 90 minutes. The experiment also suggested that the ACTH effect persisted for a short period of time (15 minutes) after its removal from the perfusing medium.

Figure 35 illustrates another early attempt to stimulate with ACTH. Here the gland was perfused for the first hour with KRBG, followed by two different doses of ACTH; 50 mU/ml KRBG during the second hour and 200 mU/ml KRBG during the third and fourth hours. This procedure elicited a gradual increase in B output which took about 60 minutes to reach a maximum level (a five fold increase), and then slowly decayed with time.

In neither of these experiments with ACTH (figures 34 and 35) was it possible to stimulate B output so that it reached the high levels usually measured at the start of the perfusion.

The irregular responses to ACTH along with the very low levels of B output indicated that the early studies were unsatisfactory. Possibly perfusate leaving the gland was being diluted by flow from extra-adrenal sources and that the net effect of this was inadequate perfusion of the gland. This belief was confirmed by the following

Figure 34. Time course of corticosterone output for an adrenal gland perfused for 45 minutes with KRBG, followed by exposure to ACTH (1000 mU/ml KRBG) for 45 minutes. The ACTH-containing perfusate was then removed and the perfusion continued for another 90 minutes with KRBG.

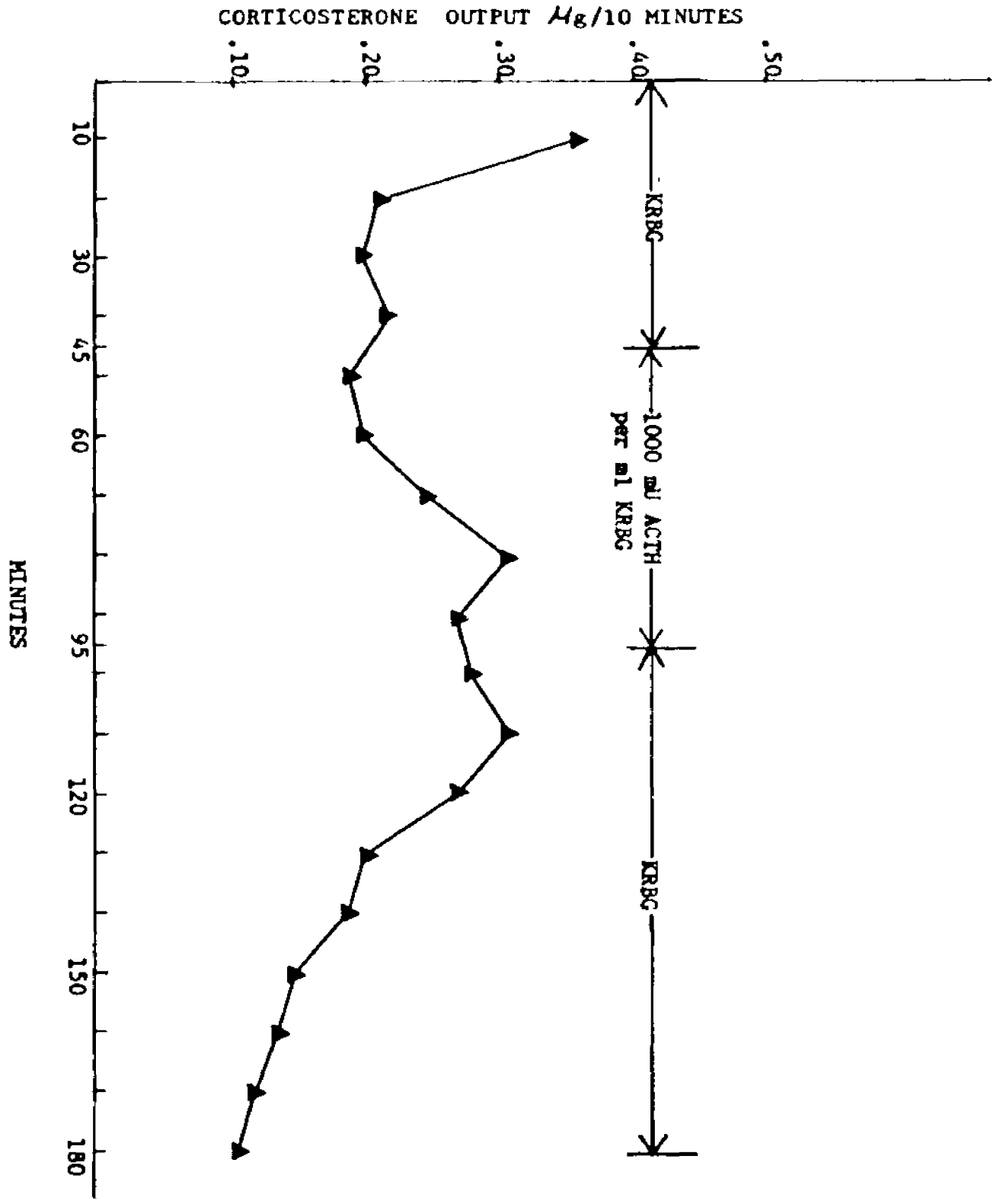
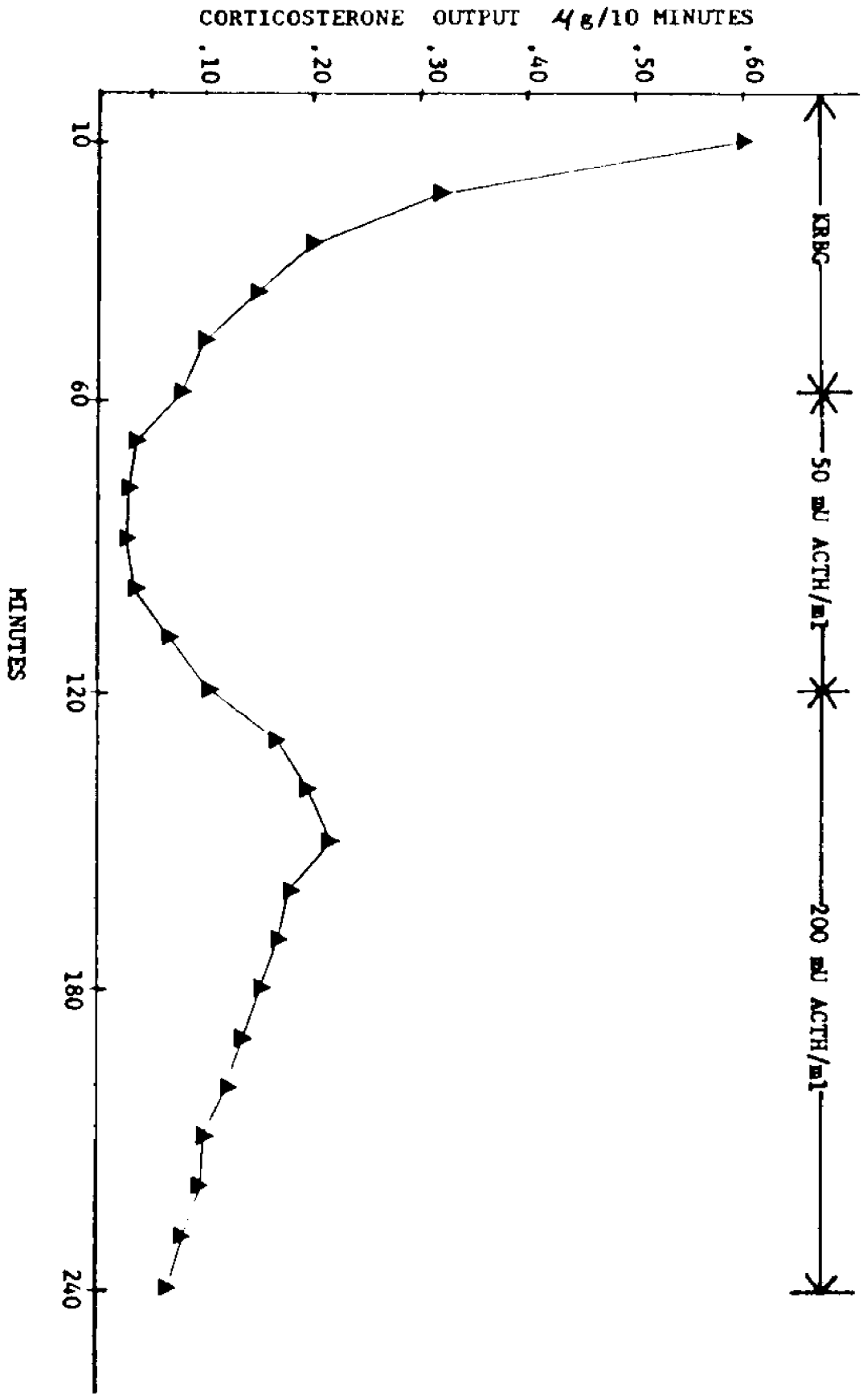

CORTICOSTERONE OUTPUT µg/10 MINUTES
.10
.20
.30
.40
.50
MINUTES
10
30
45
60
95
120
150
180
KRBG
1000 mU ACTH per ml KRBG
KRBG

Figure 35. Time course of corticosterone output for an adrenal gland perfused for 60 minutes with KRBG followed by exposure to ACTH (50 mU/ml KRBG) for 60 minutes. The ACTH concentration was then increased to 200 mU/ml KRBG and the perfusion continued for another 2 hours.


CORTICOSTERONE OUTPUT µg/10 MINUTES
.10
.20
.30
.40
.50
.60
10
60
120
180
240
MINUTES
KRBG
50 mU ACTH/ml
200 mU ACTH/ml

experiments: after perfusing with ACTH (100 mU/ml KRBG) for two hours the adrenal vein was ligated just at the point where it exits from the gland (see figure 3A), while continuing the perfusion. This experiment is illustrated in figure 36. It can be seen that B output was arrested but that flow of perfusate into the venous cannula continued even after ligating the adrenal vein. This confirmed the belief that much of the venous effluent was of extra-adrenal origin. The gland did not respond to ACTH and the output of B was low as in previous perfusions. The perfusion was continued under these conditions (adrenal vein ligated) for another two hours at which time the branches of the inferior phrenic vein and other veins which emptied into the adrenal vein were carefully ligated. This completely stopped the flow (figure 36) demonstrating that the glandular effluent was being diluted by flow coming from veins which emptied into the adrenal vein.

This experiment was repeated but all the visible venous branches which empty into the adrenal vein were ligated before the start of the perfusion. Addition of ACTH (100 mU/ml KRBG) from the start of the perfusion increased and maintained a high initial output of B for several hours as illustrated in figure 37. Corticosterone output was at least 5 to 7 fold higher than previously observed. After 3 hours of perfusion with ACTH, the adrenal vein was ligated at its exit from the gland and the perfusion continued. As a result of this manipulation the flow decreased about 90% and the output of B fell to an immeasurable amount (see figure 37). This type of experiment demonstrated that under these conditions (ligation of veins which empty into the adrenal vein) the adrenal gland is more adequately perfused

Figure 36. Time courses of corticosterone output and flow rate for an adrenal gland perfused with 100 mU ACTH/ml KRBG. After 2 hours of perfusion the adrenal vein was ligated at its exit from the gland. Perfusion was continued for another 2 hours at which time, all visible veins emptying into the adrenal vein were ligated.

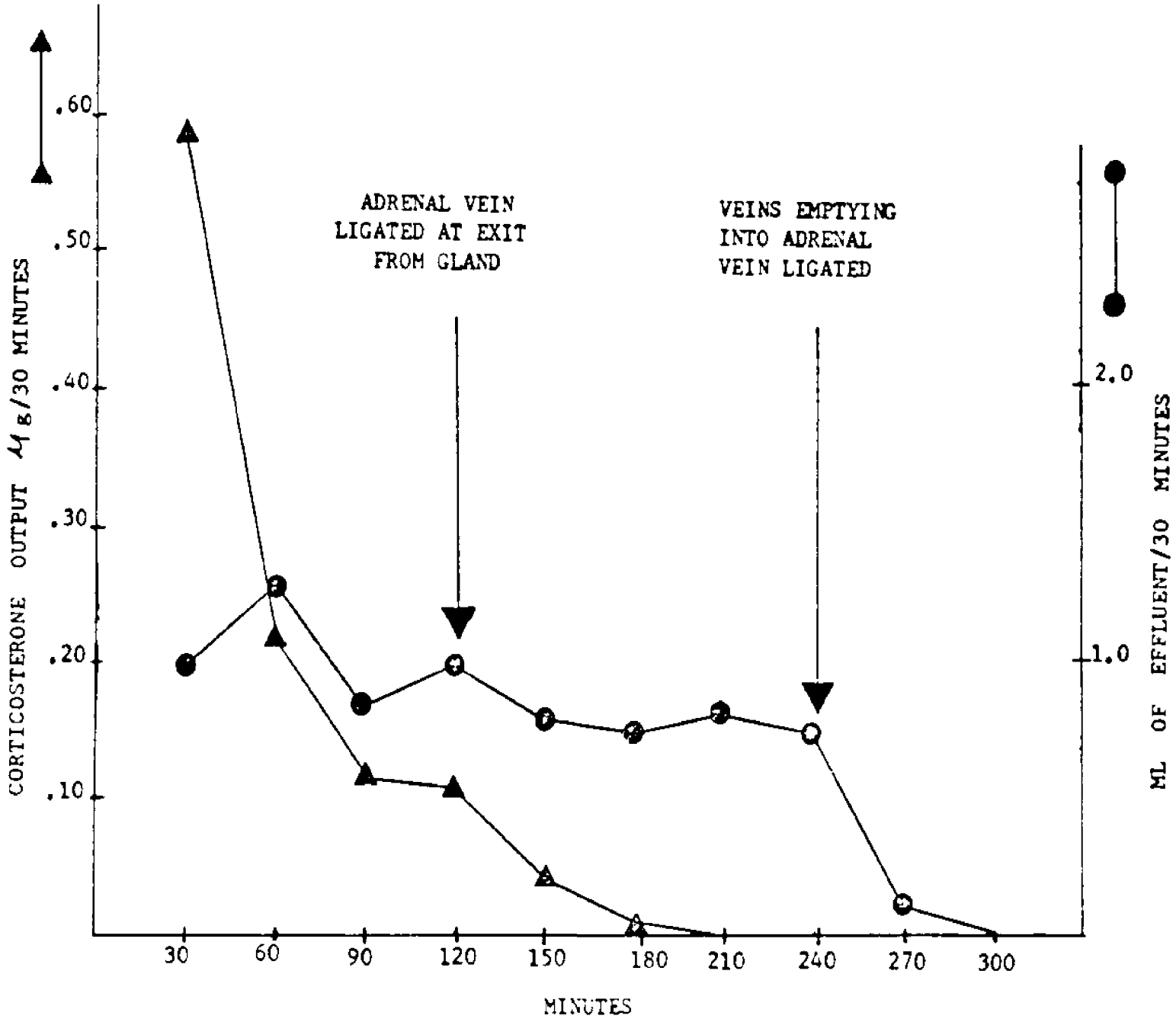
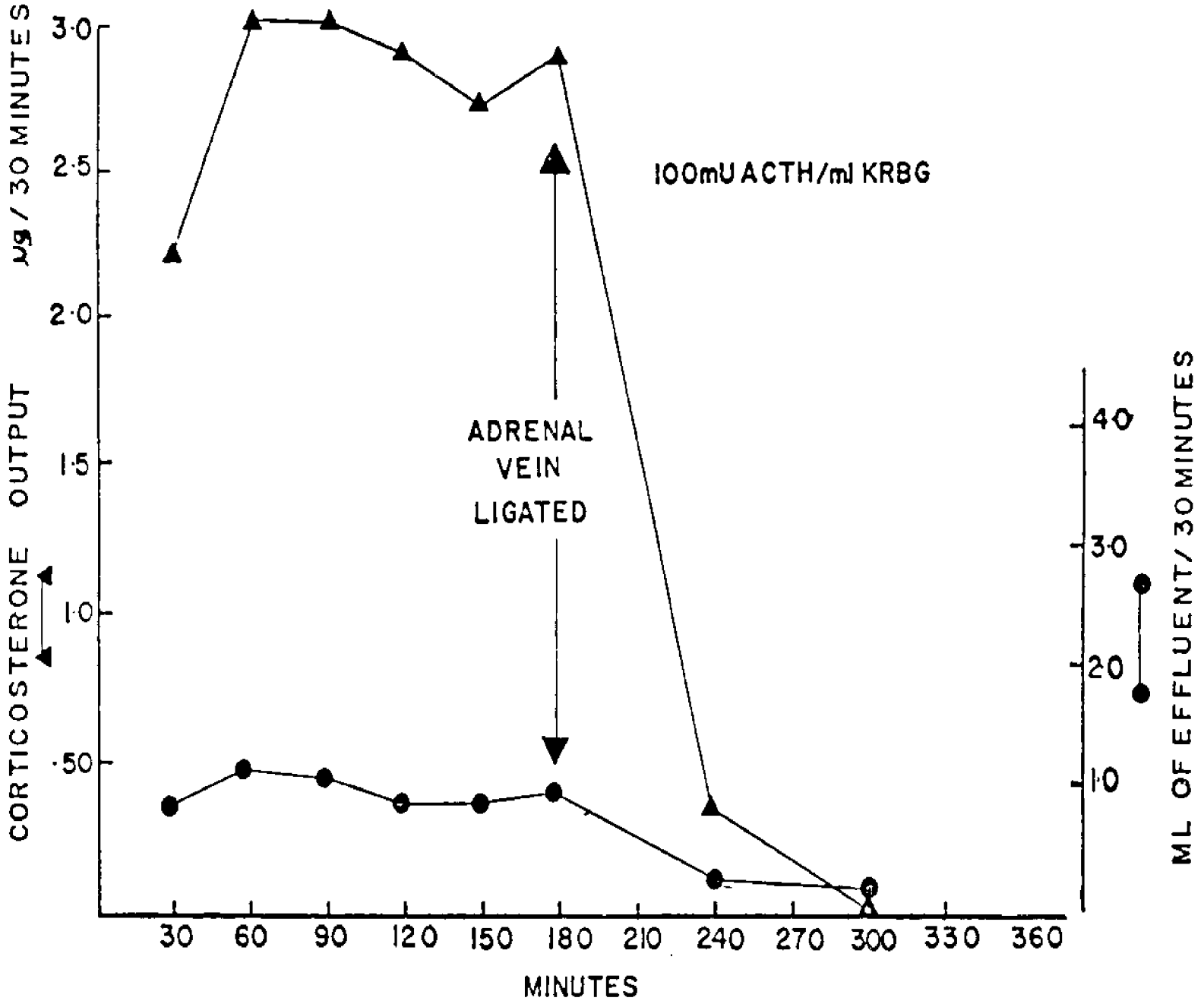

ADRENAL VEIN
LIGATED AT EXIT
FROM GLAND
VEINS EMPTYING
INTO ADRENAL
VEIN LIGATED
CORTICOSTERONE OUTPUT µg/30 MINUTES
.60
.50
.40
.30
.20
.10
ML OF EFFLUENT/30 MINUTES
2.0
1.0
30
60
90
120
150
180
210
240
270
300
MINUTES

Figure 37. Time courses of corticosterone output and flow rate for an adrenal gland perfused with 100 mU ACTH/ml KRBG. All visible veins emptying into the adrenal vein were ligated before the start of the perfusion. After 3 hours of perfusion the adrenal vein was ligated at its exit from the gland and the perfusion continued.


CORTICOSTERONE OUTPUT µg / 30 MINUTES
3·0
2·5
2·0
1·5
1·0
·50
100mU ACTH/ml KRBG
ADRENAL VEIN LIGATED
ML OF EFFLUENT/ 30 MINUTES
40
30
20
10
30 60 90 120 150 180 210 240 270 300 330 360
MINUTES

and that the venous effluent is of adrenal origin.

Extra-adrenal areas were believed to be perfused when the small venous tributaries were left patent. The small tributaries drained these (extra-adrenal) areas and emptied into the adrenal vein. The net effect was a partially perfused adrenal which would account for the low corticosterone output even though the flow rate remained within the desired range. In all the other studies described in this thesis, branches of the inferior phrenic vein and any other visible venous tributaries which emptied into the adrenal vein were always ligated.

REFERENCES

Addison, T. (1855). On the constitutional and local effects of disease of the suprarenal capsules. London: Highley Press.

Aldridge, R.R., Barrett, S., Brown, J.B., Funder, J.W., Goding, J.R., Kaltenbach, C.C. and Mole, B.J. (1970). The effect of prostaglandins on ovarian steroidogenesis in vivo. J. Reprod. Fert. 21:369-370.

Anggard, E. and Samuelsson, B. (1965). Biosynthesis of prostaglandins from arachidonic acid in guinea pig lung. Prostaglandins and related factors 38. J. Biol. Chem. 240:3518-3521.

Anson, B.J., Cauldwell, E.W., Pick, J.W. and Beaton, L.E. (1947). The blood supply of the kidney, suprarenal gland and associated structures. Surg. Gynec. Obstet. 84:313-320.

Bakker, R.F.M. and de Wied, D.(1961). The effect of corticotropin on the formation of corticosteroids in vitro. Can. J. Biochem. Physiol. 39:23-29.

Balfour, W.E. (1953). Changes in the hormone output of the adrenal cortex of the young calf. J. Physiol. (Lond.) 122:59P-60P.

Baniukiewicz, S.A., Brodie, A., Flood, C., Motta, M., Okamoto, M., Tait, J.F., Tait, S.A.S., Blair-West, J.R., Coghlan, J.P., Denton, D.A., Goding, J.R., Scoggins, B.A., Wintour, E.M. and Wright, R.D. (1968). Adrenal biosynthesis of steroids in vitro and in vivo using continuous superfusion and infusion procedures. In: Functions of the adrenal cortex. Vol. I, Ed: McKerns, K.W., pp. 153-232, Appleton Century-Crofts, N.Y.

Bar, H.P. and Hechter, O. (1969). Adenyl cyclase and hormone action. III. Calcium requirement for ACTH stimulation of adenyl cyclase. Biochem. Biophys. Res. Commun. 35:681-686.

Barthe, P., Desaulles, P.A., Schar, B. and Staehelin, M. (1964). Biological activity of synthetic β-corticotropin. Nature 202:908-910.

Behrman, H.R. and Greep, R.O. (1972). Hormonal dependence of cholesterol ester hydrolase in the corpus luteum and adrenal. Horm. Metab. Res. 4:206-209.

Beigelman, P.M., Slusher, M.A., Slater, G.G. and Roberts, S. (1956). Effect of anesthetics and collection time on corticosteroid secretion by rat adrenal. Proc. Soc. Exp. Biol. Med. 93:608-611.

Bell, P.H., Howards, K.S., Shepherd, R.G., Finn, B.M. and Meisenhelder, J.H. (1956). Studies with corticotropin. II. Pepsin degradation of β-corticotropin. J. Am. Chem. Soc. 78:5059-5066.

Ben, M., Dixon, R.L. and Adamson, R.H. (1969). Anesthesia in the rat. Fed. Proc. 28:1522-1527.

Bennett, H.S. and Kilham, L. (1940). The blood vessels of the adrenal gland of the adult cat. Anat. Rec. 77:447-471.

Bergström, S. (1967). Prostaglandins: Members of a new hormonal system. Science 157:382-391.

Bergström, S., Danielsson, H., Klenberg, D. and Samuelsson, B. (1964a). The enzymatic conversion of essential fatty acids into prostaglandins. Prostaglandins and related factors 34. J. Biol. Chem. 239:4006-4008.

Bergström, S., Danielsson, H. and Samuelsson, B. (1964b). The enzymatic formation of prostaglandin E_2 from arachidonic acid. Prostaglandins and related factors 32. Biochem. Biophys. Acta 90:207-210.

Beyer, K.F. and Samuels, L.T. (1956). Distribution of steroid 3β-OL-dehydrogenase in cellular structures of adrenal gland. J. Biol. Chem. 219-69-76.

Birmingham, M.K. and Kurlents, E. (1968). Inactivation of ACTH by isolated rat adrenals and inhibition of corticoid formation by adrenocortical hormones. Endocrinology 62:47-60.

Birmingham, M.K., Elliot, F.H. and Valère, P.H.-L. (1953). The need for the presence of calcium for the stimulation in vitro of rat adrenal glands by adrenocorticotrophic hormone. Endocrinology 53:687-689.

Birmingham, M.K., Kurlents, E., Lane, R., Muhlstock, B. and Traikov, H. (1960). Effects of calcium on the potassium and sodium content of rat adrenal glands, on the stimulation of steroid production by adenosine 3',5'-mono-phosphate and on the response of the adrenal to short contact with ACTH. Can. J. Biochem. Physiol. 38:1077-1085.

Birmingham, M.K., MacDonald, M.L. and Rochefort, J.G. (1968). Adrenal function in normal rats and in rats bearing regenerated adrenal glands. In: Functions of the adrenal cortex, Vol. II. Ed: McKerns, K.W., pp.647-689. Appleton Century-Crofts, N.Y.

Blair-West, J.R., Coghlan, J.P., Denton, D.A., Funder, J.W., Scoggins, B.A. and Wright, R.D. (1971). Effects of prostaglandin E_1 upon the steroid secretion of the adrenal of the sodium deficient sheep. Endocrinology 88:367-371.

Bradlow, H.L., Fukushima, D.K., Gallagher, T.F., Hellman, L., Hoffman, K., Li, C.H., Rosenfeld, R.S., Spencer, H. and Zumoff, B. (1963). Adrenal stimulation by adrenocorticotropic peptides. J. Clin. Endocrinol. Metab. 23:792-798.

Brown-Sequard, C.E. (1856). Recherches éxperimentales sur la physiologie et la pathologie des capsules surrénales. Arch. Générales de Medicine 8:385-410.

Bryson, M.J. and Sweat, M.L. (1968). Cleavage of cholesterol side-chain associated with cytochrome P-450, flavoprotein, and nonheme iron-protein derived from bovine adrenal cortex. J. Biol. Chem. 243:2799-2804.

Burstein, S. and Gut, M. (1969). A preliminary report on the intermediates in the conversion in vitro of cholesterol to pregnenolone in adrenal preparations. Steroids 14:207-217.

Burstein, S. and Gut, M. (1971). Biosynthesis of pregnenolone. Recent Progr. Horm. Res. 27:303-345.

Bush, I.E. (1953a). Species differences in adrenocortical secretion. J. Endocrinol. 9:95-100.

Bush, I.E. (1953b). Species differences and other factors influencing adrenocortical secretion. Ciba Foundation Colloquia on Endocrinology. London: J. & A. Churchill Ltd., 7:210-232.

Bush, I.E. (1968). General considerations affecting the use and interpretation of studies of steroid metabolism in vitro. Advances in the Biosciences 3:3-16.

Cade, R. and Perenich, T. (1965). Secretion of aldosterone by rats. Am. J. Physiol. 208:1026-1030.

Cammer, W., Cooper, D.Y. and Estabrook, R.W. (1968). Electron-transport reactions for steroid hydrolation by adrenal cortex mitochondria. In: Functions of the adrenal cortex. Vol. II, Ed: McKerns, K.W., pp. 943-992. Appleton Century-Crofts, N.Y.

Carballeira, A. and Durnhofer, F. (1967). Metabolism of cholesterol-4-^{14}C by adrenal preincubation media. Proc. Can. Fed. Biol. Soc. 174 (Abstract).

Carchman, R.A., Jaanus, S.D. and Rubin, R.P. (1971). The role of adrenocorticotropin and calcium in adenosine cyclic 3',5'-phosphate production and steroid release from the isolated, perfused cat adrenal gland. Mol. Pharmacol. 7:491-499.

Carney, J.A., Lewis, A., Walker, B.L. and Slinger, S.J. (1972). Effect of dietary rapeseed oil on the adrenocorticotrophin-induced production of prostaglandins in the rat adrenal. Biochem. Biophys. Acta 280:211-214.

Carpenter, M.P., Manning, L. and Wiseman, B. (1971). Prostaglandin synthesis in rat testis. Fed. Proc. 30:1081 (Abstract).

Carpenter, M.P. and Wiseman, B. (1970). Prostaglandins of rat testis. Fed. Proc. 29:248 (Abstract).

Carroll, K.K. (1962). Studies on the mechanisms by which erucic acid effects cholesterol metabolism. Distribution of erucic acid in adrenal and plasma lipids. Can. J. Biochem. Physiol. 40:1115-1122.

Castiglioni, A. (1941). A History of Medicine. Alfred A. Knopf, p. 428.

Cession-Fossion, A. (1969). Personal communication. Université de Liège, Liège, Belgium.

Cession-Fossion, A. (1964a). Perfusion "in vitro" des glandes surrénales du rat. Rev. Belge Path. 30:349-368.

Cession-Fossion, A. (1964b). Secretion de catecholamines par les surrénales du rat, perfusées "in situ". Rev. Franc. Etudes Clin. et Biol. IX:346-347.

Chasalow, F. and Pharris, B. (1972). Ovarian prostaglandin synthesis: Effect of aminoglutethimide and polyamines. Fed. Proc. 31:545 (Abstract).

Clayman, M., Tsang, D. and Johnstone, R.M. (1970). ACTH, corticosteroids and inhibition of protein synthesis. Endocrinology 86:931-934.

Cole, R.D., Li, C.H., Harris, J.I. and Pon, N.G. (1956). Corticotropins (ACTH). VIII. The action of pepsin on α-corticotropin and the c-terminal amino acid sequence. J. Biol. Chem. 219:903-916.

Constantopoulos, G., Carpenter, A., Satoh, P.S. and Tchen, T.T. (1966). Formation of isocaprealdehyde in the enzymatic cleavage of cholesterol side chain by adrenal extract. Biochemistry 5:1650-1652.

Constantopoulos, G. and Tchen, T.T. (1961). Cleavage of cholesterol side chain by adrenal cortex. I. Cofactor requirement and product of cleavage. J. Biol. Chem. 236:65-67.

Cook, D.M., Greer, M.A. and Kendall, J.W. (1972). The half-life of endogenous immunoreactive ACTH in rat plasma. Proc. Soc. Exp. Biol. Med. 139:972-974.

Cooper, D.Y., Narasimhulu, S., Rosenthal, O. and Estabrook, R.W. (1968). Studies on the mechanism of C-21-hydroxylation of steroids by the adrenal cortex. In: Function of the Adrenal Cortex, Vol. II, Ed: McKerns, K.W., pp.897-992. Appleton Century-Crofts, N.Y.

Cortés, J.M., Peron, F.G. and Dorfman, R.I. (1963). Secretion of 18-hydroxydeoxycorticosterone by the rat adrenal gland. Endocrinology 73:713-720.

Critchlow, V., Liebelt, R.A., Bar-Sela, M., Mountcastle, W. and Lipscomb, H.S. (1963). Sex difference in resting pituitary-adrenal function in the rat. Am. J. Physiol. 205:807-815.

Davis, W.W. and Garren, L.D. (1968). On the mechanism of action of adrenocorticotropic hormone. The inhibitory site of cyclohexamide in the pathway of steroid biosynthesis. J. Biol. Chem. 243:5153-5157.

Dawson, W., Ramwell, P.W. and Shaw, J.E. (1968). Metabolism of prostaglandins by the rat isolated liver. Brit. J. Pharmacol. 34:668P-669P.

Dear, W.E. and Guillemin, R. (1960). Adrenal sensitivity to ACTH as a function of time after hypothalamic lesion and after hypophysectomy. Proc. Soc. Exp. Biol. Med. 103:356-358.

De Wied, D., Witter, A., Versteeg, D.H.G. and Mulder, A.H. (1969). Release of ACTH by substances of central nervous system origin. Endocrinology 85:561-569.

Dexter, R.N., Fishman, L.M., Ney, R.L. and Liddle, G.W. (1967). Inhibition of adrenal corticosteroid synthesis by aminoglutethimide: studies on the mechanism of action. J. Clin. Endocrinology 27:473-480.

Douglas, W.W. and Rubin, R.P. (1961). The role of calcium in the secretory response of the adrenal medulla to acetylcholine. J. Physiol. (Lond.) 159:40-57.

Eik-Nes, K.B. (1971). Production and secretion of testicular steroids. Recent Progr. Horm. Res. 27:517-535.

Ellis, L.C. (1972). Rat testicular prostaglandin synthesis and its relationship to androgen synthesis. Fed. Proc. 31:295 (Abstract).

Espiner, E.A., Jensen, C.A. and Hart, D.S. (1972). Dynamics of adrenal response to sustained local ACTH infusions in conscious sheep. Am. J. Physiol. 222:570-577.

Estabrook, R.W., Cooper, D.Y. and Rosenthal, O. (1963). The light reversible carbon monoxide inhibition of the steroid C-21-hydroxylase system of the adrenal cortex. Biochem. Z. 338:741-755.

Farese, R.V. (1971). On the requirement for calcium during the steroidogenic effect of ACTH. Endocrinology 89:1057-1074.

Farrell, G.L., Rauschkulb, E.W. and Royce, P.C. (1955). Secretion of aldosterone by the adrenal of the dog. Effects of hypophysectomy and ACTH. Am. J. Physiol. 182:269-272.

Feldberg, W. (1940). The action of Bee venom, cobra venom and lysolecithin on the adrenal medulla. J. Physiol. (Lond.) 99:104-118.

Ferguson, J.J., Jr. (1963). Protein synthesis and adrenocorticotropin responsiveness. J. Biol. Chem. 238:2754-2759.

Ferguson, J.J., Jr. and Morita, Y. (1964). RNA synthesis and adrenocorticotropin responsiveness. Biochim. Biophys. Acta 87:348-350.

Ferreira, S.H., Moncada, S. and Vane, J.R. (1971). Indomethacin and aspirin abolish prostaglandin release from the spleen. Nature New Biology 231:237-239.

Ferreira, S.H. and Vane, J.R. (1967). Prostaglandins: Their disappearance from and release into the circulation. Nature 216:868-873.

Fichman, M.P., Litenberg, G., Brooker, G. and Horton, R. (1972). Effect of prostaglandin A_1 on renal and adrenal function in man. Circ. Res. 31:19-35.

Finn, F.M., Widnell, C.C. and Hofmann, K. (1972). Localization of an adrenocorticotropic hormone receptor on bovine adrenal cortical membranes. J. Biol. Chem. 247:5695-5702.

Flack, J.D. and Ramwell, P.W. (1972). A comparison of the effects of ACTH, cyclic AMP, dibutyryl cyclic AMP and PGE_2 on corticosteroidogenesis in vitro. Endocrinology 90:371-377.

Flack, J.D., Jessup, R. and Ramwell, P.W. (1969). Prostaglandin stimulation of rat corticosteroidogenesis. Science 163:691-692.

Flack, J.D., Ramwell, P.W. and Shaw, J.E. (1971). Endocrinological implications of prostaglandins. In: Current Topics in Experimental Endocrinology, Vol. I., Ed: Martini, L. and James, V.H.T., pp. 199-228, Academic Press, N.Y.

Flint, J.M. (1900). The blood vessels, angiogenesis, organogenesis, reticulum and histology of the adrenal. Johns Hopkins Hosp. Rep. 9:153-231.

Funder, J.W., Blair-West, J.R., Coghlan, J.P., Denton, D.A., Scoggins, B.A. and Wright, R.D. (1969). The effects of prostaglandin E_1 upon corticosteroid secretion. Aust. J. Exp. Biol. Med. Sci. 47:11-12.

Garren, L.D. (1968). The mechanism of action of adrenocorticotropic hormone. Vitamins and Hormones 26:119-145.

Garren, L.D., Ney, R.L. and Davis, W.W. (1965). Studies on the role of protein synthesis in the regulation of corticosterone production by adrenocorticotropic hormone in vivo. Proc. Nat. Acad. Sci. U.S.A. 53:1443-1450.

Gersh, I. and Grollman, A. (1941). The vascular pattern of the adrenal gland of the mouse and rat and its physiological response to changes in glandular activity. Contr. Embryol. Carneg. Instn. 29:113-125.

Gidez, L.I. and Feller, E. (1969). Effect of the stress of unilateral adrenalectomy on the depletion of individual cholesteryl esters in the rat adrenal. J. Lipid. Res. 10:656-659.

Gill, G.N. (1972). Mechanism of ACTH action. Metabolism 21:571-588.

Gill, G.N. and Garren, L.D. (1970). A cyclic-3',5'-adenosine monophosphate dependent protein kinase from the adrenal cortex: Comparison with a cyclic AMP binding protein. Biochem. Biophys. Res. Commun. 39:335-343.

Gill, G.N. and Garren, L.D. (1971). Role of the receptor in the mechanism of action of adenosine 3',5'-cyclic monophosphate. Proc. Nat. Acad. Sci. U.S.A. 68:786-790.

Gilmore, N., Vane, J.R. and Wyllie, J.H. (1968). Prostaglandins released by the spleen. Nature 218:1135-1140.

Girard, F. and Vance, V.K. (1962). Modification of Lipscomb-Nelson bio-assay for ACTH. Endocrinology 71:325-328.

Giroud, C.J.P., Stachenko, J. and Venning, E.H. (1962). Secretion of aldosterone by the zona glomerulosa of rat adrenal glands incubated in vitro. Proc. Soc. Exp. Biol. Med. 92:154-158.

Glick, D. and Ochs, M.J. (1955). Studies in histochemistry: Quantitative histological distribution of cholesterol in adrenal glands of the cow, rat and monkey, and effects of stress conditions, ACTH cortisone and deoxycorticosterone. Endocrinology 56:285-298.

Goldman, H. (1961). Endocrine gland blood flow in the unanesthetized, unrestrained rat. J. Appl. Physiol. 16:762-764.

Goldman, H. (1966). Catecholamine-induced redistribution of blood flow in the unanesthetized rat. Am. J. Physiol. 210:1419-1423.

Goodman, D.S. (1965). Cholesterol ester metabolism. Physiol. Rev. 45:747-839.

Grahame-Smith, D.G., Butcher, R.W., Ney, R.L. and Sutherland, E.W. (1967). Adenosine 3',5'-monophosphate as the intracellular mediator of the action of adrenocorticotropic hormone on the adrenal cortex. J. Biol. Chem. 242:5535-5541.

Grant, J.K. (1968). The biosynthesis of adrenocortical steroids. J. Endocrinology 41:111-135.

Grant, J.K., Dobbie, J.W., Mackay, A.M., Symington, C. and Riley, C. (1968). The production and secretion of adrenocortical steroids in man. In: Biogenesis and Action of Steroid Hormones. Eds: Dorfman, R.I., Yamasaki, K. and Dorfman, M., pp. 74-92. Geron-X Inc., Los Altos, California.

Greene, E.C. (1968). Anatomy of the rat. Trans. Amer. Phil. Soc. Vol. XXVII. p. 199, Hafner Publ. Co., N.Y.

Guillemin, R., Clayton, G.W., Smith, J.D. and Lipscomb, H.S. (1959). Measurements of free corticosteroids in rat plasma: Physiological validation of a method. Endocrinology 63:349-358.

Guillemin, R., Dear, W.E. and Liebelt, R.A. (1959). Nychthermeral variations in plasma free corticosteroid levels of the rat. Proc. Soc. Exp. Biol. Med. 101:394-395.

Halberg, F., Peterson, R.E. and Silber, R.H. (1959). Phase relations of 24-hour periodicities in blood corticosterone, mitoses in cortical adrenal parenchyma and total body activity. Endocrinology 64:222-230.

Halkerston, I.D.K. (1968). Heterogeneity of the response of adrenal cortex tissue slices to adrenocorticotrophin. In: Functions of the Adrenal Cortex, Vol. I., Ed: McKerns, K.W., pp. 399-461. Appleton Century-Crofts, N.Y.

Halkerston, I.D.K., Eichhorn, J. and Hechter, O. (1961). A requirement for reduced triphosphopyridine nucleotide for cholesterol side chain cleavage by mitochondrial fractions of ox adrenal cortex. J. Biol. Chem. 236:374-380.

Halkerston, I.D.K., Feinstein, M. and Hechter, O. (1968). Effect of lytic enzymes upon the responsivity of rat adrenals in vitro. I. Effect of trypsin upon the steroidogenic action of reduced triphosphopyridine nucleotide. Endocrinology 83:61-73.

Hall, P.F. and Young, D.G. (1968). Site of action of trophic hormones upon the biosynthetic pathways to steroid hormones. Endocrinology 82:559-568.

Harding, B.W. and Nelson, D.H. (1964). Effects of hypophysectomy on NADP and NADPH concentration and corticosteroid secretion in the rat adrenal. Endocrinology 75:501-505.

Harding, B.W., Bell, J.J., Oldham, S.B. and Wilson, L.D. (1968). Corticosteroid biosynthesis in adrenal cortex mitochondrion. In: Functions of the Adrenal Cortex, Vol. II., Ed: McKerns, K.W., pp.831-896, Appleton Century-Crofts, N.Y.

Harrison, R.G. (1951). A comparative study of the vascularization of the adrenal gland in the rabbit, rat and cat. J. of Anat. (Lond.) 85:12-23.

Harrison, R.G. and Hoey, M.J. (1960). The Adrenal Circulation. pp. 1-70, Blackwell Scientific Publications, Oxford.

Hartman, F.A. (1937). The hormones of the adrenal cortex. Cold Spring Harbor Symp. Quant. Biol. 5:289-298.

Hartman, F.A., Brownell, K.A., Hartman, W.E., Dean, G.A. and MacArthur, C.G. (1928). The hormones of the adrenal cortex. Amer. J. Physiol. 86:353-359.

Hartman, F.A., Brownell, K.A. and Liu, T.Y. (1955). Blood flow through the dog adrenal. Am. J. Physiol. 180:375-377.

Hayano, M. and Dorfman, R.I. (1953). The enzymatic C-11 β-hydroxylation of steroids. J. Biol. Chem. 201:175-188.

Hayano, M., Saba, N., Dorfman, R.I. and Hechter, O. (1956). Some aspects of the biogenesis of adrenal steroid hormones. Recent Progr. Horm. Res. 12:79-118.

Haynes, R.C., Jr. (1958). The activation of adrenal phosphorylase by the adrenocorticotropic hormone. J. Biol. Chem. 233:1220-1222.

Haynes, R.C., Jr., Koritz, S.B. and Peron, F.G. (1959). Influence of adenosine 3',5'-monophosphate on corticoid production by rat adrenal glands. J. Biol. Chem. 234:1421-1423.

Haynes, R., Savard, K. and Dorfman, R.I. (1952). An action of ACTH on adrenal slices. Science 116:690-691.

Haynes, R., Savard, K. and Dorfman, R.I. (1954). The action of adrenocorticotropic hormone on beef adrenal slices. J. Biol. Chem. 207:925-938.

Hechter, O. (1949). Corticosteroid release from the isolated adrenal gland. Fed. Proc. 8:70.

Hechter, O. and Halkerston, I.D.K. (1964). On the action of mammalian hormones. In: The Hormones, Vol. V., Eds: Pincus, G., Thimann, K.V. and Astwood, E.B., pp. 697-816. Academic Press. N.Y.

Hechter, O. and Pincus, G. (1954). Genesis of adrenocortical secretion. Physiol. Rev. 34:459-496.

Hechter, O., Jacobsen, R.P., Jeanloz, R., Levy, H., Marshall, C.W., Pincus, G. and Schenker, V. (1949). The bio-oxygenation of 11-deoxycorticosterone at C-11. J. Amer. Chem. Soc. 71:3261-3262.

Hechter, O., Jacobsen, R.P., Schenker, V., Levy, H., Jeanloz, R.W., Marshall, W. and Pincus, G. (1953). Chemical transformation of steroids by adrenal perfusion: perfusion methods. Endocrinology 52:679-691.

Hechter, O., Zaffaroni, A. Jacobsen, R.P., Levy, H., Jeanloz, R.W., Schenker, V. and Pincus, G. (1951). The nature and biogenesis of the adrenal secretory product. Recent Progr. Horm. Res. 6:215-246.

Hedge, G.A. (1972). The effect of prostaglandins on ACTH secretion. Endocrinology 91:925-933.

Hilton, J.G., Weaver, D.C., Muehlheims, G., Glaviano, V.V. and Wegria, R. (1958). Perfusion of the isolated adrenals in situ. Am. J. Physiol. 192:525-530.

Hinman, J.W. (1972). Prostaglandins. Ann. Rev. Biochem. 41:161-178.

Hochberg, R.B., Luttrell, B., Ross, D.W. and Lieberman, S. (1971). Evidence for the existence of steroidal free radicals in hormone biosynthesis. Program 53rd Meeting Endocr. Soc. U.S.A. p. A-47, (Abstract).

Hofmann, F.G. and Davison, C. (1953). Effect of ACTH and various substrates on in vitro adrenal cortical steroid synthesis. J. Clin. Endocrinol. 13:848.

Holzbauer, M. (1957). The corticosterone content of rat adrenals under different experimental conditions. J. Physiol. (Lond.) 139:294-305.

Holzbauer, M. and Newport, H.M. (1969). Adrenal secretion rates and adrenal tissue concentration of pregnenolone, progesterone, 11-β-OH-andostenedione and some other steroids in young pigs and dogs. J. Physiol. (Lond.) 200:821-848.

Holzbauer, M. and Vogt, M. (1957). Functional changes produced in the adrenal cortex of the rat by administration or by release of corticotropin. J. Physiol. (Lond.) 138:449-459.

Holzbauer, M., Newport, H.M., Birmingham, M.K. and Traikov, H. (1969). Secretion of preg-4-ene-3,20 dione (progesterone) in vivo by the adrenal gland of the rat. Nature 221:572-573.

Horton, E.W. (1969). Hypotheses on physiological roles of prostaglandins. Physiol. Rev. 49:122-161.

Horton, E.W. (1972). Prostaglandins P.59, Springer-Verlag, N.Y.

Houssay, B.A. and Lewis, J.T. (1923). The relative importance to life of cortex and medulla of the adrenal glands. Amer. J. Physiol. 64:512-521.

Huibregtse, W.H. and Ungar, F. (1970). Temporal and quantitative aspects of continuous superfusion of rat adrenals. Life Sciences 9:349-359.

Hume, D.M. and Nelson, D.H. (1954). Corticoid output in the adrenal venous blood of the intact dog. Fed. Proc. 13:73.

Jaanus, S.D. and Rubin, R.P. (1971). The effect of ACTH on calcium distribution in the perfused cat adrenal gland. J. Physiol. (Lond.) 213:581-598.

Jaanus, S.D., Carchman, R.A. and Rubin, R.P. (1972). Further studies on the relationship between cyclic AMP levels and adrenocortical activity. Endocrinology 91:887-895.

Jaanus, S.D., Rosenstein, M.J. and Rubin, R.P. (1970). On the mode of action of ACTH on the isolated perfused adrenal gland. J. Physiol. (Lond.) 209:539-556.

Jones, I.C. (1957). The Adrenal Cortex. pp.1-3. Cambridge At the University Press, Cambridge, U.K.

Karaboyas, G.C. and Koritz, S.B. (1965). Identity of the site of action of 3',5'-adenosine monophosphate and adrenocorticotropic hormone in corticosteroidogenesis in rat adrenal and beef adrenal cortex slices. Biochemistry 4:462-468.

Kass, E.H., Hechter, O., Macchi, I.A. and Mou, T.W. (1954). Changes in patterns of secretion of corticosteroids in rabbits after prolonged treatment with ACTH. Proc. Soc. Exp. Biol. Med. 85:583-587.

Kelly, L.A. and Koritz, S.B. (1971). Bovine adrenal cortical adenyl cyclase and its stimulation by adrenocorticotropic hormone and NaF. Biochim. Biophys. Acta 237:141-155.

Kendall, J.W., Jr. (1970). Personal communication. Univ. of Oregon Medical School, Dept. of Medicine.

Kloppenborg, P.W.C., Island, D.P., Liddle, G.W., Michelakis, A.M. and Nicholson, W.E. (1968). A method of preparing adrenal cell suspensions and its applicability to the in vitro study of adrenal metabolism. Endocrinology 82:1053-1058.

Koritz, S.B. (1973). Personal communication. Mt. Sinai School of Medicine, City Univ. of N.Y., Dept. of Biochemistry.

Koritz, S.B. and Kumar, A.M. (1970). On the mechanism of action of the adrenocorticotrophic hormone. The stimulation of the activity of enzymes involved in pregnenolone synthesis. J. Biol. Chem. 245:152-159.

Koritz, S.B. and Peron, F.G. (1958). Studies on the mode of action of the adrenocorticotropic hormone. J. Biol. Chem. 230:343-352.

Koritz, S.B., Peron, F.G. and Dorfman, R.I. (1957). Influence of adrenocorticotropic hormone on corticoid production and glycine-1-C^{14} incorporation into protein by rat adrenals. J. Biol. Chem. 226:643-650.

Kowal, J. (1969a). Metabolic events associated with steroid biosynthesis in adrenal tissue cultures. Trans. N.Y. Acad. Sci. 31:359-378.

Kowal, J. (1969b). Adrenal cells in tissue culture. IV. Use of an inhibitor of steroid synthesis for the study of ACTH action. Endocrinology 85:270-279.

Kramer, R.J. and Sapirstein, L.A. (1967). Blood flow to the adrenal cortex and medulla. Endocrinology 81:403-405.

Krieger, D.T. (1972). Circadian corticosteroid periodicity: critical period for abolition by neonatal injection of corticosteroid. Science 178:1205-1207.

Kunze, H. and Vogt, W. (1971). Significance of phospholipase A for prostaglandin formation. Ann. N.Y. Acad. Sci. 180:123-125.

L'Age, M., Gonzales-Luque, A. and Yates, F.E. (1970). Adrenal blood flow dependence of cortisol secretion rate in unanesthetized dogs. Am. J. Physiol. 219:281-287.

Lands, W.E.M. and Samuelsson, B. (1968). Phospholipid precursors of prostaglandins. Biochim. Biophys. Acta 164:426-429.

Lands, W., Lee, R. and Smith, W. (1971). Factors regulating the biosynthesis of various prostaglandins. Ann. N.Y. Acad. Sci. 180:107-122.

Laplante, C. and Stachenko, J. (1966). Aldosterone and corticosterone production by rat adrenal slices as a function of time of incubation. Can. J. Biochem. 44:85-89.

Lefkowitz, R.J., Roth, J. and Pastan, I. (1970). Effects of calcium on ACTH stimulation of the adrenal: Separation of hormone binding from adenyl cyclase activation. Nature 228:864-866.

Lever, J.D. (1955). Cellular-vascular relationships in the adrenal cortex as studied in the rat. In: The Human Adrenal Cortex. Ciba Foundation Colloquia on Endocrinology. Vol. VIII, pp.42-51. Little Brown & Co., Boston.

Levy, H., Jeanloz, R.W., Jacobsen, R.P., Hechter, O., Schenker, V. and Pincus, G. (1954). Chemical transformations of steroids by adrenal perfusion. J. Biol. Chem. 211:867-881.

Lewis, G.P. and Matthews, J. (1969). The cause of the vasodilation accompanying free fatty acid release in rabbit adipose tissue. J. Physiol. (Lond.) 202:95P-96P.

Li, C.H. (1962). I. Hormonal polypeptides and their functional activities. Synthesis and biological properties of ACTH peptides. Recent Progr. Horm. Res. 18:1-32.

Li, C.H. and Oelofsen, W. (1967). The chemistry and biology of ACTH and related peptides. In: The Adrenal Cortex. Ed: Eisenstein, A.B., pp.185-201, Little-Brown, Boston.

Liddle, G.W., Island, D. and Meador, C.K. (1962). Normal and abnormal regulation of corticotropin secretion in man. Recent Progr. Horm. Res. 18:125-153.

Liemann, F. (1971). The effect of a temporary interruption of the blood supply to the suprarenal gland on steroid biosynthesis and ACTH control of the adrenal cortex. Nauyn-Schmiedebergs Arch. Pharmak. 268:161-170.

Lipscomb, H.S. and Nelson, D.H. (1962). A sensitive biologic assay for ACTH. Endocrinology 71:13-23.

Lipson, L., Hynie, S. and Sharp, G. (1971). Effect of prostaglandin E_1 on osmotic water flow and sodium transport in the toad bladder. Ann. N.Y. Acad. Sci. 180:261-275.

Long, C.N.H. (1947). The relation of cholesterol and ascorbic acid to the secretion of the adrenal cortex. Recent Progr. Horm. Res. 1:99-122.

Lucis, R., Carballeira, A. and Venning, E.H. (1965). Biotransformation of progesterone-4-^{14}C and 11-deoxycorticosterone-4-^{14}C by rat adrenal glands in vitro. Steroids 6:737-756.

Macchi, I.A. and Hechter, O. (1954). Lack of influence of ACTH upon corticosteroid biosynthesis in cow adrenal homogenates. Arch. Biochem. 53:305-307.

Macho, L. and Palkovic, M. (1970). Lipolytic enzymes and the action of ACTH on lipid metabolism in rat adrenals. Hormones 1:285-292.

Macho, L. and Saffran, M. (1967). Metabolism of fatty acids in the rat adrenal gland. Endocrinology 81:179-185.

McCracken, J.A., Glew, M.E. and Scaramuzzi, R.J. (1970). Corpus luteum regression induced by prostaglandin $F_{2\alpha}$. J. Clin. Endocrinol. Metab. 30:544-546.

McDonald, I.R. and Reich, M. (1959). Corticosteroid secretion by the autotransplanted adrenal gland of the conscious sheep. J. Physiol. (Lond.) 147:33-50.

McKerns, K.W. (1968). Mechanisms of ACTH regulation of the adrenal cortex. In: Functions of the Adrenal Cortex, Vol. I., Ed: McKerns, K.W., pp. 479-537, Appleton Century-Crofts, N.Y.

McKerns, K.W. and Nordstrand, E. (1955). An in vitro assay of corticotrophin. Can. J. Biochem. Physiol. 33:209-216.

Maier, R. and Staehlin, M. (1968). Adrenal responses to corticotrophin in the presence of an inhibitor of protein synthesis. Acta Endocrinol. 58:619-629.

Margoulies, M., Coninx, P., Palem-Vliers, M. and Genard, P. (1970). Étude cinétique de la production de corticostérone par des surrénales de rats in vitro. Influence des ions Ca^{++} et de certains inhibitors de la synthese protéique sur l'action de L'ACTH. Ann. Endocrinol. 31:1215-1224.

Matsuyama, H., Ruhmann-Wennhold, A., Johnson, L.R. and Nelson, D.H. (1972). Disappearance rates of exogenous and endogenous ACTH from rat plasma measured by bioassay and radioimmunoassay. Metabolism 21:30-35.

Matthews, E.K. and Saffran, M. (1972). Effect of ions and ACTH on steroidogenesis and membrane potential of rabbit adrenocortical tissue. IV. Int. Congr. Endocrinol. Excerpta Medica Intern. Congr. Ser. 256:570 (Abstract).

Moncloa, F., Peron, F.G. and Dorfman, R.I. (1959). The fluorimetric determination of corticosterone in rat adrenal tissue and plasma: Effect of administering ACTH subcutaneously. Endocrinology 65:717-724.

Monder, C. and Kendall, J. (1972). Sulfuric acid-induced fluorescence of corticosteroids: Effects of position substituents on fluorescence. Unpublished observations.

Müller, O.A. and Scriba, P.C. (1969). Subcellular distribution and arteriovenous differences of synthetic ^{3}H-β1-23 corticotrophin-23-amide-acetate in the adrenal of rats. Acta Endocrinol. 60:463-472.

Munson, P.L. and Toepel, W. (1958). Detection of minute amounts of adrenocorticotropic hormone by the effect on adrenal venous ascorbic acid. Endocrinology 63:785-793.

Muraoka, R. (1965). Experimental study on the role of essential fatty acids and pyridoxine on adrenocortical function. Arch. Jap. Chir. 34:35-51.

Ney, R.L., Davis, W.W. and Garren, L.D. (1966). Heterogeneity of template RNA in adrenal glands. Science 153:896-897.

Orti, E., Baker, R.K., Lanman, J.T. and Brasch, H. (1965). Simple method for in vitro metabolic studies with continuous flow incubation. J. Lab. Clin. Med. 66:973-979.

Osler, W. (1896). Case of Addison's disease death during treatment with suprarenal extract. Bull. Johns Hopkins Hosp. 7:208-209.

Peng, T.C., Six, K.M. and Munson, P.L. (1970). Effects of prostaglandin E_1 on the hypothalamo-hypophyseal-adrenocortical axis in rats. Endocrinology 86:202-206.

Peron, F.G. (1961). Isolation of 18-hydroxycorticosterone from rat adrenals. Program 43rd Meeting Endocr. Soc. U.S.A. 82 (Abstract).

Peron, F.G. and McCarthy, J.L. (1968). Corticosteroidogenesis in the rat adrenal gland. In: Functions of the adrenal cortex. Vol. I, Ed: McKerns, K.W., pp.261-337, Appleton Century-Croft, N.Y.

Peron, F.G. and Robidoux, W.F. (1964). Further studies on the biosynthesis of corticoids by rat adrenal fractions and inhibition by endogenous cholesterol utilization by cobra venom. Biochim. Biophys. Acta 82:125-137.

Pharriss, B.B. (1970). The effect of prostaglandin on luteal steroidogenesis. Proc. III Intern. Congr. Horm. Ster., Hamberg, Sept. 1970. Excerpta Medica Foundation, Intern. Congr. Ser.

Pharriss, B.B., Wyngarden, L.J., Gutnecht, G.D. (1968). Biological interactions between prostaglandins and luteotropins in the rat. In: Gonadotropins. Ed: Rosenberg, E., pp.121-129, Geron-X, Inc. Los Altos, California.

Piper, P. and Vane, J. (1971). The release of prostaglandins from lung and other tissues. Ann. N.Y. Acad. Sci. 180:363-383.

Porter, J.C. and Klaiber, M.S. (1964). Relationship of input of ACTH to secretion of corticosterone in rats. Amer. J. Physiol. 207:789-792.

Porter, J.C. and Klaiber, M.S. (1965). Corticosterone secretion in rats as a function of ACTH input and adrenal blood flow. Am. J. Physiol. 209:811-814.

Ramwell, P.W. and Shaw, J.E. (1967). Prostaglandin release from tissues by drug, nerve and hormone stimulation. In: Prostaglandins. Eds: Bergström, S. and Samuelsson, B., pp.283-292, Almqvist and Wicksell, Uppsala.

Ramwell, P.W. and Shaw, J.E. (1970). Biological significance of the prostaglandins. Recent Progr. Horm. Res. 26:139-173.

Ramwell, P. and Shaw, J.E. (1971). The biological significance of the prostaglandins. Ann. N.Y. Acad. Sci. 180:10-13.

Ramwell, P.W., Shaw, J.E., Douglas, W.W. and Poisner, A.M. (1966). Efflux of prostaglandin from adrenal glands stimulated with acetylcholine. Nature 210:273-274.

Rees, L.H., Cook, D.M., Kendall, J.W., Allen, C.F., Kramer, R.M., Ratcliffe, J.G. and Knight, R.A. (1971). A radioimmunoassay for rat plasma ACTH. Endocrinology 89:254-261.

Reich, E. and Lehninger, A.L. (1955). Conversion of cholesterol to corticosteroids in adrenal homogenates. Biochim. Biophys. Acta 17:136-137.

Reichstein, T. and Shoppee, C.W. (1943). The hormones of the adrenal cortex. Vitamins and Hormones 1:345-413.

Rhodin, J.A.G. (1971). The ultrastructure of the adrenal cortex of the rat under normal and experimental conditions. J. Ultrastruc. Res. 34:23-71.

Richardson, M.C. and Schulster, D. (1972). Corticosteroidogenesis in isolated adrenal cells: Effect of adrenocorticotrophic hormone, adenosine 3',5'-monophosphate and β^{1-24} adrenocorticotrophic hormone diazotized to polyacrylamide. J. Endocrinol. 55:127-139.

Robison, G.A., Butcher, R.W. and Sutherland, E.W. (1971). Cyclic AMP and hormone action. In: Cyclic AMP pp.17-46, Academic Press,N.Y.

Rogoff, J.M. and Stewart, G.N. (1928). The influence of adrenal extract on the survival period of adrenalectomized dogs. Science 66:327-328.

Rubin, R.P. (1970). The role of calcium in the release of neurotransmitter substances and hormones. Pharmacol. Rev. 22:389-428.

Rubin, R.P., Carchman, R.A. and Jaanus, S.D. (1972). Role of calcium and adenosine cyclic 3',5'-phosphate in action of adrenocorticotropin. Nature New Biology 240:150-152.

Rubin, R.P., Jaanus, S.D. and Miele, E. (1969). Calcium dependent corticosteroid release from perfused cat adrenal gland. Experientia 25:1327-1328.

Rudman, D. and Garcia, L.A. (1966). Effect of adrenocorticotropin on the concentration of triglyceride in the adrenal gland of the hypophysectomized rat. Endocrinology 78:1087-1088.

Ryan, K.J. and Engel, L.L. (1957). Hydroxylation of steroids at carbon 21. J. Biol. Chem. 225:103-114.

Saba, N., Hechter, O. and Stone, D. (1954). Conversion of cholesterol to pregnenolone in bovine adrenal homogenates. J. Am. Chem. Soc. 76:3862-3864.

Saffran M. and Bayliss, M.J. (1953). In vitro bioassay of corticotrophin. Endocrinology 52:140-148.

Saffran M. and Rowell, P. (1969). Response of rat adrenal tissue to ACTH in a flowing system. Endocrinology 85:652-656.

Saffran, M. and Schally, A.V. (1955). In vitro bioassay of corticotropin: Modification and statistical treatment. Endocrinology 56:523-532.

Saffran, M., Grad, B. and Bayliss, M.J. (1953). Production of corticoids by rat adrenal in vitro. Endocrinology 50:639-643.

Saffran, M., Matthews, E.K. and Pearlmutter, F. (1971). Analysis of the response to ACTH by rat adrenal in a flowing system. Recent Progr. Horm. Res. 27:607-630.

Saffran, M., Ford, P., Matthews, E.K., Kraml, M. and Garbaczewska, L. (1967). An automated method for following the production of corticosteroids by adrenal tissue in vitro. Can. J. Biochem. 45:1901-1907.

Samuelsson, B. (1967). Biosynthesis and metabolism of prostaglandins. Progr. Biochem. Pharmacol. 3:59-70.

Samuelsson, B. (1969). Biosynthesis of prostaglandins. Progr. Biochem. Pharmacol. 5:109-128.

Samuelsson, B. (1972). Biosynthesis of prostaglandins. Fed. Proc. 31:1442-1450.

Sapirstein, L.A. and Goldman, H. (1959). Adrenal blood flow in the albino rat. Am. J. Physiol. 196:159-162.

Saruta, T. and Kaplan, N.M. (1972). Adrenocortical steroidogenesis: the effects of prostaglandins. J. Clin. Invest. 51:2246-2251.

Sayers, G., Beal, R.J. and Seelig, S. (1972). Isolated adrenal cells: Adrenocorticotropic hormone, calcium, steroidogenesis, and cyclic adenosine monophosphate. Science 175:1131-1133.

Sayers, G., Swallow, R.L. and Giordano, N.D. (1971). An improved technique for the preparation of isolated rat adrenal cells: A sensitive accurate and specific method for the assay of ACTH. Endocrinology 88:1063-1068.

Schimmer, B.P., Ueda, K. and Sato, G.H. (1968). Site of action of adrenocorticotropic hormone (ACTH) in adrenal cell cultures. Biochem. Biophys. Res. Commun. 32:806-810.

Schindler, W.L. (1959). Cortical secretion and steroidogenesis by the adrenal gland of the golden hamster. Ph.D. Thesis, University of California, Los Angeles.

Schönbaum, E., Birmingham, M.K. and Saffran, M. (1956). Metabolism of glucose and steroid formation by rat adrenals in vitro. Can. J. Biochem. Physiol. 34:527-533.

Schulster, D., Tait, S.A.S., Tait, J.F. and Mrotek, J. (1970). Production of steroids by in vitro superfusion of endocrine tissue. III. Corticosterone output from rat adrenals stimulated by adrenocorticotropin or cyclic 3',5'-adenosine monophosphate and the inhibitory effect of cyclohexamide. Endocrinology 86:487-502.

Schwyzer, R. and Sieber, P. (1963). Total synthesis of adrenocorticotrophic hormone. Nature 199:172-174.

Selinger, R.C.L. and Civen, M. (1971). ACTH diazotized to agarose: Effects on isolated adrenal cells. Biochem. Biophys. Res. Commun. 43:793-799.

Shaw, J.E. and Ramwell, P.W. (1967). Prostaglandin release from the adrenal gland. In: Prostaglandins. Eds: Bergström, S. and Samuelsson, N., pp.293-299, Almqvist and Wiksell, Stockholm.

Silber, R.H., Busch, R.D. and Oslapas, R. (1958). Practical procedure for estimation of corticosterone or hydrocortisone. Clin. Chem. 4:278-285.

Simpson, E.R., Cooper, D.Y. and Estabrook, R.W. (1969). Metabolic events associated with steroid hydroxylation by the adrenal cortex. Recent Progr. Horm. Res. 25:523-562.

Singer, B. and Stack-Dunne (1955). The secretion of aldosterone and corticosterone by the rat adrenal. J. Endocrinol. 12:130-145.

Smith, P.E. (1930). Hypophysectomy and a replacement therapy in the rat. Amer. J. Anat. 45:205-273.

Smith, W.L. and Lands, W.E.M. (1971). Stimulation and blockade of prostaglandin biosynthesis. J. Biol. Chem. 246:6700-6704.

Smith, J.B. and Willis, A.L. (1971). Aspirin selectively inhibits prostaglandin production in human platlets. Nature New Biology 231:235-237.

Solomon, S. Levitan, P. and Lieberman, S. (1956). Possible intermediates between cholesterol and pregnenolone in corticosteroidogenesis. Rev. Canad. Biol. 15:282-283.

Spät, A., Sarkadi, B., Intödy, Zs., Körner, A. and Szanto, J. (1971). Effect of renal papillary lipids and prostaglandin E_2 on corticosteroid production in the rat. Acta Physiol. Acad. Sci.Hung. 40:187-199.

Speroff, L. and Ramwell, P.W. (1970). Prostaglandin stimulation of in vitro progesterone synthesis. J. Clin. Endocrinol. Metab. 30:347-350.

Staehelin, M., Barthe, P. and Desaulles, P.A. (1965). On the mechanism of the adrenal gland response to adrenocorticotrophic hormone in hypophysectomized rats. Acta Endocrinol. 50:55-64.

Stark, E. and Varga, B. (1968). Effect of ACTH on target-organ blood flow with special reference to an extra-adrenal effect. Acta Med. Acad. Sci. Hung. 25:367-381.

Stone, D. and Hechter, O. (1954). Studies on ACTH action in perfused bovine adrenals: The site of action of ACTH in corticosteroidogenesis. Arch. Biochem. 51:457-469.

Strobel, G.E. and Wollman, H. (1969). Pharmacology of anesthetic agents. Fed. Proc. 28:1386-1403.

Swallow, R.L. and Sayers, G. (1969). A technique for the preparation of isolated rat adrenal cells. Proc. Soc. Exp. Biol. Med. 131:1-4.

Sweat, M.L. (1951). Enzymatic synthesis of 17-hydroxycorticosterone. J. Am. Chem. Soc. 73:4056.

Sweat, M.L. and Farrell, G.L. (1954). Decline of corticosteroid secretion following hypophysectomy. Proc. Soc. Exp. Biol. Med. 87:615-618.

Swingle, W.W. and Pfiffner, J.J. (1931). Studies on the adrenal cortex. I. The effect of a lipid fraction upon the life-span of adrenalectomized cats. Amer. J. Physiol. 96:153-179.

Sydnor, K.L. and Sayers, G. (1953). Biological half-life of endogenous ACTH. Proc. Soc. Exp. Biol. Med. 83:729:733.

Symington, T. (1962). Morphology and secretory cytology of the human adrenal cortex. Brit. Med. Bull. 18:117-121.

Symington, T., Duguid, W.P. and Davidson, J.N. (1956). Effect of exogenous corticotropin on histochemical pattern of human adrenal cortex and comparison with changes during stress. J. Clin. Endocrinol. & Metabol. 16:580-598.

Tait, S.A.S., Tait, J.F., Okamoto, M. and Flood, C. (1967). Production of steroids by in vitro superfusion of endocrine tissue. I. Apparatus and a suitable analytical method for adrenal steroid output. Endocrinology 81:1213-1225.

Tait, S.A.S., Schulster, D., Okamoto, M., Flood, C. and Tait, J.F. (1970). Production of steroids by in vitro superfusion of endocrine tissue. II. Steroid output from bisected whole, capsular and decapsulated adrenals of normal intact, hypophysectomized and hypophysectomized-nephrectomized rats as a function of time of superfusion. Endocrinology 86:360-382.

Taunton, O.D., Roth, J. and Pastan, I. (1967). ACTH stimulation of adenyl cyclase in adrenal homogenates. Biochem. Biophys. Res. Commun. 29:1-7.

Taunton, O.D., Roth, J. and Pastan, I. (1969). Studies on the adrenocorticotropic hormone-activated adenyl cyclase of a functional adrenal tumor. J. Biol. Chem. 244:247-253.

Tchen, T.T. (1968). Conversion of cholesterol to pregnenolone in the adrenal cortex: Enzymology and regulation. In: Functions of the Adrenal Cortex, Vol. I., Ed: McKerns, K.W., pp.3-26, Appleton Century-Crofts, N.Y.

Traikov, H. and Birmingham, M.K. (1966). Tests for 11 β-hydroxyprogesterone and other C-21 steroids. Can. J. Biochem. 44:291-292.

Triller, H. and Birmingham, M.K. (1965). Steroid production by incubated mouse adrenals. II. Control of steroid output. Gen. Comp. Endocrinol. 5:624-630.

Tsang, C. and Carballeira, A. (1966). Enzyme activities in the preincubation media from adrenal tissue incubations. Proc. Soc. Exp. Biol. Med. 122:1031-1035.

Tsang, C.P.W. and Stachenko, J. (1970. Effect of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and adrenocorticotrophic hormone (ACTH) on steroidogenesis by intact and sectioned rat adrenals. Steroids 16:707-722.

Uete, T., Shimogami, A. and Wasa, M. (1970). Sulfuric acid fluorescence of various steroid hormones. J. Biochem. 67:1-7.

Ungar, F. and Halberg, F. (1962). Circadian rhythm in the in vitro response of mouse adrenal to adrenocorticotropic hormone. Science 137:1058-1060.

Urquhart, J. (1965). Adrenal blood flow and the adrenocortical response to corticotropin. Am. J. Physiol. 209:1162-1168.

Urquhart, J. and Li, C.C. (1968). The dynamics of adrenocortical secretion. Am. J. Physiol. 214:73-85.

Van der Vies, J. (1960). Corticoid production in vitro as a test of adrenocortical function in rats. Acta Endocrinol. 33:59-66.

Van Dorp, D.A. (1967). Aspects of the biosynthesis of prostaglandins. Progr. Biochem. Pharmacol. 3:71-82.

Van Dorp, D.A., Beerthius, R.K., Nugteren, D.H. and Vonkeman, H. (1964a). The biosynthesis of prostaglandins. Biochim. Biophys. Acta 90:204-207.

Van Dorp, D.A. Beerthius, R.K., Nugteren, D.H. and Vonkeman, H. (1964b). Enzymatic conversion of all-cis-polyunsaturated fatty acids into prostaglandins. Nature 203:839-841.

Vane, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature New Biology 231:232-235.

Vinson, G.P. (1966). Pathways of corticosteroid biosynthesis from pregnenolone and progesterone in rat adrenal glands. J. Endocrinology 34:355-363.

Vinson, G.P. and Whitehouse, B.J. (1970). Comparative aspects of adrenocortical function. In: Advances in Steroid Biochemistry and Pharmacology. Ed: Briggs, M.H., pp.163-319, Academic Press, N.Y.

Vogt, M. (1943). The output of cortical hormone by the mammalian suprarenal. J. Physiol. (Lond.) 102:341-356.

Vogt, M. (1947). Biological assays of cortical hormones and estimation of rate of secretion of mammalian suprarenal cortex. J. Endocrinol. 5:LVII-LXI.

Vogt, M. (1951). Cortical secretion of the isolated perfused adrenal. J. Physiol. (Lond.) 113:129-156.

Vogt, M. (1953). The output of cortical hormone by the mammalian suprarenal. J. Physiol. (Lond.) 102:341-356.

Walker, B.L. and Carney, J.A. (1971). Response of rat adrenal cholesteryl esters to cold stress. Lipids 6:797-804.

Walton, G.M., Gill, G.N., Abrass, I.B.and Garren, L.D. (1971). Phosphorylation of ribosome-associated protein by an adenosine 3',5'-cyclic monophosphate-dependent protein kinase: Location of the microsomal receptor and protein kinase. Proc. Nat. Acad. Sci. U.S.A. 68:880-884.

Ward, P.J. and Birmingham, M.K. (1960). Properties of the ultraviolet-absorbing lipids produced by rat adrenals in vitro. Biochem.J. 76:269-279.

Weeks, J.R. (1972). Prostaglandins. Ann. Rev. Pharmacol. 12:317-336.

Weisbroth, S.H. (1969). The origin of the Long Evans rat and a review of the inheritance of coat colors in rats. Lab. Anim. Care 19:733-737.

Whitehouse, B.J. and Vinson, G.P. (1971). Compartmental arrangement of steroid precursors and the control of steroid hormone secretion in rat adrenal tissue in vitro. Acta Endocrinol. 68:467-476.

Wilroy, R.S., Camacho, A.M., Trouy, R.L. and Hagen, A.A. (1968). Inhibition of adrenal cortical secretion by amino-glutethimide in dogs. Endocrinology 83:56-60.

Wilson, D.B. and Kitabchi, A.E. (1971). Steroidogenesis in isolated adrenal cells (IAC) and its relation to adenyl cyclase and phosphodiesterase (PDE). Clin. Res. 19:654 (Abstract).

Wilson, D.E., Philips, C., Levine, R.A. (1971). Inhibition of gastric secretion in man by prostaglandin A_1. Gastroenterology 61:201-206.

Wright, R.D. (1963). Blood flow through the adrenal gland. Endocrinology 72:418-428.

Zaffaroni, A., Hechter, O. and Pincus, G. (1951). Adrenal conversion of C^{14} labelled cholesterol and acetate to adrenal cortical hormones. J. Am. Chem. Soc. 73:1390-1391.

Zenker, N. and Bernstein, D.E. (1958). The estimation of small amounts of corticosterone in rat plasma. J. Biol Chem. 231:695-701.

Zor, U., Kaneko, T., Lowe, I.P., Bloom, G. and Field, J.B. (1969). Effect of thyroid-stimulating hormone and prostaglandins on thyroid adenyl cyclase activation and cyclic adenosine 3',5'-monophosphate. J. Biol. Chem. 244:5189-5195.