FURTHER STUDIES ON THE CATABOLISM OF THYMIDINE AND THE REUTILIZATION OF ITS DEGRADATIVE PRODUCTS IN *TETRAHYMENA PYRIFORMIS*

by

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ABSTRACT

Previous experiments in our laboratory on the conversion of radiolabeled thymidine into biologically useful macromolecules other than DNA indicated that a reductive pyrimidine catabolic and reutilization pathway may be functioning in Tetrahymena pyriformis. In an effort to broaden the restrictive definition of nucleic acid "salvage" to include the reincorporation of such thymidine degradative fragments into biologically useful macromolecules other than nucleic acids, we initiated the present study with the radiolabeled resubstantiation of this previously proposed reductive thymidine catabolic and reutilization pathway.

To substantiate this overall reductive thymidine catabolic and reutilization pathway more rigorously, however, it was thought that at least some of the enzymes involved in these interconversions must also be demonstrated. The first enzyme in this proposed pathway, dihydrothymine dehydrogenase, was investigated. After the preliminary demonstration of this enzyme, we then became particularly interested in the unique properties of the enzyme that apparently catalyzes the critical conversion of the proposed reductive thymidine catabolic end product, B-aminoisobutyric acid, to the initial anabolic reutilization substrate, probably methylmalonic semialdehyde. We thus chose to study this enzyme in greater detail.

Although most of the studies on the further metabolism

of B-aminoisobutyric acid in other organisms suggested the transamination of this amino acid to methylmalonic semialdehyde followed by its further oxidation to methylmalonic acid, such a transaminating system could not be demonstrated. By means of a sensitive fluorometric assay system, however, we were able to demonstrate a low, but significant, amount of such B-aminoisobutyric acid oxidase activity.

In homogenates this enzymatic activity exhibited the following characteristics: (1) good activity in alkaline 0.2 M Tris-HCl buffer with a rather broad pH optimum ranging from 7.8 to 9.0; (2) optimum activity at a temperature of 37°C; (3) stimulation upon the addition of exogenous FAD; (4) inhibition upon the addition of divalent cations, EDTA, or PCMB; (5) little stimulation upon the addition of detergents; and (6) no increase in activity upon repeated freezing and thawing. Crude preparations of this oxidase were also found (1) to be relatively stable when stored up to 1 week either refrigerated or frozen; (2) to have a specific activity of 2.8 nmoles/min/mg of protein, and (3) to have a K_m of 3.6×10^{-1} M for D,L-B-aminoisobutyric acid.

Subsequent centrifugal fractionation studies indicated that a substantial amount of this oxidase activity may be associated with a subcellular organelle, probably the mitochondrion. Preliminary $(NH_4)_2SO_4$ fractionation and affinity chromatography studies also indicated that this enzyme appears to be a unique and specific oxidase whose activity is separable from other marker enzymes, including other oxidases.

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First and foremost, I wish to thank my sponsor, Dr. John Berech, for his belief in my scientific potential and for the opportunity to work in his laboratory.

I also wish to thank all the members of my committee, especially Dr. James Hogg for his careful reading of my thesis and his most valuable comments.

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LIST OF ABREVIATIONS

M&M	= materials and methods	DOC	= deoxycholate
CMP	= cytidylic acid	SDS	= sodium dodecyl sulfate
UMP	= uridylic acid	MDH	= malate dehydrogenase
TMP	= thymidylic acid	CAT	= catalase
dTMP	= deoxythymidylic acid	BDMA	= benzyldimethylamine
MMA	= methylmalonic acid	Tdine	= thymidine
BAIB	= B-aminoisobutyric acid	DHP	= dihydropyrimidine
B-ala	= B-alanine	DHT	= dihydrothymine
PEP	= phosphoenolpyruvic acid	DHTdine	= dihydrothymidine
OAA	= oxaloacetic acid	DHU	= dihydrouracil
αKG	= α-ketoglutaric acid	DHUdine	= dihydrouridine
lac	= lactic acid	DHO	= dihydroorotic acid
TCA	= trichloroacetic acid	DHP-DH	= dihydropyrimidine dehydrogenase
RRP	= Ryley-Ringer phosphate	DHT-DH	= dihydrothymine dehydrogenase
PALP	= pyridoxal 5'-phosphate	DHU-DH	= dihydrouracil dehydrogenase
EM	= electron microscopy	BUIB	= B-ureidoisobutyric acid
TEM	= transmission electron microscopy	BUPA	= B-ureidopropionic acid
val	= valine	A	= absorbance
ala	= alanine	ΔF	= change in fluorescence
asp	= aspartic acid	HVA	= homovanillic acid
glu	= glutamic acid	pHMB	= p-hydroxymercuribenzoate
met	= methionine	PCMB	= p-chloromercuribenzoate

FURTHER STUDIES ON THE CATABOLISM OF THYMIDINE AND THE REUTILIZATION OF ITS DEGRADATIVE PRODUCTS IN *TETRAHYMENA PYRIFORMIS*

INTRODUCTION

Since its axenic isolation in pure culture over 50 years ago by Lwoff,[1] *Tetrahymena pyriformis* has proved to be a useful eukaryote for the study of many biochemical processes including nucleic acid metabolism. Atypically, however, *Tetrahymena pyriformis* has been found to require for growth a preformed purine, preferably guanine, as well as a preformed pyrimidine, preferably uracil,[2,3] both of which most other organisms are quite capable of synthesizing *de novo*. Adenine is able to spare, but not replace, the guanine requirement, while cytidine or CMP, but not cytosine can replace the uracil requirement.[2,3] This rather unusual nutritional requirement has resulted in the development of *Tetrahymena pyriformis* as a useful model organism for evaluating the possible effectiveness of various pur-

ine and pyrimidine analogs for the treatment of certain human diseases. The basis for testing 8-azaguanine, the first purine analog shown to have an inhibitory effect on the growth of tumors, for example, was that it had previously been found to inhibit the growth of Tetrahymena pyriformis.[2,3]

Other aspects of pyrimidine metabolism in Tetrahymena pyriformis also appear to be unusual.[4,5] Uracil, for example, can be reversibly converted into uridine, but the uridine in turn cannot be phosphorylated into uridylic acid (UMP). Uracil, however, can be directly converted into UMP by the action of UMP pyrophosphorylase.[6,7] In addition, cytidine can be oxidatively deaminated to uridine but can form neither cytosine nor cytidylic acid (CMP).[8] Cline and Conner[9] further claim that uracil, through the deamination of cytosine, is the major pyrimidine excretory product of RNA breakdown. Previous studies from our laboratory,[10,11] however, indicated that uracil, like thymine, may also be reductively degraded to some extent.

But while the current state of knowledge concerning nucleic acid metabolism in Tetrahymena pyriformis is extensive, there still remain several important unanswered questions. For example, how is guanine converted to adenine?[12] How is uracil converted to thymine? Little is known even about the role of thymine itself in the pyrimidine metabolism of Tetrahymena pyriformis, although it is well established that thymine is not required for growth, cannot

spare uracil, and therefore cannot serve as the sole source of nucleic acid pyrimidines.[2,3]

Thymidine phosphorylase and kinase have been described along with some non-specific phosphatases capable of removing the phosphate group of thymidylic acid (TMP),[13-15] but the early failure to detect even a sparing of the pyrimidine requirement by thymine and/or thymidine was originally interpreted[16] as indicating that thymidine might be synthesized from non-pyrimidine precursors, rather than by utilization of the preformed ring. Further nucleic acid analyses, however, showed the concentration of DNA to be very low compared to that of RNA. The amount of thymine which would be required in the synthesis of this DNA, therefore, would be so small that its sparing effect on the total pyrimidine requirement could easily be overlooked. More sensitive radioactive tracer experiments eventually confirmed the assumption that thymine was indeed synthesized from uracil, without dilution of the isotope, when uracil is the only pyrimidine supplied.[17]

With the current research interest in the subcellular localization of extranuclear DNA in Tetrahymena pyriformis,[18-28] the ultimate metabolic fate of thymine in this organism takes on increased significance. The importance of thymine metabolism in this regard is due to the fact that in general cytoplasmic DNA is most sensitively localized by autoradiographic or biochemical analysis based on the incorporation of a radiolabeled nucleic acid precursor. Im-

plicit to all these studies, of course, is the assumption that the radiolabeled nucleic acid precursor--usually thymidine--is specifically incorporated into DNA[29] and not degraded with subsequent incorporation into other macromolecules. Such assumptions are usually substantiated by DNase analysis, but even the results of such treatment have been found to be variable--sometimes the label is removed, sometimes it is not.[30-32]

Until recently, whenever nucleic acid metabolism has been investigated, the discussion has almost always been limited to the "salvage" pathways first described by Kornberg. That is, those metabolic pathways whereby a purine or pyrimidine base can be aminated, deaminated, oxidized, reduced, phosphorylated, or dephosphorylated from one form to another without cleavage of the basic ring structure. Such "salvage" pathways have been described in numerous organisms, including Tetrahymena pyriformis.[2,3]

A metabolic alternative to the reincorporation of these modified purine and pyrimidine bases into nucleic acids, however, is the reutilization of fragments of these bases for the synthesis of macromolecules other than nucleic acids. This broader type of "salvage" would involve the breakdown of the purine or pyrimidine ring structure and the reutilization of at least some of the breakdown products for the synthesis of other biologically useful macromolecules such as proteins, lipids, and carbohydrates. Such "salvage" pathways have been described in a few other

organisms, although not generally in Protozoa.

Recent experiments in our laboratory[10,11] on the conversion of radiolabeled thymidine into other biologically useful macromolecules, however, initially indicated that such a pyrimidine degradation and reutilization pathway may indeed be functioning in Tetrahymena pyriformis. When these cells were grown in methyl-^{14}C-labeled thymidine, for example, considerable radioactivity was recovered in biologically useful macromolecules other than DNA, whereas, when they were grown in the presence of 2-^{14}C-labeled thymidine less radioactivity was recovered in macromolecules other than DNA and most of the non-DNA radioactivity was rapidly recovered as $^{14}CO_2$. Thus it was proposed[10,11] that these results could be most easily accounted for by assuming the reductive catabolism of the labeled thymidine and the subsequent reutilization of its radiolabeled degradative products into biologically useful macromolecules other than DNA.

According to this metabolic scheme (see Fig. 1), thymidine would be initially converted to thymine. The ring would then be reduced and cleaved. Ring cleavage would be followed by the subsequent immediate liberation of the 2-C in the form of CO_2. The remaining thymine degradation fragment, B-aminoisobutyric acid (BAIB), might then be further metabolized to methylmalonic acid (MMA) which could be reutilized along well established metabolic pathways for the synthesis of other biologically useful macromolecules with

FIGURE 1. PROPOSED PATHWAY FOR REDUCTIVE THYMINE CATABOLISM AND REUTILIZATION.

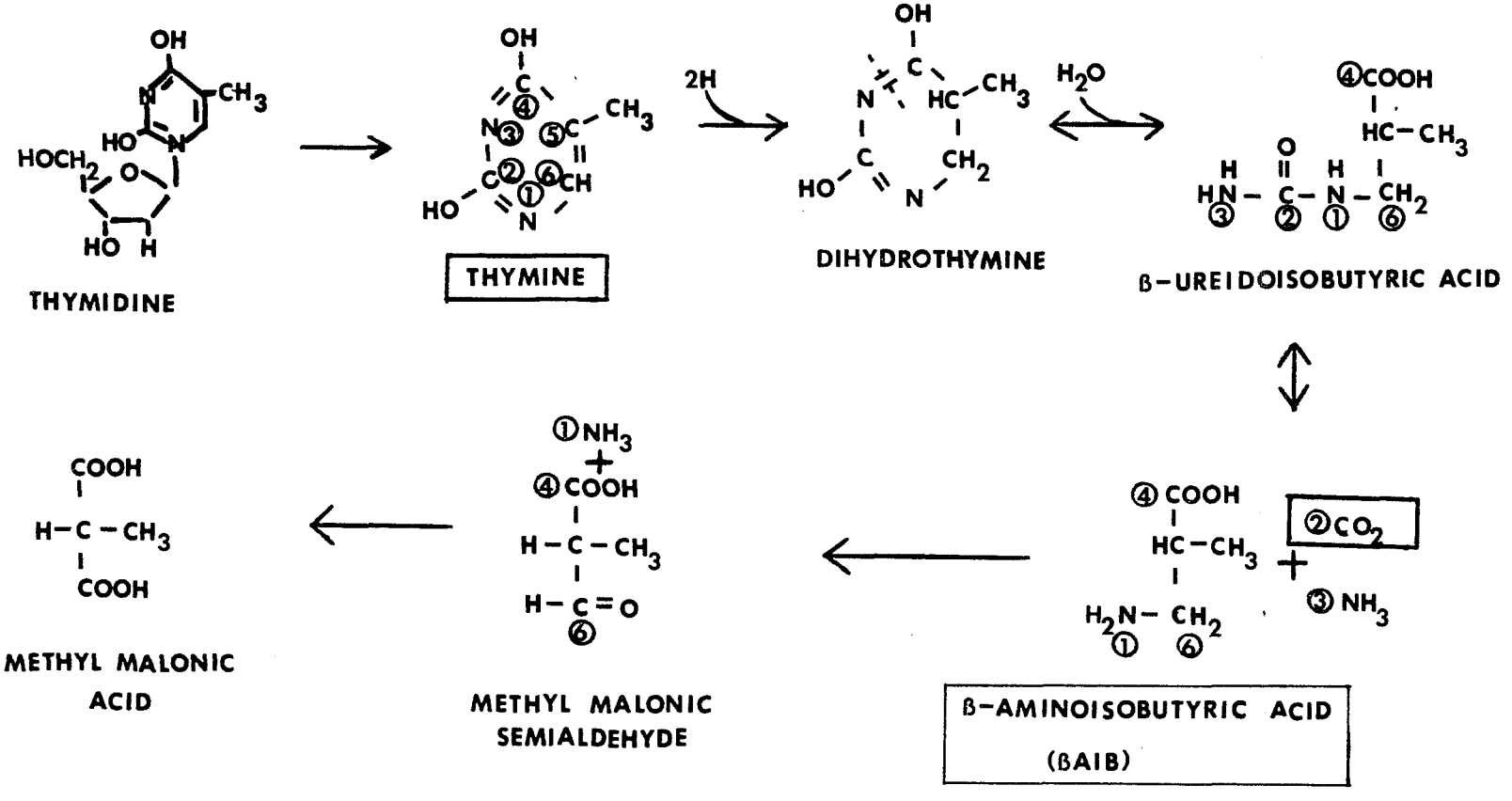
A. PROPOSED THYMIDINE CATABOLIC PATHWAY.

B. PROPOSED THYMIDINE REUTILIZATION PATHWAY.

All interconversions are known to exist in various organisms and were previously proposed[10,11] as possible pathways operating in Tetrahymena pyriformis in order to account for the recovery of thymidine radioactivity in macromolecules other than DNA. All compounds surrounded by rectangular boxes have been previously isolated[10,11] from cells grown in appropriately radiolabeled thymidine.

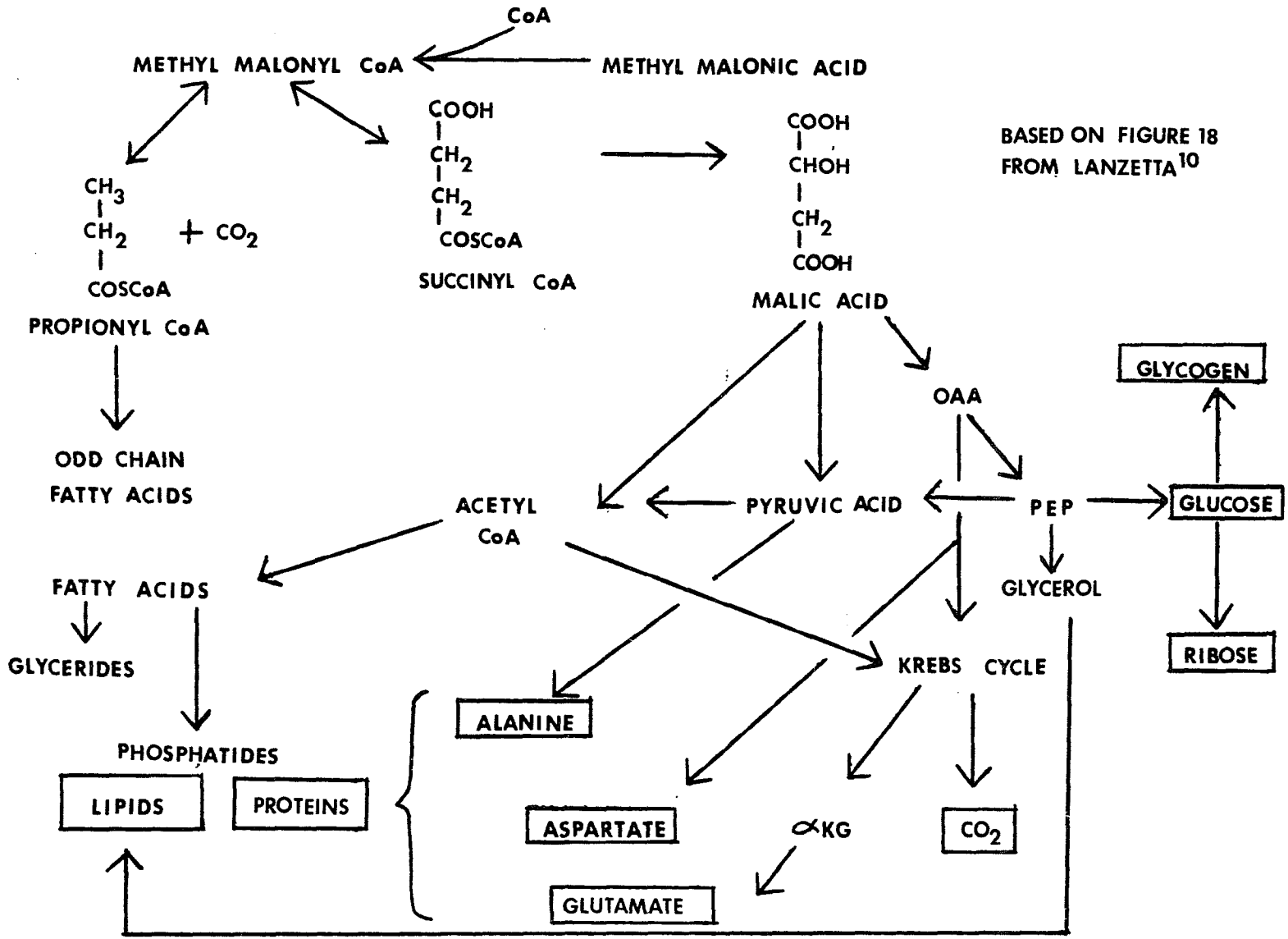
FIGURE 1(A)

PROPOSED THYMIDINE CATABOLIC PATHWAY



BASED ON FIGURE 18 FROM LANZETTA[10]

FIGURE 1(B)
PROPOSED THYMIDINE REUTILIZATION PATHWAY



the eventual liberation of the methyl-C in the form of CO_2. For example, MMA, as its CoA derivative, could be reutilized by conversion into either propionyl or succinyl CoA. Propionyl CoA could be incorporated into fatty acids which would account for the recovery of thymidine radioactivity in lipids. Succinyl CoA, on the other hand, could lead to phosphoenol pyruvic acid (PEP). PEP could then be utilized for glycerol synthesis which could alternatively account for the recovery of additional thymidine radioactivity in lipids, or be ultimately converted to glucose and glycogen through the normally operative gluconeogenic pathway which could account for the reported recovery of thymidine radioactivity in glycogen. Glucose then could be converted to ribose via the pentose phosphate cycle which would account for the recovery of thymidine radioactivity in the ribose of RNA. In addition, several Krebs cycle intermediates resulting from succinyl CoA (pyruvic acid, OAA, and αKG) could be transaminated into common amino acids (ala, asp, and glu, respectively) accounting for the recovery of thymidine radioactivity in proteins.

The present study was undertaken in an effort to further broaden the heretofore rather restrictive definition of nucleic acid "salvage" to include the reincorporation of these thymidine degradative fragments into biologically useful macromolecules other than nucleic acids. We began with the radioactive tracer resubstantiation of the proposed overall reductive thymidine catabolic and reutiliza-

tion pathway. To substantiate this pathway more rigorously, however, it was thought that at least some of the enzymes catalyzing these proposed interconversions should be demonstrated. After the preliminary demonstration of two of these catabolic enzymes, we became particularly interested in the unique properties of the enzyme that apparently catalyzes the critical conversion of the proposed reductive thymidine catabolic end product, BAIB, to the initial anabolic reutilization substrate, probably methylmalonic semialdehyde. It was this enzyme, therefore, which we ultimately chose to study in detail.

MATERIALS AND METHODS

I. RADIOACTIVE TRACER STUDIES

The following methods used in this part of the study were developed in previous work from our laboratory.[10,11]

A. Cultures

Stock cultures of Tetrahymena pyriformis strain GL were maintained on an undefined medium containing 1.1% proteose peptone (Difco), 0.1% glucose, 0.1% K_2HPO_4, 0.01% yeast extract, and 0.002 ug/ml of thiamine·HCl. This medium is a slight modification of Elliott's medium.[33] One liter nephlometer flasks (Bellco Glass Co.) containing 200 ml of this medium were inoculated with 7 ml of stock culture. Culture growth was monitored at 660 nm with a Baush and Lomb Spectronic 20 spectrophotometer. Cells were chilled to 4°C and harvested by centrifugation at 500-1,000 x g for 10 min, then washed twice in Ryley-Ringer phosphate buffer (RRP) consisting of 0.047 M NaCl, 0.002 M KCl, 0.001 M $MgSO_4$, and 0.012 M phosphate at pH 7.3.[34] Cell numbers were determined using appropriate dilutions and counting with a hemocytometer.

B. Growth in Radioactive Medium

Cells were grown in the above medium containing either 2,6-^{14}C labeled thymidine (0.25 uCi/ml) or 2-^{14}C labeled MMA (0.25 uCi/ml) for approximately the final 12 hr of culture growth. Cells were grown for 48-72 hr, depending on when the cultures reached late log phase (4-6 x 10^5 cells/

ml), at 27°C with constant gentle shaking. $^{14}CO_2$ was collected at the indicated intervals (see Fig. 4) during the first 3 hr of culture growth after the addition of the radioactive substrate by passing sterile filtered air through the culture flask and into two hyamine hydroxide (1 M solution in methanol) traps connected in series. The radioactivity from both traps was then combined and recorded as the total amount of $^{14}CO_2$ released during each time interval. After 3 hr, two additional hyamine hydroxide traps were connected in series to the culture flask to collect the $^{14}CO_2$ produced during the last 9 hr of growth. The radioactivity from both these traps was again combined and recorded as the total amount of $^{14}CO_2$ released during that time period.

The $^{14}CO_2$ trapped in the medium was then released by acid titration after the cells were harvested. A few drops of the acid-base indicator, thymol blue, were added to the cell-free radioactive medium. The medium was then titrated in a closed system with strong acid until the indicator turned distinctly pink resulting in a final pH of less than 2. At such a low pH most of the $^{14}CO_2$ produced during the course of the incubation and trapped in the medium as bicarbonate should have been liberated. This residual $^{14}CO_2$ was then collected and quantitated as described above by passing sterile air through the system into hyamine hydroxide traps and counting aliquots of the trapping solutions to determine the total amount of radioactivity so produced

(see M&M Section IH).

C. Cell Fractionation

Cells were harvested, washed, and then fractionated essentially by the procedure of Schmidt and Tannhauser.[35] Cells were suspended in an equal volume of cold 10% trichloroacetic acid (TCA). The suspension was then centrifuged and the precipitate washed twice with a volume of 5% TCA at least equal to that of the precipitate. After centrifugation the wash was added to the original 5% TCA extract. To this combined acid soluble fraction, 1.1 volumes of cold absolute ethanol was added and the precipitate collected by centrifugation. The precipitate, presumably glycogen, was dissolved in water and again precipitated by the addition of 1.1 volumes of ethanol. The precipitate was then finally dissolved in water, brought to volume, and labeled glycogen. The supernatants from these centrifugations were pooled, their volume measured, and labeled the acid soluble fraction (ASF). The remaining pellet from the original TCA extraction which contained RNA, DNA, protein, and lipids was then extracted with 5 volumes of cold absolute ethanol and centrifuged. The precipitate was subsequently extracted twice with equal volumes of a 3:1 (v/v) ether:absolute ethanol solution and finally with absolute ether. The ethanol, ether:ethanol, and ether extracts were pooled and this fraction labeled the lipid fraction. The remaining lipid-extracted pellet was weighed after air drying and then incubated for 18 hr at 37°C with 0.3 N KOH (1

ml/50 mg of pellet weight). The pellet was crushed to facilitate hydrolysis. After 18 hr the alkaline hydrolysate was acidified (to pH 1.5) with cold concentrated perchloric acid (PCA). After centrifugation the precipitate of $KClO_4$, DNA, and protein was washed with 0.1 N PCA and the wash was added to the acidified hydrolysate. The pooled supernatants were then neutralized with KOH, chilled to precipitate as much salt as possible, and centrifuged. The neutralized hydrolysate, labeled RNA, was brought to volume and the total number of $A_{260\ nm}$ units calculated after measurement of a portion of the sample in a Beckman DB spectrophotometer. The PCA precipitated pellet was suspended in 5 volumes of 5% TCA and heated for 20 min at 100°C. The hydrolysate was centrifuged, the supernatant decanted, and the precipitate again treated with 5% TCA for 20 min at 100°C. This hydrolysate was centrifuged and the supernatant added to the previous one. The precipitate was washed with cold 5% TCA and the wash was added to the above supernatants. The remaining pellet which is protein was dissolved in 0.3 N KOH and reprecipitated with a 50% solution of TCA. The supernatant containing approximately 25% TCA was pooled with the previous ones, brought to volume with 5% TCA, and labeled DNA. The remaining protein pellet was dissolved and brought to volume in 1 N KOH.

Some small amount of cross contamination of fractions was evident from previous experiments.[10,11] The fraction most consistently affected was the RNA fraction. It con-

tained glycogen which escaped the cold TCA extraction and a small amount of fragmented DNA. This fraction, therefore, was further purified.

D. Column Chromatography

The RNA fraction was subjected to DEAE cellulose column chromatography after the methods of Morisawa and Chargaff[36] and Tomlinson and Tener.[37] DEAE cellulose which was washed in 0.5 N NaOH until the wash was negative for chloride was then suspended and settled in glass distilled water until most of the fine particles were removed and the pH lowered to 9 or 10. It was then washed on a Buchner funnel until the pH was neutral, suspended in 0.1 N acetic acid, filtered, and finally suspended in 0.01 M lithium acetate buffer pH 4.5. A column 1.5 x 30 cm was loaded with DEAE cellulose to a height of at least 19 cm. The RNA fraction was layered on top of the bed and allowed to enter the column. Several ml of buffer were subsequently allowed to percolate down the column after the RNA application. The column was then filled with buffer and attached to a reservoir of buffer. Twenty ml fraction were collected. The effluent was continuously monitored at 253 nm with a LKB Uvicord I. The contaminating carbohydrate came off the column first (see Fig. 2). The buffer in the column reservoir was then changed to 0.04 M LiCl in 0.01 M lithium acetate buffer pH 4.5. The monoribonucleotides were then washed off the column (see Fig. 2) and at this point the buffer was changed again to 1.0 M LiCl in 0.01 M lithium acetate buffer pH

4.5. This last salt elution washed the few remaining oligoribonucleotides and most of the contaminating DNA off the column (see Fig. 2). The $A_{260\ nm}$ of all peak fractions was then redetermined with the Beckman DB spectrophotometer.

The monoribonucleotide and oligonucleotide (containing mostly oligodeoxyribonucleotides and a few oligoribonucleotides) fractions from the DEAE cellulose column were lyophilized, then desalted by dissolving the lyophilized sample in absolute methanol and precipitating with 5 volumes of acetone.[38] The desalting procedure was repeated, then these precipitates were brought to volume. The monoribonucleotide precipitate was labeled purified RNA, and the oligonucleotide precipitate, which was subsequently found to contain mostly oligodeoxyribonucleotides (see Results Section IA), was labeled DNA and combined with the previously prepared DNA fraction.

The glycogen fraction from the DEAE cellulose column was lyophilized, dissolved in water, and the glycogen precipitated with 1.1 volumes of cold absolute ethanol. The precipitated glycogen was added to that material obtained from the TCA extract and the combined fractions brought to volume in water and analyzed by the anthrone test (see M&M Section IG).

E. Hydrolysis

A portion of the combined glycogen fraction was reprecipitated with ethanol. To this precipitate 1 ml of 1 N H_2SO_4 was added and the solution heated for 3 hr at 100°

C.[39] The hydrolysate was neutralized with saturated $Ba(OH)_2$ and the solution freeze-thawed and centrifuged to remove the $BaSO_4$ salt. The neutralized hydrolysate was then chromatographed in order to determine that the recovered radioactivity was indeed a result of the metabolic reutilization of the tracer and not just a result of its non-specific adsorption to this macromolecular fraction.

F. Paper Chromatography

Glycogen hydrolysates were chromatographed in two solvent systems: (A) the upper phase of a butanol (4):acetic acid (1):H_2O (5) mixture[40] and (B) isobutyric acid (66): conc NH_4OH (1):H_2O (33)[41] (see Figs. 3 & 5). dTMP was run as a standard and visualized as a UV quenching spot since it had been previously reported that glycogen non-specifically binds dTMP.[42] The sugar standards were visualized by spraying the chromatograms with a solution of 0.2% p-anisidine·HCl[43] in ethanol with 4% orthophosphoric acid and additional concentrated HCl until the solution cleared. The chromatograms were heated in a drying oven at 100°C for 2 min.

G. Colorimetric Assays

The carbohydrate content of both the acid soluble and the DEAE cellulose column purified glycogen fractions was assayed by the anthrone test.[39] One ml of chilled anthrone reagent (0.2% anthrone in conc H_2SO_4) was added to 0.1 ml of sample in 0.4 ml of water on ice. The mixture was then heated for 10 min at 100°C and read at 620 nm. Standard

glucose solutions were run simultaneously.

Protein concentration was quantitated by the biuret reaction.[45,46] Human serum albumin (HSA) solutions were run simultaneously as standards.

DNA concentration was determined by the diphenylamine technique.[47] Standard DNA solutions were run simultaneously with the samples.

RNA concentration was determined with orcinol reagent.[48] Standard RNA solutions were also run simultaneously with the samples.

H. Isotope Measurements

All cell and co[illegible]assayed for radioactivity by placi[illegible]f material in Bray's counting s[illegible]a Nuclear Chicago Mark II liqu[illegible] Paper strips (1 x 2 cm) from chi[illegible]in counting vials with 1 ml of water an[illegible]before Bray's scintillation fluid was added. [illegible]adiolabeled thymidine and MMA were used as supplied from the manufacturers without further purification.

I. Repeated Experiments

The techniques used in these experiments were first worked out in several (at least 3) repeated cold experiments. As these radioactive tracer studies were in essence merely a confirmation of more rigorous previous radiolabeled work,[10,11] however, it was not considered necessary to repeat them again and hence these data represent the re-

results of a single experiment.

II. PRELIMINARY DEMONSTRATION OF THE CATAB[illegible]

A. Preparation of the Homogenates

Cultures were grown to late log phase [illegible] described (see M&M Section IA). Cells were [illegible] pended in approximately 5 volumes of cold RRP [illegible] ized on ice by 50-100 strokes of a motor-driven [illegible] tle (approximately 400 RPM) in a Potter-Elvehjem [illegible] izer. Homogenization was monitored by microscopic [illegible] tion (see M&M Section IA) and continued until at [illegible] of the cells were disrupted by this procedure. The [illegible] genate was then further diluted with at least an [illegible] ume of cold RRP.

B. Enzyme Analyses

1. Thymine Reductase (Dehydrogenase)

a. Fractionation of the Homogenate

Since it was not initially possible to demonstrate dehydrogenase in the above crude enzyme preparations, [illegible] genates were subsequently subjected to a simple differential centrifugation scheme in the hopes of eventually concentrating activity in one of the resulting fractions. The crude homogenate was thus centrifuged at 2,000 RPM [illegible] 10 min using the SS-34 rotor in a Sorvall RC-2 refrigerated ($4^{o}C$) centrifuge. The supernatant was carefully decanted and a portion was retained for enzymic analysis while the remainder was recentrifuged at 3,500 RPM (1,[illegible]

glucose solutions were run simultaneously.

Protein concentration was quantitated by the biuret reaction.[45,46] Human serum albumin (HSA) solutions were run simultaneously as standards.

DNA concentration was determined by the diphenylamine technique.[47] Standard DNA solutions were run simultaneously with the samples.

RNA concentration was determined with orcinol reagent.[48] Standard RNA solutions were also run simultaneously with the samples.

H. Isotope Measurements

All cell and column fractions were assayed for radioactivity by placing appropriate aliquots of material in Bray's counting solution[44] and counting in a Nuclear Chicago Mark II liquid scintillation counter. Paper strips (1 x 2 cm) from chromatograms were placed in counting vials with 1 ml of water and shaken for 1 hr before Bray's scintillation fluid was added. The radiolabeled thymidine and MMA were used as supplied from the manufacturers without further purification.

I. Repeated Experiments

The techniques used in these experiments were first worked out in several (at least 3) repeated cold experiments. As these radioactive tracer studies were in essence merely a confirmation of more rigorous previous radiolabeled work,[10,11] however, it was not considered necessary to repeat them again and hence these data represent the re-

results of a single experiment.

II. PRELIMINARY DEMONSTRATION OF THE CATABOLIC ENZYMES

A. Preparation of the Homogenates

Cultures were grown to late log phase and harvested as described (see M&M Section IA). Cells were then resuspended in approximately 5 volumes of cold RRP and homogenized on ice by 50-100 strokes of a motor-driven Teflon pestle (approximately 400 RPM) in a Potter-Elvehjem homogenizer. Homogenization was monitored by microscopic examination (see M&M Section IA) and continued until at least 90% of the cells were disrupted by this procedure. The homogenate was then further diluted with at least an equal volume of cold RRP.

B. Enzyme Analyses

1. Thymine Reductase (Dehydrogenase)

a. Fractionation of the Homogenate

Since it was not initially possible to demonstrate this dehydrogenase in the above crude enzyme preparations, homogenates were subsequently subjected to a simple differential centrifugation scheme in the hopes of eventually detecting activity in one of the resulting fractions. The diluted homogenate was thus centrifuged at 2,000 RPM (480 x g) for 10 min using the SS-34 rotor in a Sorvall RC-2 refrigerated (4°C) centrifuge. The supernatant was carefully decanted and a portion was retained for enzymic analysis while the remainder was recentrifuged at 3,500 RPM (1,475 x g) for 10

min. This supernatant was again carefully decanted and a portion retained for enzymic analysis while the remainder was recentrifuged at 10,000 RPM (12,100 x g) for 10 min. This supernatant was also decanted. A portion was again retained for enzymic analysis and the remainder was finally centrifuged at 31,000 RPM (100,000 x g) for 1 hr using the SW 36 rotor in a Beckman L3-40 refrigerated (4°C) ultracentrifuge. This supernatant was also decanted and retained for enzymic analysis. The pellets, however, could not be satisfactorily resuspended, and therefore were not assayed.

b. Enzyme Assay

The DHT-DH assay was based on the procedure described by Hunninghake and Grisolia.[49] The complete assay system contained: 2.875 ml of the appropriate Tetrahymena pyriformis supernatant prepared in RRP (final conc = 12 mM phosphate buffer, pH 7.3), 25 ul NADPH (final conc = 0.1 mM), and 100 ul thymine (final conc = 1.67 mM) in a total volume of 3 ml. Enzyme activity was monitored spectrophotometrically in a Beckman DB recording spectrophotometer by measuring the decrease in $A_{340\ nm}$ of the NADPH for at least 1 min after the addition of substrate. The blank contained all components except substrate (thymine). All supernatants showed considerable blank activity. Activities (see Table II), therefore, were corrected for this endogenous NADPH-DH activity by subtracting the appropriate blank reaction. No activity could be demonstrated when NADH was substituted for NADPH.

2. BAIB Metabolism

a. Transaminase Reaction

Homogenates were prepared as described above (see M&M Section IIA) except that prior to homogenization the cells were diluted with RRP to make a 2-5% suspension (1.0-2.5 x 10^6 cells/ml). Cell concentrations by volume were determined by centrifuging 1 ml of the cell suspension in a Constable protein tube at 1800 RPM (1000 x g) for 5 min using the HL-4 head in a Sorvall GLC-1 ambient temperature centrifuge. The enzyme preparation was also not further diluted after homogenization (see M&M Section IIA). This procedure resulted in a homogenate that contained 2-3 mg of protein per ml.

The BAIB transaminase analyses were based on the reagent concentrations described by Vandermeers-Piret, Camus, and Christophe[50] and the methods described by Kupiecki and Coon.[51] The complete assay system contained: 2.7 ml of the Tetrahymena pyriformis homogenate prepared as described above in RRP (final conc = 12 mM phosphate buffer, pH 7.3), 100 ul BAIB (final conc = 16.7 mM), 100 ul αKG (final conc = 5 mM), and 100 ul PALP (final conc = 33 uM) in a total volume of 3 ml.[50] The mixtures were then incubated either under N_2 or O_2 for the indicated periods initially at 25°C and then at 37°C in a Dubnoff metabolic shaker with constant gentle shaking (approximately 40 cycles/min). Two sets of controls were also rum simultaneously. One set was incubated without homogenate to check for any non-enzymatic

transamination as well as to quantitate the amount of exogenous BAIB added. The other was incubated without substrate (BAIB) to check for the non-BAIB dependent metabolism of αKG as well as to quantitate the amount of endogenous BAIB and glu present in the homogenate.

At the end of the incubation period each mixture was deproteinized by adding 10 ml of absolute ethanol. The protein precipitate was then removed by centrifuging at 10,000 RPM (12,100 x g) for 10 min, using the SS-34 rotor in a Sorvall RC-2 refrigerated (4°C) centrifuge. The supernatant was decanted and concentrated overnight by either heating in an 80°C water bath or by evaporating under reduced pressure at room temperature. The remaining supernatant was then further concentrated by lyophilizing and finally brought to a volume of 1 ml with 80% ethanol.

Originally the following transaminating system was of interest:[51]

BAIB + αKG $\rightleftharpoons$ methylmalonic semialdehyde + glu

After our intial inability to demonstrate consistent and significant changes in the amino acids and αketo acids involved in this system, however, the subsequent transaminase analyses became more exploratory and less analytical in nature.

(1) Identification and Quantitation of the Transaminating Amino Acids

Thirty-fifty ul aliquots of each of the above supernatants were spotted on Whatman #1 paper for the chromatograph-

ic separation and identification of their component amino acids. BAIB and glu standards were also spotted. The chromatograms were then developed overnight (11-13 hr) in n-propanol (80):5 N formic acid (20).[51] Amino acids were visualized by spraying the chromatogram with 0.2% ninhydrin in water saturated butanol then heating in a chromatographic drying oven (Precision Scientific) at 100°C for 5 min. The amino acids were identified by comparing their R_F with that of the standards.

Changes in amino acid concentrations were also detected with a semi-quantitative ninhydrin reagent.[52] This reagent was freshly prepared just prior to use by mixing 50 ml of 0.2 M citrate buffer, pH 5.0, containing 0.08 gm of $SnCl_2 \cdot 2H_2O$ with an equal volume of methyl cellosolve containing 2 gm of ninhydrin. Chromatograms were sprayed and heated as indicated above. The appropriate ninhydrin positive spots were cut out and eluted by shaking for 10 min[53] with 2 ml of 50% ethanol. The $A_{570\ nm}$ of the resulting blue solutions was measured against an appropriate chromatogram blank. For glu the corresponding spot in the "no enzyme" control lane was cut out, eluted, and used as the blank, while for BAIB the corresponding spot in the "no BAIB" control lane was used as the blank.

(2) Identification and Quantitation of the Transaminating αKeto Acids

The remainder of each supernatant was processed for the detection of αketo acids. One-tenth ml of a 0.1% so-

lution of 2,4-dinitrophenylhydrazine in 2 N HCl was added to a 0.3 ml supernatant aliquot chilled on ice.[51] The resulting 2,4-dinitrophenylhydrazones were then immediately extracted with 0.3 ml of chloroform. The addition of chloroform resulted in an immiscible mixture. The upper aqueous layer was carefully pipetted off and the lower chloroform layer was evaporated overnight to dryness in a fume hood. Each sample was then reconstituted in 50-100 ul of chloroform. This entire sample was then spotted on Whatman #1 paper for the chromatographic separation and identification of the 2,4-dinitrophenylhydrazone derivatives of the component keto acids. The 2,4-dinitrophenylhydrazones of standard solutions of αKG, OAA, pyruvic acid, and glyoxylic acid were also prepared and spotted. The chromatograms were then deveolped overnight (14-18 hr) in n-butanol (70):0.5 M NH_4OH (20):absolute ethanol (10).[51] The 2,4-dinitrophenylhydrazones were identified by comparing their R_F with that of the standards. On this basis, αKG and endogenous pyruvic acid were determined to be the major homogenate keto acids. Changes in these keto acid concentrations were also quantitated.[54] The appropriate 2,4-dinitrophenylhydrazone spots were cut out and eluted by shaking for 10 min with 2 ml of 1 N NaOH at room temperature. The $A_{455\ nm}$ of the resulting red solutions was measured.

A quantitative colorimetric determination of the amount of methylmalonic semialdehyde produced was also attempted based on the procedures described by Hayaishi and Nishi-

zuka[55] and by Walker.[56] A 0.5 ml aliquot of each reconstituted supernatant was brought to a pH of 4.9-5.2 by the addition of 0.5 ml of 1 M sodium acetate buffer, pH 5.2. Subsequently, 3.0 ml of diazonium reagent (prepared immediately before use by adding 6.0 ml of freshly prepared 0.5% $NaNO_2$ to 40 ml of 0.05% p-nitroaniline in 0.05 N HCl, chilling this mixture in an ice bath, and then adding 14 ml of 0.2 M sodium acetate) was added. The mixture was kept at 35°C for 30 min. The coupling reaction was then stopped by the addition of 0.1 ml of 5 N HCl, and the formazan produced was extracted by shaking with 4.0 ml of ethyl acetate. The $A_{440\ nm}$ of the resulting orange ethyl acetate extract was measured. Although this reaction is claimed to be specific for the semialdehyde,[56] αKG was also found to react strongly at the concentrations used in these experiments. It is likely, therefore, that these results reflect the overall changes in keto acid concentrations rather than just that of methylmalonic semialdehyde.

b. Oxidase Reaction

(1) 2,6-Dichlorophenolindophenol Assay System

The enzyme extract was prepared as described above (see M&M Section IIA). After the homogenate was diluted, however, it was centrifuged at 2,500 RPM (500 x g) for 10 min using the SS-34 rotor in a Sorvall RC-2 refrigerated (4°C) centrifuge. This centrifugation was necessary to clarify the enzyme preparation since the particulate matter in the homogenate interfered with the spectrophotometric

assay.

The initial BAIB oxidase assay was based on the procedure described by Müller, Hogg, and deDuve[57] for glyoxylate oxidase. The complete assay system contained: 0.1 ml of Tetrahymena pyriformis extract prepared in RRP, 1 ml BAIB (final conc = 0.1 M), 0.3 ml 2,6-dichlorophenolindophenol (final conc = 33 uM), 0.1 ml phenazine methosulfate (final conc = 1 mM), and an additional 0.6 ml RRP (final conc = 12 mM phosphate buffer, pH 7.3) to bring the total assay volume up to 3 ml. Enzyme activity was monitored spectrophotometrically in a Beckman DB recording spectrophotometer by measuring the decrease in $A_{600\ nm}$ for at least 10 min after the addition of the substrate (BAIB). The blank contained all components except substrate.

(2) Fluorometric Assay System

The enzyme extract was prepared as described above (see M&M Section IIA) except that the cells were homogenized in 0.25 M sucrose-0.1 M Tris-HCl buffer, pH 7.4,[58] instead of RRP. This homogenate was then used without further centrifugation for the initial enzyme assays.

This BAIB oxidase assay was based on the procedure described by Guilbault and Hieserman[59] for the detection of serum amino acids. Initially the complete assay system contained: 1.3 ml 0.02 M pyrophosphate buffer, pH 8.3 (final conc = 8.67 mM pyrophosphate), 1.0 ml BAIB (final conc = 0.1 M), 0.1 ml homovanillic acid (HVA) (final conc = 80 ug/ml), 0.1 ml peroxidase (50 ug/ml), and 0.5 ml of

the crude Tetrahymena pyriformis enzyme extract prepared in the above homogenization buffer to bring the total assay volume up to 3 ml. Enzyme activity was monitored fluorometrically at 10 min intervals for 90 min in a Turner model 111 filter fluorometer. A 7-60 primary filter and a 47B-2A combination secondary filter were used.

C. Repeated Experiments

The DHT-DH assay was run on numerous enzyme preparations until this activity was finally demonstrated. The data (see Table II) represent the results of this initial demonstration.

The transaminase reaction was run as described 3 times with Tetrahymena pyriformis homogenates. Initially it was run at 25°C under O_2. It was then subsequently run at 37°C under both O_2 and N_2. The data (see Table III) represent the results on one such experiment. The reaction was also run once under N_2 with mouse liver homogenate.

2,6-Dichlorophenolindophenol assays for BAIB oxidase activity were run numerous times on homogenates as well as differential centrifugal supernatants and resuspended pellets. The data (see Table IV) represent the results of one such typical experiment.

The fluorometric BAIB oxidase assay was also run on numerous enzyme preparations until this activity was finally demonstrated. The data (see Fig. 6) represent the results of this initial demonstration.

III. CHARACTERIZATION OF BAIB OXIDASE

A. The BAIB Oxidase Assay System

BAIB oxidase activity was then assayed for in a variety of buffer types (see Table V), concentrations (see Table V), and pH values (see Table V; Fig. 7); over a wide range of temperatures (see Table VI); and in the presence and absence of FAD (see Table VII), divalent cations (see Table VIII), EDTA (see Table IX), several SH reducing reagents (see Results III7), the SH blocking reagent, PCMB (see Table X); and various detergents (see Table XI). The complete BAIB oxidase system that eventually evolved from these preliminary studies contained: up to 0.5 ml of a cellular enzyme source prepared as described above (see M&M Section IIB2b(2)) in the indicated homogenization medium, 1.2 ml of 0.2 M Tris-HCl buffer, pH 8.6 (final conc = 80 mM), 0.1 ml FAD (final conc = 10^{-4} mM), 1.0 ml substrate (final conc = 0.1 M), 0.1 ml HVA (final conc = 80 ug/ml), and 0.1 ml peroxidase (final conc = 50 ug/ml) in a total volume of 3 ml. Assays using crude Tetrahymena pyriformis enzyme preparations also contained 5-10 ul of 10% Triton X-100 (final conc = 0.017-0.034%). The assay mixture was then incubated at 37°C for 90 min. Enzyme activity was monitored fluorometrically at 10-30 min intervals in a Turner model 111 fluorometer using a 7-60 primary and 47B-2A secondary filter combination. Experimental oxidase activities were corrected for endogenous oxidase activities by subtracting appropriate blank readings (see Results Section

IIB2).

B. Freeze-Thawing

Enzyme latency was assayed for according to the procedure of Leighton et al.[60] That is, at least 5 ml of a Tetrahymena pyriformis homogenate prepared in 0.25 M sucrose was rapidly frozen in a dry ice:1-methoxy-2-propanol slurry, then thawed in a 37°C water bath. An aliquot (≤1 ml) was removed and retained for enzyme assay. This freeze-thaw cycle was repeated 4-5 times. Each aliquot was subsequently assayed in the absence of Triton X-100 for BAIB as well as D-amino acid oxidase activity (see Table XII).

C. Stability

The freshly prepared Tetrahymena pyriformis homogenates used for these stability studies were divided into two portions. One of these portions was stored in the refrigerator at 4°C, while the other was stored in various ways in the freezer at -20°C. Half of the material at -20°C was stored in buffer, while the remainder was suspended in an equal volume of glycerol. Both of these enzyme preparations stored at -20°C were further subdivided into a portion which was stored in bulk and a portion which was stored in small individual aliquots. At the indicated times after preparation each of these variously stored homogenates were warmed in a 37°C water bath and then assayed for the indicated oxidase activities. Each assay system contained Triton X-100 (see M&M Section IIIA).

D. Repeated Experiments

The effect of buffer type, molarity, and pH on BAIB oxidase activity was initially determined at 25°C, then subsequently redetermined at 37°C. The data (see Table V) represent the results of the 37°C determination. The effect of pH on BAIB oxidase activity was determined at least 5 times with consistantly similar results. The data (see Fig. 7) represent the results of the average of 3 such determinations.

The effect of temperature on BAIB oxidase activity (see Table VI) was determined once.

The effect of FAD on BAIB oxidase activity was determined at least 3 times and always showed concentration independent stimulation. The data (see Table VII) represent the results of one such typical determination.

The effect of divalent cations on BAIB oxidase activity (see Table VIII) was also determined once.

The effect of EDTA on BAIB oxidase activity was determined 3 times over a wide range of concentrations. The data (see Table IX) represent a composite of these results.

The effect of PCMB on BAIB oxidase activity was determined 3 times and always showed concentration independent inhibition. The data (see Table X) represent the results of one such typical experiment.

The effect of the various detergents on BAIB oxidase activity was determined at least 3 times. The data (see Table XI) represent a composite of these results.

The effect of freeze-thawing on BAIB oxidase activity

was determined twice. The data (see Table XII) represent the results of one such typical experiment.

The stability of BAIB oxidase activity under various storage conditions was determined numerous times. That is, the refrigerated and frozen stability of BAIB oxidase activity was determined 5 times, while its freeze-thaw stability was determined 3 times, and its stability in glycerol was determined only twice. The data (see Table XIII) represent a composite of these results.

IV. SUBCELLULAR LOCALIZATION OF BAIB OXIDASE ACTIVITY

A. Centrifugal Fractionation

Eight-hundred ml cultures of Tetrahymena pyriformis were grown to late log phase in 2800 ml Fernbach flasks and harvested as described (see M&M Section IA). The washed cells (see M&M Section IA) were homogenized as described (see M&M Section IIA) in freshly prepared 0.25 M sucrose. A small aliquot of this homogenate was retained for enzymic analysis, EM, and protein determination. The remainder was fractionated by various centrifugal methods.

1. Preliminary Centrifugation

Homogenates prepared as described above were separated initially into a high speed supernatant and pellet by centrifugation at 31,000 RPM (100,000 x g) for 1 hr using the SW 36 rotor in a Beckman L3-40 refrigerated ultracentrifuge set at 4°C. The supernatant was decanted and the pellet was resuspended by repeated pipetting in at least and equal

volume of 0.25 M sucrose. Both fractions were then assayed for the indicated enzyme activities (see Table XIV).

2. Differential Centrifugation

Homogenates prepared as described above were also processed according to the differential centrifugation scheme of Smith and Law[58] (see Fig. 8). The homogenate was first centrifuged at 2,000 RPM (500 x g) for 10 min using the SS-34 rotor in a Sorvall RC-2 refrigerated centrifuge set at 4°C. The supernatant was decanted and the pellet was resuspended in at least an equal volume of 0.25 M sucrose. Both these fractions were recentrifuged at 500 x g for another 10 min. The supernatant was again decanted and the combined low speed pellets retained for further enzyme and EM analysis. A small aliquot of the resulting pooled 500 x g supernatants was also retained for enzyme analysis and the remainder was centrifuged at 11,000 RPM (14,500 x g) for 30 min in the Sorvall. This supernatant was then decanted and the pellet resuspended in at least an equal volume of 0.25 M sucrose. Both these fractions were then recentrifuged at 14,500 x g for another 30 min. The resulting intermediate speed supernatant was again decanted. The combined intermediate speed pellets were resuspended in at least an equal volume of 0.25 M sucrose and retained for further enzyme and EM analysis. A small aliquot of the pooled 14,500 x g supernatants was retained for enzyme analysis and the remainder was centrifuged at 31,000 RPM (100,000 x g) for 1 hr using the SW 36 rotor in a Beckman

L3-40 refrigerated ultracentrifuge set at 4°C. This final supernatant was decanted and assayed. The pellet was again resuspended in at least an equal volume of 0.25 M sucrose and retained for further enzyme and EM analysis.

In addition, to check for the possibility of the non-specific adsorption of soluble enzymes to subcellular organelles during this processing, exogenous hog kidney D-amino acid oxidase was added to the Tetrahymena pyriformis homogenate at approximately a concentration of 27 ug/ml before subjecting it to the centrifugal fractionation scheme outlined above. The enzyme activity of each resultant differential centrifugal fraction as well as of the high speed supernatant and pellet (see Table XV) was determined. The amount of exogenous D-amino acid oxidase activity contained in each fraction was then calculated by subtracting the endogenous D-amino acid oxidase activity of each fraction as determined from the parallel processing of an equal amount of the same Tetrahymena pyriformis homogenate without additional added exogenous D-amino acid oxidase from the total amount of D-amino acid oxidase activity (endogenous plus exogenous) in each fraction.

3. Zonal Density Gradient Centrifugation

Using the above described procedures (see M&M Section IVA 1&2), however, still did not result in a satisfactory separation of the subcellular organelle marker enzymes. With the kind assistance of Dr. Miklós Müller at the Rockefeller University, therefore, we finally resorted to the zonal den-

sity gradient centrifugation method of Müller, Hogg, and deDuve[57] to unequivocally localize the subcellular site of BAIB oxidase activity.

Tetrahymena pyriformis cultures were grown as described above (see M&M Section IVA) and harvested with a modified plankton centrifuge. The cells were washed with a few ml of distilled water and then brought to a concentration of approximately 5% (v/v) with freshly prepared 0.25 M sucrose. Cell disruption was achieved by two passes of this chilled suspension through a fritted stainless steel filter (grade G, average pore size 10 u) under light suction. When examined under a phase contrast microscope, the filtrate obtained in this manner was found to contain almost no intact cells, and no recognizable macronuclei. Fractionation by isopycnic density gradient centrifugation was performed in a Beckman model L-HV refrigerated ultracentrifuge with the Al-14 automatic zonal rotor at 4°C. The system initially consisted of 15 ml of homogenate, layered over 16 ml of a sucrose gradient extending linearly with respect to volume between density limits of 1.14 and 1.27, and itself resting on an 8 ml cushion of sucrose solution of density 1.32. The rotor was loaded at low speed, spun for 36 min at 35,000 RPM, and then decelerated for unloading. Two ml fractions were collected. Their weight and density were determined to allow calculation of their volumes as well as estimation of the average equilibrium density of the particles contained in them.

B. Enzyme Assays

The resuspended pellets and supernatants as well as the gradient fractions prepared as described above (see M&M Section IVA) were then assayed for several different enzyme activities.

1. Malate Dehydrogenase (MDH) E.C. #1.1.1.37

This Tetrahymena pyriformis mitochondrial marker enzyme was assayed for by the procedure described in the Worthington Enzyme Manual.[61] The complete assay system contained: 2.6 ml 0.1 M sodium phosphate buffer, pH 7.4 (final conc = 86.7 mM), 0.1 ml $MgCl_2$ (final conc = 3.3 mM), 0.2 ml NADH (final conc = 3.2 uM) in 0.1 M Tris-HCl buffer, pH 7.4, 5-10 ul 10% Triton X-100 (final conc = 0.017-0.034%), 5 ul enzyme preparation, and 0.1 ml freshly prepared 0.006 M OAA (final conc = 0.2 uM) in 0.1 M sodium phosphate buffer, pH 7.4, in a total volume of 3 ml. Enzyme activity was monitored spectrophotometrically in a Beckman DB recording spectrophotometer by measuring the decrease in $A_{340\ nm}$ of the NADH for at least 2 min after the addition of substrate (OAA). The blank contained all components except substrate. The activity of this enzyme is defined as the decrease in $A_{340\ nm}$ per min per ml of enzyme preparation. The total activity of each fraction is defined as its activity times its total volume. The specific activity of each fraction is defined as its change in $A_{340\ nm}$ per min per mg of protein.

2. Catalase E.C. #1.11.1.6

This peroxisomal marker enzyme was assayed for by a slight modification of the procedure described in the Worthington Enzyme Manual.[62] The complete assay system contained: 1.9 ml 0.1 M sodium phosphate buffer, pH 7.4 (final conc = 63.3 mM), 1.0 ml 1.8% H_2O_2 (final conc = 0.6%), 5-10 ul 10% Triton X-100 (final conc = 0.017-0.034%), and 0.1 ml of enzyme preparation in a total volume of 3 ml. Enzyme activity was monitored by measuring the decrease in $A_{240\ nm}$ of the H_2O_2 for at least 1 min after the addition of the enzyme preparation. The blank contained all components except enzyme preparation. The activity of this enzyme is defined as the decrease in $A_{240\ nm}$ per min per ml of enzyme preparation. The total activity and specific activity of each fraction are defined as described above (see M&M Section IVB1).

3. D-Amino Acid Oxidase E.C. #1.4.3.3

This peroxisomal marker enzyme was assayed for by the procedure of Guilbault and Hieserman[59] modified as previously described (see M&M Section IIIA) for the BAIB oxidase assay system. 1.0 ml of D-ala (final conc = 0.1 M) was used as substrate. The activity of this enzyme is defined as the change in fluorescence per 90 min per ml of enzyme preparation. The total activity and specific activity of each fraction are defined as described above (see M&M Section IVB1).

4. Lactate Oxidase (α-L-Hydroxy-Acid Oxidase) E.C. #1.1.-3.2

This peroxisomal marker enzyme was also assayed for by the procedure of Guilbault and Hieserman[59] modified as previously described (see M&M Section IIIA) for the BAIB oxidase assay system. 1.0 ml of lactic acid (final conc = 0.1 ml) was used as substrate. The activity, total activity, and specific activity of each fraction are defined as described above (see M&M Section IVB3).

5. BAIB Oxidase

This previously undescribed enzyme was also assayed for by the procedure of Guilbault and Hieserman[59] modified as previously described (see M&M Section IIIA). 1.0 ml of BAIB (final conc = 0.1 M) was used as substrate. The activity, total activity, and specific activity of each fraction are also defined as described above (see M&M Section IVB3).

Enzyme data were preferentially expressed as the per cent of homogenate activity.[63] Where this was not possible, enzyme data was alternatively expressed as the per cent of total recovered activity.

C. Electron Microscopy (EM)

The differential centrifugation pellets prepared as described above (see M&M Section IVA2) were also processed for EM (Dr. Frederick Schuster, personal communication; Betty Hershenov, personal communication; Ursula Behrens, personal communication; Janet Arce, personal communication).

1. Fixation

The pellets were fixed for 30 min on ice (Figs. 10 &

11) or 120 min at room temperature (Fig. 12) in a 1% (Fig. 12) or 2% (Figs. 10 & 11) solution of buffered glutaraldehyde. Either 0.1 M veronal acetate (Fig. 10 & 11) or 0.1 M sodium cacodylate (Fig. 12) buffer, pH 7.2-7.4, was used. These buffers also contained 0.005% (in cacodylate) or 0.05% (in veronal acetate) $CaCl_2$ to stabilize cellular membranes and thus better preserve cytoplasmic ultrastructure.

2. Post-fixation

The glutaraldehyde fixative solution was carefully decanted and the pellets were post-fixed for 20 min at room temperature (Figs. 10 & 11) or 60 min on ice (Fig. 12) with a freshly prepared 1% solution of buffered OsO_4. Again either 0.1 M veronal acetate (Figs. 10 & 11) or 0.1 M sodium cacodylate (Fig. 12) buffer, pH 7.2-7.4, containing the indicated amounts of $CaCl_2$ was used. This step was included chiefly as a means of enhancing the contrast of the subcellular organelles. The pellets were then washed twice with either distilled water (Fig. 12) or 0.1 M phosphate buffer, pH 7.2-7.4 (Figs. 10 & 11).

3. Staining with Uranyl Acetate

After washing the pellets were stained for 20 min (Fig. 12) or 60 min (Figs. 10 & 11) with a 1% solution of uranyl acetate. This solution was either made up in distilled water (Fig. 12) or in 0.1 M veronal acetate buffer, pH 7.2-7.4, containing the indicated amount of $CaCl_2$ (Figs. 10 & 11). This treatment stabilizes and increases the contrast of DNA.

4. Dehydration

The pellets were then dehydrated by a gradual transition through an increasing ethanol series. The initial alcohol concentration was 70% (Fig. 12) or 75% (Figs. 10 & 11). The pellet was usually stored overnight in the refrigerator at this initial step. The ethanol concentration was then gradually increased by two or three 5 to 10 min steps to 100%.

5. Infiltration

The transition from absolute ethanol to the Maraglas epoxy resin was initiated by a 5 min room temperature treatment of the pellet with a 1:1 (v/v) mixture of absolute ethanol:propylene oxide. Infiltration was continued with two 10 min treatments with propylene oxide followed by a 45 min treatment with a 1:1 (v/v) mixture of propylene oxide:Maraglas. The pellets were then finally allowed to infiltrate with Maraglas. They were initially treated for 1 hr with this resin, then transferred to fresh resin and treated for an additional 2-3 hr.

6. Embedding

Finally a small piece ($\leq mm^3$) of the pellet was deposited into the tip of a labeled BEEM capsule. The capsule was filled with freshly prepared embedding medium composed of either 39.5 gm Maraglas, 9.5 gm Cardolite NC-513, 5.25 gm Dibutyl Phthalate, and 1 ml BDMA (Fig. 12) or 36 ml Maraglas, 8.0 ml DER-732, 5.0 ml Dibutyl Phthalate, and 1.0 ml BDMA (Figs. 10 & 11). The first Maraglas mixture was

allowed to cure at 35°C overnight, then at 45°C during the following day, and finally at 60°C for an additional 24 hr (Fig. 12), while the other was simply hardened for at least 48 hr at 60°C (Fig. 10 & 11). After polymerization was completed, the BEEM capsules were removed and the blocks were sectioned with either a glass (Figs. 10 & 11A,B,D) or diamond (Figs. 11C & 12) knife on a Porter-Blum ultramicrotome. Gold colored sections (900-1500 A thick) were picked up on formvar-carbon coated copper mesh grids and stained with lead citrate before viewing. Low magnification observations were caried out on a Zeiss 98-2 TEM (Figs. 10 & 11 A,B,D), while higher magnification observations were carried out on a Jelco T7 TEM (Figs. 11C & 12).

D. Repeated Experiments

Homogenates were fractionated into high speed supernatants and pellets, then assayed for the indicated enzyme activities at least 4 times. The data (see Table XIV) represent the results of these four experiments.

Homogenates were fractionated at least 6 times by differential centrifugation with essentially the same results (see Fig. 9). EM analyses (see Figs. 10, 11, & 12) were run simultaneously on two of these runs.

Exogenous hog kidney D-amino acid oxidase was used to check for the non-specific adsorption of soluble enzyme to subcellular organelles twice. The data (see Table XV) represent the results of one such typical experiment.

The effect of various homogenization media on the re-

covery of marker enzyme activity (see Table XVI) was tested once.

The zonal density gradient centrifugal fractionation of the subcellular organelles (see Fig. 13) was also run once.

V. PARTIAL PURIFICATION OF BAIB OXIDASE ACTIVITY

A. $(NH_4)_2SO_4$ Precipitation

Eight-hundred ml cultures of Tetrahymena pyriformis were grown to late log phase in 2800 ml Fernbach flasks and harvested as described (see M&M Section IA). The washed cells (see M&M Section IA) were homogenized as described (see M&M Section IIA) in 0.1 M Tris-HCl buffer, pH 8.6. A small aliquot of this homogenate was retained for enzyme analysis and protein determination. The remainder was centrifuged at 3,000 RPM (1085 x g) for 10 min using the SS-34 rotor in a Sorvall RC-2 refrigerated centrifuge set at 4°C. The resulting supernatant was carefully decanted and fractionated according to an $(NH_4)_2SO_4$ precipitation scheme based in part on the procedures of Brumby and Massey.[64] All enzyme activities were assayed for as previously described (see M&M Section IVB), except that it was not thought to be necessary to include Triton X-100 in the $(NH_4)_2SO_4$ precipitate assay mixtures.

Initially attempts were made to precipitate the bulk of the BAIB oxidase activity. After some preliminary investigation on the precipitability of this oxidase activ-

ity by various $(NH_4)_2SO_4$ concentrations (see Table XVII(A)) we developed the following fractionation scheme: An appropriate amount[65] of the salt was weighed out and added to the low speed (1085 x g) supernatant prepared as described above to bring it to a concentration of 10% $(NH_4)_2SO_4$. This mixture was allowed to stand on ice for 15 min with occasional stirring until the $(NH_4)_2SO_4$ was completely dissolved. The solution was then centrifuged at 3,000 RPM (1085 x g) for 1 hr using the SS-34 rotor in a Sorvall RC-2 refrigerated centrifuge set at 4°C. The resulting supernatant was decanted. The pellet was resuspended in a minimal volume of 0.1 M Tris-HCl buffer, pH 8.6, and retained for enzyme analysis and protein determination. The total volume of the resulting 10% $(NH_4)_2SO_4$ supernatant was then measured and an additional appropriate amount[65] of the salt was weighed out and added to this supernatant to bring it to 60% $(NH_4)_2SO_4$. Again this mixture was allowed to stand on ice for 15 min with occasional stirring until the $(NH_4)_2SO_4$ was completely dissolved. This solution was then centrifuged at 14,500 RPM (25,300 x g) for 20 min using the Sorvall. The resulting supernatant was decanted, while the pellet was resuspended in a minimal volume of 0.1 M Tris-HCl buffer, pH 8.6, and retained for enzyme analysis and protein determination. This 10-60% $(NH_4)_2SO_4$ precipitate seemed to contain the bulk of the BAIB oxidase activity (see Table XVII(A)). It also contained the bulk of the D-amino acid oxidase activity (see Table XVII(A)). A final

$(NH_4)_2SO_4$ precipitate was prepared in a similar manner. That is, the volume of the resulting 60% $(NH_4)_2SO_4$ supernatant was measured and enough additional salt[65] was dissolved as described above in this supernatant to saturate it. This solution was also centrifuged at 25,300 x g for 20 min in the Sorvall. The resulting supernatant was again decanted, while the pellet was resuspended in a minimal volume of 0.1 M Tris-HCl buffer, pH 8.6, and retained for enzyme analysis and protein determination.

Subsequently attempts were made to separate BAIB oxidase activity from that of other enzyme activities, particularly D-amino acid oxidase activity. The following fractionation scheme, therefore, was developed for this purpose (see Table XVII(B)): An appropriate amount[65] of salt was weighed out and added to the low speed (1085 x g) supernatant prepared as described above to bring it to a concentration of 40% $(NH_4)_2SO_4$. This mixture was allowed to stand on ice for 15 min with occasional stirring until the $(NH_4)_2SO_4$ was completely dissolved. The solution was then centrifuged at 3,000 RPM (1085 x g) for 1 hr using the SS-34 rotor in a Sorvall RC-2 refrigerated centrifuge set at 4°C. The resulting supernatant was decanted. The pellet was resuspended in a minimal volume of 0.1 M Tris-HCl buffer, pH 8.6, and retained for enzyme analysis and protein determination. This 0-40% $(NH_4)_2SO_4$ precipitate seemed to contain substantial BAIB oxidase activity, but little of the other marker enzyme activities, including D-

amino acid oxidase activity (see Table XVII(B)). The total volume of the resulting 40% $(NH_4)_2SO_4$ supernatant was then measured and an additional appropriate amount[65] of the salt was dissolved as described above in this supernatant to bring it to 60% $(NH_4)_2SO_4$. This solution was then centrifuged at 14,500 RPM (25,300 x g) for 20 min using the Sorvall. The resulting supernatant was decanted, while the pellet was resuspended in a minimal volume of 0.1 M Tris-HCl buffer, pH 8.6, and retained for enzyme analysis and protein determination. A final $(NH_4)_2SO_4$ precipitate was prepared in a similar manner. That is, the volume of the resulting 60% $(NH_4)_2SO_4$ supernatant was measured and enough additional salt[65] was dissolved as described above in this supernatant to saturate it. This solution was also centrifuged at 25,300 x g for 20 min in the Sorvall as described above. The resulting supernatant was again decanted, while the pellet was resuspended in a minimal volume of 0.1 M Tris-HCl buffer, pH 8.6, and retained for enzyme analysis and protein determination.

B. Affinity Chromatography

Eight-hundred ml cultures of Tetrahymena pyriformis were grown to late log phase in 2800 ml Fernbach flasks and harvested as described (see M&M Section IA). The washed cells (see M&M Section IA) were homogenized as described (see M&M Section IIA) in 0.1 M Tris-HCl buffer, pH 8.6. The homogenate was centrifuged at 3,000 RPM (1085 x g) for 10 min using the SS-34 rotor in a Sorvall RC-2 refrigerated

centrifuge set at 4°C. The resulting supernatant was decanted and fractionated according to the above $(NH_4)_2SO_4$ precipitation scheme (see M&M Section VA).

A D-ala affinity column was prepared according to the company's instructions as follows: Approximately 2.5 gm of Activated CH-Sepharose 4B (Pharmacia) were weighed out and swollen in 1 mM HCl. The swollen gel was washed with an additional 500 ml (200 ml/gm) of 1 mM HCl on a coarse fritted glass filter (Corning). The washed gel was then resuspended in 15 ml of 0.1 M $NaHCO_3$ buffer, pH 8.0, and transferred to a 100 ml beaker. 0.7568 gm of D-ala was then added to the gel suspension and swirled.[66] A 1 ml aliquot of this supernatant was immediately removed and retained for enzyme assay. The suspension was then allowed to shake gently (so that the gel did not settle) for 1 hr at room temperature to permit the covalent binding of the amino acid. The gel was again filtered on the coarse fritted glass filter and the filtrate was saved for enzyme assay. The gel was resuspended in 50 ml of 0.1 M Tris-HCl buffer, pH 8.0, transferred to a 250 ml beaker, and allowed to stand at room temperature for 1 hr. This suspension was then again filtered on a coarse fritted glass filter and the filtrate was saved for enzyme assay. The filtered gel was then washed on the filter with 200 ml of 0.5 M NaCl in 0.05 M Tris-HCl buffer, pH 8.0, followed by 200 ml of 0.5 M NaCl in 0.05 M formate buffer, pH 4.0, to remove excess, non-covalently bound D-ala. The final few ml of this fil-

trate were also collected and saved for enzyme assay. The gel was finally washed with 100 ml of 0.1 M Tris-HCl buffer, pH 8.6. This filtrate was saved for enzyme analysis and the gel was resuspended and stored in this buffer.

On the basis of enzyme analysis of the various filtrates it was determined that about 16.1% of the added D-ala had been bound resulting in an adsorbent that contained approximately 18.2 umoles of D-ala/ml of gel. This was about the maximum amount of amino acid that could be expected to bind under these conditions.[67] No D-ala could be detected enzymatically in the filtrates after the salt washes.

A small column (0.9 x 6.5 cm) of this D-ala affinity gel was packed in the cold. One ml of the 0-40% $(NH_4)_2SO_4$ precipitate containing approximately 25 $A_{280\ nm}$ units was layered on top of this column. The column was then developed in the cold (4°C) with 0.1 M Tris-HCl buffer, pH 8.6. 2.5 ml fractions were collected. The effluent was continuously monitored at 253 nm with a LKB Uvicord I. The protein came off the column in one rather skewed peak. The $A_{280\ nm}$ of all peak fractions was then redetermined with the Beckman DB spectrophotometer. The enzyme activities of the peak fractions were also determined as previously described (see M&M Section IVB). Recoveries were finally expressed as the per cent of activity in the 0-40% $(NH_4)_2SO_4$ precipitate (see Table XVIII). The column was regenerated after each separation by washing with 200 ml of 0.5 M NaCl

in 0.05 M Tris-HCl buffer, pH 8.0, followed by 200 ml of 0.5 M NaCl in 0.05 M formate buffer, pH 4.0, then re-equilibrating with 200 ml of 0.1 M Tris-HCl buffer, pH 8.6.

C. Repeated Experiments

D-amino acid oxidase and BAIB oxidase activities were precipitated by various concentrations of $(NH_4)_2SO_4$ numerous (at least 10) times. The other marker enzyme activities, that is, MDH, catalase, and lactic acid oxidase, were precipitated twice. The data (see Table XVII) represent the results of one such typical experiment.

The resuspended 0-40% $(NH_4)_2SO_4$ precipitate was purified by D-ala affinity column chromatography twice. The data (see Table XVIII) represent the results of one such typical experiment.

VI. PHYSIOLOGICAL SIGNIFICANCE OF BAIB OXIDASE ACTIVITY

A. Specific Activity

A standard H_2O_2 decomposition curve was prepared by incubating varying amounts of this substrate in a peroxidase assay system (see Fig. 14). This assay system was based on the procedure of Guilbaut and Hieserman[59] and contained 1.2 ml of 0.2 M Tris-HCl buffer, pH 8.6 (final conc = 80 mM), 0.1 ml FAD (final conc = 10^{-4} mM), 1.0 ml H_2O_2 (final conc = 0.33-3.28 uM), 0.1 ml HVA (final conc = 80 ug/ml), and 0.1 ml peroxidase (final conc = 50 ug/ml) in a total volume of 3 ml. The amount of fluorescence detected was directly proportional to the amount of H_2O_2 present.

The number of nmoles of H_2O_2 decomposed in each assay system was calculated from the measurement of the $A_{240\ nm}$ of the stock solution. (The molar absorbancy for H_2O_2 at 240 nm in a 1 cm cuvette is 43.6.[62]) The number of nmoles of H_2O_2 produced as a result of BAIB oxidase activity in the Tetrahymena pyriformis homogenate under these conditions was then read off the standard curve. This activity (as nmoles/min) was finally converted to specific activity (nmoles/min/mg of homogenate protein). The specific activity of this oxidase so calculated agreed well with our previous estimate based on the assumed total enzymatic conversion of a known amount of HVA from its non-fluorescent to its fluorescent form.

B. Substrate Specificity

Determining the substrate specificity of this oxidase also became of particular interest since it had been reported that thymine was preferentially reductively catabolized to D(-)BAIB and that only this isomer could then be effectively metabolized further in mammalian systems.[68-72] As it is difficult to chemically separate these isomers from one another, however, we thought that we might be able to selectively eliminate each of them by pretreating the assay system with an appropriate exogenous enzyme prior to the addition of the Tetrahymena pyriformis homogenate. Thus, before the addition of this crude enzyme preparation, the incomplete BAIB oxidase assay system (see M&M Section IIIA) containing one of the indicated substrates (see Fig.

15) was incubated for 90 min at 37°C with either 5 ul of snake venom L-amino acid oxidase (final conc = 1.7 ug/ml) or hog kidney D-amino acid oxidase (final conc = 0.2 mg/ml). The exogenous oxidase activities of these assay systems were then measured at 10 min intervals for 90 min. It was hoped that the exogenous L-amino acid oxidase might reduce, if not eliminate, the L(+)BAIB from the assay system, while the D-amino acid oxidase might reduce, if not eliminate, the D(-)BAIB from the assay system. After these exogenous oxidase activities had leveled off, 0.1 ml of a Tetrahymena pyriformis homogenate prepared in 0.1 M Tris-HCl buffer, pH 8.6, was added to complete each assay system. It was then thought that any increase in oxidase activity in the D-amino acid oxidase system subsequent to the addition of this crude Tetrahymena pyriformis enzyme preparation might reflect the metabolism of the remaining L(+)BAIB, while a similar increase in the L-amino acid oxidase system might reflect the metabolism of the remaining D(-)BAIB.

C. Substantiation of the Overall Catabolic Pathway

The complete BAIB oxidase system (see M&M Section IIIA) was incubated for approximately 6 hr at 37°C with the indicated (see Fig. 16) dihydropyrimidine (DHP) derivatives as substrates instead of BAIB. H_2O_2 production was then measured at 10-30 min intervals. Since these DHP derivatives are considerably less soluble than BAIB, however, their concentrations (final conc = 0.0167 M) in the oxidase assay system were substantially less than that of BAIB (final conc

= 0.1 M) which might account for the apparent slowness of these reactions. We were, nevertheless, able to detect substantial oxidase activity by extending the incubation period.

D. Kinetics

After having done some initial work establishing activity dependence on enzyme preparation and substrate concentrations, preliminary studies were done on the kinetics of the indicated oxidase reactions (see Fig. 17). The linear range of activity dependence on enzyme concentration for each reaction was determined, then the appropriate substrate concentrations varied within this enzyme concentration range to obtain the necessary data to construct a Lineweaver-Burk plot of each activity. The best fitting straight line was mathematically calculated by regression analysis of the data points (Dr. Leslie Marcus, personal communication). The K_m of each reaction was then graphically determined.[73]

E. Effect of Culturing Conditions on BAIB Oxidase Activity

1. Relationship between enzyme activity and culture age

Eight-hundred ml cultures in 2800 ml Fernbach flasks were inoculated as described (see M&M Section IA). After 24 hr 200 ml of this culture were aseptically removed and harvested as described (see M&M Section IA). At four subsequent 48 hr intervals, that is, after 3, 5, 7, and 9 days, an additional 100 ml of this culture were again aseptically removed and harvested as described (see M&M Section IA).

Finally, the remaining culture was harvested as described (see M&M Section IA) 15 days after inoculation. After each harvesting the washed cells were homogenized in 0.1 M Tris-HCl buffer, pH 8.6, (see M&M Section IIA) and assayed for BAIB (see M&M Section IVB5) as well as D-amino acid (see M&M Section IVB3) oxidase activity.

2. Inducibility

To determine if BAIB oxidase activity in Tetrahymena pyriformis homogenates might be inducible, 100 ml cultures were grown to log phase in the presence of the indicated amounts of the proposed thymidine catabolic intermediates (see Table XX) in 500 ml nephelometer flasks inoculated with 10 ml of the same stock culture as described (see M&M Section IA). After harvesting the washed cells were homogenized in 0.1 M Tris-HCl buffer, pH 8.6, (see M&M Section IIA) and assayed for BAIB (see M&M Section IVB5) as well as D-amino acid (see M&M Section IVB3) oxidase activity.

F. Repeated Experiments

The standard H_2O_2 decomposition curve (see Fig. 14) was prepared once.

The stereoisomer substrate specificity of the BAIB oxidase activity (see Fig. 15) was determined twice using D-amino acid oxidase and 3 times with L-amino acid oxidase with essentially the same results.

The overall catabolic pathway (see Fig. 16) was substantiated 2-3 times with each of the indicated DHP derivatives. The results were generally as indicated, although

DHT oxidation was not always so rapid.

The kinetics of each of the indicated oxidase activities were determined numerous times. That is, the kinetics of BAIB and D-amino acid oxidase activity of Tetrahymena pyriformis homogenates were each determined 5 times, while the kinetics of BAIB oxidase activity of crude commercial hog kidney D-amino acid oxidase was determined 3 times, and that of crude commercial hog kidney D-amino acid oxidase activity itself was determined twice. The graphs (see Fig. 17) represent the best of these determinations.

The relationship between BAIB oxidase activity and culture age was determined twice. The data (see Table XIX) represent the results of one such typical experiment.

The inducibility of BAIB oxidase activity was tested 2-3 times with each of the suspected inducing substrates. Whether they were dissolved in the original culture medium or added a few hr before havesting did not seem to affect inducibility. The data (see Table XX) represent the results of one of the former such typical experiments.

VII. SOURCES OF RADIOACTIVE TRACERS, STANDARDS, ENZYMES, AND OTHER CHEMICAL REAGENTS

The 2,6-C^{14} labeled thymidine (sp. act. = 105 mCi/mM) was obtained from Nuclear-Dynamics. The 2-^{14}C labeled MMA (sp. act. = 0.05 mCi/0.6mg) was obtained from New England Nuclear.

For Bray's solution: methanol (Certified ACS Spec-

tranalyzed) was obtained from Fisher; ethelene glycol (reagent grade) and 1,4 dioxane (reagent grade) were obtained from Baker; naphthalene (Eastman grade) was obtained from Eastman; 2,5-diphenyloxazole (PPO; scintillation grade) was obtained from Amersham/Searle; and p-bis[2-(5-phenyloxazolyl)]-benzene (POPOP; scintillation grade) was obtained from New England Nuclear. Hyamine hydroxide (1 M solution in methanol) was obtained from Amersham/Serle.

B-D-glucose was obtained from Calbiochem. Shellfish glycogen was obtained from Mann. HSA (grade III) was obtained from Sigma. DNA and RNA were obtained from Mann. Anthrone (reagent grade) was obtained from Fisher. Diphenylamine and orcinol were obtained from Matheson.

Proteose peptone and yeast extract were obtained from Difco. Thiamine·HCl (vitamin B_1, B grade) was obtained from Calbiochem.

DEAE cellulose (DE 23) was obtained from Whatman.

NADPH and NADH were obtained from Sigma. Thymine was obtained from Calbiochem.

D,L-BAIB was obtained from Sigma, Calbiochem, and K & K. Glu was obtained from Mann. αKG was obtained from Sigma. OAA was obtained from Calbiochem. Pyruvic acid (sodium salt) was obtained from Eastman. Glyoxylic acid was obtained from Sigma.

2,4-Dinitrophenylhydrazine was obtained from Matheson.

2,6-Dichlorophenolindophenol was obtained from Mann. Phenazine methosulfate was obtained from Nutritional Bio-

chem.

4-Hydroxy-3-methoxy-phenylacetic acid (HVA) was obtained from Sigma. Horseradish peroxidase (type I; RZ 0.6) was also obtained from Sigma.

FAD, EDTA, B-mercaptoethanol, reduced glutathione, and dithioerythritol were obtained from Sigma. Dithiothreitol was obtained from Calbiochem. PCMB (Na salt) was obtained from Mann. Sodium dodecyl sulfate (SDS) was obtained from K & K.

1-Methoxy-2-propanol (practical grade) was obtained from Baker.

Crude hog kidney D-amino acid oxidase (0.09 units/mg) and D-ala were obtained from Sigma. Superoxol (30% H_2O_2) was obtained from Merck. Lactic acid (Li and Na salt) was obtained from Sigma.

Glutaraldehyde, sodium cacodylate, OsO_4, propylene oxide, Maraglas, Cardolite NC-513, Dibutyl Phthalate, and BDMA were all obtained from Ladd. Sodium barbital (for veronal acetate buffer) was obtained from Fisher. Uranyl acetate was obtained from Baker. DER-732 was obtained from Polysciences, Inc.

Enzyme grade $(NH_4)_2SO_4$ was obtained from Mann.

Activated CH-Sepharose 4B was obtained from Pharmacia.

Crude snake (Crotalus) venom L-amino acid oxidase (type I; 0.3 units/mg) was obtained from Sigma. L-ala was obtained from Mann. B-ala was obtained from Sigma.

DHT, DHU, DHTdine, and DHUdine were all obtained from Sigma.

Tdine was obtained from Sigma.

All other chemicals used were of analytical-reagent grade.

RESULTS

I. RADIOACTIVE TRACER STUDIES

The first part of this study involved the reinvestigation of the proposed reductive thymine catabolic and reutilization pathway by means of radioactive tracers.

A. 2,6-^{14}C-Labeled Thymidine

2,6-^{14}C-labeled thymidine was used to resubstantiate the overall pathway. Based on previous radiolabeled studies from our laboratory[10,11] the 2-^{14}C label was expected to be rapidly recovered as $^{14}CO_2$, while the 6-^{14}C label was expected to be recovered in macromolecular fractions other than DNA.

The cells were grown in 2,6-^{14}C-labeled thymidine for approximately the final 12 hr of culture growth, collected, and fractionated into their various macromolecular components as described (see M&M Section IA-D). The distribution of the recovered radioactivity is given in Table I. Slightly less than 50% of the total 2,6-^{14}C-labeled thymidine radioactivity was recovered in the DNA fraction. This percent recovery is even less than previously reported[10,11] which might be due to the fact that in these experiments the labeled thymidine was added much later in culture growth, and therefore more likely to be metabolized than incorporated into DNA.

The crude RNA fraction was placed on a DEAE cellulose column to remove contaminating carbohydrate and DNA as de-

TABLE I. INCORPORATION OF ^{14}C INTO VARIOUS CELL FRACTIONS OF TETRAHYMENA PYRIFORMIS GROWN WITH 2,6-^{14}C THYMIDINE.

Cells were grown for approximately the final 12 hr of culture in modified Elliott's medium containing 50 uCi of 2,6-^{14}C thymidine (sp. act. = 105 mCi/mM), harvested, and fractionated into the indicated macromolecular components as described in the text (see M&M Section IA-D).

% OF TOTAL ^{14}C ACT. INCORP. was calculated on the basis of the sum of the ^{14}C recovered in the component fractions.

TOTAL MACROMOLECULAR radioactivity is the sum of the ^{14}C recovered in all of the above macromolecular fractions.

TOTAL ^{14}C RECOVERED is the sum of the ^{14}C recovered in all of the above macromolecular fractions plus the total respired CO_2.

TOTAL CO_2 is the sum of the $^{14}CO_2$ produced during the first 3 hr of growth in radioactive medium plus the $^{14}CO_2$ produced during the last 9 hr of culture growth plus the $^{14}CO_2$ released after the acid titration of the cell-free medium. This $^{14}CO_2$ was produced, trapped, and measured as described in the text (see M&M Section IB).

TOTAL ^{14}C ADDED was determined by counting an aliquot of the culture medium immediately after the addition of the radioactivity. This value agreed to within 10-15% of the mathematically calculated one:

$$(2.22 \times 10^6 \text{ dmp/uCi}) \times (50 \text{ uCi}) \times \left(\frac{30,337.4 \text{ cpm}}{31,900 \text{ dpm}}\right)^{*}$$

*^{14}C counting efficiency

TABLE I

INCORPORATION OF ^{14}C INTO VARIOUS CELL FRACTIONS OF *TETRAHYMENA PYRIFORMIS* GROWN WITH 2,6-^{14}C THYMIDINE

FRACTION	TOTAL VOL (mls)	ALIQUOT (uls)	CPM	TOTAL CPM ($\times 10^4$)	% OF TOTAL ^{14}C ACT. INCORP.
Acid Soluble	34	200	1553	26.4	33.8
Lipid	13.5	200	595	4.0	5.1
RNA	4	20	66	1.3	1.7
DNA	25	250	3849	38.5	49.2
Protein	5	50	561	5.6	7.2
Carbohydrate	10	50	118	2.4	3.0
TOTAL MACROMOLECULAR				78.2	75.8
TOTAL CO_2				24.9	24.2
^{14}C TOTAL ^{14}C RECOVERED				103.1	0.9
^{14}C TOTAL ^{14}C ADDED				11320	99.1

scribed (see M&M Section ID). The results of this column fractionation are given in Fig. 2. The first relatively uncharged peak eluted off the column by the lithium acetate buffer was colorimetrically and chromatographically identified as carbohydrate. This column-recovered carbohydrate radioactivity was added to the acid-soluble carbohydrate radioactivity and the combined total entered in Table I as the amount of ^{14}C activity incorporated into the carbohydrate fraction. The second peak eluted off the column by 0.04 M LiCl was colorimetrically identified as ribonucleotides. This column-purified RNA radioactivity was entered in Table I as the amount of ^{14}C activity incorporated into the RNA fraction. The third peak to be eluted off the column by 1 M LiCl could either have been oligodeoxyribonucleotides or 2'-O-methyl oligoribonucleotides resistant to alkaline hydrolysis. Earlier methoxyl determinations of this peak, however, indicated that only 0.4-0.5% of these bases are 2'-O-methylated (Dr. John Berech, personal communication). Thus, the majority of the radioactivity in this fraction appeared to be due to radioactive oligodeoxyribonucleotides. This assumption was also colorimetrically substantiated. The radioactivity from this peak, therefore, was added to the previously prepared DNA radioactivity and this combined total was entered in Table I as the amount of ^{14}C activity incorporated into the DNA fraction.

A portion of the carbohydrate fraction was then precipitated, hydrolyzed, and chromatographed in several different

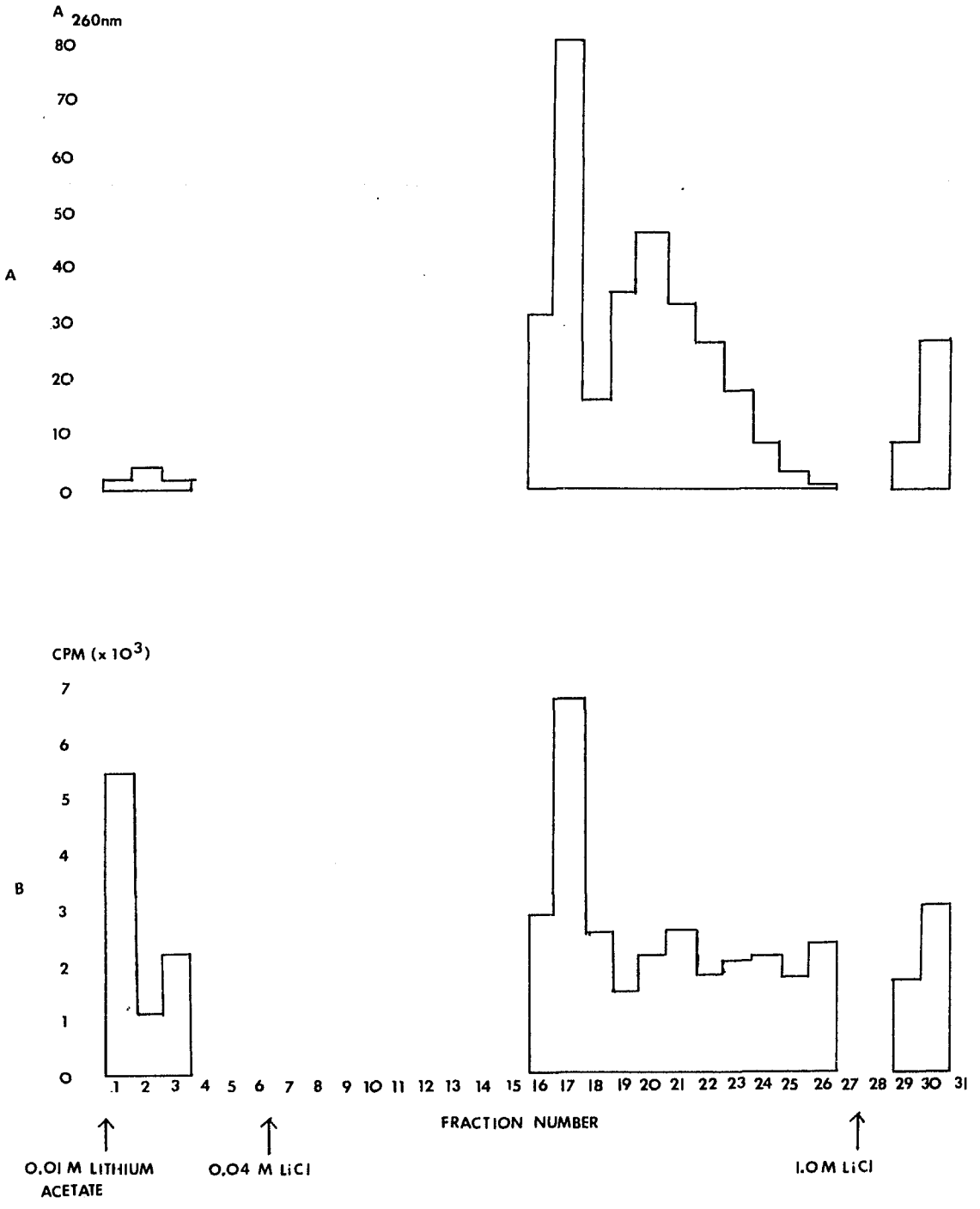
FIGURE 2. DEAE CELLULOSE COLUMN CHROMATOGRAPHY OF ALKALINE HYDROLYZED RNA FROM CELLS GROWN IN 2,6-^{14}C-LABELED THYMIDINE. This crude RNA preparation was eluted with 0.01 M lithium acetate buffer, pH 4.5, 0.04 M LiCl in 0.01 M lithium acetate buffer, pH 4.5, and 1.0 M LiCl in 0.01 M lithium acetate buffer, pH 4.5, at the points indicated.

A. Fractions were assayed for 260 nm absorbance.

B. 100 ul samples from each fraction were removed and assayed for radioactivity.

FIGURE 2

DEAE CELLULOSE COLUMN CHROMATOGRAPHY OF ALKALINE HYDROLYZED RNA FROM CELLS GROWN IN 2,6-^{14}C LABELED THYMIDINE



solvent system as described (see M&M Section IE&F). The results are given in Fig. 3. Standard dTMP was run simultaneously since it had been reported previously that glycogen specifically binds dTMP.[42] The isobutyric acid:conc NH_4OH:H_2O solvent used to develop the chromatogram illustrated in Fig. 3(B) separates glucose from all the major ribonucleotides including dTMP, ribonucleosides including thymidine, and free bases.[10,11] The thymidine radioactivity recovered in the carbohydrate fraction, therefore, is clearly associated with glucose and not thymidine or any of its closely related derivatives.

The amount of $^{14}CO_2$ produced by the cells was monitored for 3 hr after the addition of 2,6-^{14}C-labeled thymidine into the culture. The rate of this $^{14}CO_2$ release is plotted in Fig. 4.

B. 2-^{14}C-Labeled MMA

2-^{14}C-labeled MMA, a key proposed reutilization intermediate, was used to substantiate the latter part of the proposed pathway.

The cells were grown in 2-^{14}C-labeled MMA for approximately the final 12 hr of culture growth, collected, and fractionated into their various macromolecular components as described (see M&M Section IA-D). A portion of the carbohydrate fraction was then precipitated, hydrolyzed, and chromatographed in several different solvent systems as described (see M&M Section IE&F). The results are given in Fig. 5. Again the radioactivity is clearly associated with

FIGURE 3. CHROMATOGRAPHIC DISTRIBUTION OF RADIOACTIVITY FROM 2,6-^{14}C-LABELED THYMIDINE IN THE CARBOHYDRATE FRACTION. A portion of the carbohydrate fraction was precipitated, hydrolyzed, and chromatographed in:

A. THE UPPER PHASE OF A BUTANOL (4):ACETIC ACID (1):H_2O (5) SOLVENT MIXTURE

B. ISOBUTYRIC ACID (66):CONC NH_4OH (1):H_2O (33)

as described in the text (see M&M Section IE&F). The chromatogram was cut into 1 x 2 cm strips and counted (see M&M Section IH). dTMP was visulaized as a UV quenching spot (see M&M Section IF). Areas containing carbohydrate material were visulaized by running the indicated standard sugars in adjacent lanes and spraying with p-anisidine after development (see M&M Section IF).

FIGURE 3A

CHROMATOGRAPHIC DISTRIBUTION OF RADIOACTIVITY FROM 2,6-^{14}C LABELED THYMIDINE IN THE CARBOHYDRATE FRACTION

THE UPPER PHASE OF A BUTANOL (4): ACETIC ACID (1): H_2O (5) SOLVENT MIXTURE

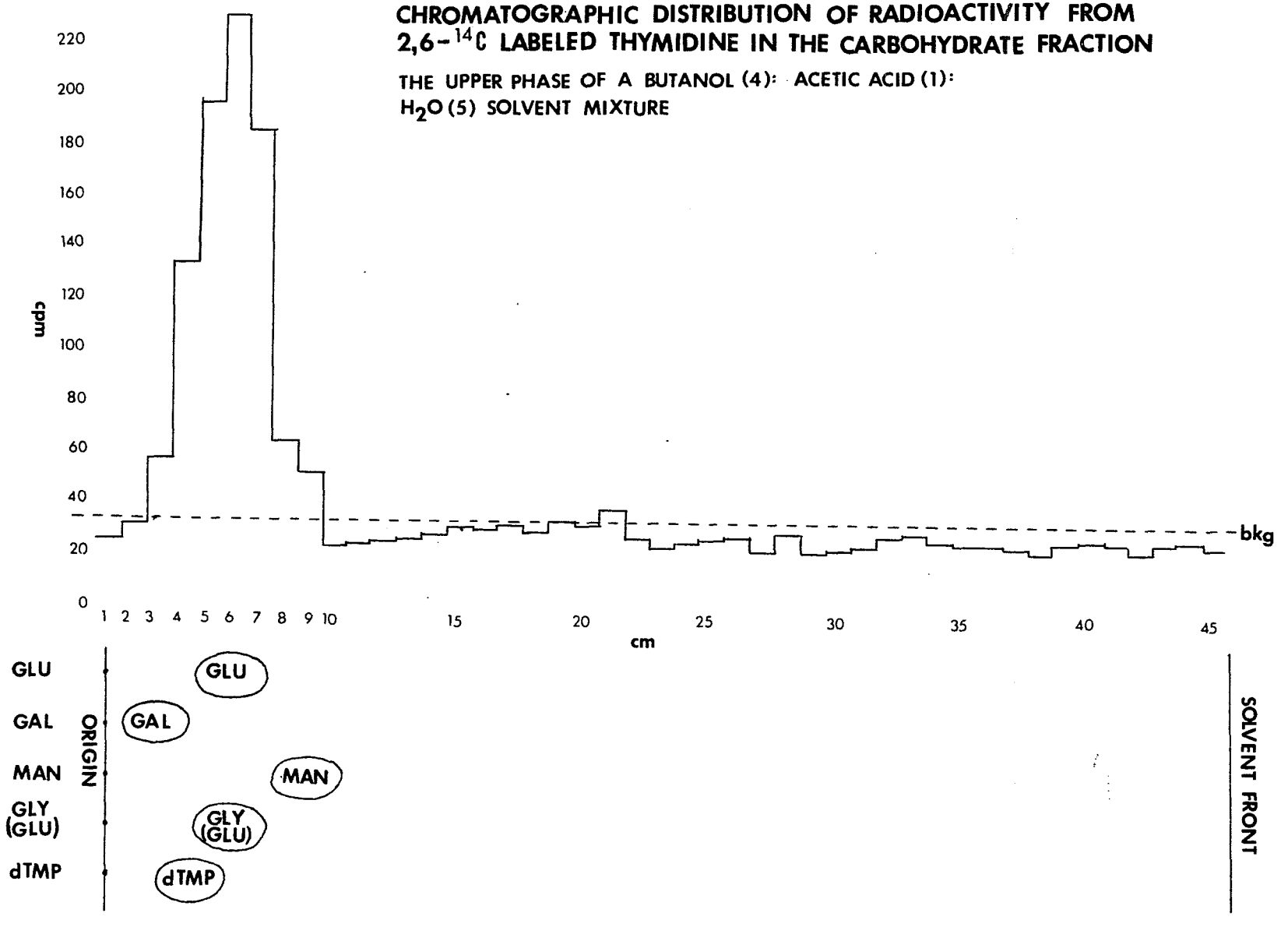
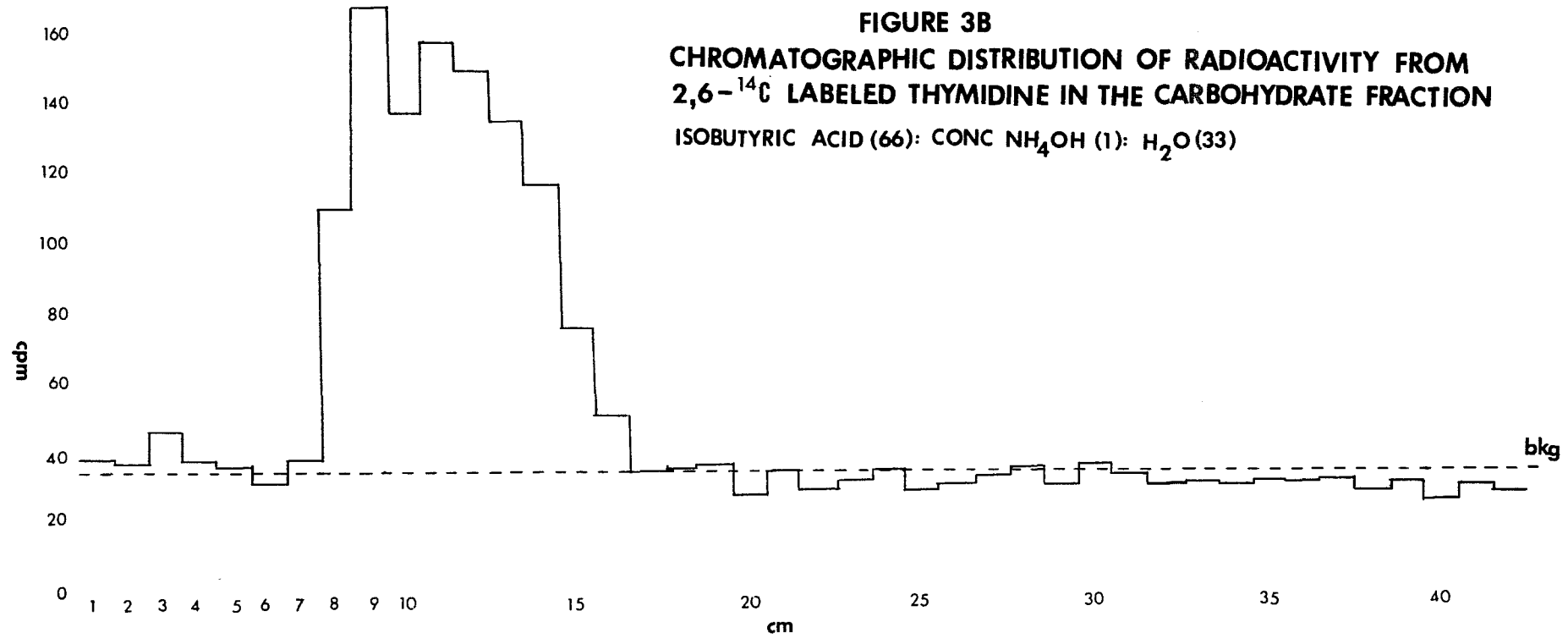


FIGURE 3B
CHROMATOGRAPHIC DISTRIBUTION OF RADIOACTIVITY FROM 2,6-^{14}C LABELED THYMIDINE IN THE CARBOHYDRATE FRACTION

ISOBUTYRIC ACID (66): CONC NH_4OH (1): H_2O (33)



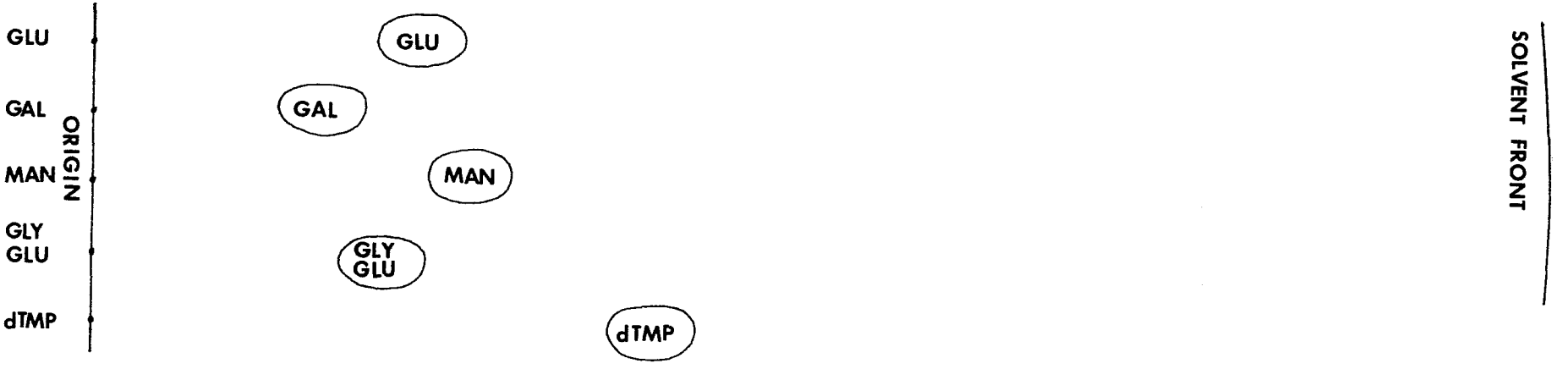


FIGURE 4. RELEASE OF $^{14}CO_2$ BY CELLS INCUBATED IN 2,6-^{14}C THYMIDINE AND 2-^{14}C MMA.

This graph compares the initial rate of $^{14}CO_2$ release by late log phase cultures incubated with 2,6-^{14}C thymidine (●—●) and 2-^{14}C MMA (■—■).

FIGURE 4

RELEASE OF $^{14}CO_2$ BY CELLS INCUBATED IN 2,6-^{14}C THYMIDINE AND 2-^{14}C MMA.

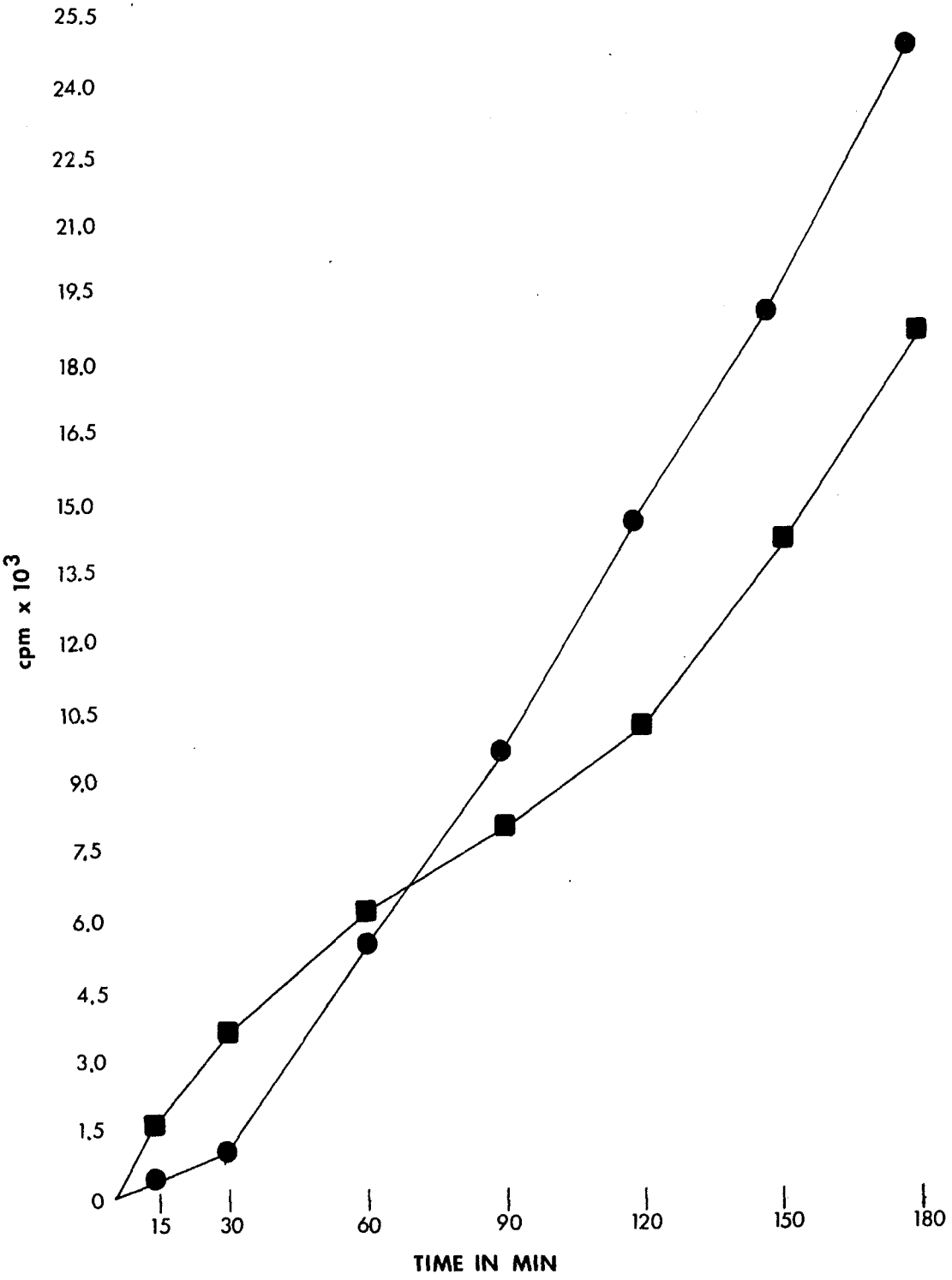


FIGURE 5. CHROMATOGRAPHIC DISTRIBUTION OF 2-^{14}C-LABELED MMA RADIOACTIVITY IN THE CARBOHYDRATE FRACTION. Details as in Fig. 3.

FIGURE 5A
CHROMATOGRAPHIC DISTRIBUTION OF RADIOACTIVITY FROM 2-^{14}C LABELED MMA IN THE CARBOHYDRATE FRACTION

THE UPPER PHASE OF A BUTANOL (4): ACETIC ACID (1): H_2O (5) SOLVENT MIXTURE

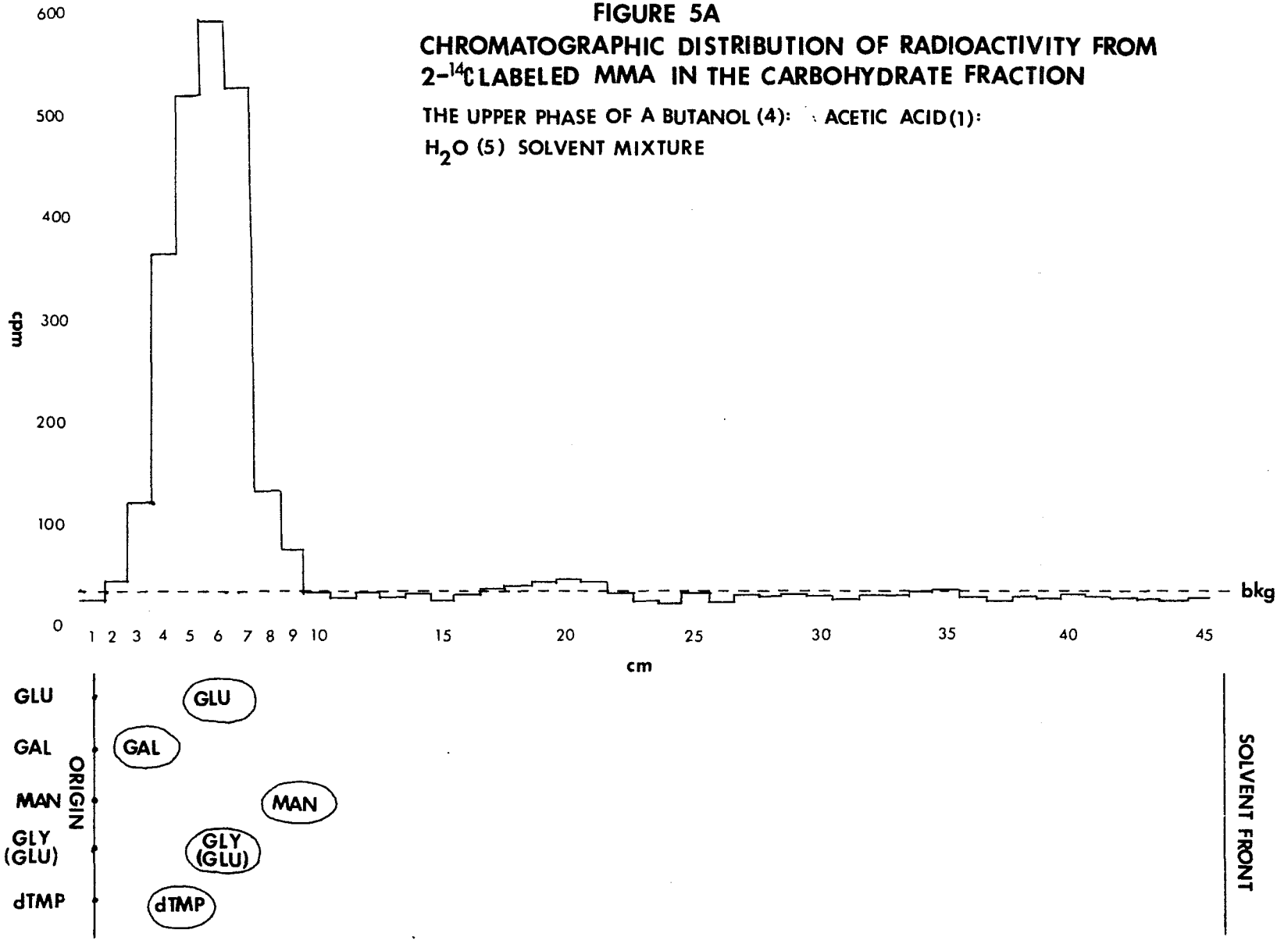
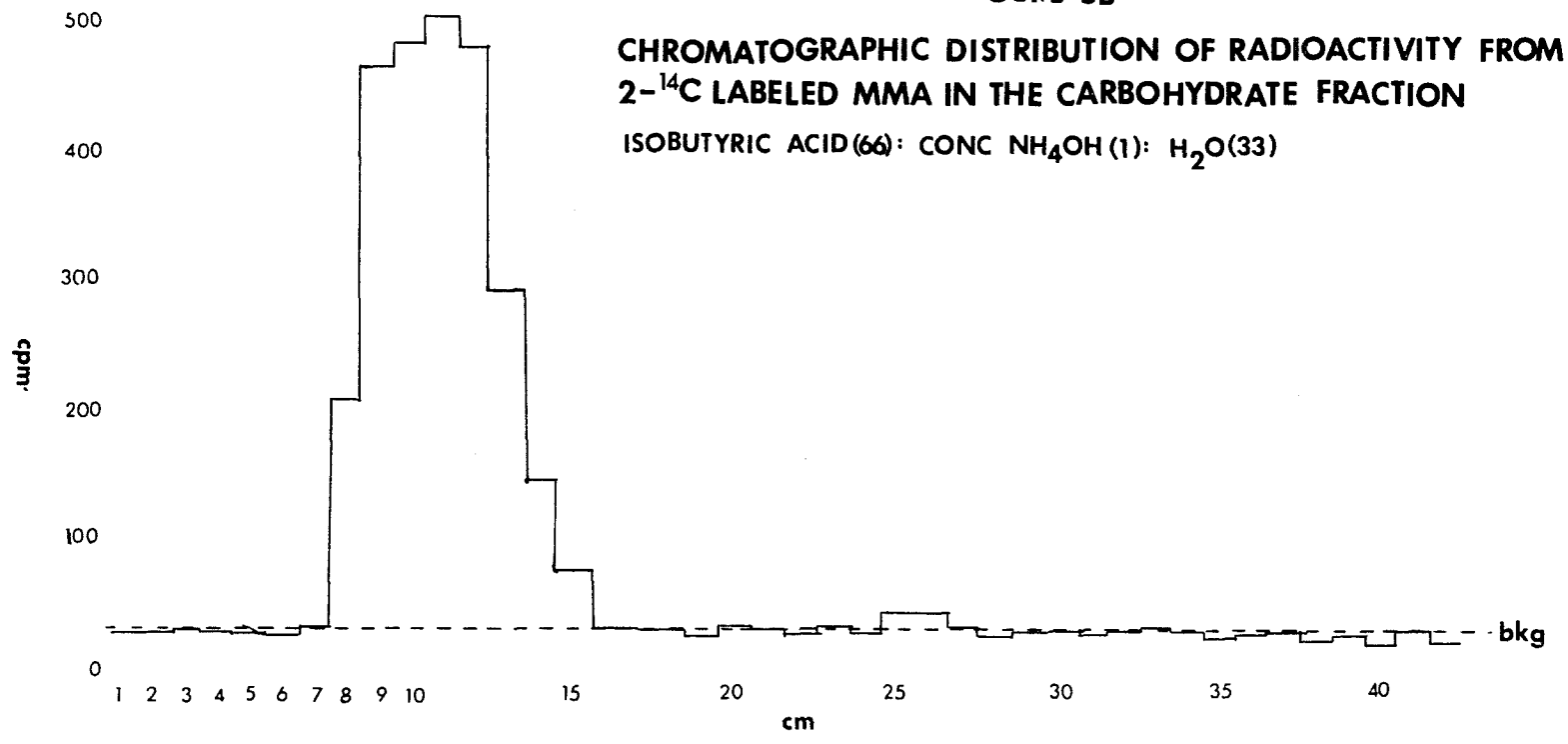


FIGURE 5B

CHROMATOGRAPHIC DISTRIBUTION OF RADIOACTIVITY FROM 2-^{14}C LABELED MMA IN THE CARBOHYDRATE FRACTION

ISOBUTYRIC ACID (66): CONC NH_4OH (1): H_2O (33)



glucose.

The amount of $^{14}CO_2$ produced by the cells was also monitored for 3 hr after the addition of 2-^{14}C-labeled MMA into the culture. The rate of this $^{14}CO_2$ release is also plotted in Fig. 4. Although the initial pattern of $^{14}CO_2$ release in these two systems is quite different, the latter part of these two graphs are approximately parallel, indicating equal rates of release[9] which is what would be expected if these two substrates were eventually metabolized in the same way.

II. PRELIMINARY DEMONSTRATION OF THE CATABOLIC ENZYMES

A. Thymine Reductase (Dehydrogenase)

After resubstantiating the proposed reductive pyrimidine reutilization pathway with radiolabeled intermediates, the first enzyme in this pathway, thymine reductase (dehydrogenase), was investigated. The possible presence of this enzyme in Tetrahymena pyriformis was suggested by a report of a reduced pyrimidine excretion product in the related ciliate Paramecium aurelia[74] as well as by previous competition experiments from our laboratory[10,11] in which unlabeled DHT added to cells grown on 2-^{14}C-labeled thymidine resulted in a reduction of the amount of $^{14}CO_2$ produced. Enzyme activity was monitored spectrophotometrically by following the decrease in 340 nm absorbance of its cofactor NADPH as described (see M&M Section IIB1b). Preliminary results (see Table II) indicated that this en-

TABLE II. THYMINE REDUCTASE (DEHYDROGENASE) ACTIVITY IN SUPERNATANT FRACTIONS OF TETRAHYMENA PYRIFORMIS HOMOGENATES. The homogenate was prepared (see M&M Section IIA) and centrifuged (see M&M Section IIB1a) at the indicated speeds as described in the text. The enzyme activity of each supernatant fraction was then assayed for as described in the text (see M&M Section IIB1b). Enzyme activities were corrected for endogenous NADPH-DH activity by subtracting the appropriate blank reaction (see M&M Section IIB1b).

TABLE II

THYMINE REDUCTASE (DEHYDROGENASE) ACTIVITY IN SUPERNATANT FRACTIONS OF *TETRAHYMENA PYRIFORMIS* HOMOGENATES

SUPERNATANT FRACTION	ACTIVITY ($\Delta A_{340\ nm}$/min/ml*)
480 x g	0.00931
1,475 x g	0.00793
12,100 x g	0.00275
100,000 x g	0

*although the protein concentration of this preparation was not determined on the basis of subsequent experiments 1 ml of this homogenate was estimated to contain approximately 10 mg of protein.

zyme activity could indeed be demonstrated in low speed supernatants of Tetrahymena pyriformis homogenates.

B. BAIB Metabolism

The catabolic part of this proposed pathway, that is, the conversion of thymine to DHT and then eventually to BAIB has already been extensively described and the enzymes involved at least partially purified from various bacterial,[75-80] mammalian,[49,50,81-101] invertebrate,[102-106] and plant[107-114] systems. Most of these studies, however, terminate with the production of BAIB and little work has been done on the further metabolism of this compound. The conversion of this catabolic end product to an anabolic substrate, possibly MMA, however, is a critical step in the overall proposed catabolic and reutilization pathway. The presence of an enzyme catalyzing such a conversion in Tetrahymena pyriformis was also suggested by previous competition experiments from our laboratory[10,11] in which unlabeled MMA added to cells growing on methyl-^{14}C-labeled thymidine again resulted in a reduction of the amount of $^{14}CO_2$ produced. Thus, encouraged by the preliminary demonstration of the first of the required enzymes in the reductive pathway and hopeful that the subsequent catabolic enzymes would also be eventually demonstrated (see Fig. 16), we decided to investigate the nature of the pivotal enzyme responsible for the further metabolism of BAIB in more detail.

1. Transaminase Reaction

Most of the few studies on the further metabolism of

BAIB in other organisms suggested the transamination of BAIB to methylmalonic semialdehyde and then its further oxidation to MMA. The possibility of such a transaminase also operating in Tetrahymena pyriformis, therefore, was investigated. The exploratory transaminating assay systems were incubated for varying periods of time and the resulting amino acids and 2,4-dinitrophenylhydrazones of the keto acids were quantitatively identified by chromatography as described (see M&M Section IIB2a). It was expected that if BAIB was indeed further metabolized in this manner, then its concentration as well as that of the coupled transaminating keto acid (αKG) should decrease with incubation time, while the concentrations of the resulting transaminated amino acid (glu) and keto acid (methylmalonic semialdehyde) should increase with incubation time. Such concomitant consistent and significant changes in the transaminating amino and keto acid concentrations, however, could not be demonstrated either by us (see Table III) or by others[51] in Tetrahymena pyriformis using this assay system. In fact, no 2,4-dinitrophenylhydrazone with the expected R_F of the methylmalonic semialdehyde derivative could even be detected. We therefore decided to investigate the alternative possibility of the further metabolism of BAIB by direct oxidation.

2. Oxidase Reaction

Oxidase activity was initially monitored spectrophotometrically by following the decrease in 600 nm absorbance

TABLE III(A). CHROMATOGRAPHIC QUANTITATION OF AMINO ACIDS FROM THE TRANSAMINASE ASSAY SYSTEM.

Homogenates were prepared as described in the text (see M&M Section IIB2a). The transaminase assay mixtures (see M&M Section IIB2a) were then incubated either under N_2 or O_2 for the indicated periods of time at 37°C as described in the text (see M&M Section IIB2a). At the end of the indicated incubation periods each mixture was deproteinized (see M&M Section IIB2a) and the concentrated supernatants (see M&M Section IIB2a) were chromatographed (see M&M Section IIB2a(1)) as described in the text. The amino acids were quantitated as described in the text (see M&M Section IIB2a(1)) by comparing the experimental absorbances with that of known concentrations of standards.

TABLE III(A)

CHROMATOGRAPHIC QUANTITATION OF AMINO ACIDS FROM THE TRANSAMINASE ASSAY SYSTEM

	AMINO ACID	umoles 0	15	30	60	90	120 MIN	NET CHANGE
					<u>Tetrahymena pyriformis</u>			
N_2	GLU	0.112	0.190	0.184	0.264	0.234	0.290	+ 0.168
	BAIB	3.750	2.071	4.104	2.873	4.459	2.295	- 1.455
O_2	GLU	0.061	0.042	0.056	0.097	0.181	0.256	+ 0.195
	BAIB	2.513	2.488	2.513	2.513	2.513	2.500	- 0.013
					Mouse Liver			
N_2	GLU	0.160	0.129	0.161	0.182	0.154	0.179	+ 0.019
	BAIB	3.675	3.458	3.483	3.958	3.300	3.117	- 0.558

TABLE III(B). CHROMATOGRAPHIC QUANTITATION OF ∝KETO ACIDS FROM THE TRANSAMINASE ASSAY SYSTEM.

Details as in Table III(A), except that at the end of the indicated incubation periods each mixture was chromatographed as described in the text (see M&M Section IIB2a(2)). The 2,4-dinitrophenylhydrazones were also quantitated as described in the text (see M&M Section IIB2a(2)) by comparing the experimental absorbances with that of known concentrations of standards. No exogenous pyruvate was added to the experimental assays. The overall change in ∝keto acids refers to that detected by the assay of Hayaishi and Nishizuka[55] (see M&M Section IIB2a(2)). Both of these ∝keto acid analyses as well as the amino acid analysis (see Table III(A)) were done on the same preparations.

TABLE III(B)

CHROMATOGRAPHIC QUANTITATION OF ∝KETO ACIDS FROM THE TRANSAMINASE ASSAY SYSTEM

	umoles						
	0	15	30	60	90	120 MIN	NET CHANGE
Tetrahymena pyriformis							
∝KETO ACIDS	11.822	10.724	7.897	8.832	8.902	14.860	+ 3.038
N_2 ∝KG	38.987	26.810	19.968	21.429	16.800	15.341	-22.645
PYRUVATE	2.100	1.313	0.656	1.588	0.893	1.378	- 0.722
O_2 ∝KETO ACIDS	7.808	10.644	7.808	11.712	11.671	11.692	+ 3.884
Mouse Liver							
∝KETO ACIDS	4.141	5.430	9.106	18.592	9.490	10.394	+ 6.253
N_2 ∝KG	10.726	11.121	11.478	10.290	13.377	11.201	+ 0.475
PYRUVATE	0.776	0.801	0.643	0.591	0.066	0.184	- 0.592

of 2,6-dichlorophenolindophenol as described (see M&M Section IIB2b(1)). These preliminary studies (see Table IV) indicated that this enzyme, like thymine reductase (dehydrogenase), could also be demonstrated in the supernatant fraction of low speed centrifugates of Tetrahymena pyriformis homogenates. The low activity of the enzyme under these assay conditions, however, made it necessary to develop a more sensitive assay system for its further more detailed study. We therefore utilized the previously described fluorometric procedure (see M&M Section IIB2b(2)) for all subsequent oxidase assays.

The initial fluorometric assays (see Fig. 6) indicated: (1) that the homogenate without any substrate produced a small but increasing amount of fluorescence during the 90 min incubation period. To correct for this endogenous activity in further experiments, therefore, the increase in fluorescence of the blank, containing an equal volume of water instead of the aqueous substrate solution, was subtracted from the total increase in experimental fluorescence during the incubation period; (2) that the increase in fluorescence over the 90 min incubation period, at least for BAIB, is essentially linear. The change in fluorescence over this period, therefore, does indeed seem to represent the measurement of a legitimate enzyme rate; and (3) that BAIB oxidase activity, although significantly above the endogenous level, is, nevertheless, still considerably less than that of D-amino acid oxidase.

TABLE IV. BAIB OXIDASE ACTIVITY IN DIFFERENTIAL CENTRIFUGAL FRACTIONS OF TETRAHYMENA PYRIFORMIS HOMOGENATES ASSAYED WITH 2,6-DICHLOROPHENOLINDOPHENOL.

The homogenate was prepared (see M&M Section IIB2b(1)) and centrifuged (see M&M Section IVA2) at the indicated speeds as described in the text. The enzyme activity of each fraction was then assayed for as described in the text (see M&M Section IIB2b(1)).

TABLE IV

BAIB OXIDASE ACTIVITY IN DIFFERENTIAL CENTRIFUGAL FRACTIONS OF TETRAHYMENA PYRIFORMIS HOMOGENATES ASSAYED WITH 2,6-DICHLOROPHENOLINDOPHENOL

FRACTION	SP. ACT. ($\Delta A_{660\ nm}$/min/mg of protein)
500 x g SUP	0.013
14,500 x g SUP	0.050
14,500 x g PPT	0.013
100,000 x g SUP	0.014
100,000 x g PPT	0.004

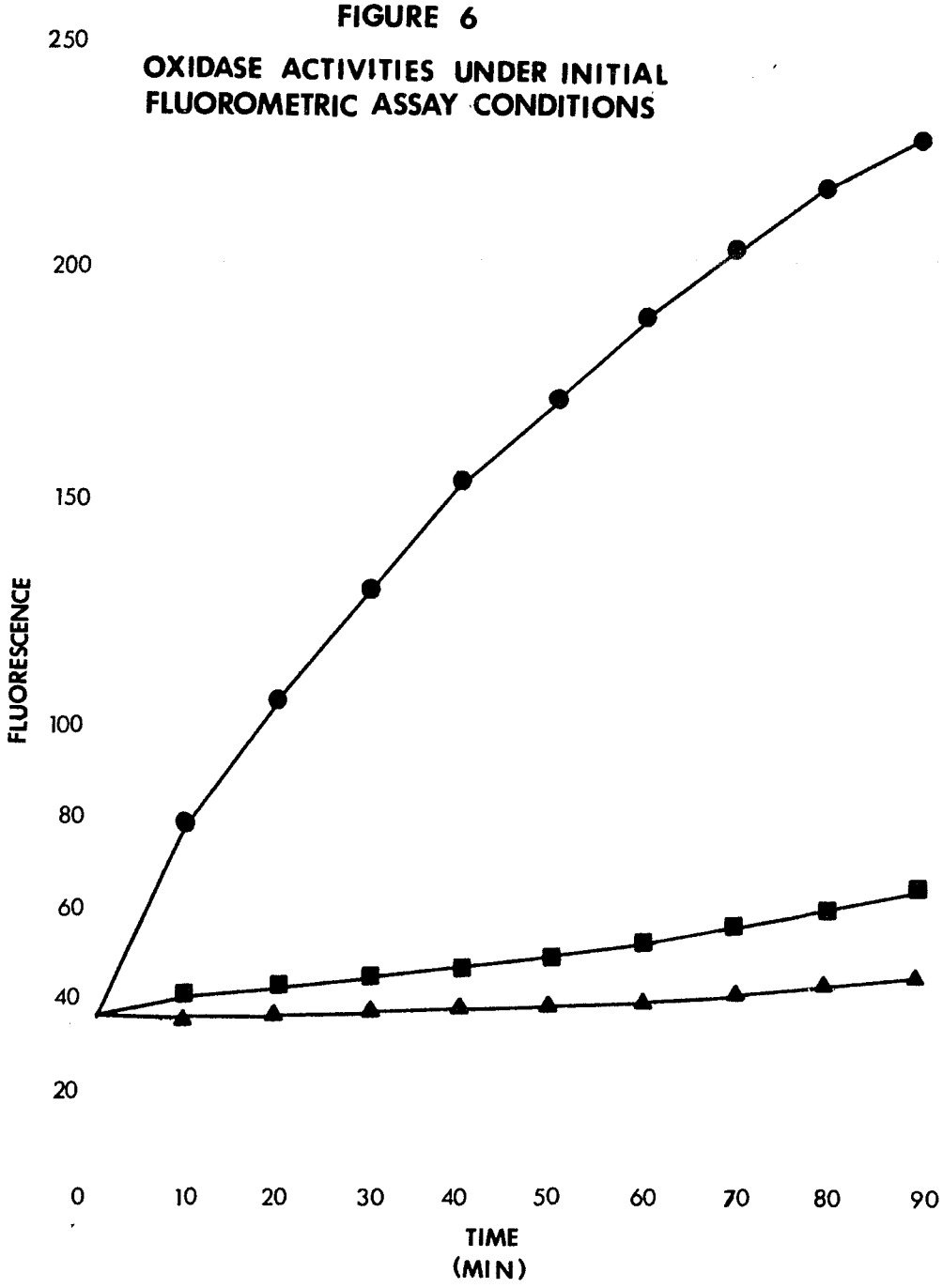
FIGURE 6. OXIDASE ACTIVITIES UNDER INITIAL FLUOROMETRIC CONDITIONS.

The homogenate was prepared and the oxidase activities were assayed for as described in the text (see M&M Section IIB-2b(2)).

SUBSTRATES: (■—■) D,L-BAIB; (●—●) D-ala; (▲—▲)endogenous.

FIGURE 6

OXIDASE ACTIVITIES UNDER INITIAL FLUOROMETRIC ASSAY CONDITIONS



III. CHARACTERIZATION OF BAIB OXIDASE ACTIVITY

After initially demonstrating BAIB oxidase activity fluorometrically in Tetrahymena pyriformis homogenates, we set about establishing the optimum assay conditions for this enzyme in the hopes of increasing its detectable activity.

1. Buffer

The first enzyme parameter investigated was the effect of buffer compound, molarity, and pH. A series of common assay buffers were tested at frequently used molarities and varying pHs within their buffering capacity (see Table V). BAIB oxidase showed good enzyme activity in alkaline 0.1 M pyrophosphate buffers. We decided not to use these highly alkaline pyrophosphate buffers, however, since highly alkaline buffers have been reported to catalyze the non-enzymatic decomposition of H_2O_2[115,116] and contaminating D-amino acid oxidase activity was also stimulated by these pyrophosphate buffers. BAIB oxidase, however, also showed good activity in alkaline 0.2 M Tris-HCl buffers, while D-amino acid oxidase was slightly less active. We therefore decided to use this buffer in all subsequent oxidase assays.

2. pH

We then investigated the pH optimum of this enzyme in more detail (see Fig. 7). We found that this enzyme activity apparently has a broad or possibly a double alkaline pH optimum. Thus we rather arbitarily chose a pH of 8.6 to be used in all subsequent oxidase assays.

TABLE V. EFFECT OF BUFFER TYPE, MOLARITY, AND pH ON BAIB OXIDASE ACTIVITY.

Homogenates were prepared in 0.25 M sucrose-0.1 M Tris-HCl buffer, pH 7.4, as described in the text (see M&M Section IIB2b(2)). 0.5 ml of this homogenate was then assayed for BAIB oxidase activity in the indicated buffers as described in the text (see M&M Section IIIA). The assays were incubated at 37°C.

TABLE V

EFFECT OF BUFFER TYPE, MOLARITY, AND pH ON BAIB OXIDASE ACTIVITY

BUFFER	MOLARITY	pH	ACTIVITY (ΔF/90 min/0.1 ml*)
Phosphate	0.05	6.5	4
		7.5	2.5
		8.7	9
	0.1	6.0	2
		7.0	2.5
		8.0	10.5
Tris	0.1	7.1	5.5
		8.0	10
		9.0	5.5
	0.2	6.9	6
		8.1	17.5
		8.9	30
Pyrophosphate	0.02	8.3	4
		9.2	12.5
		10.5	24.5
	0.1	8.3	25
		9.3	24.5
		10.5	24.5
Borate	0.1	8.1	7
		9.0	16
		10.0	8

*although the protein concentration of this preparation was not determined on the basis of subsequent experiments 0.1 ml of this homogenate was estimated to contain approximately 1 mg of protein.

FIGURE 7. THE EFFECT OF pH ON BAIB OXIDASE ACTIVITY. Homogenates were prepared in 0.25 M sucrose-0.1 M Tris-HCl buffer, pH 7.4, as described in the text (see M&M Section IIB2b(2)). 0.5 ml of this homogenate was then assayed for BAIB oxidase activity as described in the text (see M&M Section IIIA). Assays were incubated in 0.2 M Tris-HCl buffer at the indicated pHs. This graph represents the average of 3 such determinations at each pH.

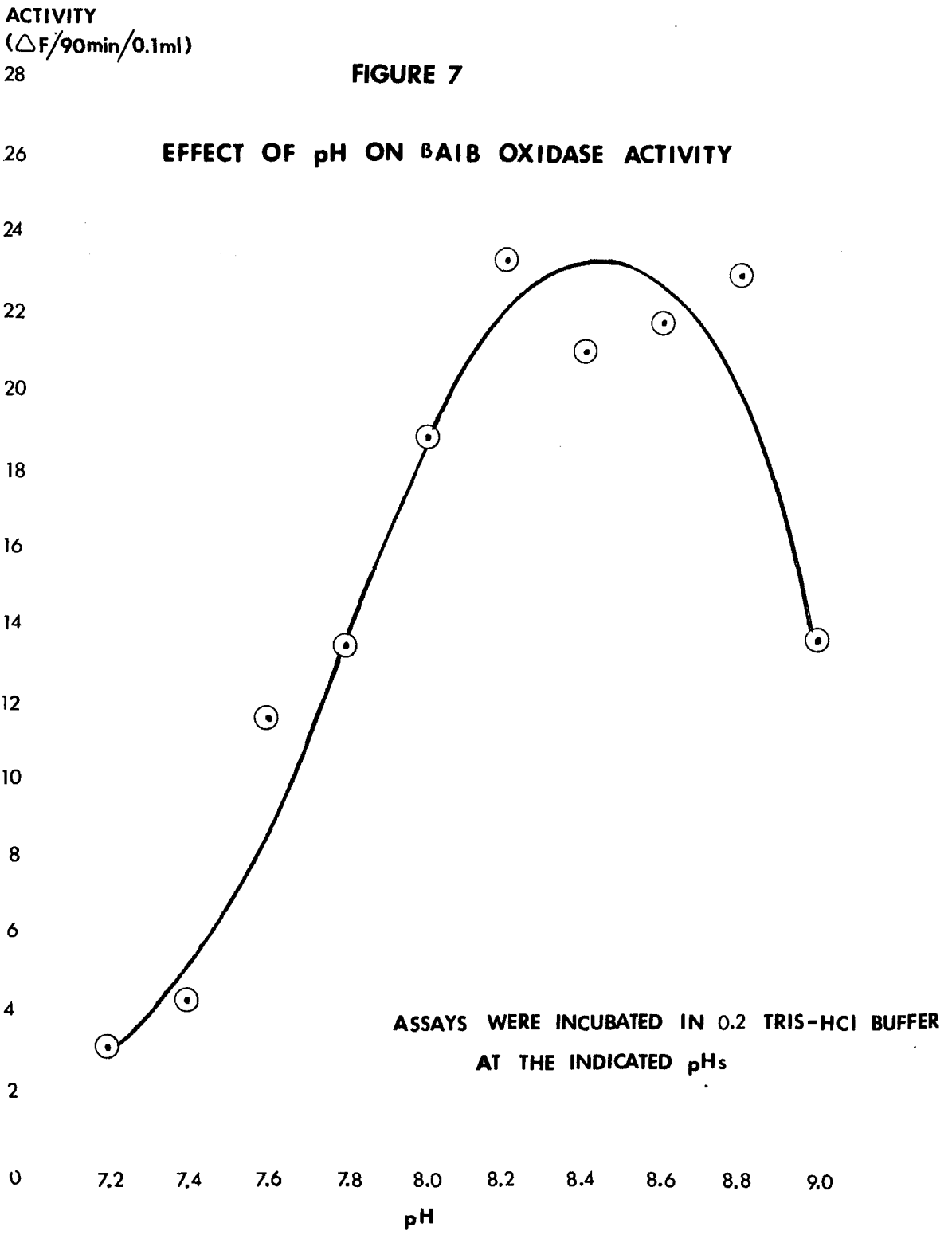


FIGURE 7

EFFECT OF pH ON ßAIB OXIDASE ACTIVITY

3. Temperature

The next enzyme parameter to be investigated was the effect of temperature on the rate of the reaction. We assayed for oxidase activity at four convienent temperatures (see Table VI). The assay system incubated at 37°C was found to be the most active. All subsequent oxidase assays, therefore, were also incubated at 37°C.

4. FAD

The effect of a common oxidase coenzyme, FAD, was also investigated. This cofactor was found to stimulate both D-amino acid oxidase and BAIB oxidase activity (see Table VII), although D-amino acid oxidase stimulation appeared to be more concentration dependent than BAIB oxidase activity. We thus rather arbitarily chose a final FAD concentration of 10^{-4} mM to be used in all subsequent oxidase assays.

5. Divalent Cations

The effect of some common divalent cations was also investigated. Both exogenous Mg^{+2} and Ca^{+2} were, however, found to inhibit BAIB oxidase and, to a lesser extent, D-amino acid oxidase activity (see Table VIII). No such exogenous divalent cations were, therefore, included in any subsequent oxidase assays.

6. EDTA

Since divalent cations were apparently inhibitory, it was thought that a chelating agent, such as EDTA, might stimulate oxidase activity by removing inhibiting divalent

TABLE VI. EFFECT OF TEMPERATURE ON BAIB OXIDASE ACTIVITY. Homogenates were prepared in 0.25 M sucrose-0.1 M Tris-HCl buffer, pH 7.4, as described in the text (see M&M Section IIB2b(2)). 0.5 ml of this homogenate was then assayed for the indicated oxidase activities in 0.2 M Tris-HCl buffer, pH 8.6, as described in the text (see M&M Section IVB3&5).

TABLE VI

EFFECT OF TEMPERATURE ON BAIB OXIDASE ACTIVITY

TEMPERATURE (°C)	ACTIVITY (Δ F/90 min/0.1 ml*) BAIB	D-ala
4	10	44.5
25	16.5	80
37	39	119.5
90	0	0

*although the protein concentration of this preparation was not determined, on the basis of subsequent experiments 0.1 ml of this homogenate was estimated to contain 1 mg of protein

TABLE VII. EFFECT OF FAD ON BAIB OXIDASE ACTIVITY. Homogneates were prepared in 0.1 M Tris-HCl buffer, pH 8.6, as described in the text (see M&M Section IIB2b(2)). 0.5 ml of this homogenate was then assayed in the presence of the above final concentrations of FAD for the indicated oxidase activities as described in the text (see M&M Section IVB3&5). Controls were incubated without added exogenous FAD.

TABLE VII

THE EFFECT OF FAD ON BAIB OXIDASE ACTIVITY

CONCENTRATION (mM)	% OF CONTROL ACTIVITY BAIB	D-ala
10^{-6}	171.2	208.1
10^{-5}	153.8	245.2
10^{-4}	150.0	383.9

TABLE VIII. EFFECT OF DIVALENT CATIONS ON BAIB OXIDASE ACTIVITY.

Homogenates were prepared in 0.1 M Tris-HCl buffer, pH 8.6, as described in the text (see M&M Section IIB2b(2)). 0.1 ml of this homogenate was then assayed in the presence of the above final concentrations of the indicated divalent cations from BAIB oxidase (see M&M Section IVB5) as well as D-amino acid oxidase (see M&M Section IVB3) activity. Controls were incubated without the indicated exogenous divalent cations.

TABLE VIII

EFFECT OF DIVALENT CATIONS ON BAIB OXIDASE ACTIVITY

DIVALENT CATION	CONCENTRATION (mM)	% OF CONTROL ACTIVITY BAIB	D-ala
Mg^{+2}	3.3	78.8	91.2
	6.6	43.9	76.7
	13.2	21.2	61.7
Ca^{+2}	6.0	97.0	73.7
	12.0	66.7	88.9

cations from the crude homogenate. Over the wide range of concentrations tested, however, EDTA also appeared to markedly inhibit BAIB oxidase and, to a lesser extent, D-amino acid oxidase activity (see Table IX). This chelating agent, therefore, was not added to any subsequent oxidase assays.

7. SH Reagents

We also thought that we might test the effects of some common reducing compounds on oxidase activity. Most of these reagents (B-mercaptoethanol, reduced glutathione, dithiothreitol, and dithioerythritol), however, were found, as previously reported,[117] to inhibit peroxidase activity under our assay conditions. We therefore could not test their effect on oxidase activity in this peroxidase coupled assay system.

8. PCMB

The effect of the SH blocking reagent, PCMB, on oxidase activity was also investigated. At the rather low concentrations used in these experiments, PCMB was found to effectively inhibit both BAIB oxidase and, to a lesser extent, D-amino acid oxidase activity (see Table X). This result suggests that free SH groups are important for the oxidase activity.

9. Detergents

The effects of various detergents (Triton X-100, Tween 80, SDS, and DOC) on oxidase activity were also investigated. Such compounds are thought to increase the detect-

TABLE IX. EFFECT OF EDTA ON BAIB OXIDASE ACTIVITY. Homogenates were prepared in 0.1 M Tris-HCl buffer, pH 8.6, as described in the text (see M&M Section IIB2b(2)). 0.1 ml of this homogenate was then assayed in the presence of the above final concentrations of EDTA for the indicated oxidase activities as described in the text (see M&M Section IVB3&5). Controls were incubated without EDTA.

TABLE IX

EFFECT OF EDTA ON BAIB OXIDASE ACTIVITY

CONCENTRATION (mM)	% OF CONTROL ACTIVITY BAIB	D-ala
0.05	22.2	81.5
0.2	14.5	89.4
2.0	22.2	86.9

TABLE X. EFFECT OF PCMB ON BAIB OXIDASE ACTIVITY.

Homogneates were prepared in 0.1 M Tris-HCl buffer, pH 8.6, as described in the text (see M&M Section IIB2b(2)). 0.1 ml of this homogneate was then assayed in the presence of the above final concentrations of PCMB for the indicated oxidase activities as described in the text (see M&M Section IVB3&5). Controls were incubated without PCMB.

TABLE X

EFFECT OF PCMB ON BAIB OXIDASE ACTIVITY

CONCENTRATION ($\times 10^{-3}$ mM)	% OF CONTROL ACTIVITY BAIB	D-ala
5	43.2	61.4
10	43.3	50.0
20	34.1	27.3

able activities of enzymes localized within subcellular organelles by disrupting the limiting membranes and thus liberating the sequestered enzyme activities. Low concentrations of Trition X-100 seem to be slightly stimulatory, although higher concentrations may be inhibitory. Tween 80, on the other hand, seems to have little effect on both oxidase activities. The strongly ionic detergents, such as SDS and DOC, however, seem to be significantly inhibitory (see Table XI). High concentrations (final conc $\geq$ 0.33%) of these detergents, moreover, greatly increase endogenous fluorescence and thus decrease the sensitivity of the assay system. Low concentrations of Trition X-100 (final conc $\leq$ 0.03%), nevertheless, were included in all subsequent oxidase assay systems where crude enzyme preparations were used as a precautionary measure.

10. Freeze-Thawing

We also investigated the effect of freezing and thawing on oxidase activity. Freeze-thawing, like detergents, is thought to increase the detectable activity of enzymes localized within subcellular organelles by disrupting the limiting membrane and thus liberating the latent enzyme activities. Repeated cycles of rapid freezing and thawing, however, did not seem to increase either BAIB oxidase or D-amino acid oxidase activity (see Table XII).

11. Stability

Finally we investigated the stability of BAIB oxidase activity under various storage conditions (see Table XIII).

TABLE XI. EFFECT OF DETERGENTS ON BAIB OXIDASE ACTIVITY. Homogenates were prepared in 0.1 M Tris-HCl buffer, pH 8.6, as described in the text (see M&M Section IIB2b(2)). 0.1 ml of this homogenate was then assayed in the presence of the above final concentrations of the indicated detergents for BAIB oxidase (see M&M Section IVB5) as well as D-amino acid oxidase (see M&M Section IVB3) activity. Controls were incubated in the absence of any detergent.

TABLE XI

EFFECT OF DETERGENTS ON BAIB OXIDASE ACTIVITY

DETERGENT	CONCENTRATION (%)	% OF CONTROL ACTIVITY BAIB	D-ala
Triton X-100	0.03	130.2	116.3
	0.33	97.1	140.0
	3.33	19.0	78.3
Tween 80	0.03	107.3	111.9
	0.33	105.4	95.0
	3.33	107.3	81.3
SDS	0.03	77.1	53.1
	0.33	42.0	18.6
	3.33	0	6.2
DOC	0.03	63.7	94.3
	0.33	67.6	20.8
	3.33	49.4	0

TABLE XII. EFFECT OF FREEZE-THAWING ON BAIB OXIDASE ACTIVITY.

Homogenates were prepared in 0.25 M sucrose as described in the text (see M&M Section IIB2b(2)). At least 5 ml of this homogenate were rapidly frozen in a dry ice:1-methoxy-2-propanol slurry, then thawed in a 37°C water bath as described in the text (see M&M Section IIIB). An aliquot of approximately 1 ml was removed and retained for enzyme assay. The remainder of the preparation was then quickly recycled the indicated number of times. Each aliquot was subsequently assayed in the absence of Triton X-100 for the indicated oxidase activities as described in the text (see M&M Section IVB3&5). The activity of each aliquot was finally expressed as the percent of the activity in the original unfrozen homogenate.

TABLE XII

EFFECT OF FREEZE-THAWING ON BAIB OXIDASE ACTIVITY

NUMBER OF CYCLES	% OF CONTROL ACTIVITY BAIB	D-ala
1	82.4	85.3
2	96.2	85.0
3	88.1	82.5
4	96.7	84.5
5		83.4

TABLE XIII. STABILITY OF BAIB OXIDASE ACTIVITY.

Homogenates were prepared in 0.1 M Tris-HCl buffer, pH 8.6, as described in the text (see M&M Section IIB2b(2)). This homogenate was then divided into two portions as described in the text (see M&M Section IIIC). One of these portions was stored in the refrigerator at 4°C (REFRIGERATED), while the other was stored in various ways in the freezer at -20° C (FROZEN; FROZEN + GLYCEROL; FREEZE-THAW; FREEZE-THAW + GLYCEROL). Half of the material at -20°C was stored in buffer (FROZEN; FREEZE-THAW), while the remainder was suspended in an equal volume of glycerol (FROZEN + GLYCEROL; FREEZE-THAW + GLYCEROL). Both of these enzyme preparations stored at -20°C were further subdivided into a portion which was stored in bulk (FREEZE-THAW; FREEZE-THAW + GLYCEROL) and a portion which was stored in small individual aliquots (FROZEN; FROZEN + GLYCEROL). At the indicated times after preparation each of these variously stored homogenates were warmed in a 37°C water bath and then assayed for the indicated oxidase activities as described in the text (see M&M Section IVB3&5). Each assay system contained Triton X-100 (see M&M Section IIIA).

TABLE XIII

STABILITY OF BAIB OXIDASE ACTIVITY

STORAGE	ENZYME ACTIVITY	% ACTIVITY REMAINING 24	48	72	96 HRS	1 WK
REFRIGERATED	BAIB	112.5	143.8			141.7
	D-ala	36.8	14.8		6.1	1.8
FROZEN	BAIB	68.3	84.0		106.1	87.3
	D-ala	61.4	36.3		27.0	3.8
FROZEN + GLYCEROL	BAIB	88.9	214.2	566.6		
	D-ala		96.4	109.4	124.0	
FREEZE-THAW	BAIB				67.1	
	D-ala		54.1	43.0	23.8	
FREEZE-THAW + GLYCEROL	BAIB			466.6		
	D-ala			84.2	111.8	

In general, BAIB oxidase, unlike D-amino acid oxidase, seems to be relatively stable during storage. In fact, its relative activity actually appears to increase upon refrigeration as well as upon freezer storage in glycerol which might reflect the inactivation of inhibitory substances or competing enzyme systems in the crude enzyme preparation during such storage.

IV. SUBCELLULAR LOCALIZATION OF BAIB OXIDASE ACTIVITY

After demonstrating the presence of an enzyme capable of oxidizing BAIB in Tetrahymena pyriformis homogenates, we became interested in its subcellular localization since such an oxidase would produce H_2O_2 as a by-product of the reaction. It is generally acknowledged, moreover, that this organism contains a highly active peroxisomal catalase capable of catalyzing the rapid decomposition of endogenous H_2O_2. In addition, it has been recently reported[118] that this organism also contains a mitochondrial peroxidase which is capable of decomposing H_2O_2. Thus it was initially thought that this oxidase might be associated with one of these subcellular organelles.

Preliminary centrifugation (see Table XIV) indicated that a considerable amount of this oxidase activity was indeed recovered in the high speed (100,000 x g) pellet. To better determine which subcellular organelles might contain this oxidase activity, homogenates were subsequently prepared (see M&M Section IVA) and fractionated by differential

TABLE XIV. SUBCELLULAR LOCALIZATION OF BAIB OXIDASE ACTIVITY BY PRELIMINARY HIGH SPEED (100,000 x g) CENTRIFUGATION. Homogenates were prepared in 0.25 M sucrose (see M&M Section IVA), then centrifuged at 100,000 x g (see M&M Section IVA1) as described in the text. Both the supernatant and resuspended pellet were then assayed for the indicated enzyme activities as described in the text (see M&M Section IVB). Individual enzyme activities (EXPERIMENT NUMBERS 1-3) were preferentially expressed as the per cent of homogenate activity.[63] Where this was not possible (EXPERIMENT NUMBER 4), individual enzyme activities were alternatively expressed as the per cent of total recovered activity.

TABLE XIV

SUBCELLULAR LOCALIZATION OF BAIB OXIDASE ACTIVITY BY PRELIMINARY HIGH SPEED (100,000 x g) CENTRIFUGATION

EXPERIMENT NUMBER	FRACTION	% HOMOGENATE ACTIVITY MDH	CAT	BAIB	D-ala	LAC
1	SUP	1.6	0.3	16.0	39.3	7.0
	PPT	47.1	61.8	32.2	33.8	41.1
2	SUP	4.4	2.0	28.3	31.9	12.7
	PPT	99.6	45.2	24.1	43.8	38.8
3	SUP			18.7	19.0	13.9
	PPT			47.5	47.5	37.9
		% TOTAL ACTIVITY				
4	SUP	15.2	0	51.4	47.6	
	PPT	84.8	100.0	48.6	52.4	

centrifugation (see M&M Section IVA2) as outlined in Fig. 8. The resulting supernatants and resuspended pellets were then assayed for their various component enzyme activities (see Fig. 9) as previously described (see M&M Section IVB). In addition, to check for the non-specific adsorption of soluble enzyme activity to pelleted subcellular organelles, exogenous hog kidney D-amino acid oxidase was added to Tetrahymena pyriformis homogenates which were then fractionated by the same differential centrifugation scheme. No membrane-bound enhancement of this oxidase activity, however, was detected (see Table XV). Thus it was concluded that any particle-associated activity was probably an accurate reflection of the in vivo localization of the enzyme and not merely the result of its non-specific adsorption to that fraction.

After the initial (500 x g) centrifugation the homogenate enzyme activities were assayed for in the resulting low speed supernatant and pellet. The total activity of the supernatant enzymes was generally greater than that of the pellet, although considerable enzyme activity, particularly MDH activity, also occassionally remained in the pellet. Electron micrographs of this pellet indicated that it was composed mostly of unbroken cells and other "cell debris" such as oral groove apparatus as well as varing amounts of contaminating free subcellular organelles (see Fig. 10).

It was originally thought that the recovery of membrane-

FIGURE 8. PREPARATION OF SUBCELLULAR FRACTIONS BY DIFFERENTIAL CENTRIFUGATION.

Homogneates were prepared in 0.25 M sucrose (see M&M Section IVA), then fractionated by differential centrifugation (see M&M Section IVA2) as described in the text.

FIGURE 8

PREPARATION OF SUBCELLULAR FRACTIONS BY DIFFERENTIAL CENTRIFUGATION

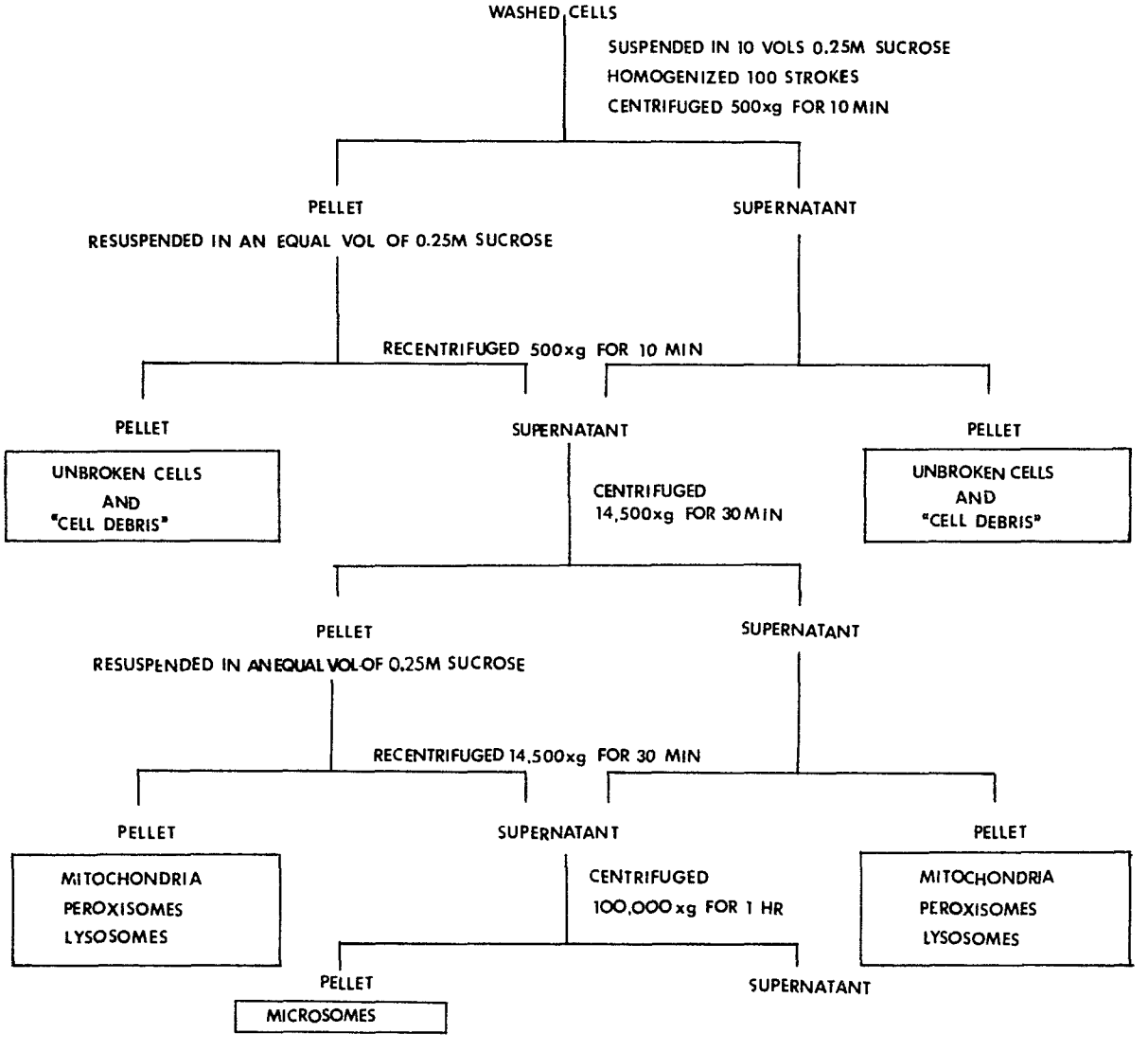


FIGURE 9. THE SUBCELLULAR DISTRIBUTION OF ENZYME ACTIVITIES FRACTIONATED BY DIFFERENTIAL CENTRIFUGATION.

Washed cells were homogenized in 0.25 M sucrose (see M&M Section IVA) and fractionated by differential centrifugation (see M&M Section IVA2) as described in the text. The resulting supernatants and resuspended pellets were then assayed as described in the text (see M&M Section IVB) for the indicated standard marker enzymes as well as for BAIB oxidase activity. MDH was assayed for as a standard Tetrahymena pyriformis mitochondrial marker enzyme. Catalase was assayed for as a standard peroxisomal marker enzyme. D-amino acid oxidase and lactate oxidase, two other standard peroxisomal marker enzymes, were also measured fluorometrically as an additional check on the BAIB oxidase assay system. Enzyme activities were finally preferentially expressed as the per cent of homogenate activity.[63]

FIGURE 9

SUBCELLULAR DISTRIBUTION OF ENZYME ACTIVITIES FRACTIONATED BY DIFFERENTIAL CENTRIFUGATION

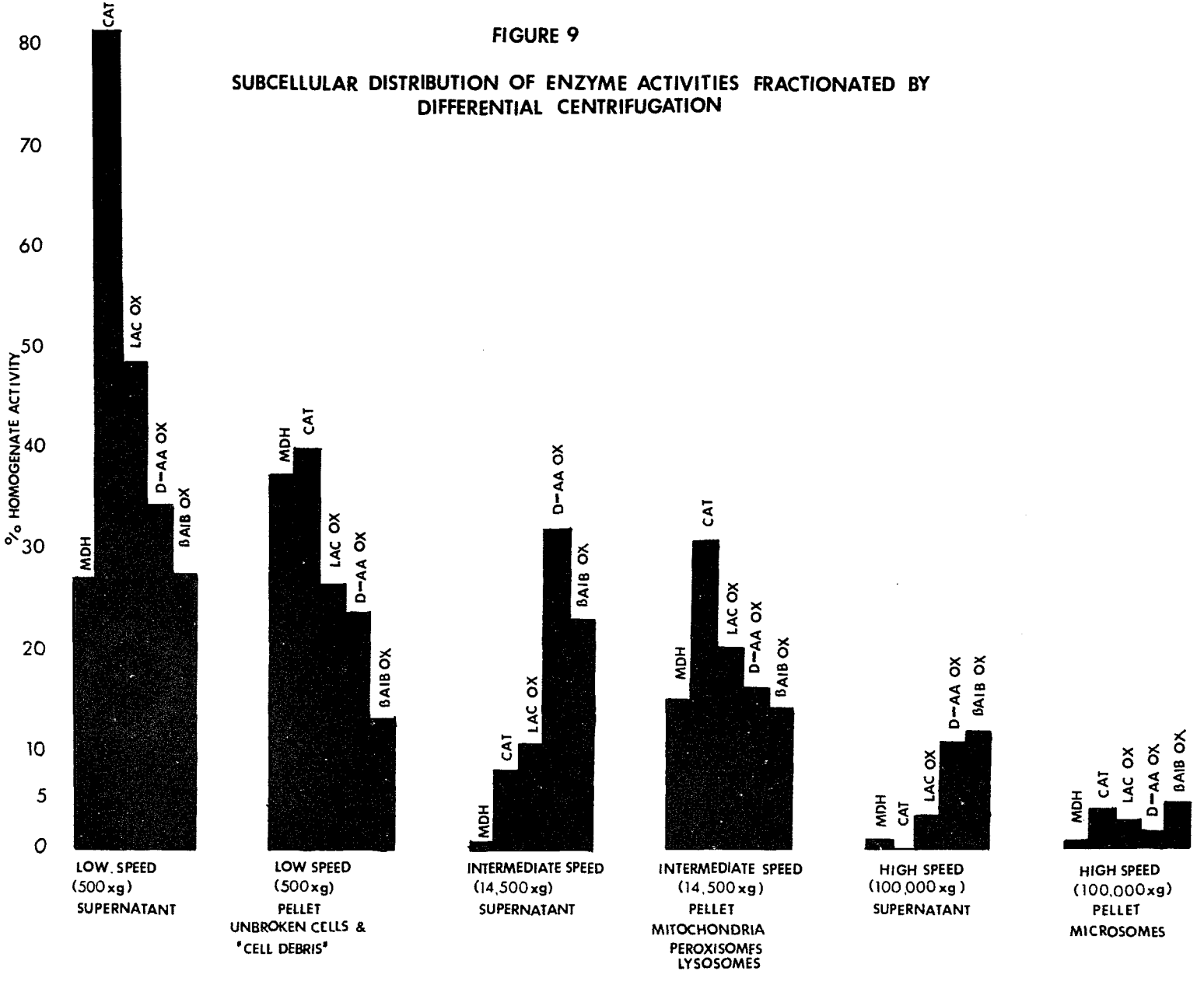


TABLE XV. AMOUNT OF EXOGENOUS D-AMINO ACID OXIDASE ACTIVITY RECOVERED IN VARIOUS DIFFERENTIAL CENTRIFUGATION FRACTIONS.

Homogenates were prepared in 0.25 M sucrose as described in the text (see M&M Section IVA). Exogenous hog kidney D-amino acid oxidase was added to the Tetrahymena pyriformis homogenate at approximately a concentration of 27 ug/ml as described in the text (see M&M Section IVA2) before subjecting it to the centrifugation schemes described in the text (see M&M Section IVA1&2). The indicated enzyme activities of (A) the high speed supernatant and pellet as well as of (B) each of the resultant differential centrifugal fractions were then determined as described in the text (see M&M Section IVB1-3). The amount of exogenous D-amino acid oxidase activity contained in each fraction was calculated by subtracting the endogenous D-amino acid oxidase activity of each fraction as determined from the parallel processing of an equal amount of the same Tetrahymena pyriformis homogenate without additional added exogenous D-amino acid oxidase from the total amount of D-amino acid oxidase (endogenous plus exogenous) in each fraction. Enzyme activities were finally expressed as the per cent of homogenate activity.[63]

TABLE XV

AMOUNT OF EXOGENOUS D-AMINO ACID OXIDASE ACTIVITY RECOVERED IN VARIOUS DIFFERENTIAL CENTRIFUGATION FRACTIONS

DIFFERENTIAL CENTRIFUGATION FRACTION		% HOMOGENATE ACTIVITY MDH	CAT	D-AMINO ACID OXIDASE ENDOGENOUS	EXOGENOUS
			(A)		
100,000 x g	SUP	3.2	5.8	43.6	140.2
	PPT	65.1	45.0	55.7	0
			(B)		
500 x g	SUP	29.2	54.2	61.7	84.2
	PPT	23.4	41.7	44.9	0
14,500 x g	SUP	0.4	11.0	25.9	150.0
	PPT	11.9	21.7	14.6	0
100,000 x g	SUP	1.0	1.9	8.9	58.2
	PPT	0.7	3.5	1.5	0

FIGURE 10. ELECTRON MICROGRAPHS OF LOW SPEED (500 x g) PELLET.

Washed cells were homogenized in 0.25 M sucrose (see M&M Section IVA) and fractionated by differential centrifugation (see M&M Section IVA2) as described in the text. The low speed (500 x g) pellet was then processed for EM, that is, it was fixed in gluteraldehyde, post-fixed in OsO_4, stained with uranyl acetate, and embedded in Maraglas as described in the text (see M&M Section IVC).

A. This fraction appeared to be composed mostly of (1) unbroken cells and other "cell debris" such as (2) oral groove apparatus. Also note: (3) occassional extracellular mitochondria and (4) aggregated membrane vessicles which might account for the occassionally high MDH activity remaining in this pellet. Magnification = 7,200 X.

B. Cross section through a whole cell. Magnification = 7,200 X.

C. Enlargement of oral groove apparatus. Magnification = 19,600 X.

FIGURE 10(A)

ELECTRON MICROGRAPHS OF LOW SPEED (500 x g) PELLET

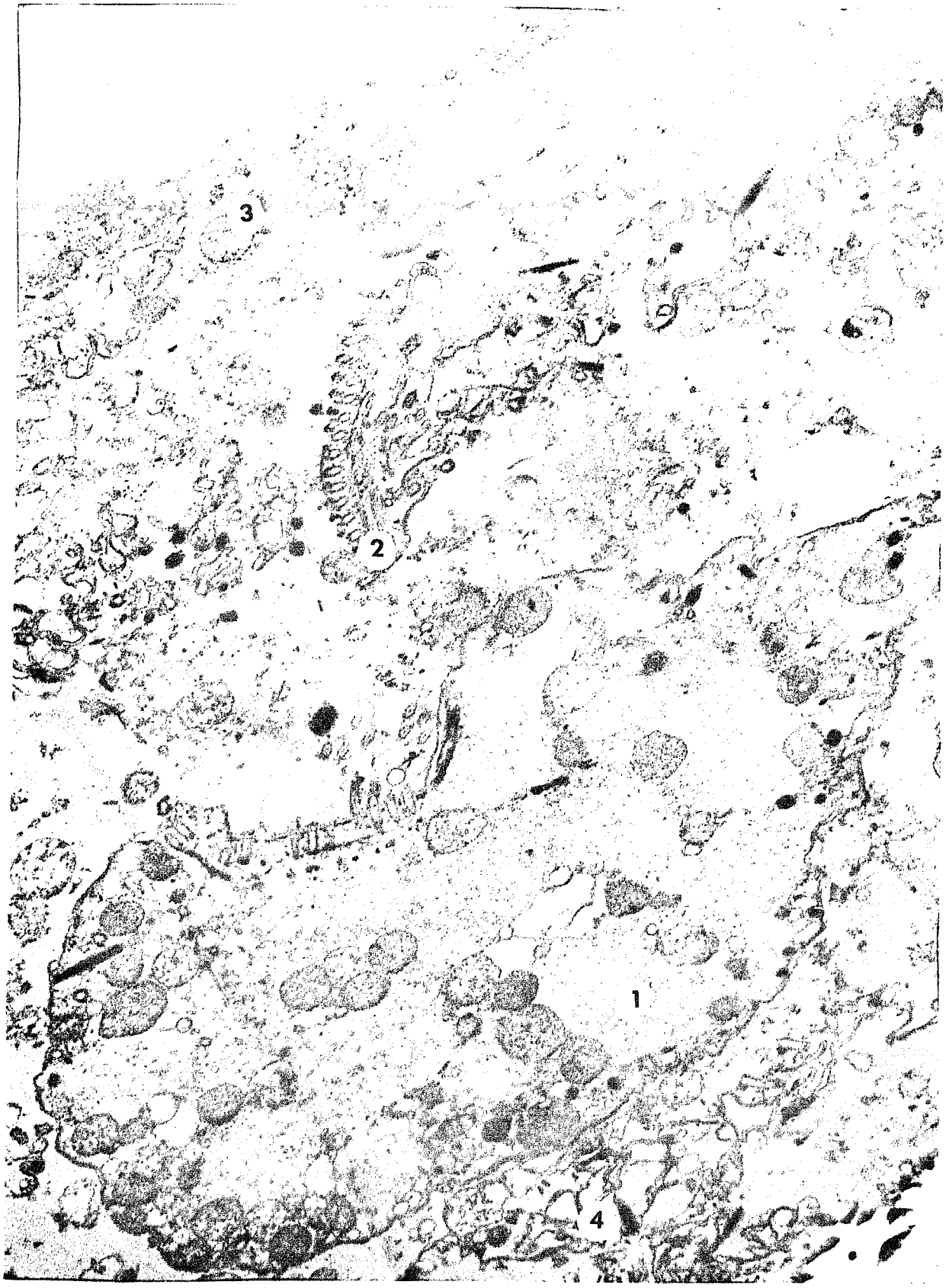


FIGURE 10(B)

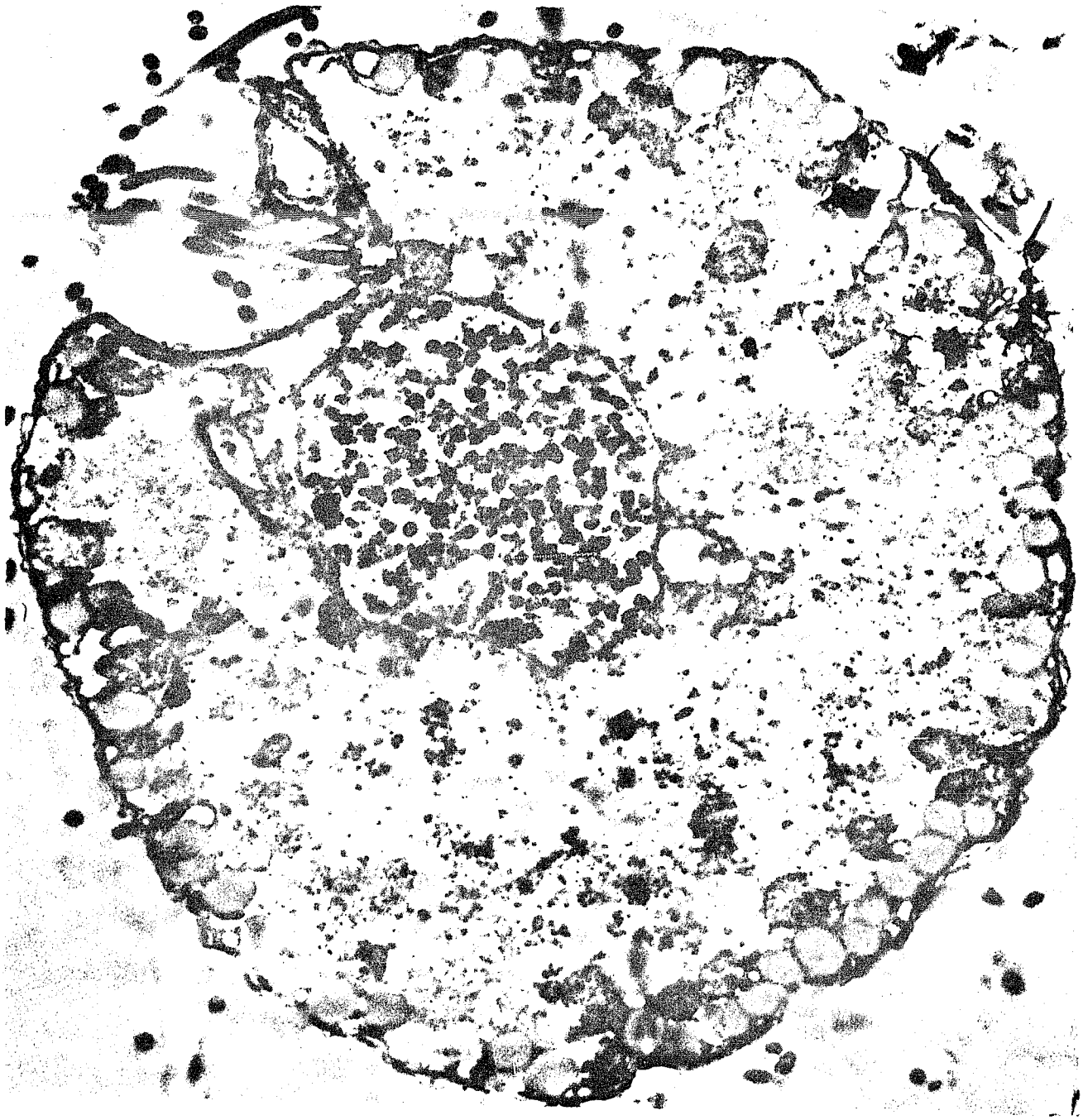
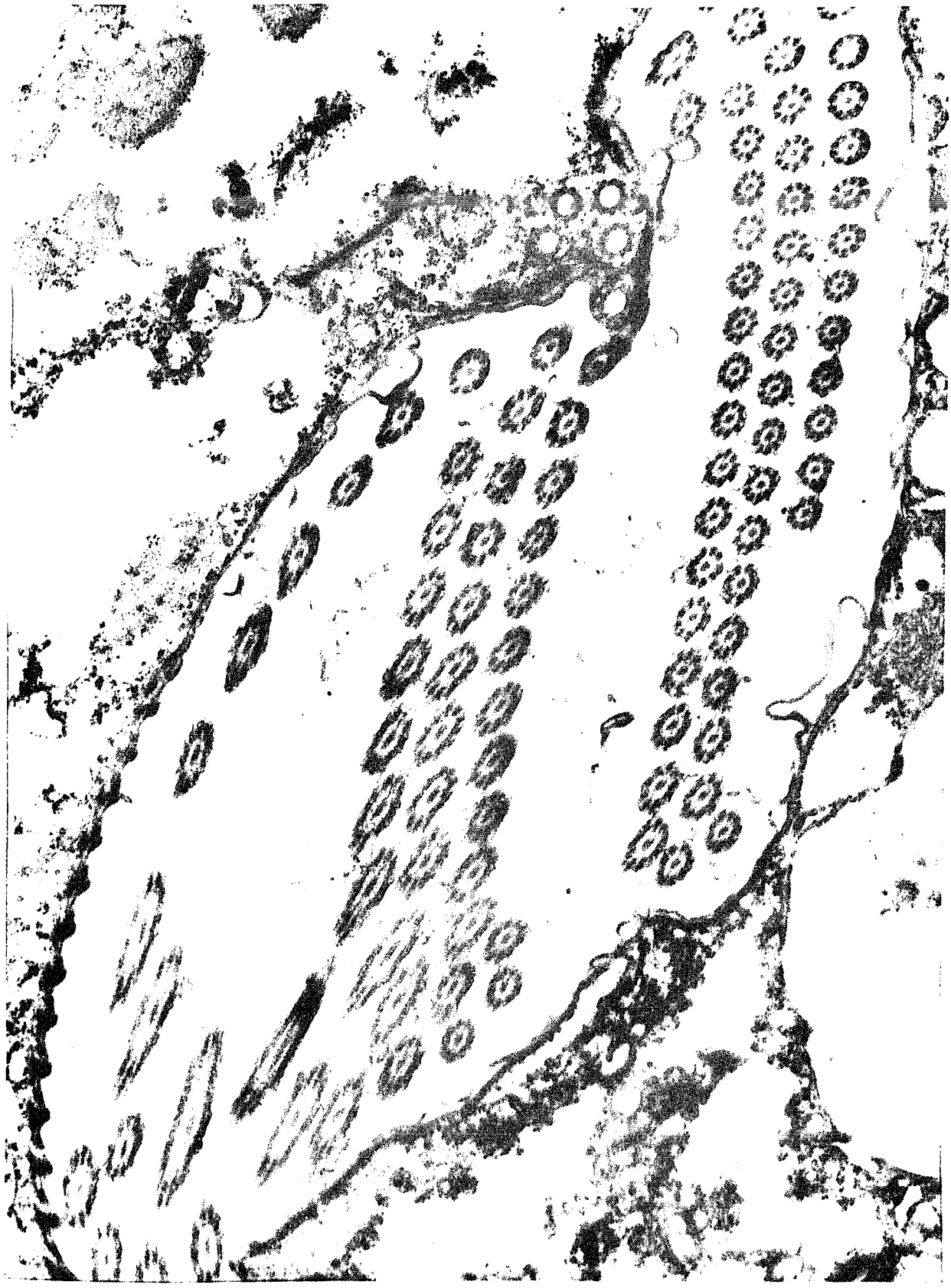


FIGURE 10(C)



bound marker enzymes in this low speed (500 x g) pellet might be due to the aggregation of the subcellular organelles during processing. To try to eliminate this aggregation and improve the recovery fo subcellular organelles in their appropriate differential centrifugal fraction, therefore, various homogenization media were tested (see Table XVI). The ionic strength of these solutions, however, was apparently too high, causing the disruption of many of the subcellular organelles, especially the peroxisomes, as indicated by the relatively small amounts of marker enzyme activity, particularly catalase, recovered in the intermediate speed (14,500 x g) pellet as well as the relatively large amount of both marker enzyme activities recovered in the high speed (100,000 x g) supernatant. We thus returned to 0.25 M sucrose as the best compromise between maintaining subcellular organelle integrity and recovering subcellular organelle enzyme activities in their appropriate centrifugal fraction.

Upon further centrifugation (14,500 x g) the low speed supernatant enzyme activities were assayed for in the resulting intermediate speed supernatant and pellet. After this centrifugation, however, the total activity of most of the marker enzymes, that is, MDH, catalase, and lactate oxidase, was greater in the pellet than in the supernatant. This indicated that mitochondria and peroxisomes were being pelleted at this speed. Preliminary electron micrographs of this pellet also indicated that it was enriched for mito-

TABLE XVI. EFFECT OF VARIOUS HOMOGENIZATION MEDIA ON THE RECOVERY OF MARKER ENZYMES.

Washed cells were homogenized in the above media (see M&M Section IVA) and fractionated by differential centrifugation (see M&M Section IVA2) as described in the text. The resulting supernatants and resuspended pellets were then assayed as described in the text for the indicated standard marker enzymes. MDH (see M&M Section IVB1) was assayed for as a standard Tetrahymena pyriformis mitochondrial marker enzyme. Catalase (see M&M Section IVB2) was assayed for as a standard peroxisomal marker enzyme. Enzyme activities were then expressed as the per cent of homogenate activity.[63]

TABLE XVI

EFFECT OF VARIOUS HOMOGENIZATION MEDIA ON THE RECOVERY OF MARKER ENZYMES

HOMOGENIZATION MEDIA	MARKER ENZYME	% HOMOGENATE ACTIVITY					
		500 x g		14,500 x g		100,000 x g	
		SUP	PPT	SUP	PPT	SUP	PPT
0.25 M SUCROSE	MDH	26.3	37.5	0.4	15.1	0.8	0.8
	CAT	81.4	39.9	7.7	30.7	0	3.9
0.25 M SUCROSE 0.1 M TRIS-HCl pH 7.4	MDH	60.0	37.6	55.3	2.2	11.8	0.2
	CAT	70.4	20.9	12.3	3.2	27.6	0
0.25 M SUCROSE 0.1 M TRIS-HCl 1 mM EDTA pH 7.4	MDH	44.6	66.0	21.8	40.6	7.2	0.4
	CAT	70.9	38.3	42.0	11.0	14.1	0
0.25 M SUCROSE 0.1 M TRIS-HCl 1 mM EDTA 10 m M NaCl pH 7.4	MDH	33.7	29.9	29.8	69.0	10.4	0.3
	CAT	61.8	11.9	22.7	4.8	9.5	0.7

chondria and other smaller membrane bound subcellular organelles (see Fig. 11). A considerable amount of the total activity of D-amino acid oxidase and BAIB oxidase, nevertheless, still remained in the supernatant. This result was somewhat surprising since D-amino acid oxidase is supposed to be a peroxisomal marker enzyme and was expected to pellet with the other marker enzymes for that subcellular organelle. It also indicated that these enzymes might not be exclusively associated with the mitochondrial-peroxisomal pellet.

After the final (100,000 x g) centrifugation the remaining intermediate speed supernatant enzyme activities were assayed for in the resulting high speed supernatant and pellet. The standard microsomal marker enzyme, L-amino acid oxidase, has been reported[119] to be absent in Tetrahymena pyriformis which is consistent with our own observations using L-ala and L-met. Electron micrographs of this pellet, however, indicated that it is indeed typically microsomal in morphology (see Fig. 12). That is, it appears to be composed of small empty membrane bound vessicles, probably ER, as well as small scattered electron dense particles, probably ribosomes. Most of the intermediate speed supernatant BAIB oxidase and D-amino acid oxidase activity, nevertheless, remained in the high speed supernatant and only a small amount was recovered in this pellet. This indicated that although a considerable amount of the recovered BAIB oxidase activity is found in the intermediate

FIGURE 11. ELECTRON MICROGRAPHS OF INTERMEDIATE SPEED (14,500 x g) PELLET.

Washed cells were homogenized in 0.25 M sucrose (see M&M Section IVA) and fractionated by differential centrifugation (see M&M Section IVA2) as described in the text. The intermediate speed (14,500 x g) pellet was then processed for EM, that is, it was fixed in gluteraldehyde, post-fixed in OsO_4, stained with uranyl acetate, and embedded in Maraglas as described in the text (see M&M Section IVC). This fraction appeared to be enriched in mitochondria and other smaller membrane-bound subcellular organelles. Note: (1) the characteristically tubular protozoan mitochondria; (2) peroxisomal-like bodies as well as (3) empty membrane-bound vessicles and (4) non-membrane-bound granular debris which indicates some subcellular organelle damage during preparation and might at least partially account for the unexpected recovery of D-amino acid oxidase activity in the soluble fraction.

A. Magnification = 7,200 X.

B. Magnification = 19,600 X.

C. Magnification = 27,750 X.

D. Magnification = 38,000 X.

FIGURE 11(A)

ELECTRON MICROGRAPHS OF INTERMEDIATE SPEED (14,500 x g) PELLET

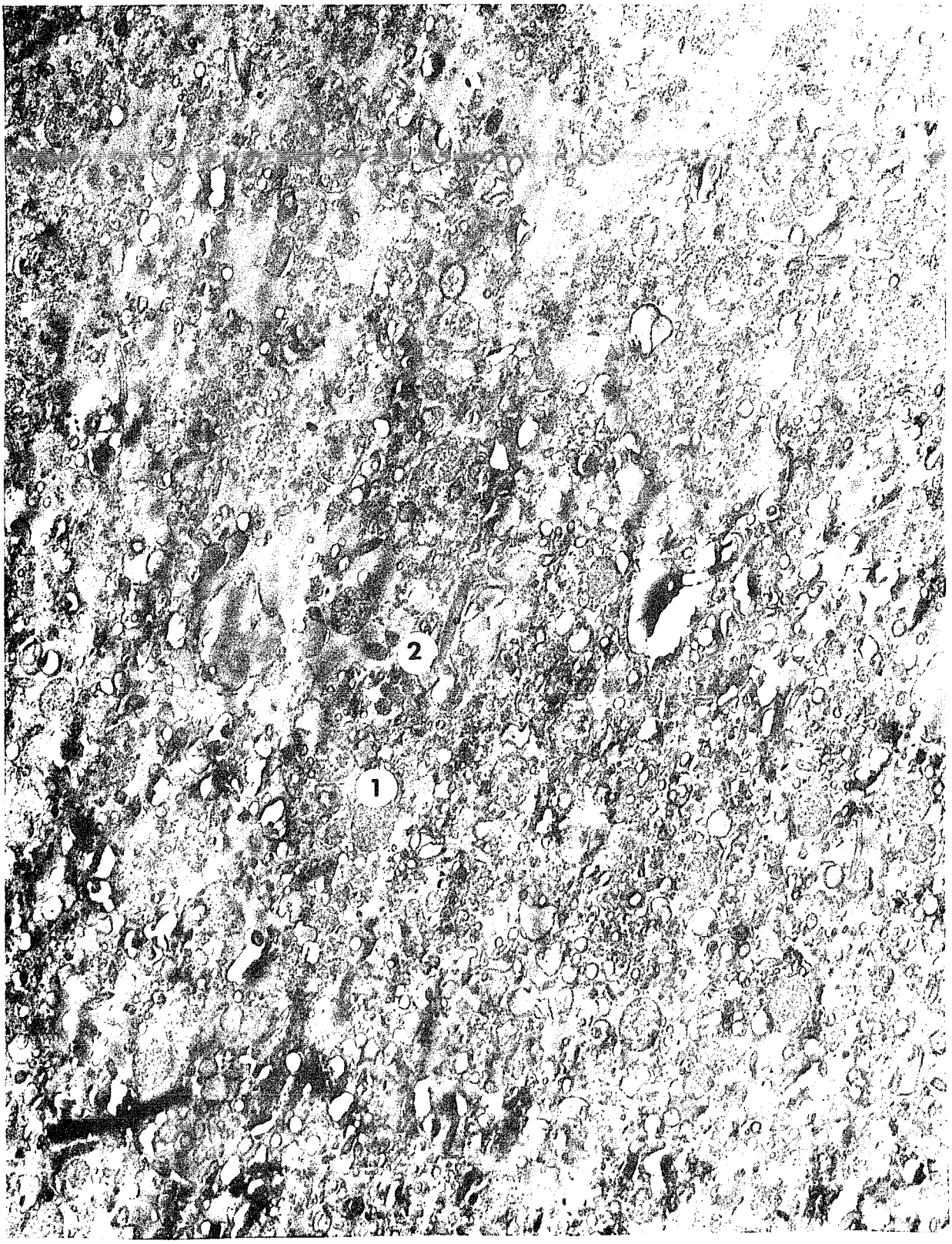


FIGURE 11(B)

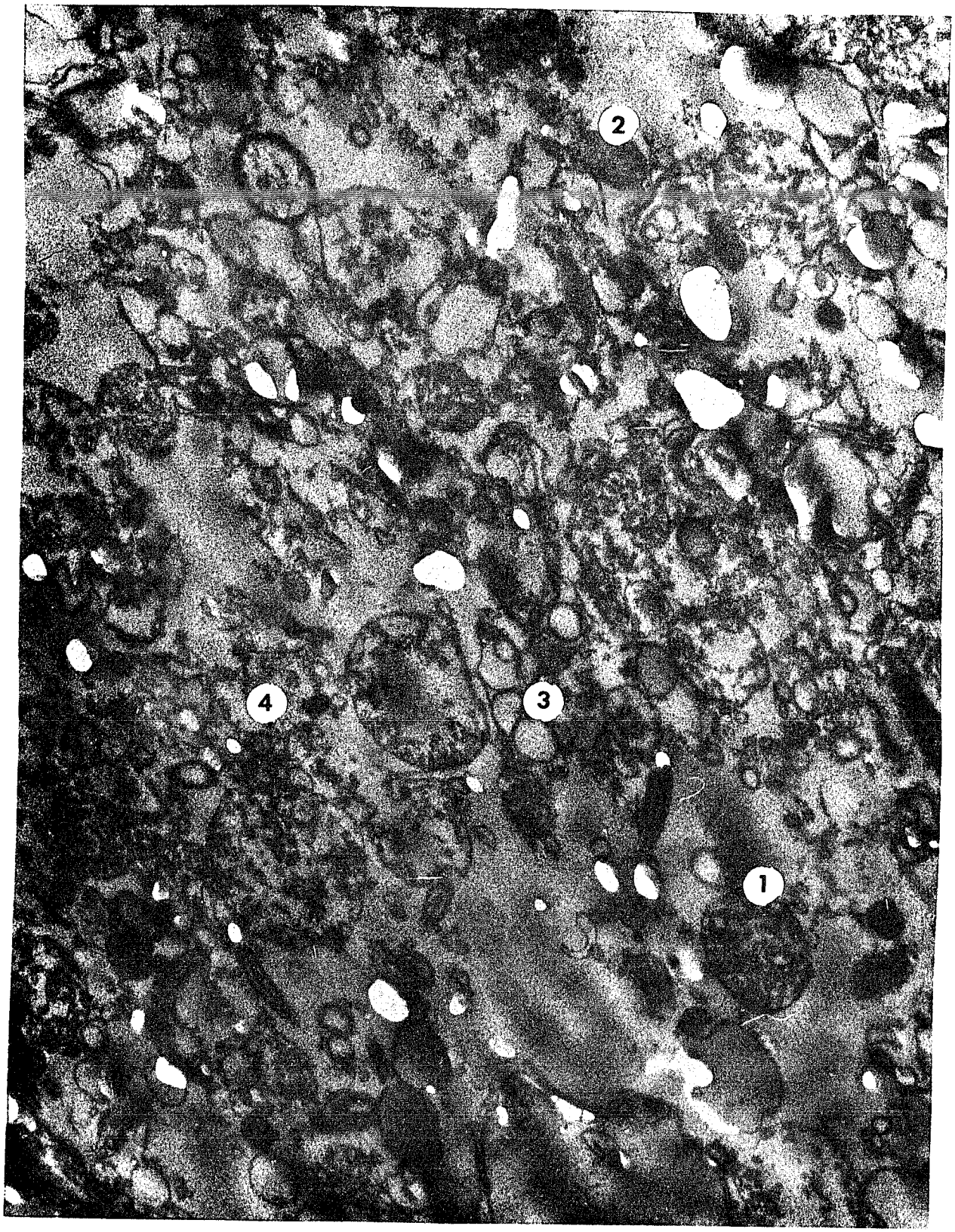


FIGURE 11(C)

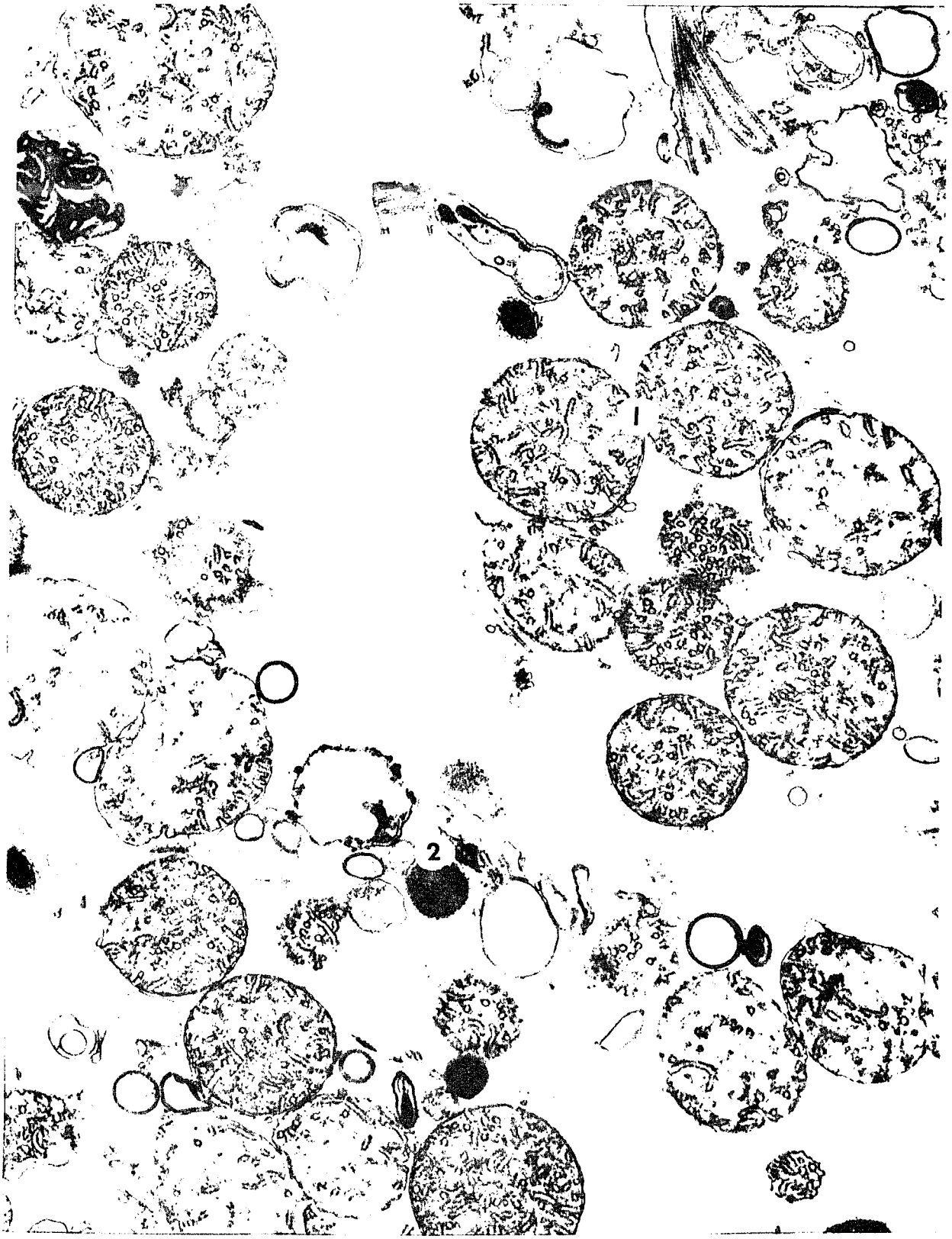


FIGURE 11(D)

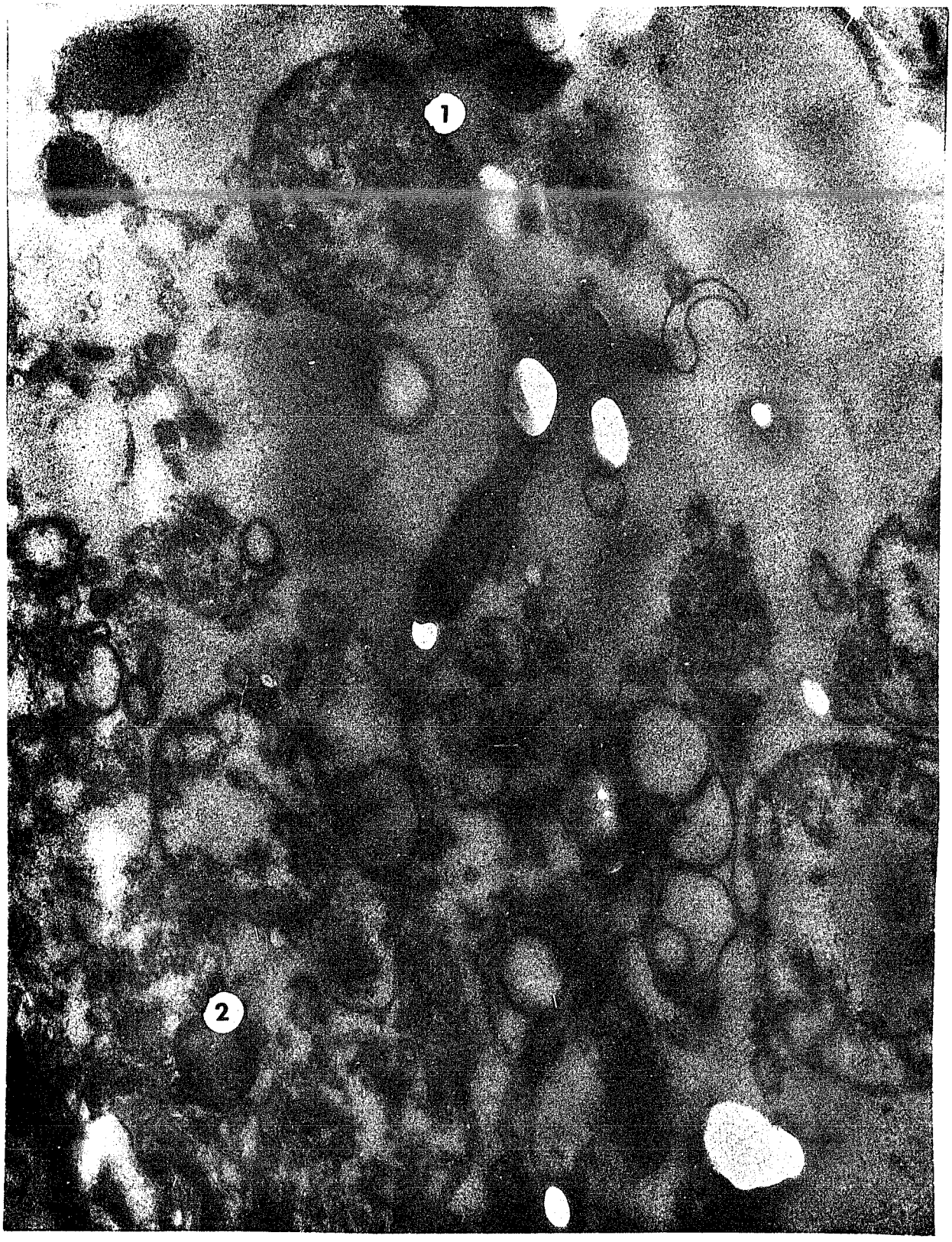
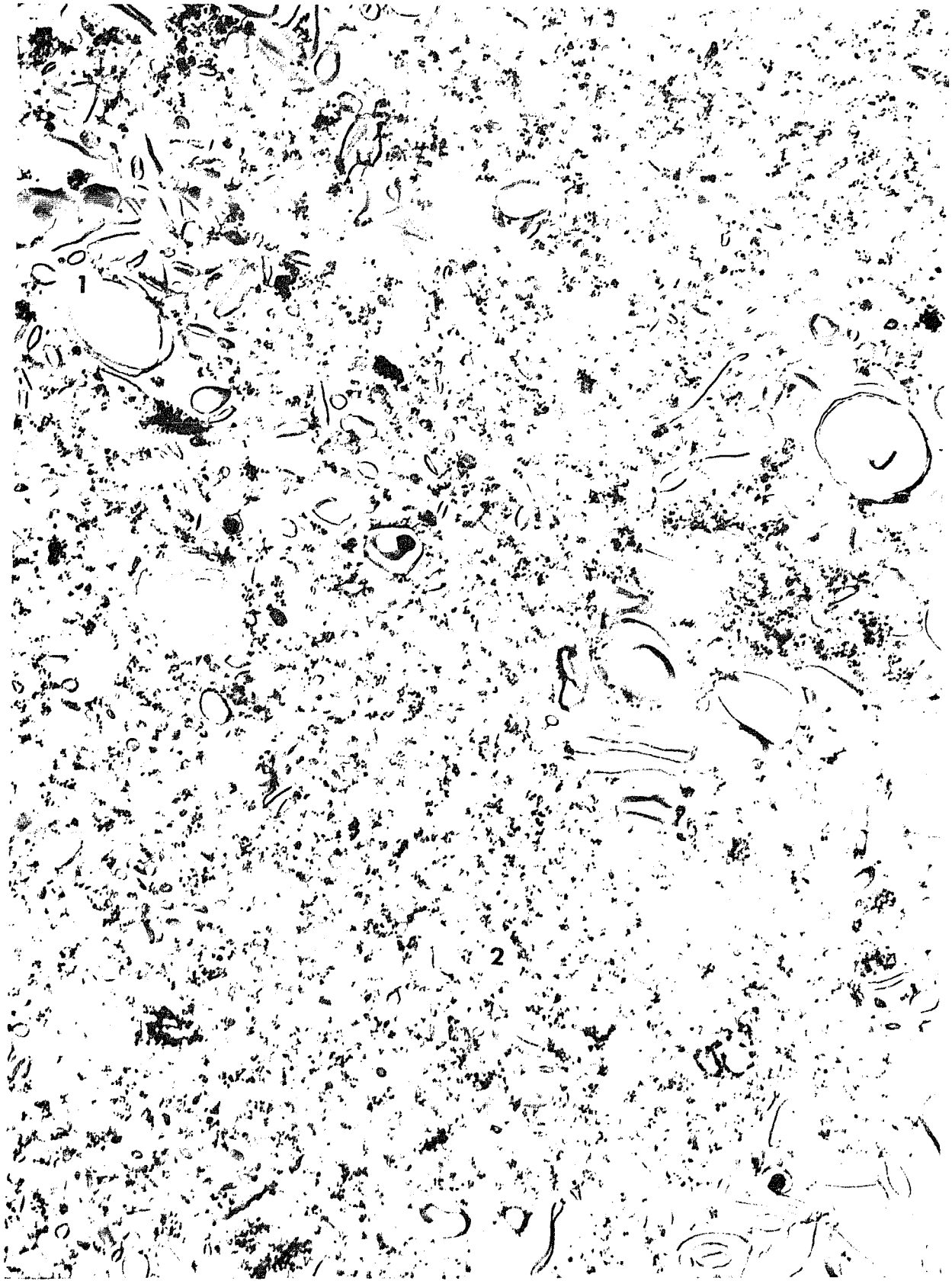


FIGURE 12. ELECTRON MICROGRAPH OF HIGH SPEED (100,000 x g) PELLET.

Washed cells were homogenized in 0.25 M sucrose (see M&M Section IVA) and fractionated by differential centrifugation (see M&M Section IVA2) as described in the text. The high speed (100,000 x g) pellet was then processed for EM, that is, it was fixed in gluteraldehyde, post-fixed in OsO_4, stained with uranyl acetate, and embedded in Maraglas as described in the text (see M&M Section IVC). This fraction appeared to be composed mostly of (1) small empty membrane-bound vessicles, probably ER, and (2) small scattered electron dense particles, probably ribosomes. Magnification = 76,500 X.

FIGURE 12

ELECTRON MICROGRAPH OF HIGH SPEED (100,000 x g) PELLET



speed pellet and therefore may be localized either within the mitochondria or peroxisomes, much of this oxidase activity is still recovered in the soluble fraction.

Finally, to determine whether the BAIB oxidase activity recovered in the intermediate speed pellet was indeed associated with either the mitochondria or the peroxisomes as well as to resubstantiate its soluble activity, the subcellular distribution of this enzyme was ultimately studied by density gradient centrifugation. Thus Tetrahymena pyriformis filtrates were prepared and centrifuged through a continuous sucrose density gradient as described (see M&M Section IVA3). Fractions were then collected and assayed for various enzyme activities as described (see M&M Section IVB).

Most of the MDH activity, as expected, was recovered at a density equivalent to that of Tetrahymena pyriformis mitochondria, while little was recovered at a density equivalent to that of the peroxisomes or as soluble activity (see Fig. 13A). Likewise, most of the catalase activity, as expected, was recovered at a density equivalent to that of Tetrahymena pyriformis peroxisomes, while little was recovered at a density equivalent to that of the mitochondria or as soluble activity (see Fig. 13B). D-amino acid oxidase shows a catalase-like distribution pattern, indicating, as expected, that it too is a peroxisomal enzyme (see Fig. 13C). In addition, however, it also shows considerable soluble activity. Similarly, BAIB oxidase shows a MDH-

FIGURE 13. THE SUBCELLULAR DISTRIBUTION OF ENZYME ACTIVITIES IN TETRAHYMENA PYRIFORMIS HOMOGNATE FRACTIONATED BY ZONAL DENSITY GRADIENT CENTRIFUGATION.

Cells were harvested, washed, and suspended in 0.25 M sucrose as described in the text (see M&M Section IVA3). They were then disrupted by filtration and their subcellular organelles separated by zonal density gradient centrifugation as described in the text (see M&M Section IVA3). After centrifugation, fractions were collected and assayed as described in the text for the indicated standard marker enzymes as well as for BAIB oxidase activity (see M&M Section IVB5). MDH (see M&M Section IVB1) was assayed for as a standard Tetrahymena pyriformis mitochondrial marker enzyme. Catalase (see M&M Section IVB2) was assayed for as a standard peroxisomal marker enzyme. D-amino acid oxidase (see M&M Section IVB3), another standard peroxisomal marker enzyme, was also measured fluorometrically as an additional check on the BAIB oxidase assay system.

The horizontal axis (%ΔV) represents the volume each fraction contains compared to the total. The vertical axis (% ACT X VOL) represents the activity each fraction contains compared to the total recovered. The broken line across the face of each figure represents the level of enzyme activity expected in each fraction if the total enzyme activity were uniformly distributed throughout the gradient.

A. Subcellular distribution of MDH activity.

B. Subcellular distribution of catalase activity.

C. Subcellular distribution of D-amino acid oxidase activity.

D. Subcellular distribution of BAIB oxidase activity.

FIGURE 13A
SUBCELLULAR DISTRIBUTION OF MDH ACTIVITY

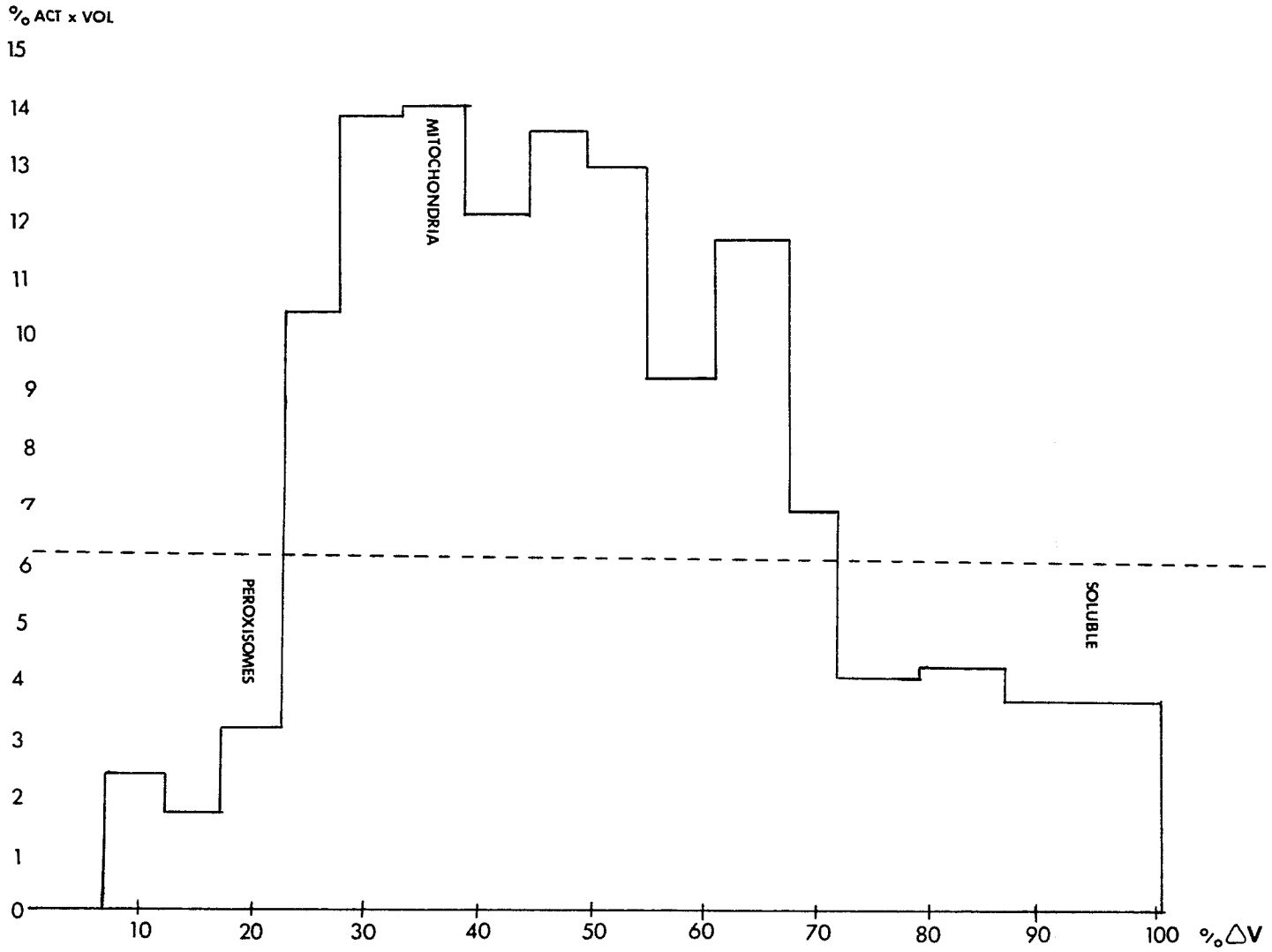


FIGURE 13B

SUBCELLULAR DISTRIBUTION OF CATALASE ACTIVITY

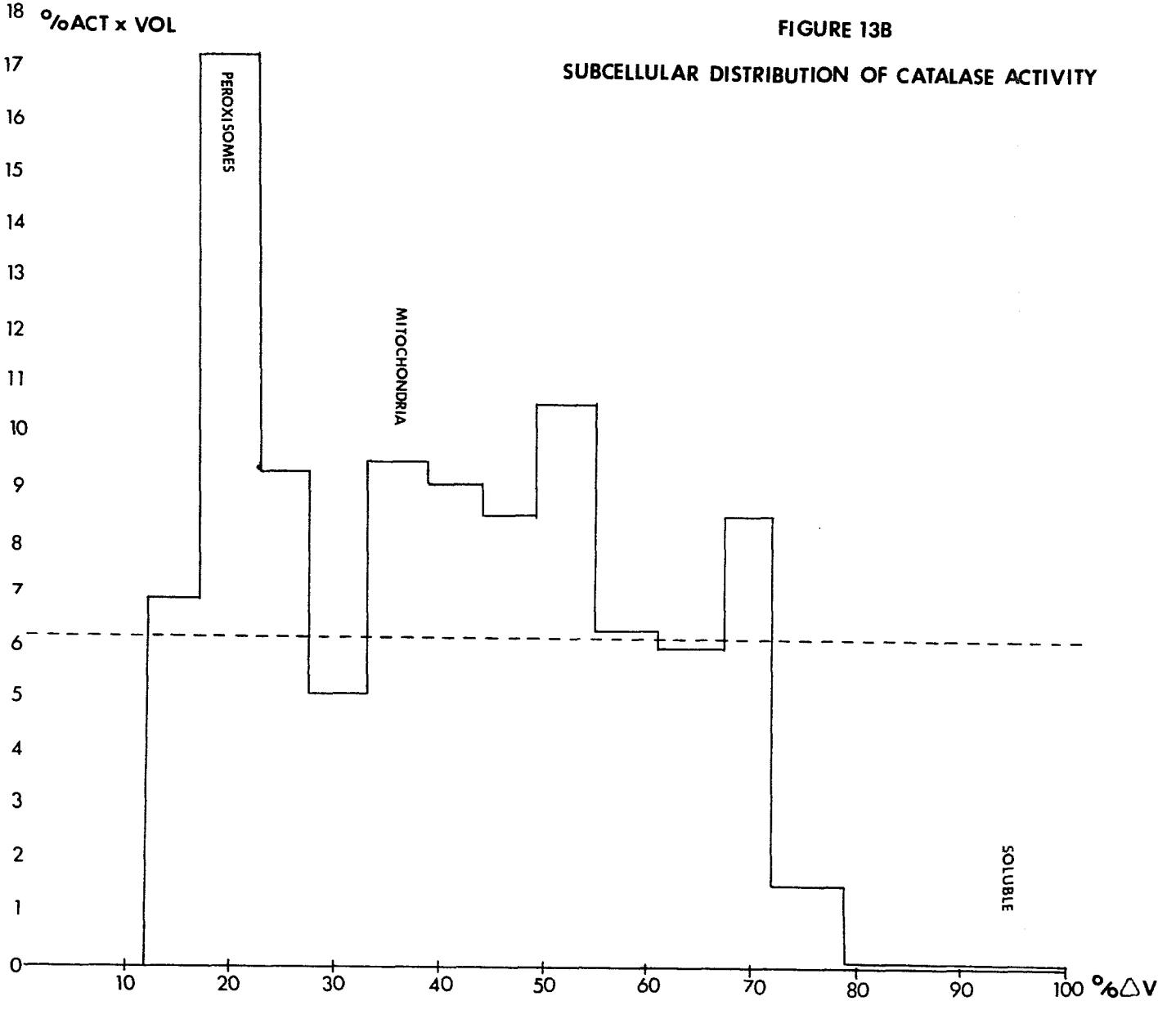


FIGURE 13C

SUBCELLULAR DISTRIBUTION OF D-AMINO ACID OXIDASE ACTIVITY

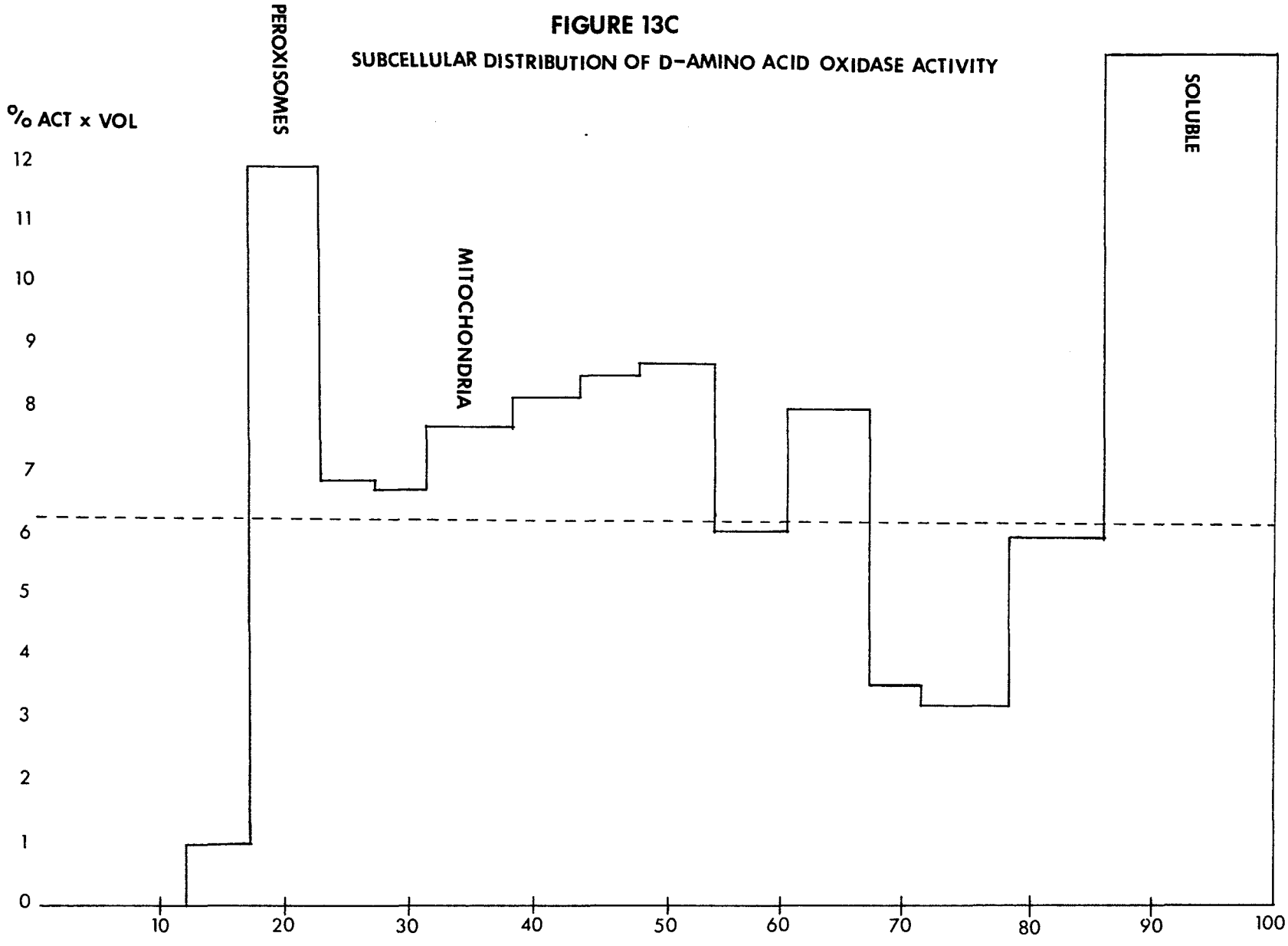
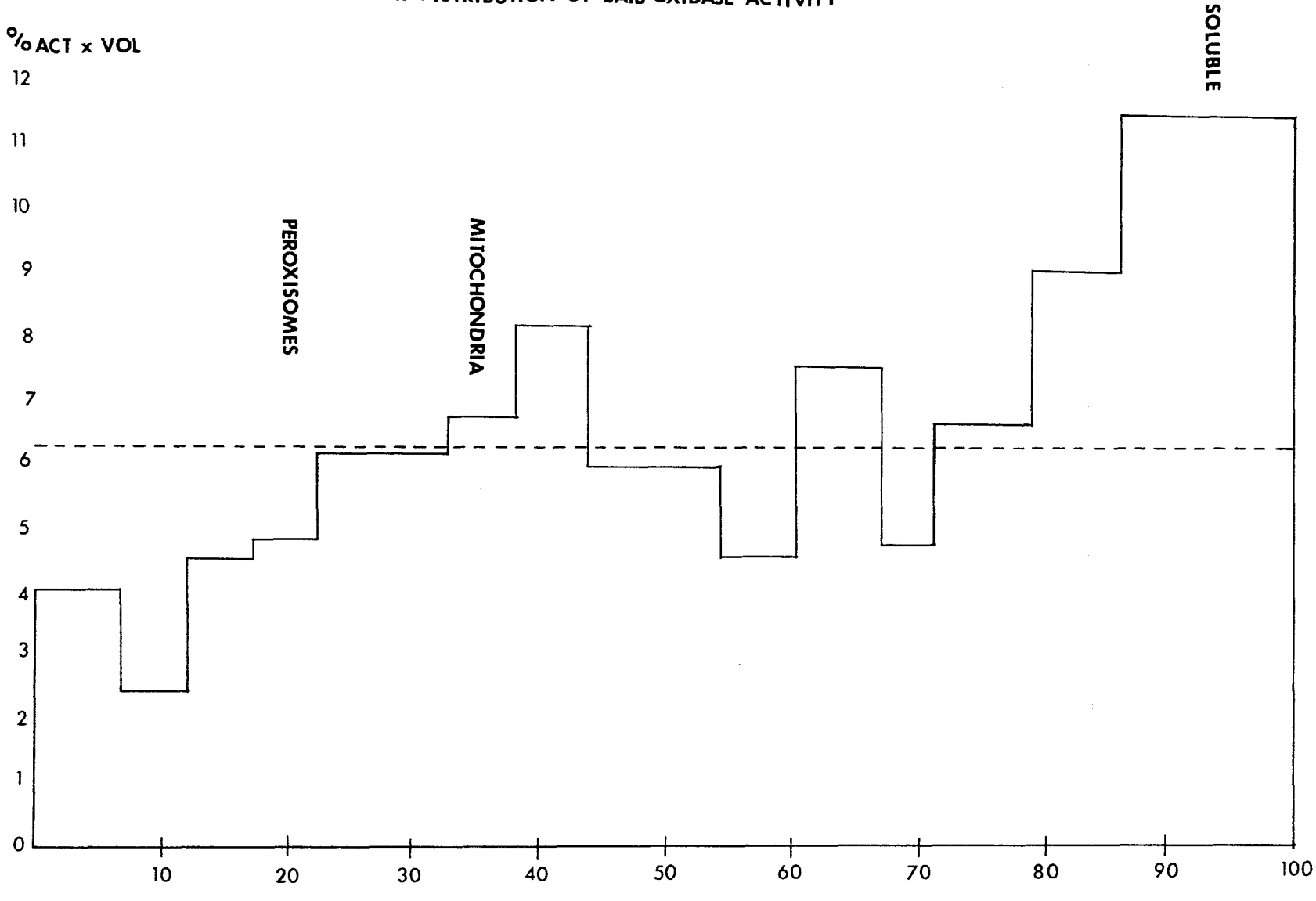


FIGURE 13D

SUBCELLULAR DISTRIBUTION OF BAIB OXIDASE ACTIVITY



like distribution pattern, suggesting that it might be a mitochondrial enzyme (see Fig. 13D), although, like D-amino acid oxidase, it also shows considerable soluble activity. These results, therefore, indicate that although a considerable amount of the BAIB oxidase activity continues to remain soluble, at least a small subcellular organelle associated activity component also appears to be localized within the mitochondria rather the the peroxisomes as originally thought (see Discussion Section IV).

V. PARTIAL PURIFICATION OF BAIB OXIDASE ACTIVITY

A. $(NH_4)_2SO_4$ Precipitation

To determine if BAIB oxidase activity is a result of a specific enzyme or the non-specific expression of some other oxidase, we decided that we must at least partially purify this activity. We began the preliminary purification of this enzyme activity by $(NH_4)_2SO_4$ fractionation. Upon initial wide range fractionation both D-amino acid oxidase and BAIB oxidase gave similar activity profiles (see Table XVII(A)). In fact, the 10-60% $(NH_4)_2SO_4$ cut effectively precipitated most of both of these enzyme activities. Subsequently more narrow range fractionation, however, indicated that these two oxidase may be separate and distinct enzymes (see Table XVII(A)). Thus if one is willing to sacrific quantity for purity, it is possible to separate BAIB oxidase from D-amino acid oxidase as well as the other marker enzyme activities by $(NH_4)_2SO_4$ precipitation

TABLE XVII. FRACTIONATION OF BAIB OXIDASE ACTIVITY BY $(NH_4)_2SO_4$ PRECIPITATION.

Cells were harvested, washed, and homogenized in 0.1 M Tris-HCl buffer, pH 8.6, as described in the text (see M&M Section VA). A small aliquot of this homogenate was retained for enzyme analysis. The remainder was centrifuged at low speed (1085 x g) as described in the text (see M&M Section VA). A small aliquot of this supernatant was also occasionally retained for enzyme analysis. The remainder was fractionated into the above $(NH_4)_2SO_4$ precipitates. These precipitates were then resuspended in buffer and assayed as described in the text (see M&M Section IVB) for the indicated enzyme activities. Enzyme activities were preferentially expressed as the per cent of the low speed supernatant activity (B). Where this was not possible, enzyme activities were alternatively expressed as the per cent of homogenate activity (A).

TABLE XVII

FRACTIONATION OF BAIB OXIDASE ACTIVITY BY $(NH_4)_2SO_4$ PRECIPITATION

(A)

$(NH_4)_2SO_4$ FRACTION	% HOMOGENATE ACTIVITY BAIB	D-ala
0 - 10 %	0.4	0.6
10 - 50 %	2.2	4.0
50 - 100%	1.5	3.9
% TOTAL RECOVERY	4.1	8.5
0 - 10 %	6.9	3.1
10 - 60 %	13.2	17.3
60 - 100%	3.0	0
% TOTAL RECOVERY	23.1	20.4
0 - 10 %	4.6	0.5
10 - 30 %	6.3	0.8
30 - 50 %	6.9	2.4
50 - 75 %	0.7	1.3
75 - 100%	1.0	0.2
% TOTAL RECOVERY	19.5	5.2

TABLE XVII

FRACTIONATION OF BAIB OXIDASE ACTIVITY BY $(NH_4)_2SO_4$ PRECIPITATION

(B)

$(NH_4)_2SO_4$ FRACTION	% LOW SPEED SUPERNATANT ACTIVITY				
	BAIB	D-ala	LAC	MDH	CAT
0 - 40 %	18.2	8.4	10.0	9.3	10.3
40 - 60 %	14.4	47.7	30.0	10.2	79.2
60 - 100%	2.9	7.0	16.0	60.8	2.1
% TOTAL RECOVERY	35.5	63.1	56.0	80.3	91.6

(see Table XVII(B)). That is, although the 0-40% $(NH_4)_2SO_4$ cut only precipitates about half of the total recovered BAIB oxidase activity, it, nevertheless, effectively isolates most of this enzyme activity from that of the other marker enzymes including the other oxidases, D-amino acid oxidase and lactate oxidase.

B. Affinity Chromatography

Subsequently we tried to further purify this BAIB oxidase activity by various ion exchange and gel filtration chromatographic techniques. None of these traditional methods, however, proved to be satisfactory for only a small amount (<10%) of the original protein activity was ever recovered. We therefore decided to try the relatively recently developed technique of affinity chromatography[66,67,120-123] to further separate D-amino acid oxidase activity from BAIB oxidase activity, if not further purify this enzyme activity itself.

A D-ala affinity column was prepared as described (see M&M Section VB). It was decided to initially try to separate D-amino acid oxidase activity from BAIB oxidase activity because the mechanism of interaction of D-amino acid oxidase with its D-amino acid substrates is well known.[124] We could thus bind a D-amino acid by its amino group via spacer to the matrix and be fairly sure that we would not greatly interfere with enzyme binding while simultaneously inhibiting catalysis (see Discussion Section V). Ultimately, however, we did hope to be able to similarly separate

BAIB oxidase activity from D-amino acid oxidase activity by preparing a BAIB affinity column.

Preliminary protein fractionation of the resuspended 0-40% $(NH_4)_2SO_4$ precipitate on such a D-ala affinity column again seemed to effectively isolate most of the BAIB oxidase activity from that of the other marker enzymes, including D-amino acid oxidase. That is, BAIB oxidase activity eluted slightly ahead of most of the other enzyme activities, while D-amino acid oxidase was somewhat retarded presumably due to its interaction with the covalently bound substrate, D-ala (see Table XVIII). The rather poor separation of D-amino acid oxidase from the other marker enzyme activities was disappointing, but probably attributable to the low substrate binding affinity of this enzyme.[67,120-123] If this is indeed the case, then separating BAIB oxidase, which has an even lower substrate binding affinity, from these other marker enzyme activities would probably not be possible as hoped on a BAIB affinity column.

VI. PHYSIOLOGICAL SIGNIFICANCE OF BAIB OXIDASE ACTIVITY

Finally, after demonstrating what we thought was the existence of a unique and specific oxidase capable of the further metabolism of BAIB in Tetrahymena pyriformis, we became interested in determining the physiological significance of this enzyme in the overall metabolism of this organism.

A. Specific Activity

TABLE XVIII. FURTHER FRACTIONATION OF BAIB OXIDASE ACTIVITY BY AFFINITY CHROMATOGRAPHY.

Cells were harvested, washed, and homogenized in 0.1 M Tris-HCl buffer, pH 8.6, as described in the text (see M&M Section VB). The homogenate was centrifuged at low speed (1085 x g) as described in the text (see M&M Section VB). The resulting supernatant was decanted and fractionated according to the previously described $(NH_4)_2SO_4$ precipitation scheme (see M&M Section VA). A D-ala affinity column was then prepared according to the supplier's directions as described in the text (see M&M Section VB). One ml of the resuspended 0-40% $(NH_4)_2SO_4$ precipitate containing approximately twenty-five $A_{280\ nm}$ units was layered on top of the column. The column was then developed in the cold ($4^{\circ}C$) with 0.1 M Tris-HCl buffer, pH 8.6. 2.5 ml fractions were collected. The effluent was continuously monitored at 253 nm with a LKB Uvicord I. The protein came off the column in one rather skewed peak. The $A_{280\ nm}$ of all peak fractions was redetermined with the Beckman DB spectrophotometer and used to calculate the per cent of the protein recovered in each fraction. Then enzyme activities of the peak fractions were also determined as previously described (see M&M Section IVB). Enzyme recoveries were finally expressed as the per cent of the total activity in the 0-40% $(NH_4)_2SO_4$ precipitate.

TABLE XVIII

FURTHER FRACTIONATION OF BAIB OXIDASE ACTIVITY BY AFFINITY CHROMATOGRAPHY

ENZYME ACTIVITY	% 0-40% $(NH_4)_2SO_4$ PPT ACT FRACTION #							% TOTAL RECOVERY
	1	2	3	4	5	6	7	
BAIB		258.0	22.7	41.3	67.5			389.5
D-ala		27.0	33.6	0	0			60.6
LAC					44.3			44.3
MDH	5.7	49.6	68.2	41.0				164.5
CAT	0	48.8	50.2	12.8	4.8			116.6
PROTEIN	0.1	46.6	6.6	1.7	0.8	0.4	0.2	56.4

Initially we were interested in determining the molar specific activity of BAIB oxidase so that we might compare its activity to that of other enzymes. A standard fluorometric H_2O_2 peroxidase decomposition curve (see Fig. 14), therefore, was prepared as described (see M&M Section VIA) and the molar specific activity of this enzyme in Tetrahymena pyriformis homogenates was calculated to be approximately:

2.8 nmoles/min/mg of protein

This is about 1/10 the reported specific activities of other Tetrahymena pyriformis oxidases.[57]

B. Substrate Specificity

1. Is D(-)BAIB preferentially oxidized?

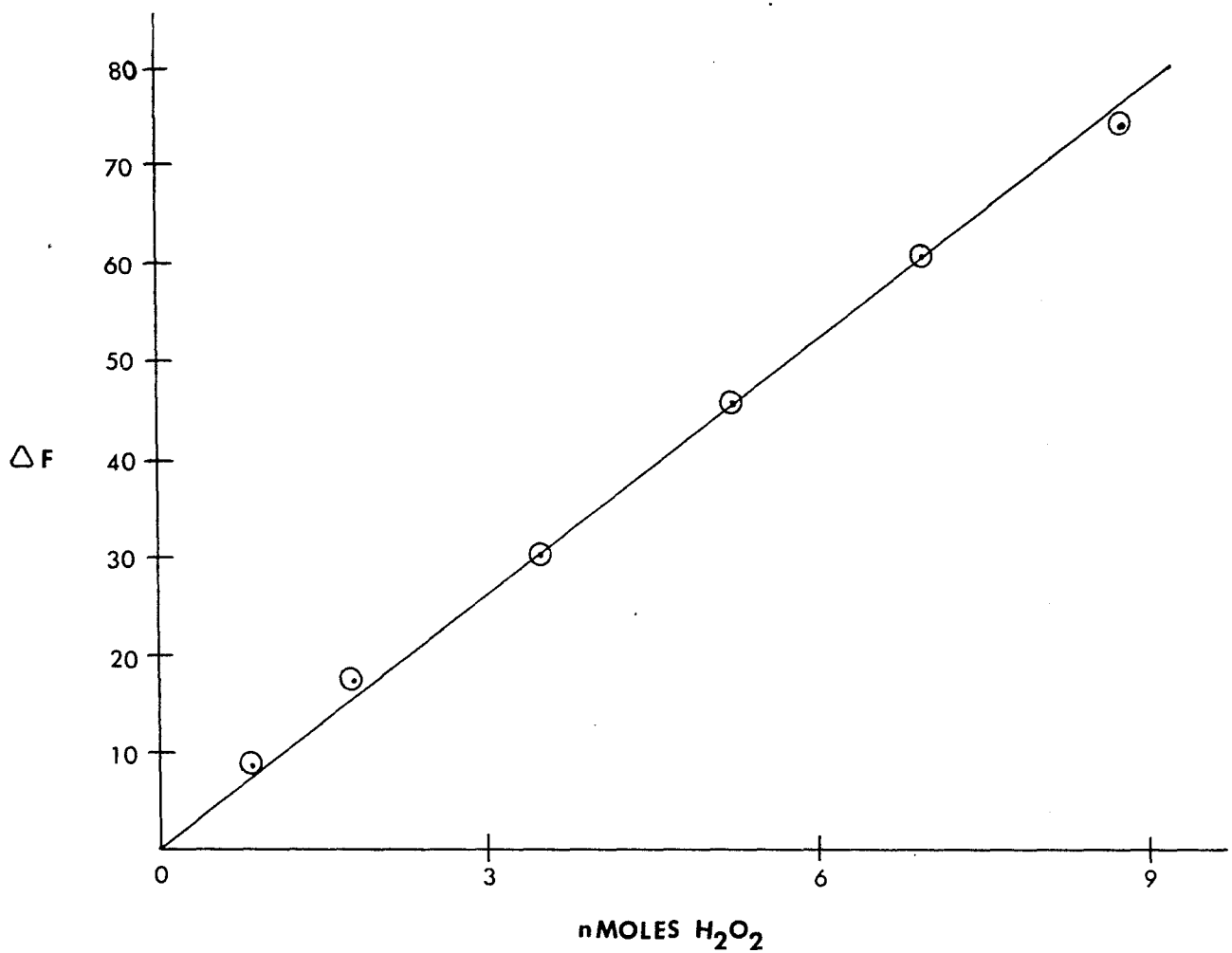
We also became interested in determining if one of the stereoisomers found in the commercial BAIB was actually the preferred substrate. This interest, as discussed above (see M&M Section VIB), was generated by reports that thymine was preferentially reductively catabolized to D(-)BAIB and that only this isomer could then be effectively metabolized further in mammalizn systems.[68-72] As it is difficult to chemically separate these isomers from one another, however, we thought that we might be able to selectively eliminate them by treating the assay system with an appropriate exogenous enzyme prior to the addition of the Tetrahymena pyriformis homogenate (see M&M Section VIB). It was hoped, therefore, that pretreating with D-amino acid oxidase might eliminate D(-)BAIB from the assay system before

FIGURE 14. STANDARD FLUOROMETRIC H_2O_2 PEROXIDASE DECOMPOSITION CURVE.

The peroxidase assay system (see M&M Section VIA) containing the indicated amounts of H_2O_2 as substrate was incubated at 37°C until the fluorescent readings stabilized (approximately 10 min). The number of nmoles of H_2O_2 decomposed in each assay system was calculated from the measurement of the $A_{240\ nm}$ of the stock solution. (The molar absorbancy for H_2O_2 at 240 nm in a 1 cm cubette is 43.6.[62]) The number of nmoles of H_2O_2 produced as a result of BAIB oxidase activity in the Tetrahymena pyriformis homogenate was then read off the standard curve. This activity (as nmoles/min) was finally converted to molar specific activity (nmoles/min/mg of homogenate protein).

FIGURE 14

STANDARD FLUOROMETRIC H_2O_2 PEROXIDASE DECOMPOSITION CURVE



the subsequent addition of the crude Tetrahymena pyriformis enzyme preparation, while pretreating with L-amino acid oxidase might eliminate the L(+)BAIB. Thus any increase in oxidase activity subsequent to the addition of the Tetrahymena pyriformis homogenate to the D-amino acid oxidase system would reflect the metabolism of the remaining L(+)BAIB, while a similar increase in the L-amino acid oxidase system would reflect the metabolism of the remaining D(-)BAIB.

Preliminary results seemed to indicate that D(-)BAIB was the preferred substrate (see Fig. 15). That is, although both exogenous enzymes showed slight BAIB oxidase activity, the subsequent addition of Tetrahymena pyriformis homogenate stimulated this oxidase activity in the L-amino acid oxidase system (see Fig. 15(A)), but not in the D-amino acid oxidase system (see Fig. 15(B)). Thus it appeared that the D(-)BAIB presumably remaining in the L-amino acid oxidase system was capable of being oxidized by the crude Tetrahymena pyriformis enzyme preparation, while the L(+) BAIB supposedly remaining in the D-amino acid oxidase system was not.

Further investigation of these commercial enzyme preparations, however, indicated that both these oxidases also appeared to be contaminated with other enzyme activities. The commercial preparation of L-amino acid oxidase, for example, also apparently contained significant D-amino acid oxidase activity (see Fig. 15(A)). Similarily, the commercial preparation of D-amino acid oxidase also apparently

FIGURE 15. STEREOISOMER SPECIFICITY OF BAIB OXIDASE ACTIVITY.

A. ASSAY SYSTEM PREINCUBATED WITH L-AMINO ACID OXIDASE. The BAIB oxidase assay system was incubated with 5 ul of crude commercial snake venom L-amino acid oxidase (final conc = 1.7 ug/ml) for 90 min prior to the subsequent addition of the Tetrahymena pyriformis homogenate as described in the text (see M&M Section VIB). Exogenous L-amino acid oxidase apparently showed some BAIB oxidase activity. Subsequent addition of 0.1 ml crude Tetrahymena pyriformis enzyme preparation, however, apparently stimulated this activity indicating that the D(-)BAIB presumably remaining in this assay system was capable of being oxidized by this homogenate.

B. ASSAY SYSTEM PREINCUBATED WITH D-AMINO ACID OXIDASE. The BAIB oxidase assay system was incubated with 5 ul of crude commercial hog kidney D-amino acid oxidase (final conc = 0.2 mg/ml) for 90 min prior to the subsequent addition of the Tetrahymena pyriformis homogenate as described in the text (see M&M Section VIB). Exogenous D-amino acid oxidase also apparently showed some BAIB oxidase activity. Subsequent addition of 0.1 ml crude Tetrahymena pyriformis enzyme preparation, however, apparently did not stimulate this activity indicating that the L(+)BAIB presumably remaining in this assay system was not preferentially oxidized by this homogenate.

SUBSTRATES: (1) D,L-BAIB; (2) D-ala; (3) L-ala; (4) D,L-ala; (5) B-ala.

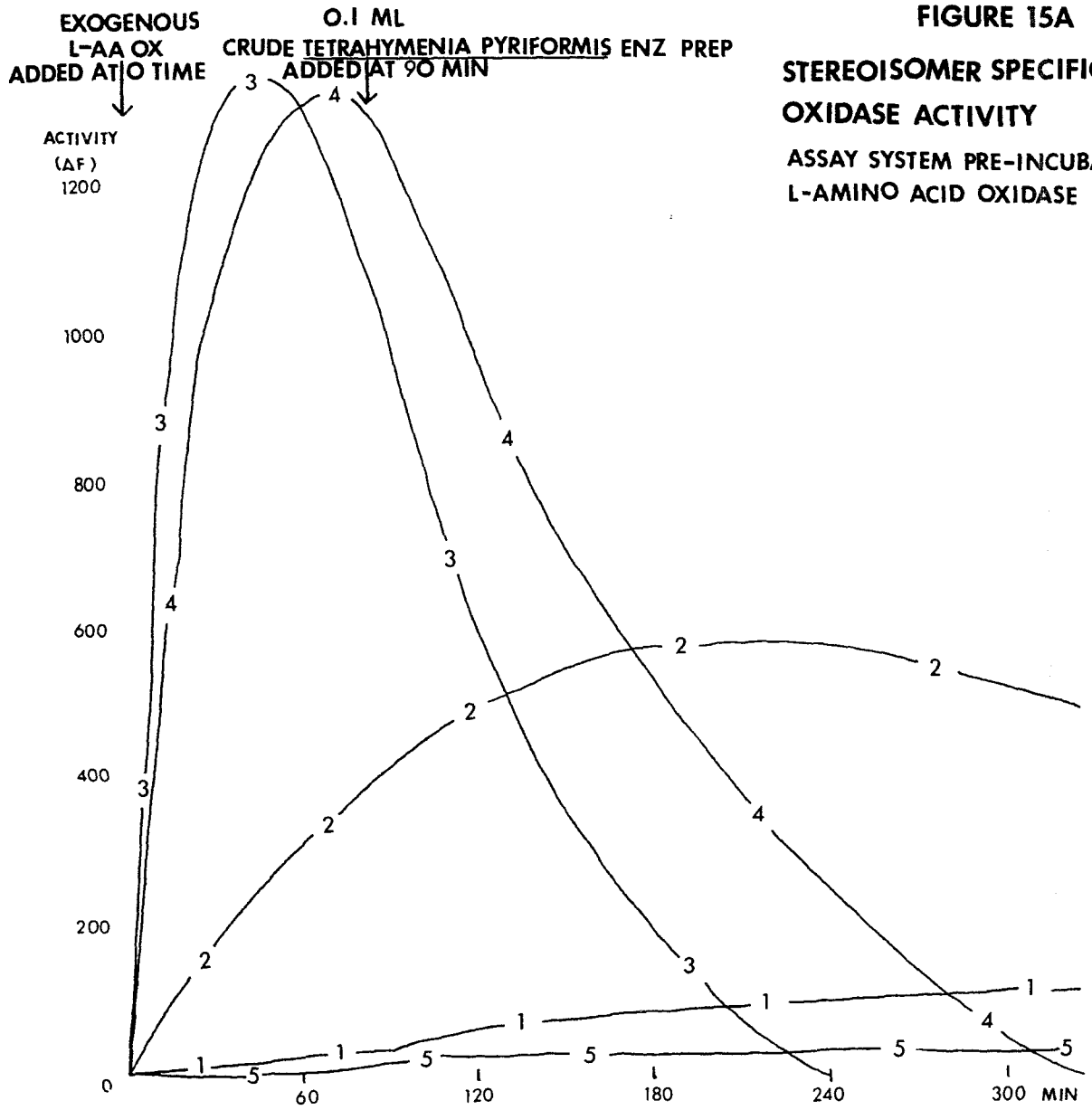


FIGURE 15A

STEREOISOMER SPECIFICITY OF βAIB OXIDASE ACTIVITY

ASSAY SYSTEM PRE-INCUBATED WITH L-AMINO ACID OXIDASE

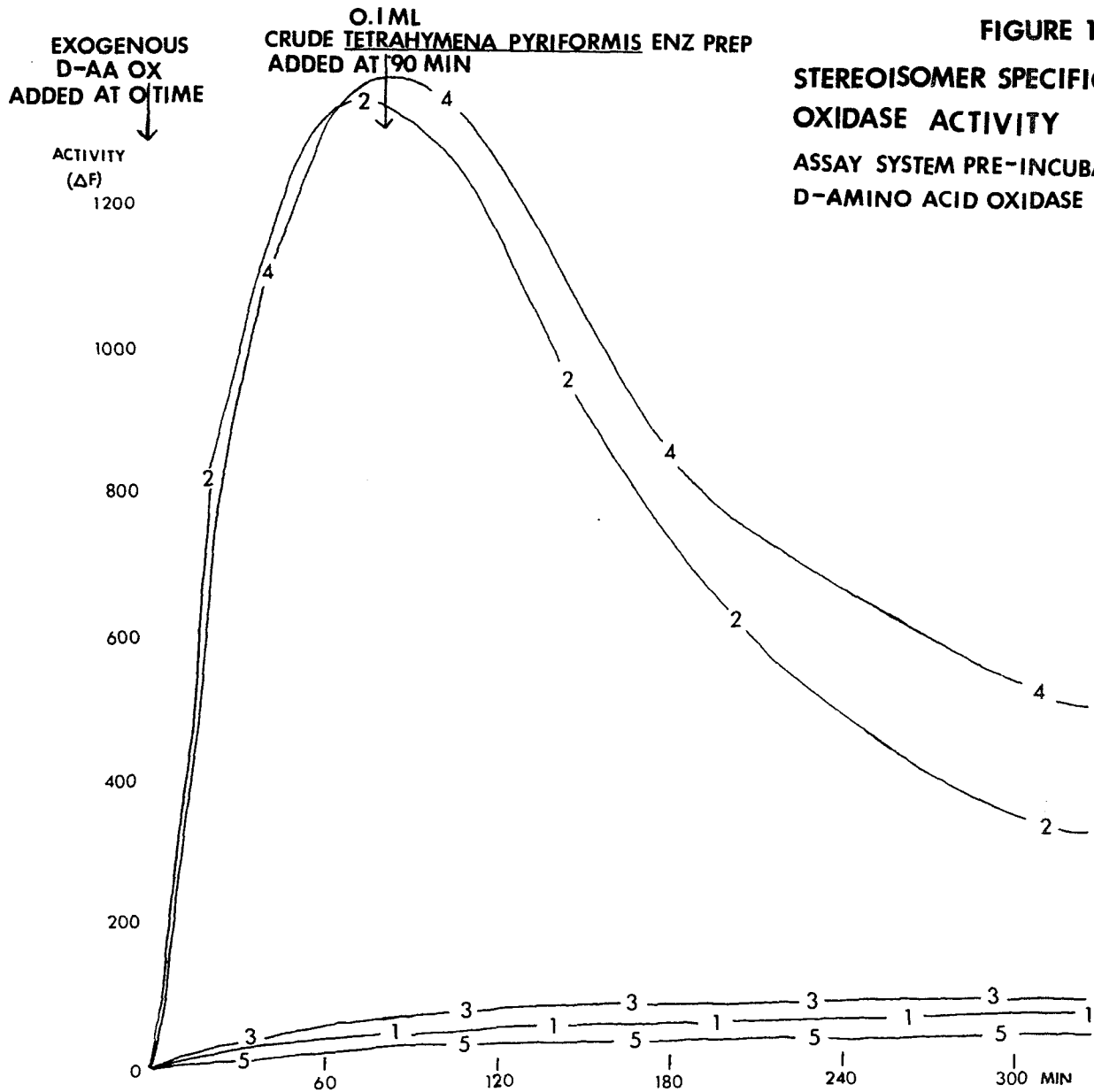


FIGURE 15B

STEREOISOMER SPECIFICITY OF βAIB OXIDASE ACTIVITY

ASSAY SYSTEM PRE-INCUBATED WITH D-AMINO ACID OXIDASE

not only contained some L-amino acid oxidase activity (see Fig. 15(B)), but also substantial L-α-hydroxy acid oxidase activity as well. Thus whether the exogenous oxidase activity in either assay system is a result of L-amino acid oxidase, D-amino acid oxidase, or some other oxidase activity is somewhat unclear. The results, nevertheless, still seem to indicate that D(-)BAIB may also be the preferred substrate for this Tetrahymena pyriformis BAIB oxidase activity.

2. Substantiation of the overall catabolic pathway

Since we had been able to demonstrate the first enzyme in the proposed reductive thymine catabolic pathway, thymine reductase (dehydrogenase), as well as develop a fluorometric assay for the fourth, BAIB oxidase, (see Table I(A)) we thought that we might also be able to demonstrate the operation of the intervening catabolic interconversions by using DHT or DHTdine as the fluorometric substrate instead of BAIB (see Fig. 16). Thus we incubated the BAIB oxidase assay system with several dihydropyrimidine derivatives as described (see M&M Section VIC). DHU and DHUdine were also tested since it had been shown in several organisms that the reduction of uracil to DHU and thymine to DHT involves the same enzyme working at different efficiencies.[49] As expected on the basis of the enzymatic experiments, DHT was the most active dihydropyrimidine substrate. The rapidity of its oxidation, however, was somewhat unexpected and may indicate the importance of the sterospecific conformation

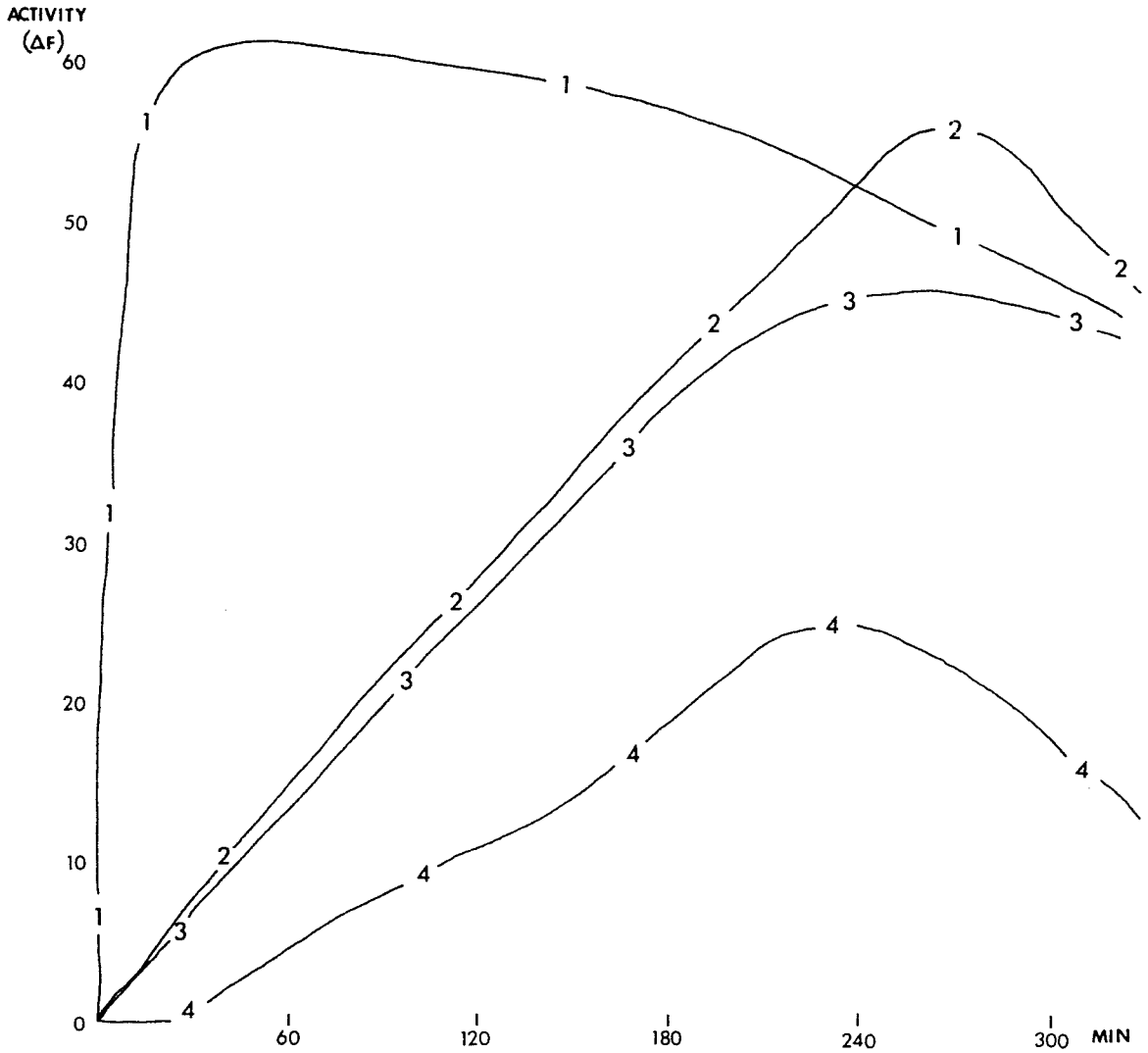
FIGURE 16. OXIDASE ACTIVITY OF DHP DERIVATIVES.

The complete BAIB oxidase system (see M&M Section IIIA) containing 0.1 ml of Tetrahymena pyriformis homogenate was incubated for approximately 6 hr at 37°C with the indicated DHP derivatives as substrate instead of BAIB as described in the text (see M&M Section VIC). H_2O_2 production was then measured at 10-30 min intervals. Since these DHP derivatives are much less soluble than BAIB, however, their concentrations (final conc = 0.0167 M) in the oxidase assay system were considerably less than that of BAIB (final conc = 0.1 M). Substantial oxidase activity, nevertheless, was detected by extending the incubation period.

SUBSTRATES: (1) DHT; (2) DHTdine; (3) DHUdine; (4)DHU.

FIGURE 16

OXIDASE ACTIVITY OF DHP DERIVATIVES



of the intermediate substrates involved in this catabolic pathway (see Discussion Section V).

C. Kinetics

Studies were also done on the kinetics of this oxidase using relatively crude Tetrahymena pyriformis enzyme preparations. A Lineweaver-Burk plot was constructed from the data to determine the K_m of this enzyme under these assay conditions (see Fig. 17(A)). The high K_m (3.6×10^{-1} M) would tend to indicate a low affinity of the enzyme for BAIB, but whether this K_m as determined in these crude preparations with the possible disruption of the spatial orientation of this enzyme and/or in the presence of inhibitors is physiologically significant will have to await further K_m determinations on more purified enzyme preparations.

For comparison kinetic studies were also done on D-amino acid oxidase. As expected the K_m (5.9×10^{-2} M) of this enzyme from relatively crude Tetrahymena pyriformis enzyme preparations is considerably lower than that of BAIB oxidase (see Fig. 17(B)), thus indicating that the former enzyme has a greater affinity for its substrate under these assay conditions than the latter. For further comparison kinetic studies were done on a crude commercial preparation of hog kidney D-amino acid oxidase. As expected the K_m (1.4×10^{-2} M) of this enzyme was even lower than that of Tetrahymena pyriformis D-amino acid oxidase (see Fig. 17(C)) indicating that this enzyme has the greatest affinity for its substrate under these assay conditions.

FIGURE 17. LINEWEAVER-BURK PLOTS OF OXIDASE ACTIVITIES. Preliminary studies on the kinetics of the indicated oxidase activities were done as described in the text (see M&M Section VID).

A. BAIB oxidase activity of Tetrahymena pyriformis homogenates.

B. D-amino acid oxidase activity of Tetrahymena pyriformis homogenates.

C. Crude commercial hog kidney D-amino acid oxidase activity.

D. BAIB oxidase activity of crude commercial hog kidney D-amino acid oxidase.

FIGURE 17A

BAIB OXIDASE ACTIVITY OF TETRAHYMENA PYRIFORMIS HOMOGENATES

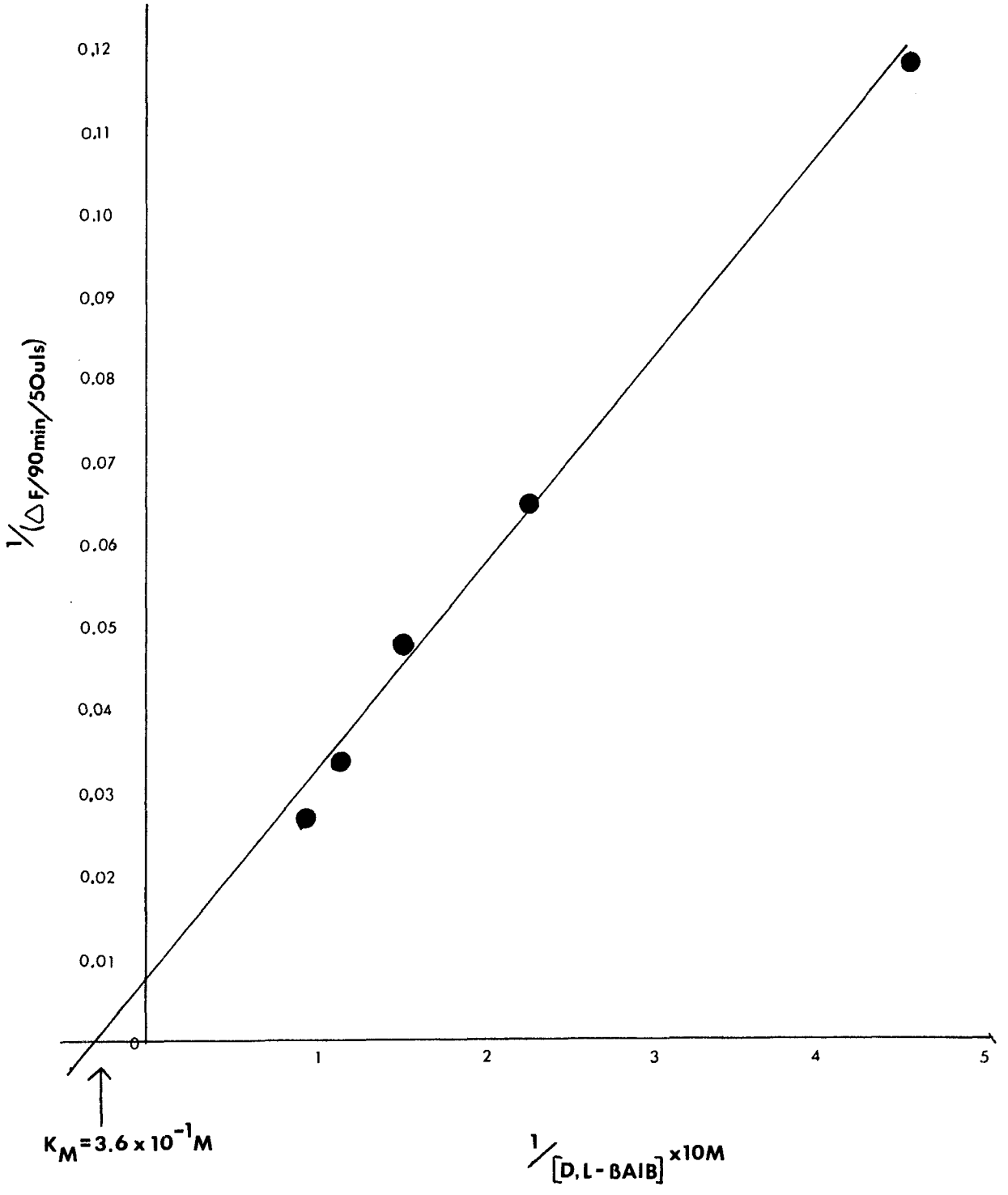


FIGURE 17 B

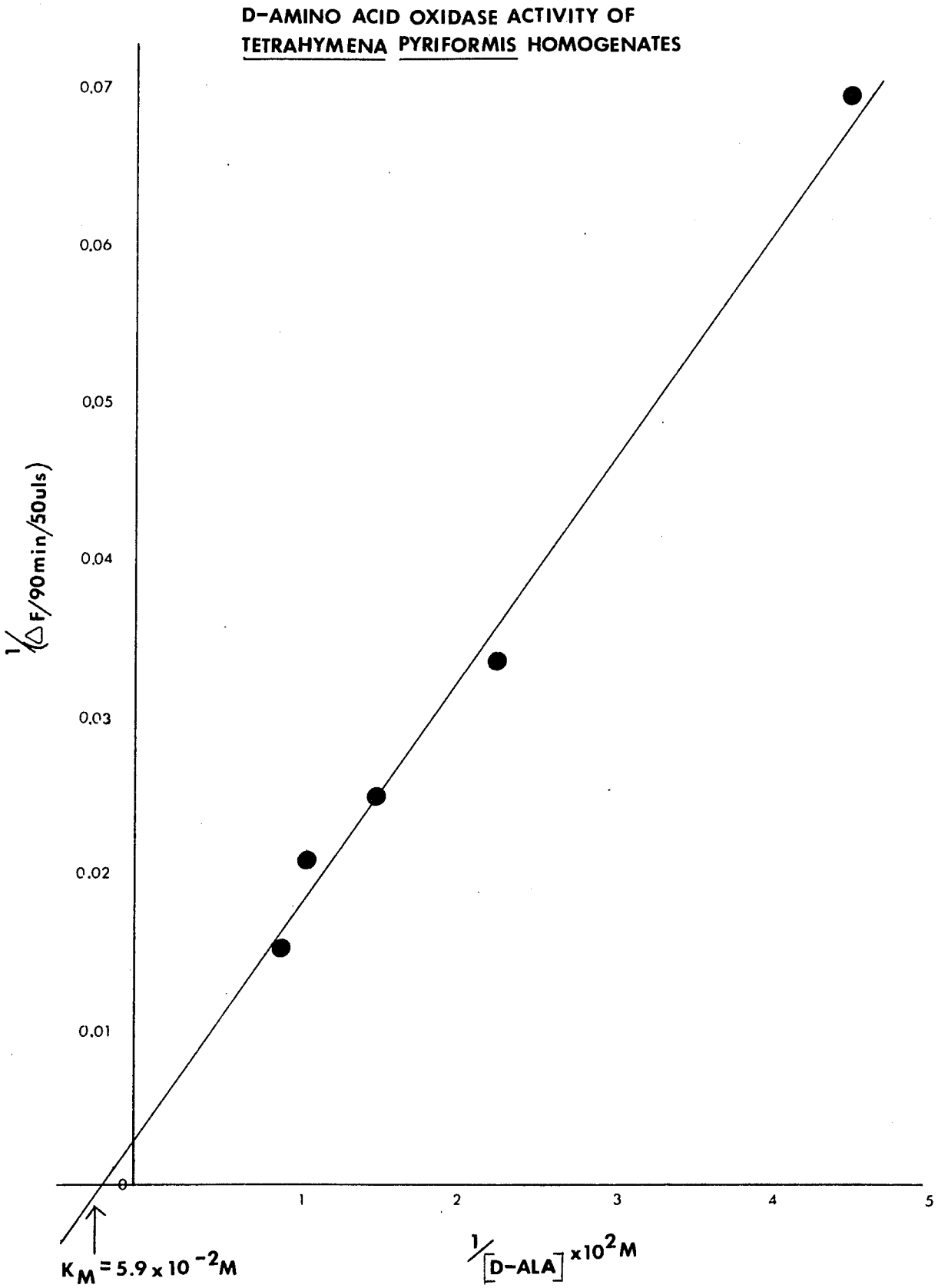

D-AMINO ACID OXIDASE ACTIVITY OF
TETRAHYMENA PYRIFORMIS HOMOGENATES
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
1/(ΔF/90min/50uls)
1
2
3
4
5
1/[D-ALA] x10^2M
K_M = 5.9 x 10^-2M

FIGURE 17C

CRUDE COMMERCIAL HOG KIDNEY D-AMINO ACID OXIDASE ACTIVITY

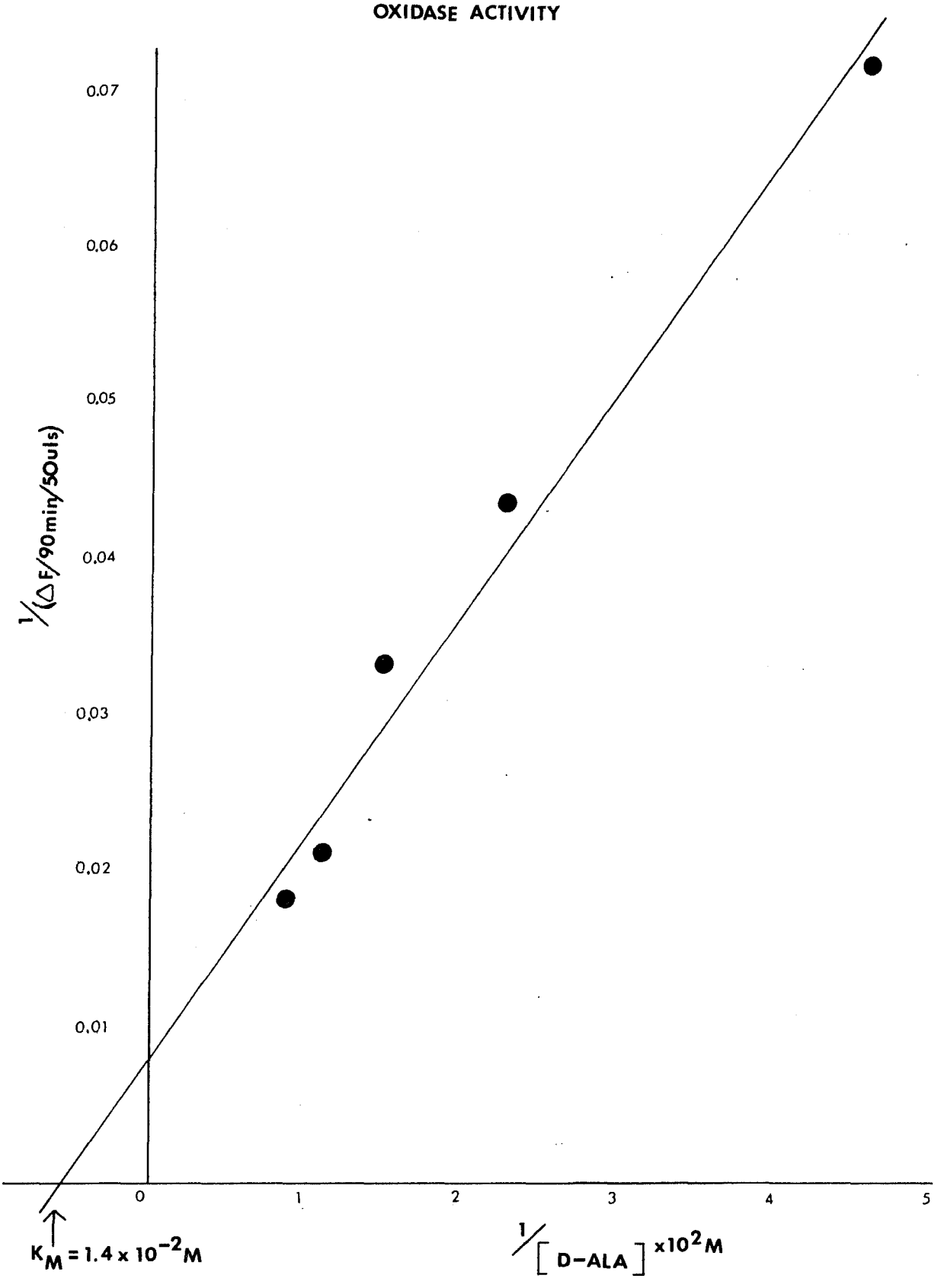
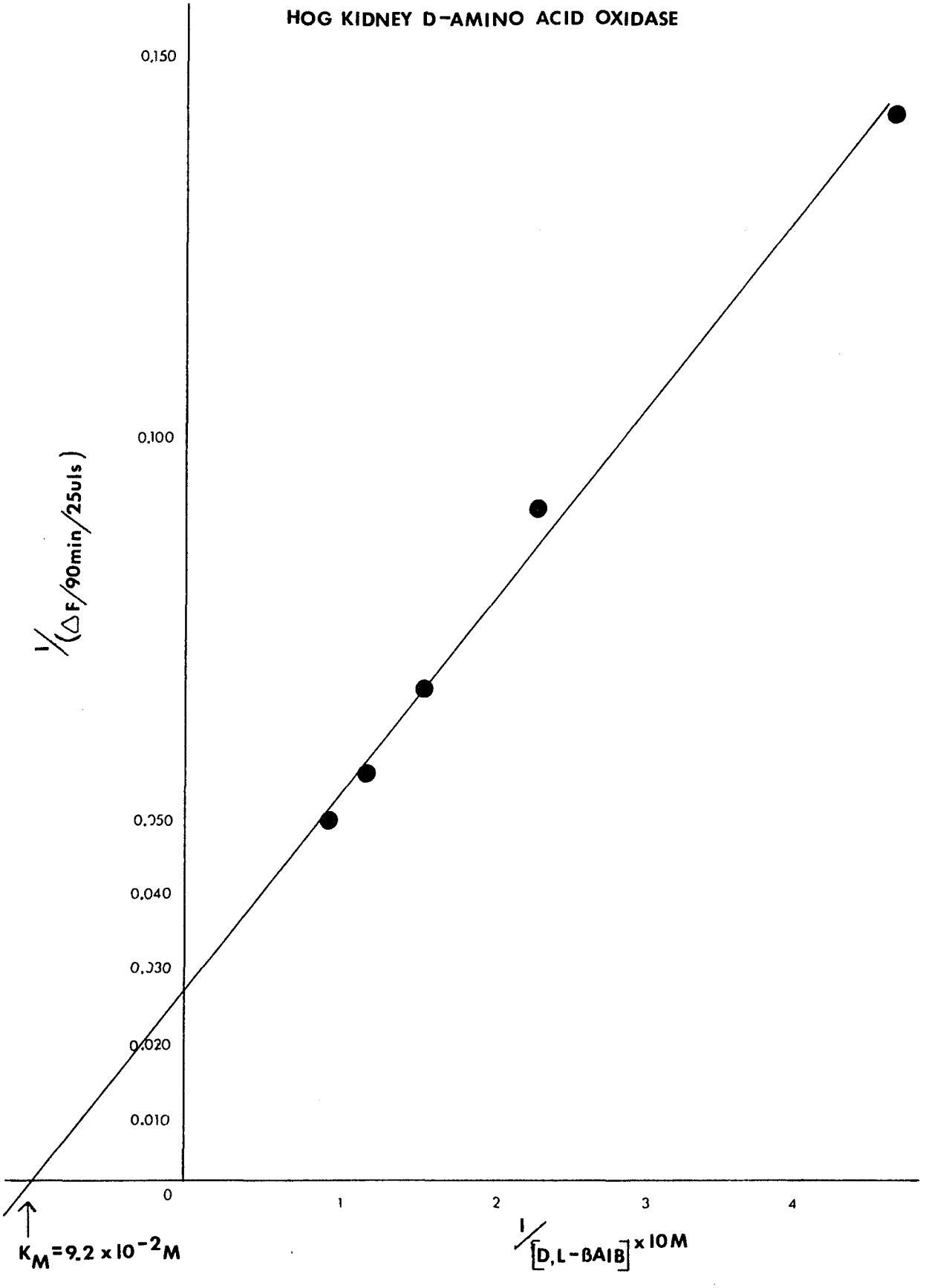


FIGURE 17D

βAIB OXIDASE ACTIVITY OF CRUDE COMMERCIAL HOG KIDNEY D-AMINO ACID OXIDASE



We also investigated the kinetics of BAIB metabolism by hog kidney D-amino acid oxidase (see Fig. 17 (D)). Such oxidase activity was suggested by the preceding substrate specificity studies (see Results Section VIB1). The K_m (9.2×10^{-2} M) of this reaction, which was even lower than that of the Tetrahymena pyriformis BAIB oxidase, indicated that perhaps one of the enzymes in the rather heterogeneous mixture of enzyme activities found in this crude commercial preparation of hog kidney D-amino acid oxidase, if not D-amino acid oxidase itself, might also be capable of oxidizing BAIB.

D. Effect of Culturing Conditions on BAIB Oxidase Activity

Ultimately we became interested in determining under what culturing conditions this enzyme might be most active so that we might be better able to speculate on its possible physiological significance in the overall metabolism of the cell. We first tried to determine if there might be any relationship between enzyme activity and culture age (see Table XIX). Interestingly enough, the total activity of this enzyme did seem to peak around 5 days of culture growth which, under these culture conditions, was about the time the cells were entering stationary phase. We also investigated the possibility that this oxidase might be inducible by culturing cells in the presence of various likely intermediates (see Table XX). Neither growing cultures in the initial presence of these additional exogenous substrates nor adding these additional exogenous substrates to cultures

TABLE XIX. BAIB OXIDASE ACTIVITY AS A FUNCTION OF CULTURE AGE.

Eight-hundred ml cultures of Tetrahymena pyriformis were inoculated and grown as described in the text (see M&M Section IA). After 24 hr 200 ml of this culture were aseptically removed (see M&M Section VIE1) and harvested (see Section IA) as described in the text. At the four subsequent 48 hr intervals indicated above, that is, after 3, 5, 7, and 9 days, an additional 100 ml of this culture were again aseptically removed (see M&M Section VIE1) and harvested (see M&M Section IA) as described in the text. The remaining culture was then harvested (see M&M Section IA) as described in the text 15 days after inoculation. After each harvesting the washed cells were homogenized in 0.1 M Tris-HCl buffer, pH 8.6, (see M&M Section IIA) and assayed for BAIB (see M&M Section IVB5) as well as D-amino acid (see M&M Section IVB3) oxidase activity as described in the text.

TABLE XIX

BAIB OXIDASE ACTIVITY AS A FUNCTION OF CULTURE AGE

CULTURE AGE (DAYS)	PROTEIN CONC (mg/ml)	ACTIVITY BAIB	ACTIVITY D-ala	SP. ACT. BAIB	SP. ACT. D-ala	TOTAL VOL (ml)	TOTAL ACTIVITY BAIB	TOTAL ACTIVITY D-ala
1	4.8	190	735	39.5	153.1	2.8	532	2058
3	5.8	75	1515	12.9	261.2	4.0	300	6060
5	14.0	220	1535	18.7	109.6	4.0	880	6140
7	10.4	135	1030	13.0	99.0	2.9	392	2987
9	8.9		580		65.2	2.7		1566
15	5.6	185	85	33.0	15.2	2.0	370	1170

TABLE XX. EFFECT OF VARIOUS PROPOSED THYMIDINE CATABOLIC INTERMEDIATES ON THE INDUCTION OF BAIB OXIDASE ACTIVITY. One-hundred ml cultures of Tetrahymena pyriformis were grown to log phase in the presence of the above indicated amounts of the proposed thymidine catabolic intermediates in 500 ml nephelometer flasks inoculated with 10 ml of the same stock culture as described in the text (see M&M Section VIE2). After harvesting the washed cells were homogenized in 0.1 M Tris-HCl buffer, pH 8.6, (see M&M Section IIA) and assayed for BAIB (see M&M Section IVB5) as well as D-amino acid (see M&M Section IVB3) oxidase activity as described in the text.

TABLE XX

EFFECT OF VARIOUS PROPOSED THYMIDINE CATABOLIC INTERMEDIATES ON THE INDUCTION OF BAIB OXIDASE ACTIVITY

ADDITIONAL EXOGENOUS SUBSTRATE	CONC (mg/ml)	% OF CONTROL SP. ACT. BAIB	SP. ACT. D-ala	TOTAL ACT. BAIB	TOTAL ACT. D-ala
DNA	0.075	75.3	108.1	77.6	111.8
	0.266	97.2	105.3	105.0	113.9
	1.097	65.6	117.2	71.3	127.2
Tdine	0.109	99.0	72.0	96.5	70.2
	0.265	91.3	96.3	85.9	90.8
	1.039	85.2	92.1	88.7	95.8
DHT	0.114	97.8	82.3	93.0	78.2
	0.249	101.7	94.5	98.2	88.2
	1.030	85.9	71.3	86.6	71.8
BAIB	0.093	93.5	101.8	80.2	87.5
	0.256	132.0	121.6	95.8	88.4
	1.006	114.2	109.6	68.8	66.1

during the last 12 hr of culture growth, however, seemed to induce BAIB oxidase activity. Whether or not this apparent lack of stimulation is significant, however, is still an open question since the possibility that this apparent lack of stimulation may merely reflect the restrictive permeability rates of these substances into the cell can not be eliminated.[88] On the basis of these data, nevertheless, this oxidase does not appear to be inducible.

DISCUSSION

I. RADIOACTIVE TRACER STUDIES

Until recently thymidine has generally been assumed to be incorporated only into DNA. Moreover, since DNA was considered a fairly stable macromolecule, not subject to constant turnover as are proteins, lipids, and carbohydrates, very little work has been done on its possible degradation and reutilization. Yet heterotrophic organisms, like Tetrahymena pyriformis, normally ingest the DNA of other organisms, and it would not be surprising, therefore, to find that they could indeed degrade and reutilize this valuable raw material, not only for the synthesis of their own nucleic acids, but also for the synthesis of other biologically useful macromolecules as well.

Most of the studies on thymine metabolism, however, have been concerned only with thymine degradation resulting in the excretion of BAIB, particularly from the point of view of human cancer research.[72,125-132] Only a few have considered the possibility of reincorporation of any of these degradation products back into other biologically useful macromolecules. Fink et al,[89] for example, first reported the recovery of radioactive glucose and alanine in the soluble fraction of mouse liver slices incubated with CH_3-^{14}C-labeled thymidine. Subsequently Bryant[133] reported the incorporation of ^3H from CH_3-^3H-labeled thymidine into mouse proteins. Piko[134] also isolated a radioactive polysaccha-

ride from mouse embryos incubated in uniformly labeled ^{14}C thymidine. Later Schneider and Greco[135] reported the recovery of substantial injected CH_3-^{14}C labeled thymidine radioactivity in rat liver lipids and proteins. Lanzetta[10] and Lanzetta and Berech[11] also recovered radioactive lipids; ala, asp, and glu from proteins; glucose from glycogen; and ribose from RNA when Tetrahymena pyriformis cultures were grown in the presence of CH_3-^{14}C-labeled thymidine. Finally, Dobson and Cooper[136] most recently reported the recovery of injected ^{14}C-labeled thymidine radioactivity in glucose isolated from mouse liver glycogen. All these results, moreover, could be most easily explained by assuming the operation of the previously proposed[10,11] thymidine catabolic and reutilization pathway (see Fig. 1) in these organisms.

Not all authors, however, agree as to the significance of the reutilization of the thymidine degradation products in accounting for the recovery of thymidine radioactivity associated with macromolecules other than DNA. Counts and Flamm,[42] for example, interpreted their detection of injected 2-^{14}C-labeled thymidine radioactivity in mouse liver glycogen as an artifact caused by the non-specific binding of thymine itself to the glycogen, rather than the result of the reutilization of any of its degradative products. Essentially this view has also been reiterated by Morley and Kingdon[137] for rat hepatocytes as well as by Goldspink and Goldberg[138] for rat diaphragm, brain, kidney, and liver.

This latter report, however, also acknowledges that labeling of at least the protein fraction probably involves both the non-specific binding of thymine or thymidine radioactivity to proteins as well as the transfer of label from thymidine into certain amino acids, which could then be incorporated into proteins.

Even Albach,[29] whose data indicated considerable incorporation of CH_3-^3H-labeled thymidine radioactivity into macromolecular fractions other than DNA, interpreted his results to conclude that there was essentially no non-DNA incorporation of radioactivity from labeled thymidine in stationary phase Tetrahymena pyriformis cultures. He attributed the activity in the acid-soluble fraction to thymidine degradation products which he presumed were excreted by the cells, and assumed the activity of the RNA fraction was due to the partial degradation and spillover of DNA into that fraction, although no chromatography was done to substantiate this conjecture. Albach's insistence that there was essentially no non-DNA incorporation of radioactivity from CH_3-^3H-labeled thymidine in Tetrahymena pyriformis, moreover, forced him to the unlikely conclusion that transmethylation from thymine accounts for the previously reported incorporation of thymine radioactivity into RNA[31] and proteins.[133] Further investigation in our laboratory,[10,11] however, revealed that the acid-soluble radioactivity was mostly due to glycogen, while the radioactivity associated with the crude RNA was mostly due to glycogen spill-

over and only a minor DNA contaminant. All of the radioactivity associated with glycogen, furthermore, was chromatographically found to be in glucose and none was due to any non-specific binding of thymine.

The preliminary radioactive tracer studies presented here thus further substantiate these earlier reports from our laboratory.[10,11] That is, once again radioactivity from ^{14}C-labeled thymidine was found in respired CO_2 (see Fig. 4) as well as in macromolecules other than DNA (see Table I). These data reinforce the hypothesis that the thymidine ring is catabolized with the subsequent liberation of at least some of the radiolabeled carbon as CO_2, while the remaining radioactive degradative fragments are reutilized for the synthesis of other non-DNA macromolecules. Chromatographic analysis of the polysaccharide fraction (see Fig. 3), moreover, indicated that the radioactivity recovered in this macromolecular fraction was clearly associated with glucose rather than dTMP or any of its closely related derivatives. In addition, the chromatographic recovery of ^{14}C radioactivity in glucose from labeled MMA, a key proposed reutilization intermediate, (see Fig. 5) also substantiated the operation of the required anabolic interconversions in Tetrahymena pyriformis. These results, therefore, further verify the operation of the previously proposed[10,11] overall thymidine catabolic and reutilization pathway in Tetrahymena pyriformis.

II. PRELIMINARY DEMONSTRATION OF THE CATABOLIC ENZYMES

Although many protozoa feed on bacteria, there have been very few studies on the metabolism of bacterial components by the protozoa. The emphasis rather has been on the metabolism of axenic cultures.[139] By using bacteria grown on radiolabeled thymine, however, it has been shown that bacterial DNA can be used as a source of protozoal DNA in Paramecium aurelia[140] and Entamoeba histolytica[141] as well as in Tetrahymena pyriformis.[142]

Exogenous pyrimidine nucleosides and free bases, however, seem to be incorporated into protozoal DNA less efficiently than whole DNA. Little incorporation of ^{14}C-thymine, for example, was detected in the nucleic acid of the ruman ciliate, Entodinium caudatum.[139] Earlier studies with Tetrahymena pyriformis,[143] which were later confirmed by previous experiments in our laboratory, indicated that thymidine was taken up 27 times more readily than thymine, but still the absolute amount of radioactive thymidine incorporated into DNA remained extremely low. What then was happening to this intracellular thymidine? Why wasn't it incorporated into DNA? How was it metabolized? Was it degraded?

Among the protozoa the existence of a reductive pyrimidine catabolic pathway has been implied in related ciliates. As mentioned previously, for example, the report of DHU as a major nucleic acid excretion product in Paramecium aurelia[74] gave impetus to our search for such a reducing en-

zyme in Tetrahymena pyriformis. Both thymine and uracil also have been reported to be quantitatively reduced to their respective DHP derivatives by Entodinium caudatum.[139]

The first direct evidence that such a reductive pyrimidine catabolic pathway may be operating in Tetrahymena pyriformis, however, came from the early metabolic studies of Heinrich et. al.[17] Recovery of $^{14}CO_2$ from cultures incubated with 2-^{14}C-uracil indicated at least a small amount of such catabolism. The actual extent of this catabolism was, however, probably greater than the recovery of $^{14}CO_2$ indicated since the residual radioactivity of the catabolic intermediates was not taken into account.[114] Unfortunately, these workers did not similarly investigate the catabolism of radiolabeled thymine. Previous studies in our laboratory,[10] however, indicated that Tetrahymena pyriformis, unlike most other eucaryotic pyrimidine catabolizing systems, degrades uracil less efficiently than thymine. These observations thus encouraged us to look for the first enzyme in the proposed reductive thymidine catabolic pathway, DHT DH, in this ciliate.

As mentioned above, most of the previous metabolic studies on pyrimidine catabolism have been primarily concerned only with degradation, usually terminating with the excretion of the resulting B-amino acid. Uracil and thymine, moreover, appear to be degraded by an analogous series of reactions, the first of which is a reduction across the 5,6-double bond.[144] The enzyme which catalyzes

this initial reduction, DHP-DH, may be the same or at least very similar[49] in many systems since the two pyrimidines have often been found to inhibit the degradation of one another in relatively pure enzyme preparations.[92,144] This as well as the other enzymes involved in the pyrimidine catabolic interconversions have already been extensively described and at least partially purified from numerous other organisms.

The first indication that a reductive pyrimidine catabolic pathway may be operating in procaryotes came from the early work of Fink et al.[75] They reported that DHT showed "growth promoting action" for a folic acid deficient Streptococcus faecalis mutant equivalent to that shown by thymine at about 1/800 the concentration. They also reported that a Pseudomonas aeruginosa mutant grown on non-nitrogenous salts, glucose, and either thymine or uracil could reductively catabolize these exogenous pyrimidines to either BAIB or B-ala, respectively. Removal of glucose from the media resulted in poor growth and barely detectable reduction, but addition of either DHP or B-amino acid supported rapid growth even without glucose. This suggested that not only could these reductive pyrimidine catabolites be used as carbon sources, but also that the first step in this degradative pathway, that is, the reduction of the pyrimidine to its respective DHP derivative, was probably the rate limiting one.

Slotnick and Weinfeld[145] also isolated several "pyrim-

idineless" mutants of Escherichia coli which required DHU or one of its subsequent degradative intermediates (BUPA or B-ala) to restore normal growth. The poor incorporation of DHU radioactivity into nucleic acid pyrimidines of these mutants compared with that of uracil radioactivity in uracil-requiring mutants indicated that although DHU supports the growth of these mutants, this activity is not directly related to nucleic acid biosynthesis. The resulting B-ala might, as was shown by Hayaishi et al[146] in Pseudomonas fluorences, be required for acetyl CoA synthesis.

Another such uracil-requiring mutant, Clostridium uracilicum, has been isolated and even more extensively studied by Campbell.[76-80] In this mutant uracil was found to be quantitatively converted to B-ala, CO_2, and NH_4.[76] DHU and BUPA were identified as intermediates.[77] The enzyme catalyzing the conversion of uracil to DHU, DHU-DH, was also eventually partially purified and characterized from these bacteria.[49,78]

Evidence that such a reductive pyrimidine catabolic pathway is operating in eucaryotes initially came from studies on mammalian systems. BAIB, for example, was first isolated, characterized, and identified in human urine by Crumpler et al.[147] Fink et al[83] also simultaneously reported its occurrence in the urine of humans as well as in rats fed large amounts of DNA. Furthermore, of the variety of pyrimidines and pyrimidine derivatives tested for their ability to cause normal rats to excrete BAIB, only thymine,

thymine-containing DNA, and DHT resulted in detectable amounts of this amino acid.

The enzyme catalyzing the first step in this pathway, that is, the conversion of thymine to DHT, moreover, has been partially purified and characterized from aqueous extracts of beef liver acetone powders[49,92] as well as from human and pig leukocytes.[148] The analogous uracil catabolizing enzyme, DHU-DH, has also been partially purified from the soluble cytoplasmic fraction (centrifuged 100,000 x g for 60 min) of rat liver homogenates.[98]

When BAIB and B-ala were identified as predominant free amino acids in several species of parasitic flatworms it was suggested that they too might arise from thymine and uracil, respectively, via the previously described reductive pyrimidine degradation pathway. Radiolabeled study of the rat tapeworm, Hymenolepis diminuta, indicated that this was indeed the case. That is, the degradation of 2-^{14}C uracil in this organism was found to produce not only radioactive DHU, BUPA, and CO_2, but also radioactive B-ala and an organic acid which was chromatographically identical with succinic acid as well as some other unidentified compounds.[106]

Finally, evidence that such a reductive pyrimidine catabolic pathway is operating in plants has also been presented. BAIB, for example, was isolated from iris bulbs.[107] Subsequently it was shown with ^{3}H-thymine that this BAIB did indeed originate from thymine.[109] Evans and Axelrod[108] also showed that 2-^{14}C-thymine as well as 2-^{14}C-uracil could

be reductively catabolized by germinating rape seedlings to the corresponding DHPs, DHT and DHU, which were in turn hydrolyzed to give BUIB and BUPA, respectively. By analogy with the previously described reactions observed in microorganisms and animal tissues, it was expected that the corresponding B-amino acids, BAIB and B-ala, would be formed. This assumption could not be substantiated in these experiments, however, since the labeled carbon was lost as $^{14}CO_2$ following decarbamylation. Later radiolabeled experiments,[112] using ^{3}H-thymine and 5,6-^{3}H-uracil, however, confirmed this expectation. Previously undescribed labeled substances were also detected with these radiolabeled substrates. This was interpreted as implying that the resulting B-amino acids were further metabolized to various organic acids. A few of the uracil-derived carboxylic acids were identified as malic, pyruvic, and citric acids.[112] Such reductive pyrimidine catabolic pathways have also been reported operating in pine tissues[110] and Chlorella fusca.[114]

In most respects, the DHT-DH preliminarily demonstrated here in Tetrahymena pyriformis is similar to the DHP-DHs described in the above eucaryotic systems. That is, it requires NADPH,[49,92,98,148] not NADH[49,78] as the procaryotic enzyme apparently does; it is active in phosphate buffer at pH 7.3;[49,78,92,148] preparations show considerable blank activity;[98] and its activity is difficult to demonstrate in crude homogenates.[94,149,150] Unlike the previously described DHP-DHs,[49,78,92,98,148] however, this activity in

Tetrahymena pyriformis appears to be particle-bound rather than soluble since its supernatant activity decreases with increasing centrifugation (see Table II). It is possible that this enzyme may also be present in a subcellular organelle in these other organisms, but could not be detected because of some inhibiting factor also associated with that subcellular fraction.[50] This inhibiting factor may be absent or easily removed by low speed centrifugation in Tetrahymena pyriformis. Alternatively, this enzyme may also be particle bound in these other organisms, but the procedures may have solubilized this activity during preparation. But whatever its subcellular localization, an enzyme capable of catalyzing at least the initial reduction of the proposed pyrimidine catabolic pathway (see Fig. 1), nevertheless, does seem to be also present in this organism.

The remaining enzymes involved in this reductive pyrimidine catabolic pathway have also been described and partially purified from various other organisms and tissues. DHP hydrase, the enzyme that catalyzes the cleavage of the DHP ring (see Fig. 1), for example, has been partially purified from Clostridium uracilicum,[79] aqueous extracts of beef liver acetone powders,[90,93,151] and rat liver.[99] The enzyme catalyzing the decarbamylation of the B-ureido amino acids (see Fig. 1) has also been detected in pigeon, dog, and rabbit liver homogenates. It has, furthermore, been partially purified from rat liver.[90,91,152] In addition, an enzyme capable of catalyzing the decarbamylation of B-

ureidopropionic acid has been partially purified from cell extracts of Clostridium uracilicum.[80]

The presence of the remaining enzymes of this reductive pyrimidine catabolic pathway, however, has not as yet been definitely established in Tetrahymena pyriformis. The recovery of thymidine radioactivity in biologically useful macromolecules other than DNA, however, suggests that this organism is indeed capable of the catabolism and reutilization of at least this pyrimidine derivative. The previously reported[10,11] recovery of thymidine radioactivity in the chromatographically identified end product of this reductive catabolic pathway, BAIB, also supports the assumption that Tetrahymena pyriformis is indeed at least able to reductively catabolize this pyrimidine. The demonstration of the first enzyme in the proposed thymidine reductive catabolic pathway, DHT-DH, further supports this assumption. Presuming that this organism is capable of catalyzing the required intermediate reductive catabolic interconversions, therefore, we decided to investigate how the reductive thymidine degradative end product, BAIB, might be further metabolized to account for the recovery of thymidine radioactivity in biologically useful macromolecules other than DNA.

Since its initial isolation, characterization, and identification by Crumpler et al[147] and Fink et al,[83] BAIB excretion has been reported in association with certain neoplastic diseases, particularly leukemia,[72,81,82,125-128] as well as with tuberculosis,[153] march hemoglobinuria,[154] liv-

er disease,[155] lead poisoning,[156] mongolian idiocy and other types of mental defects,[157,158] epilepsy,[159] and following heavy irradiation with neutrons and γ-rays.[160] It has also been reported in such diverse organisms as cat tissues,[161] iris bulb,[107] and the mollusk, Mytilus edulis.[103] A dipeptide containing this B-amino acid has also been isolated from bovine brain (γ-L-glutamyl-L-BAIB)[162] and iris bulbs (γ-L-glutamyl-D-BAIB).[163]

Fink et al[81,82,87] showed that BAIB is produced from thymine in rats. Gartler[72] demonstrated that this is also the case in humans. There was at that time, however, virtually no information concerning the quantitative importance of the degradative steps in this catabolic pathway nor for that matter was it known which direction predominated in these frequently reversible reactions.[164]

Kupiecki and Coon[51] reported that BAIB could also result from the further metabolism of B-hydroxyisobutyric acid during val catabolism. Other evidence for the formation of BAIB from val, however, has been inconclusive. Gartler[72] found no significant increase in urinary BAIB after the administration of val to human excretors and non-excretors. Armstrong et al,[165] on the other hand, fed larger amounts of val to excretor and non-excretor humans and found a marked increase in urinary BAIB only in the excretor. When they fed still larger doses of L-val to rats, however, there was no detection by paper chromatography of an increase in urinary BAIB.[166]

In most healthy persons BAIB usually is excreted only in small amounts in the urine (low excretors). Interest in BAIB itself, however, was generated by the discovery that a small proportion of otherwise normal people constantly excrete abnormally large quantities of this substance.[147] Between 6-10% of Caucasian and up to 40% of Oriental populations, for example, were found to excrete large amounts of the B-amino acid (high excretors).[167] The trait also seemed to be inherited as a simple single recessive Mendelian allele.[167] No evidence was obtained, however, that this inherited excretion is deleterious.[164]

Increased excretion could be caused either by increased formation of BAIB from its precursors or a decreased capacity to metabolize the B-amino acid. In considering the first of these possibilities, however, it was necessary to decide which of the two alternate sources of BAIB--from thymine or from val--is quantitatively more important.[164] This information was provided by the studies of Gartler[72] and Armstrong et al[165] who showed that the amount of BAIB produced from val in man is very small, only about 10%, as compared with that originating from thymine. Thus, the sequence of reactions in thymine catabolism leading to the ultimate production of BAIB and then the further metabolism of this B-amino acid did seem like a quantitatively significant pathway for further study.

BAIB isolated from the urine of high excretors was found to be the D(-)isomer.[68,70,162] Early studies also

provided evidence for the occurrence of D(-)BAIB in normal liver.[68] The metabolism of both D(-)- and L(+)BAIB was subsequently investigated by Armstrong et al[165] and they concluded that the high excretion was a result of a defect in the metabolism of D(-)BAIB. Further studies finally confirmed that the D(-)BAIB isomer was also the one formed from thymine in human liver.[68,70]

Studies were also carried out in order to determine the degree of conversion of thymine into BAIB. The results indicated that humans convert thymine into BAIB quite efficiently as compared with other animals. That is, the extent of this conversion in humans has been reported to range from 20%[127] to 50-75%[72] on a mole/mole basis. Such an extensive conversion rate, thus lends considerable support to the importance of the thymine $\rightarrow$BAIB pathway as a major one in pyrimidine catabolism.[72] The extent of this conversion in the rat, on the other hand, was calculated to be only from 0.5-2% for thymine and 3-17% for DHT.[83] With either DHT or BUIB as substrate, however, others have reported the conversion rate to be over 30%.[72,87] Similar experiments with mouse liver showed less than 2% conversion of thymine to BAIB, 35% conversion of DHT to BAIB, and 42% conversion of BUIB to BAIB.[72] Such a wide variation in the conversion rates of these thymine catabolic intermediates may indicate that this pyrimidine is metabolized at least slightly differently in each of the various animal species.

Knowledge of the further metabolic fate of BAIB, how-

ever, remains limited.[166] In the catabolism of uracil the oxidation of β-ala appears to be the rate-limiting step.[97] In the catabolism of thymine, therefore, the oxidation of BAIB may similarly be rate-limiting. Nevertheless, after injecting D,L-BAIB into normal rats the amount of BAIB recovered in the urine ranged from 5-30% and averaged about 15% of the injected dose, thus indicating that a considerable proportion of this β-amino acid is catabolized.[83] One would expect the further metabolism of BAIB, moreover, to produce MMA via methylmalonic semialdehyde.[70,166]

As mentioned above, most of the studies on the metabolism of BAIB suggest its further metabolism via transamination. One of the first such identified enzymes was the BAIB:αKG transaminase described by Kupiecki and Coon.[51] This enzyme was found to be most active in extracts of pig and rat kidney. It was also found in pig brain and liver as well as rabbit skeletal muscle. It was, moreover, partially purified from the supernatant of centrifuged (15 min at 13,000 RPM) crude pig kidney homogenate. It was not, however, detected in extracts of pig heart, Ehrlich ascites tumor, spinach leaf, or various microorganisms including Pseudomonas fluorescens, Neurospora crassa, Baker's yeast, and Tetrahymena pyriformis.

An additional question arising from the investigation of Kupiecki and Coon[51] was which optical isomer of BAIB was the actual substrate. As mentioned above, it was the D(-) isomer which had previously been isolated from the urine of

high excretors,[68,70] and had been shown to be the end product of thymine catabolism.[68] Further experiments[165] showed the D(-)BAIB was quantitatively recovered in the urine of excretors while the recovery of this optical isomer was lower in non-excretors. L(+)BAIB was catabolized in high excretors as rapidly as in low excretors. This finding was interpreted as indicating that D(-)BAIB and L(+)BAIB may be metabolized by different enzymes.[168]

In an effort to determine whether the D(-)- and L(+)-isomers were indeed oxidized by different enzymes, the BAIB:αKG transaminase described by Kupiecki and Coon[51] was further purified and characterized from hog kidney.[168] It was found that this purified enzyme catalyzed the transamination with the L(+)BAIB, while the D(-)BAIB, the natural isomer, was particularly inactive as the substrate. That is, activity for D(-)BAIB was less than 1% of that of L(+)BAIB.

An additional enzyme, BAIB:pyruvate aminotransferase, was eventually shown to be responsible for the further metabolism of D(-)BAIB in rat, guinea pig, hog, and human liver.[70] This enzyme was subsequently purified from supernatants of crude hog[70] and human[169] liver homogenates centrifuged for 20 min at 12,000 x g (mitochondrial supernatant fraction). The major differences between this enzyme and the previously described BAIB:αKG aminotransferase, moreover, appeared to be: (1) SUBSTRATE SPECIFICITY--The former enzyme was most active with D(-)BAIB and somewhat less active with B-ala. It showed almost no activity with

L(+)BAIB. It was also inactive with OAA. The products of the reaction with D(-)BAIB were shown to be L-ala and methylmalonic semialdehyde. The latter enzyme, on the other hand, was most active with L(+)BAIB and almost as active with B-ala. It showed almost no activity with D(-)BAIB. It was also inactive with pyruvate. (2) DISTRIBUTION--The former enzyme was localized almost exclusively in liver with a little activity also detected in kidney, while the latter enzyme had been reported present in liver and kidney as well as brain and some other tissues. (3) PROPERTIES DURING PURIFICATION--The former enzyme was precipitated by a lower concentration of $(NH_4)_2SO_4$ and was well adsorbed to DEAE Sephadex during purification, while the latter enzyme was not.[70]

Enzymes capable of transaminating B-ala with αKG have previously been demonstrated in rat liver and brain tissues.[95] Other mammalian tissues capable of transaminating B-ala with pyruvate have also been described.[146] In addition, Goldfine and Stadtman[170] have shown that B-ala may be further oxidized by transaminase action with either αKG or pyruvate in Clostridium propionicium. An aminotransferase which catalyzes the transamination between ω-amino acids and pyruvate has also been described by Hayaishi et al[146] in a B-ala mutant of Pseudomonas fluorescens. OAA, however, has been reported not to support the B-ala aminotransferases of Pseudomonas fluorescens,[146] beef brain,[171] or hog kidney.[51] Hatch and Stumpf,[172] observing CO_2 and B-

ala formation from ^{14}C-labeled propionate first suggested the existence of a B-ala transaminating system in plants. A B-ala:αKG aminotransferase has since been described in rape seedlings.[112] Pyruvate and OAA, however, were not active amino acceptors in this system. A B-ala aminotransferase has subsequently been identified in a subcellular particulate fraction from bean cotyledons.[173] The enzyme or enzymes involved in this system utilized pyruvate or OAA, but not αKG as an amino acceptor.

We therefore began our study of the further metabolism of BAIB with an investigation of these previously described transaminating systems. A few such aminotransferases have already been described and localized in Tetrahymena pyriformis.[174] Ala:αKG aminotransferase, for example, has been demonstrated and shown to have a distribution completely identical to that of MDH, indicating that it too is a mitochondrial enzyme. On the other hand, asp:αKG aminotransferase has also been demonstrated and shown, both by isopycnic and differential centrifugation, to have a subcellular distribution that does not correspond exactly to that of MDH. In fractions rich in peroxisomes, as shown by the distribution of catalase, an excess of this aminotransferase activity was also found. This observation indicated that a small, but significant, part of this aminotransferase activity may be localized in the peroxisomes. Such aminotransferase activity, however, could not be detected in rat liver peroxisomes.[174]

In confirmation of the earlier work of Kupiecki and Coon,[51] we too were unable to demonstrate BAIB:αKG aminotransferase activity in crude Tetrahymena pyriformis enzyme preparations (see Table III). In view of the recent finding that BAIB:pyruvate aminotransferase is more likely to be responsible for the further metabolism of the D(-)BAIB resulting from thymine catabolism than BAIB:αKG aminotransferase, however, this result is not surprising. Furthermore, although the BAIB:pyruvate aminotransferase system was not directly studied, preliminary data indicated that this transaminating system alone could not account for all the observed BAIB metabolism (see Table III). It should be pointed out, however, that of necessity amino acid and αketo acid identification and quantitation was done by paper chromatography, in spite of the relatively large error inherent in this technique.[164] This error, moreover, is really larger than is desirable for quantitative work.[127] Tests with standard amounts of BAIB indicated that the data had an accuracy of about ±20%, but owing to the frequent distortion or overlapping of colored spots on chromatograms, routine analyses could be considered as potentially in error by a factor of two or more, particularly when the density of the BAIB spot was low as compared with surrounding spots. BAIB could not be detected regularly at concentrations below 0.15 mM, and, although several of the α-amino acids could be detected at about half this concentration (due to their more intense color reaction with ninhydrin), some of these

were also more subject to interference.[83] For comparison, as well as a check on the sensitivity of the assay system, therefore, the same enzyme activity was assayed for in mouse liver where it was expected to occur.[70] In this system the amount of BAIB metabolized was roughly equivalent to the amount of pyruvate metabolized (see Table III). This result supported the reasonable reliability of the assay system. Thus it appeared that an enzyme other than the previously reported transaminases might be responsible for the further metabolism of BAIB in Tetrahymena pyriformis.

Indeed, it appeared that the enzyme responsible for the further metabolism of the thymidine catabolite, BAIB, might be an oxidase rather than a transaminase. Several other such oxidases have already been described and characterized in Tetrahymena pyriformis. These include D-amino acid oxidase,[174,175] L-α-hydroxy acid (lac) oxidase,[57,174-178] and glyoxylate oxidase.[57,174] All of these oxidases, moreover, have been reported to be associated with the peroxisome.

III. PROPERTIES OF BAIB OXIDASE ACTIVITY

An enzyme, tentatively called BAIB oxidase, has been demonstrated and characterized in Tetrahymena pyriformis homogenates. It appears, unlike D-amino acid oxidase, to be relatively stable during storage (see Table XIII). It also has been found, under conditions in which the distribution of other marker enzymes indicates minimal subcellular organelle damage, to exhibit, like D-amino acid oxidase, a

bimodal intracellular distribution (see Figs. 9 & 13). That is, about half its activity appears to be particle-bound, while the remaining half seems to be soluble. Unlike D-amino acid oxidase, however, its particle-bound activity seems to be associated with mitochondria rather than peroxisomes. Detergents (see Table XI) and freeze-thawing (see Table XII), nevertheless, seem to have little effect on increasing the activity of either this oxidase or D-amino acid oxidase prepared in sucrose.

The bulk of BAIB oxidase activity is precipitated, like D-amino acid oxidase activity, between 10-60% saturation with $(NH_4)_2SO_4$ (see Table XVII). This oxidase activity, however, can be separated from D-amino acid oxidase activity as well as from that of the other marker enzymes by precipitation between 0-40% saturation with $(NH_4)_2SO_4$, although a substantial amount of its remaining activity is precipitated with the bulk of the D-amino acid oxidase activity between 40-60% saturation with $(NH_4)_2SO_4$ (see Table XVII). This is also the $(NH_4)_2SO_4$ concentration reported to precipitate the analogous mammalian BAIB:pyruvate aminotransferase activity.[70,169]

BAIB oxidase shows good activity in alkaline 0.2 M Tris-HCl buffer (see Table V). It shows higher activity in alkaline 0.1 M pyrophosphate buffer (see Table V). The stimulatory effect on this oxidase activity by pyrophosphate buffers, in light of additional data such as the inhibition of this oxidase activity by divalent cations (see Table

VIII), might be attributed to the divalent chelating ability of this buffer. We, however, decided not to use the highly alkaline pyrophosphate buffers in this assay since, as mentioned before, highly alkaline buffers have been reported to catalyze the non-enzymatic decomposition of H_2O_2 [115,116] and contaminating D-amino acid oxidase activity also appeared to be stimulated by pyrophosphate buffers. As previously reported for D-amino acid (ala) oxidase[124] as well as BAIB:αKG[168] and BAIB:pyruvate[70,169] aminotransferases, BAIB oxidase also seems to have an alkaline pH optimum (see Fig. 7), although its broad double peaked pH profile seems unique.

That this enzyme is a true oxidase is indicated by its stimulation upon the addition of the common oxidase coenzyme, FAD (see Table VII). Its detectable activity, like that reported for D-amino acid oxidase,[179] also seems to be considerably reduced when coupled with an electron accepting dye, such as 2,6-dichlorophenolindophenol, rather than when measured in the presence of O_2 by H_2O_2 formation.

Unlike the rather innocuous effect on most of the previously described enzymes in the reductive thymidine catabolic pathway,[49,92,93,148] exogenous divalent cations, such as Mg^{+2} and Ca^{+2} seem to inhibit both BAIB and, to a somewhat lesser extent, D-amino acid oxidase activity (see Table VIII). The concentrations used in these experiments, however, were fairly high in comparison to those previously reported.[49,92]

In light of the above mentioned data, that is, the stimulatory effect of pyrophosphate buffer (see Table V) as well as the inhibitory effect of the indicated divalent cations (see Table VIII), it was thought that the commonly used chelating agent, EDTA, might stimulate oxidase activity. Over the wide concentration range tested, however, EDTA seemed to inhibit, rather than stimulate, oxidase activity (see Table IX). It is, of course, possible that this chelating agent was preferentially complexing with an as yet undetermined essential divalent cation. Alternatively, however, the strong inhibition of BAIB oxidase activity and, to a considerably lesser extent, of D-amino acid oxidase activity by EDTA in light of additional data, such as the inhibition of these enzyme activities by some detergents (see Table XI), might be attributed to the possible membrane-disrupting effects of this chelating reagent.

PCMB, a SH blocking reagent, was also found to be inhibitory to both BAIB and D-amino acid oxidase activity (see Table X). This result was expected for D-amino acid oxidase activity since it had been previously reported[179] that this enzyme contains a reactive SH group at its active site. BAIB oxidase inhibition, however, seems to be less concentration dependent than that of D-amino acid oxidase. This may indicate that there is no SH group at the active site of this enzyme, but rather PCMB inhibits by reacting with distal SH groups, thus distorting the active conformation of this oxidase and thereby decreasing its catalytic effici-

ency. At PCMB concentrations around that previously reported[51] to completely inhibit hog kidney BAIB:αKG transaminase (1.5×10^{-5} M), moreover, BAIB oxidase still retains a substantial amount of its activity.

IV. SUBCELLULAR LOCALIZATION OF BAIB OXIDASE ACTIVITY

After characterizing this enzyme capable of the further metabolism of BAIB in crude Tetrahymena pyriformis extracts, we became interested in its subcellular localization, since such an oxidase would produce the highly toxic substance, H_2O_2, as a reaction product. Tetrahymena pyriformis, along with a few other eucaryotic organisms and tissues, however, have a special subcellular organelle, the peroxisome, which contains the enzyme catalase that can catalyze the rapid decomposition of H_2O_2. It was initially thought, therefore, that BAIB oxidase activity might be localized within this subcellular organelle.

Several other oxidases, such as D-amino acid oxidase,[174,175] L-α-hydroxy acid (lac) oxidase,[57,174-178] and glyoxylate oxidase,[57,174] have already been found associated with Tetrahymena pyriformis peroxisomes. Preliminary experiments also indicated that both of the demonstrated thymidine catabolic enzymes seem to have certain organelle-associated properties. BAIB oxidase activity, for example, was found to be slightly stimulated by Triton X-100 (see Table XI) as well as upon storage (see Table XIII), suggesting latency. In addition, low speed supernatants, similar to that

of crude peroxisomal preparations,[180] appeared to contain thymine reductase (DH) as well as BAIB oxidase activity. The well-known, long established association of purine catabolic enzymes, such as xanthine oxidase and urate oxidase, with the peroxisomes of many organisms, although not Tetrahymena pyriformis,[174] also suggested a possible role for this organelle in nucleic acid catabolism. Finally, the remarkable correspondence between the organisms and tissues for which peroxisomes have been reported and those in which these reductive thymidine catabolic enzymes have been demonstrated further hinted at a possible relationship.

Preliminary centrifugation data did indicate that considerable BAIB oxidase as well as D-amino acid oxidase activity was associated with the high speed pellet (see Table XIV). Subsequent differential centrifugation studies further indicated that these particle-bound enzyme activities are apparently associated with the mitochondrial-peroxisomal pellet (see Fig. 9). Recovery of considerable amounts of these activities in the intermediate and high speed supernatants (see Fig. 9), however, suggested that these enzymes might not be localized only within a subcellular organelle, but might also be found soluble in the cytoplasm. This result would be in keeping with the subcellular localization of the previously described reductive thymidine catabolic and reutilization enzymes, but was somewhat surprising for D-amino acid oxidase since this enzyme is supposed to be a peroxisomal marker enzyme[175] and, therefore, was expected

to pellet with the other marker enzymes, that is, catalase and L-α-hydroxy acid (lac) oxidase, for this subcellular organelle.

Since D-amino acid oxidase has already been established as a peroxisomal enzyme in Tetrahymena pyriformis,[175] it was initially thought that its soluble activity might indicate that the subcellular organelles were damaged during fractionation. The low enzyme activities of the other marker enzymes recovered in the intermediate and high speed supernatants (see Fig. 9), however, indicated that the subcellular organelles had apparently not been excessively damaged by these procedures. This assumption was also supported by EM observation of the intermediate speed pellet (see Fig. 11) which further substantiated the structural integrity of the majority of the component subcellular organelles. Nevertheless, whether cell homogenates were simply centrifuged into high speed soluble and particulate fractions (see Table XIV), differentially centrifuged (see Fig. 9), or fractionated through a continuous sucrose density gradient by zonal centrifugation (see Fig. 13), a similar subcellular distribution pattern was consistently observed. That is, about half of the activity was associated with the appropriate organelle fraction, while the other half was recovered in the soluble fraction. It was thus envisioned that this apparent bimodal subcellular distribution of these two oxidase activities might reflect: (1) the partial solubilization of these enzyme activities during preparation;

(2) the existence of two distinct oxidases, each with a different subcellular localization; or (3) the adsorption of soluble enzymes to subcellular organelles during processing.

Both BAIB and D-amino acid oxidase, for example, might indeed be exclusively associated with a particular subcellular organelle *in vivo*, but may not be confined or bound as strongly as the marker enzymes, MDH and catalase, seem to be within the mitochondria and peroxisomes, respectively. During experimental processing, such as homogenization and/or centrifugation, at least some of these externally located oxidases might then be dissociated from their respective subcellular organelle without inducing the actual disruption of the organelle. The inhibition of these two oxidase activities by membrane-disrupting detergents such as SDS and DOC (see Table XI) as well as the lack of stimulation of these two oxidase activities by repeated freeze-thawing (see Table XII) might also be interpreted as indicating that the organelle associated activity of these oxidases is localized within the limiting membrane and not soluble in the subcellular organelle matrix.[181]

Another possible explanation for the bimodal distribution of BAIB oxidase activity could be the presence of two distinct cellular enzymes, each with a different subcellular localization but both capable of oxidizing this substrate. There might, for example, be a non-specific oxidase in the cytoplasm capable of oxidizing both D-ala and BAIB as well as a more specific BAIB oxidase associated

with the mitochondrion. The existence of two different BAIB oxidases, moreover, might help to explain the persistent double peaked pH profile observed for this enzyme activity.

Finally, it might also be argued from the data that both BAIB oxidase as well as D-amino acid oxidase activities are not associated with any subcellular organelle, but rather are soluble enzymes adsorbed on to subcellular organelles as an artifact of preparation. This argument is unconvincing in the case of D-amino acid oxidase since its peroxisomal localization has already been well established in Tetrahymena pyriformis[175] and it is unlikely that the small amount of cellular protein recovered at peroxisomal densities upon zonal centrifugation would be able to non-specifically adsorb much in the way of extraneous soluble enzyme activities. For mitochondrial associated enzymes, however, this non-specific adsorption argument is more tenable since a larger amount of cellular protein is recovered at these densities upon zonal centrifugation and thus the indiscriminant adsorption of soluble enzyme activities by this subcellular fraction is conceivable. This explanation for the bimodal distribution of BAIB and D-amino acid oxidase activities, however, does not seem too probable because when heterologous D-amino acid oxidase is added to cell homogenates which are then fractionated by differential centrifugation, none of this additional exogenous oxidase activity is recovered in the pellets, rather most of it re-

mains in the supernatants (see Table XV). Thus, the bimodal centrifugal distribution of this enzyme activity, whatever its origin, appears to be an accurate reflection of its actual subcellular localization.

V. PARTIAL PURIFICATION OF BAIB OXIDASE ACTIVITY

Our studies on BAIB oxidase initially indicated a number of similarities between this enzyme and D-amino acid oxidase. Both of these oxidases, for example, showed good activity in pyrophosphate buffer (see Table V) and had an alkaline pH optimum (see Fig. 7). They also were found to be stimulated by the addition of exogenous FAD (see Table VII). They were both inhibited by the addition of exogenous divalent cations (see Table VIII), EDTA (see Table IX), PCMB (see Table X), and some detergents (see Table XI). They also both failed to show any significant increase in activity upon repeated freeze-thawing. Each of these observations by itself was not considered particularly remarkable since both these oxidases probably share these properties in common with many other unrelated enzymes. Moreover, although the qualitative responses of these two oxidases are similar, their quantitative responses to many of these reagents is quite different. Whether these differences are a result of the activities of two completely different enzymes or merely the alternate expression of a single enzyme utilizing two structurally similar substrates thus remained to be determined. The consistently similar bimodal distri-

bution of these two oxidases upon differential centrifugation, which was quite distinct from the distribution of the other standard subcellular organelle marker enzymes, ultimately suggested that these two activities might indeed be catalyzed by the same enzyme. This identity was also supported by the similar behavior of these two oxidase activities upon initial $(NH_4)_2SO_4$ precipitation (see Table XVII). The high K_m of this oxidase in crude homogenates also indicated that perhaps BAIB was not the preferred substrate of this enzyme. Finally, when a crude commercial preparation of hog kidney D-amino acid oxidase was found to oxidize BAIB at about the same efficiency as Tetrahymena pyriformis homogenates (compared to D-ala activity), we decided that this possibility should be investigated in more detail.

Since the αC of BAIB is asymmetric, the question of optical isomerism must be considered. Unfortunately in most studies on the metabolism of BAIB this has largely been ignored.[164] Complicating the picture, moreover, is the observation by Fink et al[83] that only 15% of a racemic mixture of BAIB injected intraperitoneally into rats is recovered in the urine. This would suggest that both forms can be metabolized. Only the D(-)BAIB isomer,[147,164] however, has been isolated from human urine. The BAIB resulting from thymine catabolism has also been characterized as the D(-) form.[68-72] In fact, it has been proposed that isomer competition might indeed be why the reductive thymidine catabolic reactions are detected as so slow when DHT

or BUIB are used as substrates instead of thymine in humans. That is, the unnatural isomers present in the racemic mixtures of exogenous DHT and BUIB might not be converted to D(-)BAIB as efficiently as the natural ones, but when thymine is reduced only D-DHT, the natural isomer, would be produced and readily converted to D(-)BAIB. The commercial preparation of BAIB used throughout these experiments was a mixture of both the D and L isomers. We therefore initially decided to try to determine if Tetrahymena pyriformis did indeed preferentially oxidize the D(-) rather than the L(+) form of BAIB.

Preliminary results (see Fig. 15) seemed to indicate that D(-)BAIB was the preferred substrate for this oxidase activity. Unfortunately, however, this interpretation was later complicated by the discovery that the commercial enzyme preparations used in these experiments were also contaminated with various other oxidase activities.

With regard to the specificity of purified D-amino acid oxidase, few D-amino acids have been reported to fail to react, although the enzyme is strictly D specific. The active center, moreover, seems to have a strong affinity, apart from the essential COOH group, for an alkyl group of moderate chain length. Little contribution to the affinity, however, is apparently made by the part of the substrate molecule that reacts, namely the NH_3 group. The active site, therefore, is thought to contain a positively charged group which combines with the COOH group in the substrate at one

end and a hydrophobic site, binding the alkyl group (up to 4 Cs in length), at the other. The ∝C of the substrate is then thought to lie between, probably with its two C-C bonds parallel with the enzyme surface, so that the NH_3 group is directed upwards and the Hs are directed towards the flavin group which lies at the side of the substrate. Under the flavin, and masked by it, is a --SH group of the enzyme[179] which may be the primary site of PCMB inhibition (see Table X).

D(-)BAIB, therefore, seems to have many of the key properties necessary for a compatible D-amino acid oxidase substrate. That is, it has the essential terminal carboxyl group, is of a resonable hydrophobic alkyl chain length, has the proper D configuration to minimize steric hinderance, and contains oxidizable H.[179] The only question remaining, however, is: Can ∝-D-amino acid oxidase oxidize a B-amino acid at a resonable enough efficiency to account for the observed rate of further BAIB metabolism?

Assuming that BAIB oxidase activity is merely an alternative expression of D-amino acid oxidase is perhaps the simplest explanation for most of the behavioral similarities between these two enzymes, and particularly for the observation that crude commercial hog kidney D-amino acid oxidase is also apparently capable of the oxidation of BAIB. There are, however, several strong pieces of evidence against this identity and in favor of the separate and independent co-existence of these two enzymes. BAIB oxidase, unlike D-

amino acid oxidase, for example, is relatively stable during storage (see Table XIII). Furthermore, although both BAIB oxidase and D-amino acid oxidase exhibit a bimodal differential centrifugal distribution, the subcellular localization of the organelle associated activity of these two enzymes upon zonal centrifugation seems to be different. That is, BAIB oxidase activity appears to be associated with the mitochondria, while D-amino acid oxidase activity is associated with the peroxisomes (see Fig. 13). Finally, although the bulk of the activity of these two oxidases is precipitated between 10-60% $(NH_4)_2SO_4$ (see Table XVII), most of the BAIB activity can be separated from D-amino acid oxidase activity as well as from that of other standard marker enzyme activities by precipitation between 0-40% $(NH_4)_2SO_4$ (see Table XVII) and subsequent preliminary affinity chromatography (see Table XVIII). Thus it appears that although these two oxidases have a number of properties in common, BAIB oxidase is, nevertheless, a unique and specific enzyme.

An alternative explanation for the unexpected ability of the crude commercial preparation of hog kidney D-amino acid oxidase to oxidize BAIB, therefore, must be proposed. It might be, considering the relative state of impurity of this enzyme preparation (see Fig. 15), for example, that the crude commercial hog kidney D-amino acid oxidase also contains the previously reported BAIB:pyruvate aminotransferase activity[70,169] as well as L-amino acid oxidase ac-

tivity. The BAIB oxidase activity detected in this enzyme preparation might then be a result of the coupling of this transamination with the further oxidation of the product, L-ala.[70] It is unlikely, however, that this is the mechanism of the further metabolism of BAIB in Tetrahymena pyriformis, since it has been reported[119] and confirmed by us that this organism lacks detectable L-amino acid oxidase activity. Of course, it might also be that hog kidney too contains a unique and specific oxidase capable of the further direct oxidation of BAIB, but because of its relatively low activity when compared to that of the previously described transaminase as well as due to the fact that the activity of most transaminases are anaerobically determined, it has up until now been overlooked.

VI. PHYSIOLOGICAL SIGNIFICANCE OF BAIB OXIDASE ACTIVITY

It has been shown in numerous other organisms and tissues that radiolabeled thymidine may be either incorporated into DNA or reductively catabolized. These metabolic pathways now also seem to be functional alternatives in Tetrahymena pyriformis. There are, however, still several unanswered questions concerning the operation of this reductive thymidine catabolic and reutilization pathway in this organism. How quantitatively important, for example, is this pathway to the overall metabolism of this organism? What, if any, is its physiological significance?

BAIB oxidase activity, although consistently shown to

be significantly above endogenous oxidase levels (see Fig. 6), is still only about 10% of the D-amino acid oxidase activity. Since D-amino acid oxidase itself is not a very active enzyme, this means that BAIB oxidase is even a less active enzyme. On the basis of radioactive tracer experiments, however, it has been established that this enzyme is still active enough to account for all the observed conversion of radiolabeled thymidine plus a large excess of unlabeled thymidine into biologically useful macromolecules other than DNA.

Since thymine is found predominantly in DNA, it was initially thought that BAIB excretion might reflect alterations in DNA metabolism.[160] Recently, however, it has been discovered that a small amount of this pyrimidine is also found in tRNA. Thus it became of interest to determine whether the source of the excreted BAIB is one or both of these thymines. Fortunately, these two sources of thymine are methylated differently and therefore by using appropriately radiolabeled methyl donors it was possible to determine that BAIB is apparently derived from both DNA-thymine as well as tRNA-thymine. This demonstration of the dual origin of BAIB supplies a more acceptable explanation for the high urinary excretion of BAIB, especially in tumors, since DNA turnover is known to be slow compared to tRNA turnover in these tissues.[131] It also argues in favor of the further metabolism of the base rather than the nucleoside.

In organisms and tissues where this reductive catabolic pathway has been described, nevertheless, it is still generally assumed that when total DNA synthesis is decreased metabolism of thymine compounds is shunted towards BAIB production.[160] This behavior has been substantiated in differentiating, regenerating, and neoplastic tissues.[182] An inverse functional relationship, therefore, has been proposed between the opposing pathways of synthetic utilization and reductive catabolism of thymidine during these processes.

Such a relationship also appears to be characteristic of total BAIB oxidase activity during the growth cycle of Tetrahymena pyriformis cultures (see Table XIX). That is, although the specific activity of this oxidase is highest in the initial culture when it might be expected that the cells would be most actively synthesizing DNA, not catabolizing it, the total activity of this oxidase is highest on the fifth day when the culture has reached stationary phase and it would be expected that the cells would be most actively catabolizing DNA. This total oxidase activity then appears to level off with culture age, while its specific activity actually decreases. This decline in specific activity, however, may not be due to an actual decrease in the number of enzyme molecules due to protein degradation or repression, but rather may simply be a relative decrease in enzyme concentration due to the increased synthesis of additional proteins. The actual number of these enzyme mol-

ecules, therefore, may remain constant with culture age.

It is not unusual for enzymes at critical branch points to have low activities. In the catabolism of uracil, for example, oxidation of B-ala has been reported to be the rate-limiting step.[97] The enzyme which catalyzes the conversion of the thymidine reductive catabolic end product, BAIB, to its initial reutilization substrate, probably methylmalonic semialdehyde, may also be such a critical step in the overall metabolism of the cell. Thus it could be imagined that this valueable nucleic acid precursor, which Tetrahymena pyriformis can not synthesize de novo, would only be catabolized when it is present in great cytoplasmic excess such as when these ciliates are feeding on other organisms or under extremely adverse environmental conditions when these cells have been observed digesting many of their own subcellular organelles, including DNA-containing mitochondria, as just about every spare molecule in the cell is utilized for glycogen synthesis.

The specific activity of this enzyme (2.8 nmoles/min/mg of protein) is about 1/10 that reported for other Tetrahymena pyriformis oxidases.[57] Its high K_m (3.6×10^{-1} M for D,L-BAIB) also tends to indicate that it has a low affinity for its substrate. Enzymes with such a high K_m, however, are not completely unprecidented. DHP hydrase, another enzyme from the same reductive thymidine catabolic pathway, for example, has been reported to have a K_m of 1.2×10^{-1} M.[93] Moreover, if this enzyme is found, like those

previously reported, to prefer one of the optical isomers of this substrate over the other, then its true K_m should probably be at least halved. This, of course, assumes that there is no inhibition of the enzyme by the non-metabolized isomer which may not be the case. The true K_m of this enzyme, therefore, may be even lower. Whether this high K_m as determined in homogenates in the possible presence of inhibitors, as indicated by the increase in enzyme activity upon storage (see Table XIII) and affinity chromatography (see Table XVIII), or after the disruption of the possibly critical spacial orientation of the enzyme either in relation to other enzymes in this pathway or in relation to its own most active configuration on the membrane of an intact subcellular organelle, is physiologically significant or not thus remains to be investigated.

Under conditions of excessive tissue destruction, BAIB excretion has been reported in persons who are not genetic high excretors. This implies that the ability to metabolize BAIB is limited even in these persons, but at a level which permits them to handle normal turnover without appreciable excretion.[164] That is, there is only urinary spillage of BAIB in these low excretors when a subsequent step in BAIB metabolism (probably the conversion of BAIB to methylmalonic semialdehyde) is saturated.[72] This restricted ability to metabolize BAIB under these conditions may be due to limitations in the availability of various transaminating substrates, such as the necessary αketo acids, or

to some other inherent property of the enzyme. Such rigid control, whatever its origin, however, indicates the operation of an unusually delicately balanced regulatory mechanism in the functioning of this enzyme[164] as well as the possible importance of such regulation in the overall metabolism of the organism. If the enzyme itself is the source of this regulation, then the selective pressures operating to maintain such tight control at this critical metabolic interchange might even be rigid enough to survive the evolutionary transition form the transaminating systems described in most other organisms to the unique oxidase apparently functioning in Tetrahymena pyriformis and thus be an additional argument in favor of the possible regulatory significance of the seemingly low activity of this enzyme.

The nature of the metabolic interrelationship between anabolic and catabolic thymidine utilization pathways then might be such that a pool of pyrimidine derivatives accumulates as a consequence of decreased catabolic capacity. Compounds in such a pool could act as inducers for enzymes involved in polynucleotide synthesis and as repressors for catabolic enzymes. The resulting increase in anabolic enzyme formation would give rise to increased utilization of nucleic acid precursors, and inducer/repressor concentrations would diminish. This decrease could in turn cause a shift in the enzyme pattern toward equilibrium levels. At a later time the catabolic capacity could be below equilibrium and thus the anabolic enzymes would be decreased to

equilibrium levels. Consequently, a small accumulation of pyrimidine derivatives could occur, followed by increased synthesis of anabolic enzymes and repression of synthesis of catabolic enzymes.[99]

The reductive procaryotic uracil catabolic pathway in Clostridium uracilicum has, for example, been reported to be so inducible.[77] Actinomycin has also been reported to completely inhibit the in vivo rise of reductive thymidine catabolic activity as DNA synthetic activity decreases in differentiating, regenerating, and neoplastic tissues.[182] This finding further supports the inducibility of this catabolic enzyme system. In addition, it suggests that the activity of this reductive thymidine catabolic pathway during these processes may not only involve an increase in the biosynthesis of certain key enzymes, but also may require the transcription of new RNA.[182] The same authors, however, also report that the addition of actinomycin in vitro has no effect on the incorporation of thymidine into DNA or on the degradation of radiolabeled thymidine to $^{14}CO_2$.[182]

The limiting enzyme in the reductive eucaryotic uracil catabolic pathway in Chlorella fusca, on the other hand, has been reported not to be inducible.[114] BAIB oxidase activity in Tetrahymena pyriformis also does not appear to be inducible (see Table X X). That is, cells grown in the presence of either additional exogenous DNA, Tdine, DHT, or BAIB do not show any significant stimulation of BAIB oxidase activity over control specific activity.

Evidence that DHPs do occur and can serve as a source of B-amino acids in various organisms and tissues indicates that such pyrimidine degradative pathways may be of additional physiological importance.[84] It could be imagined, for example, that the presence of pyrimidines (or their simple derivatives) may interfere with the control mechanisms concerned with the synthesis of new polynucleotides especially if the appearance of these pyrimidines, as is the case in Tetrahymena pyriformis which are incapable of the de novo synthesis of these compounds and therefore must depend on their continuous exogenous supply, is not subject to the controls regulating their synthesis. The degradative pathways which provide for the disposal of waste pyrimidines, therefore, may have the important function of preventing interference with, or circumvention of, the polynucleotide synthesizing regulatory mechanism.[112] This control, moreover, could be at the translational, that is feed-back inhibition, instead of at the transcriptional, that is repressor, level. Thus even if the high K_m for BAIB oxidase in Tetrahymena pyriformis is ultimately verified, its implied low substrate affinity may have additional regulatory significance.

Finally, there is some evidence in humans that glucose might be a metabolic regulator of further BAIB metabolism,[182,183] especially in view of the relatively large amounts of this B-amino acid which are excreted after prolonged use of intravenous glucose.[183] Log phase Tetrahymena pyrifor-

mis cultures grown in the presence of 0.1% glucose have also been observed to excrete moderate amounts of BAIB when incubated under starvation conditions.[10] Thus it could be imagined that glucose actually stimulates the reductive catabolism of thymidine, although only a limited amount of the resulting BAIB could be reutilized accounting for the increased excretion of this compound. Alternatively, however, it might also be imagined that under conditions which stimulate glycogen synthesis, thymidine via the further metabolic reutilization of BAIB could also be so utilized, although again because of the relatively slow rate of its further metabolism only a limited amount of the resulting BAIB would be so reutilized. Such glucogenic regulation of the further metabolism of BAIB in Tetrahymena pyriformis would be quite interesting since one of the outstanding characteristics of these organisms is their great glyconeogenic potential.[184] The data indicating an increase in total BAIB oxidase activity as cultures enter stationary phase (see Table XIX) would be consistent with, if not directly support, this latter hypothesis.

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