The biosynthesis of betaine and related compounds in higher plants and fungi

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THE BIOSYNTHESIS OF BETAINE AND RELATED COMPOUNDS IN HIGHER PLANTS AND FUNGI

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I N D E X

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ABSTRACT

A satisfactory synthesis of crystalline betaine aldehyde was achieved and methods were developed for the isolation and characterization of choline, betaine aldehyde, betaine and related compounds. The suitability of these methods (ion-exchange column chromatography and thin-layer chromatography in particular) were demonstrated by their application to the choline oxidase system known to exist in the mitochondria of rat liver.

Attempts were made to demonstrate a similar choline oxidase system in plants. Experiments designed to demonstrate the existence of these enzymes by spectrophotometric and polarographic methods in mitochondria extracted from plant sources were largely unsuccessful. The mitochondria were structurally intact and were of high biochemical integrity. On the isolated occasions when stimulation of respiration occurred when choline was supplied as substrate, the formation of betaine aldehyde or betaine was never confirmed by the characterization techniques which had been developed.

The existence of a permeability barrier to the uptake of choline was discounted as an explanation of the 'latency' of the enzymes investigated. The methods employed to overcome such a barrier in mitochondria extracted from rat liver were without effect on the plant systems. Radioactive choline was taken up by mitochondria, from plants and a fraction of the labelled compound became closely associated with the mitochondrial membranes. However, the level of radioactivity did not increase with time indicating an upper limit of choline uptake or a continual utilization of the choline taken up. The existence of an alternative pathway for the oxidation of choline was superficially examined but no definite conclusions could be reached.

The application of polyacrylamide gel electrophoresis and incubation with tetrazolium salts for the identification of a betaine aldehyde dehydrogenase were tested initially on the supernatant enzyme known to exist in rat liver. The success of this technique led to the isolation of a specific betaine aldehyde dehydrogenase, with a requirement for NAD as a coenzyme, from several of the plant tissues examined.

INTRODUCTION

The term "betaine" coined by Scheibler (1869) for a substance isolated from sugar beet (Beta vulgaris L.) is now generally applied to N-methyl anhydrides of amino or imino-acids. They may also be regarded as quaternary ammonium bases carrying a carboxyl group - this structure expressing their chemical properties more accurately. The betaines of many of the common amino-acids are unknown naturally and only a few occur widely, the best-known being trigonelline, stachydrine and glycine betaine derived respectively from nicotinic acid, proline and glycine.

Glycine betaine is widely distributed among flowering plants (Guggenheim (1940)), occurring in all organs and can form 5% of the dry weight of the leaf, (Klein, Krisch, Pollauf and Soos (1931), Cromwell and Rennie (1953)). It is found in relatively high concentrations in members of the Chenopodiaceae and the Gramineae and Vicia species of the Leguminosae.

Stachydrine was first isolated from Stachys tubifera L. by Von Planta and Schulze (1893) and trigonelline was first found in Trigonella Foenum-graecum L. by Johns (1885). Schulze and Trier (1912) described betonicine and turicine (stereoisomeric betaines of 4-hydroxyproline in Betonica officinalis L. (Labiatae) and Pisum sativum L. Cornforth and Henry (1952) found that 3-hydroxystachydrine (the betaine of 3-hydroxyproline) constituted 10% of the dry weight of the epicarp and the endocarp in the fruit of Courbonia virgata A. Brongn. (Capparidaceae). Greshoff (1898) and Delafou, Hug and Mazzoco (1939) identified hypophorine (the betaine of tryptophan) in the seeds of various spp. of Erythrina (Leguminosae) where it can form 3% of the dry weight. Homostachydrine, the betaine of pipecolic acid occurs in Medicago sativa L. (lucerne, alfalfa) (Robertson and Marion (1959)). Blaim (1962) identified three betaines from a

number of species of seeds, glycine betaine, stachydrine and trigonelline, the latter occurring most generally in cultivated plants.

In fungi glycine betaine was found in Boletus edulis Bull. ex.Fr. (Reuter (1912)) and Amanita muscaria (L) Fr. (Kung (1914)) and hercynine the betaine of histidine was found in Boletis edulis Bull. ec Fr. (Reuter (1912)) and Boletus sulphureus Dill. ex Fr. (List (1958)). Tanret (1909) isolated ergothioneine from ergot (Claviceps pupurea) (Fr.) Tul. This is not found in higher plants but has been recorded as an animal metabolite. The corresponding amino-acid, L-thiolhistidine has not been found as a natural product. The betaine of γ-aminobutyric acid and homarine (the N-methyl betaine of picolinic acid) were found in Polyporus sulphureus Fr. (List (1958)).

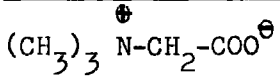
Ogino (1955) reviewed the algal literature and demonstrated stachydrine in Laminaria spp. and Chondrus ocellatus Kjellm. Stachydrine was not present in Porphyra tenera Kjellm. and no glycine betaine was found, however, trigonelline was tentatively identified. Toda (1923) isolated stachydrine from Porphyra laciniata (Lightf.) Ag.; however, Shirahama (1937) could find no evidence for the presence of glycine betaine and stachydrine in Alaria crassifolia Kjellm. and Cytophyllum hakodatensis Fr. The algae are also notable for the wide distribution of the thetins, sulphur homologues of the betaines. β-dimethyl propiothetin was found in the red alga Polysiphonia fastigiata Grev. (Haas and Russell-Wells (1923)). Challenger and Simpson (1948) demonstrated the presence of sulphonium compounds (α or β-thetin derivatives giving off dimethyl sulphide) in various algae.

The production of glycine betaine by a choline oxidase system in animal tissues is well documented.

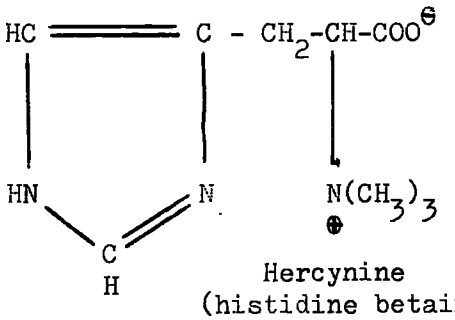
Most of the relevant experimental work has been performed on whole rats and on rat livers, however, Bilinski (1960) has also shown that choline was a precursor of betaine and trimethylamine-N-oxide in the lobster and the crab. Similar results were obtained by Farber (1960) who worked on the livers of rats, mice, chickens, dogs, hamsters, rabbits, guinea pigs, monkeys and human beings. Betaines other than glycine betaine and ergothioneine are also known to occur in the animal kingdom. These include hercynine (Ackerman and List (1958)) and homarine (Hoppe-Seyler (1933), Ackerman and List (1958)).

EXAMPLES

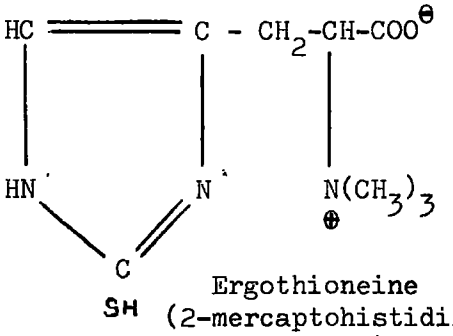
Betaines of the α-amino acids



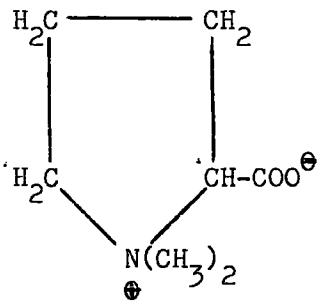
Glycinebetaine



Hercynine
(histidine betaine)



Ergothioneine
(2-mercaptohistidine
betaine)

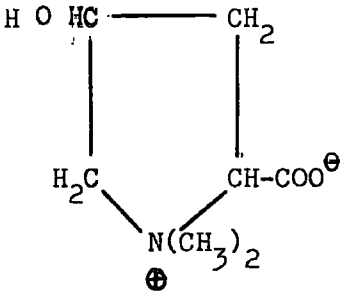


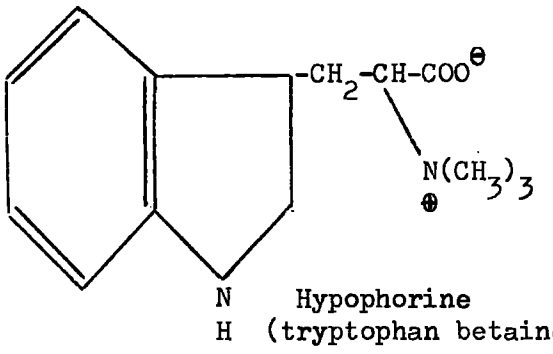
Stachydrine
(proline betaine)

Betonicine

Turicine

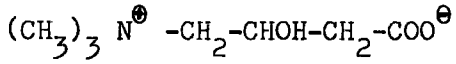
(stereoisomers)



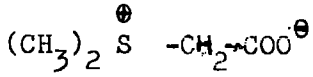


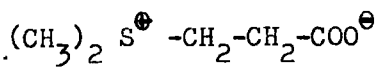
Hypophorine
(tryptophan betaine)

Betaines of the ω-amino acids



Carnitine
(β-hydroxy-γ-butyro-betaine)

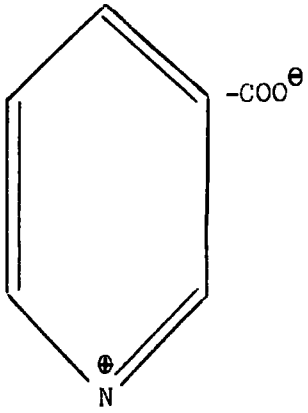




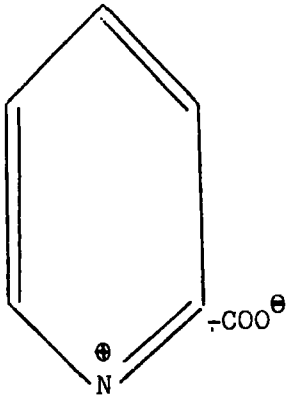
Dimethylthetin

Dimethylpropiothetin

Betaines of the pyridine-carboxylic acids



Trigonelline



Homarine

The principle importance of glycine betaine would appear to be the role it has to play as a source of methyl groups in biological methylations (Challenger (1955)). From its structure it is an apparent store of labile methyl groups and there is evidence for both demethylation and transmethylation of these methyl groups. Borsook and Dubnoff (1948) showed the presence of a betaine-homocysteine methylpherase as a soluble enzyme in liver. This was endorsed by Sloane and Boggiano (1960) who stated that this enzyme occurred in association with dimethylthetin-homocysteine methylpherase. Furthermore, Dubnoff (1949) showed methyl transfer from betaine anaerobically but from choline only aerobically indicating the non-lability of the methyl groups of the choline. Muntz (1950) in liver homogenates demonstrated that incubations with choline and homocysteine yielded dimethylglycine and not dimethylethanolamine. Du Vigneaud et al (1946) showed that homocysteine could replace methionine in the diet of the white rat only in the presence of choline or betaine. It was suggested a transmethylation was involved from choline or betaine to homocysteine and the reaction could be reversible. The methyl groups of choline, betaine, dimethylthetin and dimethyl propiothetin were shown to be oxidised in vivo in animal experiments by Ferger and du Vigneaud (1950). Sribney and Kirkwood (1954) showed that betaine was involved in transmethylations in 11-day old seedlings of barley. (^{14}C-methyl)betaine formed the source of labile methyl groups for the bio-synthesis of choline and the alkaloids N-methyltyramine and hordenine. Erickson (1960) showed the ability of homogenates of various tissues to transfer methyl groups from betaine to homocysteine. However, he was unable to demonstrate the occurrence of methyl transferases in plants.

More recent experimental work by Bowman and Rohringer (1970) examined formate metabolism and betaine formation in healthy and

rust-infected wheat. In the wheat leaves used in the study betaine was not metabolised further. These authors considered that the function of the compound was obscure. However, they state that because of its simple structure and ease of formation, together with its nitrogen content and readily accessible methyl groups it is difficult to suggest that it should represent merely a waste product of metabolism. Radioactivity from glycine-2-^{3}H, methionine - $^{14}CH_3$, serine - 3 - ^{14}C, ethanolamine - 1, 2 - ^{14}C and choline - $^{14}CH_3$, was incorporated into betaine in the dark, but was not detected in sarcosine or dimethylglycine. These results supported the view that betaine was synthesised from glycine via serine, ethanolamine and choline with methionine as the methyl donor and not by direct N-methylation of glycine.

The role of trigonelline as a precursor of pyridine nucleotides in the yeast Torula cremoris (Fcl.) Tul. and pea seedlings (Joshi and Handler (1962)) is of interest. The methyl group underwent oxidative demthylation and entered the IC carbon pool.

Richardson (1964) showed a higher concentration of betaine in the developing apical leaves than in mature leaves of Chenopodium vulvaria L. Stanek (1906, 1911), Cromwell and Rennie (1953), Simenauer (1957) and Wheeler (1964) all showed this for other plant species, (see later). Wheeler (1964, 1964 and 1969) showed that betaine was a growth regulator in a number of species. Simenauer (1957) showed that betaine was utilised during germination of sugar beet seeds. He considered that methyl groups were being removed by transmethylation but offered no evidence and no enzyme systems were isolated.

Another interesting phenomenon was seen by Rafaeli-Eshkol and Avi-Dor (1968). In a moderately halophilic bacterium BA1, betaine accelerated succinate oxidation in cells obtained from low-salt medium. In cells isolated from high-salt medium betaine showed no

stimulatory effect. However, in those grown in low-salt medium stimulation increased with increase in salt concentration. Accumulation of betaine was considerable and increased with increase in salt concentration. Betaine seems then to be exerting some effect on salt-tolerance in this organism.

From their relatively simple structure, it would seem a fairly straight-forward problem to ascertain the mode of biosynthesis of betaines. Indeed in animals their biosynthesis (particularly that of glycine betaine) has been well documented. The presence of an enzyme in mammalian liver capable of oxidising choline has been known for some time (Mann and Quastel (1937), Bernheim and Bernheim (1938)). Mann, Woodward and Quastel (1938) demonstrated that the enzyme converted choline to betaine aldehyde. As a result of liver perfusion experiments Guggenheim and Loeffler (1916) had suggested a similar reaction. Mann et al (1938), Kensler and Langemann (1951) and J. N. Williams (1952) have established that choline dehydrogenase activity was associated with the mitochondria and that it was linked to the cytochrome system. However, there still remained the problem whether the linkage was a direct one or mediated by co-factors. Strength, Christensen and Daniel (1953) believed that choline dehydrogenase was an NAD-linked enzyme as the addition of NAD stimulated choline oxidation by mitochondria. Rendina and Singer (1959) showed NAD was without effect on the oxidation of choline by soluble purified choline dehydrogenase. It maintained oxidation but had no effect on the initial stimulation. They showed this effect to be due to the removal of inhibitory betaine aldehyde by betaine aldehyde dehydrogenase which required NAD as a co-factor (Klein and Handler (1942)). This confirmed the results of Mann et al (1938) who had demonstrated an enzyme capable of converting betaine aldehyde, produced by the action of choline dehydrogenase, to betaine. Further evidence for this enzyme system was furnished by Jellinek, Strength

and Thayer (1959) and Glenn and Vanko (1959). FAD was reported to stimulate choline dehydrogenase (Rothschild, Cori and Barron (1954)) but was later shown to have no stimulatory effect on the enzyme by Singer and Kearney (1959). Most of this work has been performed with rats, rat liver or mitochondria isolated from rat liver. However, Sidransky and Farber (1960) worked on livers of rats, mice, chickens, dogs, hamsters, rabbits, guinea pigs, monkeys and human beings and found marked species differences in the ease of induction of the choline oxidase system of choline deficient fatty liver.

Packer, Estabrook, Singer and Kimura (1960) showed that the choline oxidase system of rat liver comprised the same type of cytochrome components as the succinate and NADH oxidase systems. They also stated that the flavo-protein and the flavin coenzyme were tightly bound. Kimura, Singer and Lusty (1960) and Kimura and Singer (1959) had furnished kinetic evidence that choline and succinate dehydrogenases fed electrons into functionally equivalent respiratory chains inter-connected between cytochrome c_1 and oxygen. However, between the flavo-protein and the cytochrome c_1 there seemed to be a cytochrome b peculiar to the choline oxidase system. This was autoxidisable under certain conditions.

J. N. Williams, Jnr and Sreenivasan (1953) and Ebisuzaki and J. N. Williams, Jnr. (1955) both showed that solubilization and purification of the enzyme was hampered by lack of an assay method and failure of conventional extraction procedures. Kun and Singer quoted in Kearney and Singer (1956) suggested the use of phenazine methosulphate reduction as an assay method. Rendina and Singer (1959) found this dye to be the only one suitable for assay of soluble succinate dehydrogenase. The spectrophotometric adaptation of phenazine methosulphate reduction was suggested by Arigoni and Singer (1962) and Rendina and Singer (1958). Solubilization of an

active enzyme was claimed by Rendina and Singer (1958-1959) using phospholipases. Karzenovsky and Auda (1958) have claimed success using a non-ionic detergent but it is doubtful whether the enzyme was truly solubilized in this manner.

Guha and Wegmann (1963) using histochemical techniques showed the localisation of choline oxidase in rat liver and kidney. Rothschild, Cori and Barron (1954) found that the enzyme had a requirement for Magnesium ions. They showed an optimum pH of between 6.8-7.3. Above pH 8.0 there was no activity. They also showed that the enzyme was inhibited by -SH reagents and copper complexing agents and was reactivated by betaine aldehyde and glutathione. Kagawa and Lardy (1965) showed a requirement for permeability of the mitochondrial membrane to choline. G. R. Williams (1960) had also shown that there was a competition for entrance between choline and other cations in mitochondria isolated from predominantly ionic media. He suggested the biological significance of this was difficult to estimate. However, it could mean that direct oxidation of choline was quantitatively less significant in the metabolism of this compound than has been assumed. In fact the metabolic pattern of choline could resemble that found in animals which did not possess a choline oxidase.

Yue, Russell and Mulford (1966) showed an uncoupling effect on mitochondria by amino compounds in in vitro systems. Göschke, Burkard, Gey and Pletscher (1963) showed the inhibitory effect of potent amine oxidase inhibitors on the choline oxidase system.

Much less work has been done on betaine aldehyde dehydrogenase as a single enzyme. Wohlrab (1964) carried out histochemical studies on the enzyme using tetrazolium salts. Glenn and Vanko (1959) claimed the existence of a specific and non-specific aldehyde dehydrogenase from rat liver preparations. However, Yue et al (1966)

isolated mitochondria from rat liver which were unable to oxidise betaine aldehyde.

In higher plants the picture is far from clear. Klein and Linser (1932, 1935) claimed that ornithine and proline stimulated the formation of trigonelline in Dahlia variabilis Hort. and Trigonella Foenum-graecum L. and of stachydrine in Stachys palustris L., S. recta L. and Galeopsis ochroleuca Lam. However, the observed increases were small and Leete, Marion and Spenser (1955) using seedlings of Medicago sativa L. found no evidence of stachydrine formation from administered ^{14}C-labelled ornithine. However, Weihler and Marion (1958) demonstrated that pyridoxal and folic acid were required for this reaction to take place. Zeijlemaker (1953) found that the pea plant converted nicotinic acid to trigonelline.

Barrenscheen and Valyi-Nagy (1942) reported the stepwise methylation of glycine to betaine in homogenates of etiolated wheat seedlings supplied with methionine as a methyl group donor. Cromwell and Rennie (1954) were unable to confirm these results. In their experiments they were unable to show any substantial conversion of glycine in combination with methionine into betaine. Possible precursors of choline were fed to etiolated wheat seedlings grown under sterile conditions. The feeding of glycine alone and in combination with methionine brought about a slight increase in the choline content of the tissues but a slight decrease in the content of betaine. The feeding of dimethyl glycine alone or in combination with methionine resulted in slight losses of choline, but a slight increase in the content of betaine was observed when dimethyl glycine was fed in combination with formate.

Preliminary experiments carried out by Cromwell and Rennie (1953a) on roots and leaves of seedling plants of Beta vulgaris L. from which homogenates were prepared seemed to show the presence of an

enzyme which oxidised added choline to betaine. Dialysed homogenates of roots of Beta vulgaris were incubated with a buffered solution containing cytochrome c. After a period of 24 hours at 20°C the protein was precipitated by heating. After filtration, unchanged choline was precipitated by the addition of ammonium reineckate. The filtrate was acidified and kept at 4°C for 30 mins. during which time betaine reineckate precipitated. The bases were isolated, recrystallised and characterised. Homogenates of young leaves of sugar beet were inactive under all conditions. It was concluded that betaine was formed in the root tissues of sugar beet by oxidation of choline and translocated upwards into the leaves.

However, subsequent work showed that the activity of these homogenates was due to bacterial action. The experiments were repeated by Cromwell and Rennie (1954) and it was found that when thymol was used as an antiseptic during the incubation period it did not prevent bacterial activity. This activity was shown to be responsible for the conversion of choline into betaine, for when toluene was used instead of thymol no conversion of added choline occurred, either in homogenates of young roots of Beta or in homogenates of etiolated wheat seedlings. Homogenates sterilised by passage through a Seitz filter also failed to bring about the conversion of added choline into betaine.

Other workers have also pointed out the danger of spurious results being obtained as a consequence of the activity of micro-organisms during prolonged incubations. In particular Stein von Kamienski (1958) and Richardson (1964) have demonstrated the readiness with which choline was attacked by micro-organisms.

Cromwell and Rennie (1954) examined the effects of infiltration of possible precursors of betaine into leaves of Beta vulgaris and Atriplex patula. They observed that infiltration of choline and betaine aldehyde resulted in a significant increase in the betaine

content of the tissues. In addition little variation from controls was found when solutions of glycine in combination with methionine were infiltrated into the leaves. When they kept leaves infiltrated with solutions of choline in an atmosphere of nitrogen during the experimental period no significant increase in their betaine content was recorded. But when leaves infiltrated with solutions of choline were exposed to light during the experimental period they observed a greater increase in betaine content than in leaves kept in darkness during the experimental period. They concluded that the synthesis of betaine in the tissues of higher plants took place largely as a result of the oxidation of choline.

Byerrum, Sato and Ball (1956) demonstrated that the methyl group(s) of betaine-methyl-^{14}C were incorporated into the N-methyl group of nicotine in tobacco, (Nicotiana rustica L. var humilis). Betaine-methyl-^{14}C was fed to three-month-old tobacco plants growing in a nutrient solution. After a 7-day feeding period the plants were removed and nicotine was extracted and isolated as the dipicrate. It was found that the nicotine was radioactive after betaine-methyl-^{14}C had been supplied. To determine the location of the label the nicotine molecule was chemically cleaved (Brown and Byerrum (1952)), and the methyl group was recovered as methyl triethyl ammonium iodide. The amount of radioactivity associated with this compound confirmed that the radioactivity of nicotine following feeding of methyl labelled betaine was in the N-methyl group.

Byerrum et al (1956) also attempted to ascertain whether or not choline might first be oxidised to betaine before it could yield its methyl group(s) for nicotine synthesis. They fed tobacco plants with choline-methyl-^{14}C and subsequently isolated the quaternary ammonium bases from these plants using the ammonium reineckate procedure. Investigation of these bases by paper chromatography and autoradiography indicated that after feeding choline-methyl-^{14}C

to tobacco plants radioactive betaine and dimethyl glycine arose.

However, they were unsuccessful in their attempts to demonstrate the activity of a choline oxidase system in various preparations made from the leaves and roots of tobacco. Delwiche and Bregoff (1958) found that crude homogenates of the leaves of Beta vulgaris were capable of synthesising serine from glycine-2-^{14}C but there was no evidence of any conversion of choline to betaine. Cromwell and Richardson (1966) working with cuttings, leaf discs and sterile excised roots of Chenopodium vulvaria showed that the major radioactive compound isolated from these tissues after being fed with choline-methyl-^{14}C was betaine. Traces of two other radioactive compounds were also isolated and were tentatively identified as dimethyl glycine and trimethylamine-N-oxide-^{14}C. Nevertheless, all attempts made by Richardson (1964) to demonstrate the production of betaine from choline by preparations of the leaves of Chenopodium vulvaria were unsuccessful.

de Heus (1959) observed that in the fungus Aspergillus niger, van Tiegh stepwise methylation did not occur but there was aerobic oxidation of choline to betaine. This work was supported by Hogg and Richardson (1968) who studied the biosynthesis of betaine in Neurospora crassa (Shear and Dodge). The radioactive quaternary ammonium compounds extracted from mycelia after 3-days' growth in a medium containing (^{14}C-methyl) choline chloride were separated by column chromatography. Two major radioactively labelled quaternary ammonium compounds were obtained. The elution volumes, electrophoretic mobilities and paper chromatographic behaviour of these compounds in a number of solvent systems, indicated that they were betaine and choline. Culture filtrates were examined for the presence of radioactive quaternary ammonium compounds but only (^{14}C methyl) choline was recorded.

Buffa and Velutti (1957) examined the utilisation and metabolism of choline by various strains of Pseudomonas aeruginosa (Schrocter)

Migula. Ten strains of the organism were tested and all of them grew with choline as the sole source of C and N. The organism was also able to grow on ethanolamine, betaine, N-dimethylglycine and sarcosine, but not on methylaminoethanol and dimethylaminoethanol. The latter compound was shown to be an inhibitor of choline utilisation.

The enzyme system involved in the utilization of choline was found to be adaptive. The enzyme disappeared rapidly from the adapted cells grown in the absence of the inducing substrate.

Choline oxidation by washed cell suspensions of the organism was investigated using paper chromatography and autoradiography. The disappearance of choline from the incubation medium was accompanied by a transient accumulation of a compound which behaved chromatographically like betaine. No accumulation of N-dimethylglycine or sarcosine was recorded but a faint radioactive spot appeared which had the same R_F value of the former compound.

There is reasonable evidence then to suggest that glycine betaine found in plant tissues is derived from choline via the activity of an oxidase system. In experiments with various whole plants and micro-organisms Cromwell and Rennie (1954), Cromwell and Richardson (1966), de Heus (1959), Hogg and Richardson (1968) and Buffa and Velutti (1957) have all cited evidence for an in vivo enzyme system capable of oxidising choline to glycine betaine. However, Cromwell and Rennie (1954), Byerrum, Sato and Ball (1956), Delwiche and Bregoff (1958) and Richardson (1964) using crude homogenates or preparations from plant tissues, as described previously in the text, have all been unable to demonstrate an in vitro system capable of this oxidation. Moreover, the ease with which choline is degraded by micro-organisms is a problem in the elucidation of such an enzyme system.

In view of the association of the choline oxidase system with

the mitochondria in animals it seemed reasonable in this investigation to attempt to obtain active mitochondria from suitable plant sources and to determine whether an enzyme system similar to that occurring in animals could be demonstrated.

MATERIALS AND METHODS

Plant Materials

Species of higher plants used in these investigations were sugar beet (Beta vulagaris L.), stinking goosefoot (Chenopodium vulvaria L.), spinach beet (Spinacea oleracea L.), mung bean (Vignia radiata L./Phaseolus aureus L.) broadbean (Vicia faba L.), pea (Pisum sativum L.) and Atriplex hastata L. All seeds were obtained from Carters Tested Seeds Ltd., P.O. Box 568, Raynes Park, London, S.W.20. Atriplex hastata plants were collected from the wild when available. Beta vulgaris, Spinacea oleracea and Chenopodium vulvaria were sown in pots and kept in heated greenhouses during the winter months. These were transferred outdoors in March or April. These plants served as sources of mature tissue. Etiolated seedlings of sugar beet, spinach beet, mung bean, broad bean and the pea were also grown as sources for plant extracts. In the cases of sugar beet, spinach beet and mung bean, the seeds were placed on a layer of muslin in perforated plastic trays and grown in the dark at 27-28°C. The seedlings were irrigated intermittently from an overhead spray of water for 4-7 days.

The broad bean and pea seedlings were grown by placing the seeds in trays containing moist Vermiculite to a depth of about 5 cms. The trays were kept in the dark at 27-28°C, and intermittently watered for about 7 days. All the seeds used for these short term supplies of etioliated tissue were surface sterilised by rapidly shaking with 10% w/v calcium hypochlorite for approximately 15 min. and thoroughly washed with distilled water after this treatment. When these seedlings were harvested either the hypocotyls or the roots, depending on the tissue source required, were separated from the remainder of the seedling (in some cases the whole seedling

was utilised) and any showing signs of infection were discarded. The selected tissue was thoroughly washed with distilled water prior to use.

Fungal cultures

Neurospora crassa wild-type Shear et B.O. Dodge (I.M.I. 75721) was obtained from the International Mycological Institute. The chol$^-$ strains of Neurospora crassa Shear et B.O. Dodge CBS 238.55 and CBS 280.48 were obtained from the Centraalbureau Voor Schimmel-cultures. Baarn, The Netherlands. Type CBS 238.55 corresponds to the strain Mucka 47904 or chol-2 (Horowitz et al., 1945) and type CBS 280.45 corresponds to the strain Beadle 344 86A (= ATCC 9277), or chol-1 (Horowitz and Beadle, 1943). Aspergillus niger Van Tieghem strain I.M.I. 59374 obtained from the International Mycological Institute was also used as a source of tissue.

Culture Media

The liquid culture medium utilised for the Neurospora crassa strains was based on that described by Fincham and Boylen (1954) a modified Fries minimal medium No. 3 (FMM) containing 0.5% (w/v) choline chloride and 1% (w/v) sucrose. The sample cultures were grown and sub-cultured on either potato glucose agar slopes or potato dextrose agar slopes at around 27°C.

FMM

10	g	sucrose
5	g	choline chloride
5	g	ammonium tartrate
1	g	NH_4NO_3
1	g	KH_2PO_4
0.5	g	$MgSO_4.7H_2O$
0.1	g	$CaCl_2.4H_2O$
0.1	g	NaCl
5	µg	biotin
1	ml	trace elements

The solution was made up to 1 l. with distilled water. The trace elements were AC microelements a modification of those described by Kratz and Myers (1955).

H_3BO_3	2.86 g/l.	
$MnCl_2.4H_2O$	0.181 g	
$ZnSO_4.7H_2O$	0.222 g	+ Fe (Edta form)
$CuSO_4.5H_2O$	0.079 g	
$Na_2MoO_4.2H_2O$	0.027 g	

The sucrose, choline and biotin were autoclaved at 10 lbs/sq.in. for 20 mins. separately. The remainder of the medium was autoclaved for 15 mins. at 15 lbs/sq. in. The flasks were inoculated from cultures grown on the agar slopes. The cultures were grown at 25°C. until they yielded a thick hyphal mat.

Aspergillus niger when used was grown in liquid culture in a medium based on that described by Watson & Smith (1967).

Glucose	15 g/l
Choline chloride	10 g
NH_4NO_3	3 g
KH_2PO_4	2 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	12 mg
$ZnSO_4.7H_2O$	16 mg
$CuSO_4.5H_2O$	2 mg
$MnCl_2.4H_2O$	5 mg
$CaCl_2.4H_2O$	20 mg

Chemicals

Except for those listed below chemicals were obtained from British Drug Houses Ltd., or Koch-Light Laboratories Ltd., Colnbrook, Bucks., and were of analytical grade where possible.

Bovine Albumin, fraction V powder

β-NAD from yeast, grade III

Adenosine-5-diphosphate disodium salt, grade I

MTT tetrazolium 3-(4, 5 - dimethyl thioazolyl-2)-2, 5-diphenyl tetrazolium bromide)

NBT (Nitro Blue Tetrazolium) 2, 2 - di - p - nitrophenyl-5, 5^1 - diphenyl-3, 3^1-(3, 3^1 dimethoxy-4, 4^1 - diphenylene) ditetrazolium chloride

were obtained from Sigma Chemical Co. Ltd.

The two tetrazolium salts were also obtained from C. F. Boehringer and Soehne GmbH Mannheim, Germany.

Silica Gel G. nach Stahl	E. Merck Laboratory chemicals
Silica Gel HR nach Stahl	
Cellulose powder MN 300	Macherey-Nagel & Co.
Ilford Industrial G X-ray film	Ilford Ltd., Ilford, Essex.
Zeo-Karb 226 100-mesh beads (hydrogen form) (obtained in the graded form as a gift to M.R. from Dr. K. Blau, Kings College, London).	Permutit Co. Ltd.
Amberlite CG.-50 100-200 mesh chromatographic grade, Type 1	Rohm & Haas Co., Philadelphia, Pa., U.S.A.
Microcapillary tubes (chromatography)	Microcaps, Fa., Drummond Scientific Co., Broomhall, U.S.A.
Choline chloride (methyl-C^{14}) (specific activity 54 mC/mM)	The Radiochemical Centre, Amersham, Bucks., U.K.
Choline-1.2-C^{14} Bromide (Specific activity 117mC/mM)	Tracerlab, Waltham, Mass. 02154, U.S.A.
2, 2-diethoxyethyltrimethyl-ammonium iodide	Aldrich Chemical Co. Inc. Milwaukee, Wisc., U.S.A.

2, 5-diphenyloxazole — Thorn Electronics Ltd.

1, 4-bis 2-(4-methyl-5-phenyloxazolyl) benzene — Thorn Electronics Ltd.

Synthesis of Chemicals

Betaine aldehyde was synthesised by a modification of the method described by Jellinek et al (1959). 2, 2-diethoxyethyltrimethylammonium iodide was dissolved in water and converted to the chloride by treatment with silver chloride. The aqueous filtrate was evaporated in vacuo at 50°C. The residue, a yellow syrup, was frozen in liquid nitrogen and concentrated hydochloric acid added slowly, keeping the mixture frozen. The entire reaction vessel was then flushed with nitrogen for 30 minutes. The system was then evacuated and the contents heated to 55°C and the hydrolysis allowed to continue at this temperature for 2-3 hours. The excess acid was removed in a vacuum desiccator containing sodium hydroxide. The syrup was then reduced to small volume by rotary evaporation and recrystallised from one volume of glacial acetic acid and 7 volumes of acetic anydride. The recrystallisation was performed three times. The identity of the colourless crystalline solid was determined by infra-red spectroscopy and comparison with published data.

Initial syntheses followed the procedure of Jellinek et al (1959) and yielded a compound identified as a polymerised form of the aldehyde. This was confirmed by comparison with a sample supplied by F. Wohlrab.

Melting point determinations:-

Betaine aldehyde chloride	150°C	Reported m.pt. 123 - 144°C
Polymer	No melting at 250°C	

Betaine aldehyde reineckate	Blackened at 215°C, decomposed at 255°C (complete) and melted into a charry froth at 295°C
Picrate of 2,4-dinitrophenylhydrazone of betaine aldehyde (prepared after Jellinek et al.)	Melting point 160°C Reported m. pt. 181 - 3°C

Choline sulphate was prepared by the method of Schmidt & Wagner (1904) as suggested by Orsi & Spencer (1964). 5.0 g of dry choline chloride was added to 5.5 ml of concentrated H_2SO_4 and the mixture heated on a boiling water bath for 4 hours. After this time the mixture was cooled and poured into a large excess of cold absolute ethanol with atirring. A white crystalline precipitate appeared. This was collected and dissolved in a small amount of water and reprecipitated with ethanol. This was repeated until there was no trace of free inorganic sulphate in the filtrate (tested with $BaCl_2$).

Better yields and more consistent results are obtained if the base used is dry and in the salt form. When the base or liquid form was the only one available the initial reaction mixture was 1 g of base + 2.7 ml concentrated H_2SO_4. For bases other than choline the mixture was left in the cold for 24 hours and then subjected to precipitation with ethanol.

Plant Mitochondria Extraction Methods

(1) Extraction procedure for plant mitochondria based on the method of Ikuma & Bonner (1967).

Extraction Medium	0.3M	Mannitol
	0.1%	BSA
	0.05%	cysteine
	1 mM	Edta

pH adjusted to 7.2 with 6N KOH.

Wash Medium	0.3M	Mannitol
	0.1%	BSA
	1 mM	Edta

pH adjusted to 7.2 with 6N KOH.

Reaction Medium	0.3M	Mannitol
	0.01M	KCl
	0.01M	KPO_4 buffer pH 7.2
	5 mM	$MgCl_2$

pH adjusted to 7.2 with 6N KOH.

All solutions were stored at 0-4°C.

The tissue was harvested and initially macerated in a household mincer. The macerate was ground using a mortar and pestle with 5 volumes of Extraction Medium and a little acid-washed sand. Care was taken not to over-macerate to maintain the integrity of the organelles. The brei was adjusted to pH 7.2 with 6N KOH and filtered through a double layer of muslin and the filtrate was centrifuged at 1,000 g x 15 min. to remove larger cell debris. The supernatant was retained and centrifuged at 10,000 g x 15 min. The pellet was retained and resuspended in Wash Medium (≏20 volumes) using a ground glass homogeniser and resedimented at 6,000 g x 15 min. The pellet was retained and resuspended in the Reaction Medium using the ground glass homogeniser. The whole operation was performed at 0-4°C.

(2) Extraction procedure for plant mitochondria using the method of Stokes, Anderson & Rowan (1968).

Medium 1	0.35M	Mannitol
	0.35M	Sucrose
	10 mM	Phospate buffer pH 6.5
	0.1%	BSA
	1.0 mM	Edta
	2.0 mM	$Na_2S_2O_5$

Medium 2

As above but Edta omitted and the concentration of metabisulphite reduced to 1.0 mM.

Medium 3

Similar to medium 2 but with the metabisulphite omitted.

Medium 4

Similar to medium 3 but with o.5 mM Edta added.

The solutions were stored at 0-4°C. and the whole extraction procedure performed at 0-4°C. The tissue was macerated in the household mincer and then using a mortar and pestle with a little acid-washed sand in 10 volumes of medium 1. The brei was filtered through a double layer of muslin and the pH of the filtrate adjusted to approximately neutrality. The cell debris was removed by centrifugation at 600 g x 10 min. and the mitochondria pelleted at 8,000 g x 10 min. from the supernatant. The pellet was resuspended in about 20 volumes of medium 2 using a ground glass homogeniser and the mitochondrial fraction again recovered by centrifugation at 8,000 g x 10 min. This pellet was suspended as before in a small volume of medium 3, aliquots of which were added to medium 4 as the reaction medium in the reaction vessel. The use of this method was an attempt to minimise the inhibition of mitochondrial enzymes that could be caused by the presence of phenolic compounds during the extraction. This was a real problem in most of the tissues used.

(3) Rapid isolation method after Palmer (1967)

Medium 1	0.5M	sucrose
	0.05M	tris pH 7.8
Medium 2	0.4M	sucrose
	0.05M	tris pH 7.2

The whole procedure was performed at 0-4°C. The plant tissue was minced and macerated as before in 10 volumes of Medium 1. The

resulting homogenate was then stirred and filtered through 4 layers of muslin and the resulting solution centrifuged at 3,000 g x 10 min. to remove all debris and nuclei. The supernatant was retained and centrifuged at 15,000 g x 25 min. and the pellet which formed was resuspended in 20 volumes of medium 2 using a ground glass homogeniser. The mitochondria were then sedimented at 15,000 g x 25 min. and finally resuspended in a small volume of medium 2.

The attraction of this method was the speed of extraction of the organelles, allowing comparative experiments to be performed. Also the speed of extraction must mean there is less chance of damage to the enzymes of the mitochondria from quinones and fatty acids which may be present in the tissues.

(4) Extraction procedure for plant mitochondria based on the method of Sarkissian & Srivastava (1969).

Grinding Buffer	0.25M	Sucrose
	1 mM	Edta
	0.075%	BSA
	67 mM	KPO_4 buffer pH 7.2

The whole procedure was again carried out at 0-4°C. The plant tissue was minced then ground using a mortar and pestle with acid-washed sand in 10 ml of grinding buffer/g of tissue. The homogenate was strained through 2 layers of muslin and cellular debris removed at 1,000 g x 15 min. The supernatant was centrifuged at 40,000 g x 2 min. The pellet was washed with fresh grinding buffer but not suspended. The tubes were placed back in the rotor head at 90° to the previous spin and centrifuged at 20,000 g x 2 min. The pelleted mitochondria were suspended in a small volume of 0.3M Mannitol. This method is again rapid and claims to separate the mitochondrial pellet from starch (by rotating the tubes) but met with little success in this investigation.

(5) Preparation of plant mitochondria from green leaf tissue after Zelitch & Barber (1960).

Grinding medium	0.45M	sucrose
	0.05M	Mannitol-borate buffer pH 7.2
	0.03M	potassium citrate
	0.01M	Edta
	0.05M	Tris buffer pH 8.3
Washing medium	0.3M	sucrose
	2×10^{-4}M	Edta
	0.05M	Tris buffer at pH 7.5

The extraction procedure was performed at 0-4°C. Leaf tissue was minced and then ground using a mortar and pestle with acid-washed sand using about 5 volumes of grinding medium. The ground tissue at a final pH of 7.2 was expressed through 2 layers of muslin and centrifuged at 600 g x 5 min. The supernatant was retained and centrifuged at 10,000 g x 20 min. The pellet was suspended in wash medium and resedimented at 10,000 g x 20 min. The mitochondria were resuspended in a small volume of wash medium. This method yielded a final pellet containing large amounts of chloroplasts.

The Extraction of Mitochondria from Fungal Hyphae

(1) Isolation of mitochondria as by Watson & Smith (1967). The whole procedure was performed at 0-4°C. Hyphal mats were harvested and washed with cold tap water then cold distilled water and then homogenised in a ground glass homogeniser.

Grinding medium	0.5M	Mannitol
	4 mM	Edta pH 7.0

The homogenate was filtered through a double layer of moist muslin and the pH of the filtrate which was 6.5-6.8 was not further

adjusted. The filtrate was centrifuged at 800 g x 5 mins, and the residue resuspended in extraction medium and again centrifuged at 800 g x 5 min. The two supernatants were combined and the pellet collected at 12,000 g x 10 min. The pellet was then suspended in a wash medium containing 0.5M Mannitol and 0.5mM Edta pH 6.5 and the suspension was centrifuged at 800 g x 5 min. The supernatant was retained and the mitochondria collected at 12,000 g x 8 min. The final suspension was in 0.5M Mannitol.

(2) Isolation of Mitochondria as by Hall and Greenawalt (1964).

The extraction was performed at 0-4°C.

Preparation Medium	0.25M	sucrose
	0.005M	Edta
	0.15%	BSA

The hyphae were ruptured in a ground glass homogeniser and the mitochondria were collected by differential centrifugation between 1,500 g and 8,000 g. A second 1,500 g centrifugation before washing and collecting the mitochondria at 8,000 g removed excessive cellular debris. The mitochondrial pellet was resuspended in a small volume of preparation medium.

(3) Rapid preparation of Neurospora crassa mitochondria using the method of Hall and Baltscheffsky (1968). The procedure was performed at 0-4°C. The hyphal mat was collected by pouring cultures through a nylon bag (double layer, mesh about $50/cm^3$). The hyphae were then resuspended in 200 ml of sorbitol 0.6M. The mat was broken up with scissors and poured through the bag again, and then resuspended in 200 ml of a preparation medium.

Preparation Medium	0.25M	sucrose
	0.005M	Edta
	0.3%	BSA

The hyphae were broken up in a ground glass homogeniser (fairly loose, medium coarse). Cell wall breakage was checked under a light microscope. The even suspension was then poured through the nylon bag to remove whole cells, cell walls, etc., and centrifuged at 10,000 g x 5 min. The pellet was resuspended in 100 ml of preparation medium and centrifuged at 2,000 g x 1 min, and the supernatant was retained and centrifuged at 10,000 g x 5 min. and the pellet was resuspended in 3 ml of preparation medium.

The last method gave relatively active mitochondria but the others were unsuccessful in these investigations.

For comparative purposes mitochondria were isolated from rat liver by differential centrifugation in isotonic sucrose (0.25m) as described by Schneider and Hogeboom (1950). Rat livers (each rat liver ≏ 10g) were cooled in a beaker in an ice bath. The whole procedure was performed at 0-4°C. The livers were macerated in a chilled mortar with a little acid-washed sand and 9 volumes of 0.25M sucrose. The macerate was passed through 3 layers of muslin and the filtrate centrifuged at 2,000 g x 10 min. to sediment the muclei and red blood cells. The sediment was washed twice in 0.25M sucrose and recentrifuged at 2,000 g x 10 min. The supernatant and washings were combined and centrifuged at 9,200 g x 10 min. The sediment was washed twice in 0.25M sucrose and resedimented at 9,200 g x 10 min. The mitochondrial pellet was resuspended in a small volume of 0.25M sucrose:-

Reaction Medium — G. R. Williams (1960)

0.255M	sucrose
0.0225M	KCl
0.001M	KPO_4 buffer pH 7.2
2×10^{-5}M	DNP

Acetone powder extractions of plant mitochondria were performed using the method of Hiatt and Evans (1960). These extracts were stored in vacuo at -20°C. Similar extracts of rat liver mitochondria were shown to retain their activity when stored (Williams and Sreenivasan 1953). The plant mitochondria were isolated after the method of Ikuma and Bonner (1967). The mitochondria were dispersed in 20 volumes of cold acetone (stored at -20°C.) and resedimented. The powder was then dispersed in 20 volumes of cold diethyl ether (stored at -20°C.) and resedimented. The powder was then air-dried for 15 mins and the ether removed at the vacuum pump and the powder stored in vacuo at -20°C.

The Preparation of Electron Micrographs from Mitochondrial Pellets.

(1) The pellet was fixed in 4% glutaraldehyde in 0.1M phosphate buffer pH 7.0 for 3 hrs.

(2) The sample was then washed in the same buffer (3 changes of 10 min. each).

(3) Post-fixing was performed in 1% Osmium tetroxide in 0.1M phosphate buffer pH 7.0 for 30 mins.

(4) The sample was dehydrated in ethanol series 25%, 50%, 75% and twice in 100% (20 mins. in each).

(5) The sample was transferred to propylene oxide for 20 mins.

(6) After embedding in Araldite, sections were cut using the LKB Ultratome.

(7) The sections were then examined in an AEI EM_6B. Electron Microscope.

The Assay of Mitochondrial Activity

(1) Spectrophotometry

Various methods for the assay of mitochondria have been attempted and modified for the particular problems involved. Electron accepting dyes have been used in animal work to assay

both the primary enzyme and the oxidase complex and the considerations involved have been reviewed by Singer and Kearney (1955) with respect to succinic dehydrogenase activity. Williams and Sreenivasan (1953) developed a spectrophotometric assay for choline dehydrogenase based on the reduction of 2, 6 - dichlorophenolindophenol.

Acetone powders of the mitochondrial preparations were homogenised in 0.1M sodium phosphate buffer at pH 6.8 for rat liver mitochondria and pH 7.2 for plant mitochondria. Freshly prepared plant mitochondria were suspended in Reaction Medium after Ikuma and Bonner (1967) and rat liver mitochondria suspended in the Reaction Medium after G. R. Williams (1960).

2, 6 - dichlorophenolindophenol	10 mg % solution
Choline	2% solution

(Both made up in 0.1M sodium phosphate buffer.)

Assay Procedure

The cuvette contained:-

0.6 ml	2, 6 - DCPIP
0.6 ml	buffer
0.8 ml	water (distilled)
0.2 ml	substrate (distilled water control)
1.0 ml	mitochondrial preparation

The change in O.D. was measured at 607 mμ, in a Unicam SP 800. N-methyl phenazonium methosulphate was employed. The assay medium without the 2, 6 - DCPIP but containing the N-methyl phenazonium methosulphate (2 mg/cuvette) was also employed and the change in O.D. at 387 mμ measured.

(2) Polarography

Polarographic estimations of mitochondrial and enzyme preparations were performed using a simple platinum/silver oxygen

electrode (Rank Bros., Bottisham, Cambridge). Reaction media were allowed to equilibrate before substrates and co-factors were introduced into the reaction chamber via a movable well with a small opening. The electrode was designed for following the uptake or production of oxygen by cell suspensions, subcellular particles or enzyme systems. The principle of operation was that first described for the Clark electrode. Oxygen diffused through a thin (0.0005 cm) teflon membrane and was reduced at a platinum surface immediately in contact with the membrane. The other half-cell was also incorporated in the incubation vessel and was composed of a silver-silver chloride electrode (this was modified in later models).

The electrode was set up by first detaching the base of the incubation vessel by unscrewing the perspex locking nut.. Sufficient saturated potassium chloride solution was added to wet the silver and platinum electrodes. A 1 cm square of lens tissue with a 1 mm diameter hole in the centre was placed over the platinum electrode so that the hole was over the electrode. A 1 cm square of teflon was placed immediately on top of this and locked into place by screwing down the incubation vessel. Care had to be taken that no air bubbles became trapped and that the membrane was not twisted. The electrode was placed on a magnetic stirrer. The vessel was enclosed in a water jacket enabling water of a known and constant temperature to be circulated.

The platinum electrode is polarised at -0.6v with respect to the silver chloride. The current flowing in these conditions being about 1 H amp in stirred air-saturated water at 30°C.

The current flowing was proportional to the activity (partial pressure) of oxygen in solution over a wide range.

The sensitivity control resistor was a 10 turn helipot. The

choice of value for the sensitivity control resistor depended on the span of the recorder. A suitable value was given by:-

$$R = 2 \times \text{span of recorder in (mv)} \times 10^3 \text{ ohms.}$$

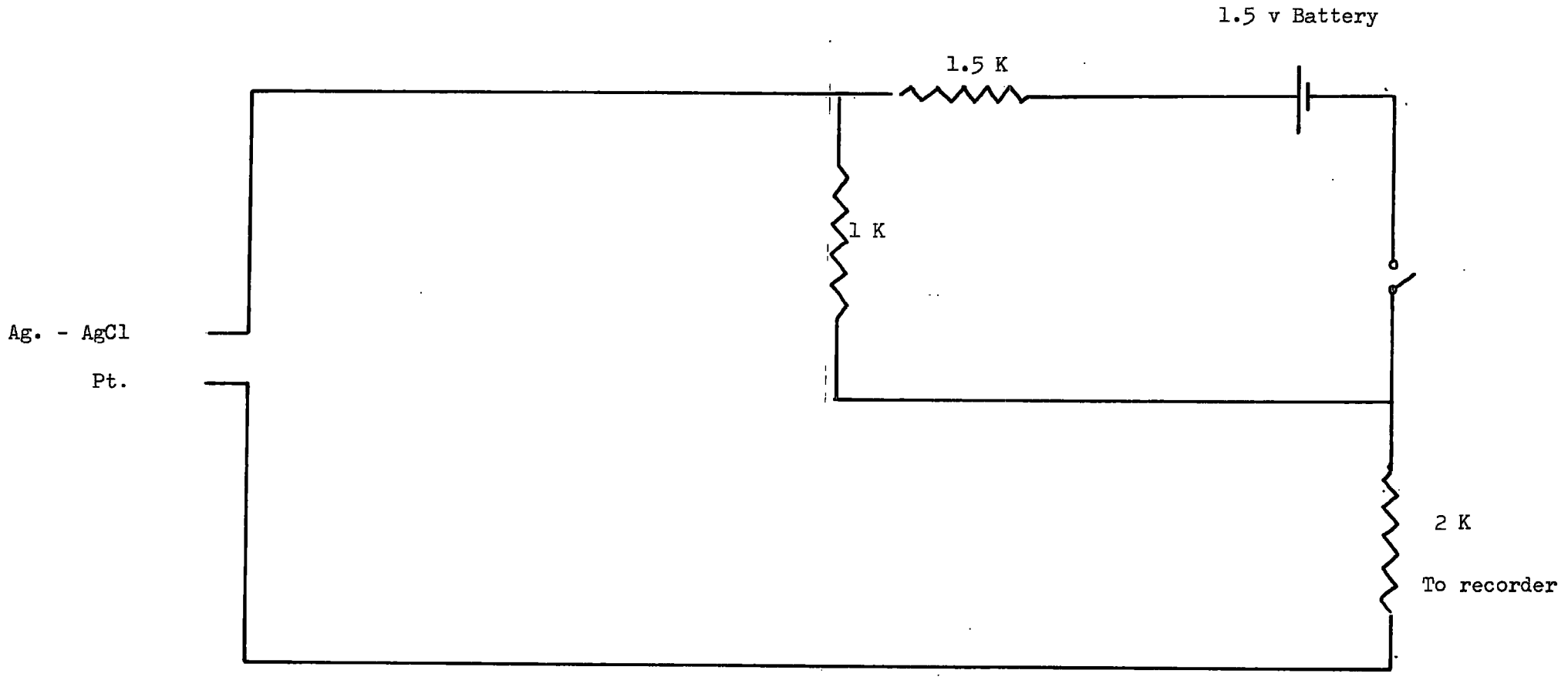
Ideally R should not exceed 20K as this would alter the polarising voltage. Any potentiometer recorder with a sensitivity better than 2 Kmv full scale and which would accept a source impedance of 1K/mv span was suitable. For the best results a response time of 1-2 secs. was advisable. The recorder used during these investigations was a Vitatron linear/logarithmic integrating recorder (Dieren, Holland).

To set up the apparatus the Ag-AgCl electrode was connected to the positive side of the potential divider and the platinum to the negative. Air-saturated medium was added to the incubation vessel and the perspex cover replaced and the magnetic stirrer started. The sensitivity control was adjusted to give a suitable deflection on the recorder. When a steady state was achieved the experiment could be commenced.

To test for residual current a solution of sodium dithionite was added. The electrode current should fall within 5 secs. to zero or nearly so. If this did not occur the membrane was changed and the electrical connections checked. Noise on the recorder response was usually due to a "leaky" membrane. This was remedied by changing the membrane.

Suitable data for the oxygen content of solutions were referred to in the International Critical Tables and the Handbook of Physics and Chemistry.

Radioactive incubations were performed and followed in the polarograph. ^{14}C-methyl choline and 1,2, ^{14}C-choline were used as tracers. After the incubations had been performed the mixture was deproteinised by heating to 100°C for about 10 mins. The filtrate was subjected to the Reineckate procedure.



Suggested Circuit Diagram

Chemical Methods of Analysis of Quaternary Ammonium Compounds.

(1) Ion-exchange Column Chromatography

Zeo-Karb 226 resin 100 mesh beads, hydrogen form, was regenerated and buffered at pH 7.3 with phosphate-citrate buffer (McIlvaine 1921) as described by Cromwell and Richardson (1966). The weakly cationic resin Amberlite C.G.-50 Chromatographic grade, type 1, 100-200 mesh was treated as above and the elution profile of the compounds from the column proved to be very similar. The resin was regenerated by suspension in 1 l of distilled water and 40% sodium hydroxide. The resin was stirred continuously and more alkali added to a pH of approximately 11. The resin was then stirred for a further three hours. The resin was then washed by decanting with 10 l of distilled water, the pH of the filtrate falling to about 10. The resin was converted to the acid form by washing with 10 l of 3N-HCl. The resin was again washed with 10 l of distilled water. The resin was then suspended in twice its own volume of phosphate-citrate buffer pH 7.3 and stirred overnight adjusting the pH with additions of solid analar sodium hydroxide until it remained unchanged at 7.3. The resin was stored at $0^{\circ}C$ under a layer of toluene to minimise bacterial contamination.

The column was poured in a thick slurry and allowed to settle to final column dimensions of 1.5 cm x 125 cm or 1.6 cm x 120 cm depending on which was required. The sample for analysis was introduced into the top of the column in a small volume (5-7 ml) of phosphate citrate buffer pH 5.3. This was washed into the column with another small volume of buffer. A reservoir of buffer was set up giving a hydrostatic head and continuous flow. The compounds were eluted off at room temperature and samples of a given volume were collected using a fraction collector (Beaumaris Co.). The usual flow rate obtained was around 1 ml/min.

(2) Determination of Quaternary Ammonium Compounds

Column effluent fractions were analysed by measuring the absorbance of their periodide derivatives at 365 mμ. Aliquot samples (0.5-1.0 ml) of the effluent fractions were made up to 2 ml with 2N HCl (samples containing betaine aldehyde were adjusted to pH 7.3 as the formation of the periodide of this compound was greatly reduced under acid conditions. 1 ml of a reagent (10 g of resublimed iodine + 12.4 g of KI in 1 l of water) was then added to the solutions which were shaken and placed in an ice-bath for 20 mins. 10 ml of 1,2 - dichloroethane were then added and the two layers were mixed by bubbling a fine stream of nitrogen through them for exactly 30 secs. The absorbance of the organic layer was determined in a Unicam S.P. 800 at a wavelength of 365 mμ within 10 min. and compared with standard curves obtained previously for each of the quaternary ammonium compounds.

Table 1 gives the elution volumes of the quaternary ammonium and related compounds under investigation by column chromatography on Zeo-Karb 226 and Amberlite C.G. 50 resin. The samples applied contained 0.5-3.0 mg of the compound. The elution volume was taken as the total volume eluted at the peak in a column of 1.6 cm bore and a resin depth of 120 cm filled with resin buffered with phosphate-citrate buffer pH 7.3 and eluted with buffer pH 5.3. The eluate was collected as soon as the compound had been applied.

Table 2 shows the optical densities of periodide derivatives of quaternary ammonium and related compounds. Each value given in these tables was the mean of five separate determinations.

Isolation of Quaternary Ammonium Compounds from Radioactive Incubations.

The supernatants were acidified with a little concentrated hydrochloric acid and the bases precipitated as their water-insoluble

TABLE 1

COMPOUND	ELUTION VOLUME (ml)
Betaine	110
Betaine aldehyde	450
Choline	350
Choline sulphate	230
Dimethylethanolamine	480
Dimethylglycine	200
Phosphorylcholine	80
Trimethylamine	380
Trimethylamine-N-oxide	100
Tetramethylammonium hydroxide	280

Table 1 demonstrates the elution volumes of the quaternary ammonium compounds under investigation obtained during column chromatography on Zeo-Karb 226 and Amberlite C.G. 50 resin. A column of 1.6 cm bore and a resin depth of 120 cm was used. The resin was buffered with phosphate-citrate buffer pH 7.3 and eluted with the same buffer at pH 5.3.

TABLE 2

COMPOUND	$E^{\mu\ mole}_{1\ cm}$ at 365 mμ
Acetylcholine	2.825
Betaine	1.006
Betaine aldehyde	1.476
Choline	2.134
Dimethylethanolamine	0.400
Dimethylglycine	0.610
Phosphorylcholine	0.195
Trimethylamine	0.925
Trimethylamine-N-oxide	1.117
Tetramethylammonium hydroxide	2.700

Table 2 - Optical densities of periodide derivatives of trimethylamine and related quaternary ammonium compounds.

reineckate derivatives by the addition of excess saturated ammonium reineckate solution. A little betaine or choline was added as a "carrier" compound. The reineckates were collected by centrifugation and converted to the free bases by the method of Byerrum, Sato and Ball (1956). The reineckates were dissolved in the minimum volume of acetone and excess saturated silver nitrate solution was added and the precipitate of silver reineckate filtered off. Excess silver was removed as the chloride by adding excess concentrated hydrochloric acid. The filtrate was evaporated to dryness on a rotary evaporator. The residue was taken up in a small volume of water and evaporated to dryness in a vacuum desiccator over sodium hydroxide (to remove excess hydrochloric acid).

Thin-layer chromatography. (Table 3)

The method used was essentially that employed by Eneroth & Linstedt (1965) for the separation of compounds related to carnitine. Plates of Silica Gel G with an approximate wet thickness of 0.4 mm were prepared using a Unoplan leveller (Shandon Scientific Co., London). 40 g of the powder were mixed in an electrical mixer with 80 ml of water for 2 x 30 secs. before pouring into the spreader. When the plates were completely dry, samples of the compound under investigation were spotted onto the plates using a microcapillary. Solvent systems investigated were:

(1) Methanol-acetone-HCl (90:10:10 v/v)

(2) Methanol-acetone-HCl (90:10:4 v/v)

(3) Methanol-0.88 ammonia (75:25 v/v)

(4) Methanol-dioxane-0.88 ammonia (30:45:25 v/v)

For two-dimensional separations solvent (3) was used for the first dimension and was followed by solvent system (2) in the second dimension. After the two dimensions had been developed the plate was dried and placed in a desiccator containing iodine vapour from

TABLE 3

COMPOUND	SOLVENTS			
	1	2	3	4
Betaine	0.55	0.48	0.47	0.10
Betaine aldehyde	0.48	0.42	0.06	0.03
Choline	0.37	0.31	0.01	0.01
Choline Sulphate		0.32	0.45	
Dimethylethanolamine		0.47	0.57	
Dimethylglycine	0.59	0.51	0.60	0.18
Phosphorylcholine		0.28	0.01	
Trimethylamine-N-oxide		0.90	0.77	
Tetramethylammonium hydroxide		0.90	0.77	

Table 3 gives the R_F values obtained by thin-layer chromatography on Silica Gel G of quaternary ammonium and related compounds.

Solvent 1 Methanol-acetone-HCl (90:10:10 v/v)

2 Methanol-acetone-HCl (90:10:4 v/v)

3 Methanol-0.88 Ammonia (75:25 v/v)

4 Methanol-Dioxane-0.88 Ammonia (30:45:25 v/v)

The compounds were detected using Iodine vapour.

a few crystals at the bottom. Spots of the quaternary ammonium compounds appeared after a few minutes as brown areas which were outlined with a fine needle (the brown colour fades rapidly).

Paper Electrophoresis (Table 4)

The method used was a slight modification of that reported by Brockhuysen, Dierickx and Deltour (1961), and Deltour, Brockhuysen and Dierickx (1960) and Brockhuysen and Deltour (1961) for the separation of carnitine and several other quaternary ammonium compounds. Electrophoresis was carried out on strips (40 x 410 mm) of filter paper (Schleicher and Schull No. 2043) impregnated with 0.3M acetic acid-pyridine buffer pH 4.0, for 90 mins. at 340 V. Aqueous samples of the bases (containing approximately 100 µg) were applied from a capillary tube as a thin streak to a line a known distance from the end of the paper strip. The paper strips were then painted with 0.3M acetic acid-pyridine buffer pH 4.0, to within 1-2 cm of the origin. The solvent rose gradually up to the origin by capillary action and in so doing compacted the origin into a sharper band. The ends of the strips were allowed to dip into the anode and cathode chambers of the electrophoresis apparatus filled with equal volumes of the 0.3M acetic acid-pyridine buffer pH 4.0. The end of the paper strip nearest to the origin was placed in the anode compartment. After electrophoresis the strips were removed and dried in a current of circulating air for 24 hours to remove all traces of the pyridine which would otherwise interfere with the location of the quaternary ammonium bases. No corrections were made for electro-endosmosis.

Ion-exchange resin Paper Chromatography (Table 5)

The chromatography of the quaternary ammonium compounds was investigated on two types of ion-exchange resin paper. "Amberlite" WA-2 contains a resin in which a carboxylic acid group is the functional part. It behaves in a similar fashion to Zeo-Karb 226.

TABLE 4

COMPOUND	ELECTROPHORETIC MOBILITY (Distance moved in cms)
Betaine	5.0
Betaine aldehyde	11.0
Choline	12.2
Dimethylglycine	5.5
Phosphorycholine	4.5
Trimethylamine	18.2
Trimethylamine-N-oxide	14.8
Tetramethylammonium hydroxide	16.4
Dimethylethanolamine	11.0
Choline Sulphate	4.0

Electrophoresis was carried out on strips (40 x 410 mm) of filter paper (Schleicher and Schull No. 2043) impregnated with 0.3M acetic acid-pyridine buffer pH 4.0 for 90 mins. at 340 V.

TABLE 5

COMPOUND	R_F value Amberlite SA - 2	R_F value Amberlite WA - 2
Betaine	0.92	0.82
Betaine aldehyde	0.51	0.39
Choline	0.59	0.29
Dimethylglycine	0.86	0.85
Phosphorylcholine	0.95	
Trimethylamine	0.58	0.25
Trimethylamine-N-oxide	0.38	0.41

Table 5 shows the R_F values obtained for the quaternary ammonium compounds using exchange paper chromatography. Amberlite WA - 2 paper was impregnated with 0.2M phosphate-citrate buffer pH 7.3 and the papers were developed with the same buffer at pH 5.3. Amberlite SA - 2 paper was developed using IN-HCl as the solvent.

and Amberlite C.G.-50. The paper was impregnated with 0.2M phosphate-citrate buffer pH 7.3 and the papers were developed with the same buffer at pH 5.3. "Amberlite" SA-2 contains a sulphonated copolymer resin which, in the hydrogen form, is similar in acidity to sulphuric acid. These papers were pretreated with IN-HCl to remove a substance which gave rise to anomalous blackening of X-ray films. The papers were washed with IN-HCl and distilled water and dried before use. IN-HCl was used as the developing solvent.

Paper Chromatography (Table 6)

The papers used throughout were Whatman No. 3 MM. The compounds under investigation were applied using micro-capillaries. The papers were developed by descending chromatography. The following solvent systems (all single phase) were used.

(1) Ethanol-0.88 ammonia 95:5 (Bregoff, Roberts and Delwiche (1953)).

(2) n-butanol-acetic acid-water 4:1:2 (Iwamoto (1958)).

(3) n-butanol-ethanol-28% ammonia 8:1:3 (Fleming (1960), Fowler (1962)).

(4) n-butanol-acetic acid-water 4:4:1 (Munier and Macheboeuf (1949)).

(5) Pyridine-sec.-butanol-water 6:3:1 (Neilsen (1963)).

(6) n-butanol-ethanol-acetic acid-water 9:1:1:2 (Byerrum, Sato and Ball (1956)).

Radioactive Analysis

Radioactive bases isolated from incubation mixtures by the Reineckate method and separated by ion-exchange column chromatography were determined by the liquid scintillation method described by Richardson (1966) using a Beckman liquid scintillation spectrometer

TABLE 6

COMPOUND	1	2	3	4	5	6
Betaine	0.53	0.31	0.29	0.47	0.14	0.50
Betaine aldehyde	0.75 (0.48)	0.36	0.56	0.61	0.20	0.79 (0.50)
Choline	0.65	0.32	0.23	0.63	0.22	0.76
Dimethylethanolamine	0.43		0.36	0.35		0.71
Dimethylglycine	0.52	0.31	0.28	0.41	0.33	0.45
Phosphorylcholine	0.55		0.42	0.25	. .	
Trimethylamine	0.49		0.25	0.55	0.35	0.56
Trimethylamine-N-oxide	0		0.36	0.52	0.32	0.55
Tetramethylammonium hydroxide	0.40			0.40	0.30	

Number	SOLVENT SYSTEM (all single phase)	
1	n-butanol-acetic acid-water	4:1:2 v/v
2	Pyridine-sec-butanol-water	6:3:1 v/v
3	n-butanol-ethanol-acetic acid-water	9:1:1:2 v/v
4	95% ethanol-0.88 ammonia	95:5 v/v
5	n-butanol-ethanol-28% ammonia	8:1:3 v/v
6	n-butanol-acetic acid-water	4:4:1 v/v

Table 6 demonstrates the R_F values observed for the quaternary ammonium and related compounds following paper chromatography. The general reagent used for the detection of the spots was I_2(0.02%) in petroleum ether (b.p. below 40°C.) (Brante (1949)). Figures in parentheses indicate anomalous stained areas.

series 200 B (Beckman Instruments Inc., Fullerton, Calif., U.S.A.). The scintillation fluid consisted of 6 g of 2, 5-diphenyloxazole and 0.6 g of 1, 4-bis 2-(4-methyl-5-phenyloxazolyl)-benzene in 1 l of toluene.

0.3 ml aliquots of the column fractions were pipetted into a mixture of 5 ml scintillation fluid and 5 ml of ethanol in scintillation bottles.

Radioautographs of all chromatograms of ^{14}C-labelled compounds were made with Ilford Industrial G X-ray films. X-ray films were affixed to TLC plates or paper electrophoresis strips under the recommended safe-light precautions and allowed to develop for about a week in total darkness. The radioautographs were developed under safe-light conditions for 5 mins. in X-ray Phenisol developer (1 part developer / 4 parts water), washed twice in distilled water and transferred to fixer for 5 mins. The position of radioactive compounds was shown by blackened areas on the plates.

Recently Wilken (1970) developed a solvent system, n-butanol/ethanol/acetic acid/H_2O 8:2:1:3 v/v and showed a separation of ^{14}C-labelled betaine, betaine aldehyde and choline when subjected to descending paper chromatography for 20 hours.

Mitochondrial Permeability

Williams (1960) has observed that choline failed to stimulate the respiration of rat liver mitochondria diluted with 1.0 mM phosphate buffer made isotonic with 0.15M solutions of LiCl, NaCl, KCl, CsCl, NH_4Cl, KI, K_2SO_4 and tetraethylammonium bromide. In a lightly buffered sucrose medium the mitochondria respired actively with choline as substrate. Choline respiration was nearly completely inhibited by concentrations of KCl higher than 0.1M. This was first thought to be a permeability factor but was obviously more complex than this simple explanation.

Treatment of Mitochondria

(1) Pretreatment with 0.06% sodium deoxycholate at 0°C for 5 mins.

(2) Repeated freezing and thawing.

(3) Addition of 1.0 mM Ca^{++} to reaction medium.

(4) Ageing of mitochondria.

(5) Pretreatment with 0.1% Tergitol.

(6) Dimethylsulphoxide (concentrations 1-25%) added to the reaction medium. (Delmer and Mills, (1969)).

Various workers had shown that changes in water content and optical density ran parallel. The effect on the optical density at 520mμ was observed when mitochondria were incubated in a medium containing:-

0.2M KCl

25mM tris-chloride pH 7.4

0.3M mannitol

2mM-50mM choline (as required)

The penetration of radioactive choline into mitochondria was investigated by incubating mitochondria with 1, 2 ^{14}C-choline for varying periods. The mitochondria were pelleted, washed and re-pelleted several times and then subjected to freezing and thawing very rigorously and finally extracted. The extracts and the washings were separately analysed for radioactive choline content, using methods previously described.

Histochemical Localisation of Dehydrogenases

The method used was that of Seligman and Rutenberg (1951). Tissue sections of fresh plant material were prepared and incubated in reaction medium. The reaction medium consisted of:-

0.05M substrate

0.1% MTT tetrazolium or Nitro BT

0.05M Sodium phosphate pH 7.3

Trace quantities of phenazonium methosulphate were added to act as an electron carrier. Incubations were performed in the dark and in vials to minimise atmospheric oxidation. The duration of the incubation varied from 5-20 mins. at room temperature. Control incubations were performed containing no added substrate in the reaction medium. After the incubations the tissue sections were mounted in 50% glycerine and examined for dark blue staining spots indicating the presence of dehydrogenases.

An alternative reaction medium was also employed (Koenigs (1966)) consisting of:-

1.0M substrate solution

0.067M Phosphate buffer pH 7.0

1mg/ml Nitro BT.

50mg/ml NAD.

Supernatant Protein Extraction

Pilot experiments were performed on about 10 g. of leaf tissue of Beta vulgaris, Chenopodium vulvaria and etiolated seedlings of Vigna radiata (Phaseolus aureus).

0.05M Tris-HCl buffer pH 7.6 - 8.0

1.0mM Edta was the extraction medium used.

The tissue was macerated using a mortar and pestle with a little acid-washed sand and a small volume of extractant to form a thick paste. The paste was expressed through three layers of muslin and the supernatant retained. This supernatant was centrifuged at 50,000 g for 1 hour. Samples of the resulting supernatant were layered on polyacrylamide gels for electrophoresis. The resultant gels were incubated with tetrazolium salts to detect the presence of dehydrogenases. Large scale experiments were performed using various extraction media. The buffers used were either 0.05M Tris-HCl pH 7.6 - 8.0 or sodium phosphate pH 7.6 - 8.0. Edta,

cysteine, ascorbic acid, BSA, potassium metabisulphite from 1 - 10mM were used in different combinations.

Browning occurred in all extracts from higher plant tissues. This was taken to be due to the presence of polyphenols and their oxidation by endogenous phenol oxidases. To counteract this the following treatments were attempted. To the extraction medium were added:-

(1) 1% /w/v) insoluble PVP (polyvinylpyrrolidone)

(2) 1% (w/v) Polyclar AT

(3) 1% (w/v) soluble PVP

(4) Metabisulphite (20mM)

The extraction of enzymes and subcellular organelles from plant tissue and the various components of extracting media were reviewed by Anderson (1968). Large scale extractions of tissue usually consisted of 100 g quantities of mature leaves and 500 g quantities of etiolated seedlings. These were minced before maceration using a mortar and pestle with acid-washed sand and 2 ml extractant/g of tissue (fresh weight), or in a Waring blender where appropriate. The macerates were allowed to extract for varying lengths of time up to about 15 mins. The macerates were then expressed through layers of muslin and centrifuged at 23,000 g x 1 hour. The straw-coloured supernatant was retained for assay. The whole procedure was performed at 0-4^{o}C.

Extracts of fungal hyphae were made either by the method of Sanwal and Lata (1961) or that of Barratt and Strickland (1963).

The Extraction Medium as described by Sanwal and Lata (1961)

0.1M tris-HCl pH 8.0

β-mercaptoethanol 5 x 10^{-4}M

The Extraction Medium based on that of Barratt and Strickland (1963)

0.1M sodium phosphate buffer pH 7.5

20mM metabisulphite

Extraction method after Sanwal and Lata (1961):-

Hyphal mats were collected, washed repeatedly with distilled water and the excess water expressed. The mass of cells was frozen ($-20^{o}C$) before maceration using a pestle and mortar with acid-washed sand and the mass extracted with 3 volumes of buffer. The mass was expressed through 3-4 layers of muslin and the filtrate centrifuged at 11,000 g for 15 mins. The opalescent supernatant was used for assay and subsequent concentration.

The extraction method was in substance that described by Barratt and Strickland (1963):-

Mycelia were harvested by vacuum filtration and washed with 0.1M sodium phosphate buffer pH 7.5, frozen and stored at $-20^{o}C$. The frozen mycelia were ground in extraction medium in a mortar with acid-washed sand and the mass extracted with about 3 volumes of extractant. The mass was expressed through muslin and the filtrate centrifuged at 38,000 g x 30 mins. and the supernatant retained. Nucleic acids were removed by adding protamine sulphate 15 mg/100 mg of protein and precipitated at $0^{o}C$ by adjusting the pH to 6.5 with IN-acetic acid. The supernatant was retained, the precipitate being removed by centrifugation.

For comparative purposes a supernatant extract was prepared from rat liver using the method of Rothschild and Barron (1954). The livers from 2 or 3 rats were cooled in ice, cut into small pieces and homogenised in a chilled mortar with a little acid-washed sand in 10 volumes of ice-cold KCl-$KHCO_3$ solution containing 80 ml 0.15M KCl and 20 ml 0.15M $KHCO_3$. The suspension was centrifuged at 600 g x 20 mins. The supernatant fluid was then centrifuged at 152,000 g x 40 mins. The bottom portion containing mitochondria and microsomes and the upper fat layer were both discarded. The optically clear fluid was retained for assay.

The Folin Method for estimation of protein

(Lowry, Rosebrough, Farr and Randall (1951))

Solutions (1) 2% $Na_2 CO_3$ in O.IN NaOH

(2) 1% $CuSO_4 . 5H_2O$

(3) 2% Sodium potassium tartrate

Alkaline Copper solution

50 ml of (1)

0.5 ml of (2) made up when required and then

0.5 ml of (3) discarded after use.

Folin Ciocalteu phenol reagent diluted 1:1 (IN)

A standard solution of bovine serum albumin was made up giving 1 mg/ml, i.e. 1,000 µ g/ml. Table 7 gives the range of dilutions for the calibration curve. The reference cell contained 1 ml of distilled water. To the samples, 4 ml of alkaline copper solution were added and thoroughly mixed and allowed to stand at room temperature for 10 mins. 0.4 ml of Folin's reagent was added, mixed and allowed to stand for 1 hour. The optical density was measured against the reference sample at 500 mµ on the Unicam SP 800. Duplicates were prepared. The instrument was adjusted to zero using the distilled water as a reference blank.

Protein was precipitated from undiluted extracts with TCA (final concentration 10% w/v) and the precipitate washed twice with acetone. The washed precipitate was extracted with O.IN-NaOH at 50° for 2 hours, and an aliquot used for estimating protein by the method of Lowry et al. (1951) using BSA as standard.

Spectrophotometic Assay

(1) Rat Liver Supernatant Rothschild and Barron (1954)

tris buffer pH 8.84	1.8 ml
NAD	0.2 ml containing 0.3mM
0.1M cysteine	0.15 ml
0.1M $MgCl_2$	0.15 ml

TABLE 7

Amount of Standard Solution	mls 1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
H_2O	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
Amount of Protein	μg 1000	900	800	700	600	500	400	300	200	100

The range of dilutions of a standard solution of bovine serum albumin for a calibration curve of protein concentrations.

0.54M NaCl	0.3 ml
Enzyme extract	0.3 ml

At zero time 0.1 ml of 0.1M betaine aldehyde was added and the reaction was followed spectrophotometrically at 330mμ - 340 mμ .

(2) Higher Plants and Fungal Supernatant.

Reaction medium was essentially the same as above but a range of buffer pH's was employed. A range of concentrations of NAD was also used.

Polyacrylamide Gel Electrophoresis

The method used was that of Ornstein and Davis (1962) as modified by Fox, Thurman and Boulter (1963).

Solutions	(A)	NHCl	48 ml
		tris	36.6 g pH 8.9
NNNN' tetramethyl 1:2 diaminoethane		temed	0.46 ml
		Solution made up to 100 ml with distilled water.	
	(B)	NHCl	48 ml
		tris	5.98 g pH 6.7
		Solution made up to 100 ml with distilled water.	
	(C)	acrylamide	30 g
NN' methylene bis-acrylamide		bisacryl-amide	0.8 g
		Solution made up to 100 ml with distilled water.	
	(D)	acrylamide	10 g
		bisacryl-amide	2.5 g
		Solution made up to 100 ml with distilled water.	
	(E)	riboflavin	4mg
		H_2O	100 ml

(F) sucrose 40%

Solution 1 contains 1 part A

2 parts C pH 8.7 - 9.0

1 part H_2O

Solution 2 contains 1 part B

2 parts D pH 6.5 - 6.8

1 part E

4 parts F

Buffer used in the electrophoresis of the gels.

Tris 6.0 g

glycine 28.8 g usually diluted 5:1 with distilled

H_2O up to 100 ml water

One volume of solution 1 was added to 1 volume of a solution containing 0.14 g/100 ml of ammonium persulphate. The mixture was introduced into standard lengths of perspex tubing to a standard depth and allowed to polymerise. A drop of water was placed on the top of the gel to give a smooth interface on polymerisation. After polymerisation the water was shaken off and solution 2 added to about 1 cm depth. This layer was polymerised (again with a drop of water on the top) in the light. The layer changes to an opaque grey colour.

The tubes were placed in the apparatus and the electrode reservoirs filled with buffer. The compound under analysis was taken up in 40% sucrose solution and layered onto the top of the gels. Bromophenol blue was added to the upper reservoir as a marker and electrophoresis performed at a current of 5mA/tube. The concentration of protein in the sample varied from 50-300µg. The electrophoresis was performed in a cold room at a temperature of 0-4°C, to cut down the denaturation of protein caused by ohmic heating.

The Detection of Dehydrogenases using Tetrazolium Salts.

(After Laycock, Thurman and Boulter (1965)).

Incubation medium:- 0.1M sodium phosphate buffer pH 6.5

substrate 5mg/ml

NAD 1mg/ml

N-methylphenazonium methosulphate 0.1mg/ml

Tetrazolium salt 1mg/ml

either MTT tetrazolium or Nitro Blue tetrazolium.

The gels were incubated in the medium at room temperature in the dark until a blue band developed. The gels were then placed in a large volume of water to allow the colour to develop. Control gels were incubated and the presence of a diaphorase was allowed for. The electron carrier N-methyl phenazonium methosulphate was very light sensitive and sensitive to atmospheric oxidation. The initial yellow solution was oxidised via the green semiquinone to the blue pyocyanin (both poor electron carriers in this reaction.)

Staining for Total protein

The protein stains employed were 0.5% Amido black in 7% acetic acid, and 0.25% Coumassie blue in methanol-H_2O-acetic acid (5:5:1 v/v). The gels were placed in the solutions for 20 mins. They were then washed in their particular solvent and destained in the same solvent by electrophoresis at about 1 amp current, until the individual protein bands could clearly be discerned. The gels were scanned using a Joyce-Loebl Chromoscan densitometer.

Concentration of Protein Extracts

(1) Ammonium sulphate fractionation

Protein samples were treated with $(NH_4)_2SO_4$ to 30% saturation and stirred at 0^oC for 15 mins. The precipitate was collected by centrifugation and the supernatant then treated

with $(NH_4)_2SO_4$ to 60% saturation and the precipitate again collected by centrifugation. The two precipitates were dissolved in a small volume of extractant buffer and dialysed against distilled water at $0^{\circ}C$ overnight.

(2) Polyethylene glycol

Protein samples were poured into dialysis tubing and placed in bowls surrounded by the polymer flakes. This concentrated the extract by absorbing water out of the samples. The procedure was performed at $0^{\circ}C$ and the chemical absorbent changed fairly regularly for efficiency.

(3) Ultrafiltration

The protein extracts were concentrated using the Ultrafiltration cell Amicon Diaflo Model 401 using the PM 10 membrane. There was an optimal concentration factor as when the extract had been brought to small volume, precipitation of protein occurred. The procedure was performed at $0^{\circ}C$.

Lipid Determination

Examination of the choline esters and total lipids extractable from various materials as described by Kaschnik, Peterlik and Weiss (1969) was performed. The tissue samples were extracted for 30 secs. using a top drive blender in 2:1 $CHCl_3$/MeOH. The macerate was allowed to stand for 30 mins. The extract was submitted to thin-layer chromatography on Silica Gel HR Merck. The chromatogram was developed in $CHCl_3$/MeOH/H_2O, 70:30:5.

Methods of detection of Lipids. (reviewed by Randerath (1963)).

(1) Sulphuric acid.

The plates were sprayed with 50-100% H_2SO_4 and warmed on a hot plate. The degree of charring can be used quantitatively using a densitometer.

(2) Potassium dichromate/conc. sulphuric acid.

The reagent-lipid reaction gave light-brown spots on a white

background. On heating the lipids give characteristic colours.

(3) Rhodamine B,

0.05% solution of Rhodamine B in ethanol. Red/violet spots were produced which fluoresce red in UV light.

(4) Bromothymol Blue.

(a) 50 mg bromothymol blue, 1.25g boric acid, 8 ml IN NaOH and 112 ml distilled water.

(b) 40 mg bromothymol blue and 100 ml 0.IN NaOH,

Ochre spots were produced (monoglycerides and polyene acids as pale yellow spots with a white edge). The plates were exposed to ammonia vapour if the spots were not sufficiently visible.

(5) Phosphomolybdic acid.

Plates were sprayed with 10% (w/v) solution of phosphomolybdic acid in ethanol and heated in a drying cabinet to 120°C.

(6) Iodine vapour.

Brown spots were formed by unsaturated lipids. Colours are also given by saturated lipids containing nitrogen and esters of fatty acids.

(7) Iron-hydroxamate.

(a) 20g hydroxylamine hydrochloride dissolved in 50 ml water and diluted to 200 ml with 95% ethanol.

(b) 50 g KOH dissolved in the minimum volume of water and diluted to 500 ml with 95% ethanol.

Reagent (a) was mixed with 2 volumes of reagent (b), filtered and used to spray the dried chromatogram. After drying, the chromatogram was sprayed with a solution of ferric chloride (6 g anhydrous $FeCl_3$, dissolved in 20 ml 10N HCl and shaken with 300 ml ether until a homogeneous

solution was obtained.) The esters gave purple spots on a yellow background.

(8) Munier and Macheboeuf Modification of the Dragendorff reagent (as described by Randerath (1963)), specific for choline phosphatides.

Solution 1 1.7 g basic bismuth nitrate in 100 ml 20% acetic acid.

Solution 2 40 g KI in 100 ml water.

20 ml Solution 1 mixed with 5 ml Solution 2 and 70 ml water. Orange/orange-red spots appeared immediately or on gentle warming.

Table 8 gives the thin-layer chromatographic R_F values of lipids and lipid constituents extracted from plant sources and reference compounds.

Lipids were extracted from fungal hyphae incubated in vivo with radio-active choline; mitochondria and microsomal fractions from the above were prepared in the normal way and the pellets extracted for total lipid. Mitochondria from all sources used and incubated with radio-active choline in the polarograph were pelleted and extracted for lipid contents. The extracts were analysed by thin-layer chromatography on Silica Gel HR and radioautographs developed as previously described.

A final method of detection of ^{14}C-labelled compounds produced in vivo or in incubation experiments was attempted. This substantially followed the method of Feige, Gimmler, Jeschke and Simonis (1969). A preceding purification of the plant extracts from interfering compounds was deemed to be unnecessary. The extracts were brought to small volume by rotary evaporation, spotted onto cellulose MN 300 plates (15 g/90 ml water) and submitted to thin-layer chromatography.

TABLE 8

COMPOUND	R_F Value	Solvent System $CHCL_3$-MeOH-H_2O 70:30:5 v/v
Acetylcholine		0.25
Choline		0.12
Lecithin		0.58 (0.28)
Lysolecithin		0.21
Phosphatidyleth-anolamine		0.78
Sphingomyelin		0.35

The figures in parentheses give the R_F of an anomalous spot

Table 8 gives the R_F values determined by thin-layer chromatography on silica gel HR of lipids and lipid constituents extracted from plant sources and reference compounds.

Spots were detected using one of the methods previously described.

Vertical Development:-

Isobutyric acid - n-butanol-isopropanol-n-propanol-H_2O-NH_3-

Edta (1,000:30:30:140::380:40:0.5 v/v/v/v/v/v/g)

Lateral Development:-

I n-butanol-n-propanol-n-propionic acid-H_2O (400:175:285:373 v/v)

II n-butanol-acetic acid-H_2O (5:1:4 v/v) upper phase used.

The running time was about 5 hours/development. The plates were left overnight at room temperature between developments. Radioautographs of the plates were prepared as previously described.

Utilization of Choline, Betaine Aldehyde and Betaine by Choline ($chol^-$) mutants of Neurospora crassa.

250 ml Erlenmeyer flasks containing 25 ml of F.M.M. and the various additives choline, betaine and betaine aldehyde at a final concentration of 10 μ g/ml, all presterilised separately by autoclaving, were inoculated with mycelium from the two mutants. Flask samples were taken at regular intervals and the pads were removed, pressed out on filter paper and dried at 90° and then weighed to the nearest half mg. This method was essentially that of Horowitz and Beadle (1943).

Growth of the mutants on solid media was measured by means of the Petri plate method described by Horowitz, Bonner and Houlahan (1945) after Thompson, Isbell and Mitchell (1943). Petri plates containing the same basal medium as used for liquid medium + 2.5% agar were inoculated with segments of mycelium from agar slopes of the mutants. The rate of linear growth of the organism over the agar plate was determined by measuring the diameter of the circle formed by the growing mycelium after regular intervals of time at 25°C.

RESULTS AND DISCUSSION

Infra-red Spectroscopy

Figures 1-4 show the infra-red spectra of samples of betaine aldehyde synthesised by a modification of the method of Jellinek, Strength and Thayer (1959) as described by Speed and Richardson (1968). Figure 5 is the infra-red spectrum of a sample of polymerised betaine aldehyde supplied by F. Wohlrab (Pathol. Inst. Univ. Leipzig Cl., Leibigstr. 26). In Fig. 1 the sample used was from one of the earliest attempts at synthesis. The starting compound used was amino-acetal (Koch-Light Labs.) after Jellinek et al (1959). No peak was produced at 1728 cm^{-1} and only a small peak was formed at 788 cm^{-1}. It was suggested by Jellinek et al (1959) that the loss of both of these peaks was caused by hydration. Although precautions were taken to avoid hydration during the synthesis, this possibility cannot be eliminated.

The sample used in Fig. 2 was from preliminary attempts to synthesis betaine aldehyde using the intermediate 2,2-diethoxyethyl-trimethylammonium iodide (see Materials and Methods) as the starting compound. A very small peak was formed at 1728 cm^{-1} and a broad peak at 788 cm^{-1}. Fig. 3 was a sample produced as for Fig. 2 but which was not respired by rat liver mitochondria. No peak was formed at 1728 cm^{-1} and there was only a relatively small peak at 788 cm^{-1}. Fig. 4 was a sample synthesised after modification of the method. The period of hydrolysis was increased by 50% and fewer recrystallisations were employed. Large peaks were produced at 1728 cm^{-1} and 788 cm^{-1}. Fig. 5 was the polymerised sample supplied to compare with Fig. 3. A peak was produced at 1728 cm^{-1}, but there was no peak formed at 788 cm^{-1}.

According to the interpretation presented by Jellinek et al (1959) the peak at 1728 cm^{-1} was due to the carbonyl group of betaine

FIG. 1

Infra-Red Spectrum of the product of an attempted Synthesis of betaine aldehyde during the early part of this investigation (starting compound aminoacetal).

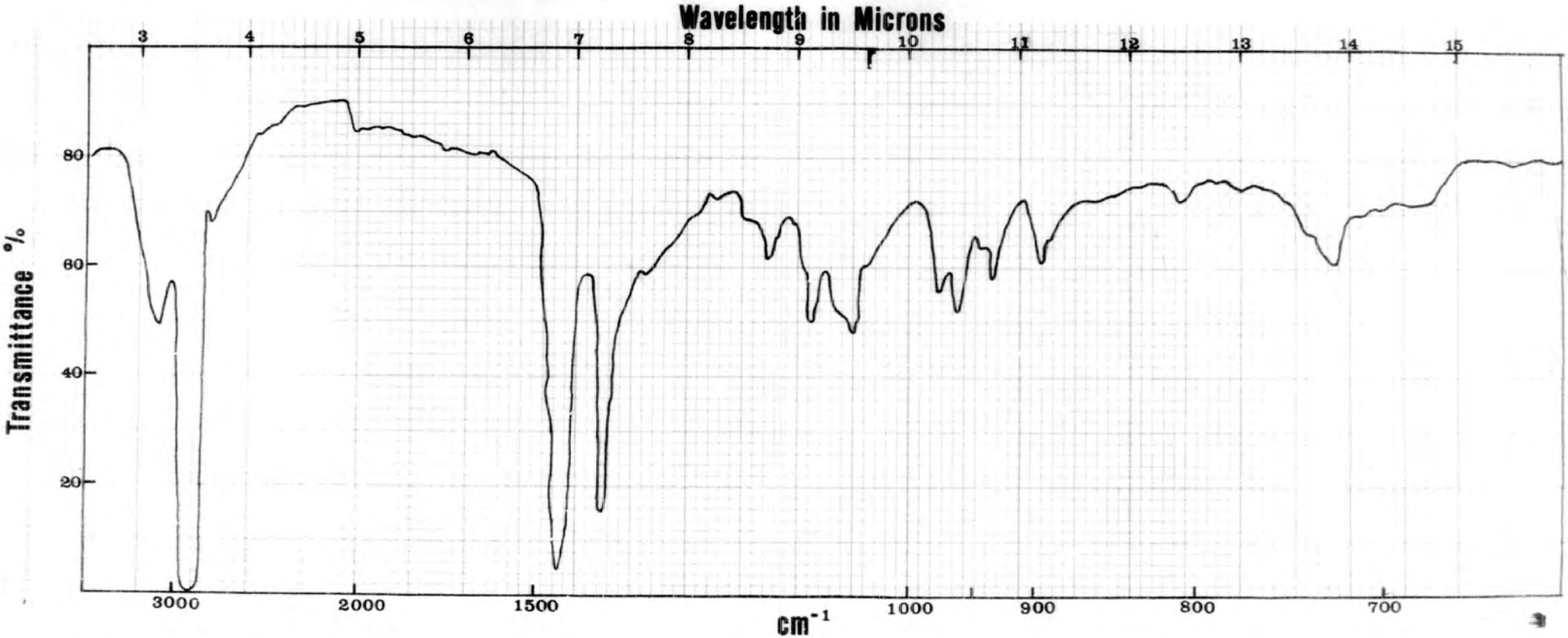

Wavelength in Microns
3
4
5
6
7
8
9
10
11
12
13
14
15
Transmittance %
80
60
40
20
3000
2000
1500
1000
900
800
700
cm-1

FIG. 2

Infra-Red Spectrum of a product from preliminary attempts to synthesise betaine aldehyde using 2,2-diethoxyethyltrimethyl-ammonium idodide as the starting compound.

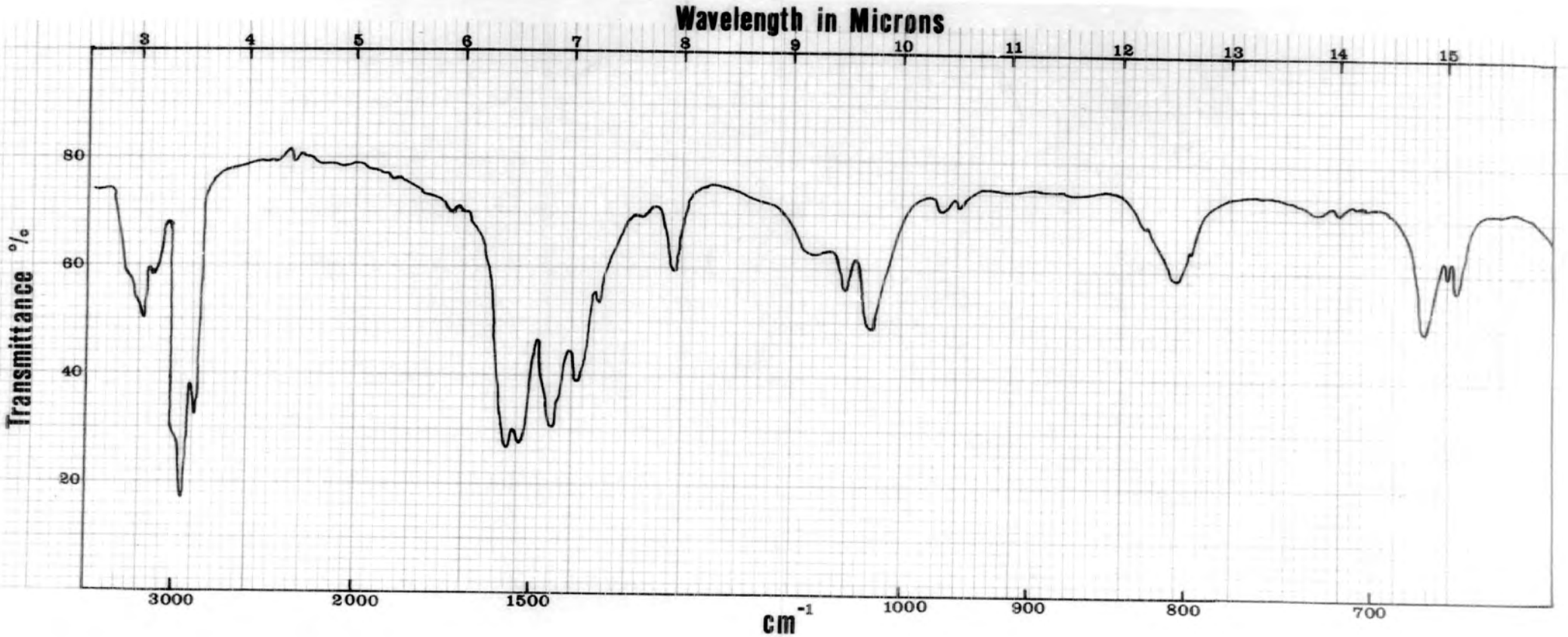

Wavelength in Microns
3
4
5
6
7
8
9
10
11
12
13
14
15
Transmittance %
80
60
40
20
3000
2000
1500
1000
900
800
700
cm-1

FIG. 3

Infra-Red Spectrum of a product synthesised as in Fig. 2 but not respired by rat liver mitochondria.

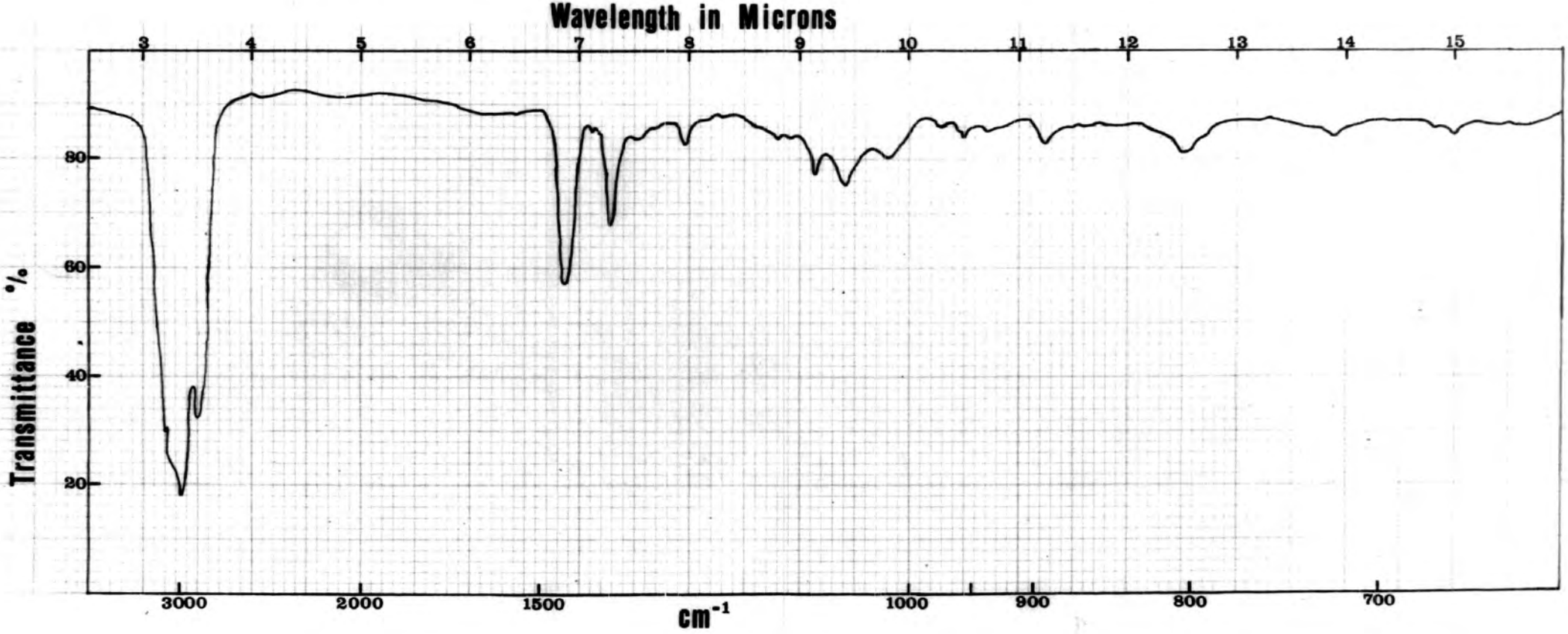

Wavelength in Microns
3
4
5
6
7
8
9
10
11
12
13
14
15
Transmittance %
80
60
40
20
3000
2000
1500
1000
900
800
700
cm-1

FIG. 4

Infra-Red Spectrum of a product resulting from the modified synthesis procedure.

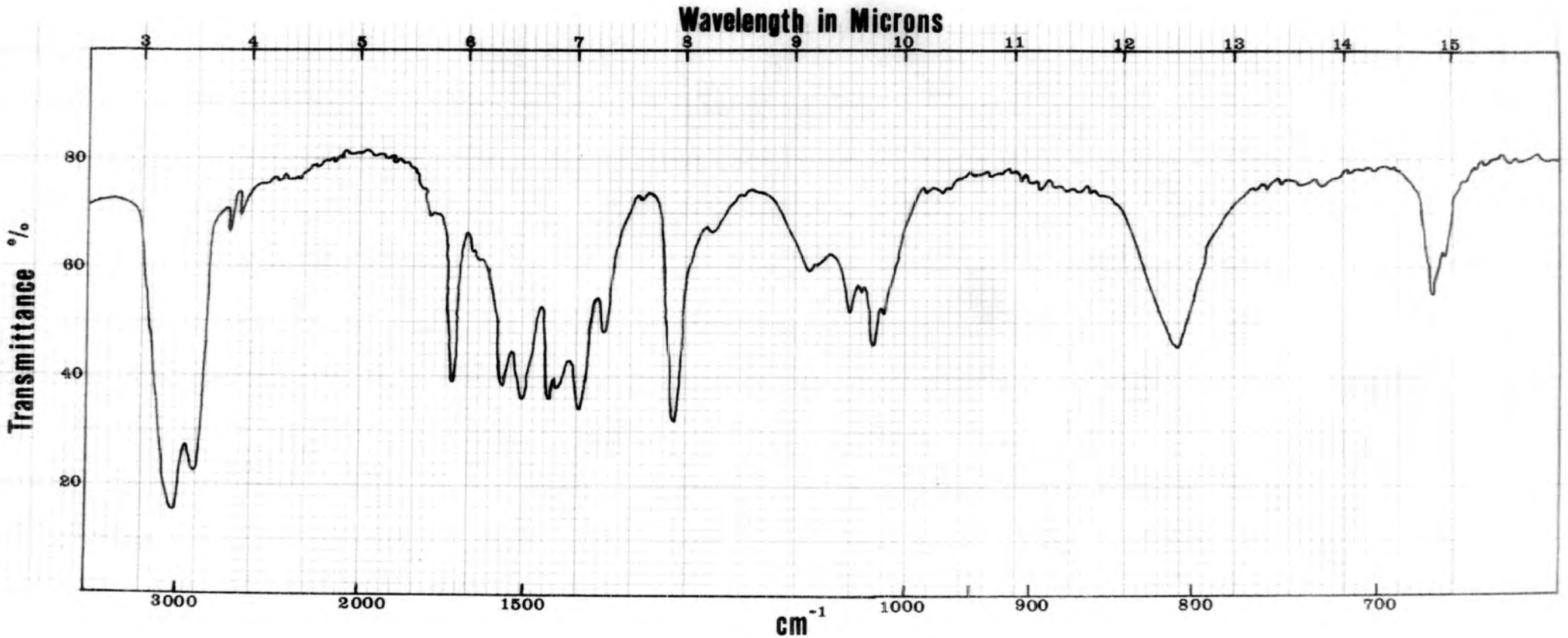
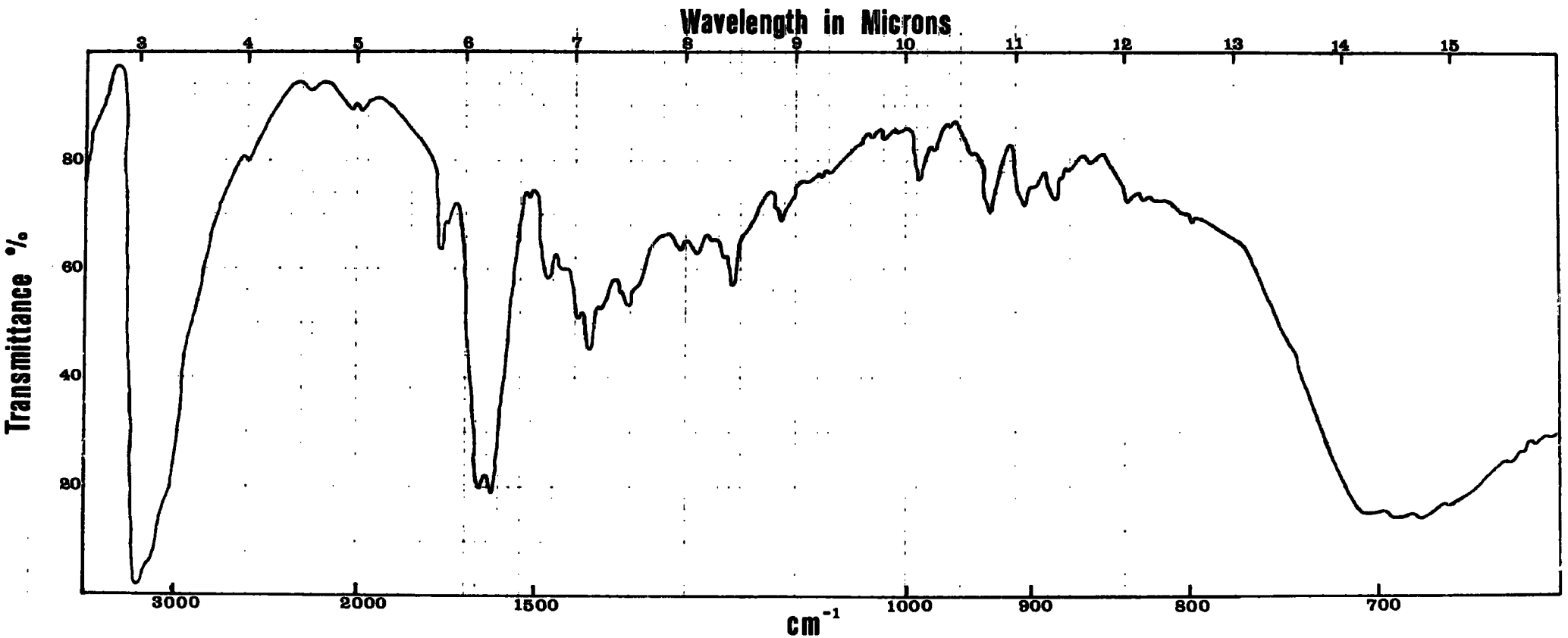

Wavelength in Microns
3
4
5
6
7
8
9
10
11
12
13
14
15
Transmittance %
80
60
40
20
3000
2000
1500
1000
900
800
700
cm-1

FIG. 5

Infra-Red Spectrum of a polymer sample of betaine aldehyde (as supplied by F. Wohlrab).


Wavelength in Microns
3
4
5
6
7
8
9
10
11
12
13
14
15
Transmittance %
80
60
40
20
3000
2000
1500
1000
900
800
700
cm-1

aldehyde. Fig. 3 did not show this peak; however, the polymerised sample supplied showed this peak, but lacked the 788 cm^{-1} peak. This may have some bearing on the mode of polymerisation of betaine aldehyde or could reflect on the description of the supplied sample as a polymer.

Relative Growth Rates of chol-1 and chol-2 mutants of Neurospora crassa.

It is widely accepted that in animals choline is oxidised to betaine via the intermediate betaine aldehyde (Jellinek et al., (1959)) and that the process involves the activity of the enzymes choline dehydrogenase (Rendina and Singer (1959)) and betaine aldehyde dehydrogenase (Glenn and Vanko (1959)). de Heus (1954) has suggested that the biosynthesis of betaine in the fungus Aspergillus niger occurred via the aerobic metabolism of choline. Hogg and Richardson (1968) have shown that (^{14}C-methyl) choline was readily oxidised to (^{14}C-methyl) betaine by in vivo cultures of Neurospora crassa but were unable to demonstrate the occurrence of the possible intermediate betaine aldehyde in these cultures. Initial attempts to demonstrate the activity of the enzymes choline dehydrogenase and betaine aldehyde dehydrogenase in in vitro preparations of Neurospora were unsuccessful. It was, therefore, felt of interest to determine whether chol$^-$ mutants of Neurospora crassa would grow with betaine aldehyde as the sole addition to minimal media.

The relative growth rates of chol-1 and chol-2 in minimal liquid culture medium (MM) and in the media supplemented with choline, betaine aldehyde and betaine are shown in Fig. 6 (a, b). It was found that both chol-1 and chol-2 made very slow but continuous growth in unsupplemented liquid culture. This growth rate was only slightly increased by the addition of betaine to the medium. This slight activity of betaine is in contrast to the findings of Horowitz and Beadle (1943), who reported the complete inactivity of this compound. The activity observed here for betaine is of the same order as that previously

FIG. 6a

Growth rates of chol-1 mutant in minimal liquid culture medium and in the medium supplemented with choline, betaine aldehyde and betaine, at a concentration of 10μg/ml. The hyphal mats were removed, pressed out on filter paper and dried at 90°C and then weighed to the nearest 0.5 mg.

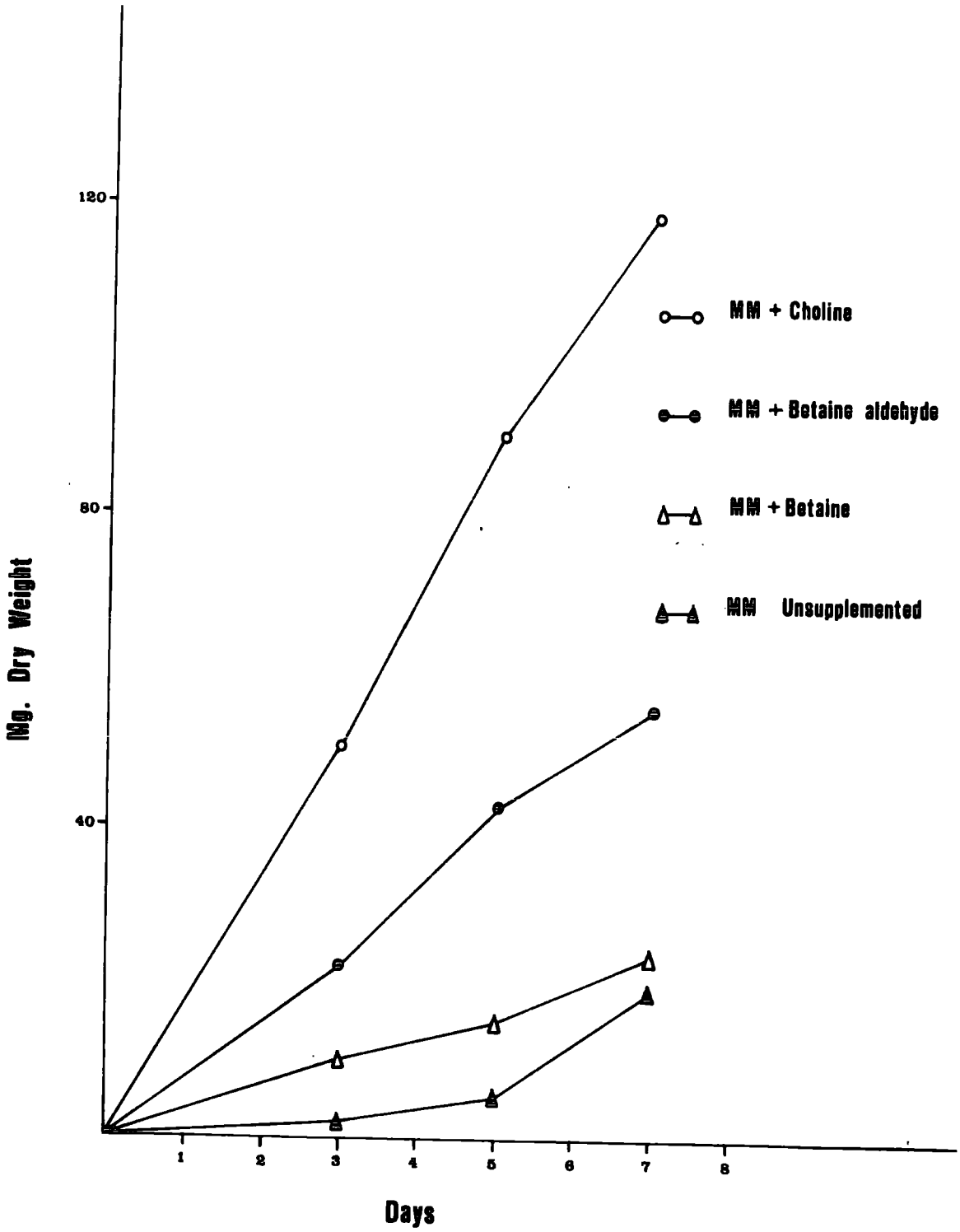
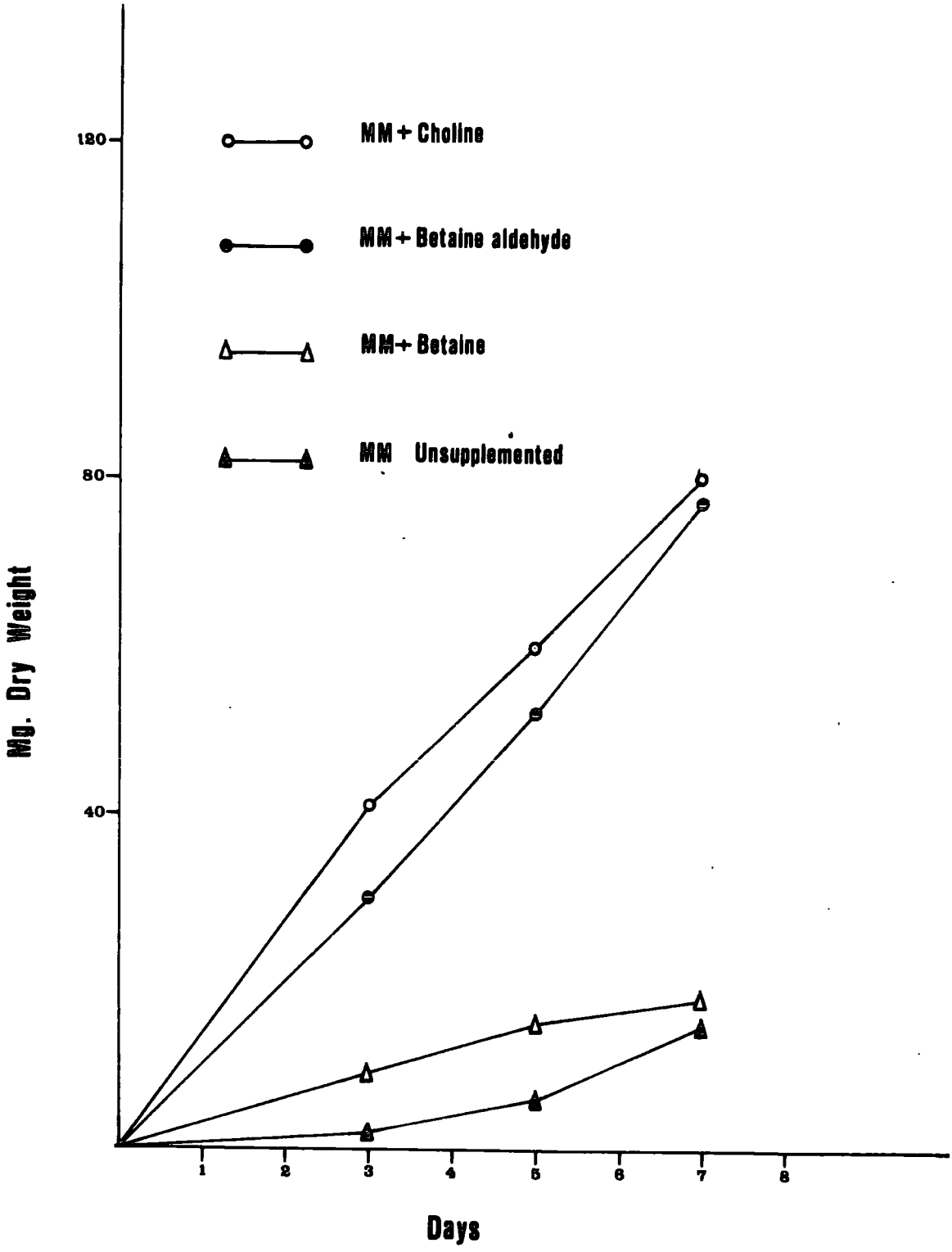

120
80
40
Mg. Dry Weight
1
2
3
4
5
6
7
8
Days
MM + Choline
MM + Betaine aldehyde
MM + Betaine
MM Unsupplemented

FIG. 6b

Growth rates of chol-2 mutant in minimal liquid culture medium and in the medium supplemented with choline, betaine aldehyde and betaine, at a concentration of 10μ g/ml. The growth rates were measured as for Fig. 6a.

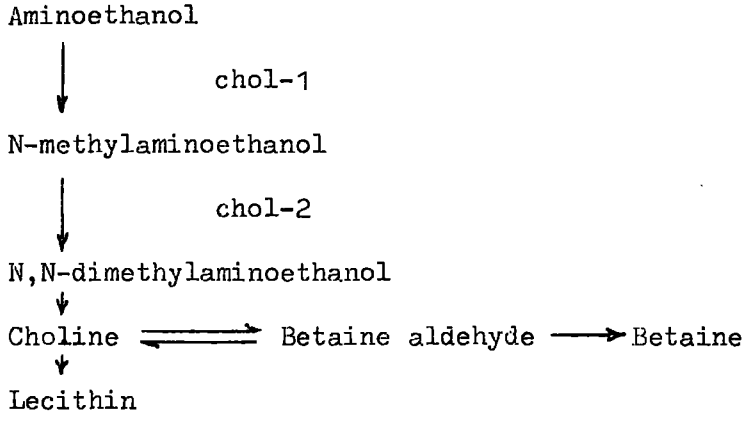

MM + Choline
MM + Betaine aldehyde
MM + Betaine
MM Unsupplemented
120
80
40
Mg. Dry Weight
1
2
3
4
5
6
7
8
Days

observed for methionine (Horowitz et al., (1945)), and it may be that both compounds have a sparing action on the small amounts of choline present in the inoculum. This assumption is in accord with the known ability of betaine and methionine to act as donors of labile methyl groups (Challenger (1955)).

Betaine aldehyde at a concentration of 10μ g/ml was almost as active as the same concentration of choline in promoting the growth of chol-2. In the case of chol-1, however, betaine aldehyde was only approximately 50% as active as choline. The reasons for this differential response of the two mutants is not understood, but it may be related to the fact that chol-2 is not completely deficient in ability to synthesise choline in liquid culture (Horowitz (1946)).

The relative growth rates of these mutants on solid minimal medium supplemented with choline, betaine aldehyde and betaine were essentially similar to thos in liquid culture (Fig. 7 (a, b)).

In Neurospora crassa betaine aldehyde may undergo reduction to choline via the activity of the enzyme choline dehydrogenase. The choline thus produced by a reversal of the suggested normal pathway may be utilised for synthesis of the phospolipid lecithin and hence support the growth of the mutants.



The relative inability of betaine to support growth may be explained by the fact that the action of betaine aldehyde dehydrogenase does not appear to be reversible (Rothschild and Barron (1954)).

FIG. 7a

Growth rates of chol-1 mutant on solid minimal medium and on the medium supplemented with choline, betaine aldehyde and betaine, at a concentration of 10μg/ml.

The rate of linear growth of the organism over the agar plate was determined by measuring the diameter of the circle formed by the growing mycelium after regular intervals.

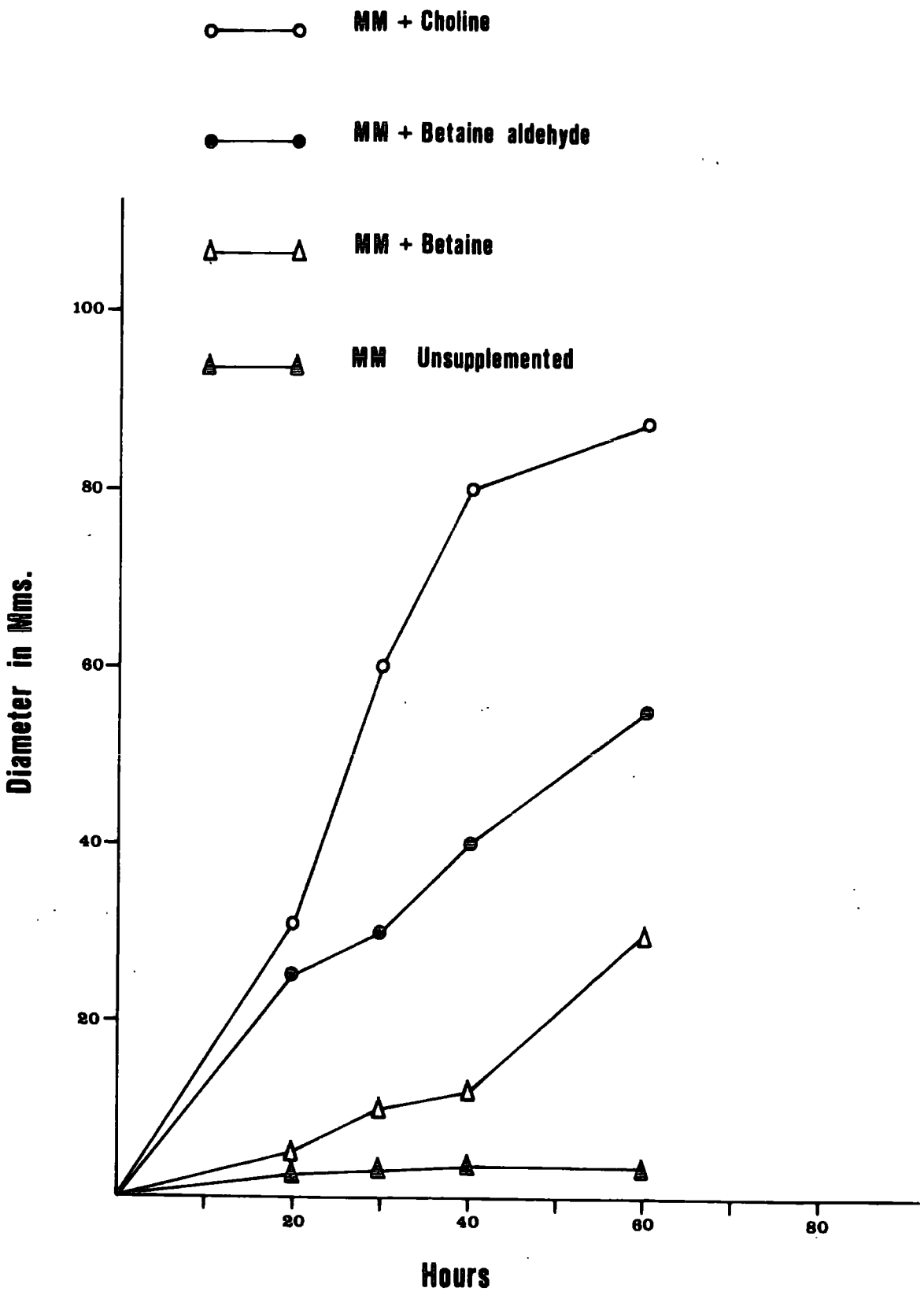
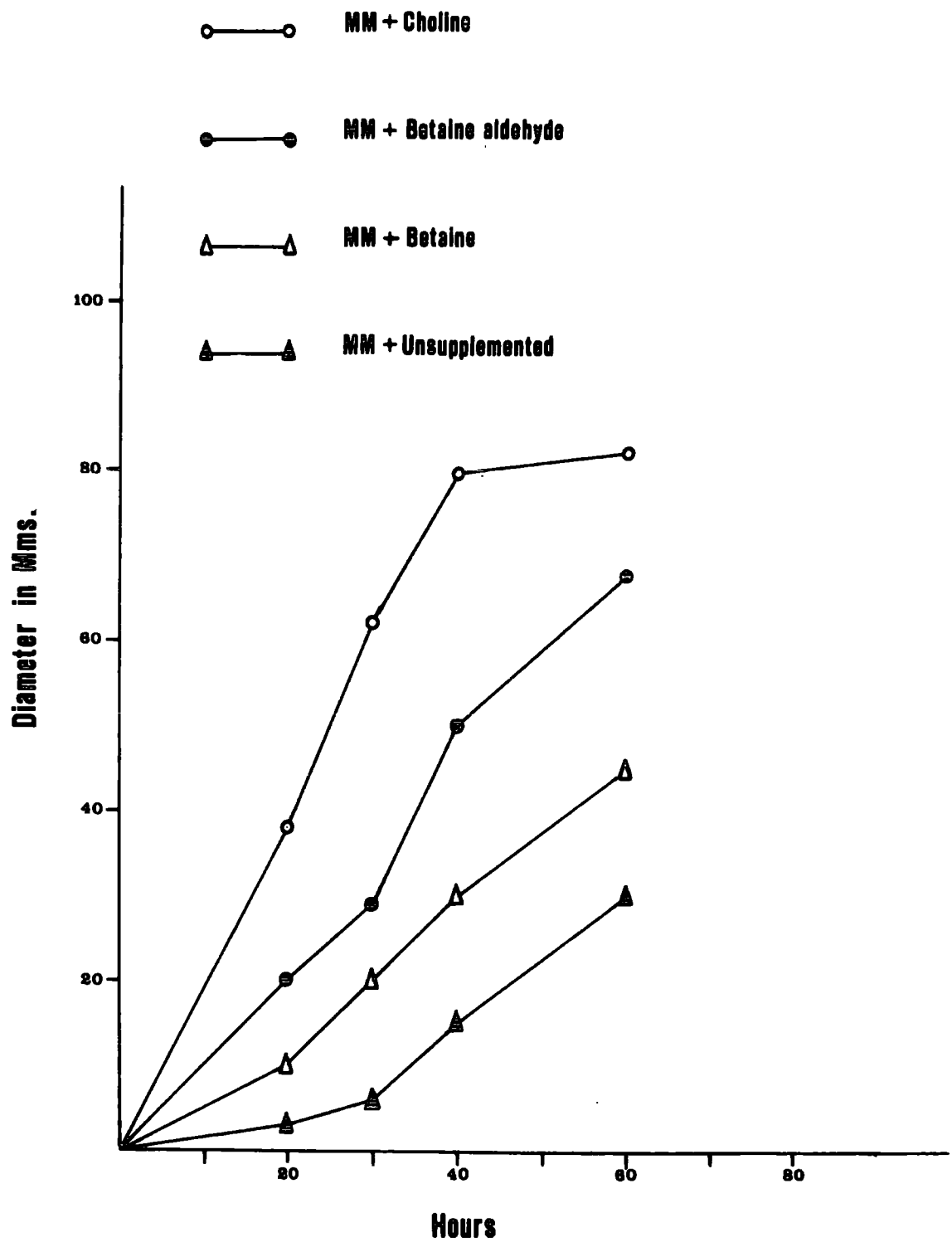

MM + Choline
MM + Betaine aldehyde
MM + Betaine
MM Unsupplemented
Diameter in Mms.
100
80
60
40
20
20
40
60
80
Hours

FIG. 7b

Growth rates of chol-2 mutant on solid minimal medium and on the medium supplemented with choline, betaine aldehyde and betaine, at a concentration of 10μ g/ml.

The growth rates were measured as in Fig. 7a.


MM + Choline
MM + Betaine aldehyde
MM + Betaine
MM + Unsupplemented
Diameter in Mms.
100
80
60
40
20
20
40
60
80
Hours

Spectrophotometric Assay of Mitochondria for the determination of Choline Dehydrogenase activity.

Figs. 8 and 9 demonstrate results obtained from rat liver mitochondria and mung bean mitochondria using dye reduction as a measure of activity of choline dehydrogenase. Fig. 8 confirmed the results of Williams and Sreenivasan (1953) working on rat liver mitochondria. The dye was reduced to the leuco-form over a period of 8 minutes. The control cuvette was identical with the reaction cuvette except that it contained distilled water instead of substrate. Fig. 9 demonstrates a typical result obtained when plant mitochondria were incubated with 2,6-dichlorophenolindophenol and N-methyl phenazonium methosulphate (acting as an electron carrier). The latter used alone, showed a high level of atmospheric oxidation which made it unsuitable in this type of reaction. The two dyes used in conjunction gave the more promising result, however the level of endogenous dye reduction was usually so great as to obscure any difference between the control and the reaction cuvette. Similarly dilution of the extract only served to make the difference in levels of dye reduction insignificant (see Fig. 9).

Attempts to solubilise the enzyme choline dehydrogenase by treatment with acetone, deoxycholate, etc., as described in the Methods section were unsuccessful. No significant dye reduction was ever recorded with such extracts. Williams and Sreenivasan (1953) have reported the apparent stability of choline dehydrogenase in acetone powder extracts of rat liver mitochondria stored at $5^{\circ}C$. However, all acetone powder extracts of both plant and rat liver mitochondria prepared during this investigation were inactive when assayed for respiratory activity.

Singer and Kearney (1957) observed the marked difference between the absorption of the oxidised and reduced forms of phenazine metho-

FIG. 8

The Spectrophotometric assay of rat liver mitochondria for the determination of choline dehydrogenase activity, using the reduction of 2, 6-DCPIP. The change in O.D. was measured at 607 mμ. The reference cuvette was identical with that of the reaction cuvette except distilled water was used in place of substrate.

The reaction cuvette contained:-

0.6 ml	2, 6 - DCPIP (10 mg % solution)
0.6 ml	0.1 M sodium phosphate buffer
0.8 ml	distilled water
0.2 ml	choline chloride (2% solution)
1.0 ml	mitochondrial suspension
2 mg	N-methyl phenazonium methosulphate

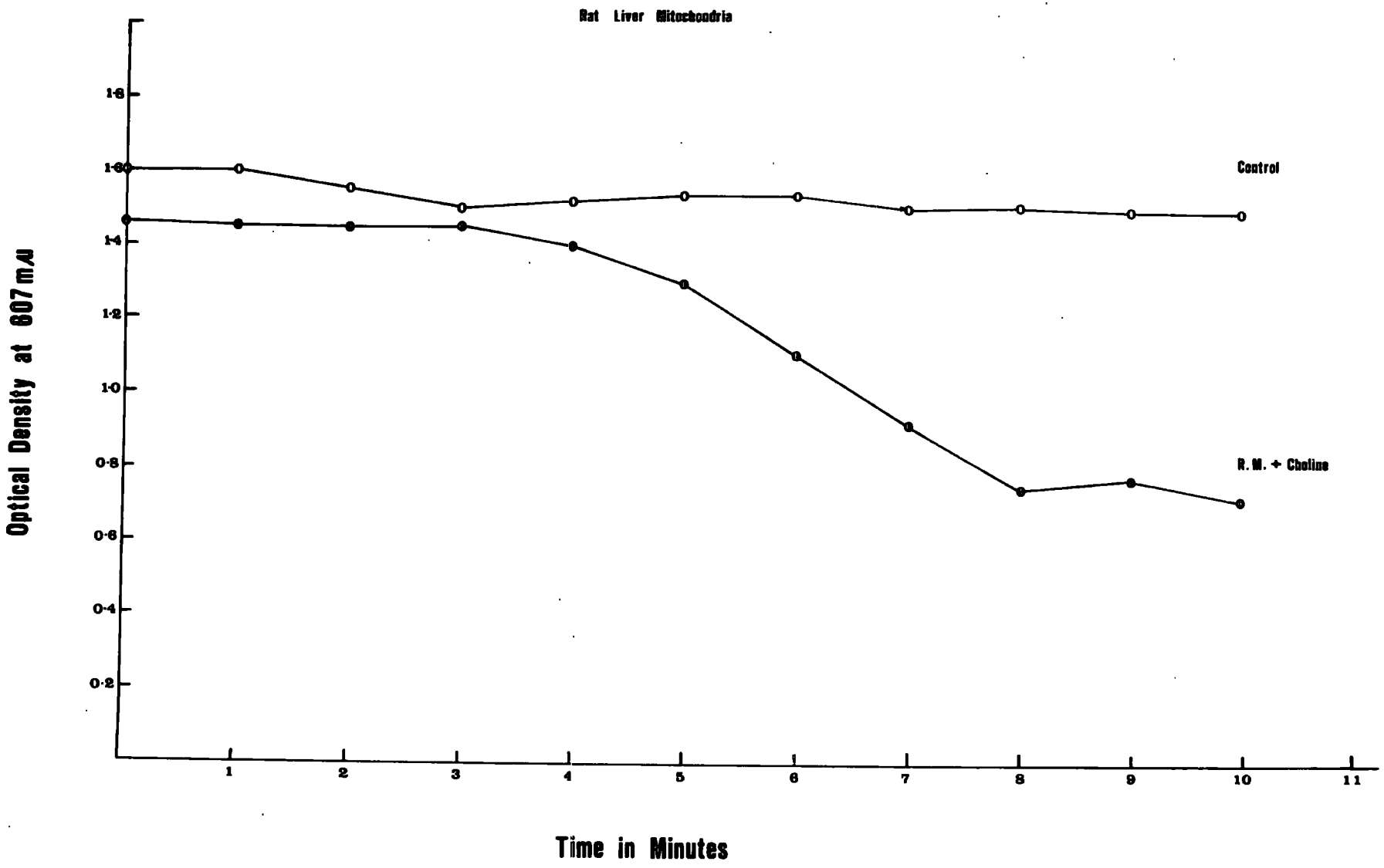
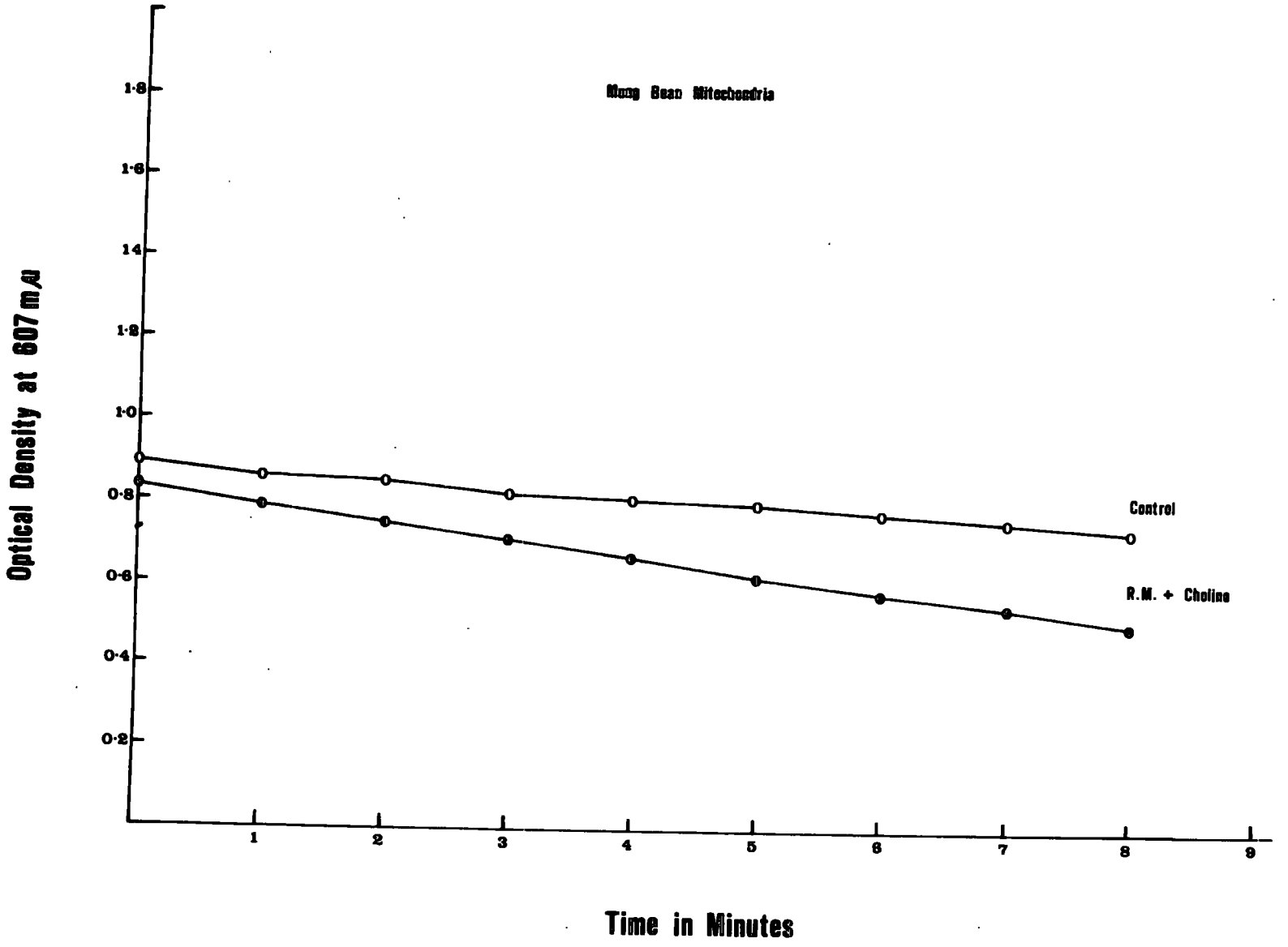

Rat Liver Mitochondria
Optical Density at 607 mμ
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
1
2
3
4
5
6
7
8
9
10
11
Time in Minutes
Control
R. M. + Choline

FIG. 9

Spectrophotometric assay of mung bean hypocotyl mitochondria for the determination of choline dehydrogenase activity using the reduction of N-methyl phenazonium methosulphate. The change in O.D. was measured at 607 mμ.

The reaction cuvette contained:-

0.6 ml	2, 6 - DCPIP (10 mg % solution)
0.6 ml	0.1 M sodium phosphate buffer
0.8 ml	distilled water
0.2 ml	choline chloride (2% solution) (distilled water control)
1.0 ml	mitochondrial preparation
2 mg	N-methyl phenazonium methosulphate


Mung Bean Mitochondria
Optical Density at 607 mμ
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
1
2
3
4
5
6
7
8
9
Control
R.M. + Choline
Time in Minutes

sulphate at 387 mμ and suggested the possibility of a sensitive spectrophotometric method for the assay of the primary succinic dehydrogenase. However, certain properties have limited its usage. Turbidity often developed when the dye was reduced in anaerobic conditions. Singer and Kearney (1957) suggested that this was probably due to the relative insolubility of the leuco-dye in aqueous media. They proposed that the reduction of the phenazonium methosulphate was coupled to cytochrome c or 2, 6-dichlorophenolindophenol and the absorption of the latter compounds could be measured. Kun and Singer quoted in Kearney and Singer (1956) had already suggested the use of phenazine methosulphate reduction as an assay method for choline dehydrogenase activity.

Significantly Singer and Lusty (1960) observed that although in mitochondrial fragments and other types of particulate preparations phenazine methosulphate is the only electron acceptor known to measure the full activity of succinic dehydrogenase (Guiditta and Singer (1959), in intact mitochondria the phenazine assay registered a small and variable fraction of the succinic dehydrogenase activity. They showed that the addition of low (0.76mM) concentrations of Ca^{++} proved to be the most convenient treatment for increasing the rate of the succinate - phenazine methosulphate reaction to a level which equalled or approximated to that found in damaged mitochondria. The major cause of the depressed rate of the succinate - phenazine methosulphate reaction in undamaged mitochondria was a permeability barrier towards the dye.

Singer and Kearney (1957) concluded that phenazine dyes did not appear to penetrate freely into mitochondria, while other electron acceptors had indeterminate reaction sites in the respiratory chain and did not measure the full activity of the dehydrogenase.

There seemed to be no reliable method for the assay of the full succinic dehydrogenase activity in mammalian mitochondria with an intact permeability barrier. For this reason the use of phenazine methosulphate as a terminal electron acceptor or as a carrier to other dyes or oxygen in the assay of other dehydrogenases in mitochondria must be viewed with caution.

Polarographic Assay of Mitochondrial Activity

Figs. 10, 11 and 12 demonstrate the polarographic traces obtained with rat liver mitochondria when choline, succinate or betaine aldehyde were employed as substrates for respiration. Rapid respiration was recorded with all the substrates, the greatest activity being obtained with succinate. Fig. 13 demonstrates the polarographic trace obtained with mung bean mitochondria isolated after the method of Ikuma and Bonner (1967) and incubated in the reaction medium as described by them. A relatively high rate of endogenous respiration was usually recorded using this method of extraction. This trace was typical of the earliest mitochondrial extracts; no stimulation of respiration was recorded when choline was added. The mitochondria respired succinate and this respiration was stimulated by additions of ADP.

The trace obtained when mung bean mitochondria were pre-treated with deoxycholate is shown in Fig. 14a. The results from Fig. 13 suggested a permeability barrier to choline as described by Williams (1960). The deoxycholate treatment was similar to that suggested by Williams and is described fully in the methods section. The treatment did not affect the stimulation of respiration by choline. Mitochondrial respiration was no longer stimulated by ADP and only marginally stimulated by succinate. Fig. 14b is the trace obtained for the respiratory activity of mung bean mitochondria pre-treated with 0.1% tergitol for 10 mins. 0.05M tris-HCl buffer pH 7.4 was

FIG. 10

Polarographic assay of the respiratory activity of rat liver mitochondria with choline as substrate.

The reaction mixture contained:-

3 ml	aerated reaction medium
1 ml	mitochondrial suspension

Choline was added to a final concentration of 4mM.

In all polarographic assays, endogenous oxidation, where present, was allowed to reach a constant rate before external substrates were added.

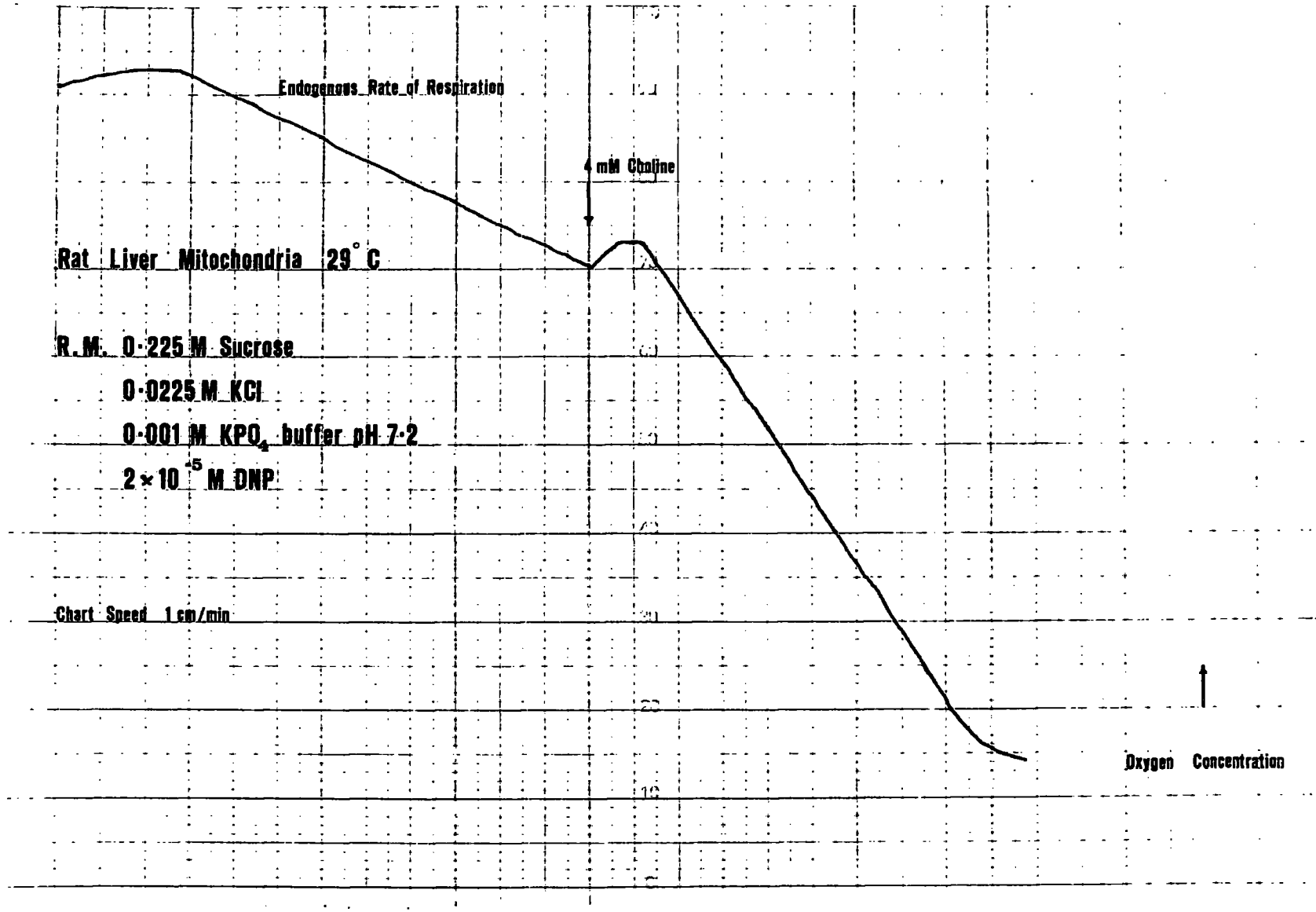

Endogenous Rate of Respiration
4 mM Choline
Rat Liver Mitochondria 29° C
R.M. 0·225 M Sucrose
0·0225 M KCl
0·001 M KPO_4 buffer pH 7·2
2×10^{-5} M DNP
Chart Speed 1 cm/min
Oxygen Concentration

FIG. 11

Polarographic assay of the respiratory activity of rat liver mitochondria with choline and succinate as substrates.

The reaction mixture contained:-

3 ml	aerated reaction medium (see Fig. 10)
1 ml	mitochondrial suspension

Substrate was added to a final concentration of 2mM (choline) or 10mM (succinate).

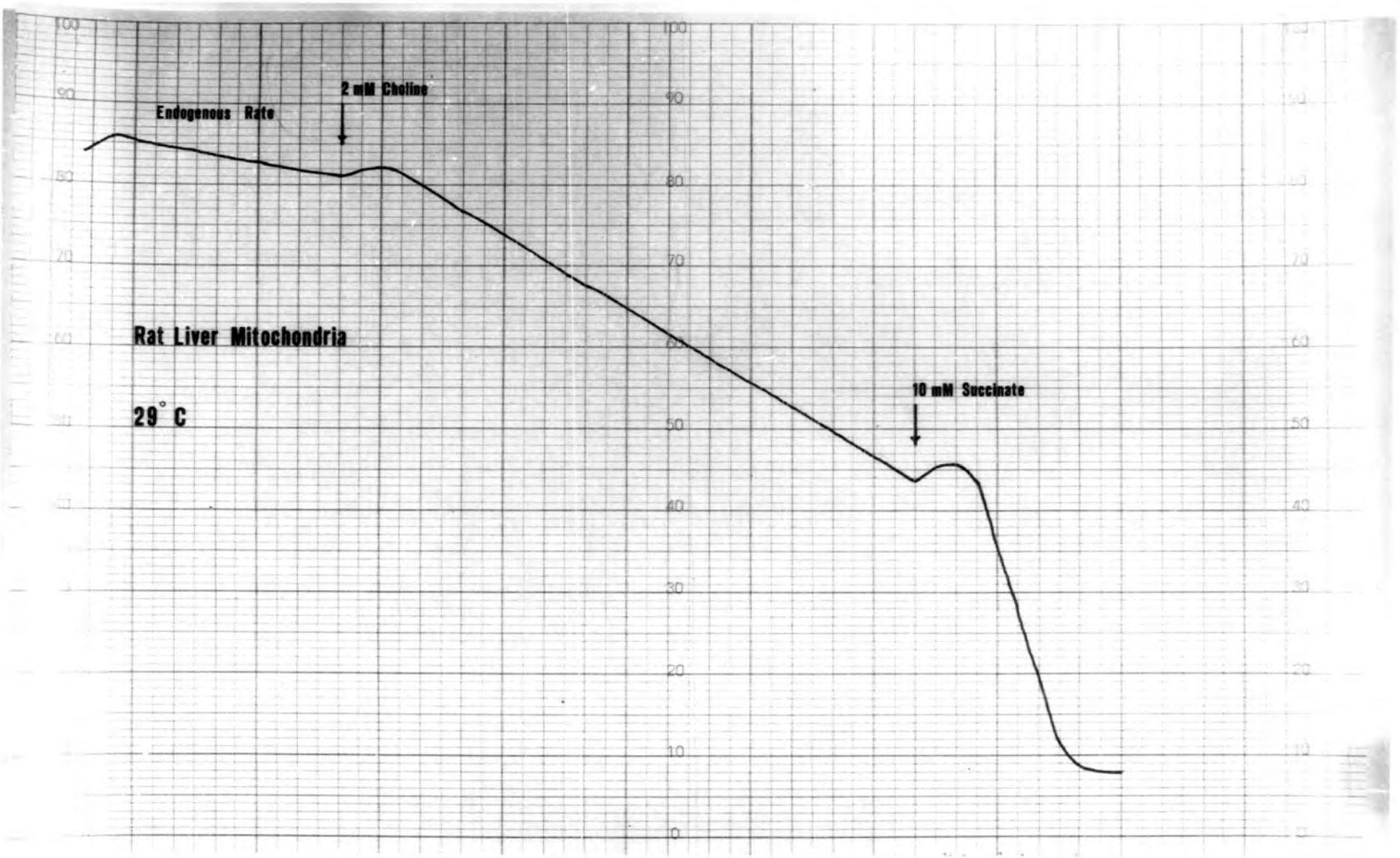

Endogenous Rate
2 mM Choline
Rat Liver Mitochondria
29° C
10 mM Succinate

FIG. 12

Polarographic assay of the respiratory activity of rat liver mitochondria with betaine aldehyde as substrate.

(Reaction mixture as described for Figs. 10 and 11)

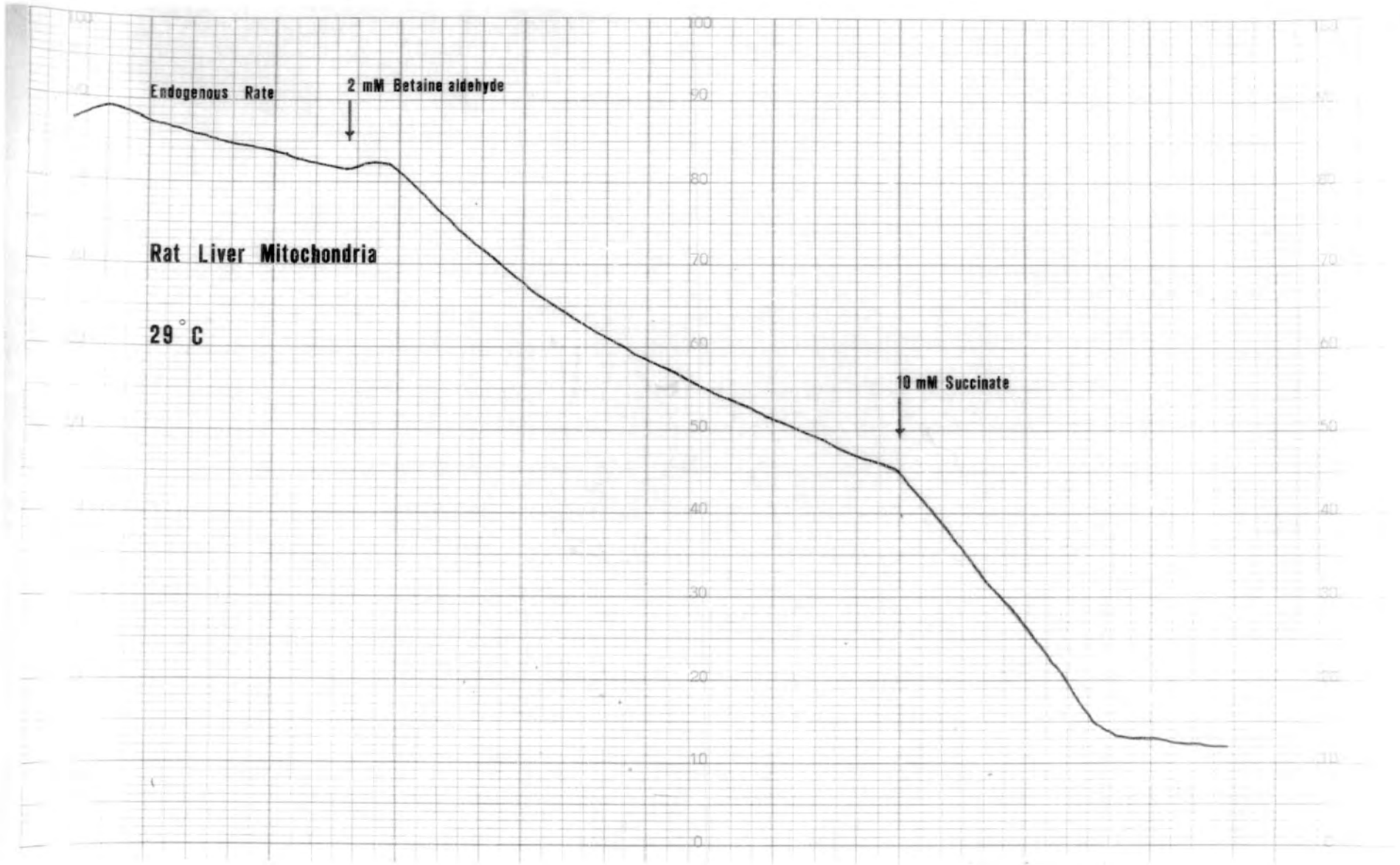

Endogenous Rate
2 mM Betaine aldehyde
Rat Liver Mitochondria
29 °C
10 mM Succinate

FIG. 13

Polarographic assay of the respiratory activity of mung bean hypocotyl mitochondria.

The reaction mixture contained:-

0.3 M	Mannitol
0.01 M	KCl
0.01 M	KPO_4 buffer (pH 7.2)
5 mM	$MgCl_2$
1 ml	mitochondrial suspension

Substrates were added as indicated.

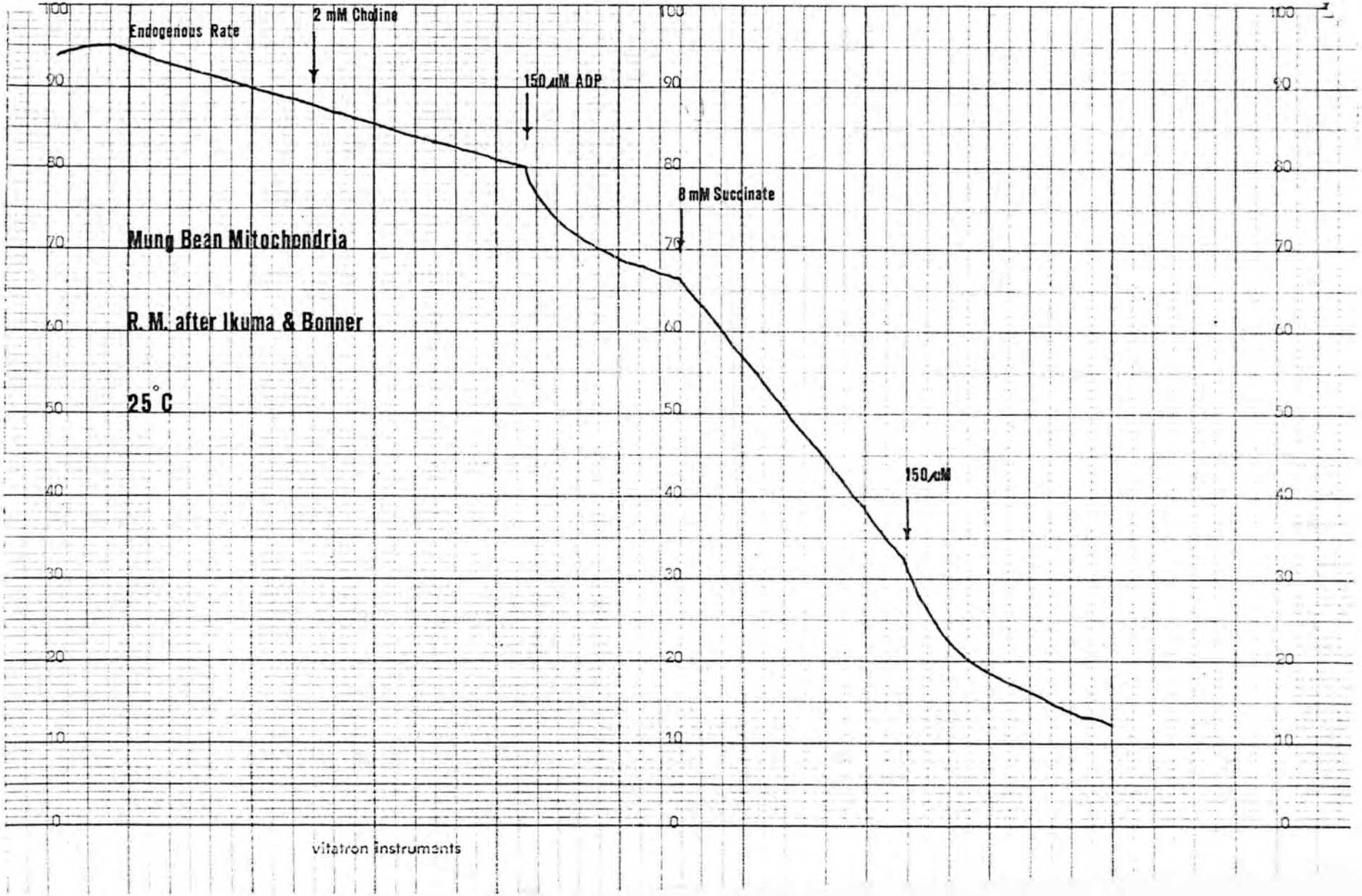
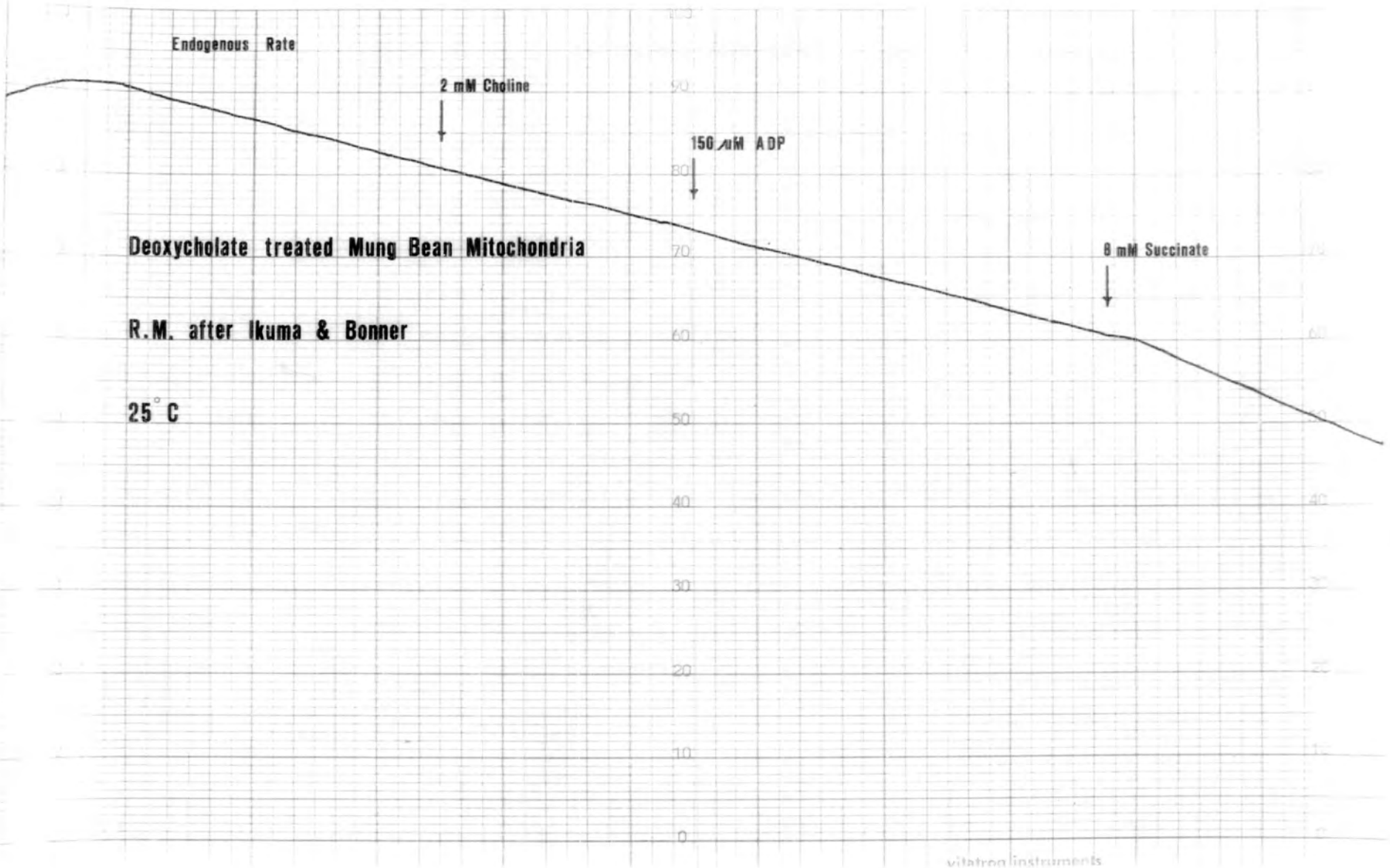

Endogenous Rate
2 mM Choline
150 µM ADP
8 mM Succinate
150 µM
Mung Bean Mitochondria
R. M. after Ikuma & Bonner
25° C
vitatron instruments

FIG. 14a

Polarographic assay of the respiratory activity of mung bean hypocotyl mitochondria pre-treated with deoxycholate. (See Fig. 13 for reaction mixture)


Endogenous Rate
2 mM Choline
150 µM ADP
8 mM Succinate
Deoxycholate treated Mung Bean Mitochondria
R.M. after Ikuma & Bonner
25° C
90
80
70
60
50
40
30
20
10
0

used instead of the 10 mM potassium phosphate described by Ikuma and Bonner (1967). A high endogenous rate of respiration was recorded and apparent stimulation of respiration by choline and ADP was observed. Active mitochondria obtained by this method were then incubated with ^{14}C-methyl choline but the only radio-active quaternary ammonium compound recovered subsequently was the ^{14}C-methyl choline administered.

Figs. 15 and 16 show traces obtained when mung bean mitochondria were "aged" by allowing freshly prepared mitochondria to respire in oxygenated reaction medium for 1 - 3 hours (at 0-4^{o}C). This was largely an attempt to reduce the rate of endogenous respiration. The interesting result was that as well as the endogenous rate being reduced, stimulation of respiration by succinate and NADH was recorded, and this was stimulated further by additions of ADP. The traces obtained indicated that the mitochondria, despite the aging treatment were tightly coupled. Fig. 17 demonstrates a polarographic trace of sugar beet mitochondria isolated by the method of Ikuma and Bonner (1967). A very high endogenous rate of respiration was recorded and the addition of choline had no stimulatory effect. An addition of NADH caused an immediate increase in oxygen uptake.

Fig. 18 shows a trace obtained with sugar beet mitochondria using a reaction medium after Kensler and Langemann (1951) containing 0.033M $KHPO_4$, 0.000027M $CaCl_2$ and 0.000016M cytochrome c. Exogenous cytochrome c was added to ascertain whether the endogenous cytochrome c necessary for the terminal oxidative process could have been depleted by the extraction procedure. The results demonstrated that the exogenous cytochrome c had no effect on choline oxidation. The ability of the mitochondria to respire succinate in this reaction medium was unimpaired. A polarographic trace of the respiration of 3-hour-old mitochondria prepared from sugar beet leaves is shown

FIG. 14b

Polarographic assay of the respiratory activity of mung bean hypocotyl mitochondria pretreated with 0.1% tergitol.

The reaction mixture (aerated) contained:-

0.3 M	Mannitol
0.01 M	KCl
0.05 M	Tris - HCl buffer pH 7.4
5 mM	$MgCl_2$
1 ml	Mitochondrial suspension

Substrates were added as indicated.

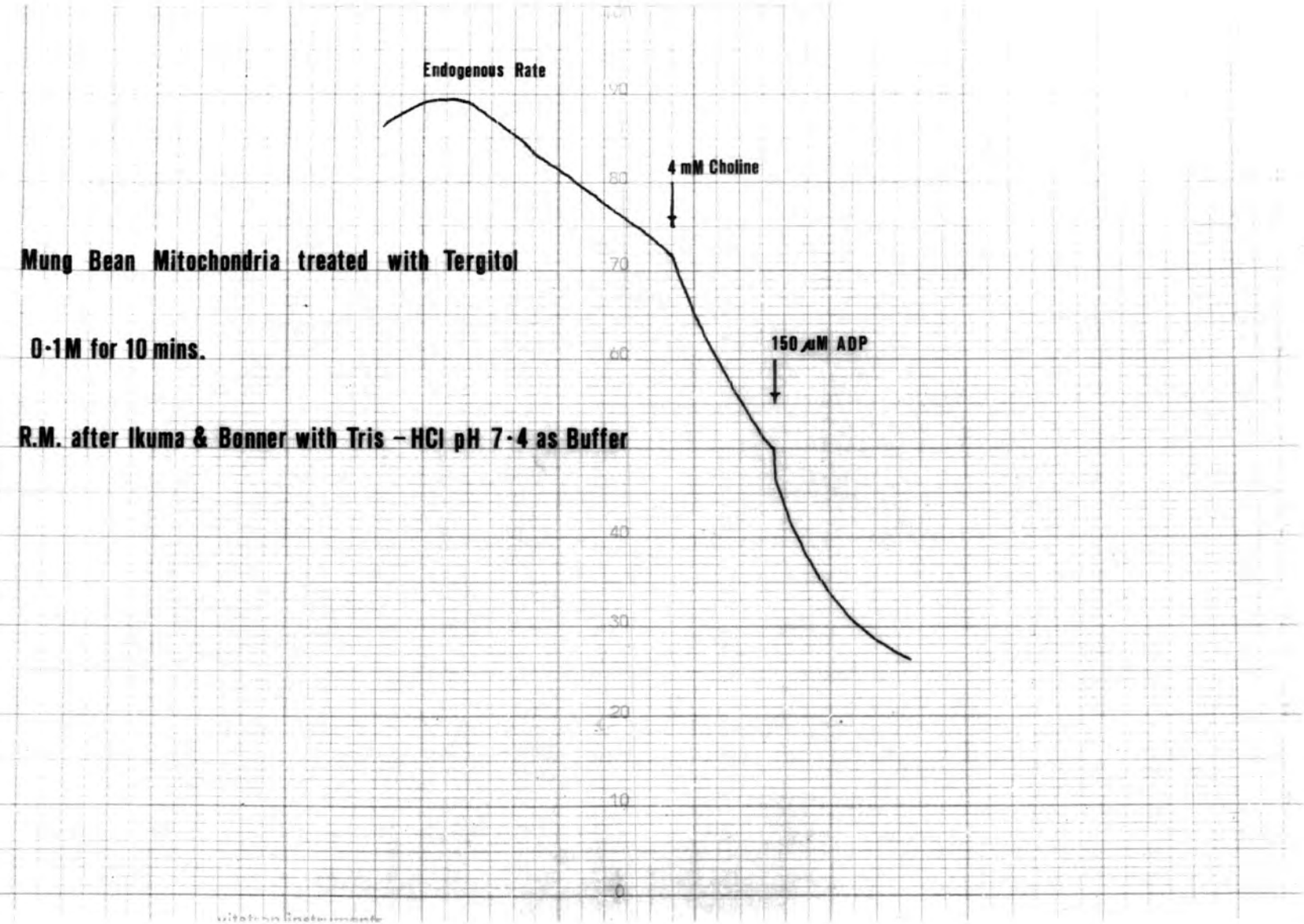

Endogenous Rate
4 mM Choline
150 µM ADP
Mung Bean Mitochondria treated with Tergitol
0·1M for 10 mins.
R.M. after Ikuma & Bonner with Tris – HCl pH 7·4 as Buffer
80
70
60
40
30
20
10
0

FIG. 15

Polarographic assay of the respiratory activity of "aged" mung bean hypocotyl mitochondria.

The reaction mixture (aerated) contained:-

0.3 M	mannitol
0.01	KCl
0.01	KPO_4 buffer pH 7.2
5 mM	$MgCl_2$
1 ml	Mitochondrial suspension.

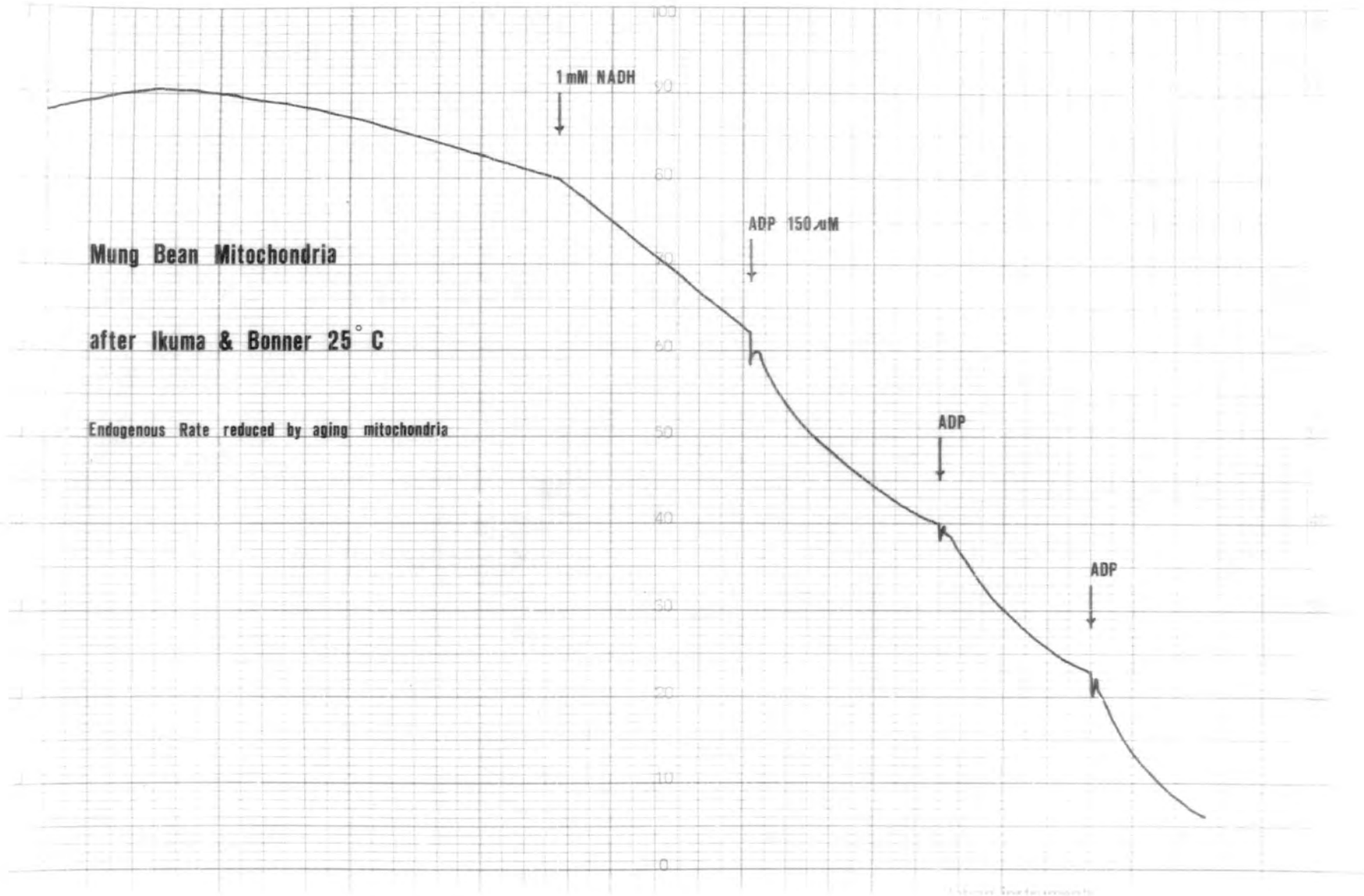

1 mM NADH
ADP 150 uM
ADP
ADP
Mung Bean Mitochondria
after Ikuma & Bonner 25° C
Endogenous Rate reduced by aging mitochondria

FIG. 16

Polarographic assay of the respiratory activity of "aged" mung bean hypocotyl mitochondria.

Reaction mixture as for Fig. 15.

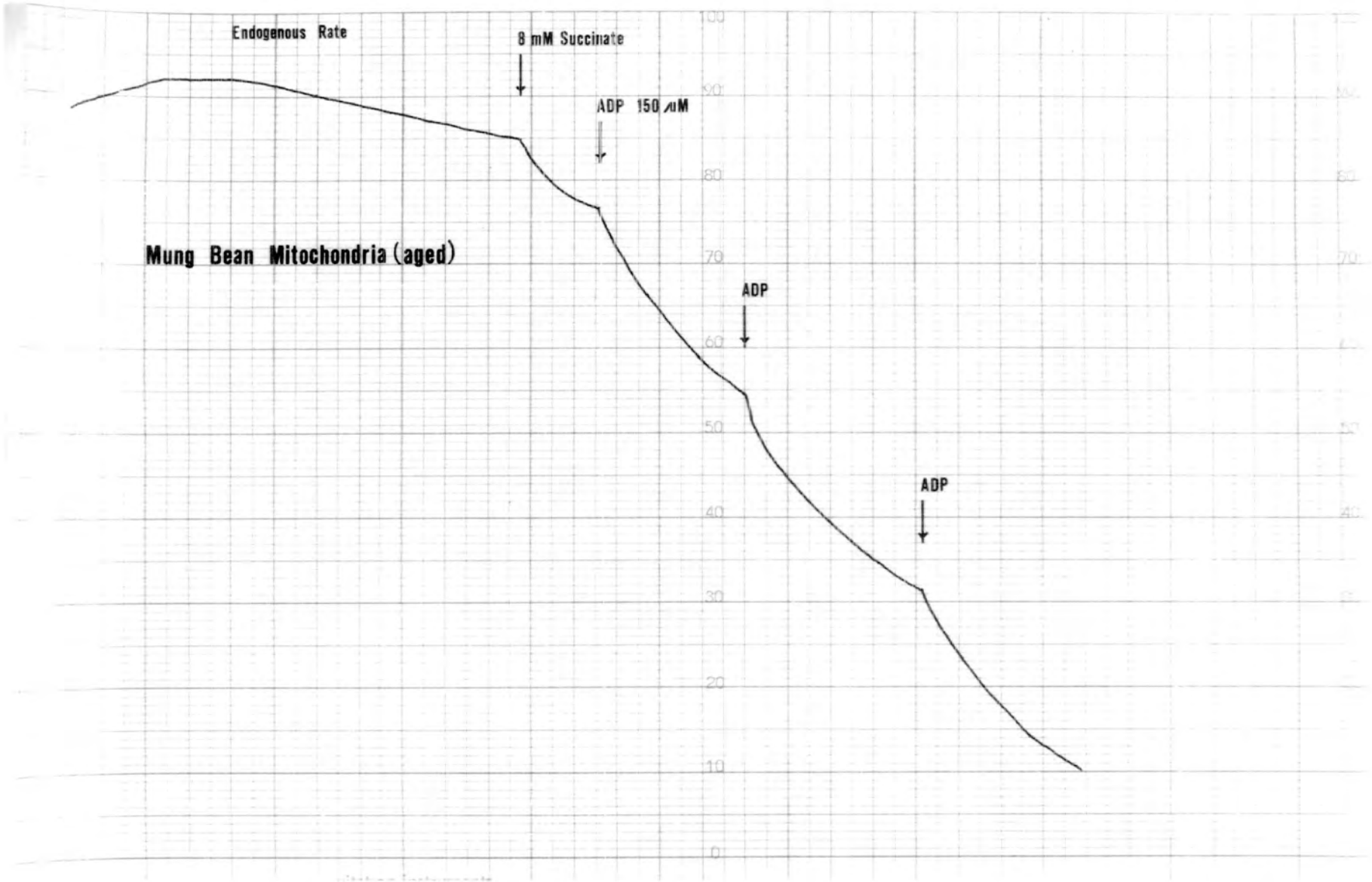

Endogenous Rate
8 mM Succinate
ADP 150 nM
Mung Bean Mitochondria (aged)
ADP
ADP
100
90
80
70
60
50
40
30
20
10
0

FIG. 17

Polarographic assay of the respiratory activity of sugar beet leaf mitochondria.

Reaction mixture as for Fig. 15.

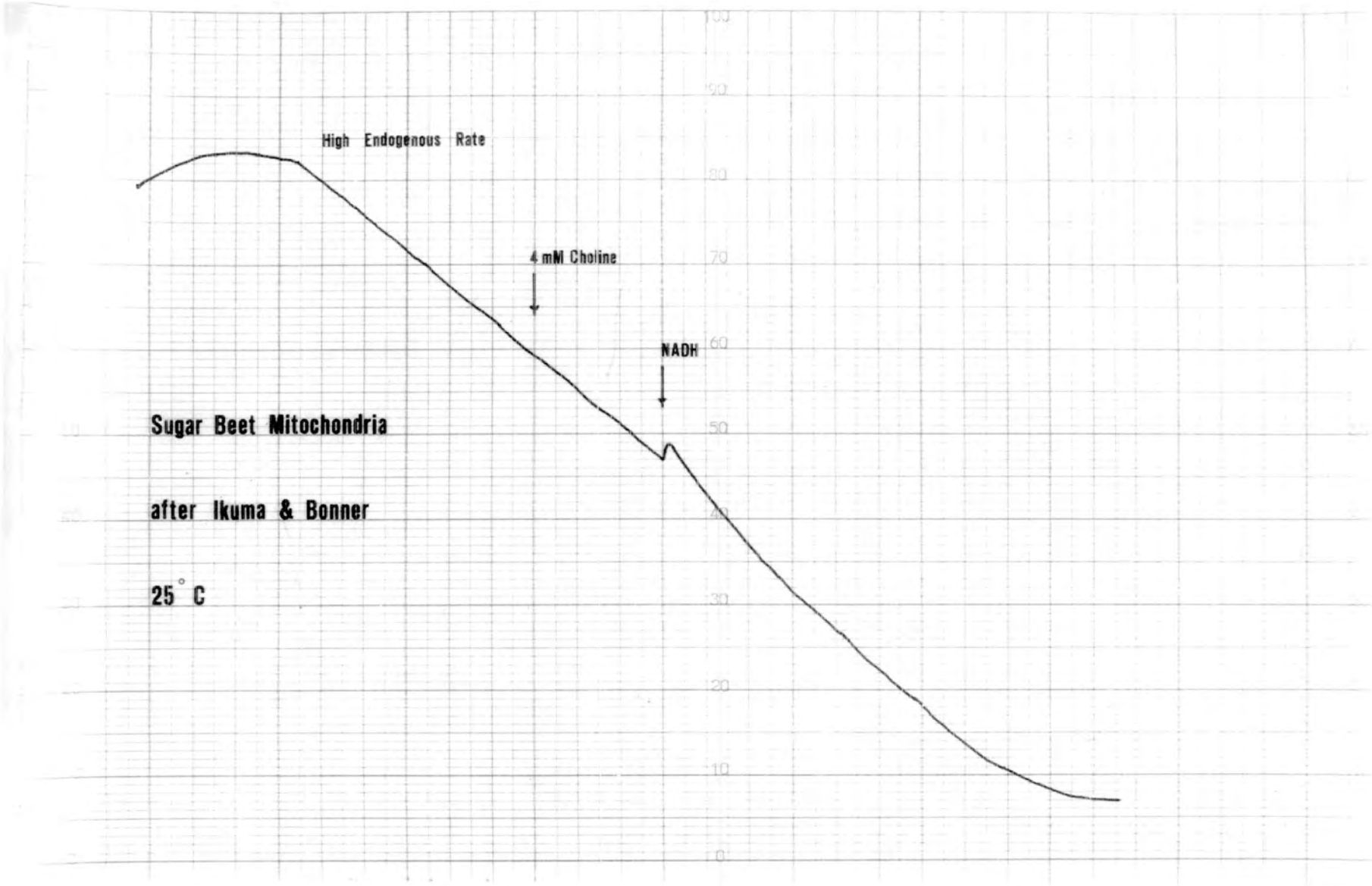
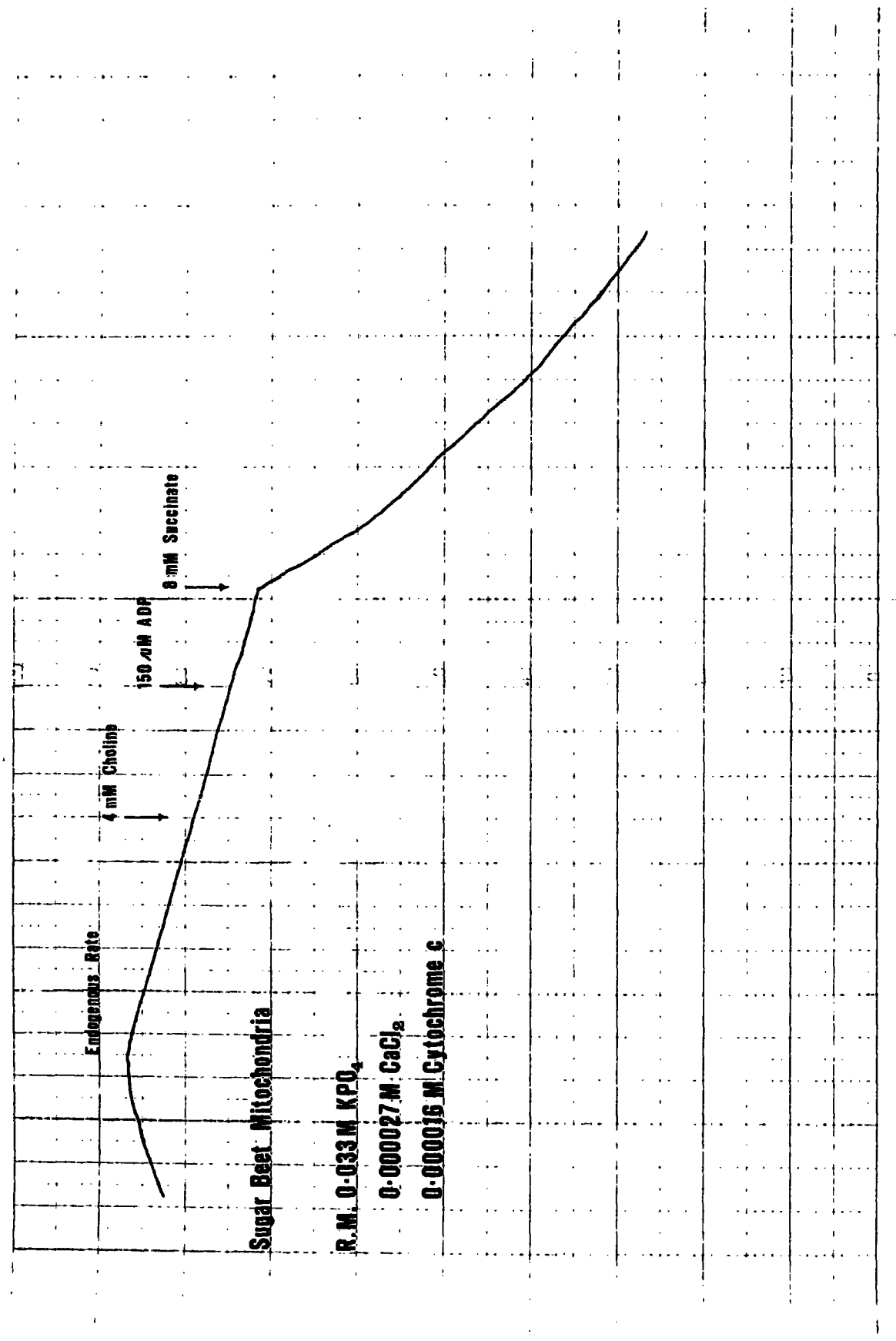

High Endogenous Rate
4 mM Choline
NADH
Sugar Beet Mitochondria
after Ikuma & Bonner
25° C

FIG. 18

Polarographic assay of sugar beet leaf mitochondria using a reaction medium containing cytochrome c.

The reaction mixture contained:-

3 ml	aerated reaction medium
1 ml	mitochondrial suspension


Endogenous Rate
4 mM Choline
150 nM ADP
8 mM Succinate
Sugar Beet Mitochondria
R.M. 0·033 M KPO_4
0·000027 M $CaCl_2$
0·000016 M Cytochrome c

in Fig. 19. Even after this length of time the endogenous rate of respiration was considerable. An apparent stimulation of respiration was recorded when choline was added to the incubation vessel. The rate of respiration was further increased when NAD was added. This could be interpreted as evidence for the presence of betaine aldehyde dehydrogenase requiring NAD as a cofactor.

Because of the inability to demonstrate the activity of a choline dehydrogenase from mitochondrial extracts in the early stages of this investigation it was felt to be of interest to examine the structural integrity of mitochondria extracted from the plant sources. Plate 1 is an electron micrograph of mitochondria extracted from mung bean hypocotyls using the method of Ikuma and Bonner (1967). The mitochondria shown have intact outer membranes and suggest a high structural integrity. However, the method of fixing, etc., of mitochondrial extracts tends to distort the physical nature of mitochondria. It is now possible to define precisely the criteria of biochemical integrity of mitochondria. These criteria include good respiratory control by ADP concentrations. This control must include not only acceleration of substrate oxidation rate by ADP addition, but also inhibition of oxidation rate when the ADP concentration in the reaction medium reaches zero. Externally added NAD should have no influence on the rate of oxidation of NAD-linked substrates and added cytochrome c should not influence any substrate oxidation rate. Respiratory control should be retained for some hours following isolation.

Bonner (1967) explained the development of the extraction procedure described by Ikuma and Bonner (1967). The concentration of mannitol in the extraction medium did not appear to be critical, but the 0.3M chosen had proved suitable for a wide range of plant tissues. The addition of phosphate buffer to the extraction medium,

FIG. 19

Polarographic assay of "aged" sugar beet leaf mitochondria.

The reaction mixture (aerated) contained:-

0.3 M	mannitol
0.01	KCl
0.01	KPO_4 buffer pH 7.2
5 mM	$Mg\ Cl_2$
1 ml	mitochondrial suspension

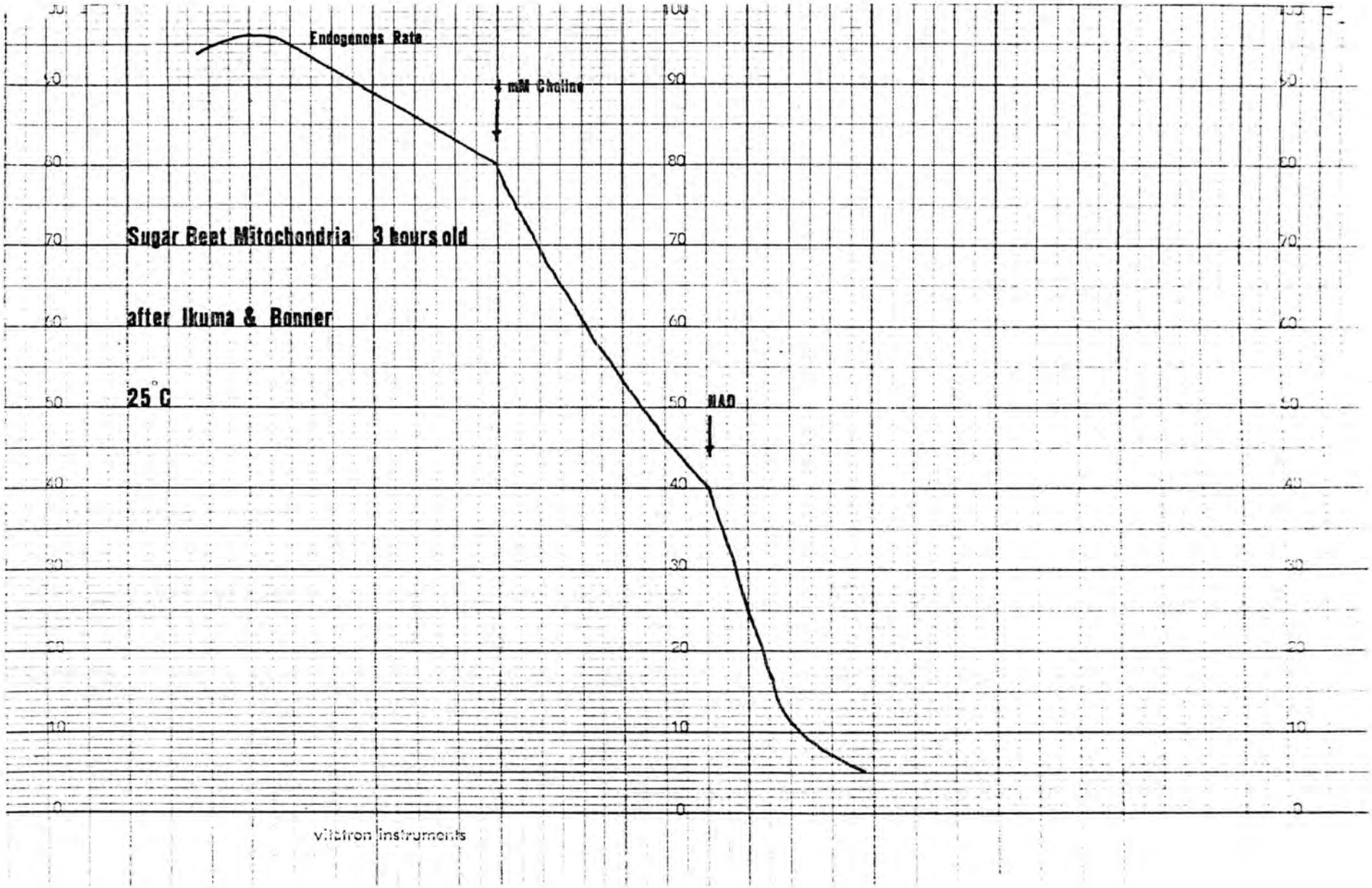
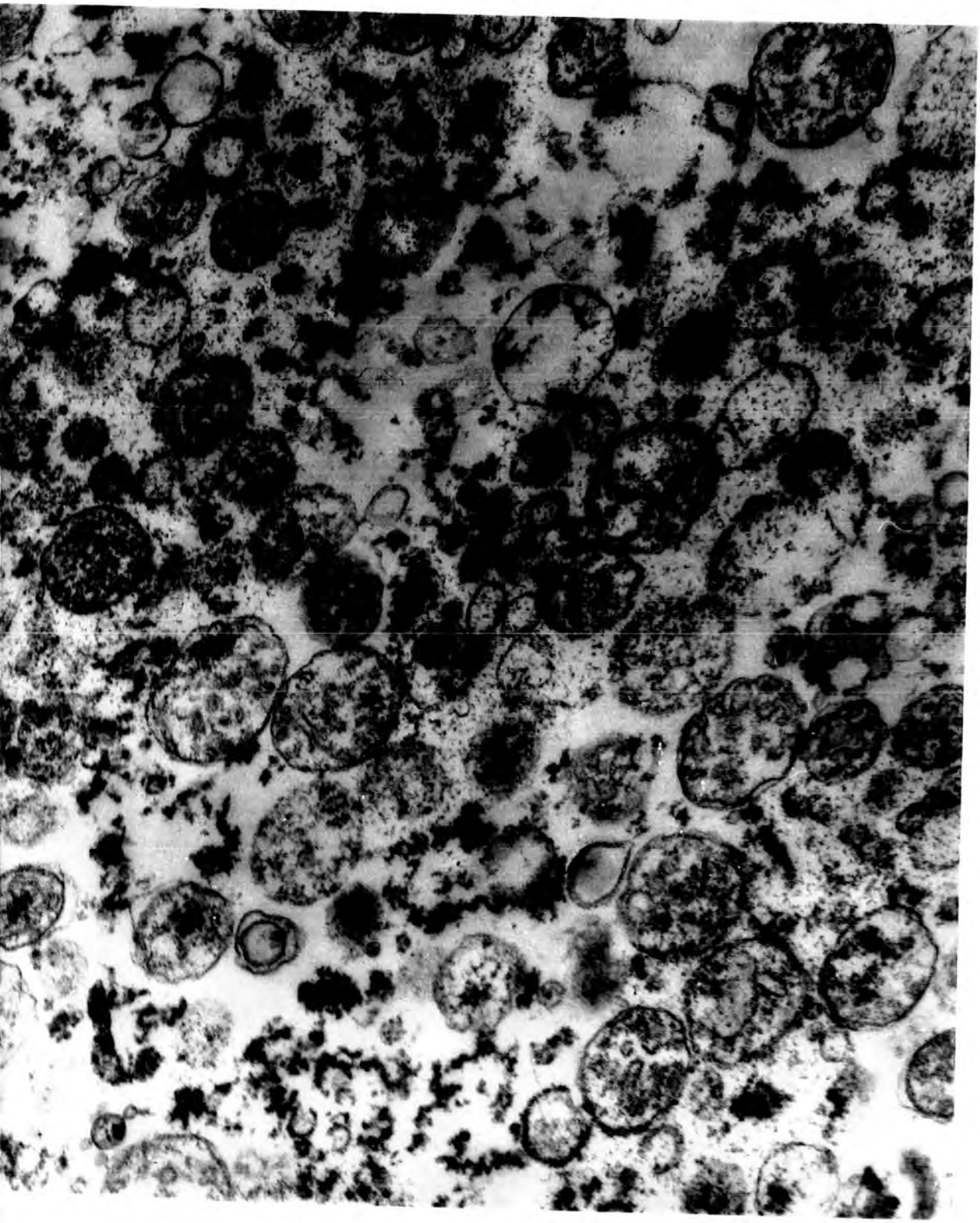

Endogenous Rate
4 mM Choline
Sugar Beet Mitochondria 3 hours old
after Ikuma & Bonner
25°C
NAD
vittron instruments

PLATE 1

An electron micrograph of mitochondria extracted from mung bean hypocotyls.



recommended by Millerd, Bonner, Axelrod and Bandursky (1951) and Axelrod (1955) has been shown to be detrimental. Phosphate extracts cytochrome c and also leads to mitochondrial rupture. Tsou (1952) experimented on the relative efficiency of extraction of cytochrome c with electrolytes and non-polar compounds and Fig. 20 sets out the efficiency of extraction of cytochrome c from heart-muscle mince and indicates that the use of boric acid buffer or sucrose in an extractant medium would have no "leaching" effect of endogenous cytochrome c from mitochondria. Zelitch (1967) advocated the use of a borate buffer in conjunction with a tris buffer for the extraction of mitochondria from green leaves. The inclusion of a mild reducing agent in the grinding medium was necessary to minimise the concentration of oxidising compounds, e.g. quinones (which are formed when cells are ruptured).

Bovine serum albumin has the capacity to bind a variety of substances and its presence in the grinding medium is required for the isolation of good mitochondria from most plant tissues, (Price and Thimann (1954); Crane (1957); Throneberry (1961); Wiskich and Bonner (1963); Lane, Hobson, Young and Biale (1965)). The importance of pH control is well documented and is discussed by Wiskich and Bonner (1963). Stokes, Anderson and Rowan (1968) have advocated the use of metabisulphite in the isolation of mitochondria from potato tuber tissue to prevent damage by phenolic compounds during extraction. During extraction of plant tissues, endogenous phenolics are rapidly oxidized to form quinones, condensed tannins and brown pigments. The oxidation is catalysed by phenoloxidases but only occurs to an appreciable extent when the tissues are homogenised because the phenolics are spatially separated from the phenoloxidases in intact tissue. The products of the phenoloxidase reaction powerfully inhibit plant enzymes and subcellular organelles.

FIG. 20

Relative efficiency of extraction of cytochrome c from heart-muscle mince using various extractants, after Tsou (1952).

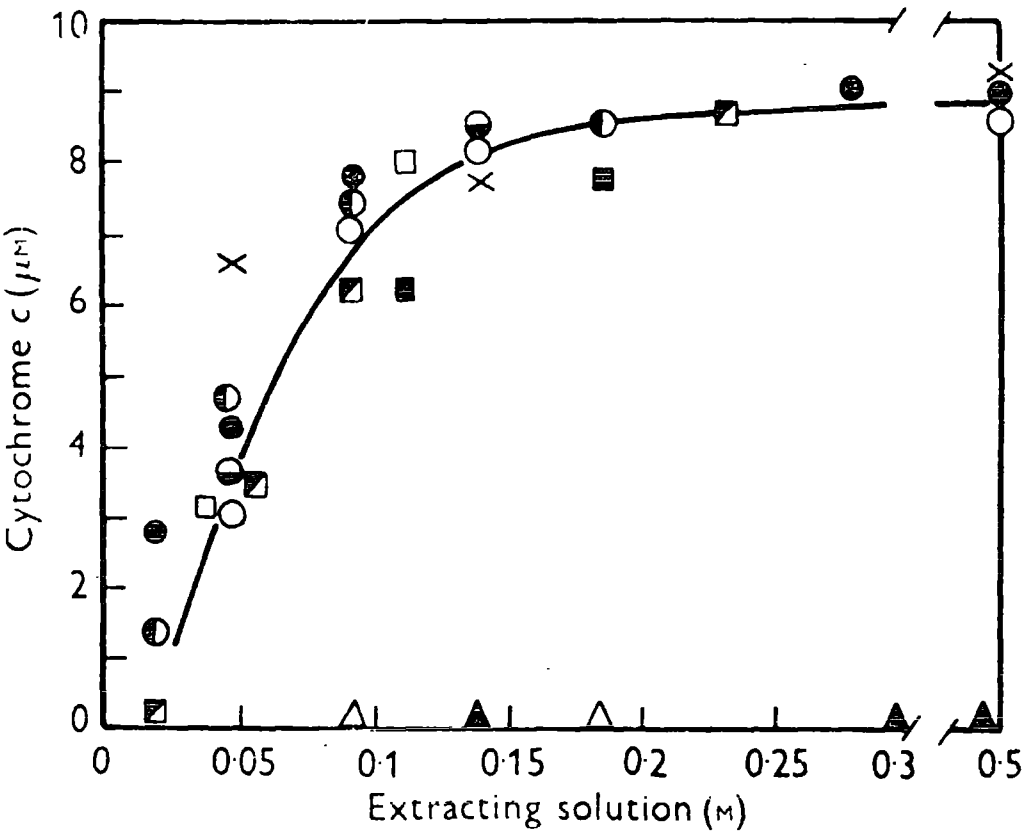


Fig. 20 Extraction of cytochrome *c* from heart-muscle mince (for method see text). ●, KCl; ○, NaCl; ◖, LiCl; ◟, KNO_3; phosphate buffer: ▬, pH 6·2; ◪, 7·3; □, 8; ×, Na_2SO_4; △, boric acid titrated to pH 7·3; ▲, sucrose.

Anderson (1968) reviewed the effectiveness of reducing agents and other compounds on the efficiency of extraction of enzymes from tissues containing phenolics. To predict the suitability of various agents available and to increase the efficiency of extraction from specific tissues a number of variables must be defined.

1. The o-diphenoloxidase activity of the tissue.
2. The subcellular location of o-diphenoloxidase.
3. The nature and the amount of the phenolics and tannins in the tissue.
4. The mechanism of action of o-diphenoloxidase(s) and which of the endogenous phenolics is a substrate for the oxidase.
5. The mechanism of action of the various compounds for preventing accumulation of the products of o-diphenoloxidase.
6. The susceptibility of the enzyme being extracted to products of the o-diphenoloxidase reaction.

In the absence of this information the methods available must be experimentally compared for efficiency of extraction. However, Anderson (1968) was able to suggest a tentative guide. Reducing agents (cysteine, thioglycollate, metabisulphite, diethyldithiocarbamate and dithionite) have proved effective for the extraction of enzymes and subcellular organelles from tissues containing o-diphenoloxidase substrates of low M.W. Verleur (1965) had suggested that cysteine would be ineffective for the preparation of coupled mitochondria from plant tissues containing high concentrations of endogenous phenolics. Equally high concentrations of cysteine would have to be used, but cysteine is oxidised rapidly by plant mitochondria (Hackett, Haas, Griffiths and Niederpruem (1960)), which may lead to high background oxidation in experiments. However,

there is reason to believe that metabisulphite should be effective for preparing mitochondria from tissues containing high concentrations of endogenous phenolics, (Stokes, Anderson and Rowan (1968)). Polymers, in contrast to the reducing agents, powerfully bind tannins and oxidation products of o-diphenoloxidase activity of high M.W. and polymers would appear to be of most use in tissues containing such compounds. The ability of polymers to bind phenolics of low M.W. is not proved. BSA is routinely included in media for extracting plant mitochondria (Verleur (1965); Ikuma and Bonner (1967); Stokes, Anderson and Rowan (1968)), because of its capacity to absorb free fatty acids which would otherwise be absorbed by and uncouple plant mitochondria (Dalgarno and Birt (1962)). Weinbach and Barbus (1966) demonstrated that BSA powerfully binds phenolics and reverses the uncoupling effects of phenol of low M.W. Watson and Smith (1967) suggested that the effectiveness of BSA in improving the properties of mitochondria from Aspergillus niger could be due to binding of phenolics in addition to binding of free fatty acids.

So the apparent inability to demonstrate the occurrence of a choline dehydrogenase in mitochondrial extracts cannot be explained away by suggesting structural damage. Fig. 21a demonstrates a trace of the respiration of sugar beet mitochondria with betaine added as a possible substrate. Previous work had suggested the presence of dimethylglycine in plant isolates and Buffa (1957) has presented evidence that choline degradation in Pseudomonas pyocyanea proceeded through betaine, N-dimethylglycine and sarcosine. No increase in oxidation was recorded when mitochondrial extracts of any of the plant species used were incubated with betaine. However, malate was readily respired. Fig. 21b is a polarographic trace of the respiration of spinach beet mitochondria isolated after Ikuma and Bonner (1967). A high endogenous rate of respiration was recorded

FIG. 21a

Polarographic assay of the respiration of mitochondria isolated from sugar beet leaves.

The reaction mixture (aerated) contained:-

0.3 M	mannitol
0.01	KCl
0.01	KPO_4 buffer pH 7.2
5 mM	$MgCl_2$
1 ml	mitochondrial suspension

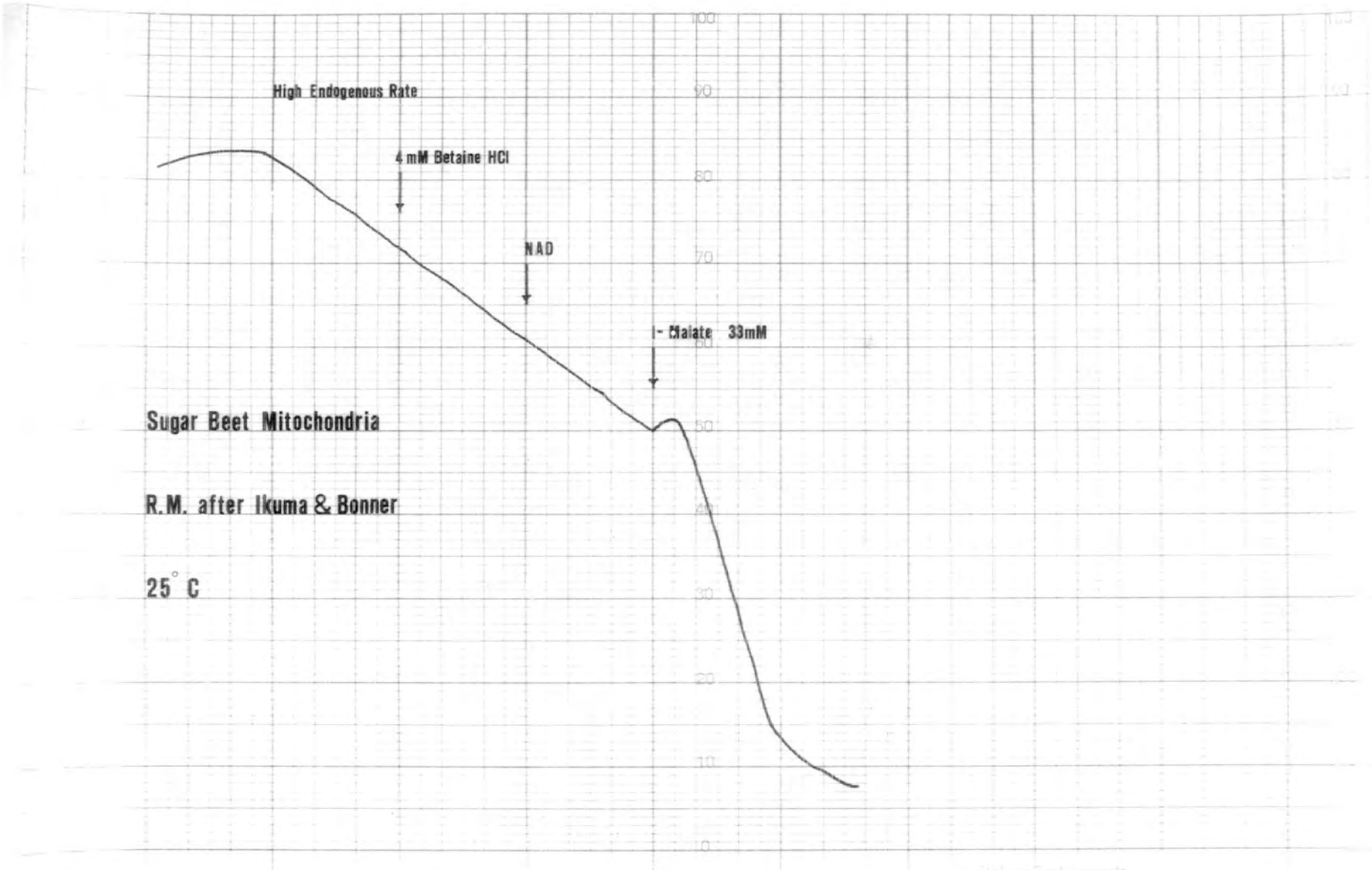
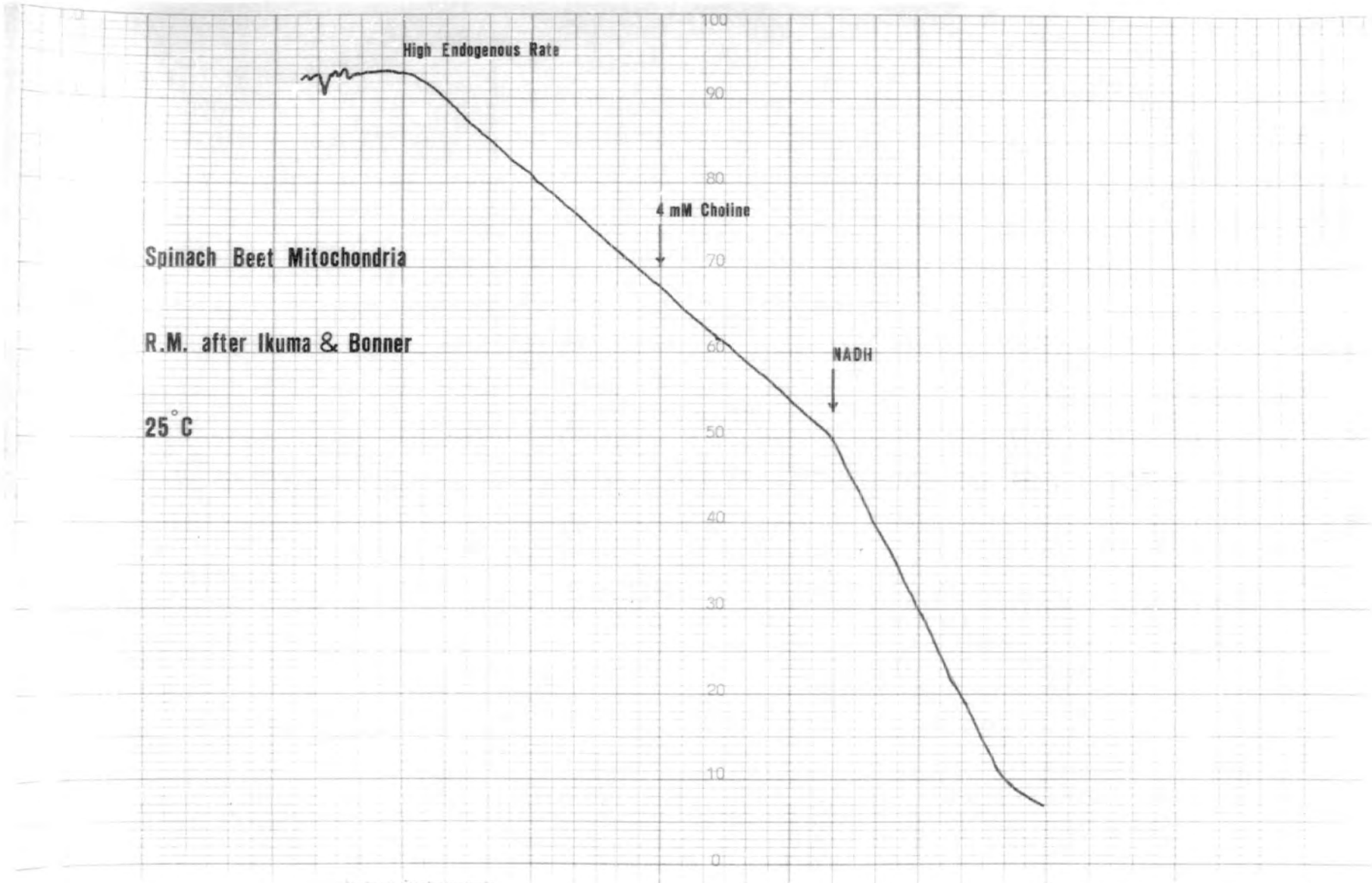

High Endogenous Rate
4 mM Betaine HCl
NAD
l- Malate 33mM
Sugar Beet Mitochondria
R.M. after Ikuma & Bonner
25° C
100
90
80
70
60
50
40
30
20
10
0

FIG. 21b

Polarographic assay of the respiration of spinach beet leaf mitochondria.

The reaction mixture (aerated) contained:-

0.3 M	mannitol
0.01	KCl
0.01	KPO_4 buffer pH 7.2
5 mM	$MgCl_2$
1 ml	Mitochondrial suspension extracted using the method of Stokes, Anderson and Rowan (1968).


High Endogenous Rate
4 mM Choline
NADH
Spinach Beet Mitochondria
R.M. after Ikuma & Bonner
25° C
100
90
80
70
60
50
40
30
20
10
0

and no stimulation of respiration was observed when choline was added. An addition of NADH immediately increased the rate of respiration. A trace of the respiration of mitochondria isolated from leaves of Chenopodium vulvaria by the method of Ikuma and Bonner (1967), is shown in Fig. 22. An initial increase in the rate of respiration was recorded when choline was first added. No further increase was recorded when other additions of choline, betaine aldehyde and ADP were made.

Mitochondria isolated from choline mutants and wild type strains of Neurospora crassa by all three methods described in the Methods Text demonstrated the ability to respire malate, succinate and NADH. There was no evidence that choline and betaine aldehyde were respired.

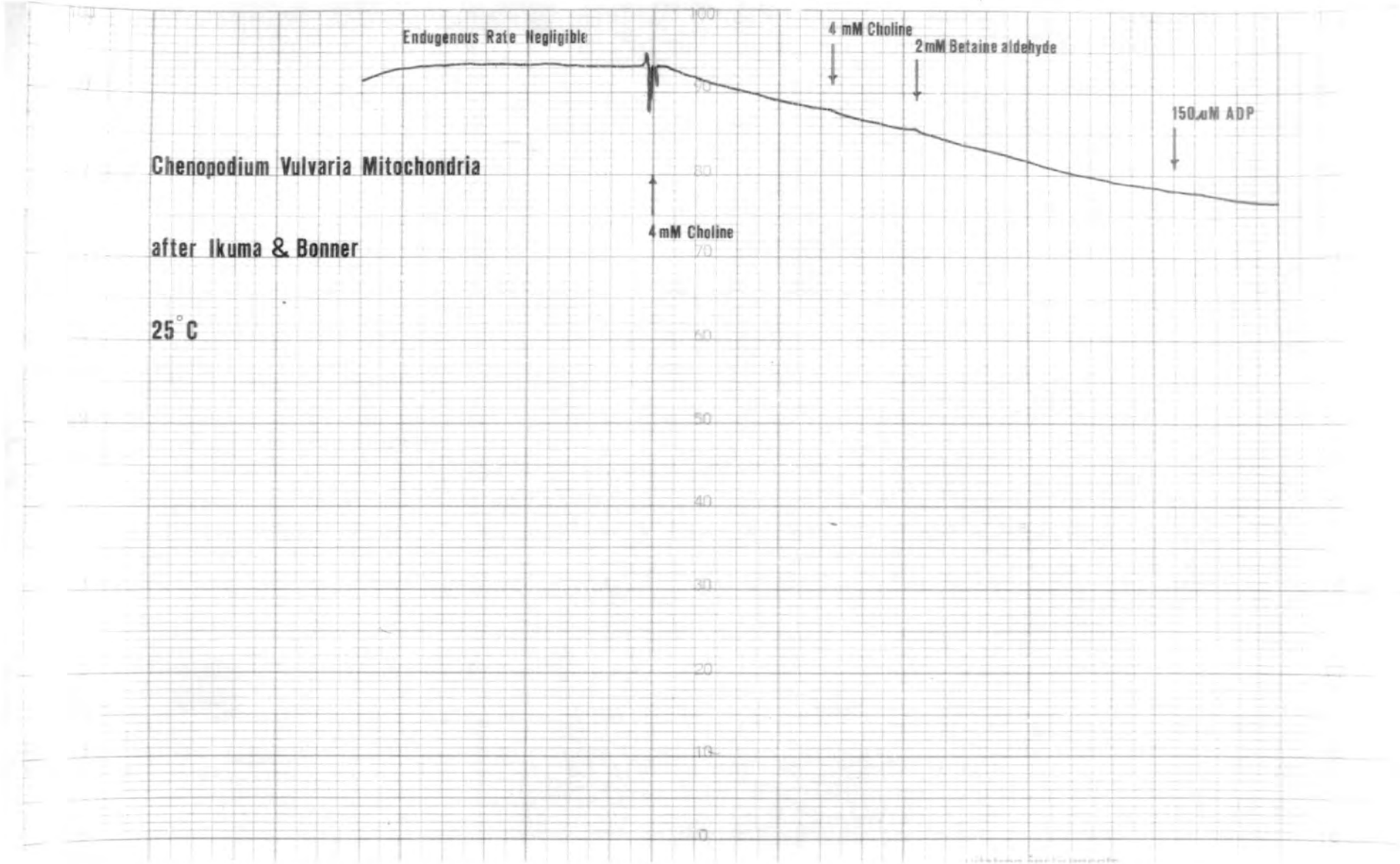
Of the various methods used for the isolation of plant mitochondria that of Ikuma and Bonner (1967) was generally the most successful in producing active mitochondria. The extraction procedure after Stokes, Anderson and Rowan (1968) was also successful when isolating mitochondria from green tissue. The method of Zelitch and Barber (1960) for the preparation of plant mitochondria from green leaf tissue was unsuccessful. Such mitochondrial pellets were found to be contaminated with many intact and broken chloroplasts. The rapid isolation method after Palmer (1967) yielded mitochondrial pellets with a very high endogenous rate of respiration. Aging of these mitochondria for several hours did not deplete the endogenous substrate levels to any significant degree.

Pilot experiments with mitochondria extracted from Pisum sativum L. and Vicia faba L. demonstrated respiratory activity with succinate, malate and NADH as substrates, but gave no suggestion of the presence of an active choline dehydrogenase system. Other organs of the various plant sources were investigated as potential sources

FIG. 22

Polarographic assay of the respiration of mitochondria isolated from Chenopodium vulvaria leaves.

(See Fig. 21b for Reaction Mixture etc.)


Endugenous Rate Negligible
4 mM Choline
2mM Betaine aldehyde
150 uM ADP
Chenopodium Vulvaria Mitochondria
after Ikuma & Bonner
25° C
4 mM Choline
100
90
80
70
60
50
40
30
20
10
0

of active mitochondria. Young roots were found to be an excellent source of active mitochondria but in preliminary experiments the addition of choline as a substrate did not give rise to an increase in respiration. Mitochondria were also extracted from leaves of Atriplex hastata L. when available. Unlike Chenopodium vulvaria L. no evidence was obtained for the presence of an active choline dehydrogenase system in these preparations.

Despite the fact that the evidence for the presence of a choline dehydrogenase system has not been absolutely confirmed by spectrophotometric analysis and polarography there are suggestions of activity (Figs. 14, 19 and 22). Mitochondria extracted from plant tissues were shown to be physically intact by electron micrographs, and their biochemical integrity was demonstrated by the respiratory control shown by the addition of ADP; the addition of cytochrome c did not influence the oxidation rate of any substrate; and the retention of respiratory control for several hours following isolation. However, their inability to use choline as a respiratory substrate was marked. Consequently, the previously described techniques of determination of quaternary ammonium compounds have been applied to incubations of the mitochondria isolated from the various plant sources. These results were then compared and contrasted with the evidence suggested by the spectrophotometric and polarographic data.

Application of Column Chromatography and Thin-Layer Chromatography for the determination of Quaternary Ammonium compounds formed during Mitochondrial incubations.

The radio-active base(s) were isolated and applied to the ion-exchange resin column as described in detail in the Methods Section. Betaine, dimethylglycine, choline and betaine aldehyde are well resolved in that order by a buffered column of Amberlite C.G.-50 using 600 ml of phosphate-citrate buffer pH 5.3 as demonstrated by

Fig. 23. Fig. 24 demonstrates the separation obtained for the radio-active products of the oxidation of ^{14}C-methyl choline by rat liver mitochondria. Rapid conversion of choline to betaine aldehyde by the enzyme choline dehydrogenase and the further conversion of betaine aldehyde to betaine by the enzyme betaine aldehyde dehydrogenase was confirmed. Fig. 25 shows the radio-active products resulting from the incubation of ^{14}C-methyl or 1, 2-^{14}C-choline with mitochondria isolated from mung bean hypocotyls and is typical of the results obtained with the plant sources described in the Methods section. Fig. 26 however is typical of the results obtained with mitochondria isolated from both sugar beet and spinach beet. This demonstrates a peak corresponding to that of betaine and another peak, which from its elution volume appears to correspond to that of dimethylglycine. Control incubations of mitochondria inactivated by boiling also yielded similar results. Obviously the second peak is not derived from mitochondrial oxidation and may be due to bacterial contamination during the incubation period. Fig. 26a, however, is the column profile of radioactive compounds from a sample of 1, 2-^{14}C-choline (i.e. not supplied to incubations of mitochondria). A betaine contaminant and an unidentified contaminant were in evidence. The supplied 1, 2-^{14}C-choline was no longer homogeneous despite the great care to handle and store such compounds in such a way as to minimise the possibilities of bacterial contamination. The second contaminant although corresponding to dimethylglycine in its elution volume did not co-chromatograph with dimethylglycine during thin-layer chromatography or paper electrophoresis.

Isolated radioactive base(s) were applied to TLC plates and subjected to two-dimensional chromatography as described in the Methods section. The plates were attached to photographic film.

FIG. 23

Column profile of betaine dimethylglycine, choline and betaine aldehyde on Amberlite C.G.-50 buffered at pH 7.3 (phosphate-citrate pH 5.3 was the eluting buffer).

Column dimensions 1.6 cm x 120 cm.

(For experimental details of column chromatography see Materials and Methods Text p.34).

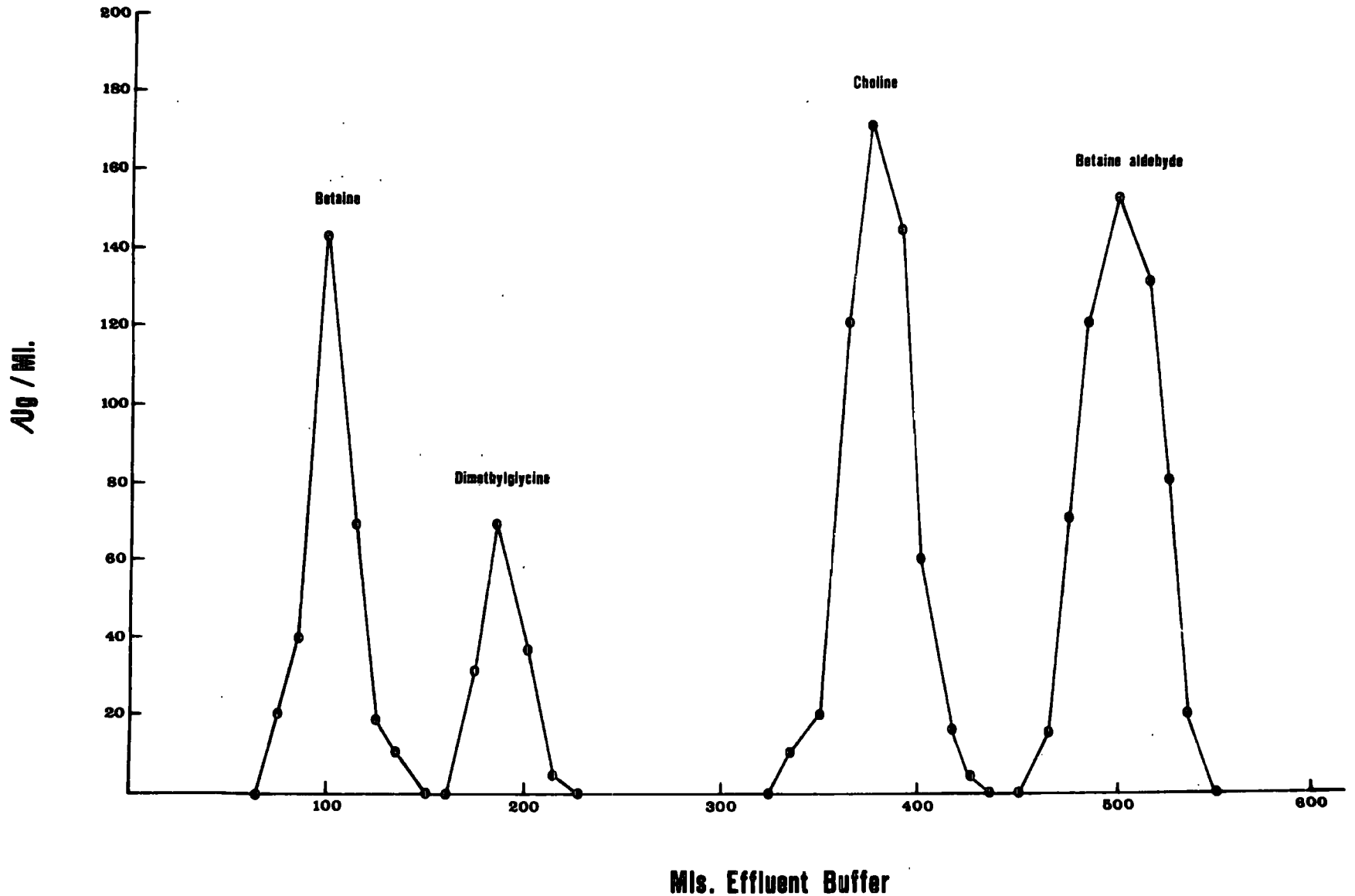

Betaine
Dimethylglycine
Choline
Betaine aldehyde
µg / ml.
Mls. Effluent Buffer
200
180
160
140
120
100
80
60
40
20
100
200
300
400
500
600

FIG. 24

Separation of radioactive quarternary ammonium products of the oxidation of ^{14}C-methyl choline by rat liver mitochondria by column chromatography (Amberlite C.G.-50.)

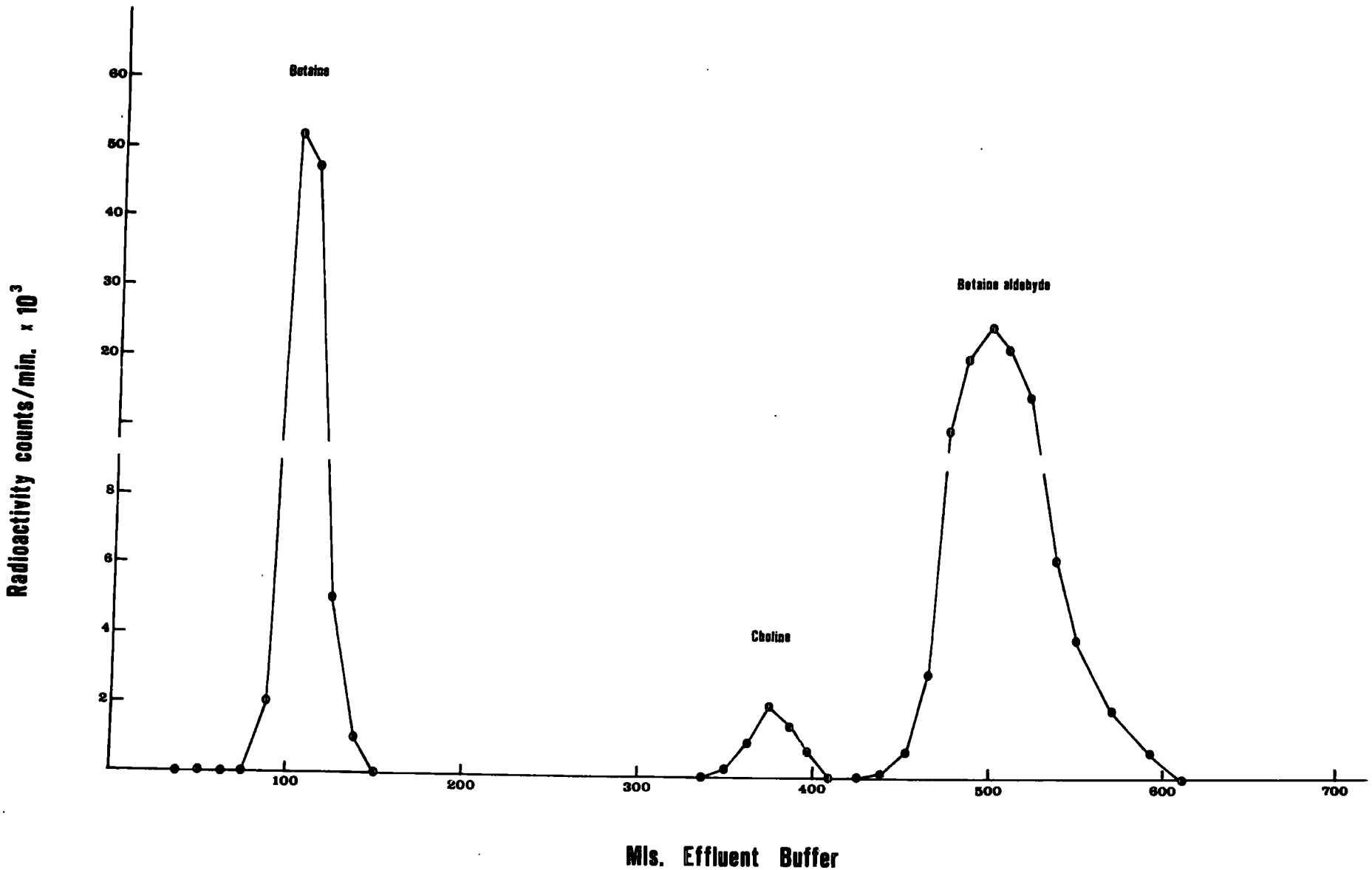

Betaine
Choline
Betaine aldehyde
Radioactivity counts/min. x 10³
Mls. Effluent Buffer
60
50
40
30
20
8
6
4
2
100
200
300
400
500
600
700

FIG. 25

Separation of the radioactive quaternary ammonium products resulting from the incubation of ^{14}C-methyl or 1,2 -^{14}C-choline with mitochondria isolated from mung bean hypocotyls by column chromatography.

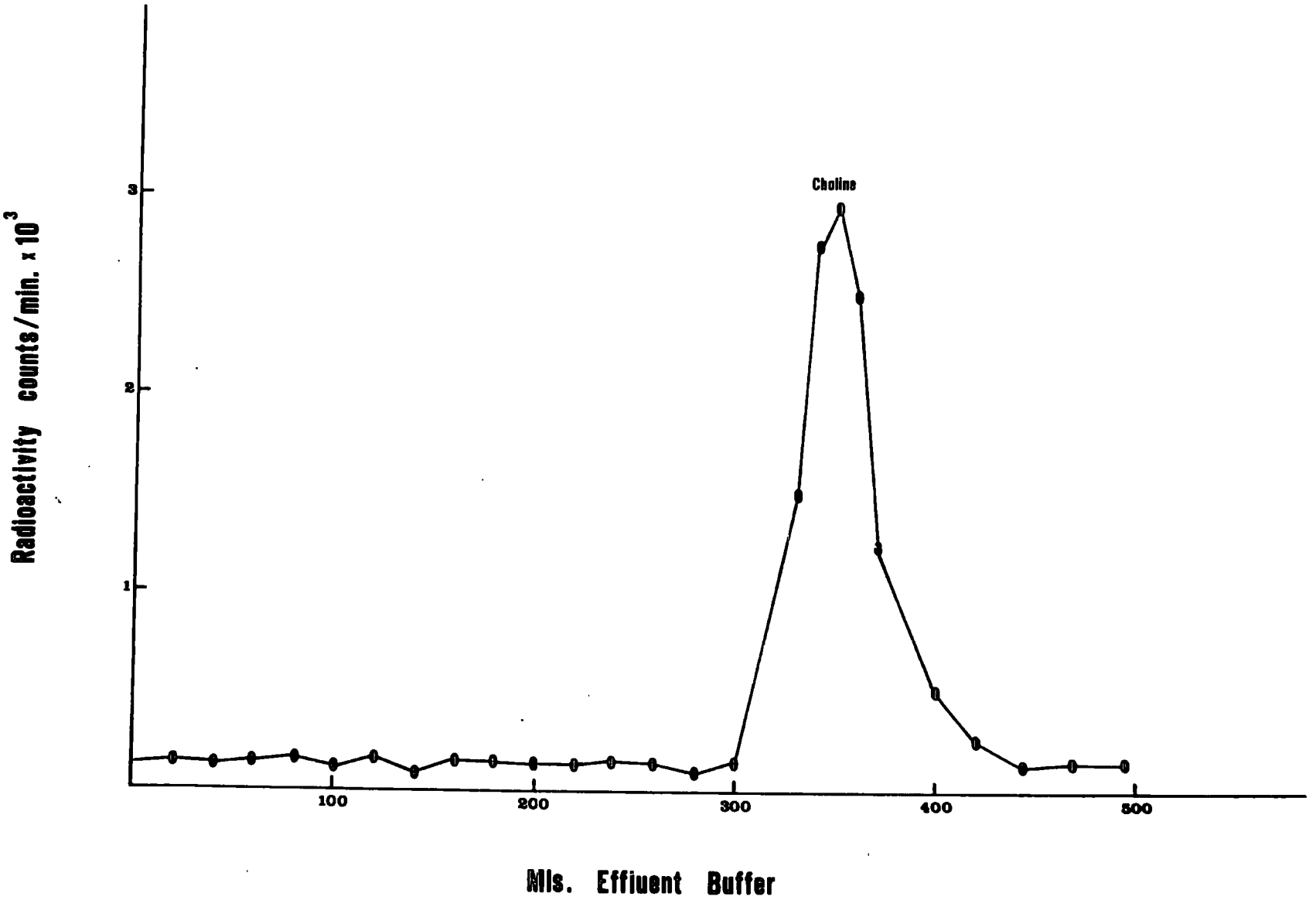

Radioactivity counts/min. x 10³
3
2
1
Choline
100
200
300
400
500
Mls. Effiuent Buffer

FIG. 26

Separation of the radioactive quaternary ammonium products resulting from the incubation of 1, 2 -^{14}C-choline with sugar beet leaf mitochondria by column chromatography.

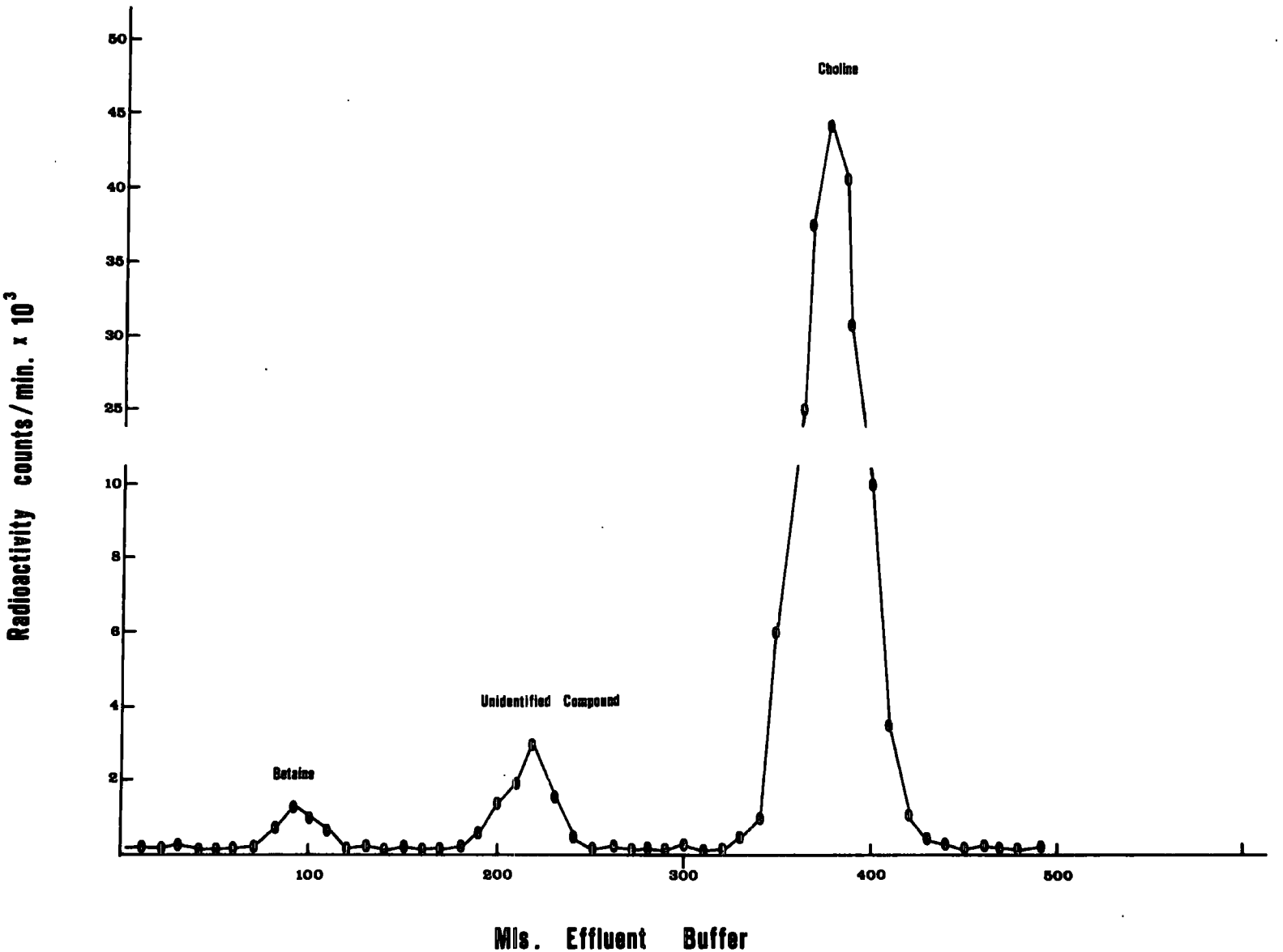
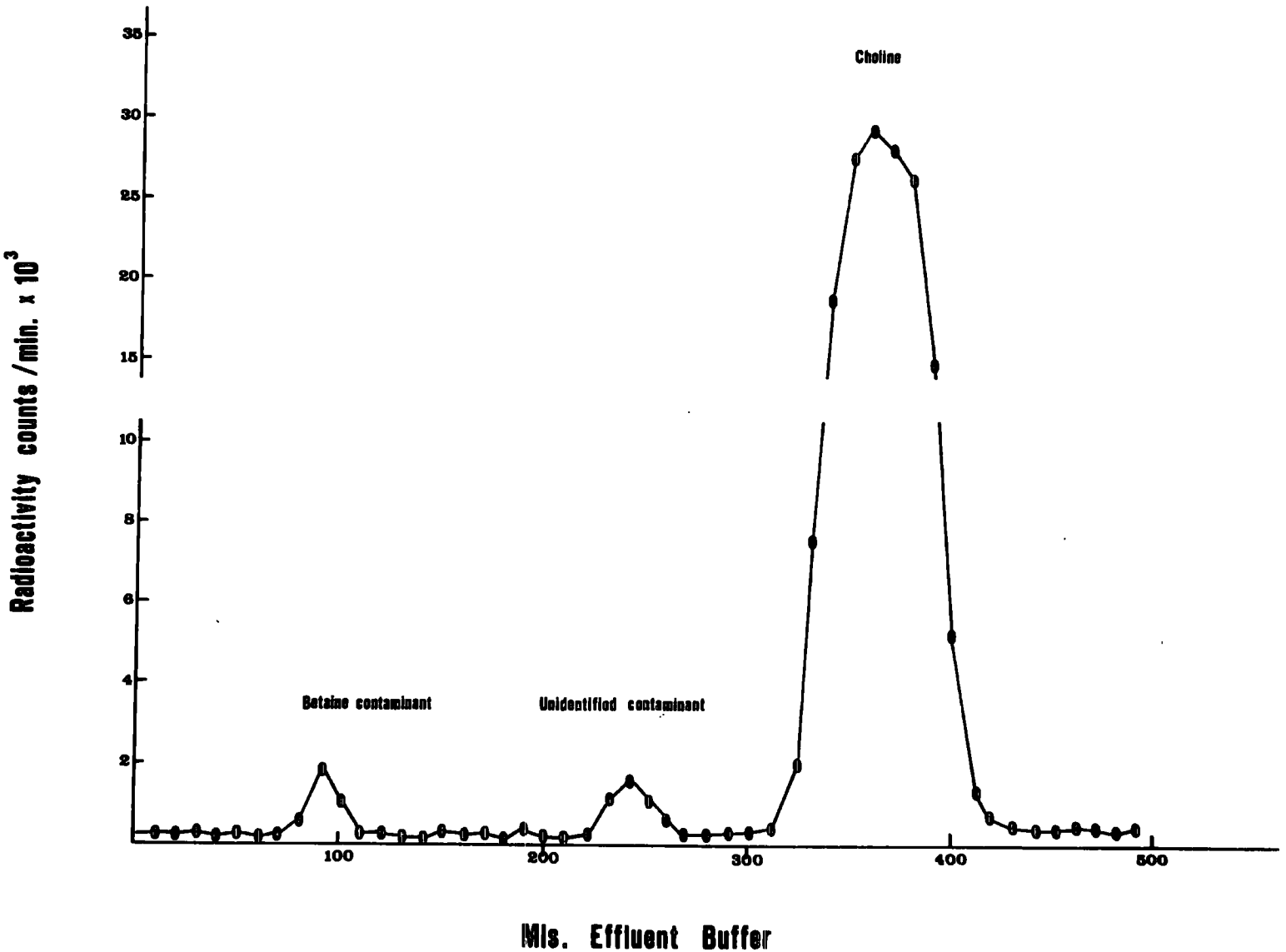

Radioactivity counts/min. x 10³
50
45
40
35
30
25
10
8
6
4
2
Choline
Unidentified Compound
Betaine
100
200
300
400
500
Mls. Effluent Buffer

FIG. 26a

Column profile of the radioactive quaternary ammonium compounds separated from a sample of 1, 2 - ^{14}C-choline (not incubated) by column chromatography.


Radioactivity counts /min. x 10³
35
30
25
20
15
10
8
6
4
2
Choline
Betaine contaminant
Unidentified contaminant
100
200
300
400
500
Mls. Effluent Buffer

and autoradiographs allowed to develop in complete darkness for about a week. Fig. 27 is an autoradiograph of the quaternary ammonium compounds resulting from the incubation of rat liver mitochondria with ^{14}C-methyl choline. The three radioactive spots corresponded to choline, betaine aldehyde and betaine confirming the presence of an active choline dehydrogenase system (the two enzymes choline dehydrogenase and betaine aldehyde dehydrogenase) in rat liver mitochondria. Figs. 28 (a), (b) and (c) are the individual compounds separated by column chromatography (Amberlite C.G.-50). The fractions containing radio-activity in the three peaks were pooled and subjected to the reineckate procedure. The quaternary ammonium compounds thus isolated were subjected to TLC on Silica Gel G, and autoradiographs developed as before.

Fig. 29 shows the radioactive products of the incubation of 1, 2-^{14}C-choline with sugar beet mitochondria. Two spots were tentatively identified, one as choline and the other as betaine aldehyde. However, column chromatography did not confirm this - only one peak corresponding to the elution volume of choline was in evidence. This may indicate the presence of a degradation byproduct in the radioactive choline sample or breakdown of the reisolated base after incubation. Similarly, the two spots formed by radio-active products of the incubation of 1, 2-^{14}C-choline with mito-chondria isolated from leaves of Chenopodium vulvaria as shown in Fig. 30 were unconfirmed by column chromatography. Fig. 31 shows the radioactive products of the incubation of 1, 2-^{14}C-choline with mitochondria extracted from mung bean hypocotyls. Again two spots were separated, the identification of which was not supported by column chromatographic results.

Fig. 32 shows the results of two dimensional TLC of the products of the incubation of 1, 2-^{14}C-choline with mitochondria extracted

FIG. 27

Autoradiograph of the quaternary ammonium compounds* resulting from the incubation of rat liver mitochondria with ^{14}C-methyl choline.

* Separated by T.L.C. as described in the Methods text.

(For experimental details of T.L.C. see Materials and Methods p.38 , and for details of radioactive analysis see p.43).

1. MeOH — NH_3 75/25 v/v ⟶

2. MeOH — Acetone — HCl 90/10/4 v/v ⟶

FIG. 28a

Autoradiograph of choline re-isolated from the incubation with rat liver mitochondria using the reineckate procedure and separated by column chromatography before being subjected to thin-layer chromatography.

1. **MeOH — Acetone — HCl** 90/10/4 v/v

Choline

1. **MeOH — NH_3** 75/25 v/v

FIG. 28b

Autoradiograph of betaine aldehyde isolated from the incubation with rat liver mitochondria using the reineckate procedure and column chromatography before being subjected to thin-layer chromatography.

Betaine aldehyde

1. MeOH — NH_3 75/25 v/v ⟶

2. MeOH — Acetone — HCl 90/10/4 v/v ⟶

FIG. 28c

Autoradiograph of betaine isolated from the incubation with rat liver mitochondria using the reineckate procedure and column chromatography before being subjected to thin-layer chromatography.

Betaine

2. **MeOH — Acetone — HCl** 90/10/4 v/v

1. **MeOH — NH_3** 75/25 v/v

FIG.29

Autoradiograph of the quaternary ammonium compounds resulting from the incubation of 1, 2 -^{14}C-choline with sugar beet leaf mitochondria.

1. **MeOH — NH_3** 75/25 v/v —→

2. **MeOH — Acetone — HCl** 90/10/4 v/v ←—

FIG. 30

Autoradiograph of the quaternary ammonium compounds resulting from the incubation of 1, 2 - ^{14}C-choline with mitochondria isolated from leaves of Chenopodium vulvaria.

1. **MeOH — NH_3** 75/25 v/v

2. **MeOH — Acetone — HCl** 90/10/4 v/v

FIG. 31

Autoradiograph of the quaternary ammonium products of the incubation of 1, 2 - ^{14}C-choline with mung bean hypocotyl mitochondria.

1. MeOH — NH_3 75/25 v/v ⟶

2. MeOH — Acetone — HCl 90/10/4 v/v ⟶

FIG. 32

Autoradiograph of the quaternary ammonium products of the incubation of 1, 2 -^{14}C-choline with mitochondria extracted from the mycelia of Neurospora crassa.

1. **MeOH — NH_3** 75/25 v/v ⟶

2. **MeOH — Acetone — HCl** 90/10/4 v/v ⟶

from the mycelia of Neurospora crassa. Two radioactive spots were in evidence but in this case the second spot was hardly separated from the spot corresponding to the position attained by choline. On Figs. 29 - 32 to a greater or lesser extent a radio-active blackening of the film occurred at the origin. This was an interesting phenomenon as of all the quaternary ammonium compounds examined in this study, none was found to remain at or near the origin in both the solvent systems. On the other hand this may be an overloading effect, the concentration of compound being too great to run with the solvent.

Figs. 33 and 34 show two TLC separations of the radioactive products of the incubation of 1, 2-^{14}C-choline with spinach beet mitochondria. These are anomalous results compared with those obtained in all other experiments. Fig. 33 shows three fogged areas and bad tailing between two of the spots. Two of these correspond to the position that would be attained by choline and betaine aldehyde. Fig. 34 shows four fogged areas (as well as fogging at the origin). Two spots corresponded to choline and betaine aldehyde; the others were not identified. These results again were not confirmed by prior column chromatography of these incubations.

The autoradiographs contrasted with the results obtained from column chromatography. The applied radioactive choline was the only compound eluted off the column in any significant amount (counts of less than twice background were regarded as insignificant). These anomalous results may reflect incomplete elution of compounds from ion-exchange columns or may be the result of breakdown of choline after isolation by the reineckate procedure. Known quantities of radioactive choline, betaine aldehyde (formed by incubating radioactive choline with rat liver mitochondria) and betaine applied to Amberlite C.G.-50 columns were eluted quantitatively. Samples of the three radioactive compounds precipitated with ammonium reineckate and

FIG. 33

Autoradiograph of the quaternary ammonium products of the incubation of 1, 2 - ^{14}C-choline with spinach beet leaf mitochondria.

1. MeOH — NH_3 75/25 v/v ⟶

2. MeOH — Acetone — HCl 90/10/4 v/v ⟶

FIG. 34

Autoradiograph of the quaternary ammonium products of the incubation of 1, 2 -^{14}C-choline with spinach beet leaf mitochondria.

1. **MeOH — NH_3** 75/25 v/v →

2. **MeOH — Acetone — HCl** 90/10/4 v/v ↑

reisolated before being applied to TLC plates gave no anomalous fogged spots during autoradiography.

Because of the apparent anomalies between autoradiography and column chromatography the method developed by Feige, Gimmler, Jeschke and Simonis (1969) which permitted the separation of labelled intermediates of plant metabolism by TLC on cellulose layers without requiring a preliminary purification of the plant extracts from interfering compounds, was utilised. It was also of interest to ascertain whether choline was being degraded to form compounds other than related quaternaries. The method was adapted for incubations of plant mitochondria with 1, 2 -^{14}C-choline and Fig. 35 is an autoradiograph of the products of the incubation of mitochondria extracted from Neurospora crassa with 1, 2 - ^{14}C-choline. Three fogged areas were separated, none of which were positively identified. Extracts from other plant mitochondrial incubations gave no satisfactory results.

Mitochondrial pellets extracted from all plant sources and mycelia of Neurospora crassa were extracted after the method of Kaschnik, Peterlik and Weiss (1969). The mitochondria had been incubated with 1, 2 -^{14}C-choline for varying periods of time. The mycelia were harvested from flasks whose growth medium had been supplemented with 1, 2 -^{14}C-choline. The extracts were submitted to thin-layer chromatography on Silica Gel HR Merck. Extracts from mung bean mitochondria yielded spots which corresponded with marker spots of Phosphatidylethanolamine (R_F = 0.80) and choline (R_F = 0.14). More spots were located at or near the solvent front and were probably neutral fats and free fatty acids. Extracts from Neurospora crassa mycelia and sugar beet seeds yielded spots corresponding to phosphatidylethanolamine and neutral fats and free fatty acids. Table 8 gives reference values for R_F's of certain lipids. (See page 58).

FIG. 35

Autoradiograph of the products* resulting from the incubation of 1, 2 - ^{14}C-choline with mitochondria extracted from mycelia of Neurospora crassa.

*Separated by TLC after Feige, Gimmler, Jeschke and Simonis (1969) as described in the Methods text.

Isobutyric acid — n-butanol — isopropanol — n-propanol — H_2O — NH_3 — Edta

1000/30/30/140/380/40/0·5 v/v/v/v/v/v/g

1. n-Butanol — n-propanol — n-propionic acid — H_2O 400/175/285/373 v/v
2. n-Butanol — acetic acid — H_2O 5/1/4 v/v upper phase

Extracts incubated with 1, 2-^{14}C-choline were submitted to TLC and autoradiographs allowed to develop for about a week in the dark. The only fogged spot which developed approximated to an R_F value corresponding to that of choline. This may have been free choline or may have been derived from hydrolysis of lecithin in the extraction procedure. No other blackened spots were in evidence.

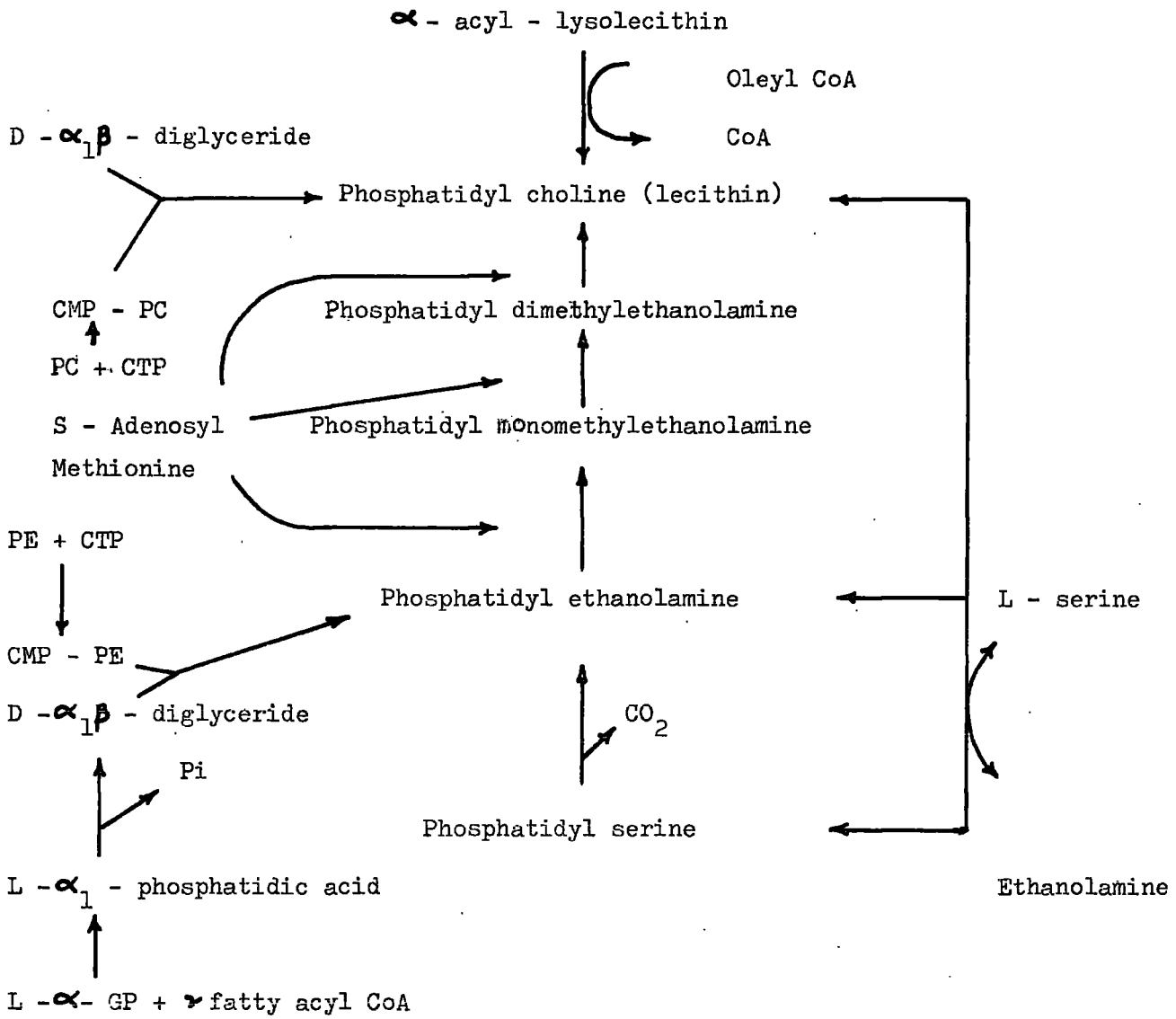
The incorporation of choline into phosphatides is of particular importance in fat metabolism. Pozsar (1957) studied lecithin synthesis in dehydrating seeds of Helianthus annuus and Phaseolus vulgaris and observed that the choline level decreased simultaneously with the dehydration of the seeds and lecithin synthesis. This emphasises the flux between free and bound choline. Street, Kenyon and Watson (1946) described a method for the estimation of free choline in plants, based on the conversion of choline to trimethylamine by treatment with dilute alkaline permanganate under controlled conditions. Using this method they produced figures for the choline-N content of the organs of Atropa belladonna, Atropa acuminata, Hyoscyamus muticus and Solanum tuberosum. Ramasarma and Wetter (1957) demonstrated a choline kinase of Brassica campestris. This enzyme phosphorylated choline, dimethylethanolamine and diethylethanolamine but not ethanolamine. It was suggested that this constituted the first stage of the synthesis of lecithin. Hanahan and Chaikoff (1947 and 1948), Tookey and Balls (1956), Kates (1957), Einset and Clark (1958), Davidson and Long (1958) all demonstrated the presence of phospolipases in carrot, cabbage and cotton seed capable of catalysing the hydrolysis of choline from lecithin leaving phosphatidic acid. Acker and Ernst (1954) and Smith (1954) demonstrated the presence of a phospholipase in cereals and the latex of Hevea brasiliensis Muell respectively.

Kornberg and Pricer (1952) first demonstrated that phosphoryl choline, doubly labelled with ^{14}C and ^{32}P was incorporated as a unit

into the phosphatides of rat liver preparations. Previously Riley (1944) as a result of in vivo experiments had suggested that phosphoryl choline was not a precursor of choline phosphatides. Kennedy (1954) originally reported that ^{14}C-choline was more readily incorporated into lecithin than ^{14}C-phosphoryl choline; however, subsequent work (Kennedy and Weiss (1956)) established that free choline was incorporated into some choline-containing lipid other than lecithin. It would seem that phosphoryl choline was formed in most tissues by phosphorylation of choline in the presence of ATP and the enzyme choline phosphokinase. Wittenberg and Kornberg (1953) partially purified a yeast enzyme which catalysed this reaction. Kennedy and Weiss (1956) discovered the participation of cytidine nucleotides. The various pathways for lecithin synthesis are shown (see p. 121) schematically and little may be said regarding their relative importance.

The Uptake and Binding of Choline by Mitochondria

The relative inability to demonstrate a choline dehydrogenase system in mitochondria may indicate a permeability barrier to choline, or strong binding of choline and breakdown products within the mitochondria. Chappell and Greville (1963) worked on the influence of the composition of the suspending medium on the properties of mitochondria and suggest possible explanations for the "latency" of an enzyme. In most instances it is probably a consequence of impermeability of the mitochondrial membrane, to one or more of the reactants or products. Latency of the dehydrogenases may well be due to a common cause, namely impermeability of the membrane to NAD and $NADH_2$ (Bendall and de Duve, (1960)). The extent of uptake of sucrose from the suspending medium appears to be very variable and may depend on the amount of swelling and damage initially present (Tadeschi (1961)). In the slow, extensive swelling which comes after any adjustment to



Alternative pathways of biosynthesis and interconversions of the phosphatidyl compounds containing nitrogenous bases.

external osmotic pressure, external solutes, e.g. sucrose or potassium chloride enter the mitochondria (Jackson and Pace (1956), Tedeschi (1961)).

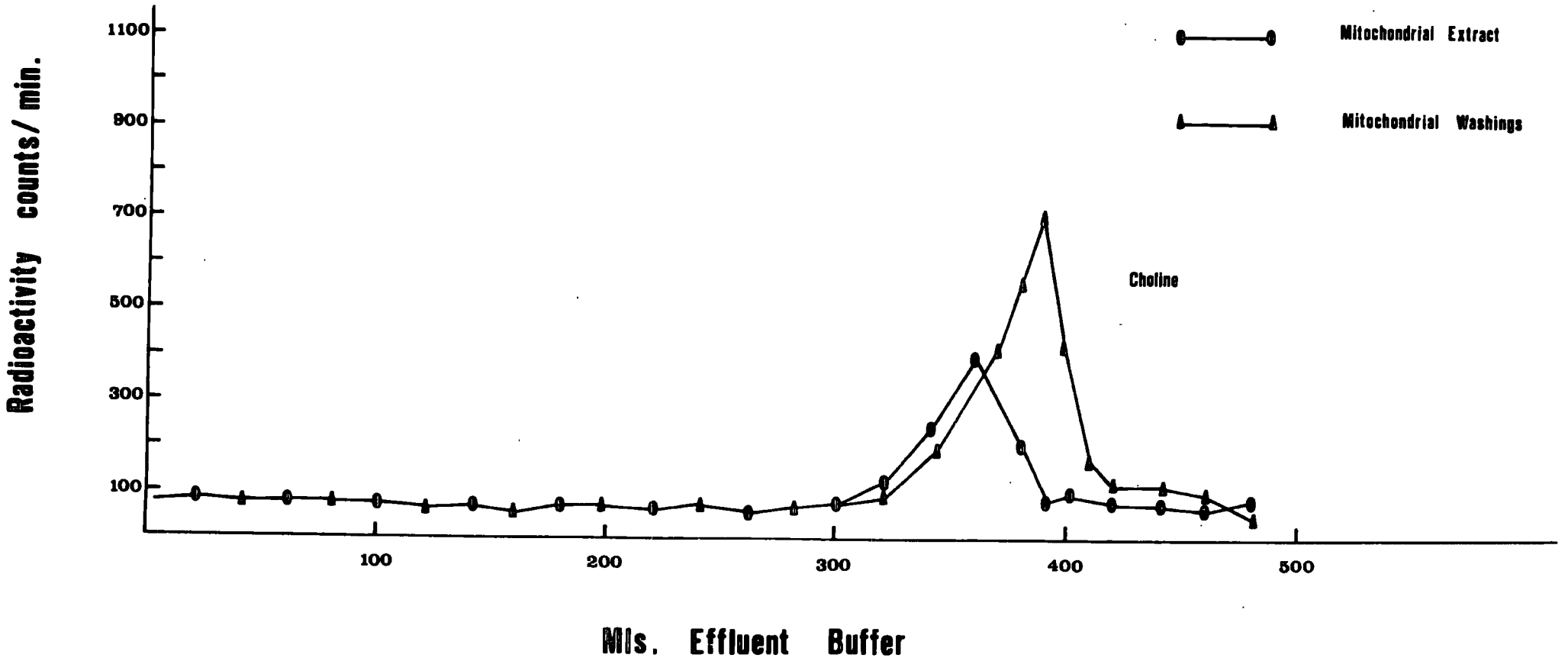
It was felt of interest to establish whether choline could induce increases in mitochondrial volume (measured by a decrease in optical density). Mitochondria extracted from all plant sources were suspended in the reaction medium described in the Methods section and held at $0^{\circ}C$. Samples of mitochondria were taken and introduced into a 1 mm spectrophotometric cell and the extinction at 520 mμ measured. The effect of the addition of concentrations of choline (2 mM - 50 mM) was observed. Apart from an initial increase in optical density, no other observed change in optical density was observed in any of the aged samples or with any of the concentrations of choline employed.

Mitochondria were subjected to pretreatment with sodium deoxycholate, tergitol and dimethyl sulphoxide before incubation; or where incubated with calcium ions and dimethyl sulphoxide, added to the incubation medium with 1, 2-^{14}C-choline as the supplied substrate. The respiratory activity was monitored polarographically and after the required time the mitochondria were pelleted and extracted to assess choline uptake. The pelleted mitochondria were washed and repelleted several times (and the washings retained) and the pellet subjected to rigorous freezing and thawing, and the extracts and washings were analysed separately for radioactive choline content. Fig. 36 shows the column profile of quaternary ammonium compounds isolated from mung bean mitochondria after incubation with 1, 2-^{14}C-choline. These mitochondria were pre-incubated with dimethylsulphoxide and as may be seen a greater amount of the radioactive choline was found associated with the mitochondrial washings, which may be contrasted with Fig. 37. This column profile of quaternary ammonium compounds isolated from

FIG. 36

Column profile of the radioactive quaternary products of the incubation of 1, 2 - ^{14}C - choline with mung bean hypocotyl mitochondria*.

* preincubated with dimethyl sulphoxide.


Radioactivity counts/ min.
1100
900
700
500
300
100
100
200
300
400
500
Mls. Effluent Buffer
Mitochondrial Extract
Mitochondrial Washings
Choline

Neurospora crassa mitochondria shows a larger amount of the radioactivity residing in the mitochondrial extract. This contrast was predictable in that dimethyl sulphoxide had been used by Delmer and Mills (1969) to increase membrane permeability; however, some radioactivity still resided in the mitochondria and was only released after severe disruption of the membranes. Pretreatment of the mitochondria with sodium deoxycholate resulted in a column profile similar to Fig. 37, i.e. no apparent effect on the membrane permeability. Similarly, 1 mM Ca^{++} did not appear to increase the level of uptake by choline. However, pretreatment of the mitochondria with tergitol resulted in all significant radioactivity being associated with the mitochondrial washings. Aging of mitochondria had no observable effect up to a point when no choline was found in the mitochondrial extracts. This occurred after six hours onwards and probably coincides with physiological inactivation.

While stating that 1 mM Ca^{++} did not increase the level of choline uptake the significant thing was that the level ofradio-activity extracted from active mitochondria did not increase with time. This must mean that either uptake only occurs to a particular choline concentration or that the choline is continuously converted to compounds not precipitated by the reineckate procedure.

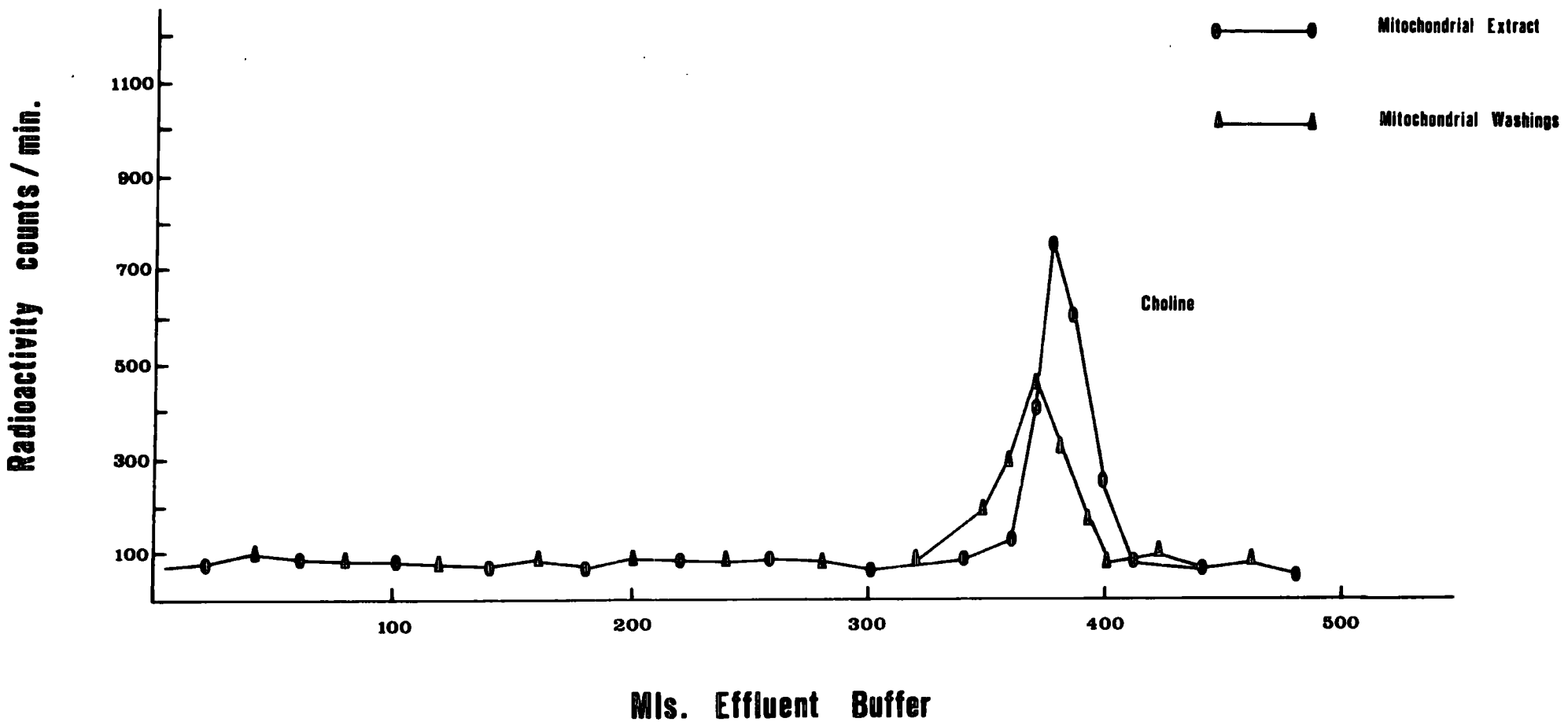
From these results it would appear that mitochondrial membranes are permeable to choline and that some of the choline taken up becomes associated with the mitochondrial membranes in such a way that release of the choline only occurs after rigorous disruptive treatment. This association may suggest a particular site for choline metabolism within the mitochondria; however, the nature of the metabolic process cannot be suggested with the results so far obtained.

Histochemical Localization of Dehydrogenases

The method used was that of Seligman and Rutenberg (1951) as

FIG. 37

Column profile of the radioactive quaternary products of the incubation of 1, 2 - ^{14}C - choline with mitochondria from the mycelia of Neurospora crassa.


Mitochondrial Extract
Mitochondrial Washings
Choline
Radioactivity counts / min.
1100
900
700
500
300
100
100
200
300
400
500
Mls. Effluent Buffer

described in the Methods section. Studies on the localization of dehydrogenases in tissue slices gave reasonable results when glutamate and malate were supplied as substrate. Sites of activity were indicated by blue spots within the cells of the tissue. However, when betaine aldehyde or glyceraldehyde was used as substrate the level of background staining was such that no conclusion could be drawn as to the presence of the particular dehydrogenase. The level of atmospheric oxidation precluded the use of this technique as a pilot experiment for the selection of plants or particular organs as a source of mitochondria or supernatant extract.

Analysis of Supernatant Protein Extraction for the Presence of a Betaine Aldehyde Dehydrogenase.

Spectrophotometric Assay

Initially the detection of dehydrogenase activity was attempted using NAD reduction as the criterion with the relevant substrate supplied. This assay was acceptable where, as in the case of rat liver supernatant, the endogenous rate of NAD reduction was at a low level and when betaine aldehyde was added in the recommended concentrations the optical density decreased rapidly to zero 1 - 2 min. However, supernatants extracted from plant sources usually exhibited a high endogenous rate of NAD reduction and the addition of betaine aldehyde and glyceraldehyde (concentrations from 10 mM - 100 mM) had no significant effect on the rate of NAD reduction. With plant extracts a range of NAD concentrations was used up to a final concentration of 150μM. NAD reduction was halted by additions of betaine aldehyde over a range of buffers from pH 7.2 - 9.1. NAD reduction resumed above this pH but then only at a minimal rate. But protein extracts from Neurospora crassa mycelia did show a marginally increased rate of NAD reduction over the background value when 33 mM betaine aldehyde was added at a pH of 8.1.

This assay technique was disappointing in that it was not even possible to use it as a rough assay to confirm that extracts from plant sources were active before embarking on the more lengthy procedures developed to determine dehydrogenase activity.

Polyacrylamide Gel Electrophoresis

Disc electrophoresis was developed by Ornstein and Davis (1962) for the analytical separation of protein mixtures. By this method proteins are concentrated into thin starting zones and then separated by the combined action of electrophoresis and molecular sieving. Disc electrophoresis is usually performed in small columns of polyacrylamide gel consisting of 3 sections:

(1) A large-pore anti convection gel into which the protein sample is introduced.

(2) A large-pore spacer gel in which the sample is electrophoretically concentrated.

(3) A small-pore gel in which the sample is separated electrophoretically and by molecular sieving.

Electrode compartments, electrodes and a power supply complete the arrangement. In this investigation the sample was layered on in 40% sucrose (no sample gel was employed). In fact the use of sucrose as the layering medium can obviate the use of a spacer gel but during this investigation the use of a spacer gel was preferred in general.

At the beginning of the electrophoretic run the trailing ion constituent is in the two electrode compartments and the leading ion is in the spacer and running gels. The buffer is in all sections of the apparatus and there is a pH discontinuity at the spacer gel/running gel interface.

As the run progresses a schlieren boundary is observed to move down from the top of the sample gel, or sucrose layer. Analysis has shown that this is the leading ion/trailing ion boundary. If the

sample is coloured (bromophenol blue was used as a marker dye in this investigation), the sample can be seen to be swept up by this boundary and concentrated in it. As the combined boundary passes the pH discontinuity at the spacer gel/running gel interface, the schlieren boundary is seen to break up. The leading ion/trailing ion boundary continues while the protein zone migrates more slowly and is left behind. With time the protein zone broadens as it moves and may diffuse or be resolved into a number of boundaries.

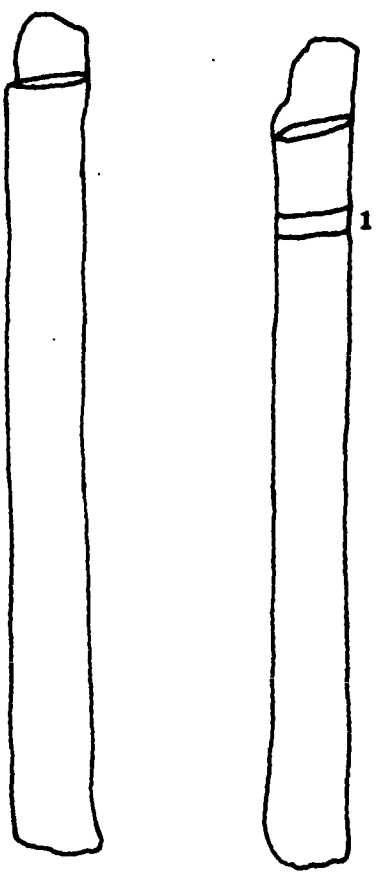
Staining of the gel for protein at the completion of the run reveals that this is due to separation of the protein into its various fractions. The comparison of this separation with those obtained in free-boundary electrophoresis and ultra-centrifugation confirmed that disc electrophoresis separated proteins both according to charge and size (Williams and Reisfeld (1962)). The extent of this size separation was shown to depend upon the concentration of monomer used in the polymerisation of the running gel. The concentration generally used and that used in this investigation was 7%. Chloride was the leading ion, glycine the trailing ion and tris (hydroxymethyl) aminomethane the buffer counterion employed.

The technique was tested for feasibility in the first instance by extracting supernatant from rat liver. The extract was prepared after the method of Rothschild and Barron (1954). The electrophoretogram demonstrated the presence of a betaine aldehyde dehydrogenase in rat liver cytoplasm. Fig. 38 is a diagram of gels incubated with MTT tetrazolium and (a) betaine aldehyde (b) glyceraldehyde as substrate. Glenn and Vanko (1959) have reported an NAD-dependent non-specific aldehyde dehydrogenase in rat liver mitochondria which respired glyceraldehyde particularly and other related aldehydes as well as betaine aldehyde. The supernatant enzyme would appear to be specific from these results. Glyceraldehyde did not act as a substrate in

FIG. 38

Diagram of gels of rat liver supernatant subjected to electrophoresis and incubated with MTT tetrazolium and (a) glyceraldehyde, (b) betaine aldehyde, as substrate.1 betaine aldehyde dehydrogenase.

(For experimental details see Materials and Methods p. 52)



A B

tetrazolium salt incubations either in electrophoretograms derived from rat liver or plant sources.

Supernatant extracts were prepared and concentrated in the manner described in the Methods text. The extracts were subjected to polyacrylamide gel electrophoresis and the gels were then incubated with MTT. tetrazolium salt or Nitro Blue tetrazolium to stain for the presence of dehydrogenases. Gels used as controls in this reaction were treated similarly in all respects to the other gels except that no substrate was supplied to the incubation medium. Attempts to record the presence and position of stained bands by photography were not always successful as faintly visible bands did not always appear on the photographs. The gels were, however, scanned wherever possible on a densitometer and the densitometer scans were related to the stained gels. Protein concentrations varied from 50 - 300μg. Gels stained for total protein even when considerably concentrated never contained less than 14 separable and stainable protein bands. The activity of the plant extracts was based on control gels stained for the presence of active glutamate dehydrogenase. Electrophoretic mobilities which were evaluated as the mean of many determinations never differed by greater than $\pm$ 3%. (see table 9).

Fig. 39 is a diagram of the gel obtained when supernatant extracted from the mycelia of a wild type strain of Neurospora crassa was subjected to electrophoresis and subsequently stained for the presence of betaine aldehyde dehydrogenase. Two bands were observed; the larger more densely stained, faster moving band corresponded to glutamate dehydrogenase. This protein band was often in evidence as there appeared to be sufficient substrate present in supernatant extracts to give the colour reaction. The less distinct band corresponds to the position of betaine aldehyde dehydrogenase. Extracts from choline mutants of Neurospora crassa showed no evidence

TABLE 9

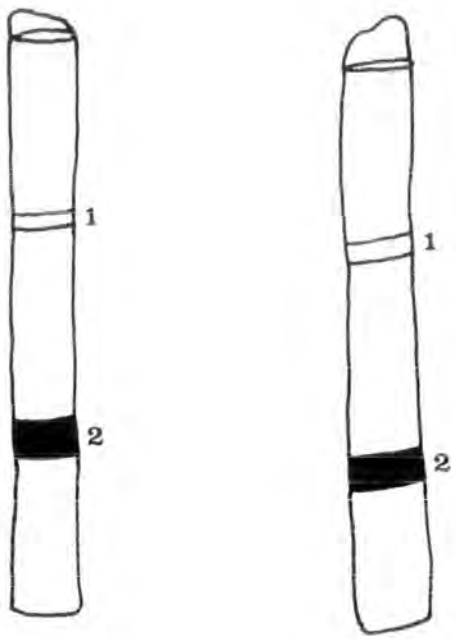
Plant Source	*Electrophoretic Mobility. Betaine aldehyde dehydrogenase	Electrophoretic Mobility. Glutamate dehydrogenase
Vignia radiata L. (Phaseolus aureus)	-	0.32
Beta vulgaris L.	0.55	0.18
Spinacea oleracea L.	0.38	0.12
Chenopodium Vulvaria L.	0.55	0.18
Neurospora crassa L.	0.31	0.69
Rat liver	0.14	-

Table 9 demonstrates the Electrophoretic mobilities of protein bands on polyacrylamide gels. The protein bands were confirmed to be either betaine aldehyde or glutamate dehydrogenase when incubated with tetrazolium salts, and the relevant substrate.

*Alternatively described as the Retardation Value.

FIG. 39

Diagram of gels of supernatant extracted from the mycelia of wild type strain of Neuorspora crassa, subjected to electrophoresis, and stained for the presence of betaine aldehyde dehydrogenase. (1) betaine aldehyde dehydrogenase. (2) glutamate dehydrogenase.


1
2
1
2

of betaine aldehyde dehydrogenase activity. Over-staining of the gel was caused by atmospheric oxidation of the two dyes in the reaction medium. The concentration of N-methyl phenazonium methosulphate would appear to be particularly crucial in this reaction. This was reduced by degrees because as well as causing overstaining the concentration of the dye determined the rate of the staining reaction.

Figs. 40a and b are densitometer scans of gels of supernatant extracted from Neurospora crassa subjected to electrophoresis and stained for the presence of betaine aldehyde dehydrogenase. These gave peaks corresponding to the position of the stained bands.

Fig. 41 is a diagram of an electrophoretogram of supernatant extracted from leaves of sugar beet and demonstrates a marked difference in electrophoretic mobility of the betaine aldehyde dehydrogenases of sugar beet from that of Neurospora crassa. Glutamate dehydrogenase separated from these extracts had a lower electrophoretic mobility than betaine aldehyde dehydrogenase separated from the same source. This again is in direct contrast to the situation found in Neurospora crassa. Fig. 42a is a diagram of an electrophoretogram of supernatant from sugar beet leaves. A highly stained formasan band was formed with betaine aldehyde as substrate. This gel showed a high degree of background staining making the densitometer scan (Fig. 42b) a little less meaningful.

Electrophoretograms obtained from supernatant extracts from leaves of Chenopodium vulvaria demonstrated the presence of a betaine aldehyde dehydrogenase having an electrophoretic mobility identical to the band obtained from sugar beet (see Fig. 43a). The amount of overstaining again was enough to mask the densitometer scan (Fig. 43b). Glutamate dehydrogenase extracted from sugar beet and Chenopodium vulvaria exhibited almost exactly similar electrophoretic mobilities.

Figs. 44 and 45 are diagrams of electrophoretograms of spinach

FIG. 40a

Densitometer scan of a gel of supernatant extracted from *Neurospora crassa* subjected to electrophoresis and stained for the presence of betaine aldehyde dehydrogenase.

(1, 2 show the position of stained bands.)

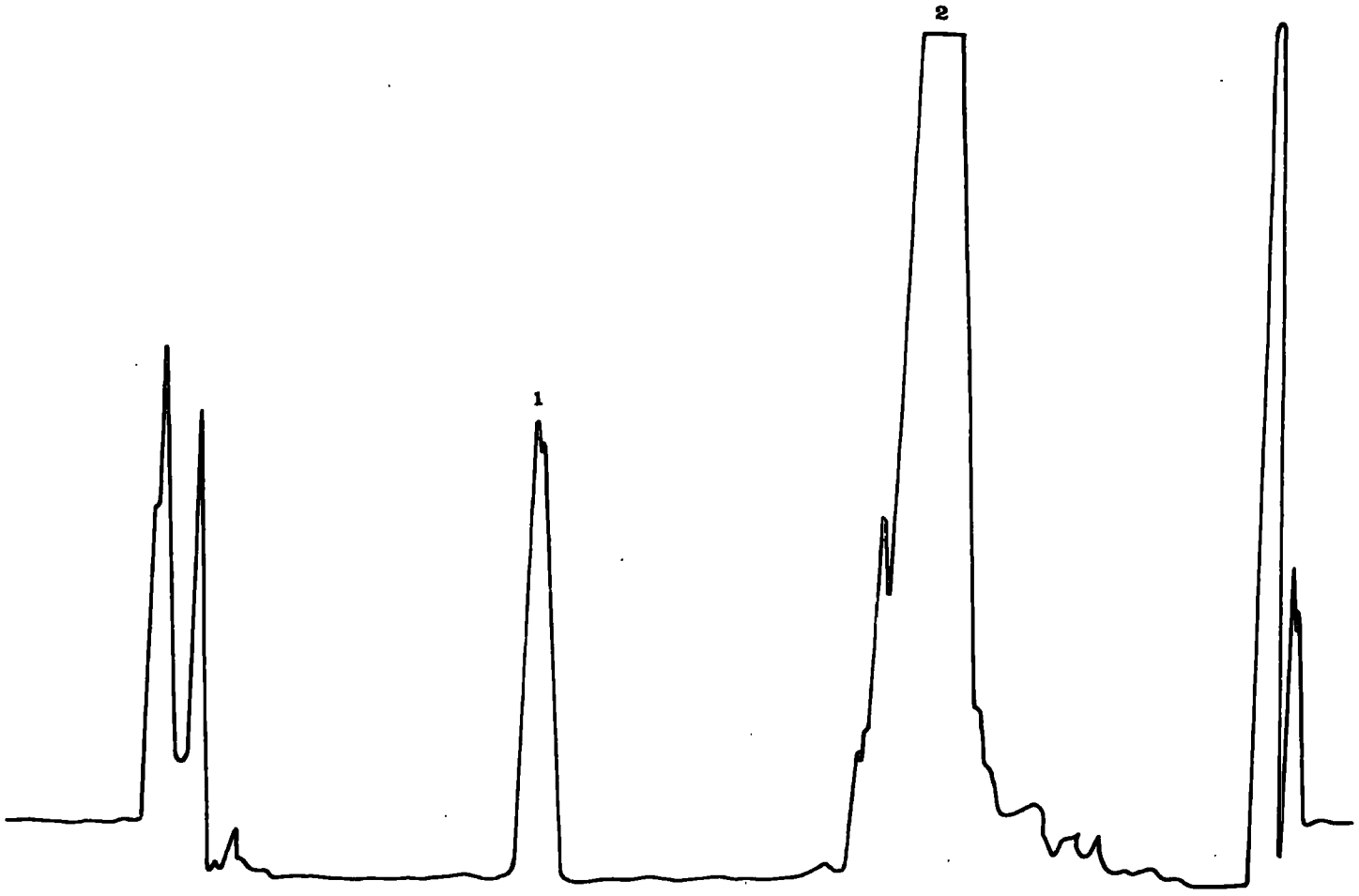

1
2
Electrophoretic Mobility

FIG. 40b

Densitometer scan of a gel of supernatant extracted from Neurospora crassa subjected to electrophoresis and stained for the presence of betaine aldehyde dehydrogenase.

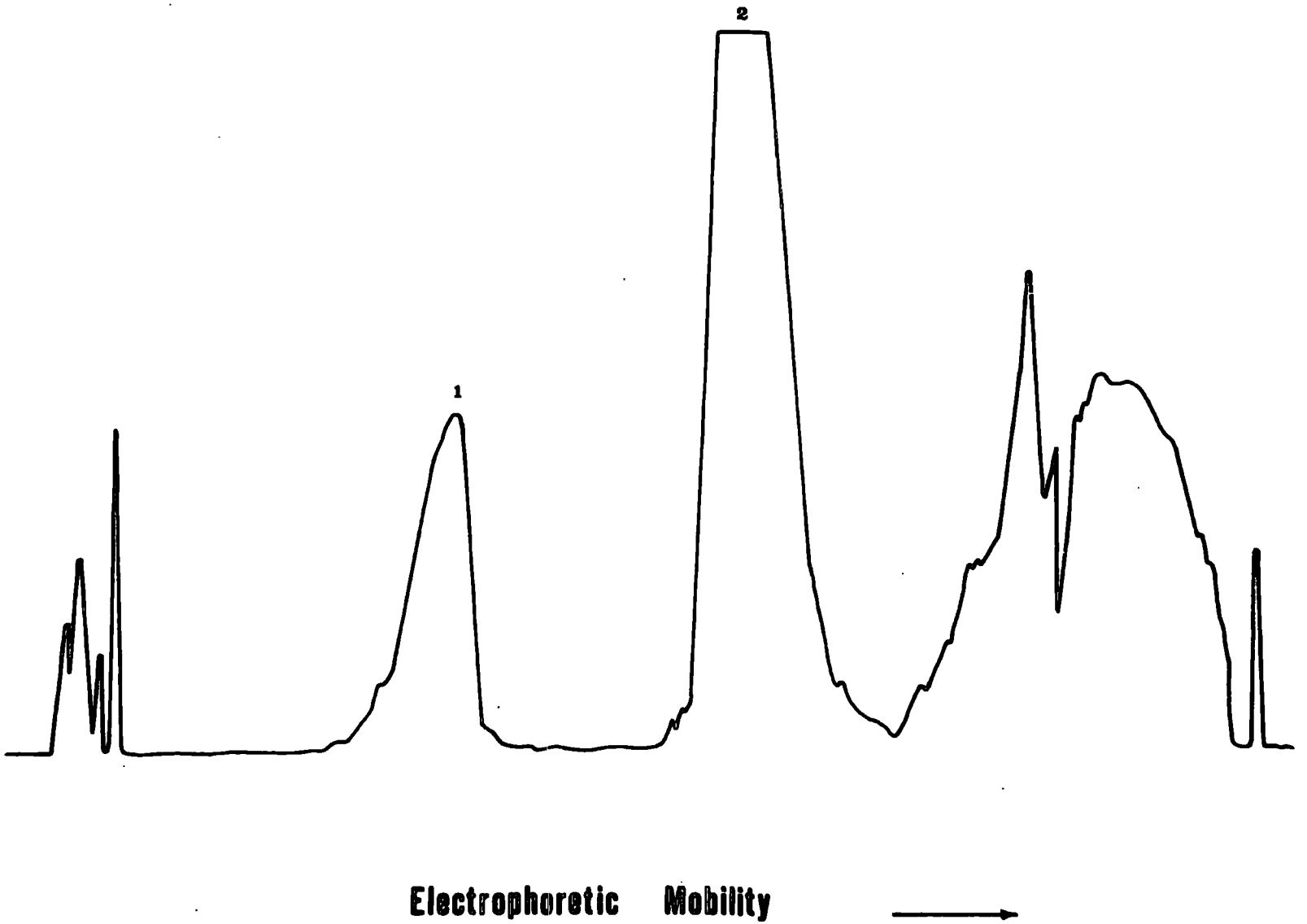

2
1
Electrophoretic Mobility

FIG. 41

Diagram of stained electrophoretograms of supernatant extracted from sugar beet leaves (A) incubated with glutamate, (B) incubated with betaine aldehyde, (C) no substrate. (1) betaine aldehyde dehydrogenase. (2) glutamate dehydrogenase.

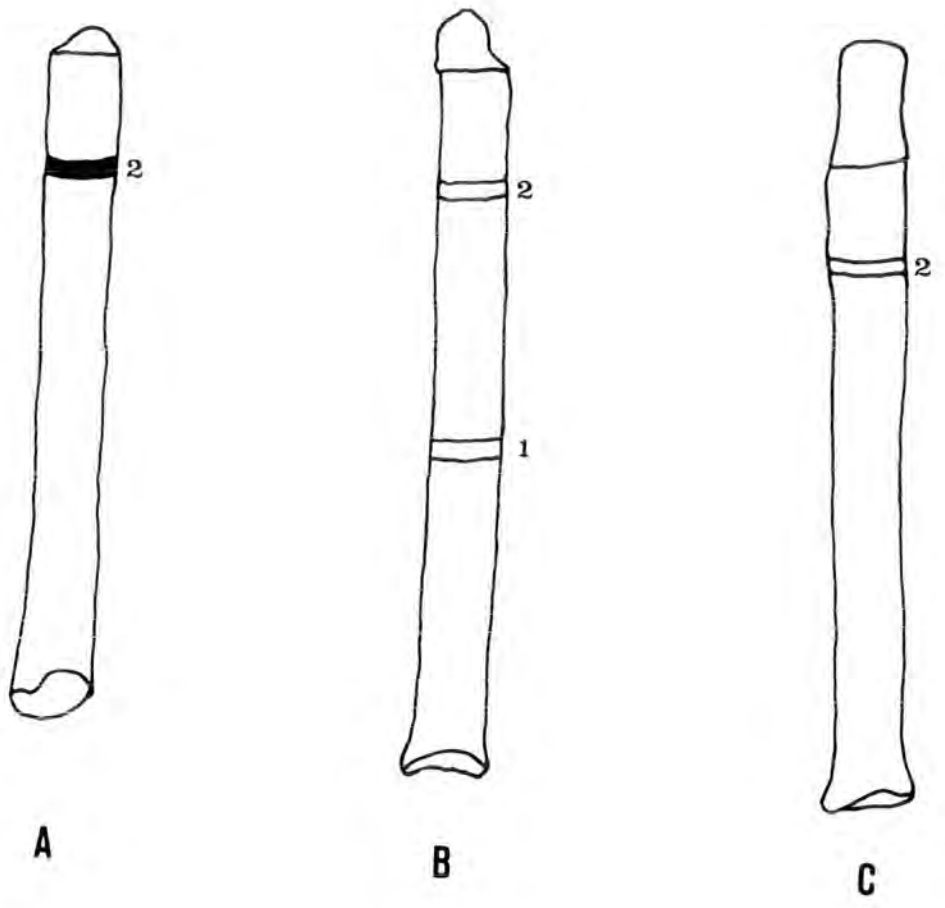

2
2
1
2
A
B
C

FIG. 42a

Diagram of stained electrophoretograms of supernatant extracted from sugar beet leaves. (1) betaine aldehyde dehydrogenase.

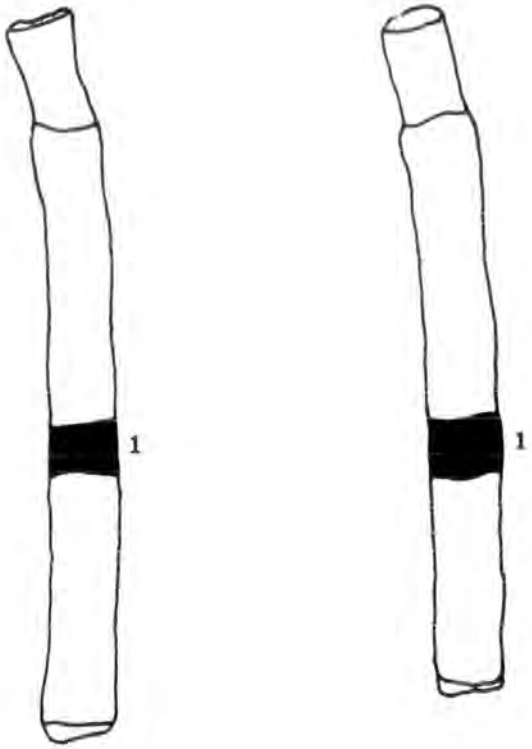
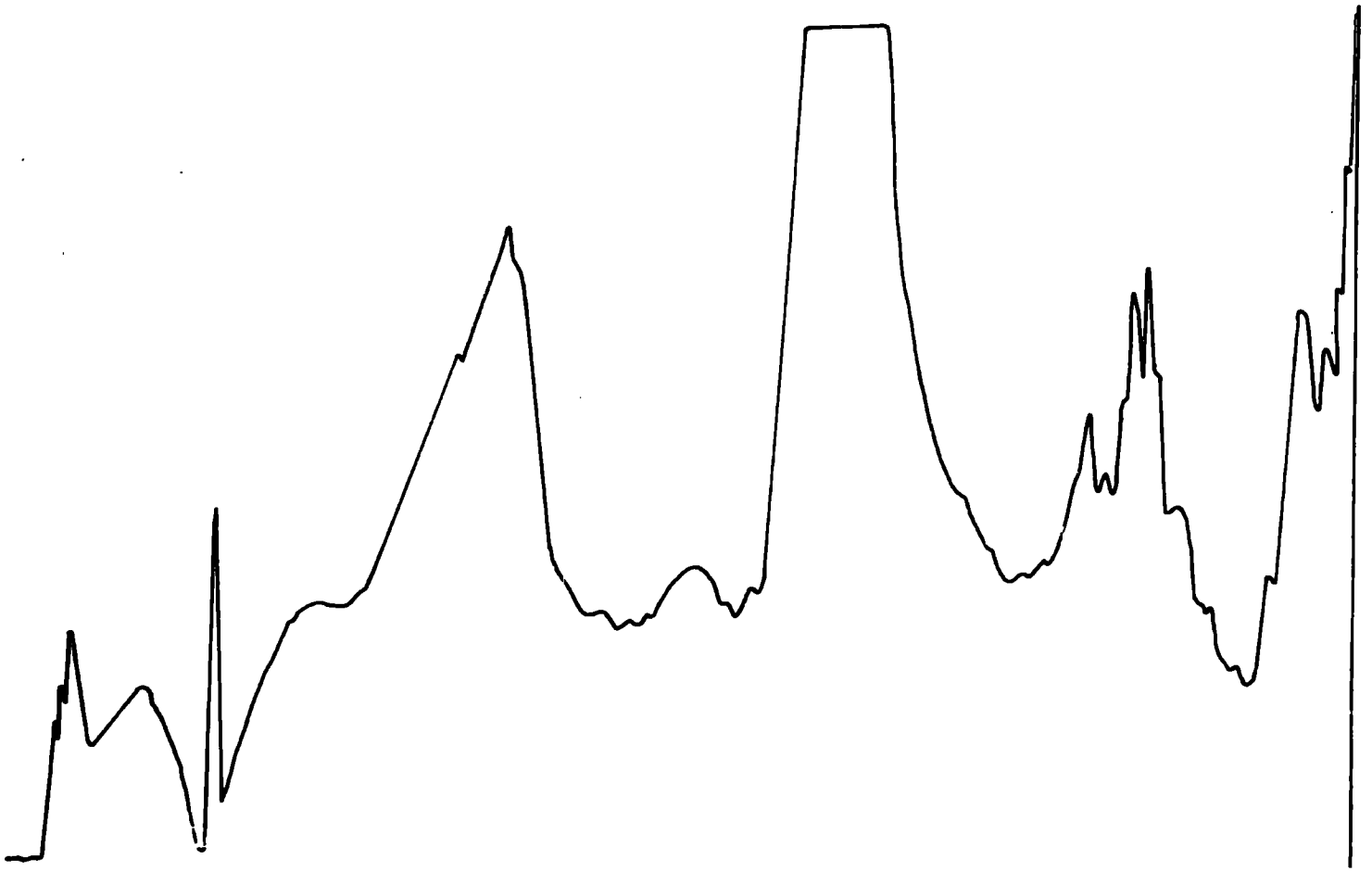

1
1

FIG. 42b

Densitometer scan of the electrophoretograms in Fig. 42a.



Electrophoretic Mobility →

FIG. 43a

Diagram of stained electrophoretograms of supernatant extract from leaves of Chenopodium vulvaria. (1) betaine aldehyde dehydrogenase.

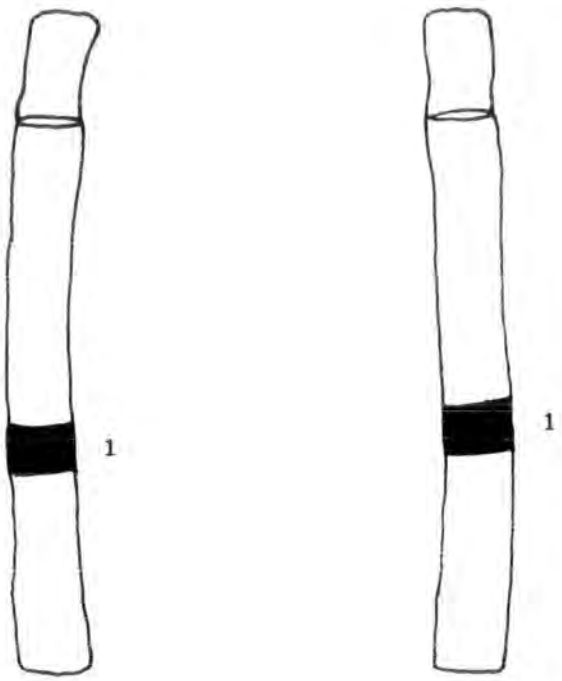
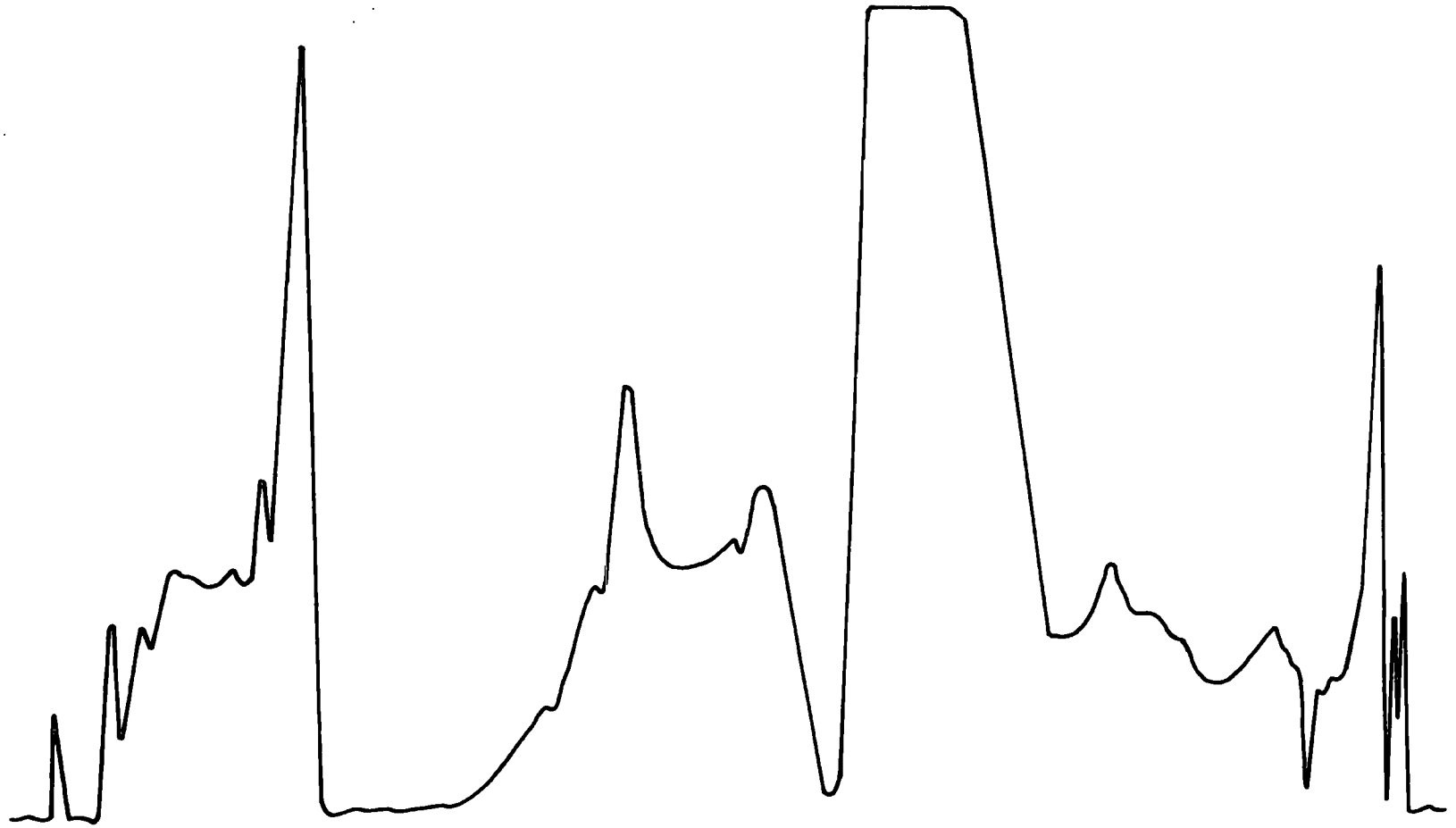

1
1

FIG. 43b

Densitometer scan of the stained gel used for Fig. 43a.



Electrophoretic Mobility →

FIG. 44

Diagram of stained electrophoretograms of spinach beet leaf supernatant. (A) no substrate, (B) glutamate, (C) incubated with glyceraldehyde, (D) betaine aldehyde. (3 glutamate dehydrogenase, 2 isoenzyme).

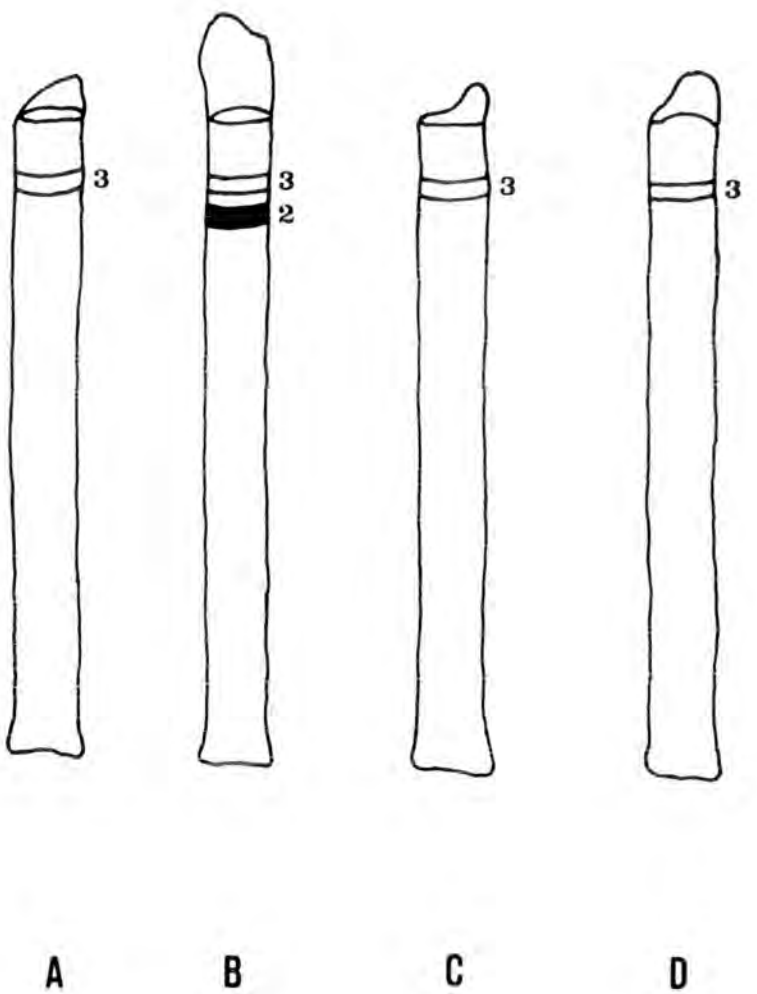
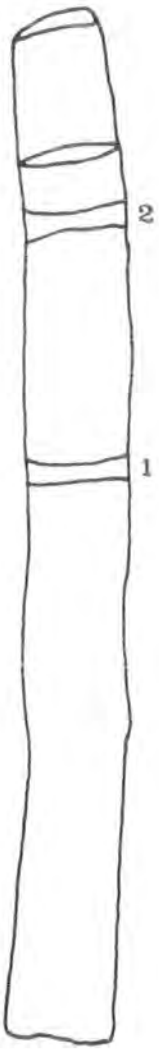

3
3
2
3
3
A
B
C
D

FIG. 45

Diagram of a stained electrophoretogram of spinach beet leaf supernatant. (1) betaine aldehyde dehydrogenase, (2) glutamate dehydrogenase.


2
1

beet supernatant. On these gels the R_F values for betaine aldehyde dehydrogenase and glutamate dehydrogenase were removed from those of sugar beet and Chenopodium vulvaria.

Anomalous bands are seen on these gels R_F values 0.06 - 0.08. They may be other forms of glutamate dehydrogenase. Isoenzymes of glutamate dehydrogenase have been reported by several workers. Van der Helm (1962) resolved the glutamate dehydrogenases of human tissues into separate molecular forms by agar-gel electrophoresis. Thurman, Palin and Laycock (1965) subjected albumins extracted from mature seeds of Vicia faba and Pisum sativum to polyacrylamide gel electrophoresis and detected by the tetrazolium staining procedure seven isoenzymes of glutamate dehydrogenase. In their studies of isoenzymes involved in glutamate metabolism in legume root modules, Grumier and Fottrell (1966) have demonstrated that glutamate dehydrogenase was separated by starch gel electrophoresis into three isoenzymes. Macko, Honold and Stahmann (1967) have shown isoenzymes to be present in wheat seedlings. Yue (1969) has studied glutamate dehydrogenase of several different plants and resolved the enzyme into separate molecular forms using polyacrylamide gel electrophoresis and has compared the isoenzymic patterns detected by the tetrazolium technique. The isoenzymes were found to have the same coenzyme specificity and to localize in the mitochondrial fraction of the cell in all the plants examined.

Yue (1969) gave evidence through electrophoretograms of extracts from mitochondrial and supernatant fractions that glutamate dehydrogenase was localized in the mitochondria, but showed activities detected in the supernatant fraction extracted from mung bean. However, they corresponded to the particulate enzymes as far as R_F values were concerned. Figs. 46a and 46b are densitometer scans of supernatant fractions from mung bean hypocotyls. A protein band

FIG. 46a

Densitometer scan of a gel of supernatant extracted from mung bean hypocotyls subjected to electrophoresis and stained for the presence of betaine aldehyde dehydrogenase.

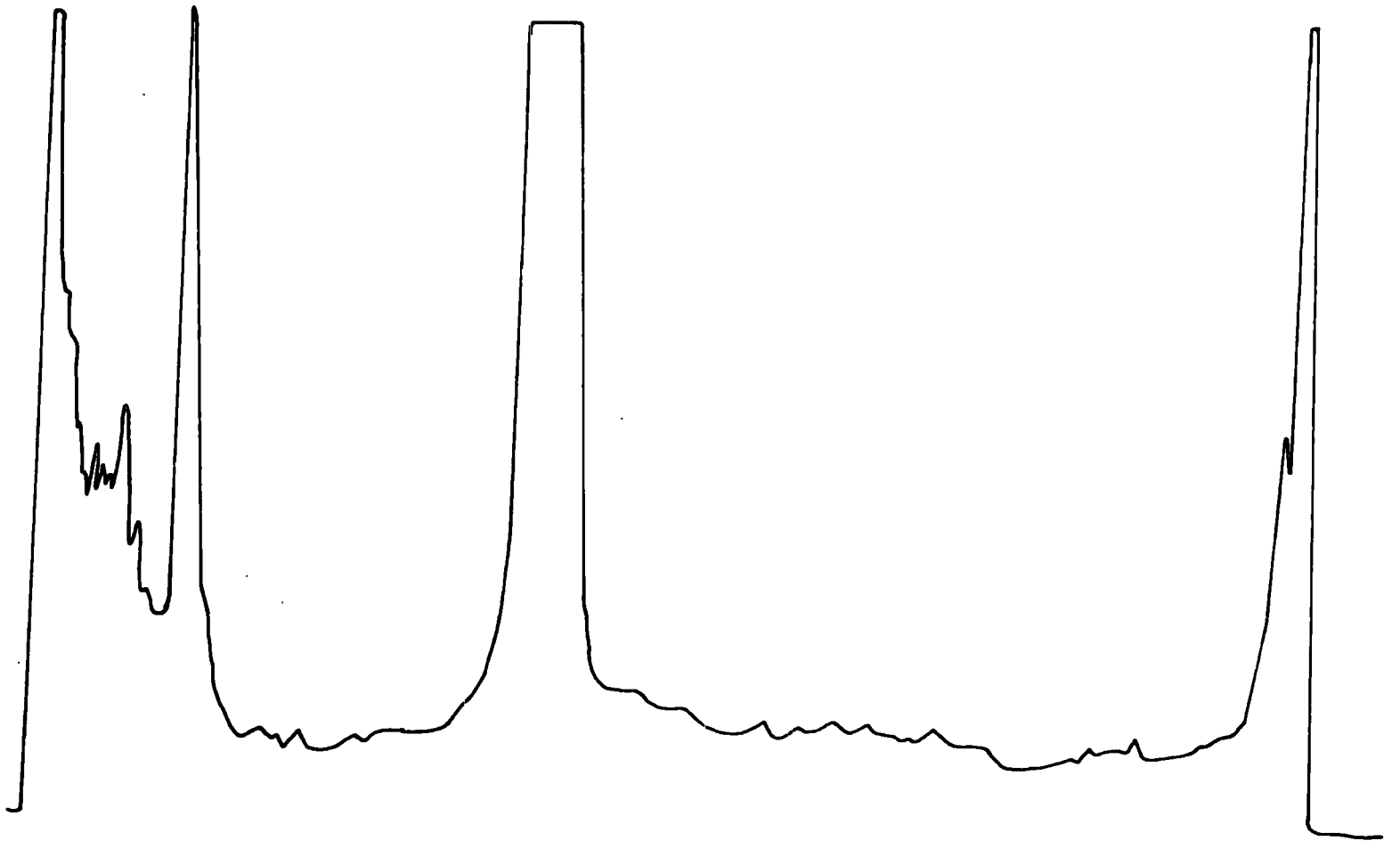
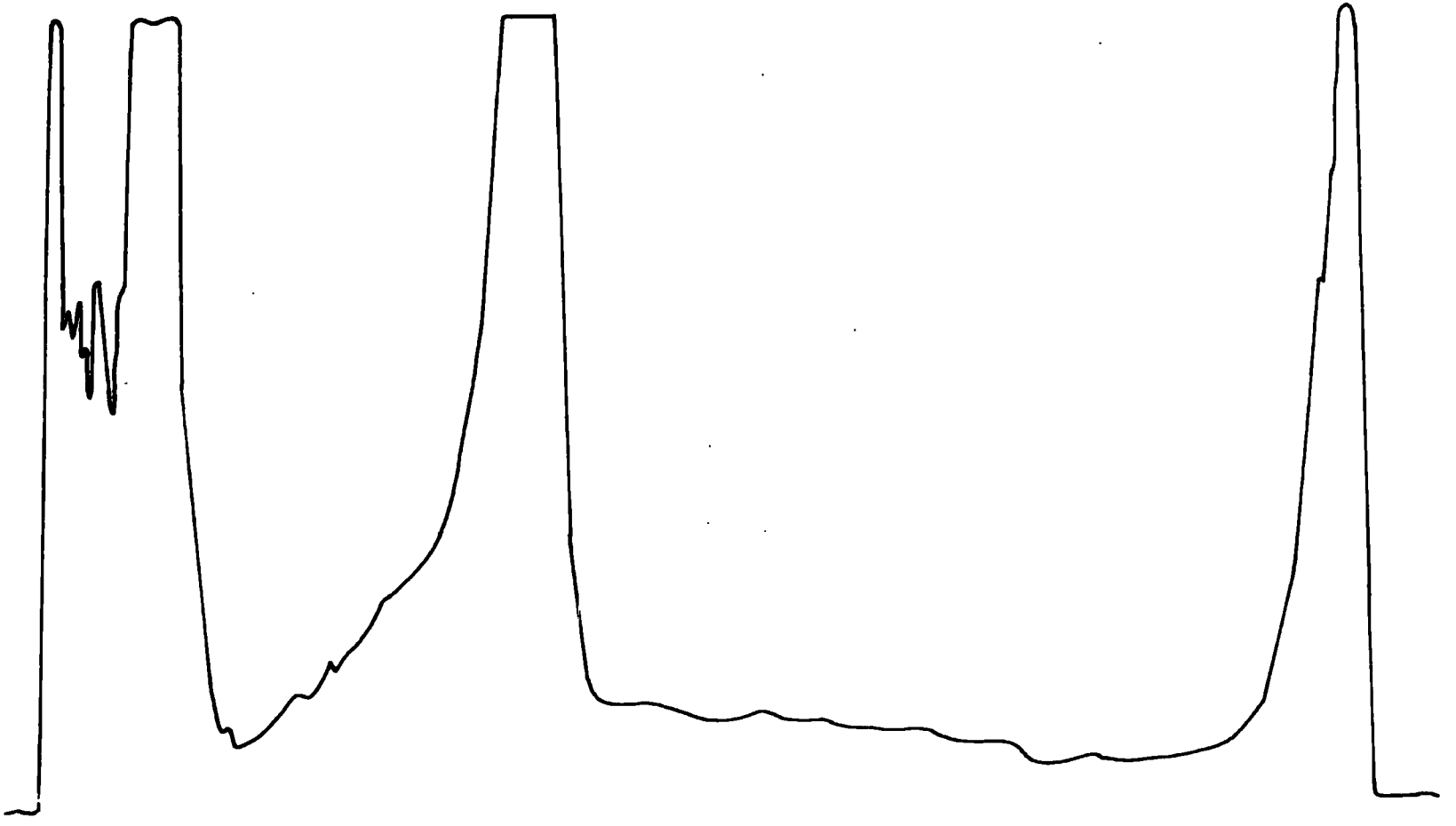

Electrophoretic Mobility

FIG. 46b

Densitometer scan of a gel of supernatant extracted from mung bean bypocotyls subjected to electrophoresis and stained for the presence of betaine aldehyde dehydrogenase.



Electrophoretic Mobility ⟶

staining for glutamate dehydrogenase is shown having an R_F value of 0.32 which is slightly removed from the isoenzymes extracted by Yue (R_F values 0.13 - 0.30). However, glutamate dehydrogenase isolated from Neurospora crassa (NAD specific) was the only band with an R_F value greatly removed from this range.

There is evidence of leaching of glutamate dehydrogenase from the mitochondria of plants during isolation (Bone (1959)). Bone (1959), Das and Roy (1961), Davies (1956), and Ritenour, Joy, Bunning and Hageman (1967) have all reported that glutamate dehydrogenase is localized in the mitochondrion. However, Yakovleva, Kretovich and Gil'manov (1965) have found evidence that glutamate dehydrogenase of corn roots exists in two forms, one associated with particles sedimenting at 4000 g (mitochondria) and the other with the supernatant after centrifugation at 20,000 g.

Attempts to use the presence of malate dehydrogenase as an indicator of active extracts was not dependable in that extracts showing no evidence of malate dehydrogenase activity did show the presence of glutamate dehydrogenase. Thus, the weight of opinion suggested that this enzyme was associated with the mitochondrion and any glutamate dehydrogenase activity was derived by leaching of the enzyme from the mitochondrion. This may be so but the relatively high activity of glutamate dehydrogenase found in these investigations seems to cast doubt on this explanation.

The anomalous bands described, which prompted the discussion of glutamate dehydrogenase may alternatively be the result of a diaphorase system which requires NADH as a coenzyme. Such bands usually took a relatively long time to develop (a matter of hours) which may be due to the time lag of NADH generation by NAD - requiring enzymes in the tetrazolium reaction.

A band staining for betaine aldehyde dehydrogenase was never

demonstrated in preparations from mung bean. Supernatants prepared from Pisum sativum and Vicia faba yielded glutamate dehydrogenases similar to each other but removed from those of other species used in these investigations but no evidence of betaine aldehyde dehydrogenase activity was ever found.

Betaine aldehyde dehydrogenase and glutamate dehydrogenase both had a specific requirement for NAD. No activity was observed when NADP was substituted in tetrazolium incubations.

Concentration of the extracts by ammonium sulphate fractionation was successful in the case of rat liver, but in extracts from plant sources activity appeared to be lost during the lengthy dialysis. This could have been due to the loss of some cofactor but this was never confirmed. The use of polyethylene glycol to absorb water from the extract contained in dialysis tubing appeared to work quite successfully but activity of the extracts was impaired. The acquisition of an Amicon Diaflo Model 401 Ultrafiltration cell enabled the application of the Ultrafiltration technique to the concentration of extracts. This was found to be by far the most successful method of concentration. The use of the PM_{10} membrane (allowing the filtration of molecules of up to 10,000 Molecular Weight) did not result in loss of enzyme activity in the concentrate which contrasted with the results of dialysis. This could suggest that the impairment of activity of the supernatants during ammonium sulphate fractionation occurred before dialysis, or was dependent on the time factor involved.

Previously cited evidence for the presence of a choline dehydrogenase system existing in mitochondrial extracts of higher plants was largely negated by the results of this investigation. On the isolated occasions when stimulation of respiration occurred when choline was supplied as substrate, the formation of betaine aldehyde or betaine was never confirmed by the characterization techniques which had been developed. The mitochondrial extracts were shown to be structurally intact by electron micrographs and their biochemical integrity was confirmed by their independence of exogenous cytochrome c and their ability to respire, malate, succinate and NADH. The stimulation of respiration by additions of ADP indicated that the mitochondria were tightly coupled.

The existence of a permeability barrier to choline was discounted in that radioactive choline was taken up by mitochondria, a fraction of which became closely associated with the mitochondrial membranes. However, the level of radioactivity reached an upper limit indicating that choline entry was permitted up to this level or that the choline was utilized or incorporated in some other biochemical system whose intermediates were not precipitated by the reineckate procedure.

Although an aldehyde dehydrogenase associated with mitochondrial extracts was never confirmed, a specific betaine aldehyde dehydrogenase requiring NAD as a coenzyme was isolated from several plant sources. The R_F values of the enzyme isolated from Chenopodium vulvaria and Beta vulgaris were found to be closely similar.

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