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A COMPARATIVE STUDY OF THE MITOCHONDRIA OF THE SMALL INTESTINE OF VERTEBRATES

by

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This thesis contains no material previously submitted for a degree in any University either by me or by any other person, except when due reference is made in the text of the thesis.

P. J. Stanbury.

PREFACE

The work described in this thesis was carried out in the Zoology Department of the University of Adelaide, and was supported by a University Research Grant for which I am grateful.

I wish to thank Professor W. P. Rogers of the Zoology Department and Professor R. K. Morton of the Department of Agricultural Chemistry for their advice and supervision throughout this work. I also wish to thank the members of the Zoology Department for their interest and advice. I especially wish to thank Dr. H. G. Andrewartha who read the manuscript of this thesis several times and who suggested many improvements.

I am grateful to Dr S. G. Tomlin who placed the electron microscope in the Deparment of Physics at my disposal.

SUMMARY

1. Light and electron microscopy of the intestinal cells of the rat, guinea pig, chicken, lizard, toad, and carp showed that the intestinal mitochondria of these animals looked similar. A mitochondria-free zone was seen next to the brush border. Dense granules were seen in the mitochondria.
2. Separated intestinal cells of the rat and the lizard had similar QO_2s. The activation energy of the reactions involved in the oxygen uptake was similar in both species. However, the enzymes in the intestinal cells of the lizard were more heat labile; and although the addition of succinate increased the QO_2 in both species, glucose increased the QO_2 in the rat but not in the lizard.
3. Intestinal mitochondria isolated in 0.25 M sucrose were swollen and in many instances had ruptured to form particles similar to some of those described by Green and his co-workers.
4. When the succinoxidase activity of the mitochondria isolated from the intestine of the above six animals was compared no activity could be detected in the mitochondria isolated from the rat. Fatty acids, apparently liberated by a lipase during the homogenization of the cell, were found to be inhibiting cytochrome oxidase.
5. The intracellular distribution of some of the enzymes of the Krebs cycle was compared. The diaribution of isocitric dehydrogenase and possibly of aconitase was different in the intestinal cells of the guinea pig and the lizard.
6. P/O ratios of 1.5 - 1.6 with succinate and 2.5 - 2.7 with alpha ketoglutarate as substrate were observed in the intestinal mitochondria of the guinea pig, chicken, lizard, and toad. At low Mg^{++} concentrations (0.045 M) thyroxine caused uncoupling of oxidative phosphorylation.
7. The endogenous and thyroxine-induced swelling of the

mitochondria of intestine and liver was compared. Tentative hypotheses have been put forward to explain why liver mitochondria are more susceptible to thyroxine-induced swelling than are intestinal mitochondria from the same species, and to explain why some mitochondria contract in the presence of thyroxine.

CONTENTS

ABBREVIATIONS

ADP	= adenosine diphosphate
ATP	= adenosine triphosphate
Co A	= coenzyme A
DPN	= diphosphopyridine nucleotide
DPNH	= reduced diphosphopyridine nucleotide
Intestinal homogenate	= the homogenate of the columnar absorbing cells of the mucosa of the small intestine
Intestinal mitochondria	= the mitochondria of the columnar absorbing cells of the mucosa of the small intestine
P_i	= inorganic phosphate
TPN	= triphosphopyridine nucleotide
TPNH	= reduced triphosphopyridine nucleotide
Tris	= tris-hydroxymethyl-aminomethane
Versene	= ethylene diamine tetra-acetic acid

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1. The discovery of mitochondria

Although other scientists may have observed mitochondria before 1890 (Cowdry, 1924), Altman (1890) was the first to descibe them in detail. Altman proposed that mitochondria were the fundamental units of metabolic function. He called them bioblasts (after the Greek words meaning life and germ) and believed that they were elementary micro-organisms embedded in a lifeless ground substance. This view was not universally accepted. In the resultant controversy several hypotheses about the role of "bioblasts" in cellular metabolism were advanced. Soon after, however, Altman's bioblasts were discredited. This followed the discovery that several structures previously thought to be characteristic of cells were artifacts which could be produced at will by the use of the appropriate method of fixing and staining.

In 1900 Benda demonstrated the presence in cells of some granules, without realizing that they were the same as those described by Altman. Benda called these granules

mitochondria (Gr.: mitos, filament; chondros, granule). This is the name in use today, although about fifty synonyms have been proposed (Bourne, 1942).

The first cytologist to examine the mitochondria of the small intestine in detail was Asher (1908). He reported that the mitochondria of this tissue were fewer and harder to stain than those found in glandular cells. Asher also found that the mitochondria of the small intestine increase in number when a fasted animal is re-fed. Three years later Champy (1911) pointed out that the intestinal mitochondria are found at both ends of the columnar cells. He suggested that this might be associated with the fact that these cells absorb substances from the intestine and secrete them into the capillaries.

In the early 1900's the precise function of mitochondria was not clear. On the one hand mitochondria seemed to occur in all embryonic cells; and in the early stages of development they seemed to be the only formed elements in the cytoplasm. Mitochondria never seemed to arise de novo, but always by the duplication of pre-existing units (Meeves, 1908). Mitochondria seemed to be associated with the origin of various cellular components (e.g. myofibrils, secretory granules, neurofibrils, plant plastids, pigments, the Golgi apparatus etc.); nearly a hundred structures were said to arise from mitochondria (Cowdry, 1924). These observations led to the theory that mitochondria are associated with histogenesis.

On the other hand, Kingsbury (1912) argued that because mitochondria occur in most living organisms, they probably have a function that is essential to all living matter - such as oxidation or reduction. A year later, Warburg (1913)

showed that the ability of a tissue brei to take up molecular oxygen resided almost entirely in its particulate elements. These observations led to the theory that mitochondria were concerned with cellular respiration.

A number of other hypotheses about the function of mitochondria were put forward. Meeves (1918) suggested that mitochondria might be the material basis of heredity. Portier (1919) and Wallin (1922, 1923) independently advanced the hypothesis that mitochondria might be bacteria adapted to an intracellular existence. It was also suggested that mitochondria were the site of enzyme synthesis (Marston, 1923, 1926: Cowdry, 1926; Horning, 1928). However, in spite of numerous hypotheses, little of importance was actually discovered and the idea began to spread that the study of mitochondria was a rather unprofitable field of research (Hoerr, 1943).

But about this time, i.e. 25 years after Warburg's important discovery, Keilin (1929, 1933) found that the cytochrome pigments were associated with some insoluble particles. It is now known that Keilin's particles were fragments of mitochondria which retained some of the activity of the original mitochondrion. Keilin was one of the firt biologists to recognize that the succinic and cytochrome oxidases were integrated complexes of enzymes arranged in a unique spatial pattern. This concept was confirmed by the work of others (e.g. Szent Gyorgi, 1937; Stotz, Sidwell, and Hogness, 1938; Stern, 1939) and was a big step forward toward our present concept of the mitochondrion.

Another very important contribution was the development of a method for isolating whole mitochondria from the cell. Bensley and Hoerr first isolated mitochondria by differential

centrifugation in 1934, but the details of their method were not published until 1943 (Hoerr, 1943; Lazarow, 1943). The gradual perfection of their method by numerous scientists (e.g. Claude, 1944, 1946; Hogeboom, Schneider, and Palade, 1948; Witter, Watson, and Cottone, 1955; Hogeboom, 1955; Novikoff, 1956) has permitted the isolation and study of mitochondrial from many animal tissues.

2. Intestinal mitochondria

It is surprising that so little attention has been paid to the mitochondria of the intestine compared with those of other tissues, especially as a considerable amount of literature has been published on the anatomy, biochemistry, and physiology of the intestine. Much of what is known about intestinal mitochondria has been found out incidentally, during the course of investigations designed for another purpose. For example, mitochondria have been isolated from the intestine during the course of experiments on the distribution of enzymes (Crane and Sols, 1953; Morton, 1954); and Dalton (1951), Zetterqvist (1956), and Palay and Karlin (1959 a, b) have published electron micrographs of the intestinal mitochondria while studying the whole columnar cell. But very little research has been directed solely at the intestinal mitochondrion.

Crane and Sols (1953) found that the mitochondria from the intestine of the rat contained 73% of the cell's hexokinase activity. Morton (1954) found that only a small proportion of the alkaline phosphatase activity of the intestinal cell was located in the mitochondria. Dalton (1951), in the early days of electron microscopy, published some micrographs of the intestinal mitochondria of mice.

Later, in a monograph about "The ultrastructural organization of the columnar absorbing cells of the mouse jejunum" Zetterqvist (1956) included a section on mitochondria. He found that the mitochondria in the intestine of mice were basically similar in structure to the mitochondria in other mammalian tissues. This has been confirmed recently by Palay and Karlin (1959).

The mitochondria of any two vertebrate tissues are unlikely to be radically different from each other. For this reason, and because the intestinal mitochondria have received relatively little attention, it is worth while to review some of the information available about the mitochondria of other tissues. It is with this that the remainder of the Introduction is primarily concerned.

3. The structure of mitochondria

When the method of isolating mitochondria by differential centrifugation became widely used, biologists began to wonder whether the structure or function of mitochondria changed during their isolation (Danielli, 1946; Bradfield, 1950; Schneider and Hogeboom, 1951). As mitochondria are too small for their internal structure to be seen with the light microscope, this question was not answered until the electron microscope became readily available to biologists - about 1950. Much of the credit for describing the ultrastructure of mitochondria goes to two laboratories - those of Palade and Sjostrand. Palade (1952) described mitochondria as structures containing lamellae and bounded by a single outer membrane. He termed the lamellae cristae mitochondrales. In the next year Sjostrand (1953 a, b) suggested that mitochondria had a double membrane and this was confirmed by Palade (1953).

(Palade made his observations on the mitochondria of the muscle, nerve, liver, and kidney of rats, and Sjostrand on the mitochondria of the kidney and pancreas of mice and the retina of guinea pigs.)

For some years there was a controversy whether the cristae mitochondrales were merely adjacent to (Rhodin, 1954; Sjostrand, 1956), or actually a part of (i.e. infoldings of) the inner of the two limiting membranes (Palade, 1953; Dempsy, 1956; Low, 1956; Dalton and Felix, 1957). The latter view is now believed to be the correct one (Ekholm and Sjostrand, 1957).

Several laboratories have estimated the dimensions of mitochondria; these measurements have varied as new and better apparatus has become available. However, there have been few major disagreements and the measurements accepted today are shown in Figure 1. Zetterqvist's (1956) measurements of the intestinal mitochondria are similar - see Table 1

TABLE 1

DIMENSIONS OF THE INTESTINAL MITOCHONDRIA OF MICE
(after Zetterqvist, 1956)

Length		3 μ[K]
Width		0.28 $\pm$ 0.002 μ
Mitochondrial membrane	Outer membrane	60 A'
	Osmiophilic interspace	55 A'
	Inner membrane	60 A'
		170 $\pm$ 5 A'
Cristae	Membrane	65 A'
	Osmiophilic interspace	75 A'
	Membrane	65 A'
		210 $\pm$ 6 A'
Distance between cristae		200 - 600 A'
Diameter of dense granules		200 - 600 A'

[K] longest section measured

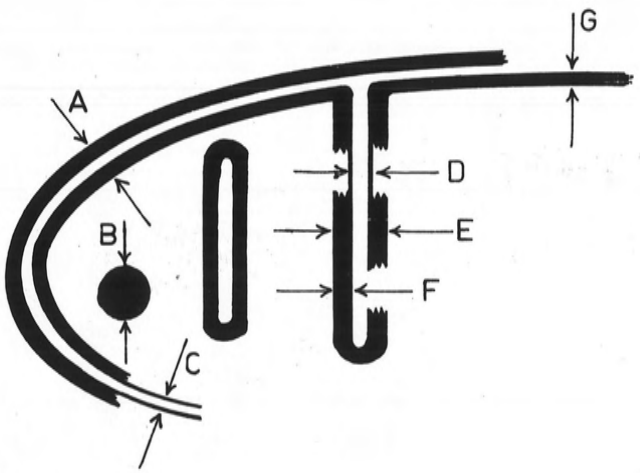


Figure 1.- Measurements of a generalized mitochondrion. A = 150 A'; B = 200 - 600 A'; C = 60 A'; D = 70 A'; E = 180 A'; F = 55 A'; G = 50 A'.

In the ground substance or matrix of some types of mitochondria dense granules about 400 A' in diameter can be seen. These granules are common in the mitochondria of pancreas (Sjostrand and Hanzon, 1954), liver (Palade, 1953), and intestine (Weiss, 1955; Zetterqvist, 1956; Palay and Karlin, 1959 a, b).

4. The electron transport system and oxidative phosphorylation

One of the most constant and characteristic features of mitochondria is that they contain the enzymes associated with electron transport and oxidative phosphorylation. The study of these enzymes was given a special impetus about ten years ago when Keilin's classical picture of electron transport (Keilin, 1929, 1930; Keilin and Hartree, 1937, 1938, 1939, 1940) was modified by Slater (1949 a, b, c; 1950 a, b) and Chance (1952, 1954) (see Figure 2).

It is now known that electron transport is initiated by two main types of substrate:

(i) pyruvate and the Krebs cycle acids;
and (ii) fatty acids.

Mitochondria are capable of the complete oxidation of pyruvate provided they are supplemented with a "sparker" of fumarate. This is so, even though some of the enzymes involved in the Krebs cycle, such as the condensing enzyme and isocitric dehydrogenase, are not located exclusively in the mitochondrion.

The oxidation of fatty acids involves two dehydrogenations; in the first, two electrons are transferred directly to one of three flavoproteins depending on the length of the carbon chain of the fatty acid concerned. DPN is not involved (c.f. the oxidation of succinate). In the second dehydrogenation,

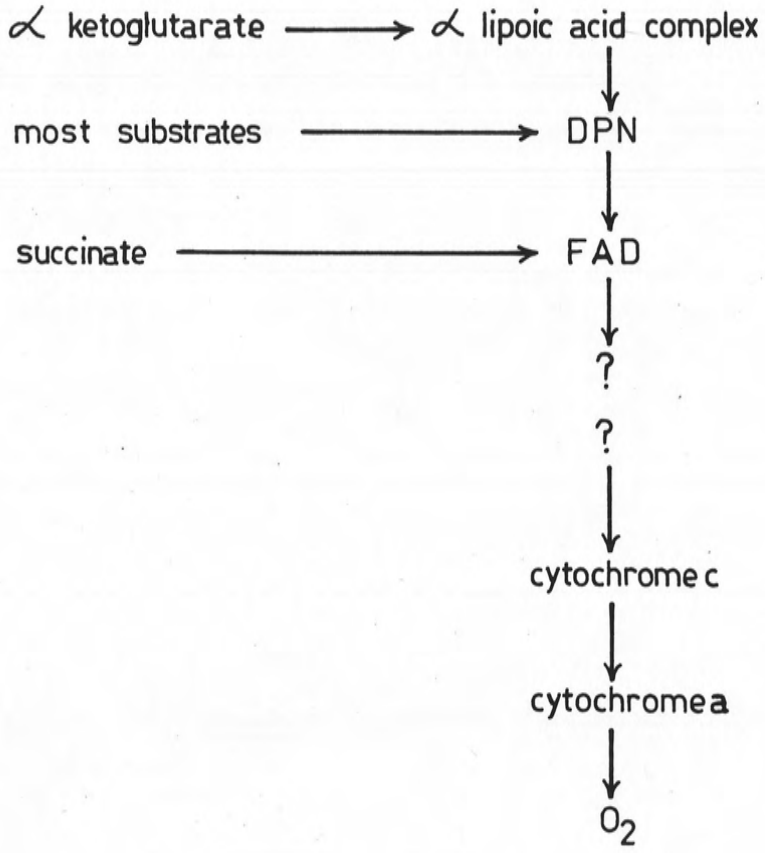


Figure 2.- A diagram representing the state of knowledge about the electron transport system in 1954. At this time biochemists were not certain how electrons were transferred from FAD to cytochrome c; several substances had been suggested - vitamins K and E, cytochrome b, and Slater's factor.

the electrons are transfered through DPN in the more usual manner (Crane, Hauge, and Beinert, 1955; Beinert and Crane, 1956).

The various pathways of the electron transport system are indicated diagramatically in Figure 3. Although most of the enzymes involved are thought to be present in mitochondria (Kennedy and Lehninger, 1948; Schneider, 1948), some of the enzymes may occur in other parts of the cell as well (Langdon, 1957).

The distribution and functional arrangement of the enzymes involved in electron transport in intestinal cells has not been examined in detail. It seems unlikely, however, that the distribution or arrangement of such fundamental enzymes would differ markedly in different cells. Minor differences may occur.

The electron transport system allows the energy released upon the combination of hydrogen and oxygen to be made available in small amounts. Under suitable conditions the energy may be trapped in the cell in the form of the potential energy of a phosphate bond (Kalchar, 1937; Belitzer and Tsibakowa, 1939; Ochoa, 1941). This process is called oxidative phosphorylation. Up to three reactive phosphoryl groups can be formed as two electrons are transfered from the substrate (DPNH) to molecular oxygen; the P/O ratio is said to be three. There is some controversy over the position in the electron transport chain of the three phosphorylations, especially as the precise form of the electron transport chain is not yet clear. It is known that one phosphorylation takes place during the transfer of electrons from DPN to FAD, another between FAD and cytochrome c, and the third during the oxidation of cytochrome c (Chance and Williams, 1953; Slater, 1956; Myers and Slater, 1957 a, b). Each

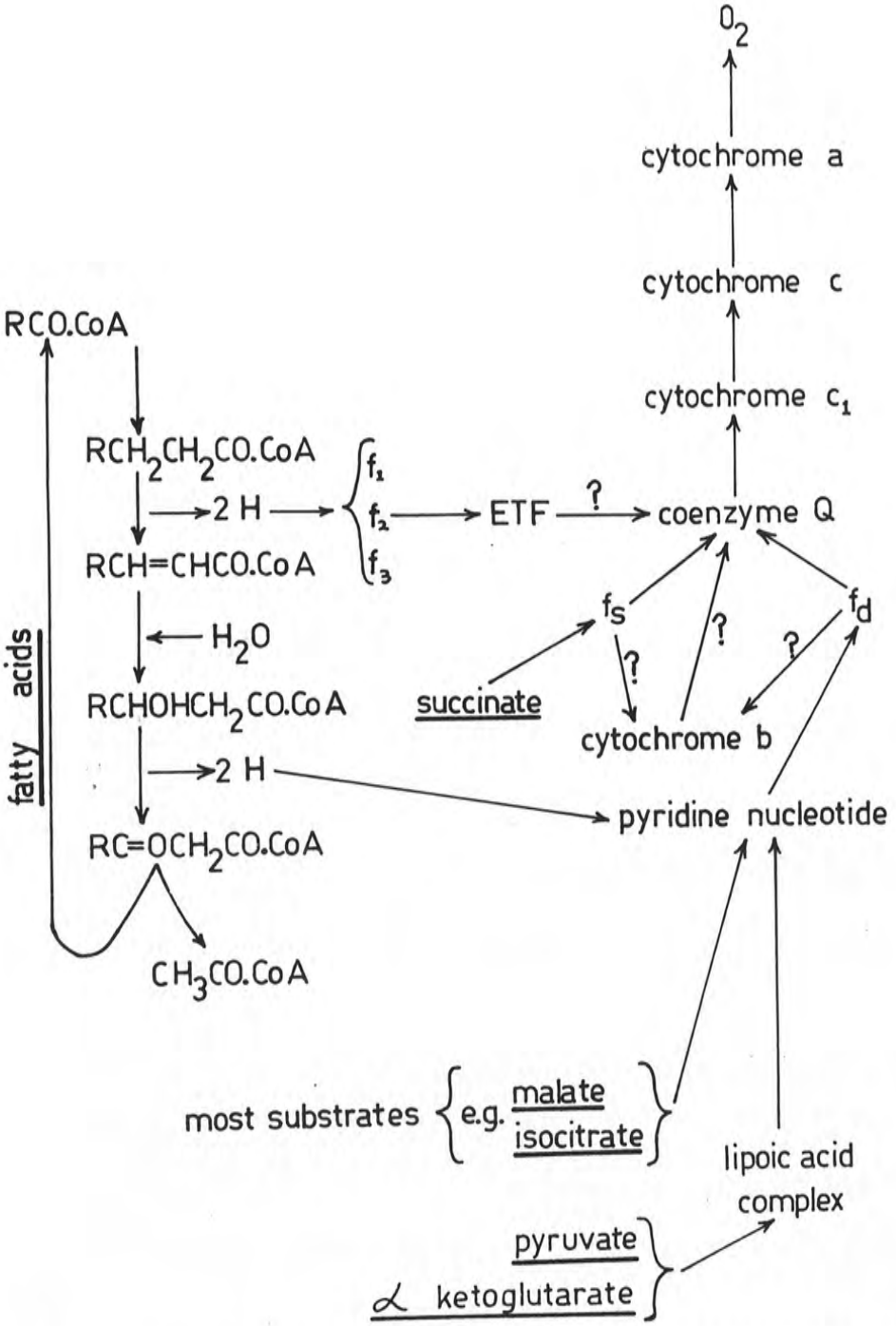


Figure 3.- A diagram representing the path of electrons in the electron transport chain. Substrates are underlined. There is some controversy about the position of cytochrome b; most biochemists consider it to be involved in the main pathway, but others relegate it to a less important position. ETF (electron transfering flavoprotein) is a fourth flavoprotein involved in fatty acid oxidation.

phosphorylation is catalysed by a separate enzyme with a characteristic pH optimum (Hulsmann and Slater, 1957 a, b), but the overall reaction is the same in each instance. It is:

$$ADP + P_i \xrightarrow{\text{energy}} ATP$$

The term oxidative phosphorylation implies that for phosphorylation to occur, oxidation must be proceeding simultaneously. This is so, although the converse does not appear to apply. Numerous compounds have been found which, *in vitro*, allow oxidation to occur without accompanying phosphorylation. Some of these compounds are listed in Table 2. Whether or not uncoupling of oxidative phosphorylation occurs *in vivo* is not clear, although in the "best" preparations of mitochondria oxidation will not take place without accompanying phosphorylation (Lardy and Wellman, 1952).

Of the uncoupling agents listed in Table 2 (overleaf), the only one that has been considered to be a physiological uncoupler is thyroxine. The hypothesis that thyroxine is a physiological uncoupler has been questioned as:

(i) larger than physiological amounts of thyroxine are required to uncouple oxidative phosphorylation;

(ii) thyroxine promotes mitochondrial swelling thus causing structural damage (Tapley, 1956);

and (iii) if the effect of structural damage is minimized by using mitochondrial fragments, thyroxine does not uncouple oxidative phosphorylation (Tapley and Cooper, 1956 a; Lehninger, 1956).

But these criticisms are not irrefutable. For instance:

(i) if thyroxine is not the physiological uncoupler, then lack of the enzyme/s, or an inclement environment in the experimental medium, may prevent

TABLE 2

VARIOUS INHIBITORS OF OXIDATIVE PHOSPHORYLATION

AGENTS	EFFECT ON MITOCHONDRIAL SWELLING	REFFERENCE
Anti-coagulents		
Dicoumerol	Inhibits	Tapley, Cooper, and Lehninger 1955
Anti-biotics		
Gramicidin	No effect	Cross, Taggert, Covo, and Green 1949
Antimycin A	Inhibits	Jalling, Low, Ernster, Lindberg 1957
Anaestehetics		
Ether		Brody and Brain, 1951
Amytal		Wolpert, Trutt, Bell, and Krantz 1956
Dyes		
Methylene blue		Judah and Williams-Ashman, 1951
Cresyl blue		Lehninger, 1949
Heavy metal ions		
Ag^{+}		Chapell and Greville, 1954
Cu^{++}		Ernster, 1956
Cd^{++}		Jacobs, Jacob, Sanadi, Bradley 1956
Ca^{++}	Promotes	Lehninger, 1956
As^{+++}	Promotes	Lehninger, 1956
Hg^{++}	Promotes	Lehninger, 1956
Zn^{++}		Mahler, 1957
Hormones		
Progesterone		Wade and Jones, 1956
Thyroxine	Promotes	Klemperer. 1955
Miscellaneous agents		
(a) Chemical		
Dinitrophenol	Inhibits	Loomis and Lipman, 1948
Pentachlorphenol	Inhibits	Weinbach, 1956
Chloropromazine		Abood, 1955
Carcinogens		Kielly, 1957
(b) Physical		
Sonic vibration		Hogeboom and Schneider, 1950 b
Ultraviolet light		Beyer, 1958
X-ray radiation		Van Bekkum, 1955
Electrical stimulation		Abood, 1954
Detergents		Zellestrom and Ernster, 1956

the active compound being formed (from thyroxine) except in small amounts;

(ii) the swelling produced by thyroxine may be better controlled *in vivo*, so that irreversible structural changes do not occur;

and (iii) mitochondrial fragments are only parts of the whole, and it may be that the control of oxidative phosphorylation is only possible when the surrounding membranes of the mitochondrion are more or less intact.

There are several pieces of evidence which suggest that the swelling induced by thyroxine on the mitochondria of liver, at least, has physiological significance:

(i) microscopic examination of the liver of hypothyroid rats showed the mitochondria to be swollen (Aebi and Abelin, 1953) and the fine structure disorganized (Schultz, Low, Ernster, and Sjostrand, 1957);

(ii) derivatives of thyroxine which increase the basic metabolic rate also promote swelling, and the activities are roughly proportional (Lehninger, 1960 *a*);

and (iii) mitochondria from hyperthyroid rats swell faster than normal mitochondria, while mitochondria from hypothyroid rats are resistant to spontaneous swelling (Tapley, 1956).

Mitochondria isolated from the kidney also swell in the presence of thyroxine, but little or no swelling occurs in mitochondria isolated from skeletal or cardiac muscle, although oxidative phosphorylation is still uncoupled (Vitale, Nakamura, and Hegsted, 1957). Intestinal mitochondria have not been examined.

Thyroxine (or its derivatives) is not thought to be the primary agent in uncoupling oxidative phosphorylation. Thyroxine is believed to affect the supply of some other compound more intimately connected with oxidative phosphorylation - such as ADP (Chance and Williams, 1955, 1956; Lehninger, 1960 b).

Some of the ways in which oxidative phosphorylation might be controlled are shown in Figure 4.

5. Sub-mitochondrial particles

Only when mitochondria are *in situ* are they structurally intact. As soon as a mitochondrion is isolated (in 0.25 or 0.44 M sucrose) it starts to swell and disintegrate. Sub-mitochondrial particles can be isolated containing the electron transport chain, but not necessarily the associated dehydrogenases and phosphorylative enzymes (Green, Mackler, Repaske, and Mahler, 1954). These particles can be broken by chemical agents or by sonic vibration into still smaller particles containing smaller pieces of the electron transport chain.

In general, sub-mitochondrial particles have a narrower function and a different structureto mitochondria. Figure 5 shows how some of the larger sub-mitochondrial particles can be formed. Some of these can carry out oxidative phosphorylation (phosphorylating electron transport particles, or PETP; and heavy electron transport particles, or ETP_H), whereas others can not (electron transport particles, or ETP).

Green (1959 a, b) has suggested that PETP, or miniature mitochondria, may also be formed by the twisting of a mitochondrion upon itself (see Figure 6), but this has

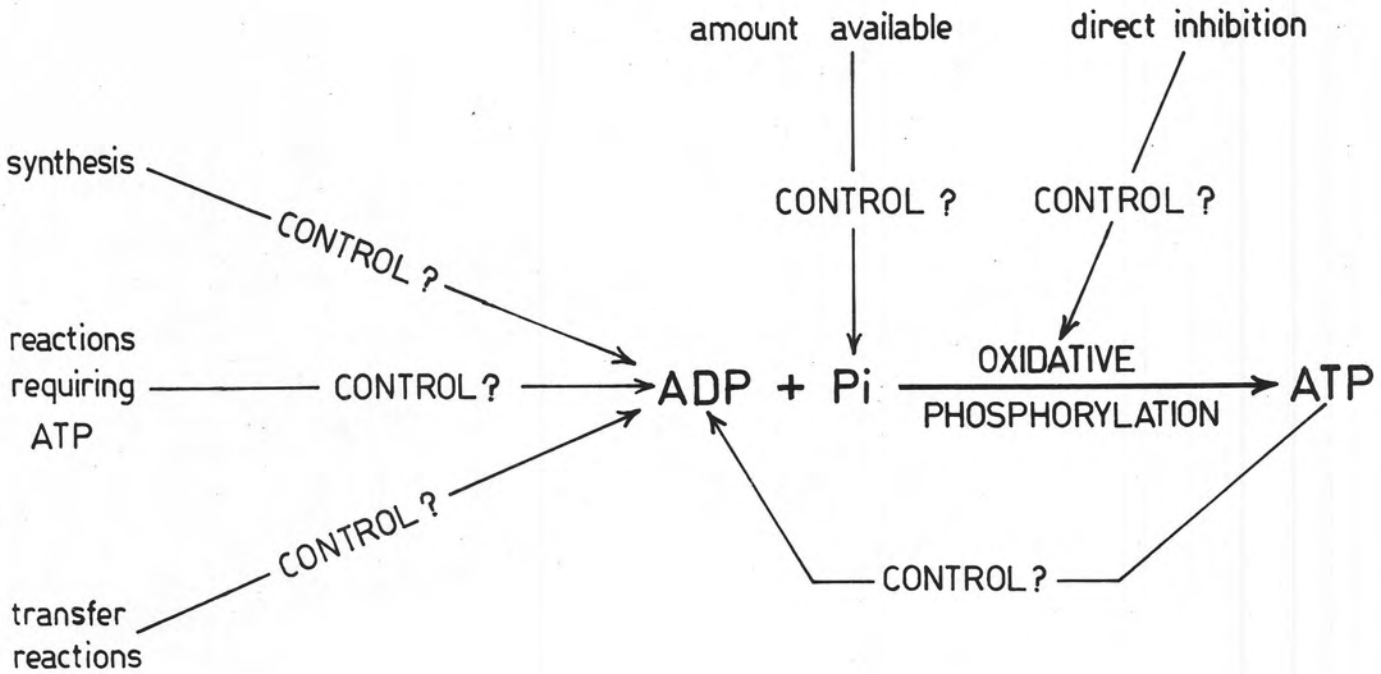


Figure 4.- A diagram showing the reactions which may control oxidative phosphorylation.

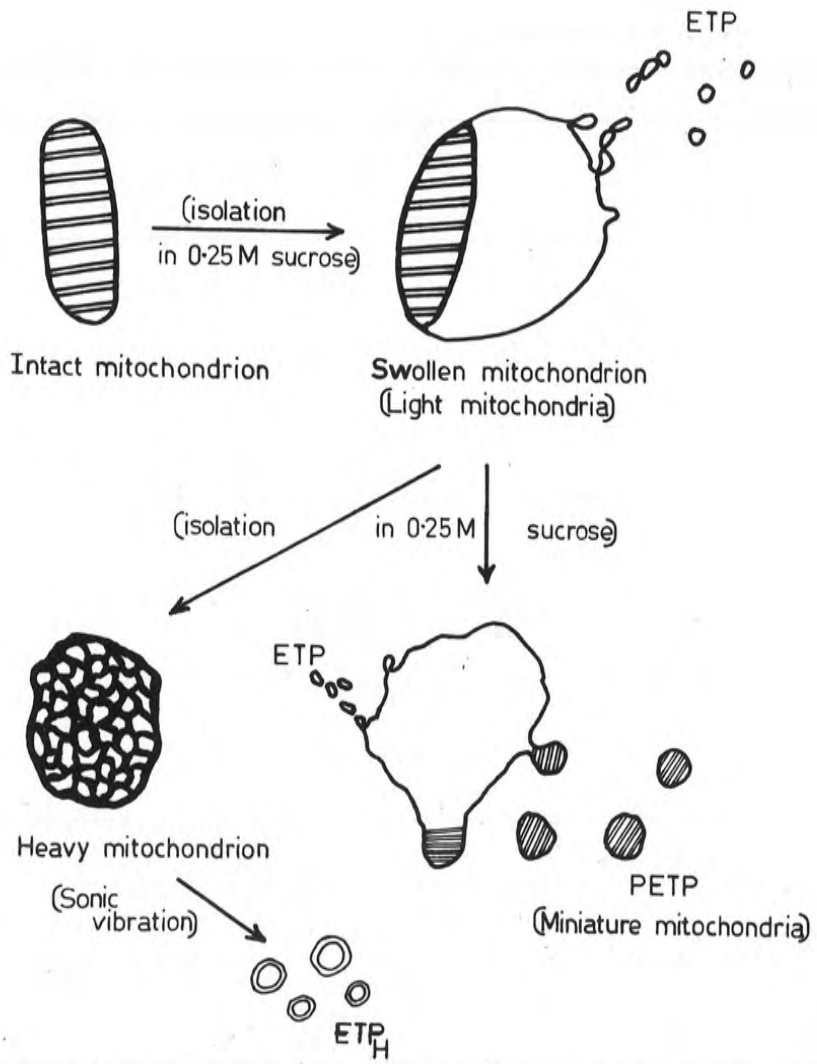


Figure 5.- A diagram showing the degradation of a mitochondrion into ETP, PETP, and other fractions. (after Green, 1958)

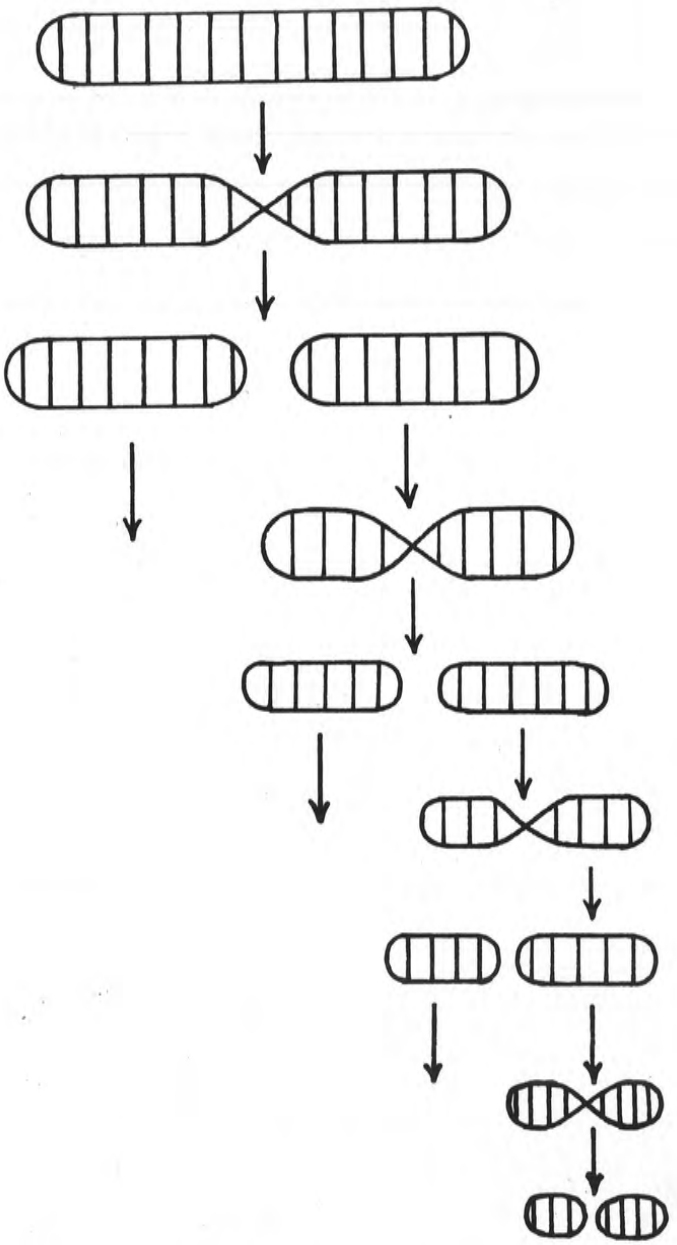


Figure 6.- A diagram showing the way in which miniature mitochondria (i.e. phosphorylating electron transport particles) may arise. This process has been called miniaturization (Green 1959 a).

not been confirmed.

The biochemical characteristics of the major and some of the minor sub-mitochondrial particles are summarized in Table 3 (overleaf).

Green (1956 b, a) has suggested that ETP is the basic unit of the mitochondrion and that the structural and functional linking of thousands of these particles forms the polymer which is known as a mitochondrion. It is possible that this is so, but as yet no suggestion has been put forward to explain how the other enzymes known to be present in mitochondria (but not in ETP) are arranged.

The chemicals used to produce sub-mitochondrial particles (amyl and isobutyl alcohol; lauryl sulphate, cholate, and deoxycholate) are all substances which extract lipids; and it is thought that many of the enzymes of the particles are held together in a functional manner by lipoproteins.

One lipoprotein associated with the electron transport chain is of especial interest as it contains a lipid soluble quinone which is capable of undergoing oxidation and reduction (Crane, Hatefi, Lester, and Widmer, 1957; Jarnfelt, Basford, Tisdale, and Green, 1958; Green, Jarnfelt, and Tisdale, 1959; Hatefi, Lester, Crane, and Widmer, 1959). This quinone, coenzyme Q, is thought to be one of the compounds which regulates the rate of oxidative phosphorylation. In particles capable of oxidative phosphorylation, inorganic phosphate has the effect of freezing the quinone in its reduced form, whereas ADP reverses this effect (Hatefi, 1959).

As might be expected, there has been less success in putting the mitochondrion together than there has in breaking it up. However, some progress has been made and this is summarized below:

TABLE 3

THE PREPARATION AND PROPERTIES OF SUB-MITOCHONDRIAL PARTICLES

PARTICLE	PREPARATION	PROPERTIES	
		Structural	Enzymatic
PETP	Miniaturization[1] Light mitochondria[2]	Miniature mitochondria	P/O ratios similar to mitochondria but phosphorylation slower due to loss of bound pyridine nucleotide. Added pyridine nucleotide increases oxidation but not phosphorylation.
ETP_H	Sonic vibration of heavy mitochondria[3]	Double membrane vesicular particles	Can oxidize only succinate of the Krebs cycle acids. Bound pyridine nucleotide lost. Can oxidize DPNH (unlike intact mitochondria). Rates of oxidation and phosphorylation are 3 - 4 times higher than those of the parent mitochondrion. $(f_s, f_d, Q, b, c_1, c, a)$
ETP	Sonic vibration of heavy mitochondria in absence of Mg^{++}[4] Differential centrifugation of light mitochondria in 15% ethanol[5] or high levels of phosphate[6] Extraction of mitochondria at pH 8.5[7]	Single membrane vesicular particles	As ETP_H but with no phosphorylation, and ETP is twice as active as regards oxidation. ETP_H requires cytochrome c for maximum activity (like mitochondria) but ETP has no such requirement. $(f_s, f_d, Q, b, c_1, c, a)$
DPNH oxidase	Fragmentation of ETP by deoxycholate[8]		(f_d, Q, b, c_1, c, a)
Succinic DPNH bc complex OR Succinic DPNH dehydrogenase OR DPNH-DC-SDC	Fractionation of mitochondria or ETP with tert-amyl alcohol[9] or isobutyl alcohol[10] Fractionation of ETP with cholate and ammonium SO_4[11] or deoxycholate[12]	Red particle	These particles catalyse reactions 5 times faster than mitochondria. The first two methods of preparation produce degraded particles such as: (f_s, f_d, Q, b, c_1, c). The last two methods of preparation produce more degradation: (f_s, f_d, Q, c_1, c), (f_s, Q, c_1, c), and (f_d, Q, c_1, c).
Cytochrome oxidase	Fractionation of DPNH oxidase with cholate and trypsin[13]	Green particle	This particle contains 3 atoms of copper per molecule of (a).

KEY: f_s = succinic dehydrogenase; f_d = DPNH dehydrogenase; Q = coenzyme Q; c_1 = cytochrome c_1; c = cytochrome c; a = cytochrome a

1. Green, Lester, and Ziegler, 1957.
2. Green and Crane, 1958.
3. Linnane and Ziegler, 1958.
4. Linnane, 1958.
5. Ziegler, Lester, and Green, 1956.
6. Mackler and Green, 1956.
7. Crane, Glenn, and Green, 1956.
8. Crane and Glenn, 1957.
9. Green, Mii, and Kohout, 1955.
10. Basford, Tisdale, Glenn, and Green, 1957.
11. Rabinowitz and de Bernhard, 1957.
12. Crane and Glenn, 1957.
13. Mackler and Penn, 1957.

(i) Keilin (1929, 1930; Keilin and Hartree, 1938, 1940) found that in his particulate enzyme preparations in which the cytochrome c component was masked or deficient, the addition of soluble cytochrome c greatly increased the catalytic activity of the preparation;

(ii) more recently, Keilin and King (1958) have been able to remove reversibly f_s (succinic dehydrogenase) from a particle similar to ETP;

(iii) if the succinic DPNH bc complex is supplemented with the green particle (cytochrome a and bound copper) a slow oxidation of succinate or DPNH by molecular oxygen can be observed. The rate is increased 5 or 10 fold by the addition of cytochrome c. If coenzyme Q is removed from the complex, then it also must be added before oxidation will occur (Crane, Widmer, Lester, and Hatefi, 1959);

and (iv) when coenzyme Q is removed from ETP, succinic DPNH bc complex, or (f_s,Q,c_1,c) by isooctane extraction, the capacity of these particles to oxidize succinate is lost. The original activity is regained upon the re-addition of the coenzyme (Green, 1959 b).

It should be stressed that, at present, the aim of this reconstruction process is not to produce a man-made, major piece of structural biochemistry, but to learn more about the way in which the mitochondrion, and especially ETP, is constructued. And from the results of these experiments, and from those of the degradation processes described above, a considerable amount has already been learnt.

6. Enzyme systems associated with mitochondria

(a) Krebs cycle

Green (1951, 1959 a) has suggested that none of the enzymes of the

Krebs cycle are located outside mitochondria. But if numerous enzyme distribution studies (see Table 4) are valid it would seem that certain parts of the Krebs cycle may occur outside mitochondria - in liver cells at least.

TABLE 4

THE PERCENTAGE RECOVERY FROM THE MITOCHONDRIA (COMPARED TO THE WHOLE CELL) OF SOME OF THE ENZYMES OF THE KREBS CYCLE

ENZYME	LIVER OF	% RECOVERY	REFERENCE No.
Succinic dehydrogenase	rat	59	1
Isocitric dehydrogenase	mouse	12	2
Aconitase	rat	16	3
Fumarase	mouse	55	4
Oxaloacetic oxidase	rat	45	5
alpha ketoglutaric oxidase	rat	20	6
DPNH cytochrome c reductase	mouse	28	7
DPNH cytochrome c reductase	rat	27	8
TPNH cytochrome c reductase	mouse	49	9
TPNH cytochrome c reductase	rat	57	1
Cytochrome c	rat	48	10
Cytochrome oxidase	mouse	76	11
Cytochrome oxidase	rat	75	1

References:

1. deDuve, Pressman, Gianetto, Wattiaux, and Appelmans, 1955.
2. Hogeboom and Schneider, 1950 a.
3. Dickman and Speyer, 1954.
4. Kuff, 1954.
5. Schneider and Potter, 1949.
6. Siekevitz, 1952.
7. Hogeboom and Schneider, 1950 b.
8. Hogeboom, 1949.
9. Hogeboom and Schneider, 1950 a.
10. Beinert, 1951.
11. Strittmatter and Ball, 1954.

And in the few studies of enzyme distribution in other types of cells similar results have been obtained. The distribution within the intestinal cell of the enzymes of the Krebs cycle has not been examined in detail.

(b) Glycolysis

The reactions of glycolysis are believed to take place mainly in the soluble fraction of the cell. However, as glycolysis takes place twice as fast in unfractionated tissue (LePage and Schneider, 1948), the other parts of the cell may be involved as well. Some of the reactions (e.g. the oxidation of 3 phosphoglyceraldehyde, lactic acid, and dihydroxy-acetone phosphate) have been shown to occur quite readily in mitochondria isolated from the kidney of rabbits (Kaplan, Still, and Mahler, 1951). And the whole of glycolysis seems to occur in the isolated mitochondria of a few tissues, e.g. rat brain (Gallager, Judah, and Rees, 1956), mouse liver (Hesselbach and duBuy, 1953; duBuy and Hesselbach, 1958), Cloudman S 91 melanoma from mice (Hochstein, 1957), and bacteria (Gale and Folkes, 1954; Gale, 1956). The intracellular distribution of the glycolytic enzymes in the intestine has not yet been examined.

(c) Protein synthesis

The contribution of mitochondria towards the synthesis of protein appears to vary in different tissues. The mitochondria in the liver of rats seem to do no more than produce the energy for protein synthesis and transfer it to a suitable cofactor (Siekevitz, 1952); while the mitochondria in the kidney, pancreas and muscle of rats appear to be able to synthesise protein by

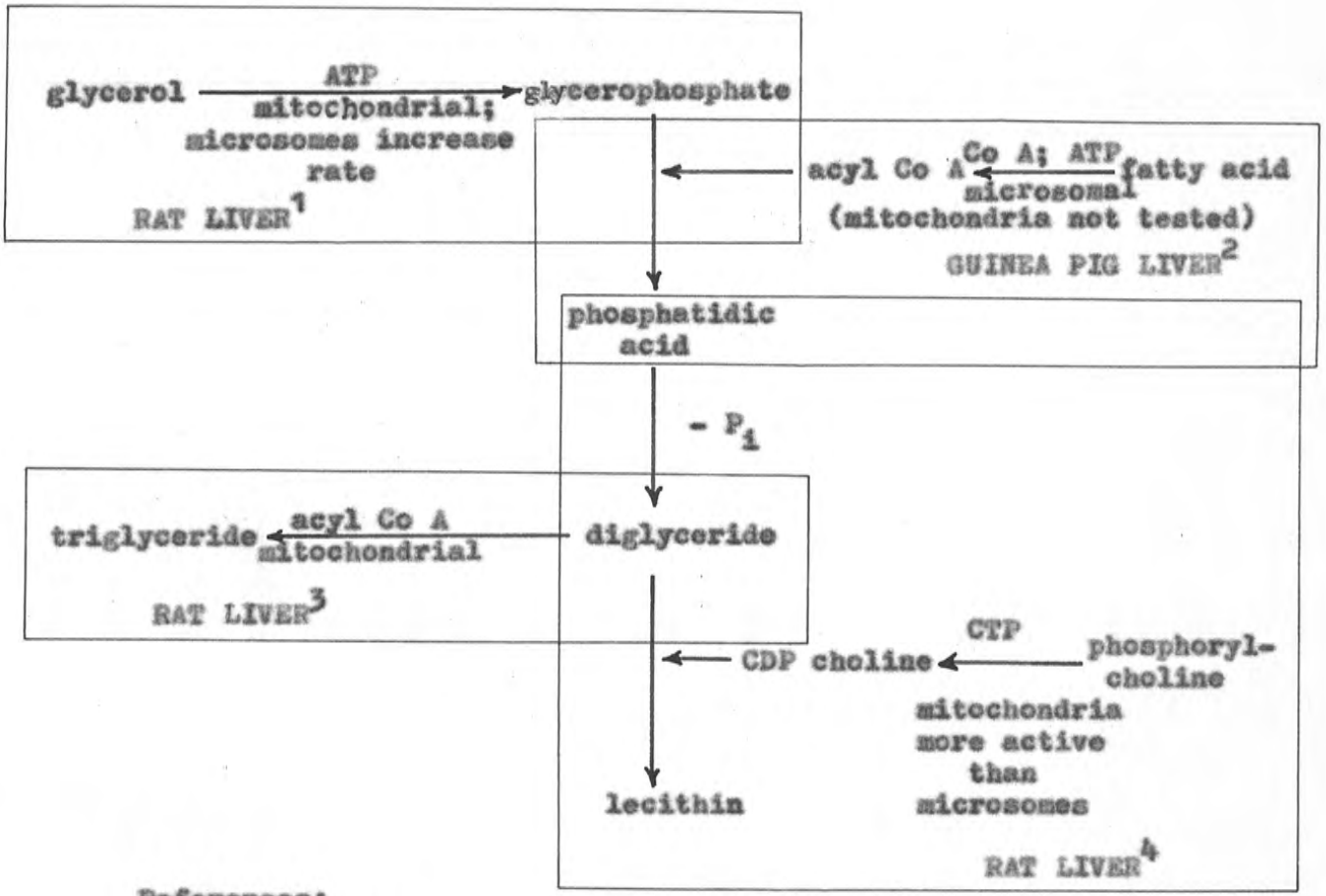
themselves (Simpson and McLean, 1955; McLean, Cohn, Brandt, and Simpson, 1958). Isolated mitochondria of calf heart have been shown to synthesise cytochrome c, too (Bates and Simpson, 1959). The contribution of intestinal mitochondria toward protein synthesis is unknown.

(d) Fatty acid and lipid synthesis

The sequence of reactions involved in the synthesis of fatty acids was thought to be the reverse of that for their oxidation - which occurs in mitochondria - except that TPN, rather than a series of flavoproteins was thought to be involved in the reduction of the double bonds. However, recent work has shown that the first step in the synthesis of fatty acids is the carboxylation of acetyl Co A to malonyl Co A, followed by the condensation with another acetyl Co A unit (Steberl, Wasson, and Porter, 1960). Exactly what happens after this remains to be elucidated.

The site of synthesis of fatty acids is not clear. On the one hand, Langdon (1957) has reported that mitochondria are not necessary for fatty acid synthesis in the liver of rats and pigeons; a finding similar to that of Popjack and Tietz (1955), who worked with the mammary gland of rats. On the other hand, Brady and Gurin (1952), and Dituri and his co-workers believe that both the soluble fraction and mitochondria are required for fatty acid synthesis in the liver of rats (Dituri, Shaw, Warms, and Gurin, 1957). In plants (avacado) the synthesis of fatty acids appears to be inhibited by the soluble fraction (Stumpf and Barber, 1957).

Present knowledge of the intracellular location of the enzymes involved in the synthesis of lipids is summarized in Figure 7.



References:

1. Kennedy, 1953.
2. Kornberg and Prier, 1953.
3. Stein, Tietz, and Shapiro, 1957.
4. Rodbell and Hanahan, 1955.

Figure 7.- A diagram summarizing present knowledge about the synthesis of lipids

The extent to which the mitochondria of the intestine participate in the synthesis of lipids and fatty acids is unknown, although large amounts of fatty acids are synthesised in the intestine (Coniglo and Cate, 1958; Neiderhiser and Wells, 1959).

(e) Miscellaneous synthetic systems

The other synthetic reactions which are known to require mitochondria for their completion can be divided into two types:

(i) those that are associated with mitochondria only because of a requirement for ATP (or another "high-energy" compound);

and (ii) those that appear to take place wholly within the mitochondrion.

Some of the more important type (i) reactions are listed in Table 5.

TABLE 5

SYNTHETIC SYSTEMS THAT ARE ASSOCIATED WITH MITOCHONDRIA DUE TO A REQUIREMENT FOR ATP

SYNTHESIS	REACTION OCCURING INSIDE MITOCHONDRIA	REF. No.
Urea cycle	CO_2 + NH_3 + ATP → carbamyl PO_4 + ADP carbamyl PO_4 + ornithine → citrulline	1, 2
Cholesterol synthesis	ATP + CO_2 + hydroxyisovalerate → hydroxymethylglutarate and a later step: mevalonic acid + 2ATP → 2 ADP + mevalonic acid pyroPO_4	3 4
Sulphate metabolism	SO_4 + ATP → adenyl phosphoSO_4 + PP adenyl phosphoSO_4 + ATP → phosphoadenylphosphoSO_4	5

References:

1. Grisolia and Cohen, 1951.

2. Reichard, Smith, and Hanahoff, 1955.

3. Block, 1956.

4. Cornforth, Cornforth, Popjack, and Gore, 1958.

5. Lipmann, 1958.

Type (ii) reactions are less common. They include the transaminases and the nucleotide kinases. The latter are responsible for the formation during oxidative phosphorylation of

triphosphates other than ATP, and have been said to control the level of ADP in mitochondria (Siekevitz and Potter, 1955).

7. The formation of mitochondria

Mitochondria may increase in number by dividing. This hypothesis was first put forward by Meeves (1908). But although mitochondria in the liver cells of vertebrates (Fawcett, 1955) and in tissue cultures (Dempsy, 1956) have been observed to divide, it seems that mitochondria can also increase in other ways. For example, Harvey (1946) showed that in the eggs of Arbacia, mitochondria can arise de novo in cytoplasm free of mitochondria; and when mitochondria can be marked in some way - as with the pancreatic cells of rats fed on a sugar and water diet (Weiss, 1953), or with the unfertilized ova of rabbits (Dempsy, 1956) - simple division can not explain the observed facts.

Chantrenne (1947) and Zollinger (1950) have suggested that mitochondria are formed from microsomes. In 1953 Eichenberger published some electron micrographs of the kidney of mice in which this process was apparently occurring. Lindberg and Ernster (1954) summarized it in a tentative scheme reproduced in Figure 8. However, experiments with Protozoa in which P^{32} was used to trace the path of phosphorus from microsomes to mitochondria did not support this hypothesis (Jeener and Szafarz, 1950; Jeener, 1952). And Eichenberger's evidence has received little support from recent electron microscopy.

Another suggestion is that mitochondria arise from "microbodies". These are described, in mouse kidney, as oval

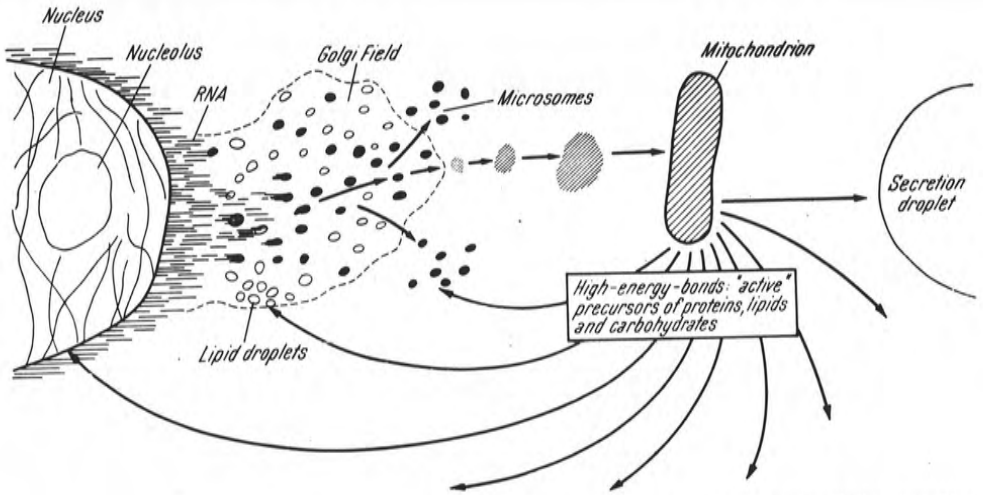


Fig. 32. Tentative scheme summarizing present information about the genesis and function of cytoplasmic particles and their interaction with other cell constituents.

Figure 8.- Lindberg and Ernster's (1954) tentative scheme summarizing the genesis of cytoplasmic particles.

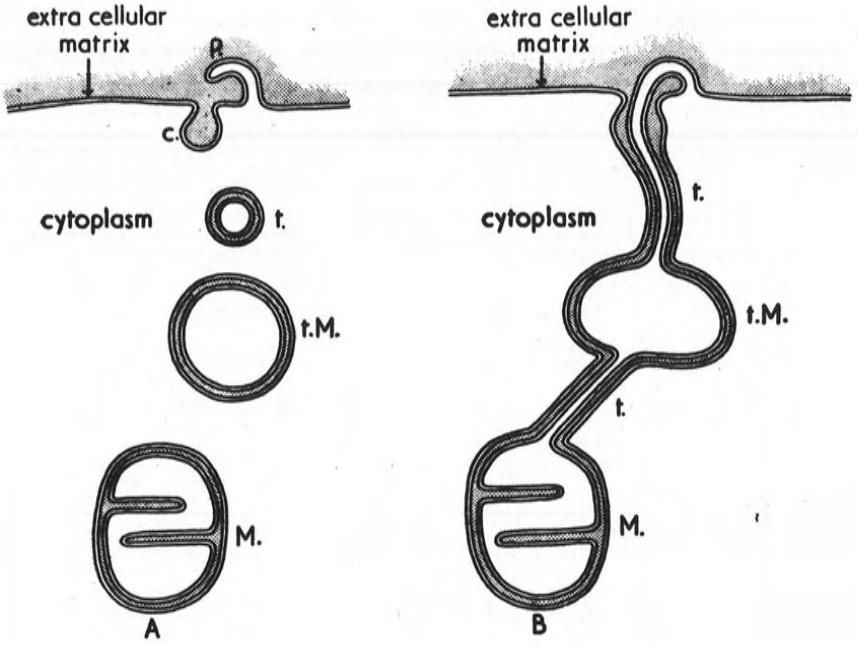
structures, 0.1 to 0.5 μ in size, consisting of a single membrane surrounding a finely granular substance (Rhodin, 1954). Microbodies are found in liver, pancreas, and tumour cells in large numbers when mitochondria are re-appearing after having been destroyed (Rouiller and Bernhard, 1956; Ferreira, 1957; Oberling, 1959).

Another recent suggestion is that mitochondria are budded off from the nuclear membrane (Causey and Hoffman, 1955). Observations supporting this hypothesis have been made in several types of cells - rat thymus lymphocytes, mouse lymph node, spermatagonia and spermatocytes of rat testis, and the meristematic cells of onion root tip (Hoffman and Grigg, 1958). In 1959 Robertson published a diagram illustrating a series of events by which mitochondria might originate from cell surfaces. This is reproduced in Figure 9.

It is not known how the mitochondria of the intestine are formed. But one would expect intestinal mitochondria to be formed in the same way as mitochondria from other sources, even though the intestinal mitochondria may contain different kinds of enzymes to other mitochondria.

8. Conclusion: the concept of mitochondria

Before 1950 it was fairly clear whether or not a particular structure should be called a mitochondrion. At that time mitochondria were defined as semi-solid bodies, 1 - 2 μ in size, which, in the living cell, stained with Janus Green B (Abercrombie, Hickman, and Johnson, 1951). Since then more has been learnt about mitochondria and this term now has a much wider meaning. It is quite possible today for biologists to misunderstand one another when they discuss "mitochondria" because the similarities between the mitochondria of different



Text-fig. 16. This diagram illustrates a way in which mitochondria might originate from cell surfaces. To the left there is shown a segment of the surface of a muscle fibre in which structures which have been directly observed have been drawn. To the right these structures are related speculatively.

Figure 9.- Robertson's (1959) diagram illustrating one way in which mitochondria may arise.

cells has tended to obscure the fact that there may be differences.

It is not only that a biochemist, for instance, thinks of mitochondria in terms of enzymes while a cytologist is more interested in fine structure; though, of course, it is essential that the biochemist should realize that the enzyme in which he is especially interested is just one of an integrated structural sequence. The real problem lies in the comparison of the mitochondria of different tissues and species, and of "mitochondria" subjected to different treatments.

Although the mitochondria of a single cell may differ slightly in size and shape (Dempsy, 1956), the mitochondria of plants and animals seem to be basically similar in structure. A general definition of mitochondria could, without difficulty, include most of the minor variations that have been found. Variations such as the peculiarly shaped tubular cristae of Protozoa (Sedar and Rudzinska, 1956; Pappas and Brandt, 1959); the very large mitochondria from the flight muscles of Diptera and Hymenoptera (Williams, 1953); the concentric mitochondria of some snails (Beams and Tahmisian, 1954); and the irregularly shaped cristae of retinal rod mitochondria (Sjostrand, 1956). What would be difficult, would be to decide:

(i) when the particles that give rise to mitochondria should be called mitochondria;

(ii) whether the dissimilar structures of similar function in yeast and bacteria (see Table 6) should properly be called mitochondria;

and (iii) whether it is confusing to call mitochondria by that name after their properties have been changed by unskillful removal from the cell.

Because the early stages in the formation of mitochondria have not been adequately described, point (i) is only academic

TABLE 6

A SHORT DESCRIPTION OF THE "MITOCHONDRIA" OR BACTERIA AND YEAST

SOURCE	DESCRIPTION	REFERENCE
Bacteria Type 1	The protoplast membrane has been found to contain many of the enzymes that are found in the mitochondria of animals. Electron transport particles can be prepared from the membrane which are similar to those obtained from animals. The bacterial particles exhibit oxidative phosphorylation (although most bacterial preparations have a lower P/O ratio than is found in animals).	1, 2, 3, 4
Type 2	Shinohara has described some structures in the cytoplasm of bacteria which are similar in structure to the mitochondria of animals. These structures are enclosed by a membrane and contain many parallel, dense lines separated by clear spaces.	5, 6, 7
Yeast	The presence of mitochondria in yeast has been confirmed by numerous staining methods and by electron microscopy. They are spherical, approximately 0.2 - 0.3 μ in diameter and contain similar enzymes to those found in animal mitochondria. Their internal ultrastructure is unknown.	8, 9, 10

References:

1. Weibull, 1953.
2. Storck and Sachsman, 1957.
3. Cota-Robles, Marr, and Nilson, 1958.
4. Marr, 1960.
5. Shinohara, Fukushi, and Suzuki, 1957.
6. Murray, 1960.
7. Glauert and Hopwood, 1959.
8. Lindergren, 1949.
9. Bartholomew and Levin, 1955.
10. Linnane and Still, 1955.

at the present time. But confusion can very easily occur when comparing the ultrastructure of yeast or bacteria with that of unicellular animals and plants; or when comparing the activity of similar enzymes. This last point is of especial importance.

Before one can attempt to estimate the activity of an enzyme, the cell may have to be disrupted and the structure in which the enzyme is localized isolated. Several difficulties arise when the structure to be isolated is a mitochondrion. Firstly, when mitochondria are isolated from the cell so that they look the same as they did *in vivo*, they exhibit considerably less enzymatic activity than damaged or swollen mitochondria (Green 1956 *a*, *b*; Ziegler and Linnane 1958). That is to say, the activity of mitochondrial enzymes is usually estimated in mitochondria which are different from those found in the cell. And secondly, before the activity of enzymes of similar function but from different sources can be compared, one must decide either to examine the different mitochondria in a common medium, or to vary the medium so that it corresponds as nearly as possible to the *in vivo* environment. In actual fact, even in the few species in which the extracellular fluid has been analysed, very little is known of the composition of the intracellular fluid; moreover, even if it were known it is unlikely that it could be reproduced as the environment in the cell is complicated by the presence of membranes and other devices which allow local and transient concentrations of ions and other substances. So mitochondria from different sources are usually examined in a common medium, which is varied only when the usual one prooves unsatisfactory. In order to demonstrate oxidative phosphorylation in preparations made from *Ascaris*, it is necessary to add a non-dialyzable component of the perienteric fluid (Chin and Bueding, 1954); and mitochondria

isolated from several species of insects require serum albumin for "full" activity (Sacktor, 1954; Lewis and Slater, 1954).

This gives rise to a third difficulty in experimenting with mitochondria (apart from the fact that several sorts of common media are used). It is this. How slowly can an enzyme system catalyse a reaction before it becomes "unsatisfactory"? Normally, when an enzyme is studied in vitro, efforts are made to obtain the maximum possible activity by providing optimum conditions of pH, temperature, and substrate and ionic concentration. Yet the physiological activity of an enzyme is probably less dependent on its theoretical maximum capacity than on a pacemaker reaction in the associated enzyme sequence. This pacemaker reaction, in its turn, is probably controlled by an hormone or a physiological barrier such as a membrane.

The existence of these difficulties in experimenting with mitochondria emphasises the need for care when interpreting the results of experiments in which enzyme activities are compared. Estimates of enzyme activity should be accompanied by an account of the method by which they were obtained. Unless this is done the estimates obtained by different methods may seem to contrdict one another even before any comparisons can be made between different types of mitochondria.

The concept of mitochondria was clear when it was simple. At the present time the term mitochondrion by itself has too broad a meaning. Biologists think of mitochondria in different ways: some imagine mitochondria as an ever changing piece of structural biochemistry completely integrated with the cytoplasm; others imagine a cellular particle with discrete boundaries through which interchanges occur only with

difficulty; and others imagine mitochondria as the damaged particle that they have isolated and forced to work in an unnatural environment. Although none of these ways of thinking about mitochondria may be correct, they are all useful, providing a straight comparison is not made between the results obtained from one sort of mitochondria and those from another sort. (By sort I mean mitochondria with a particular experimental history.)

II. MATERIALS AND METHODS

1. Chemicals

"Analytical Reagent" quality chemicals (from The British Drug Houses, Ltd., Poole, England) were used in this investigation except where specifically stated to the contrary. Coenzymes were obtained from the Sigma Chemical Company (Missouri, U.S.A.). Double glass-distilled water was used for preparing reagents and for rinsing glassware.

2. Experimental animals

The animals used in the experiments, and their approximate ages, are shown in Table 7. Male animals were used throughout this investigation. The fish, birds, and mammals were fasted for at least three hours before death. Toads and lizards were taken straight from the cage in which they lived; the toads were obtained commercially and had not been

TABLE 7

THE ANIMALS USED IN THIS INVESTIGATION

CLASS	ANIMAL	AGE AT DEATH
Pisces	Carassius auratus (golden carp)	2 - 3 years
Amphibia	Bufo marinus (toad)	mature
Reptilia	Tiliqua rugosa (sleepy lizard)	4 - 12 years
	Tiliqua scincoides (blue tongue lizard)	4 - 12 years
Aves	Gallus domesticus (chicken)	3 - 4 months
Mammalia	Cavia percellus (guinea pig)	3 - 6 months
	Rattus norvegicus (hooded rat)	3 - 4 months

fed for 3 - 4 weeks. Most of the animals were killed by a sharp blow behind the head, but the carp and chicken were decapitated.

3. Histological methods

The intestine was fixed either by potassium dichromate and osmic acid (Altmann, 1890), or by potassium dichromate and formalin (Regaud, 1909). Both methods gave similar results but as Altmann's method was quicker it was used more often. The time between the death of the animal and the placing of the material into the fixative was usually less than a minute.

The tissue was dehydrated by passing it through tert-butyl alcohol as shown in Table 8.

TABLE 8

COMPOSITION OF THE ALCOHOLS USED TO DEHYDRATE THE FIXED INTESTINE

TOTAL % OF ALCOHOL	COMPOSITION OF ALCOHOL (IN PARTS)			
	H_2O	95% alcohol	abs. alcohol	tert-butyl alcohol
50	50	40	--	10
70	30	50	--	20
80	15	50	--	35
95	--	45	--	55
100	--	--	25	75

This unusual series of alcohols was used because it had been reported to dehydrate material containing mucus better than the more usual series of ethyl alcohols (Nizammuddin, 1960). The intestine was kept in each percentage of alcohol for two to three hours.

The material was cleared for ten hours in cedar wood oil, and then embedded in paraffin wax (with a melting point of 58° C) in the usual manner.

A "Sartorius" microtome was used for cutting sections 5 - 7.5 μ thick. The block was cooled to - 5° C before cutting the thinner sections.

The mitochondria in the intestinal sections were stained with Fast Green FCF (Peterson, Conn, and Melin, 1934) by a method similar to that used by Harman (1950). However, as the sections fixed with osmic acid were found to be rather dark, they were bleached with hydrogen peroxide before they were stained. The sections were also counter-stained for a shorter time, and a celloidin-ether bath was used to ensure that the sections remained on the slides while they were being stained. The time-schedule of the staining proceedure is shown in Table 9.

4. Photomicrography

Most of the photomicrographs were taken with a Leitz Dialux microscope. Two cameras were used; a Leica 1 F together with a Leitz MIKAS camera attachment, and a Rolleicord Va with a Franke and Heidecke microscope attachment. Ilford Pan F film was used in the Leica and Ilford FP3 for the Rolleicord. The films were developed in Kodak Microdol and were printed on Kodak F3 or F5 paper according to the degree of contrast required.

TABLE 9

TIME-SCHEDULE OF THE STAINING PROCEEDURE

BATH	TIME IN MINUTES
Xylol 1	2
Xylol 2	2
Abs. alcohol	3
Celloidin-ether	2
Bleach { 1 part 100 vol H_2O_2 / 2 parts 80% alcohol	15, in bright light
70% alcohol	2
50% alcohol	2
4% (W/V) solution of Fast Green FCF in 10% (V/V) aniline water	6, stain initially at 62° C
Distilled water	½
Saturated aqueous picric acid	10
Distilled water	½
1% phosphomolybdic acid	1
Distilled water	1
1% (W/V) safranine in 50% alcohol	2 - 3
50% alcohol	2
70% alcohol	2
85% alcohol	2
90% alcohol	2
95% alcohol	2
Abs. alcohol	3
Mounted in "Xam"	

The magnification was estimated by a stage micrometer. The phase contrast micrographs were taken with the Leica on a Reichert Neozet microscope.

5. The isolation medium

(a) for whole cells:

The QO_2 of whole cells was estimated in Krebs' modified Ringer which consists of:

1000 ml 0.9% NaCl
40 ml 1.15% KCl
10 ml 3.84% $MgSO_4$
and 300 ml phosphate buffer, pH 7.4
(prepared by dissolving
17.8 g $Na_2HPO_4.2H_2O$ and
20 ml N HCl in 1000 ml H_2O).

(b) for homogenized cells and mitochondria:

A buffered sucrose solution containg ethylene diamine tetra-acetic acid (versene) was used as a medium in which to homogenize the cells of the intestine and to isolate the mitochondria. This solution consisted of 0.25 M sucrose containing 0.01 M versene and 0.01 M phosphate buffer at pH 7.6. The pH of the final solution was adjusted to 7.4 by adding KOH.

In the remainder of this thesis the buffered sucrose solution containing versene is referred to, for the sake of conciseness, as sucrose solution.

6. Removal of columnar cells from the intestine

After the body cavity of the animal had been opened, the small intestine was removed (though see Section 8, p. 41) and placed on a sheet of laminex. Fat and any other material adhering to the outside of the intestine was removed as completely as possible. Approximately 25 ml sucrose solution at 37° C was forced through the small intestine in order to remove any material in the process of being digested. The

intestine was then slit along its length and strips of tissue 5 - 7 cm long cut from it. These were allowed to fall into ice-cold sucrose solution. After washing, the strips were transferred to fresh, cold sucrose solution.

The columnar cells were removed by allowing an intestinal strip to hang vertically by holding it at one end with a pair of forceps, while at the same time drawing the strip through the partially closed jaws of a second pair. This scraping process often had to be repeated several times before most of the columnar cells were removed from the wall of the intestine. The scrapings were allowed to fall into ice-cold sucrose or into a chilled homogenizer.

The intestine of the carp was small and fragile and it was difficult to scrape the columnar cells from the wall of the intestine without breaking it. Consequently, after the intestine of the carp had been opened lengthwise, it was cut into small (about 1.0 cm) sections and the entire intestine (with the absorbing cells still attached to the wall) homogenized.

7. Methods of separating columnar cells

Two methods were devised to separate single cells from the intestinal scrapings:

Method (a) The scrapings were put into an 100 ml beaker and about 20 volumes of cold sucrose solution added. This mixture was stirred with a "Z" shaped glass rod at 120 - 200 r.p.m. with an electric motor. The whole apparatus was kept at 1 - 5° C in order to keep cytolysis to a minimum. It took 10 - 15 minutes to separate the intestinal cells of the species examined. Liver cells may also be separated by this method, provided the liver is cut into small cubes, although

a longer stirring time is required (approximately 30 minutes). Method (b): The scrapings from the intestine may also be separated into single cells by using an homogenizer (glass-tephlon - see p. 43). The scrapings were put into the homogenizer and 3 or 4 volumes of sucrose added. The pestle of the homogenizer was inserted and moved slowly up and down by hand. The pestle was not allowed to rotate. Two or three complete movements were usually sufficient to separate the cells. Microscopical examination of the separated cells showed that relatively few cells were ruptured.

Method (b) was used to separate the cells for the manometric experiments as it was much quicker than the stirring method.

8. Irrigation of the intestine

The intestine of mammals (especially that of rats) contains a considerable amount of mucus. It was found that some of this mucus could be removed by allowing sucrose solution at 37° C to run through the gut before it was taken from the body. This was done in the following manner.

The animal was given an intraperitoneal injection of nembutal (30 mg/kg body weight). At second stage anaesthesia the body cavity was opened and the gut was severed just below the stomach and just above the large intestine. The intestine was displaced as little as possible in order to avoid breaking blood vessels. A cannula was introduced into the top of the small intestine and about 500 ml of warm (37° C) sucrose solution was passed through the small intestine by means of the apparatus shown in Figure 10. This took about 10 minutes. The apparatus consisted of a reservoir held about 3 feet above the animal; the reservoir was connected to the cannula in the intestine by plastic tubing. Sucrose was

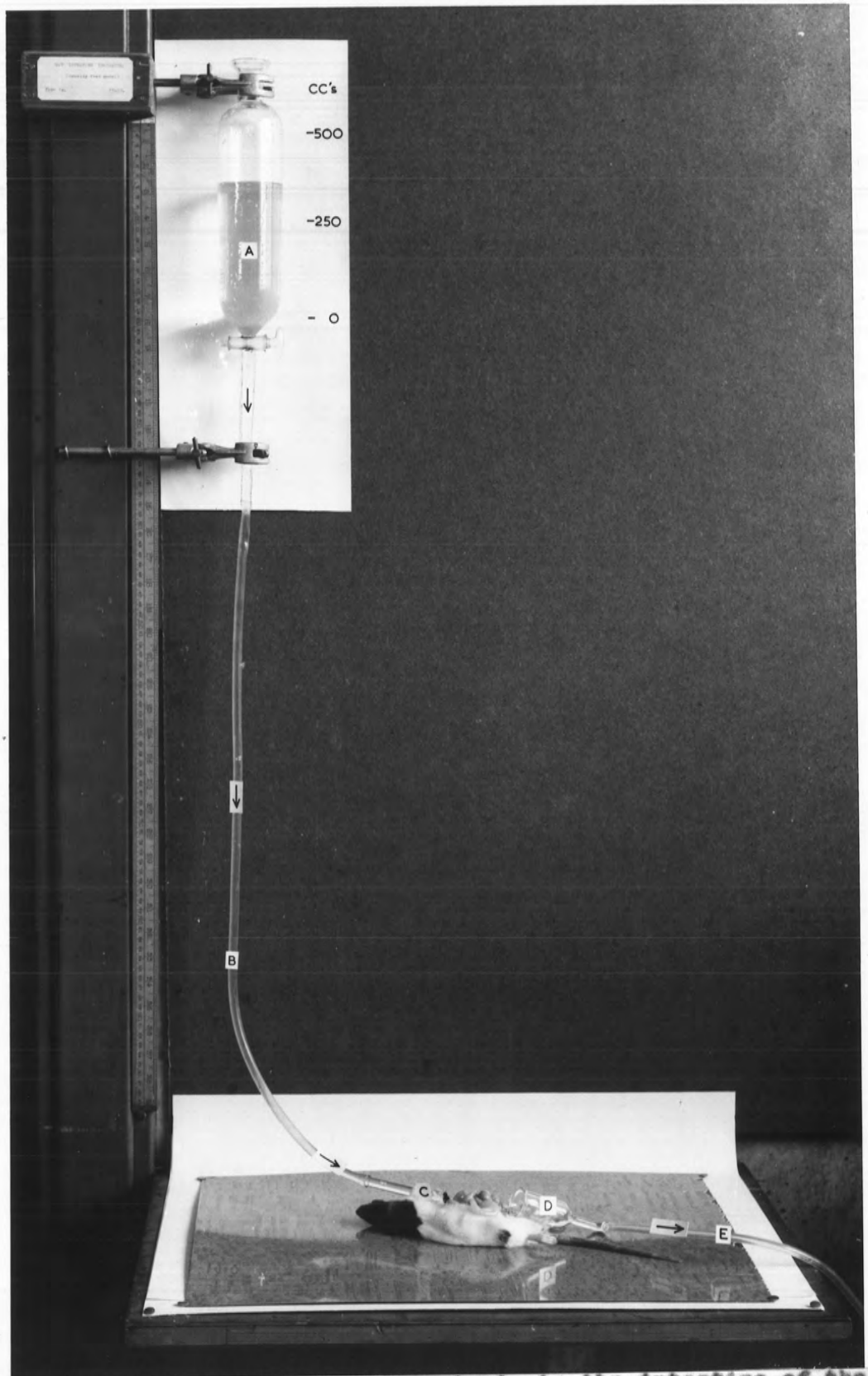


Figure 10.- The apparatus used to irrigate the intestine of the rat. Sucrose was passed from the reservoir, A, to the intestine by means of the tube, B, and the cannula, C. Sucrose which had passed through the intestine was allowed to drain into a thistle funnel, D, and was removed by suction along the tube, E.

allowed to flow through the intestine under the force of gravity. The sucrose that had passed through the intestine was collected in a thistle funnel and removed by suction.

As well as removing some of the mucus, this proceedure made the stripping of the cells from the intestinal wall much easier.

9. Homogenization

A glass-tephlon homogenizer was used. It was obtained from the Arthur H. Thomas Company, Philadelphia, U.S.A. It held 10 ml and the clearance of the tephlon pestle was 0.004 - 0.006 inches.

The scrapings from the intestine were allowed to fall into a chilled homogenizer, the sides of which had been rinsed with sucrose solution so that the scrapings slid to the bottom of the vessel. Three to four volumes of sucrose solution were added and the cells homogenized in a cold room at - 5° C. The pestle was turned with a 1/75 h.p. motor which was capable of a speed of 2000 r.p.m. The speed of the motor could be varied. The time required to break the majority of the cells was from 60 - 90 seconds at the highest speed of which the motor was capable under load.

When mitochondria were being prepared (as opposed to homogenates) sufficient material could not always be obtained from one animal. Three carp intestines, or the intestinal scrapings from two toads were necessary to isolate a sufficient quantity of mitochondria from these animals.

10. Centrifugation

An equal quantity of cold buffered sucrose was added to the homogenate before it was centrifuged at 500 g for 20

minutes. The supernatant was recentrifuged at 15,000g for 30 minutes. All centrifugation was done at 0° C in an International centrifuge (model PR 2). Lusteroid centrifuge tubes were used.

11. Manometry

Standard Warburg constant volume respirometers with a cup volume of approximately 8 c.c. were used for the manometric experiments. They were calibrated by the method of Zwart (1952).

The cup contents were:

In the main compartment
- 0.4 ml enzyme preparation
- 0.6 ml incubation medium
- 0.1 ml substrate (or sucrose)

In the centre well
- 0.05 ml 5N KOH
- 0.5 x 1.0 cm piece of fluted filter paper (Whatman No. 40).

12. Manometric estimation of succinoxidase activity

The incubation medium for the estimation of succinoxidase activity was:

20 µmol. succinate
10 µmol. $MgCl_2$
10 µmol. ATP
50 µmol. phosphate buffer, pH 7.4
75 µmol. KCl
1 µmol. DPN
and 1 µmol. cytochrome c

13. Estimation of dry weight

The dry weight of a preparation was obtained by placing

the whole contents of a manometer cup (except the filter paper) onto a watch glass of known weight and drying it in an oven at 100 - 105° C for twelve to fifteen hours. The dried preparation was then removed and cooled in a desiccator. It was weighed immediately it was cool. Allowance was made for the weight of salt in the incubation medium.

14. Estimation of nitrogen and protein

Three methods were used to measure the protein content of samples. They were:

(i) tryptophane, tyrosine, and nucleotide as a measure of protein content (Lerner and Barnum, 1949; Harman and Feiglson, 1952);

(ii) a method involving the Biuret reaction;

and (iii) the Kjeldahl method.

Although the first two methods were quicker, the last one was found to give the most consistent results and this method was the one regularly used.

15. Isooctane extraction of mitochondria

Two methods for extracting mitochondria with isooctane were devised:

Method (a): The mitochondrial pellet was resuspended in 2.5 ml sucrose. 2.5 ml isooctane was added and the suspension shaken by hand in a Cold room (- 5° C) for 1 minute. The mitochondria were then reprecipitated by centrifugation at 15,000 g for 15 minutes. After the isooctane had been removed and kept, the mitochondria were resuspended in 2.5 ml sucrose and 2.5 ml isooctane was added as above. The whole cycle was repeated twice more. Thirty minutes were allowed for the final centrifugation. The precipitate from this

centrifugation was washed several times with cold sucrose, resuspended with fresh sucrose, and tested for enzyme activity. The three fractions were pooled.

Method (b): The mitochondrial pellet was resuspended in 2.5 ml sucrose. The resuspended mitochondria were then shaken with 10 ml of isooctane for 30 minutes at 2° C. The shaking was done by rotating a tube (three inches long) containing the mitochondria at 15 r.p.m. The apparatus used is shown in Figure 11. The tube was shaken vigourously by hand for one minute at the beginning and the end of the mechanical shaking. The mitochondrial suspension was then recentrifuged at 15,000 g for 30 minutes. The isooctane was removed with a Pasteur pipette. The mitochondrial pellet was washed several times with cold sucrose, resuspended with fresh sucrose, and tested for enzyme activity.

As method (a) took much longer and was less efficient than method (b), the latter was the one routinely used.

16. Spectrophotometric detection of unsaturated fatty acids

Unsaturated fatty acids were detected by their absorption of ultraviolet light (Duel, 1951). The material suspected to contain unsaturated fatty acids was dissolved in isooctane and its absorption spectrum followed between 300 and 220 mμ.

17. Chromatographic separation of fatty acids

Long chain fatty acids were separated by paper chromatography. The method used was similar to that suggested by Kaufman and Nitsch (1954). Whatman No. 4 filter paper was impregnated with kerosene, and spotted with both standard and unknown. The mobile phase, 85% aqueous acetic acid saturated with kerosene, was allowed to run for 30 cm. The fatty acids

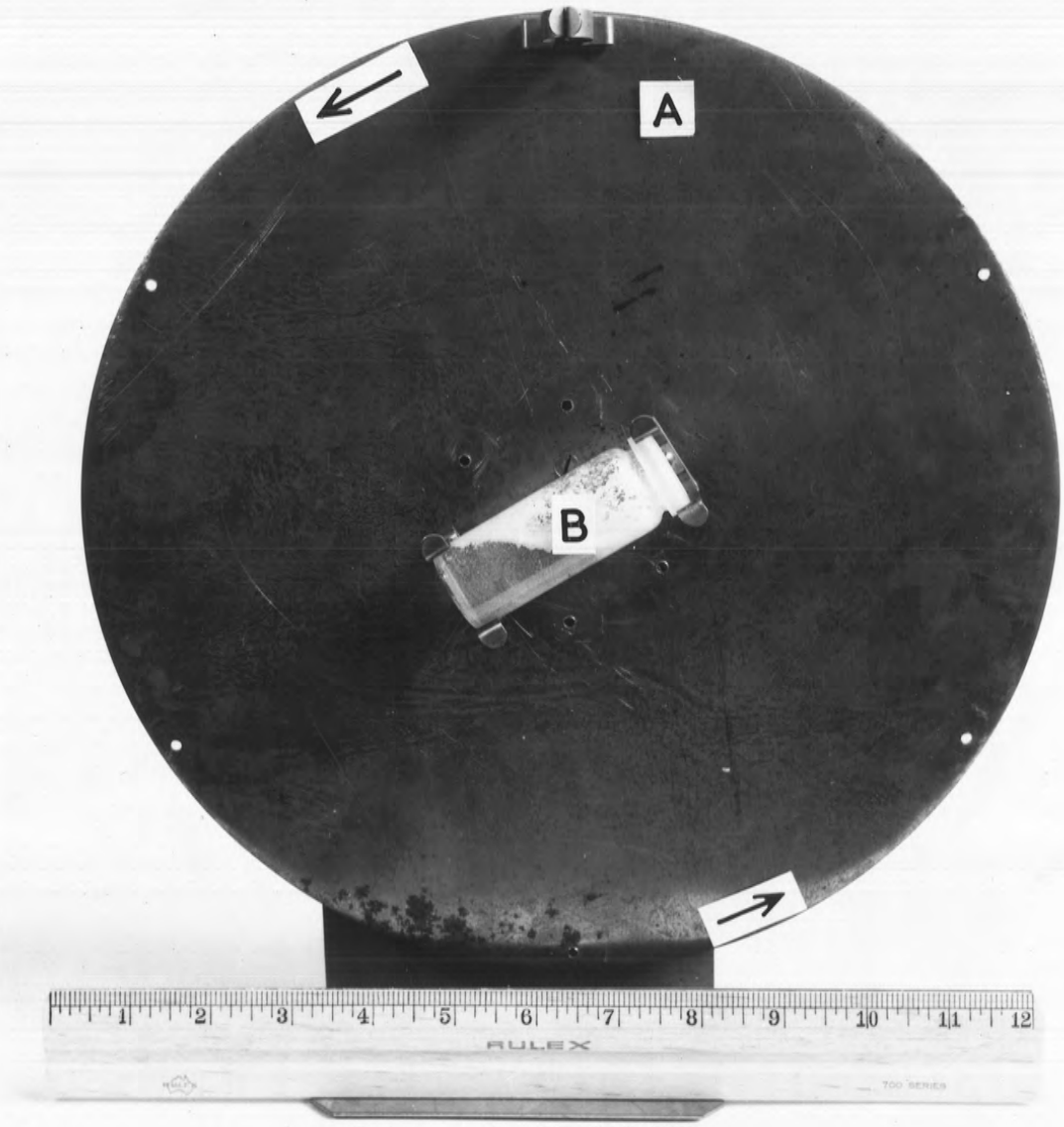


Figure 11.- The apparatus used for the isooctane extraction of mitochondria. The isooctane and the mitochondrial suspension was placed in the tube, B, and rotated in the position shown at 15 r.p.m. with an electric motor (hidden behind the plate). Mixing was more effective in this position than when the tube was clamped at position A.

were identified by converting them into copper salts by soaking the paper for 45 minutes in copper acetate (10 ml saturated copper acetate plus 240 ml water). After the excess copper acetate had been removed by washing, the copper bound by the fatty acids was detected with potassium ferrocyanide (3.5%). Purple or pinkish-brown spots were formed on a pink background.

18. Cytochemical detection of succinoxidase activity

Succinoxidase activity was detected cytochemically by the tetrazolium method developed by Antopol, Glaubach, and Goldman (1948), and modified by Seligman and Rutenberg (1951). This method depends on the reduction of one of the tetrazolium compounds by an hydrogen donor, or in a specific system by succinic dehydrogenase.

The tetrazolium compound used in the present investigation was 2:3:5 triphenyl tetrazolium chloride (British Drug Houses Laboratory Reagent). The composition of the incubation medium used was:

10 ml 0.2% triphenyl tetrazolium chloride
10 ml 0.2M sodium succinate
10 ml 0.2M phosphate buffer, pH 7.4
0.2 ml 0.3M calcium chloride
2.0 ml 0.6M sodium bicarbonate
6.8 ml distilled water

The tissue was incubated anaerobically for 1 hour at 37° C.

Frozen sections (approximately 25 u thick) were cut on a Reichert freezing microtome cooled by the evaporation of liquid CO_2.

19. Spectrophotometric estimation of succinic dehydrogenase activity

The succinic dehydrogenase activity of mitochondria was compared by measuring the rate of reduction of 2:6 dichlorphenol indophenol by the mitochondria in the presence of succinate. Any cytochrome oxidase activity present was inactivated by KCN.

The following proceedure was adopted: 0.3 ml 0.1M neutralized KCN, 0.05 ml 1 $x10^{-3}$ dichlorphenol indophenol, 0.4 ml 0.5M phosphate buffer (pH 7.4), 0.1 or 0.05 ml mitochondria, and 2.2 ml distilled water was put into a cuvette. The reaction was started by adding 0.1 ml 0.2M potassium succinate. The optical density of the solution was read at 30 second intervals for 5 minutes in a spectrophotometer set at 600 mμ. The blank contained no succinate.

20. Manometric estimation of cytochrome oxidase

Cytochrome oxidase was estimated manometrically by the method suggested by Umbreit in his book, "Manometric Techniques" (Umbreit, Burris, and Stauffer, 1957).

21. Electron microscopy

(a) Fixation

Slices of intestine (1 - 2 mm thick) and small mitochondrial pellets were fixed in buffered osmium tetroxide. This was prepared in accordance with Sjostrand's (1954) modification of Michaelis' formula (Michaelis, 1931):

Stock Buffer Solution A

Sodium acetate	9.714 g
Veronal sodium	14.714 g
Distilled water to make	500 ml

Stock Buffer Solution B

Sodium chloride	40.3 g
Potassium chloride	2.1 g
Calcium chloride	0.9 g
Distilled water to make	500 ml

Buffered Osmium Tetroxide Solution

Solution A	10.0 ml
Solution B	3.4 ml
0.1 N HCl	11.0 ml
Osmium tetroxide	0.5 g
Distilled water to make	50 ml

The pH of the buffered osmium tetroxide solution was 7.2 - 7.4, and its tonicity was 0.34 M. The intestinal material was fixed for two hours at 5° C, except that of the carp, which only required 15 minutes fixation.

(b) Embedding

After fixation the material was treated in the following manner:

Wash in running water	2 hours
10% methyl alcohol	15 minutes
25% methyl alcohol	15 minutes
50% methyl alcohol	30 minutes
75% methyl alcohol	30 minutes
90% methyl alcohol	30 minutes
Abs. alcohol	30 minutes
Abs. alcohol	30 minutes
Abs. alcohol/methacrylate	3 hours
Methacrylate	3 hours
Polymerization with fresh methacrylate	24 - 30 hours at 48° C, or 6 - 8 hours at 60° C.

The polymerization was carried out in No. 4 gelatin capsules.

(c) Sectioning

The methacrylate block containing the specimen was trimmed under a dissecting microscope so that the intestinal material was at the apex of a four sided pyramid with a flat top about 0.3 mm square. The slope of the faces of the block varied between 70° and 90°.

The material was sectioned on a "Serval Porter-Blum" microtome with hand made glass knives. Sections 60 - 90 mu thick were considered suitable for viewing in the electron microscope. They were collected (after they had been expanded with toluene) from a 20% acetone solution onto grids coated with 0.45% parlodion.

(d) Glass knives

Glass knives were made in the laboratory from 10" x 2" x ¼" strips of xanthelius glass by two different methods. The sequence of operations for both methods is shown in Figure 12. In the first method, the glass was scored on one side only. The glass was broken along these score lines with a pulling motion with a pair of "Red Devil" glass pliers. The second method was similar except in the manner of breaking the glass. In this method the scores were made in alignment on both sides of the glass. The glass was broken by pressure on an uneven surface. A match stick was placed on the bench and the glass was arranged so that it lay with the score marks on top of the match. The glass was then covered with a flexible wooden ruler and pressure applied with a hand on each side of the score.

The second method usually produced better knives. However, with some batches of glass the first was more efficient.

Reservoirs to hold the acetone onto which the sections were floated, were made by gluing a piece of plastic tubing

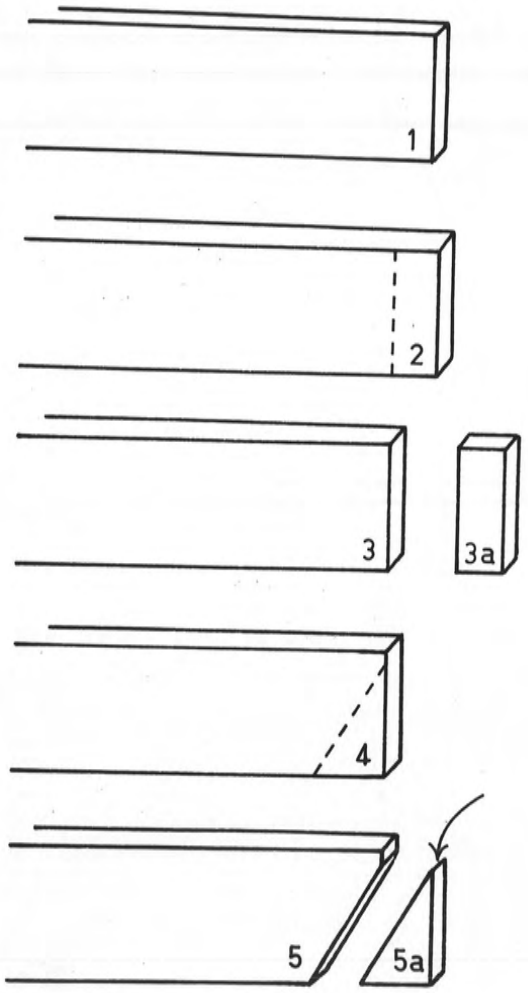


Figure 12.- The sequence of operations in making glass knives. 1 represents a strip of xanthelius glass 10" x 2" x $\frac{1}{4}$". In 2 the glass has been scored with a glass cutter along the dotted line. The small piece, 3a, is then broken off with a pair of glass pliers. In 4 the remaining glass has been scored again so that the glass knife, 5a, can be broken off. The knife edge is arrowed.

to the glass knife as shown in Figure 13.

(e) The electron microscope

A Philips desk-type electron microscope was used, the Metalix 11980. The degree of magnification was estimated by reference to polystyrene latex spheres, 1,100 or 2,600 A' in diameter.

(f) Photographs

Photographs were taken on Ilford X ray film 5B11 and developed in D 76. The negatives were printed on Kodak bromide paper. This was developed in Dektol where normal contrast was required, or in Ilford ID 13 when extra contrast was desired.

22. Spectrophotometric estimation of enzymes

The following methods were used to estimate the distribution of enzymes within the cell:

(a) Diaphorase

The method developed in the laboratories of Edelhoch and Mahler was used (Edelhoch, Hayaishi, and Teply, 1952; Mahler, Sarkar, Vernon, and Alberty, 1952). Each cuvette contained:

100 µmol. tris buffer, pH 7.4
0.1 µmol. 2:6 dichlorphenol indophenol
6.0 µmol. KCN
0.3 µmol DPNH

and enzyme to a total volume of 3 ml.

The reaction was initiated by the addition of DPNH. The optical density at 600 mµ was read at 30 second intervals for 5 minutes against a blank which contained no DPNH.

(b) DPNH-cytochrome c reductase

Mahler's modification of Edelhoch's method (Edelhoch, Hayaishi, and Teply, 1952; Mahler, Sarker, Vernon, Alberty, 1952)

Figure 13.- This figure shows how a plastic tube was glued to the glass knives to form a reservoir.

was used to estimate DPNH-cytochrome c reductase. Each cuvette contained:

50 µmol. phosphate buffer, pH 7.4
6 µmol. KCN
0.1 ml 1% aqueous cytochrome c
0.6 µmol. DPNH
and enzyme in a total volume of 3 ml.

The reaction was initiated by the addition of the DPNH. The optical density at 550 mµ was read at 30 second intervals for 5 minutes against a blank which contained no DPNH.

(c) Aconitase

The method of Racker (1950) was used to estimate aconitase. Each cuvette contained:

2.9 ml 0.03 M citrate in 0.05 M phosphate buffer
0.1 ml enzyme.

The reaction was initiated by the addition of the enzyme. The optical density at 240 mµ was read at 30 second intervals for 5 minutes against a blank which contained only buffer and enzyme.

(d) Isocitric dehydrogenase

The method used to estimate isocitric dehydrogenase was that of Ochoa (1948). Each cuvette contained:

75 µmol. glycyl glycine buffer, pH 7.4
6 µmol. KCN
2 µmol. $MnCl_2$
0.2 µmol. TPN
0.6 µmol. d-l isocitrate
and enzyme in a total volume of 3 ml.

The reaction was initiated by the addition of isocitrate. The optical density at 340 mµ was read at 30 second intervals against a blank which contained no TPN.

(e) alpha ketoglutarate dehydrogenase

The method used to estimate alpha ketoglutarate dehydrogenase was that of Kaufman (1955). Each cuvette contained:

100 µmol. phosphate buffer, pH 7.4
6 µmol. KCN
8.3 µmol. cysteine
2.5 µmol. alpha ketoglutarate
0.13 µmol. Co A
0. 3 µmol. DPN

and enzyme in a total volume of 3 ml.

The reaction was initiated by the addition of the alpha ketoglutarate. The optical density at 340 mµ was read at 30 second intervals for 5 minutes against a blank which contained no alpha ketoglutarate.

(f) Fumarase

Racker's (1950) method was used to estimate fumarase. Each cuvette contained:

30 µmol. phosphate buffer, pH 7.4
20 µmol. malate

and enzyme in a total volume of 3 ml.

The reaction was initiated by the addition of the malate. The optical density at 240 mµ was read at 30 second intervals against a blank which contained no malate.

(g) Malic dehydrogenase

The assay method devised by Mehler and his co-workers was used (Mehler, Kornberg, Grisolia, and Ochoa, 1948). Each cuvette contained:

75 µmol. tris buffer, pH 7.4
6 µmol. KCN
0.2 µmol. DPNH
0.8 µmol. oxaloacetate

and enzyme in a total volume of 3 ml.

The reaction was initiated by the addition of the oxaloacetate. The optical density at 340 mμ was read at 30 second intervals for 5 minutes against a blank which contained no oxaloacetate.

23. Oxidative phosphorylation

The intestinal mitochondria were suspended in sucrose solution and incubated for 5 minutes at 37° C in a medium consisting of:-

0.1 ml 10^{-3} M thyroxine (or water)
0.1 ml 0.5 M phosphate buffer, pH 7.4
0.1 ml 2.5×10^{-4} M cytochrome c
0.1 ml 0.2 M tris buffer, pH 7.4
0.1 ml 0.1 M NaF
0.05 ml 0.835 M glucose
0.05 ml 0.1 M ATP
0.1 ml 0.1 M $MgCl_2$ (or 0.05 M)
0.1 ml hexokinase (20 mg/ml)
0.2 ml mitochondrial suspension.

The substrate, 0.1 ml 0.75 M potassium succinate (or alpha ketoglutarate) was then added and the oxygen consumption measured over a period of 30 minutes. The amount of inorganic phosphate present was estimated as soon as the substrate was added, and at the end of the 30 minutes. This was done, after the protein had been precipitated with trichloracetic acid, by the method of Fiske and Subbarow (1925). The P/O ratio was then calculated.

24. Spectrophotometric measurement of mitochondrial swelling

The mitochondria were isolated in buffered sucrose solution without versene. They were suspended in this medium and kept at 0° C until used (up to 2 hours). Aliquots of the

suspension were added to cuvettes containing buffered sucrose (the standard) and various concentrations of thyroxine in buffered sucrose. The changes in optical density were read at 520 mm for 20 minutes. Enough suspension was added to give an initial optical density of from 0.3 to 0.6.

Temperature control was not possible. The temperature at which the experiments were carried out was 30° ± 2.5° C - room temperature during the evenings of the Australian summer. The temperature did not vary more than one degree during any one experiment.

III. RESULTS

1. Light microscopy of the intestinal cell

The structure of the intestinal cells of rats, chicken, lizards, toads, and carp looked similar under the microscope (see Figures 14 - 22).

The cells were elongate. They contained an oval nucleus which was usually nearer the basal end of the cell. In some nuclei the nucleolus could be seen; this was often compact and granular, but occasionally it was diffuse. In sections stained with Fast Green FCF the brush border was seen as a striated structue; in living cells it was black under phase contract illumination, but appeared structureless and transparent in bright field illumination.

The mitochondria were seen as dense, elongate, round, or oval structures. They were found at both ends of the cell, but not immediately adjacent to the brush border. The intestinal cells of toads (which had not been given food for three to four weeks) appeared to have fewer mitochondria per cell than the other animals.

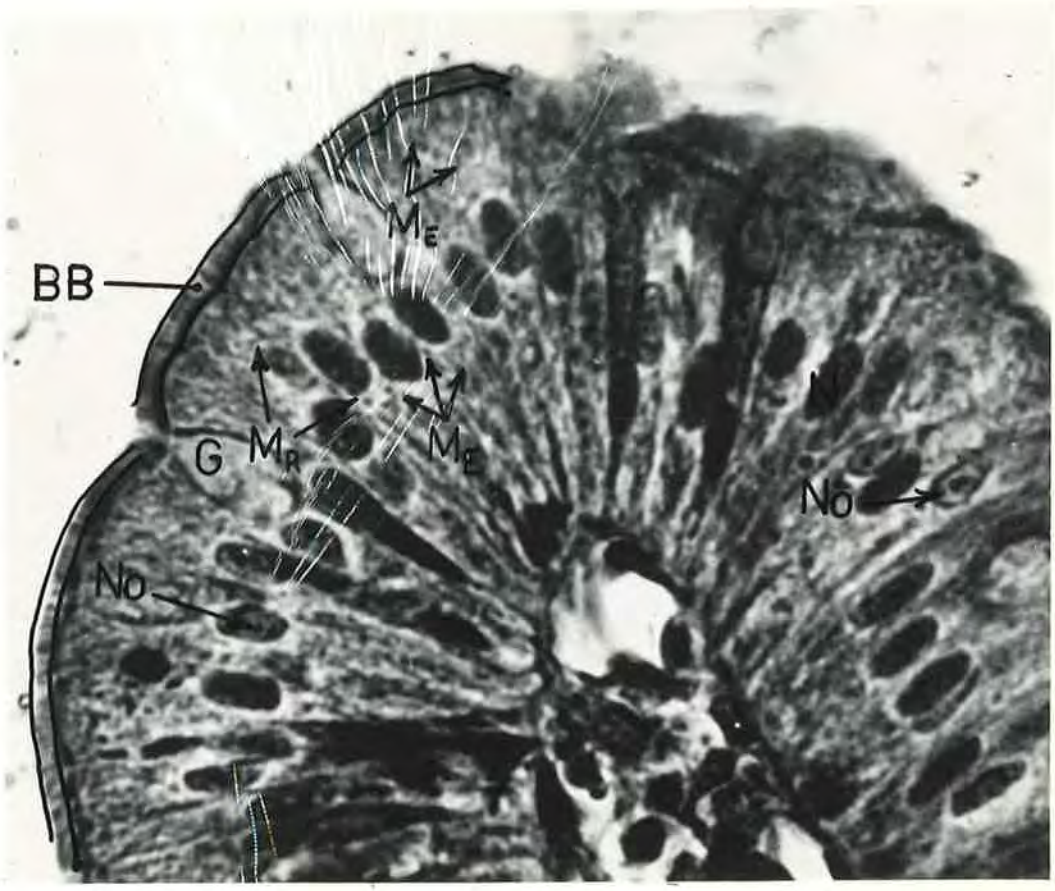


Figure 14.- A section through the apex of an intestinal villus showing columnar absorbing cells, goblet cells (G), round mitochondria (M_R), elongate mitochondria (M_E), nuclei (N), nucleoli (No), and the brush border (BB).

Animal: Gallus domesticus

Stain: Fast Green FCF

Magnification: 4,700 X

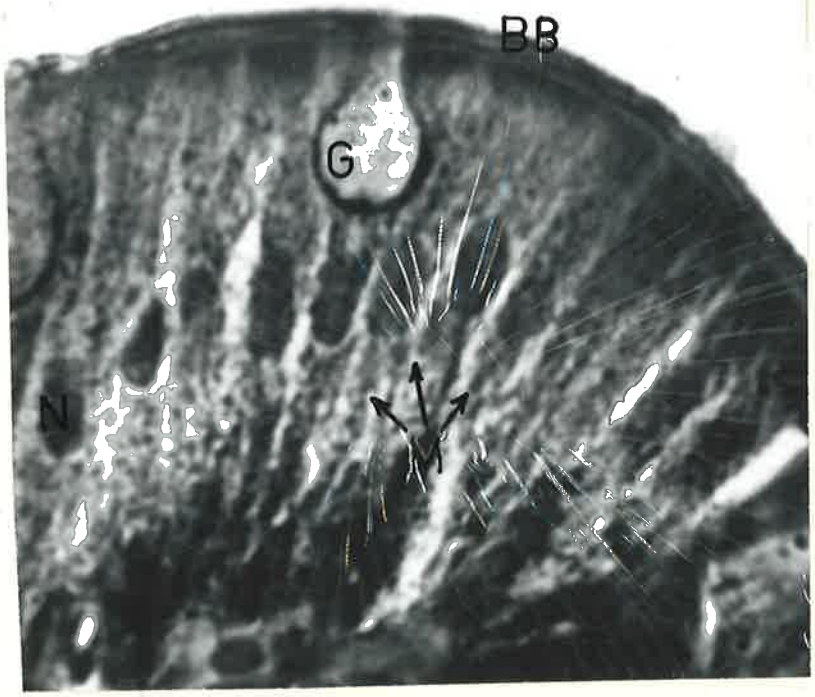


Figure 15.- A section through the apex of an intestinal villus showing columnar absorping cells, a goblet cell (G), mitochondria (N), nucleus (N), and the brush border (BB).

Animal: Carassius auratus

Stain: Fast Green FCF

Magnification: 5,500

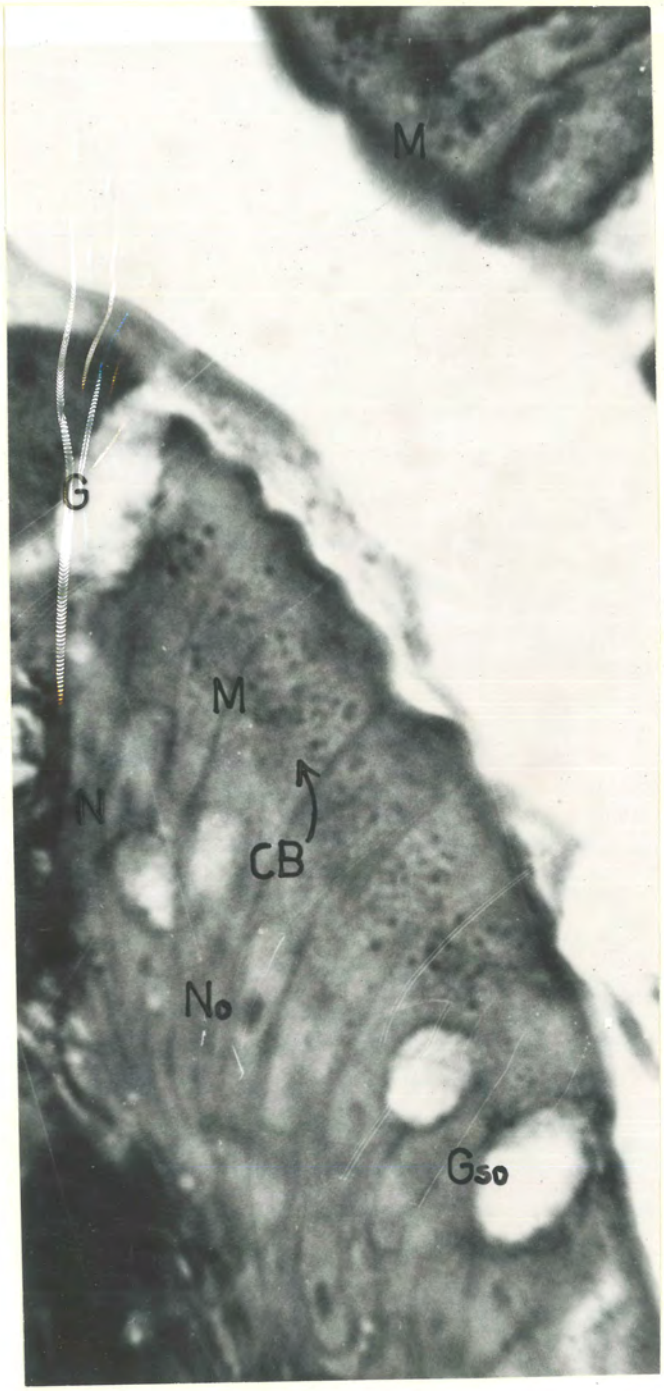


Figure 16.- A section of part of an intestinal villus showing mitochondria (M), goblet cells - sectioned through the middle (G), and obliquely sectioned (G_{SO}), nuclei (N), nucleolus (No), and cell boundaries (CB).

Animal: Bufo marinus

Stain: Fast Green FCF

Magnification: 6,150 X

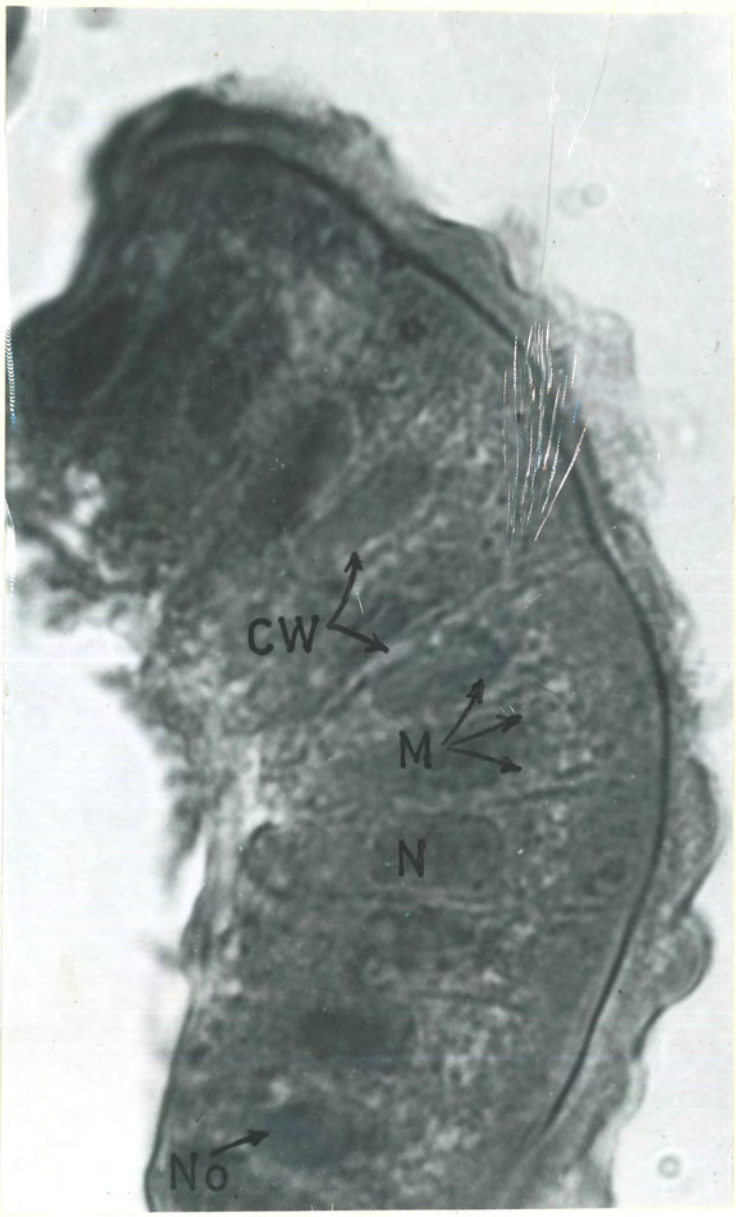


Figure 17.- A section through columnar absorbing cells from the intestine showing mitochondria (M), cell borders (CB), nuclei (N), and nucleolus (No).

Animal: Rattus norvegicus

Stain: Fast Green FCF

Magnification: 4,700 X

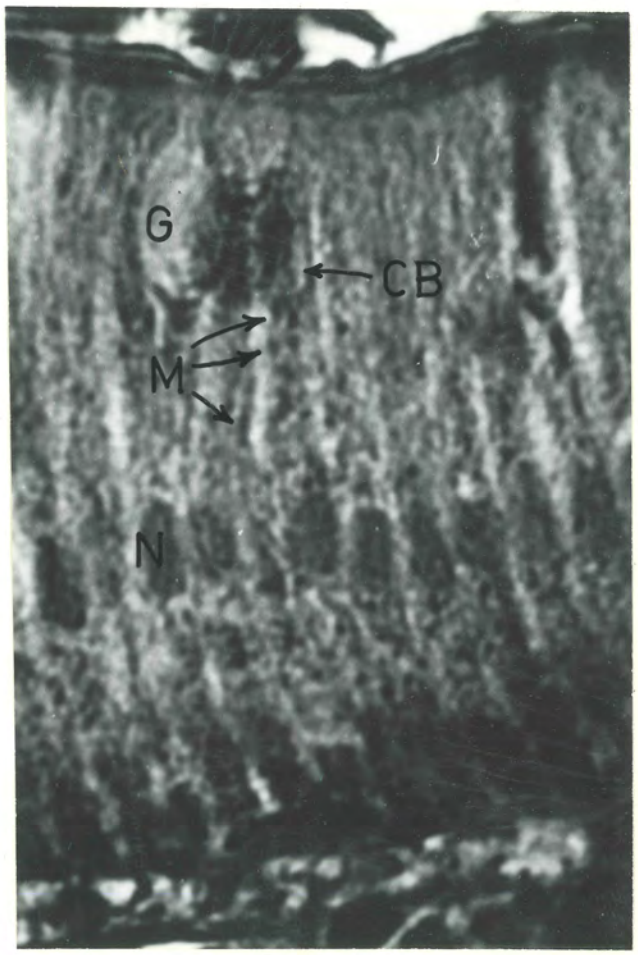


Figure 18.- Section of columnar absorbing cells showing mitochondria (M), nuclei (N), and cell borders (CB). A goblet cell (G) can also be seen.

Animal: Carassius auratus

Stain: Fast Green FCF

Magnification: 5,500 X

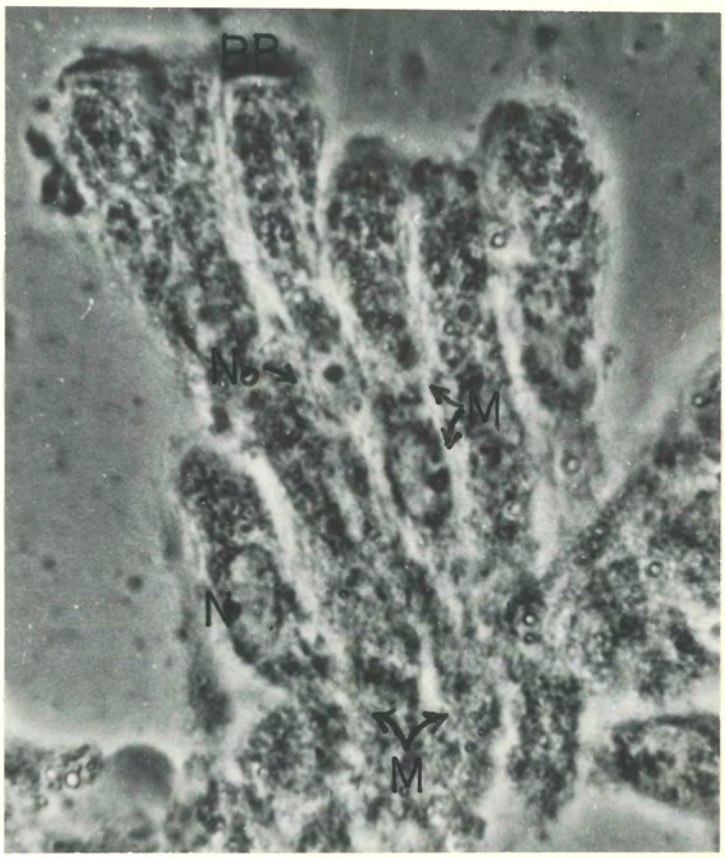


Figure 19.- A photomicrograph of living intestinal cells showing mitochondria (M), a nucleus (N), a nucleolus (No), and the brush border (BB).

Animal: Tiliqua rugosa

Treatment: Phase contrast

Magnification 4,000 X

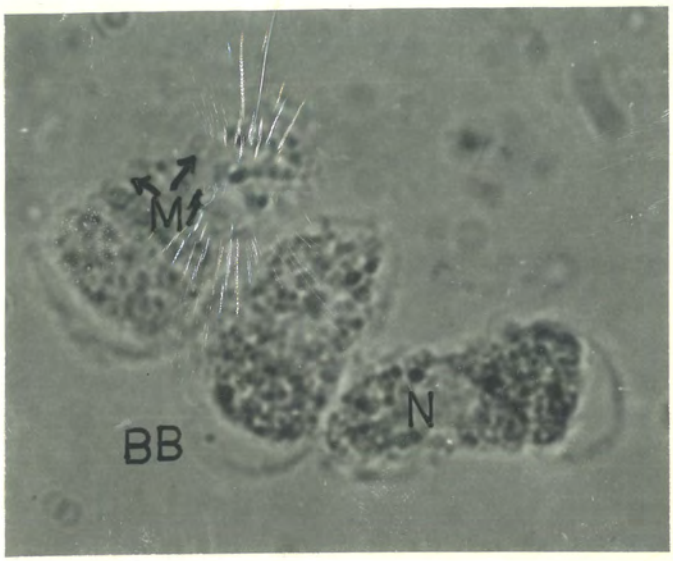


Figure 20.- A photomicrograph of living intestinal cells showing mitochondria (M), nuclei (N), and the brush border (BB).

Animal: Rattus norvegicus

Stain: Janus Green B

Magnification: 3,600 X

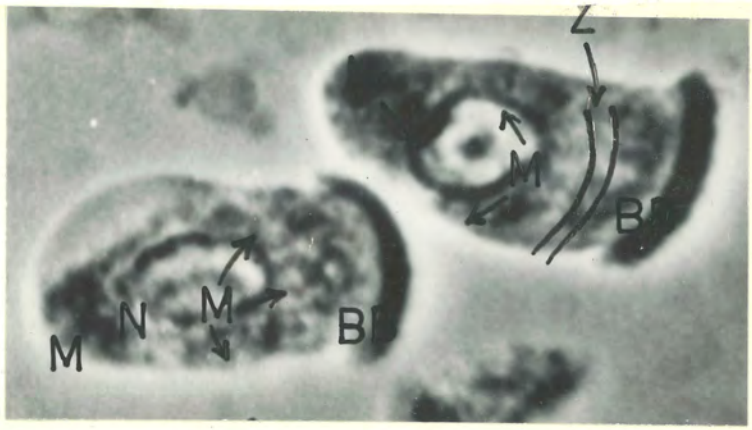


Figure 21.- A photomicrograph of living intestinal cells showing mitochondria (M), nucleus (N), nucleolus (No), brush border (BB), and an area free of mitochondria (Z).

Animal: Rattus norvegicus

Treatment: Phase contrast

Magnification: 4,000 X

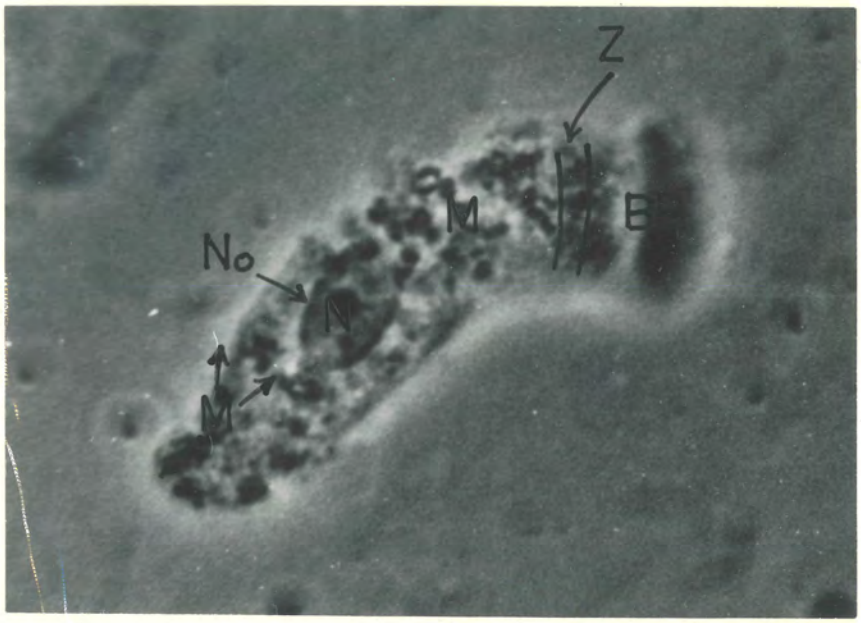


Figure 22.- A photomicrograph of a living intestinal cell showing mitochondria (M), nucleus (N), nucleolus (No), brush border (BB), and an area free of mitochondria (Z).

Animal: Rattus norvegicus

Treatment: Phase contrast

Magnification: 4,600 X

These experiments showed that, as far as could be judged with the light microscope, the intestinal cells of the species examined were similar in structure to those which have been studied previously (for references see Macklin and Macklin, 1932), although elongate mitochondria did not appear to be confined to the apical portion of the cell as some of the earlier reports had suggested. No differences could be detected between the mitochondria of the various species.

2. Experiments with whole cells

As the distribution and number of mitochondria in the intestinal cells examined looked similar to those which have been examined previously, it was decided to compare the endogenous respiration of the intestinal cells, and to find out whether this endogenous respiration was affected by the addition of substrate or changes in temperature. This was intended to be only a brief examination; the aim was simply to find out if the intestinal cells behaved as one might predict - i.e. "normally". Two species were examined: Rattus norvegicus (a homiotherm) and Tiliqua rugosa (a poikilotherm).

The QO_2 of the intestinal cells of the rat and the lizard was found to vary from 3.7 to 11.0 and from 5.8 to 8.5 respectively. The values obtained are shown in Tables 10 and 11.

It has been shown before that it is difficult to obtain reproducible values for the QO_2 of the intestinal mucosa (Dickens and Weil-Malherbe, 1941; Nakamura, Pichette, Broitman, Bezman, Zamcheck, and Vitale, 1959). There are several possible explanations for this:

(i) the physical condition of the individual animal may affect the QO_2;

(ii) a quantity of intestinal cells is difficult to

TABLE 10

OXYGEN CONSUMPTION OF THE INTESTINAL CELLS OF THE RAT

The QO_2's are expressed as ulitres oxygen consumed per mg dry weight of tissue per hour. The oxygen consumption was measured in Krebs' modified Ringer in a Warburg manometer cup at approximately 1 atmosphere pressure and at 37° C. The gas phase was air.

EXPERIMENT	- QO_2	ARITHMETIC MEAN
1	3.7	7.65
2	4.7	
3	5.3	
4	6.9	
5	7.7	
6	8.2	
7	8.7	
8	8.8	
9	9.2	
10	9.8	
11	11.0	

TABLE 11

OXYGEN CONSUMPTION OF THE INTESTINAL CELLS OF THE LIZARD

Legend as Table 10

EXPERIMENT	- QO_2	ARITHMETIC MEAN
1	5.8	7.3
2	7.2	
3	7.2	
4	7.6	
5	8.5	

replicate because the cells clump together;

(iii) the cells in the centre of a clump may contribute less toward a reaction than those on the outside;

and (iv) if the cells contain an inhibitor, accidental breakage of a few cells during an experiment would produce an unusually low QO_2.

But perhaps the most likely explanation of the variation is this. Different regions of the intestine have different QO_2's. Dickens and Weil-Malherbe (1941) found values* of 7.6 for the duodenum, 14.1 for the jejunum, and 3.0 for the ileum. Consequently, if cells from one part of the intestine happened by chance to predominate in a manometer cup, then the QO_2 of the tissue in that cup would appear to be different from the QO_2 obtained from other cups. One would expect errors of this kind to cancel one another out if the figures were averaged.

The arithmetic mean of Dickens' figures is 8.25 and that of the figures obtained in this investigation is 7.65. (Because the former were estimated in the presence of glucose and the latter without added substrate, these means are not strictly comparable, but see the Discussion, p. 135).

The arithmetic mean of the values obtained for the QO_2 of the lizard is 7.3. Statistical analysis showed that this mean was not significantly different from that of the rat. (The probability calculation is shown in the Appendix, p. 162).

The apparent variability of the QO_2 of intestinal cells made it difficult to compare accurately the rate of oxygen consumption at different temperatures. However, in both the

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*These figures are calculated for measurement of the oxygen consumption over 60 minutes.

rat and the lizard, the graph of oxygen consumption against time was linear at 30' and 37' C whereas at 45' C the initial high rate of respiration fell. This is shown in Figures 23 and 24. The oxygen consumption, summed over 1 hour, of the intestinal cells of the lizard incubated at 45' C was less than that at 37' C, whereas the oxygen consumption of the cells of the rat was greater at 45' C.

The rapid decline in the oxygen consumption of the cells incubated at 45' C is possibly the result of the derangement or partial denaturation of some enzyme/s. Rats never experience as high a temperature in their natural environment and even the tissues of a lizard basking in the noon-day sun would be unlikely to reach 45' C. This temperature, therefore, would be well outside the physiological range and some denaturation might be expected.

The fact that the cells of the rat respire relatively faster at 45' C than the cells of the lizard may be associated with the fact that rats are homiothermic and lizards poikilothermic. As the body temperature of a lizard would normally be below that of a rat, the lizard may have evolved a lower optimum temperature for maximum enzyme efficiency. It is also possible that the temperature coefficient for the denaturation of enzymes is higher in the lizard than the rat. If this is so, small increases in temperature above the optimum would have a greater effect on the respiration of the cells of the lizard than of those of the rat.

In 1889 Arrhenius formulated an equation relating the rate of chemical reactions to temperature, and he later (1907) pointed out that the rate of many life processes increases with temperature according to this equation. The equation has been used since by enzymologists to determine the "activation energy" of enzymes or groups of enzymes. The activation energy of enzymes has been shown to remain the same whether the enzyme is partially purified, or present in dead cells, or in living cells

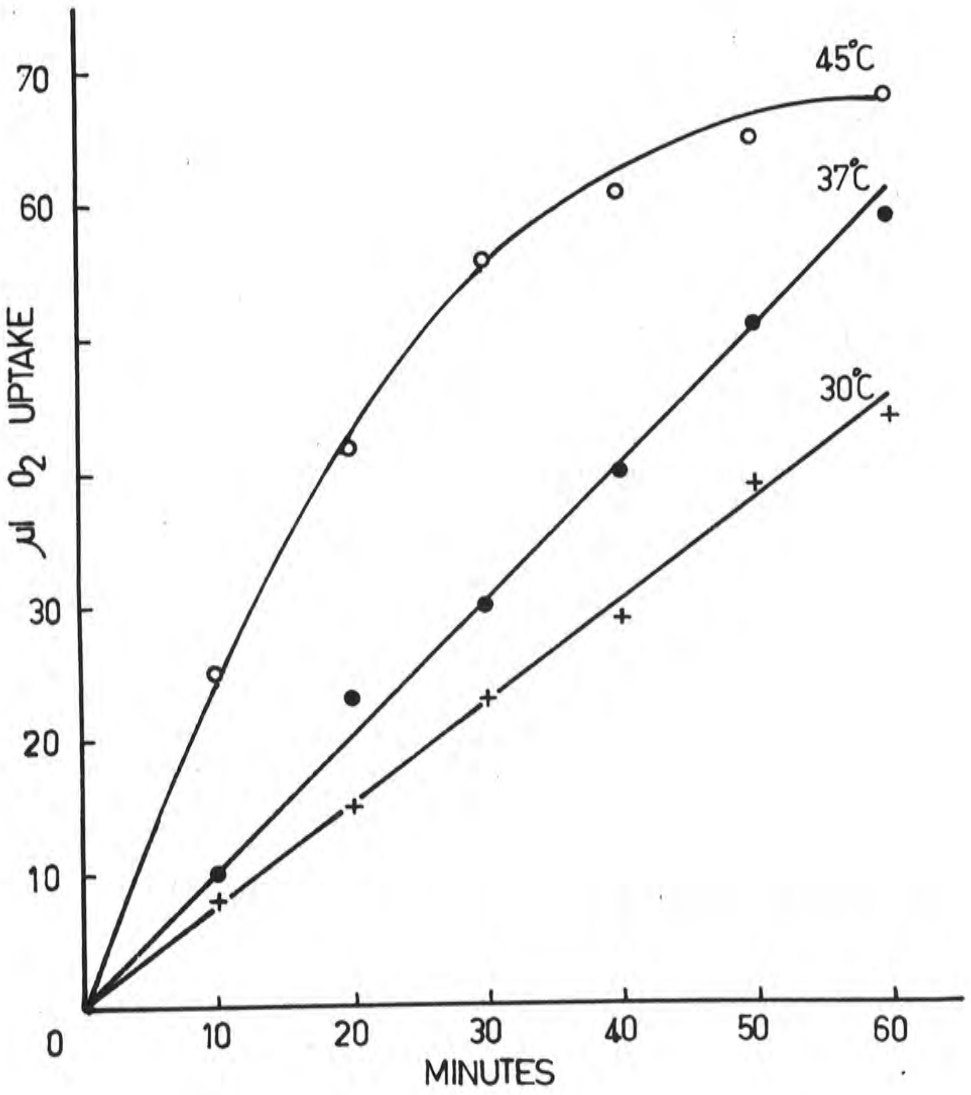

Figure 23.- The effect of temperature on the oxygen consumption of the intestinal cells of the rat incubated in Krebs' modified Ringer.

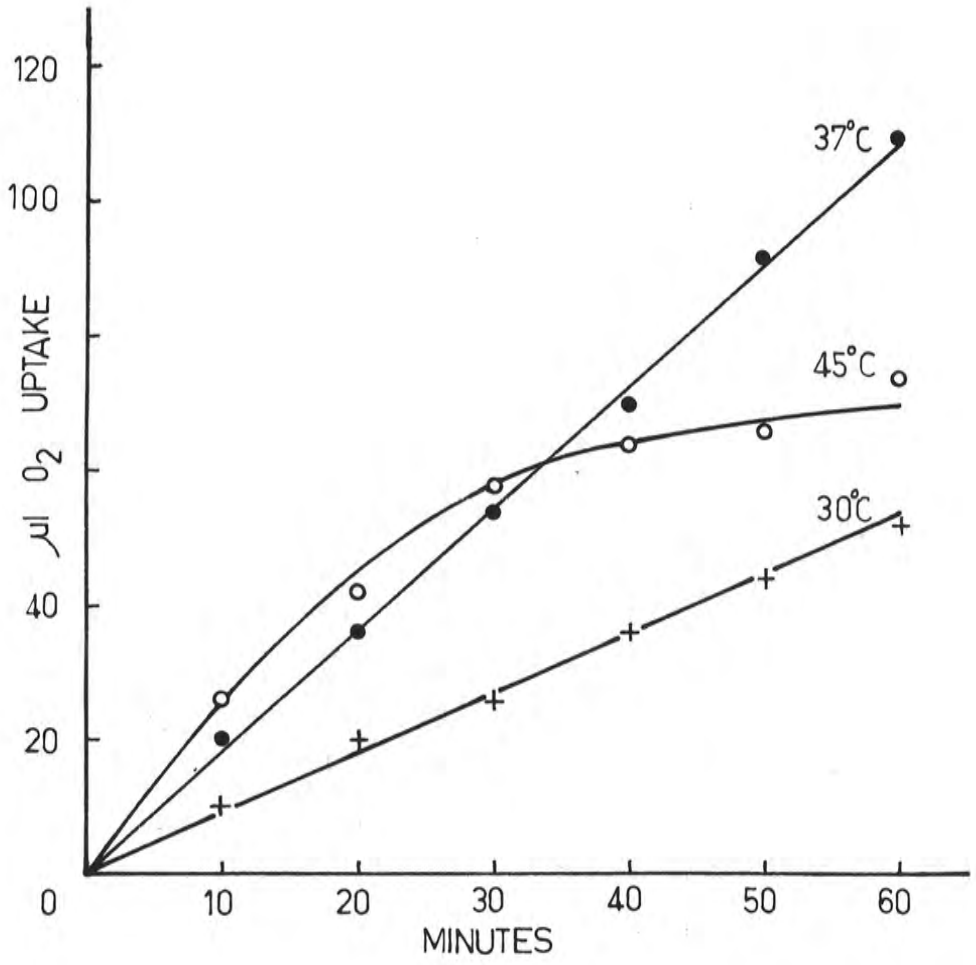

Figure 24.- The effect of temperature on the oxygen consumption of the intestinal cells of the lizard incubated in Krebs' modified Ringer.

(Sizer, 1938, 1939). In most instances analagous enzymes from different species have had similar activation energies (Bodansky, 1939; Sizer, 1943).

It was thought worth while to see whether this constant was the same for the respiration of the intestinal cells of the rat and lizard.

The differential form of the equation proposed by Arrhenius (1889) is:

$$\frac{d \ln k}{d T} = \frac{A}{R T^2}$$

where k is the rate of the reaction, T is the absolute temperature, R is the gas constant, and A is the activation energy. Integrating this equation gives:

$$\ln \frac{k_2}{k_1} = \frac{A}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

$$\text{or} \quad A = R \cdot \frac{\left(\ln \frac{k_2}{k_1}\right)}{\left(\frac{1}{T_1} - \frac{1}{T_2}\right)}$$

Converting to natural logarithms to logarithms to the base 10, the equation becomes:

$$A = 2.303R \cdot \frac{\left(\log \frac{k_2}{k_1}\right)}{\left(\frac{1}{T_1} - \frac{1}{T_2}\right)}$$

$$\text{As} \quad \log \frac{k_2}{k_1} = \log k_2 - \log k_1$$

$$\text{and} \quad R = 1.986$$

$$A = 4.58 \frac{\left(\log k_2 - \log k_1\right)}{\left(\frac{1}{T_1} - \frac{1}{T_2}\right)}$$

$\log k_2 - \log k_1$ and $\frac{1}{T_1} - \frac{1}{T_2}$ can be determined from a graph of log k against $\frac{1}{T}$ and so A, the activation energy, can be found.

Figure 25 is a graph of the logarithm of the rate of oxygen consumption of whole intestinal cells (log k) against the reciprocal of absolute temperature (1/T). It was plotted using the values obtained from the experiments on the variation of oxygen consumption with change of temperature. The values have been taken straight from the graphs of oxygen consumption against time (Figures 23 and 24, pp. 71 and 72) just before the point of inflection of the 45° C curve. Table 12 contains the values of $\log k_2 - \log k_1$ and $\frac{1}{T_1} - \frac{1}{T_2}$ determined from the graph of log k against 1/T (Figure 25).

TABLE 12

VALUES OF $\log k_2 - \log k_1$, $\frac{1}{T_1} - \frac{1}{T_2}$, AND A FOR THE OXYGEN CONSUMPTION OF THE INTESTINAL CELLS OF THE RAT AND THE LIZARD

ANIMAL	$\log k_2 - \log k_1$	$\frac{1}{T_1} - \frac{1}{T_2}$	A IN CALS/MOLE
Rat	0.40	0.00014	13,100
Lizard	0.43	0.00015	13,200

The constant, A, for the rat was calculated to be 13,100 and for the lizard 13,200.

The value of A thus appeared to be the same for both the rat and the lizard. This constant probably does not represent the activation energy for the dehydrogenation of an individual substrate, but is more likely to correspond to the activation energy of a pacemaker reaction controlling the rate of oxidation of several substrates. The constant may also be influenced by a non-enzymatic process, such as the diffusion of oxygen.

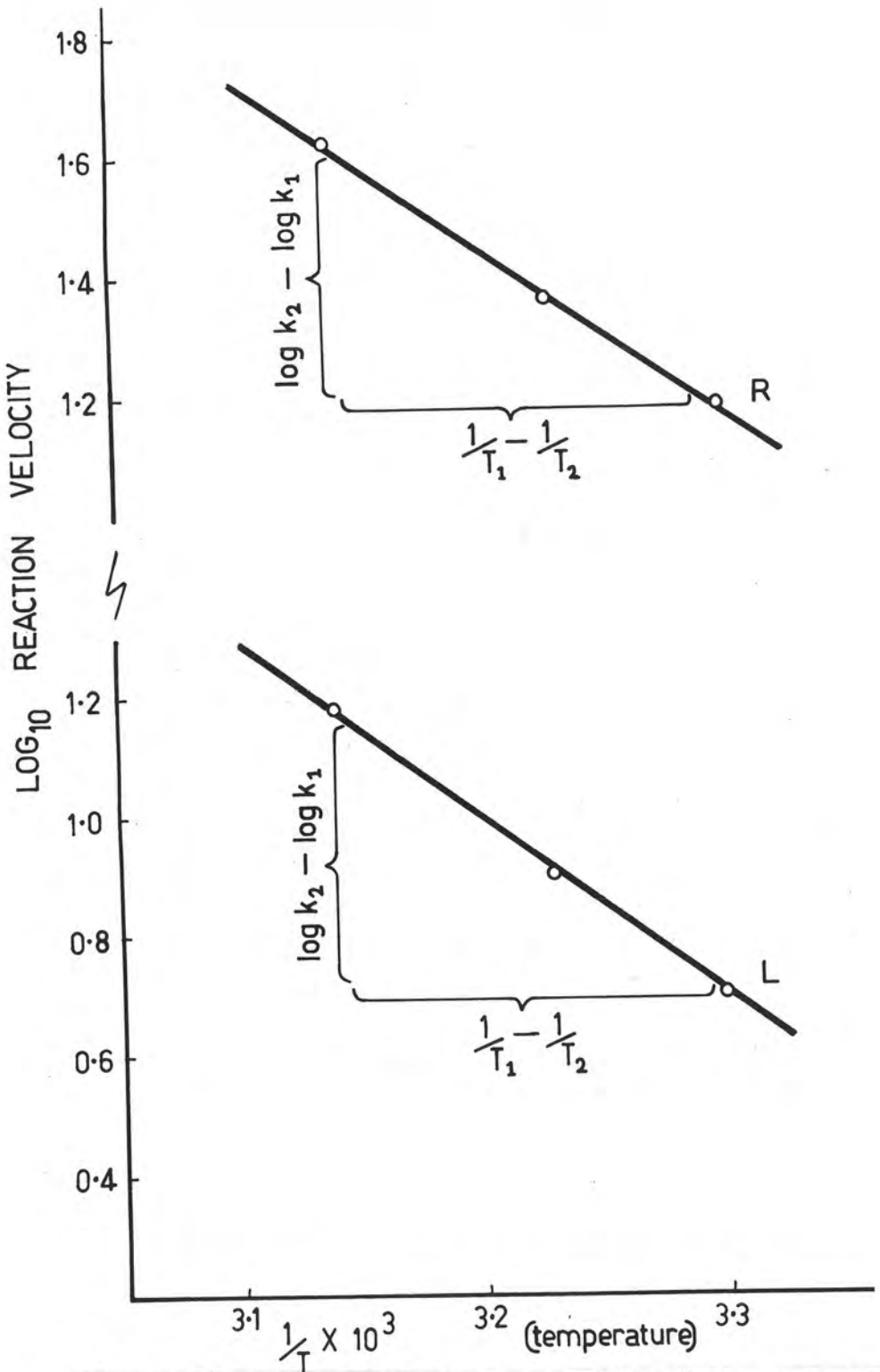

Figure 25.- Graph of the logarithim of the rate of oxygen consumption of whole intesinal cells (log k) against the reciprocal of the absolute temperature (1/T). Graph R is that for the intestinal cells of the rat, and graph L for the lizard.

The addition of succinate to a suspension of intestinal cells increased by approximately 25% the rate of endogenous respiration of the cells of both the rat and the lizard. Addition of glucose had a similar effect in the rat but not in the lizard. Neither citrate or fumarate appeared to have any effect on the endogenous respiration of the intestinal cells of either animal.

The response of the intestinal cells to some substrates and not to others may be due to differences in permeability of the cells to different substrates. Most membranes are relatively impermeable to the Krebs cycle acids, which are highly ionized (Davson and Danielli, 1943).

However, the results of the experiments with lizards can not be explained wholely by differences in permeability of the cells. One would expect glucose to be freely available (i.e. permeable) to the cell, but no stimulation of the endogenous respiration was observed when glucose was added to the respiring intestinal cells from the lizard.

3. The succinoxidase activity of the mitochondria of the rat intestine

After the brief comparison of the behaviour of whole intestinal cells from different species, experiments were designed to compare the activity of the mitochondria isolated from these cells. It was intended to use the succinoxidase activity of mitochondria isolated from the intestine of the rat as the standard reference or starting point as:

(i) succinoxidase activity is so characteristic of mitochondria that in the past this activity has been regarded practically as the basis of the definition of mitochondria;

(ii) the rat is perhaps the most widely used experimental

animal;

and (iii) the succinoxidase of many tissues of the rat have been measured, so that comparisons between the intestine and other tissues could be made easily.

However, no appreciable* succinoxidase activity could be demonstrated manometrically in mitochondria isolated from the intestinal cells of rats. Yet a similar weight of liver mitochondria prepared from the same individual showed succinoxidase activity (see Figure 26); and so did the mitochondria isolated from the intestine of lizards, chickens, and guinea pigs. This difference between the intestinal mitochondria of the rat and of other animals, and between mitochondria from liver and the intestine of rats was unexpected because succinate had stimulated the respiration of the <u>whole</u> intestinal cells of the rat. Further, no one has yet found, in any species, mitochondria that lack succinoxidase. It seemed that the apparent difference must have been due to some factor which only indirectly affected the succinoxidase activity.

The two most likely types of inhibition were thought to be:

(i) an inhibition due to a technical oversight during the preparation of the mitochondria - an oversight of some feature peculiar to the intestine of the rat - which once recognized could be obviated by altered technique;

or (ii) an inhibition caused by a naturally occuring factor operating in such a manner that succinoxidase activity could never be measured by the manometric method used.

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*1 out of 16 experiments showed approximately 10% of the activity that would have been expected had liver mitochondria been used.

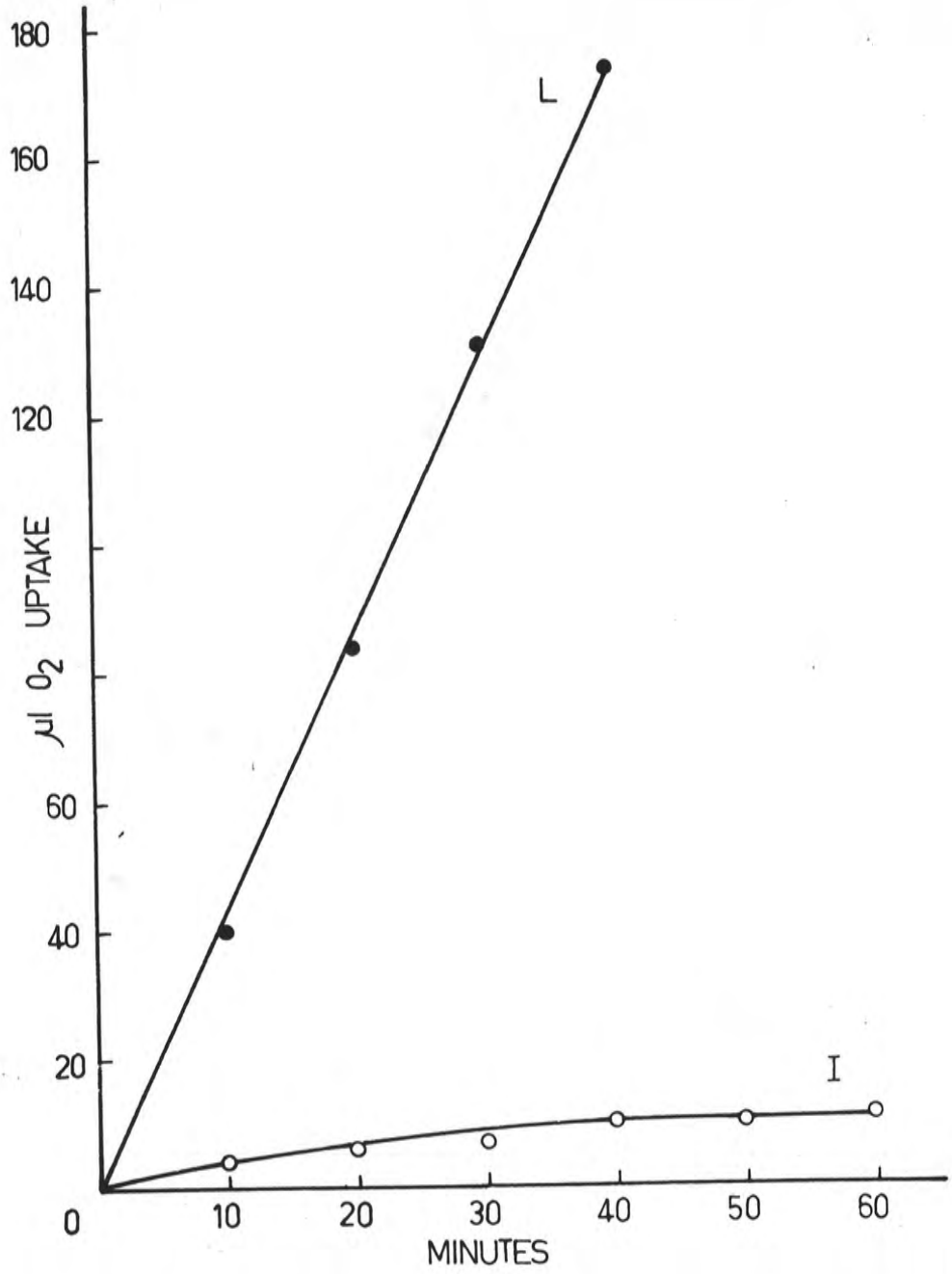

Figure 26.- The apparent difference in succinoxidase activity between the mitochondria isolated from the intestine (I) and the liver (L) of the rat. The succinoxidase activity was estimated manometrically by measuring the ulitres O_2 consumed by the mitochondria in a medium containing succinate.

The first alternative was thought more likely and the following points of experimental technique were re-examined:

(i) temperature: preparations of mitochondria from the intestine of the rat were not subject to higher temperatures - either during homogenation or centrifugation - than the other preparations. The temperature did not exceed 3° C throughout the operations. The intestinal mitochondria of rats were considered unlikely to be more heat sensitive than the other types of mitochondria which had been prepared successfully.

(ii) pH: the pH of intestinal homogenates was found to be similar to that of liver homogenates of the rat (7.2 - 7.4). The intestinal cells of the rat were thought, therefore, to be unlikely to release more acid upon homogenization than other cells.

(iii) fractionation of cells: If the intestinal mitochondria of the rat had sedimented irregularly upon centrifugation it would have been possible for an apparent loss of succinoxidase activity to occur. Consequently, the mitochondrial suspension was examined by light microscopy to see whether it contained mitochondria. Structures resembling mitochondria were found to be present. Succinoxidase could not be demonstrated in the non-mitochondrial fractions of the cell - the cell wall-nuclear precipitate and the supernatant-microsome fraction.

and (iv) rupture of cells: In order to find out if the way in which the cells were ruptured had any affect on the succinoxidase activity of the homogenate (or mitochondria), different methods of homogenizing the cell were tried. Both "vitamixing" in a

M.S.E. blendor and homogenizing with powdered glass ruptured the intestinal cells faster than the usual method of homogenizing (see p. 43), but neither gave a preparation showing more succinoxidase activity.

The oxygen consumption of the whole homogenate (measured manometrically in a medium containing succinate) was found to be only a fraction - usually less than 1/5, but the homogenates had an oxygen uptake even more variable than that of the whole cells - of that obtained with an equal weight of intact cells. Whatever type of homogenization was used, the more cells that were broken, the lower was the succinoxidase activity.

When intestinal homogenates were added to homogenates of liver cells the total oxygen consumption was less than expected. This is shown graphically in Figure 27. This discovery suggested that the intestinal homogenate contained an inhibitor of succinoxidase.

During these experiments it had been noticed that the intestinal homogenates of the rat (and the mitochondrial suspensions prepared from them) appeared to contain a considerable amount of mucus. When these intestinal homogenates were left to stand for 15 minutes at room temperature thay became very viscus indeed compared to homogenates prepared from the liver of rats or from the intestine of lizards or chickens. Even at 0° C the intestinal homogenates of rats were viscus, and higher than usual centrifugal forces were required to obtain firmly packed precipitates.

It was thought that this mucus might be inhibiting succinoxidase in a physical manner - a blanketing action preventing gaseous exchange. Consequently, attempts were made to remove the mucus. The most efficient way to do

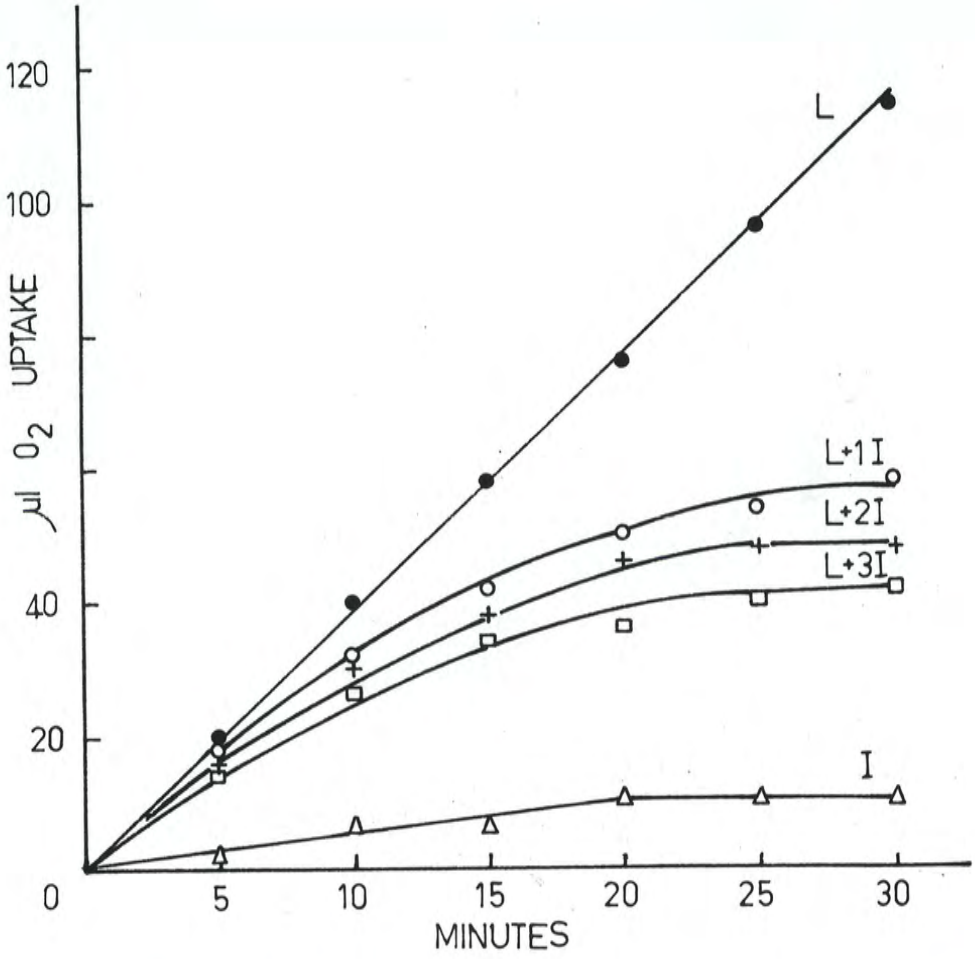

Figure 27.- The effect of an intestinal homogenate from the rat on the oxygen consumption of a liver homogenate from the same animal. L represents the oxygen consumption of a liver homogenate, and I the oxygen consumption of an intestinal homogenate. L + I, L + 2I, and L + 3I represent the oxygen consumption of a 1:1, a 1:2, and a 1:3 mixture of liver and intestinal homogenates. The oxygen consumption was measured in a medium containing succinate.

this would have been to have broken it down with enzymes. Trypsin, chymotrypsin, and papain have been shown to reduce the viscosity of duodenal mucus (Hartiala and Grossman, 1952). But it was essential that the enzymatic activity of the homogenate should be preserved, and so more delicate methods had to be discovered.

The first method tried consisted of persuading the intestine to secrete most of its mucus before the cells were isolated. A warm salt solution (0.9% NaCl buffered with 2% phosphate buffer to pH 7.4 at 37° C) containing 5% eugenol (4 allyl, 2 methoxy phenol) was used to wash out the intestine instead of the ice-cold sucrose solution that had hitherto been used. The reasons for choosing a solution of this composition were:

(i) the secretion of mucus has been found to be a function of temperature (Best and Taylor, 1955);

(ii) the shock of the ice-cold sucrose solution might have caused the living mucosa to contract and so trap mucus previously secreted at the bottom of the spaces between the villi. This trapped mucus would be removed with the intestinal lining and would be fully dispersed only after the homogenization process;

(iii) a salt instead of a sucrose solution was used because the physiological release of mucus would be more likely to be stimulated by an ionic rather than a non-ionic solution;

and (iv) Hollander and Lauber (1948) found that eugenol stimulated the secretion of the gastric mucus of dogs more than any other substance that they tried.

The use of the modified irrigation medium, however, did not prevent "gelling" of the intestinal homogenates of the rat and no improvement in oxygen uptake was obtained.

After the homogenate was considerably diluted (1 part in 50 and 1 part in 100) the mucus was less noticable, but the mitochondria prepared from such homogenates showed no more activity than before.

In 1952, Hartiala and Grossman reported that cysteine and glutathione accelerated the rate of decline of the viscosity of mucus from the duodenum of dogs, producing an effect comparable with that of proteolytic enzymes. When mitochondria were isolated from the intestine of the rat using an isolation medium containing 5 mg/ml cysteine, less mucus appeared to be present and more firmly packed precipitates were obtained. At first it seemed that this proceedure had allowed some succinoxidase activity to be demonstrated. However, it was found that the oxygen uptake observed was not due to the oxidation of succinate, but to the oxidation of residual cysteine. Originally it was thought that the cysteine was reducing the cytochrome c in the incubation medium, and the cytochrome was then being re-oxidized by cytochrome oxidase. However, later results (see pp. 93 to 94) made this explanation improbable, and it now seems that the cysteine must have been oxidized by metal ions in the incubation medium.

The results of this series of experiments showed that:

(i) the inhibition of succinoxidase activity in homogenates and mitochondrial suspensions prepared from the intestine of the rat was not caused by any of the more obvious errors in technique that might have occurred;

and (ii) the mucus present was not inhibiting succinoxidase to any extent as: (a) the experiments with cysteine showed that oxygen uptakes could occur in the presence of mucus; and (b) even when most of the mucus was

removed, as in the dilution experiments, no succinoxidase activity could be detected.

Thus, the second possibility mentioned above (p. 77) remained - that the inhibition was caused by a naturally occurring factor in such a manner that succinoxidase could never be measured by the manometric method used. This possibility was tested by using a cytochemical method (Seligman and Rutenberg, 1951) to detect the presence of succinic dehydrogenase. Suspensions of whole cells, frozen sections of cells, and homogenates prepared from the intestine of the rat, reduced 2:3:5 triphenyl tetrazolium chloride in the presence or absence of succinate. Figures 28 and 29 show reduced tetrazolium in the intestinal cells. The intestinal mitochondria of the rat, however, only reduced the dye in the presence of succinate.

These results showed that the mitochondria isolated from the intestine of the rat do contain a succinic dehydrogenase. As whole intestinal cells, and sections and homogenates of these cells, reduce the dye in the absence of succinate there must be sufficient endogenous substrate present in these preparations to allow the reaction to occur; this endogenous substrate probably includes succinate among other compounds.

Having found that succinic dehydrogenase was present in the intestinal cells of rats, it was assumed that these cells upon homogenization must release an inhibitor which affects some part of succinoxidase between succinic dehydrogenase and oxygen. The next experiments were designed to find out something about the size and lability of this inhibitor.

Intestinal homogenates which had been placed in a boiling water bath for three minutes inhibited liver homogenates just as actively as unboiled intestinal homogenates. Intestinal

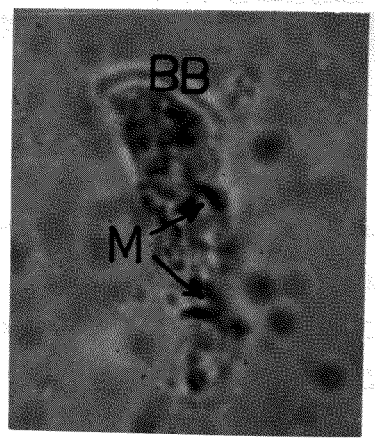

Figure 28.- An intestinal cell stained with 2:3:5 triphenyl tetrazolium showing rod-shaped areas of succinoxidase activity, presumably mitochondria (M). The brush border (BB) can also be seen.

Animal: Rattus norvegicus

Magnification: 1,750 X

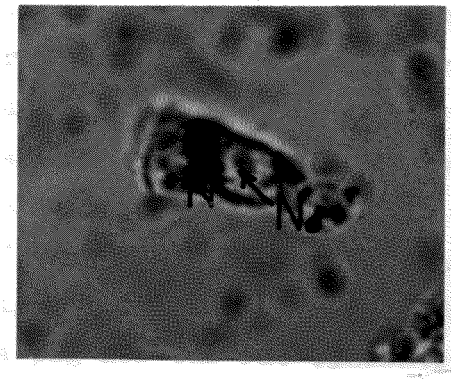

Figure 29.- An intestinal cell stained with 2:3:5 triphenyl tetrazolium showing the nucleus (N) and nucleolus (No) around which can be seen the densely-staining areas of succinoxidase activity (mitochondria).

Animal: Rattus norvegicus

Magnification: 1,750 X

homogenates which had been dialysed for 24 hours at 3° C and acid-treated homogenates - homogenates which had been shaken with HCl to a final concentration of 2 N, dialysed for 24 hours, and the pH finally adjusted to 7.4 - also inhibited liver homogenates.

The inhibitory factor then was not dialysable; nor was it a molecule particularly affected by heat or by incubation in an acid medium. As one would expect most proteins to be denatured by boiling, it seemed likely that the inhibitory factor was either a polysaccharide or a fat. And as, on the whole, polysaccharides are more easily broken down in acid than fats, it was decided to work on the assumption that the inhibitor was a fat.

Equal wet weights of intestinal mitochondria from various animals were shaken with isooctane. The isooctane was examined by spectrophotometry to find if there were any unsaturated fatty acids present. Isooctane that had been shaken with intestinal mitochondria from the rat contained approximately three times as much unsaturated fatty acid as did that in which had been shaken the same amount of mitochondria isolated from chicken, guinea pigs, lizards, or toads, or as the mitochondria isolated from the liver of rats. The results of one of these experiments is shown in Figure 30. Neither the intestinal mitochondria of the guinea pig nor the rat exhibited succin-oxidase activity after the treatment with isooctane.

When the isooctane extract obtained from the intestinal mitochondria of the rat was evaporated by passing a stream of air at room temperature across the surface of the liquid, a pale yellow sludge remained. The sludge was shown by paper chromatography to contain compounds with R_f values similar to those of oleic and linoleic acids (see Figure 31). Approximately

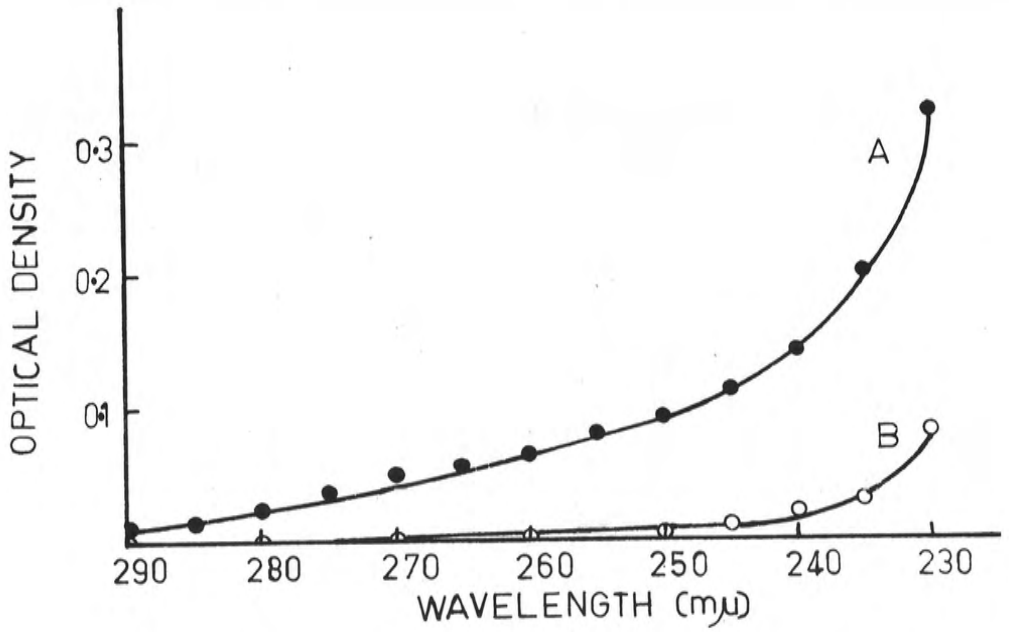

Figure 30.- The optical density of an isooctane extract of intestinal mitochondria isolated from the rat, A, and from the guinea pig, B.

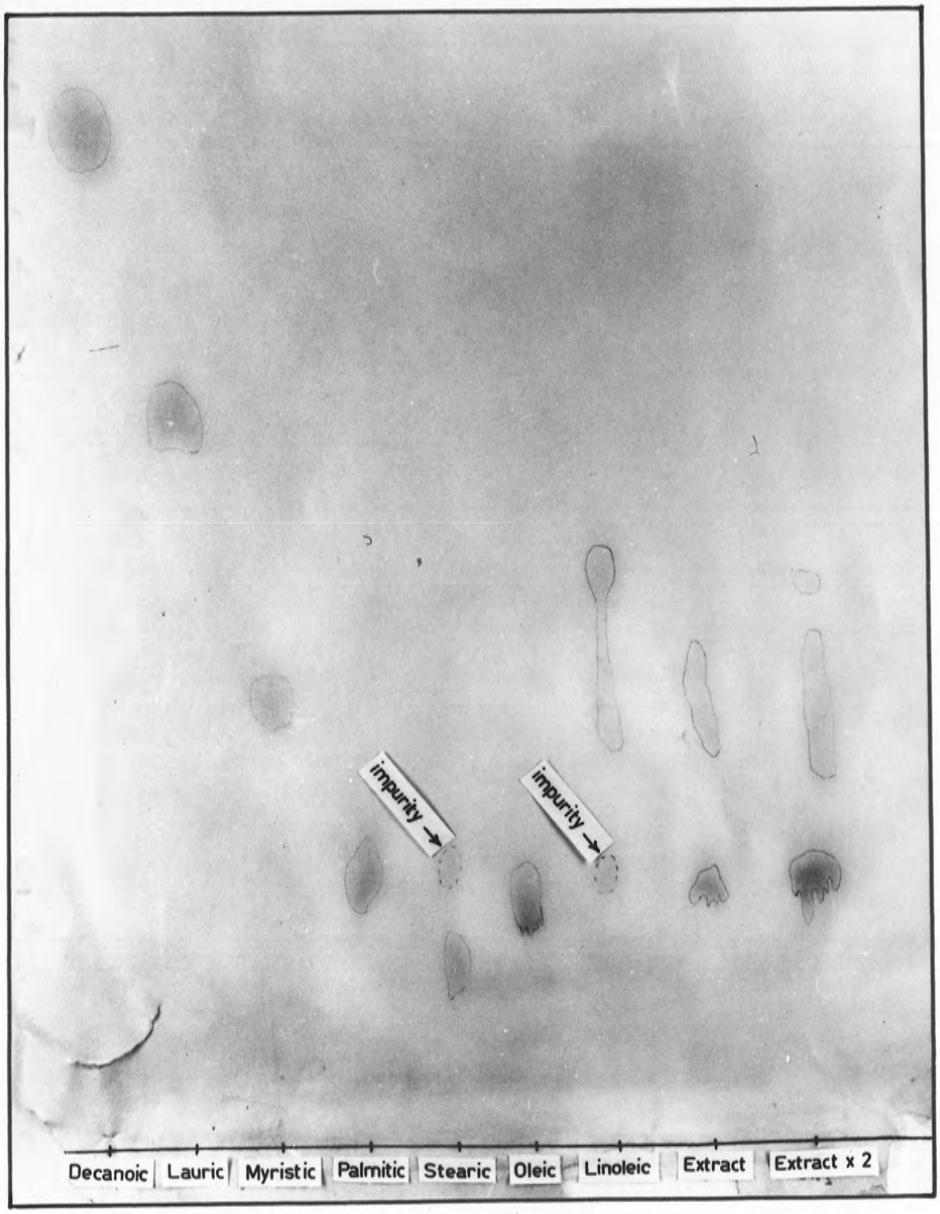

Figure 31.- A chromatogram of long chain fatty acids and of the material extracted from the intestine of the rat. The fatty acids used as standards were of "laboratory reagent" quality and contained some impurities. The method of Kaufman and Nitsch (1954) was used which produces pinkish-brown spots on a pink background. The paper becomes very fragile as it is soaked in acetic acid and paraffin before a lengthy developing process.

twice as much oleic as linoleic acid appeared to be present. Larger amounts of the sludge could be obtained by shaking whole homogenates with isooctane: similar compounds were found to be present.

When the fatty acids isolated from a known weight of rat intestinal cells were dissolved in phosphate buffer, pH 7.4, and added to mitochondria isolated from an equivalent weight of cells from the intestine of the guinea pig, the succinoxidase activity of the mitochondria was inhibited by up to 95%. But when only 1/10 of this amount of inhibitor was added to the system, an increase in oxygen uptake was observed. This is shown in Figure 32. Similar results were obtained on adding the inhibitor to mitochondria isolated from the intestine of chicken and lizards. The isooctane extract also inhibited the oxygen uptake of homogenates of the intestinal cells of guinea pigs, chicken, and lizards.

A decrease in succinoxidase activity could also be produced by adding commercially manufactured oleic acid (of laboratory reagent purity) to a mitochondrial suspension. The oleic acid was converted to a neutral solution of sodium oleate before it was added. The results of adding sodium oleate to intestinal mitochondria prepared from the guinea pig is shown in Figure 33. Similar results were obtained by adding sodium oleate to intestinal mitochondria isolated from the lizard. The amount of oleic acid necessary to inhibit the oxygen uptake by 95% was approximately 0.005 ml sodium oleate per mg mitochondrial nitrogen. The addition of 0.0005 ml sodium oleate per mg mitochondrial nitrogen did not increase the oxygen uptake but inhibited it by approximately 50%.

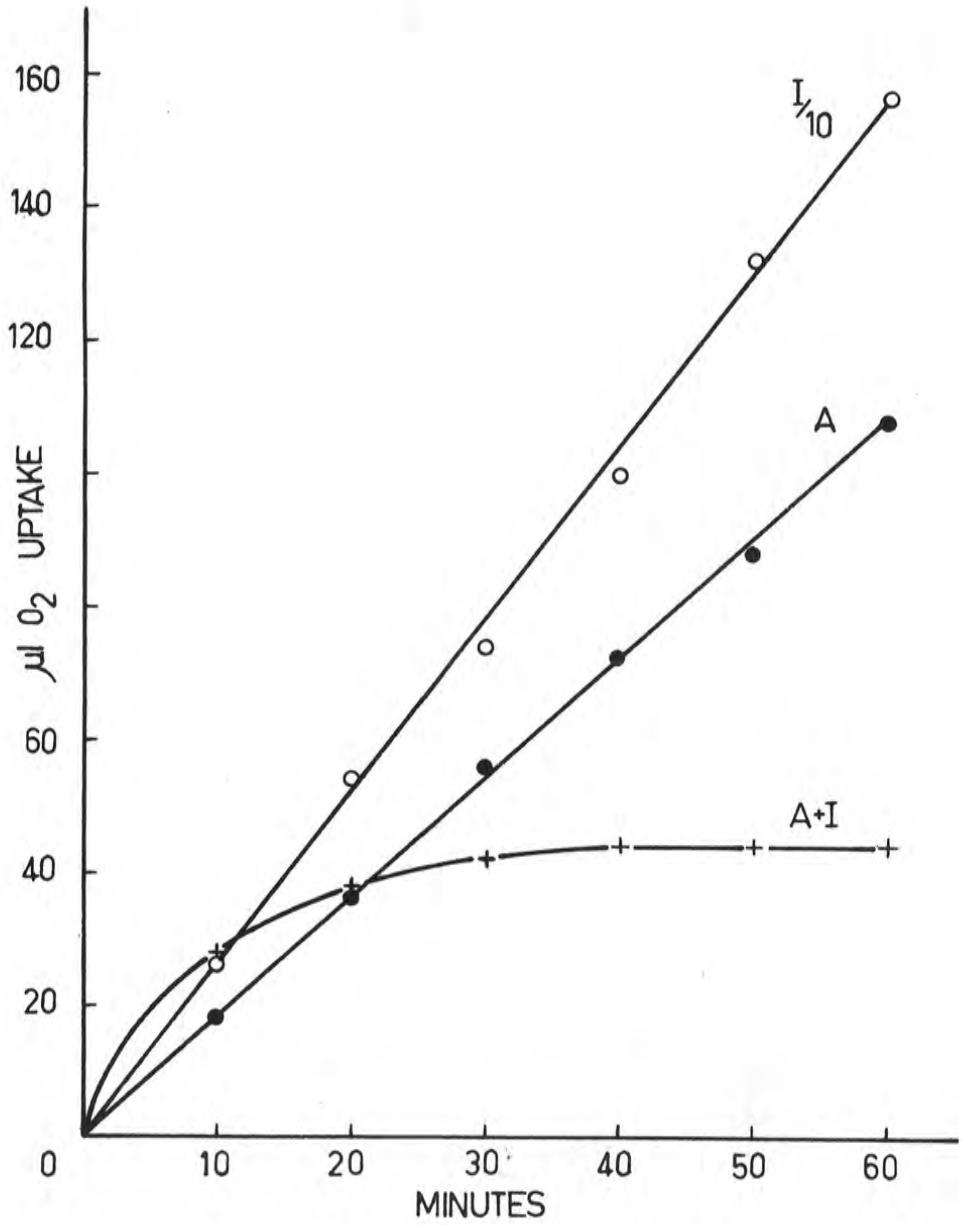

Figure 32.- The effect of the inhibitor isolated from the intestine of the rat on the succinoxidase activity of the intestinal mitochondria of the guinea pig. A = no inhibitor added; A + I = addition of an equivalent amount (see p.89) of inhibitor; I/10 = addition of 1/10 of the equivalent amount of inhibitor. The succinoxidase activity was estimated manometrically by measuring the ulitres O_2 consumed by the mitochondria in a medium containing succinate.

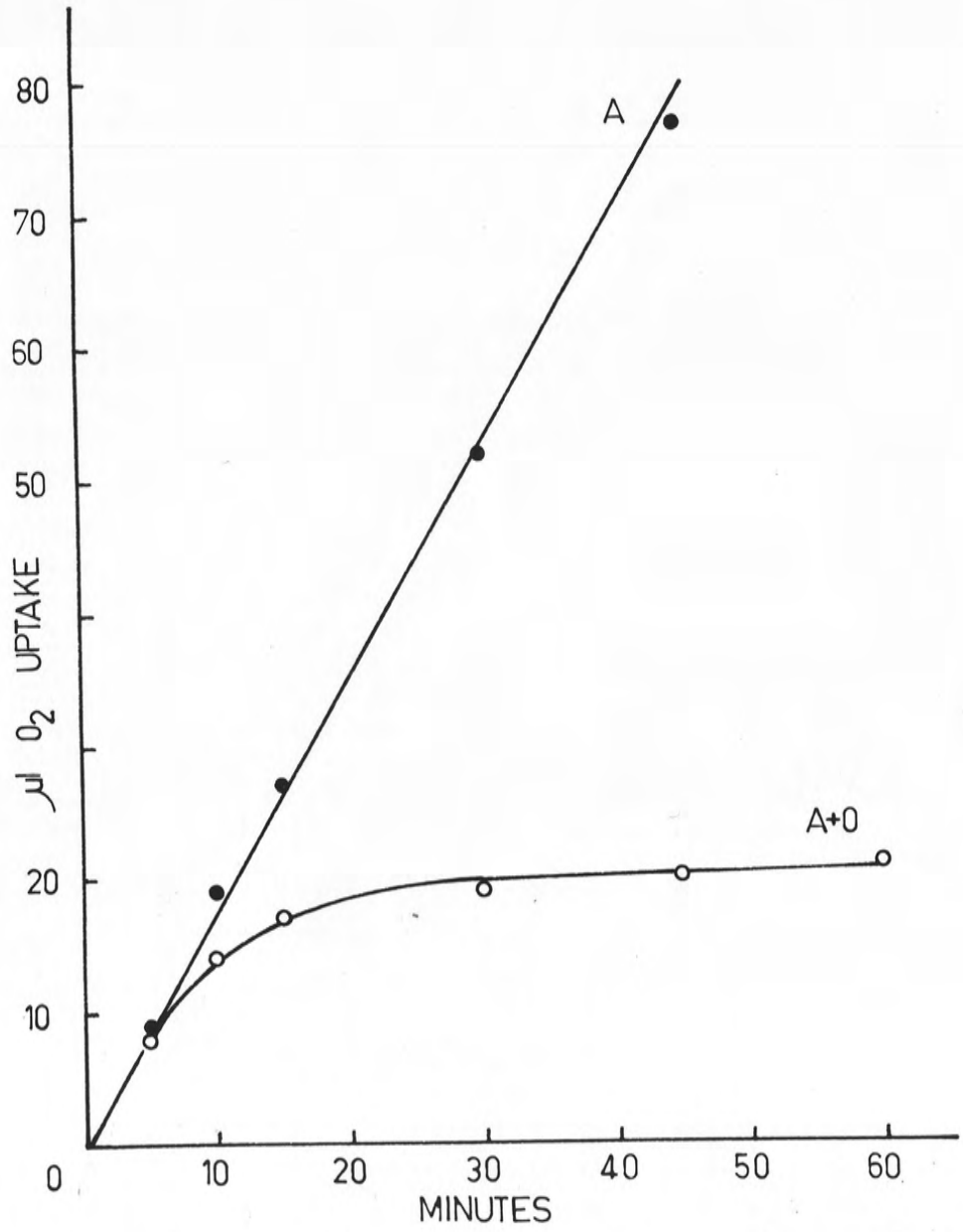

Figure 33.- The effect of sodium oleate on the succinoxidase activity of mitochondria isolated from the intestine of the guinea pig. A = oxygen consumption of unsupplemented mitochondria; A + O = oxygen consumption of mitochondria plus 0.005 ml sodium oleate per mg mitochondrial nitrogen. The succinoxidase activity was estimated manometrically by measuring the ulitres O_2 consumed by the mitochondria in a medium containing succinate.

The increase in oxygen uptake observed on adding small amounts of the natural inhibitor to mitochondria may have been due to the fatty acids being oxidised. But other explanations are just as pausible. For example:

(i) fatty acids are known to uncouple oxidation from phosphorylation in the mitochondria isolated from the kidney and brain of rats (Scholefield, 1956) and uncoupling of oxidative phosphorylation is known to result in increased oxidation rates (Sickevitz and Potter, 1953);

or (ii) small amounts of oleate have been shown to cause the liver mitochondria of rats to swell (Lehninger and Remmert, 1959), and swollen mitochondria may be more permeable to substrate or product, thus allowing an enzyme reaction of occur more quickly;

or (iii) small amounts of C_6 - C_{16} fatty acids increase the rate of those reactions in which the phosphate of ATP is exchanged (Ahmed and Scholefield, 1960), and it is possible that this might increase the oxygen uptake of a system;

or (iv) during the isooctane extraction, coenzyme Q might have been extracted as well as fatty acids. If this were so, coenzyme Q might have stimulated the oxygen uptake before the fatty acids were present in sufficient quantity to inhibit the reaction.

Small quantities of "laboratory reagent" fatty acid did not increase the oxygen uptake. Whether small enought amounts of fatty acid were used, or whether the laboratory reagent is unable to stimulate oxygen uptake, is not known. The latter hypothesis is consistent with explanation (iv) above.

In a paper that arrived in Australia at the time when the last of these experiments were being carried out, Nakamura and his co-workers (Nakamura, Pichette, Broitman, Bezman, Zamcheck, and Vitale, 1959) reported that certain fatty acids, especially linoleic, oleic and stearic acids, inhibited succinoxidase in homogenates prepared from the intestine of the rat. The inhibitory effect of oleic acid on a succinoxidase preparation from ox heart has also been reported (Edwards and Ball, 1954).

The component of the succinoxidase system which donates its electrons to 2:3:5 triphenyl tetrazolium was recently shown to be cytochrome oxidase (Nachlas, Morgulies, and Seligman, 1960). The reduction of tetrazolium by mitochondria isolated from the intestine of the rat (see p. 84) therefore suggests that the fatty acid inhibitor in the intestinal cells of the rat inhibits the succinoxidase system between cytochrome oxidase and oxygen.

When the cytochrome oxidase activity of mitochondria isolated from the intestine of the rat was compared manometrically with that of mitochondria isolated from the intestine of the lizard, it was found that the latter had at least ten times more activity. Table 13 shows the values obtained.

TABLE 13

COMPARISON OF THE CYTOCHROME OXIDASE ACTIVITY OF THE INTESTINAL MITOCHONDRIA OF THE RAT AND THE LIZARD

The cytochrome oxidase activity was measured manometrically by the method of Umbreit (Umbreit, Burris, and Stauffer, 1957).

Animal from which mitochondria taken	ul O_2 uptake	mg dry wt./ml mit. suspension	Specific activity
rat	5	71	7
lizard	47	62	76

Single experiments with the intestinal mitochondria isolated from the guinea pig and from the toad showed that the cytochrome oxidase activity of these mitochondria was also more than ten times that of the intestinal mitochondria of the rat.

These results confirmed the hypothesis that the inhibitor of succinoxidase released upon the homogenization of the intestinal cells of the rat acts at the level of cytochrome oxidase.

4. The succinoxidase activity of the intestinal mitochondria of some other vertebrates

The succinoxidase activity of mitochondria isolated from the intestine of various vertebrates is shown in Table 14.

TABLE 14

THE $QO_2(N)$ IN THE PRESENCE OF SUCCINATE OF THE INTESTINAL MITOCHONDRIA OF VARIOUS VERTEBRATES

The components of the medium in which the oxygen consumption was measured is given on p. 44.

EXPERIMENT	CARP	TOAD	LIZARD	CHICKEN	GUINEA PIG	RAT
1	307	172	405	447	372	15 in 16 experiments
2	333	223	451	518	403	
3	361	240	462	522	428	
4	380	-	501	570	448	
5	398	-	518	571	465	
Arithmetic mean	356	212	467	526	420	-

These figures have been statistically analysed (in the Appendix, p. 163) and the estimate of the values of the QO_2 s within 95% confidence limits is shown in Table 15.

TABLE 15

ESTIMATE OF THE VALUES OF THE $QO_2(N)$s WITHIN 95% CONFIDENCE LIMITS

MITOCHONDRIA FROM INTESTINE OF	$QO_2(N)$
carp	343 - 369
toad	187 - 237
lizard	454 - 480
chicken	513 - 539
guinea pig	407 - 433

The difference between the QO_2s of the various species was shown to be highly significant (Appendix, p. 164).

The supernatant fraction did not consume oxygen in the presence of succinate. Neither mitochondria nor supernatant showed any oxygen uptake in the absence of succinate. Addition of supernatant (0.1 ml) to mitochondria (0.3 ml) had no effect on oxygen consumption in the absence of succinate; in the presence of succinate, however, the oxygen uptake of the combined system was approximately 5% more than the sum of the parts. The $QO_2(N)$ values were calculated from results obtained with mitochondria unsupplemented with supernatant.

In order to get some idea of the succinoxidase activity of mitochondria isolated from the intestine of the rat, the succinic dehydrogenase activity of these mitochondria was compared spectrophometrically with that of mitochondria isolated from the intestine of guinea pigs and from the liver of rats. The results are shown in Table 16.

TABLE 16

SUCCINIC DEHYDROGENASE ACTIVITY OF THE INTESTINAL MITOCHONDRIA OF THE RAT AND THE GUINEA PIG, AND OF THE LIVER MITOCHONDRIA OF THE RAT

The succinic dehydrogenase activity was estimated by measuring the rate of reduction of 2:6 dichlorphenol indophenol by the mitochondria in the presence of succinate.

MITOCHONDRIA ISOLATED FROM	UNITS OF ENZYME ACTIVITY*	ml MITOCHONDRIAL SUSPENSION	DRY WT. OF 1.0 ml MITOCHONDRIAL SUSPENSION	SPECIFIC ACTIVITY
rat liver	0.067	0.05	0.0715	187
rat intestine	0.069	0.1	0.0656	104
guinea pig intestine	0.064	0.1	0.0632	101

*Units of enzyme activity are defined as a change in optical density of 1.0 in 5 minutes.

The succinic dehydrogenase activity of the intestinal mitochondria of the rat and the guinea pig was approximately equal; the mitochondria from rat liver were nearly twice as active.

5. Electron microscopy

After the experiments on the succinoxidase activity of isolated intestinal mitochondria had been completed, it became important to know whether the intestinal mitochondria had been damaged during the isolation process. The mitochondria were examined under the electron microscope for changes in ultra-structure that might indicate changes in physiological activity. Mitochondria inside the intestinal cell were examined first, and then isolated mitochondria.

Electron microscopy of the intestinal cells showed that the mitochondria in these cells were similar in shape and size to those in other types of cells.

The intestinal mitochondria were usually oval or elongate. Occasionally, mitochondria were seen which appeared to be round; these may have been elongate mitochondria sectioned obliquely. The round mitochondria did not predominate in any part of the cell.

The mitochondria were usually between 1.0 and 1.6 μ long. This measurement is the length of a section through a mitochondrion and the mitochondrion itself may be longer. The longest mitochondrion seen was 2.4 μ long. The average width of the mitochondria was 0.3 μ. There was no marked difference between the measurements of the intestinal mitochondria of the various species studied.

The intestinal mitochondria were composed of a double outer membrane enclosing a number of double inner membranes, or cristae mitochondrales. The cristae were usually situated in a transverse position; occasionally, however, they ran obliquely or even along the length of a mitochondrion. In some mitochondria the cristae mitochondrales were seen to be continuous with the inner layer of the outer membrane. Around the cristae mitochondrales was a structureless material which appeared mid-grey under the electron microscope. In this ground substance or matrix dense granules up to 300 A' in diameter were frequently found in all the species examined, but the granules were not so numerous as to be found in every mitochondrial section.

A selection of electron micrographs of mitochondria in the whole intestinal cell is shown in Figures 34 to 44 (pp. 98 to 105).

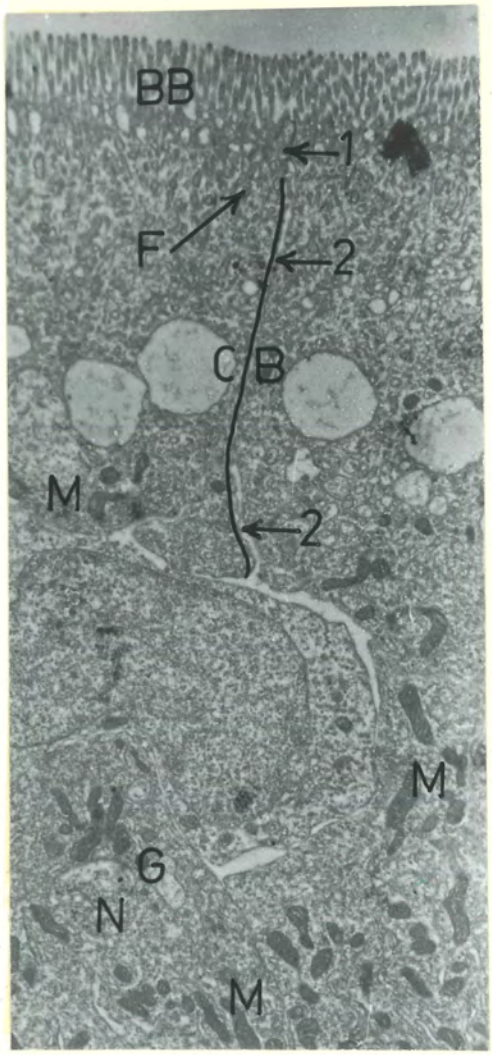

Figure 34.- A survey picture showing the brush border (BB), food droplets (F), mitochondria (M), a cell boundary (CB) - single at 1 and double at 2, nucles (N), Golgi apparatus (G), and an intrusive cell (IC).

Animal: Carassius auratus

Magnification: 7,000 X

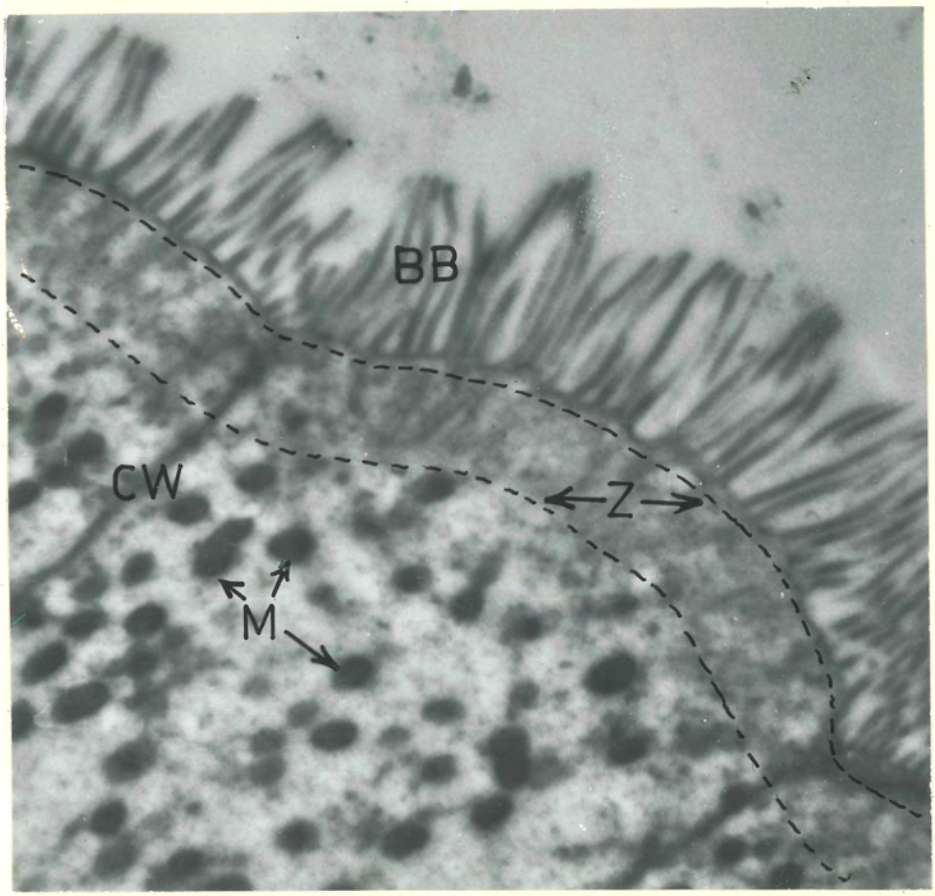

Figure 35.- A section through the brush border (BB), and top of an intestinal cell showing the granular zone in which mitochondria (M) were seldom detected. The cell walls (CW) can also be seen.

Animal: Gallus domesticus

Magnification: 15,000 X

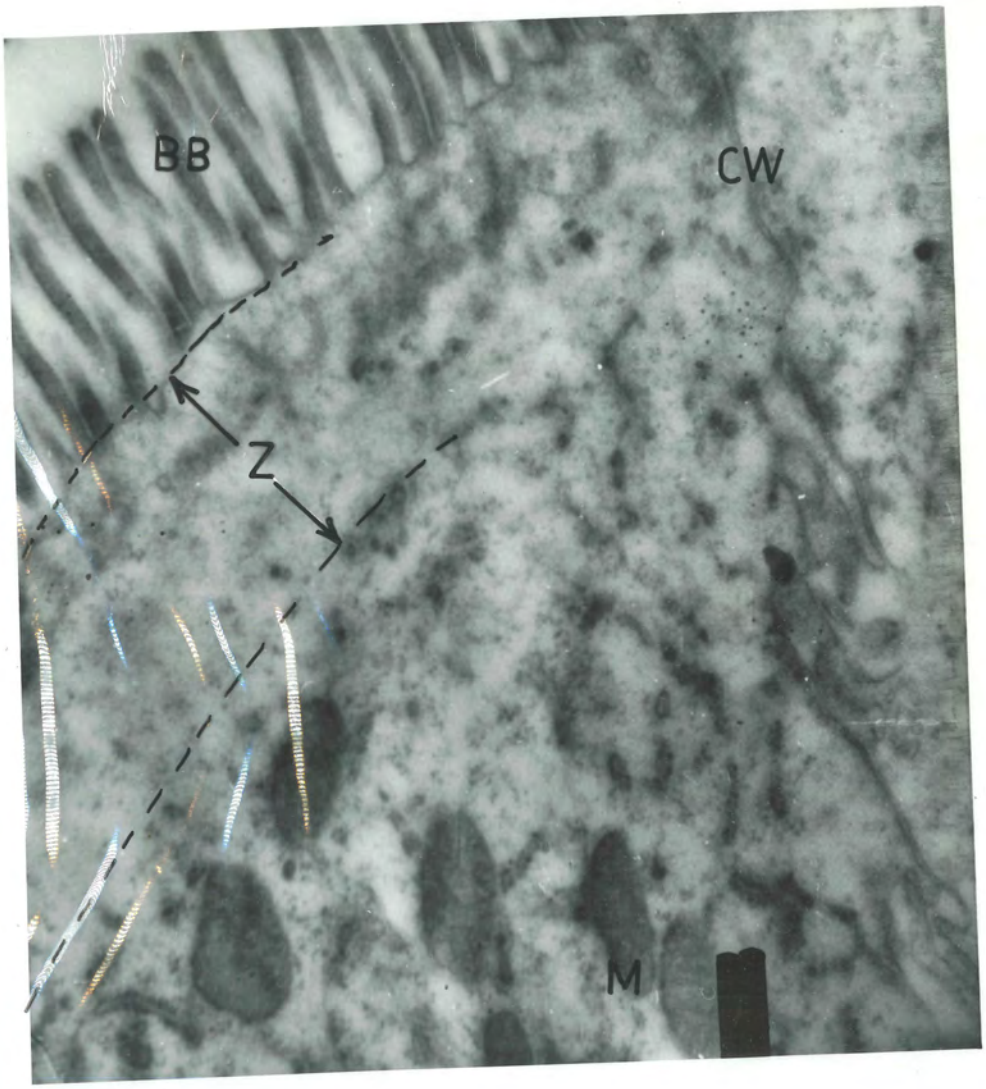

Figure 36.- A section through the brush border (BB) and top of an intestinal cell showing the granular zone (Z) in which mitochondria (M) were seldom seen. The cell wall (CW) can be seen to be double.

Animal: Rattus norvegicus

Magnification: 22,500 X

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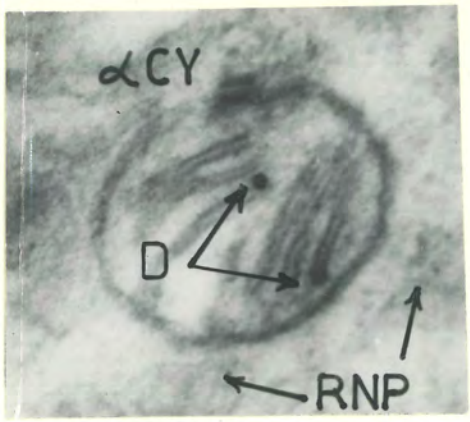

Figure 37.- A section through a mitochondrion containing two dense granules (D). cytomembranes can be seen in the top left hand corner of the picture (αCY), as well as scattered ribonucleoprotein granules (RNP).

Animal: Gallus domesticus

Magnification: 50,000 X

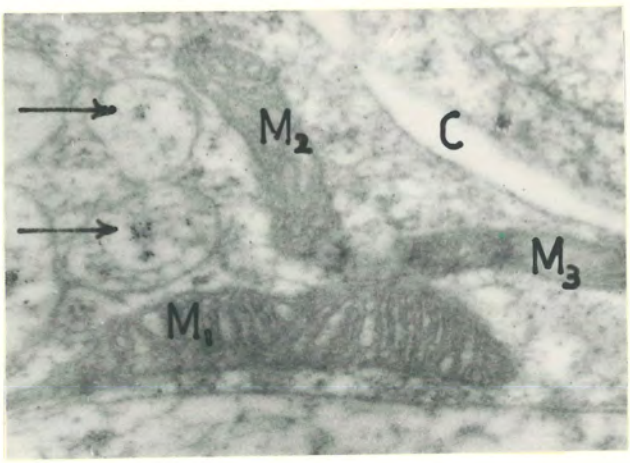

Figure 38.- A section through three mitochondria (M). In M_1 the cristae run transversely, and in M_2 they run along the length of the mitochondrion. An intercellular space (C) can also be seen. The two structures arrowed may be early stages in the formation of mitochondria.

Animal: Carassius auratus

Magnification: 37,500 X

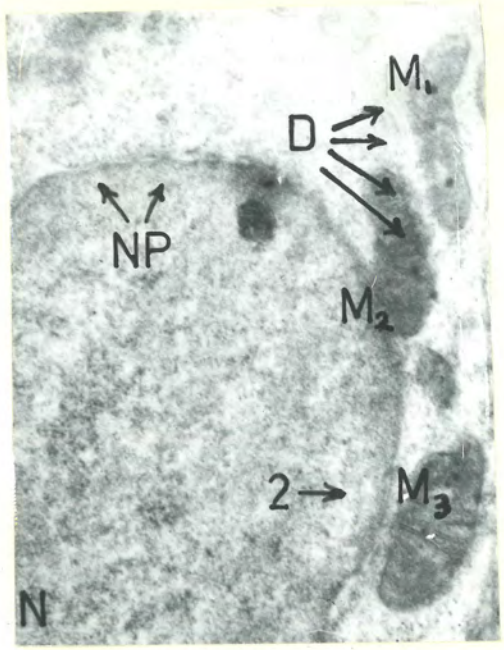

Figure 39.- A section through a nucleus (N) and three mitochondria (M). M_2 lies very close to the nuclear membrane (c.f. nuclear membrane theory of mitochondrial origin). The double wall of the nuclear membrane can seen at places (2) as well as nuclear pores (NP). M_1 contains at least 4 dense granules (D). M_3 also contains dense granules.

Animal: Gallus domesticus

Magnification: 27,500 X

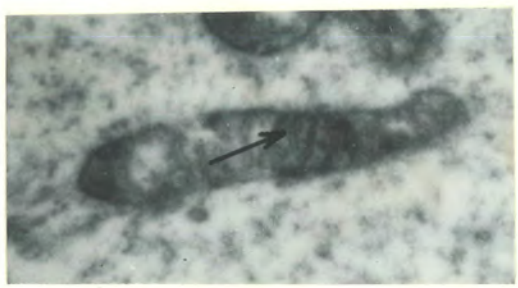

Figure 40.- A section through a mitochondrion. A cristae can be seen curving around a dense body.

Animal: Rattus norvegicus

Magnification: 35,000 X

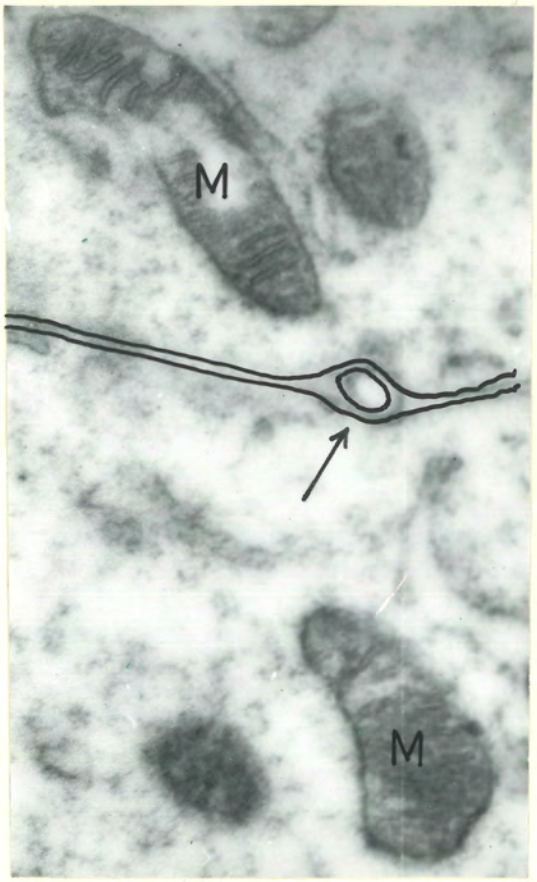

Figure 41.- A section through two mitochondria. The structure arrowed may give rise to the unidentified bodies shown in Figures 53 and 55, pps. 112 and 113; see the discussion on p. 114.

Animal: Tiliqua rugosa

Magnification: 40,000 X

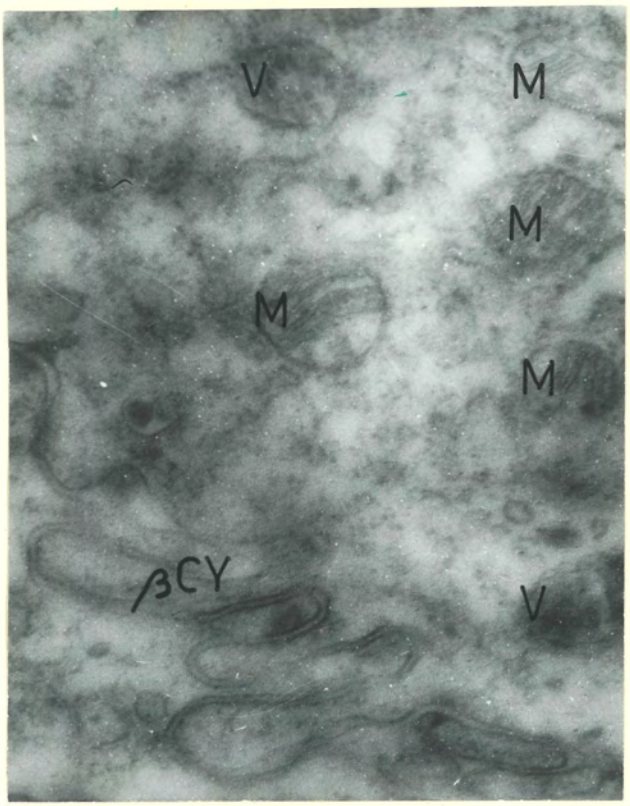

Figure 42.- A section showing mitochondria (M), cytomembranes (β CY), and vacuole containing bodies (V).

Animal: Cavia percellus

Magnification: 37,500 X

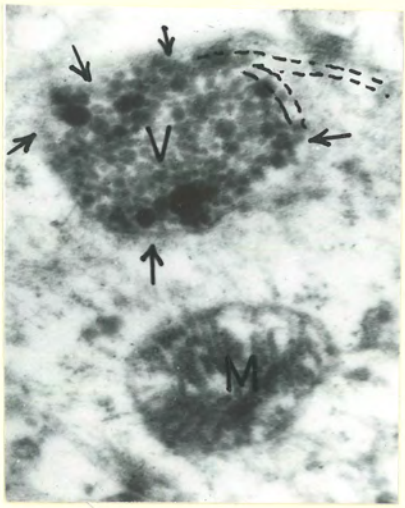

Figure 43.- A section through a mitochondrion (M) and a vacuole-containing body (V) with surrounding membrane (arrowed).

Animal: Gallus domesticus

Magnification: 42,500 X

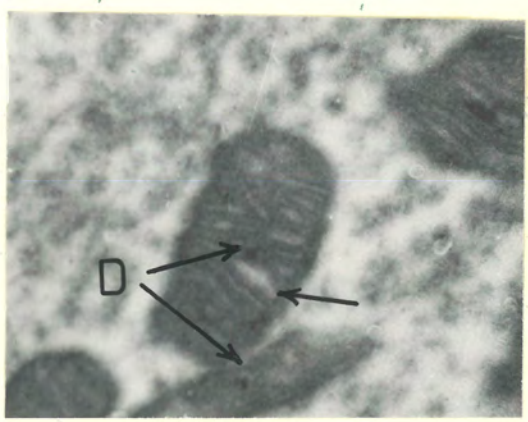

Figure 44.- A section through mitochondria containing dense granules (D). In one place (X) the cristae appears to be formed by an infolding of the inner outer membrane.

Animal: Tiliqua scincoides

Magnification: 42,500 X

These observations are similar to those of Zetterqvist (1956), who examined the intestinal mitochondria of mice. Zetterqvist found that the intestinal mitochondria of mice were similar in structure to other types of mitochondria. The longest intestinal mitochondrion found by Zetterqvist was 3.0 μ long; the mean width of the mitochondria was 0.28 μ.

Intestinal mitochondria which had been isolated in 0.25 M sucrose solution looked different under the electron microscope to mitochondria in the cell. This is shown in the electron micrographs in Figures 45 to 54 (pps. 107 - 113).

The arrangement of the cristae was irregular: in some mitochondria all the cristae were on one side and appeared to be flattened against the mitochondrial wall; other mitochondria were crescent-shaped and the cristae were squashed between the walls of the mitochondria; and in other mitochondria the cristae themselves were ring-shaped. Even when the cristae appeared to cover most of the available area in an isolated mitochondrion, the arrangement of the cristae was different to that in mitochondria in the intact cell. The cristae in the isolated mitochondria appeared to intersect many times, whereas in "intact" mitochondria only a few intersecting cristae were found. The cristae of isolated mitochondria were thicker than those in "intact" mitochondria, and in isolated mitochondria the characteristic mitochondrial membranes were not easily distinguished either in the cristae or in the mitochondrial wall.

The diameter of isolated mitochondria varied from 0.4 to 1.4 μ.

In some mitochondria the wall appeared to have ruptured, and the mitochondrial contents appeared to be spilling out.

Other components of the mitochondrial pellet which could be

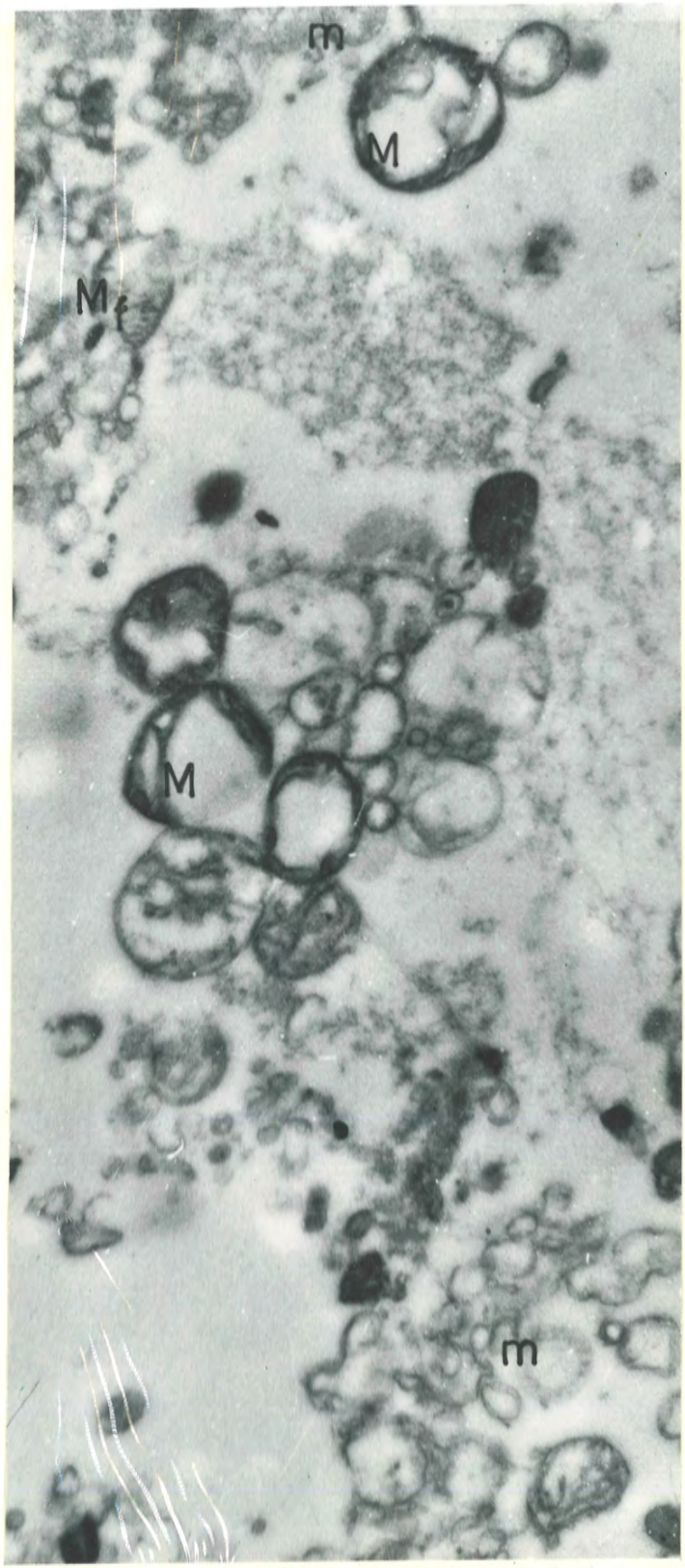

Figure 45.- A section through a mitochondrial pellet showing mitochondria (M) with their cristae flattened against the mitochondrial wall, microsome vesicles (m), and fragments of mitochondria (M_f).

Animal: Rattus norvegicus

Magnification: 43,000 X

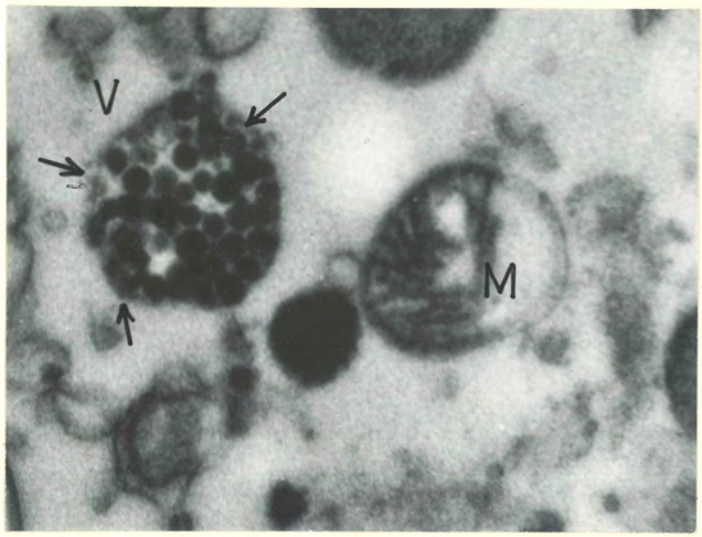

Figure 46.- A micrograph of an isolated mitochondrion (M) showing the cristae migrating to the outer walls. V is a vacuole-containing body surrounded by a membrane (arrowed).

Animal: Rattus norvegicus
Magnification: 45,000 X

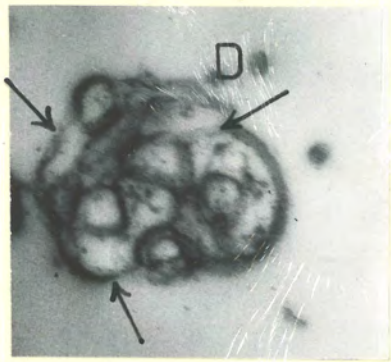

Figure 47.- A micrograph of an isolated mitochondrion, the outer membrane of which, at the places arrowed, appears to be in the process of rupturing. Some circular (in section) cristae and some small, spherical, opaque bodies can be seen (D).

Animal: Cavia percellus

Magnification: 45,000 X

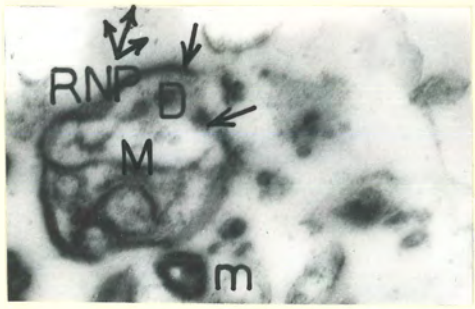

Figure 48.- A micrograph of an isolated mitochondrion (M) with a ruptured wall (arrowed). A microsome vesicle (m) and some ribonucleoprotein granules (RNP) can be seen. Small sperical opaque bodies (D) appear to be about to escape from the mitochondrion.

Animal: Cavia percellus

Magnification: 45,000 X

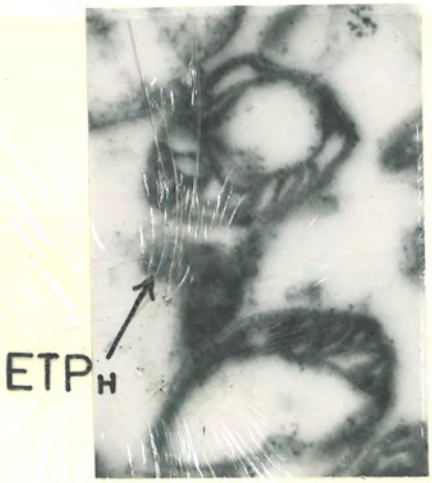

Figure 49.- Isolated ring shaped mitochondria. A double-walled electron transport particle can also be seen (ETP$_H$).
Aniaml: Gallus domesticus
Magnification: 45,000 X

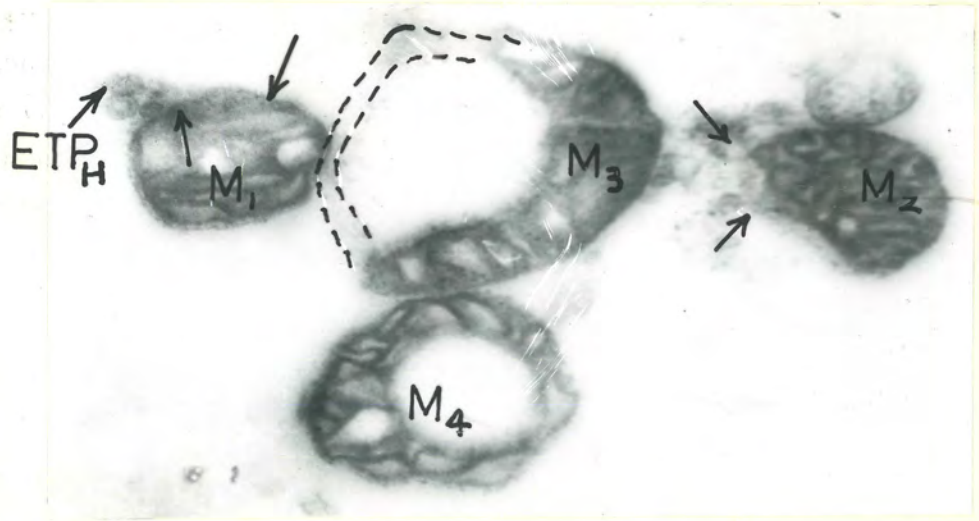

Figure 50.- Isolated mitochondria. The outer membranes of M$_1$ and M$_2$ appear to be breaking (arrowed). Near M$_1$ some double-walled electron transport particles (ETP$_H$) can be seen. A third mitochondrion (M$_3$) is elongate. Its shape is probably secondary - the result of the rupture of a ring-shaped mitochondrion such as M$_4$ - rather than being the normal shape of an isolated mitochondrion.
Animal: Carassius auratus
Magnification: 47,000 X

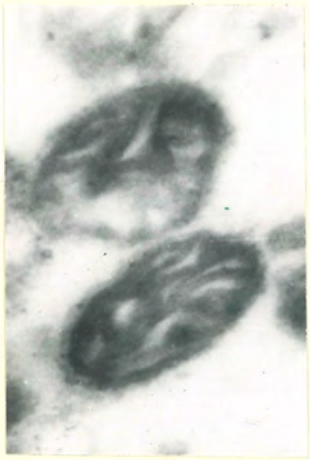

Figure 51.- Isolated mitochondria.

Animal: Carassius auratus

Magnification: 42,500 X

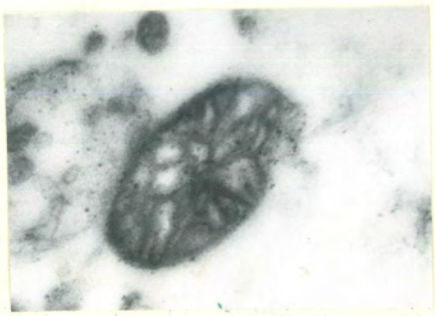

Figure 52.- An isolated mitochondrion.

Animal: Gallus domesticus

Magnification: 42,500 X

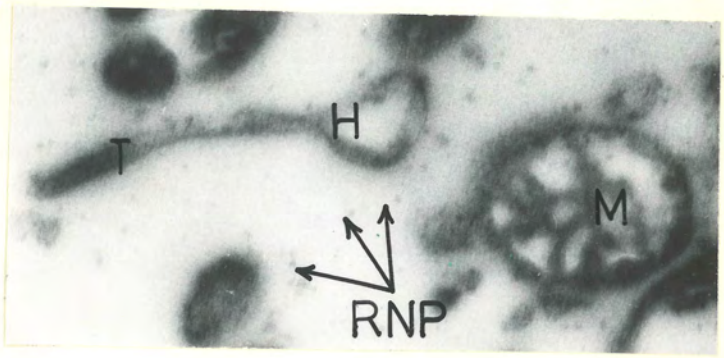

Figure 53.- A section showing an isolated mitochondrion (M), some ribonucleoprotein granules (RNP), and an unidentified structure consisting of a circular hollow head (H) to which is joined a straight tail approximately 1 μ long (T).

Animal: Tiliqua rugosa

Magnification: 50,000 X

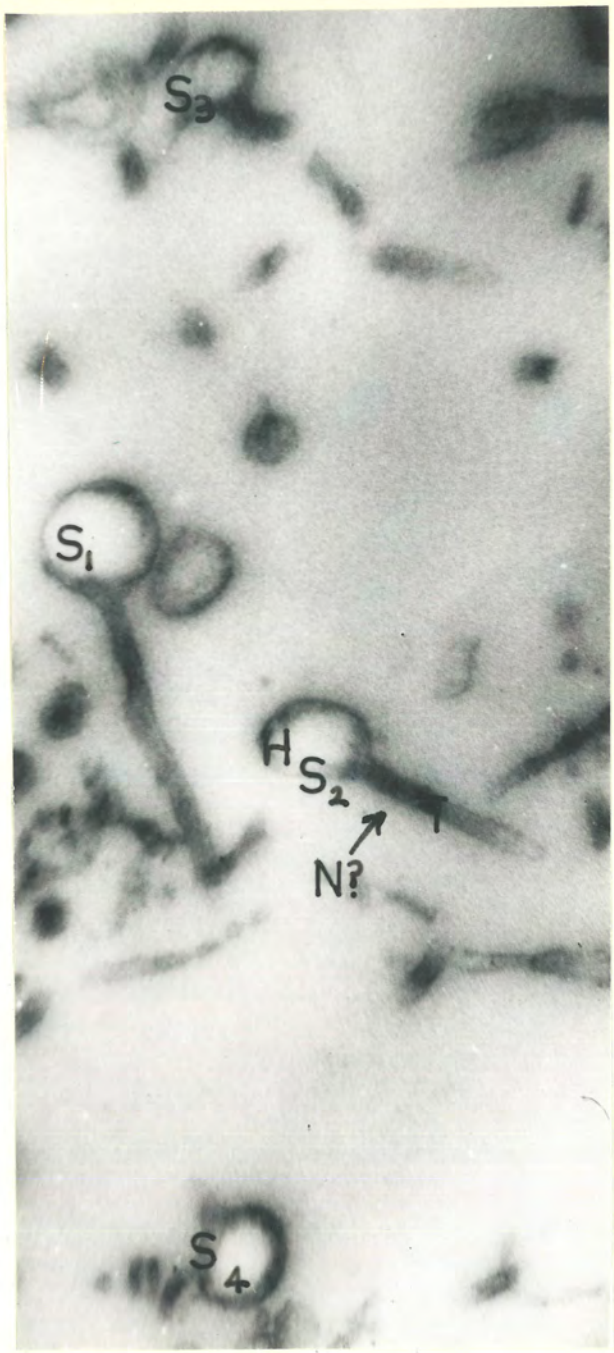

Figure 54.- A section through some unidentified structures, S_1 and S_2. They consist of a circular hollow head (H) and a straight tail (T), which in S_2 contains an opaque body similar to a bacterial nucleus (N?). S_3 and S_4 appear to similar structures which have been sectioned obliquely.

Animal: Tiliqua rugosa

Magnification: 55,000 X

identified were microsomes, vaccuole-containing bodies, and round hollow structures up to 0.2 μ in diameter. The latter usually appeared to have a double membrane and were tentatively identified as heavy electron transport particles.

Among the unidentified objects was a structure that looked like a heavy electron transport particle with a tail about 1 μ long (see Figures 53 and 54, pps. 112 and 113). This structure was found most commonly in the mitochondrial pellet prepared from the intestinal cells of the lizard, although it was also seen in the mitochondrial pellets of the guinea pig and the rat.

This tailed structure looked like a bacterium, especially as it sometimes contained a "nucleus". But it seemed unlikely that they were bacteria as:

(i) although some species of Clostridium look similar they do not have a hollow "head" (spore);

(ii) the chance of finding a species of Clostridium (tetani, say) in the intestine would be small;

(iii) any bacteria in the scrapings from the intestine should have been removed by the first centrifugation during the preparation of the mitochondrial pellet;

and (iv) the unidentified structures were about four times smaller than normal Clostridium tetani.

The unidentified bodies were probably only fragments of mitochondria, or some other normal cellular structure, such as the endoplasmic reticulum or the cell wall (c.f. Figure 41, p. 103).

6. The distribution within the intestinal cell of enzymes associated with the Krebs cycle

Although mitochondria are capable of the complete oxidation of the acids of the Krebs cycle, the enzymes of this cycle are not located exclusively in the mitochondrion. The intracellular distribution of these enzymes differs according to the type of cell; for instance, mitochondria isolated from the liver of rats account for 16% of the total aconitase activity of the cell (Dickman and Speyer, 1954), whereas mitochondria isolated from the brain of rabbits account for 86% of the total aconitase activity (Shepherd and Kalnitsky, 1954). The distribution of the enzymes of the Krebs cycle in the intestinal cell has not been examined.

It seemed of interest, therefore, to see whether there were differences in the distribution of these enzymes in the intestinal cells of different species, and to compare the distribution with that in other types of cell.

The intestinal cells of the lizard and the chicken were examined. The cells were homogenized and the mitochondrial and supernatant fractions isolated. The enzymatic activity of each fraction was estimated, and compared with the activity of the combined fraction - i.e. the supernatant fraction of the first centrifugation (which consisted of the whole homogenate minus unbroken cells, cell walls, and nuclei). The results are shown in Tables 17 and 18.

As it was not possible to do a large number of experiments it was necessary to analyze the results statistically to find out if the apparent differences between the arithmetic means obtained for the enzymes of each species were significant. The "t" test was used (see the Appendix, p. 165). The results are summarized in Table 19.

TABLE 17

THE DISTRIBUTION OF ENZYMES OF THE KREBS CYCLE IN THE INTESTINAL CELLS OF CHICKEN

The figures are recovery % (enzyme activity of fraction/enzyme activity of homogenate.

ENZYME	EXPERIMENT	MITOCHONDRIA	SUPERNATANT	GAIN/LOSS
DPNH cytochrome c reductase	1	24	54	- 22
	2	29	52	- 19
	Arithmetic mean	27	53	- 20
Isocitric dehydrogenase	1	23	69	- 8
	2	20	79	- 1
	3	24	72	- 4
	4	21	63	- 16
	5	17	86	- 3
	Arithmetic mean	21	74	- 6
Aconitase	1	33	92	+ 25
	2	34	78	+ 12
	Arithmetic mean	34	85	+ 19
Fumarase	1	30	63	- 7
	2	22	69	- 9
	Arithmetic mean	26	66	- 8
Malic dehydrogenase	1	15	88	+ 3
	2	18	87	+ 5
	Arithmetic mean	17	88	+ 4
Diaphorase	1	31	70	+ 1

TABLE 18

THE DISTRIBUTION OF ENZYMES OF THE KREBS CYCLE IN THE INTESTINAL CELLS OF THE LIZARD

The figures are recovery % (enzyme activity of fraction/enzyme activity of homogenate.

ENZYME	EXPERIMENT	MITOCHONDRIA	SUPERNATANT	GAIN/LOSS
DPNH cytochrome c reductase	1	26	73	- 1
	2	22	56	- 22
	3	27	56	- 17
	4	26	58	- 15
	Arithmetic mean	25	61	- 15
Isocitric dehydrogenase	1	14	85	- 1
	2	15	75	- 10
	3	13	85	- 2
	4	13	85	- 2
	Arithmetic mean	14	83	- 4
Aconitase	1	30	80	+ 10
	2	29	76	+ 5
	Arithmetic mean	30	78	+ 8
Fumarase	1	21	68	- 11
	2	14	76	0
	Arithmetic mean	18	72	- 6
Diaphorase	1	19	85	+ 4
	2	25	70	- 5
	Arithmetic mean	22	78	0
ketoglutarate dehydrogenase	1	21	69	- 10
Malic dehydrogenase	1	16	93	+ 9

TABLE 19

RESULTS FROM THE STATISTICAL ANALYSIS OF TABLES 17 AND 18

The complete analysis is shown in the Appendix, pp. 165 and 166.

ENZYME	ANIMAL	% IN MITOCHONDRIA	HAS A DIFFERENCE BETWEEN SPECIES BEEN SHOWN?
DPNH cytochrome c reductase	chicken	27	no
	lizard	25	
Fumarase	chicken	26	no
	lizard	18	
Malic dehydrogenase	chicken	17	no
	lizard	16	
Isocitric dehydrogenase	chicken	21	yes
	lizard	14	
Aconitase	chicken	34	doubtful
	lizard	30	
Diaphorase	chicken	31	no
	lizard	22	

When this series of experiments was started it was planned to examine more enzymes and more species. However, the work was abandoned at this stage because the value of the results did not justify the work required to get them. Although the results obtained from the chicken can be compared with those from the lizard, the results can not be compared directly with the work of other biologists in which the cellular fractions have been isolated under different conditions. In the present investigation the mitochondrial fraction was contaminated with microsomal material (see p. 114) which may have contributed

some of the activity attributed to "mitochondria". (Of course, the method of isolating the mitochondria could have been changed, but it was thought undesirable to do this half-way through the investigation.) Furthermore, the spectrophotometric methods used to estimate the activities of the enzymes showed up the individual variation among animals so that several runs of an experiment were necessary to obtain reliable results.

The percentage enzyme recovery in intestinal mitochondria of some of the enzymes investigated is compared in Table 20 with the percentage enzyme recovery in mitochondria isolated from other tissues. All the figures of DPNH cytochrome c reductase are similar, but there is considerable variation among the others.

TABLE 20

A TABLE COMPARING THE PERCENTAGE ENZYME RECOVERY IN MITOCHONDRIA ISOLATED FROM VARIOUS TISSES

TISSUE	ANIMAL	ENZYME			
		DPNH cytochrome c reductase	Fumarase	Isocitric dehydrogenase	Aconitase
Intestine	Chicken	27	26	21	34
	Lizard	25	18	14	30
Liver	Rat	23[1], 28[2], 38[3]	-	-	16[9]
	Mouse	28[4]	55[5]	12[7]	-
Brain	Rabbit	-	67[6]	58[8]	86[8]
Spleen	Rat	30[3]	-	-	-

References:

1. Strittmatter and Ball, 1954.
2. deDuve, Pressman, Gianetto, Wattiaux, and Appelmans, 1955
3. Eichel, 1957.
4. Hogeboom and Schneider, 1950 b.
5. Kuff, 1954.
6. Shepherd and Kalnitsky, 1954.
7. Hogeboom and Schneider, 1950 a.
8. Shepherd, 1955.
9. Dickman and Speyer, 1954.

7. Oxidative phosphorylation and its uncoupling by thyroxine

The ratio of phosphorus to oxygen used during oxidative phosphorylation has been estimated in mitochondria isolated from mammalian tissues and from insects. Mitochondria from other sources have been examined only in isolated instances. Even in mammals, one tissue, the liver, has received most of the attention, while the intestinal mitochondria have been neglected altogether. It was therefore decided to estimate the P/O ratios of intestinal mitochondria to see whether the ratios were similar to those of other mitochondria.

Thyroxine has been shown to uncouple oxidative phosphorylation, but whether uncoupling is responsible for the symptoms of thyrotoxic animals is not known. As the thyroid appears to have become more efficient in controlling the basal metabolic rate during the evolution of vertebrates - although a hormone of similar structure seems to be involved throughout - this seemed a good opportunity to see whether the effect of thyroxine on oxidative phosphorylation was different in the lower vertebrates to that in mammals. If it proved to be different, then it might be argued that the uncoupling effect of thyroxine was possibly the physiological one.

The P/O ratios were estimated in the intestinal mitochondria of the guinea pig, chicken, lizard, and toad. Two different substrates were used: succinate, which theoretically has a P/O ratio of 2; and alpha ketoglutarate, which has a P/O ratio of 4. As the magnesium concentration has been shown to affect the efficiency of the uncoupling of oxidative phosphorylation by thyroxine (Lehninger, 1956), two different concentrations of Mg^{++} (0.09 and 0.045 M) were used in the incubation medium. The final concentration of thyroxine used was 9×10^{-5} M.

The P/O ratio with succinate as substrate was 1.6 for the intestinal mitochondria of the chicken, lizard, and toad; and 1.7 for those of the guinea pig. It is extremely doubtful that the figure obtained for the guinea pig was significantly different. With half the concentration of Mg^{++} (i.e. 0.045 M instead of 0.09 M) the P/O ratio fell to approximately 1.5. With alpha ketoglutarate as substrate the ratios recorde were 2.4 for the toad, 2.5 for the chicken, and 2.6 for the guinea pig and lizard. Once again the differences are probably not significant. With half the amount of Mg^{++}, the P/O ratio fell to approximately 2.4. These figures are set out in Table 21.

TABLE 21

THE INFLUENCE OF Mg^{++} AND THYROXINE ON OXIDATIVE PHOSPHORYLATION IN INTESTINAL MITOCHONDRIA

The medium consisted of: 0.1 ml 10^{-3} M thyroxine (or water), 0.1 ml 0.5 M phosphate buffer, pH 7.4, 0.1 ml 2.5 x 10^{-4} M cytochrome c, 0.1 ml 0.2 M tris buffer, pH 7.4, 0.1 ml 0.1 M NaF, 0.05 ml 0.835 M glucose, 0.05 ml 0.1 M ATP, 0.1 ml 0.1 M $MgCl_2$ (or 0.05 M), 0.1 ml hexokinase (20mg/ml), and 0.2 ml mitochondrial suspension.

INTESTINAL MITOCHONDRIA OF	SUBSTRATE	P/O RATIOS			
		0.09 M Mg^{++}		0.045 M Mg^{++}	
		Control	+thyroxine	Control	+thyroxine
Guinea pig	Succinate	1.7	1.5	1.4	0.8
	α ketoglutarate	2.6	2.3	2.4	1.4
Chicken	Succinate	1.6	1.5	1.5	1.0
	α ketoglutarate	2.5	2.2	2.4	1.3
Lizard	Succinate	1.6	1.7	1.5	0.8
	α ketoglutarate	2.6	-	2.5	1.7
Toad	Succinate	1.6	1.4	1.6	0.6
	α ketoglutarate	2.4	2.2	2.4	1.5

The P/O ratios for succinate found experimentally agreed well with the expected ratio of 2. The P/O ratio for alpha ketoglutarate was lower than expected. The reason for this may be

that the phosphorylation which occurs between DPN and flavoprotein is more easily uncoupled than other phosphorylations. But it is more likely that the P/O ratio was lower than expected because of unselective ATP-ase activity. As the oxygen consumption of mitochondria with alpha ketoglutarate as substrate is less than that with succinate as substrate (approximately one third as much with intestinal mitochondria), the total amount of phosphate esterified is less. Consequently, the same amount of ATP-ase activity has a larger effect of the P/O ratio of alpha ketoglutarate than on the P/O ratio of succinate.

Thyroxine lowered the P/O ratio of intestinal mitochondria by lowering the rate of phosphorylation. The oxygen consumption of the mitochondria was not affected. At the higher concentration of Mg^{++} thyroxine did not uncouple oxidative phosphorylation nearly as much as it did at the lower concentration (see Table 21). The percentage uncoupling was approximately 10 and 40% respectively; more accurate figures are given in Table 22.

TABLE 22

THE INFLUENCE OF Mg^{++} ON THE UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY THYROXINE IN INTESTINAL MITOCHONDRIA

For details of the way in which the P/O ratios were estimated see Table 21, p. 121.

INTESTINAL MITOCHONDRIA OF	SUBSTRATE	% UNCOUPLING BY THYROXINE IN A Mg^{++} CONCENTRATION 0.09 M	0.045 M
Guinea pig	Succinate	12	43
	α ketoglutarate	12	42
Chicken	Succinate	6	33
	α ketoglutarate	12	46
Lizard	Succinate	-6	47
	α ketoglutarate	--	32
Toad	Succinate	13	63
	α ketoglutarate	8	38

This is in agreement with the degree of uncoupling obtained with similar magnesium concentrations with other types of mitochondria (Lehninger, 1956).

The possibility that the uncoupling effect of thyroxine is due to the removal of Mg^{++} from the medium by the formation of an undissociated Mg-thyroxine complex has been rejected by Tapley and Cooper (1956 a). They considered that the concentration of Mg^{++} and thyroxine present during uncoupling experiments was not compatible with this concept.

No significant difference could be detected between the P/O ratios of the intestinal mitochondria isolated from the four species examined. The addition of thyroxine uncoupled oxidative phosphorylation a similar amount to that observed with the mitochondria of other tissues. The reponse of the intestinal mitochondria to thyroxine appeared to be similar in the four species studied.

8. Thyroxine and mitochondrial swelling

Thyroxine causes some mitochondria to swell (Klemperer, 1955; Tapley, 1956). At first this was thought to be responsible for the uncoupling of oxidative phosphorylation that occurs when thyroxine is added to respiring mitochondria. It was shown later that the effect of thyroxine is not a direct one, because thyroxine can not uncouple oxidative phosphorylation in sub-mitochondrial fragments (Tapley and Cooper, 1956 a; McMurray, Maley, and Lardy, 1958). Furthermore, Tapley and Cooper (1956 b) have shown that not all mitochondria swell in the presence of thyroxine. For example, the mitochondria isolated from the brain, spleen, heart, diaphragm, and testis of the rat swell very little in the presence

of (3×10^{-5} M) thyroxine. And as thyroxine has been shown to uncouple oxidative phosphorylation in mitochondria from skeletal and cardiac muscle it would seem that the uncoupling caused by thyroxine is not necessarily accompanied by mitochondrial swelling.

The intestinal mitochondria of the rat, guinea pig, chicken, toad, and carp were examined to see whether they swelled in the presence of thyroxine. The swelling was compared with that of liver mitochondria isolated from the same animal. A series of concentrations of thyroxine was used. The results are shown in Figures 55 to 59, and are summarized below.

Mitochondria isolated from the liver of the rat swelled considerably more in the presence of 1×10^{-5} and 1×10^{-3} M thyroxine than they did endogenously (the control consisted of phosphate-buffered sucrose without versene, see the Methods, p. 57). The swelling was fastest during the first five minutes; it was relatively slow after ten minutes, although swelling could still be detected. The intestinal mitochondria of the rat did not swell or contract, either endogenously or in the presence of up to 1×10^{-3} M thyroxine. These results are shown in Figure 55.

The mitochondria isolated from the liver of the guinea pig behaved similarly to those isolated from the liver of the rat. They swelled considerably in the presence of 1×10^{-5} or 1×10^{-3} M thyroxine even though the endogenous swelling was marked. In 1×10^{-7} M thyroxine some swelling was noticed. The intestinal mitochondria swelled relatively little. Addition of 1×10^{-5} and 1×10^{-3} M thyroxine increased the swelling, but the overall swelling was much less than that of the liver mitochondria (see Figure 56).

Mitochondria were difficult to isolate from the liver of chicken in the quantities usually expected from the liver of higher vertebrates. After homogenization the suspension seemed

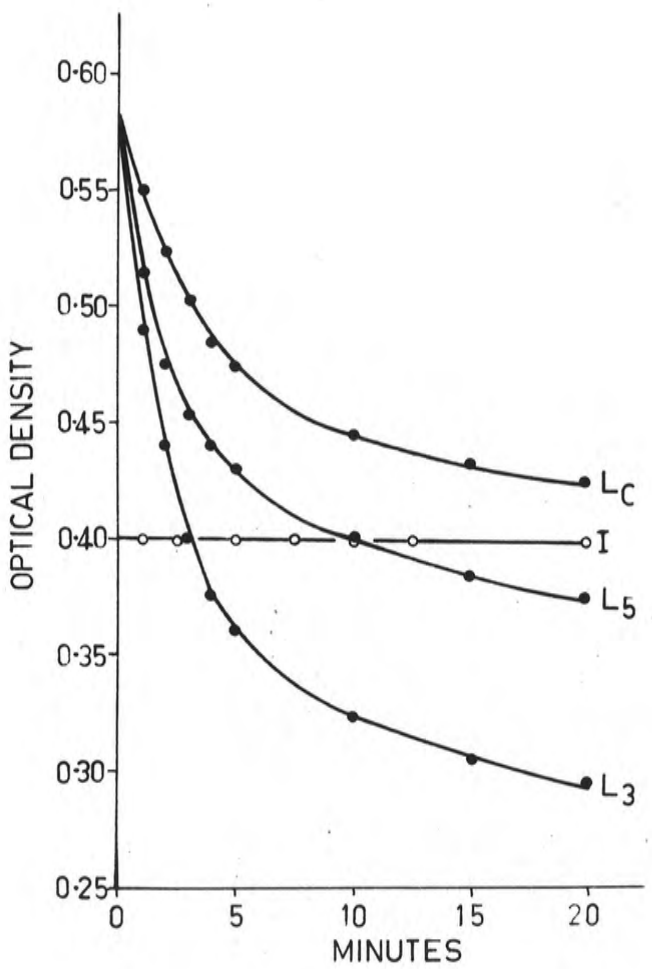

Figure 55.- The effect of thyroxine on the swelling of mitochondria isolated from the liver (L) and the intestine (I) of the rat. L_C = endogenous swelling; L_5 = swelling in the presence of 1×10^{-5} M thyroxine; L_3 = swelling in the presence of 1×10^{-3} M thyroxine; the intestinal mitochondria (I) did not swell either endogenously or in the presence of up to 1×10^{-3} M thyroxine. For experimental details see p. 57.

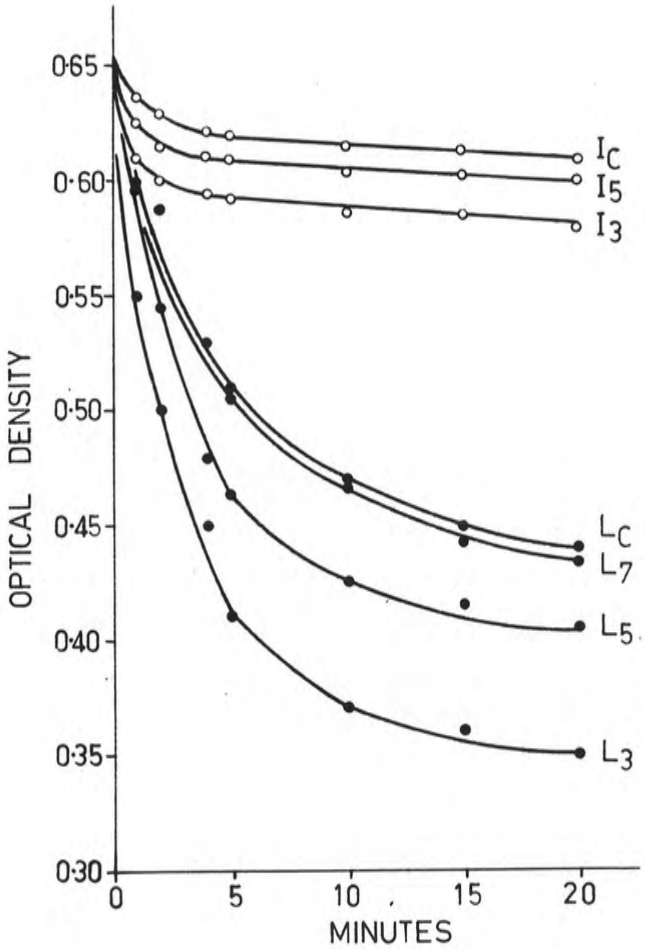

Figure 56.- The effect of thyroxine on the swelling of mitochondria isolated from the liver (L) and the intestine (I) of the guinea pig. Subscript C = endogenous swelling; subscript 7, 5, and 3 = swelling in the presence of 1×10^{-7}, 1×10^{-5}, and 1×10^{-3} M thyroxine respectively. **For experimental details see p. 57.**

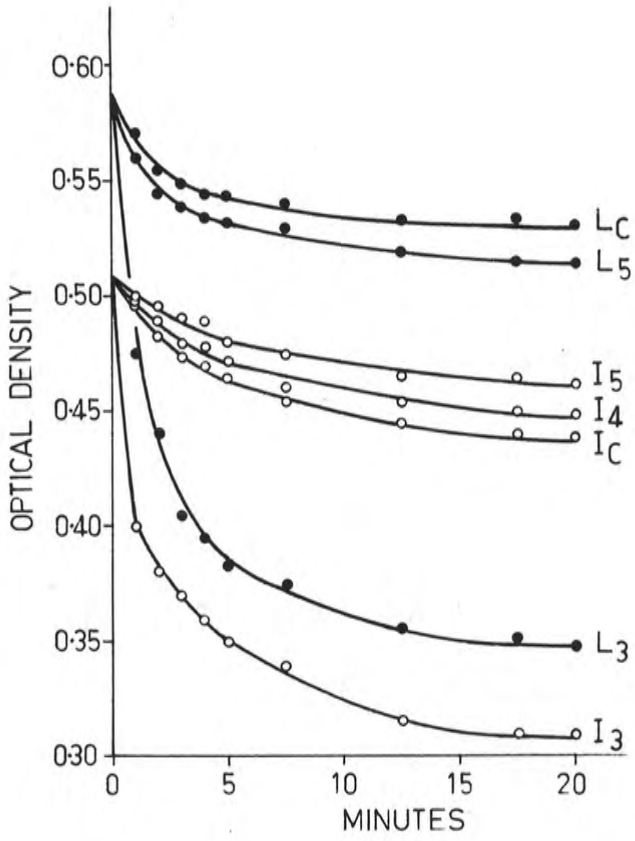

Figure 57.- The effect of thyroxine on the swelling of mitochondria isolated from the liver (L) and the intestine (I) of the chicken. Subscript C = endogenous swelling; subscript 5, 4, and 3 = swelling in the presence of 1×10^{-5}, 1×10^{-4}, and 1×10^{-3} M thyroxine respectively. **For experimental details see p. 57.**

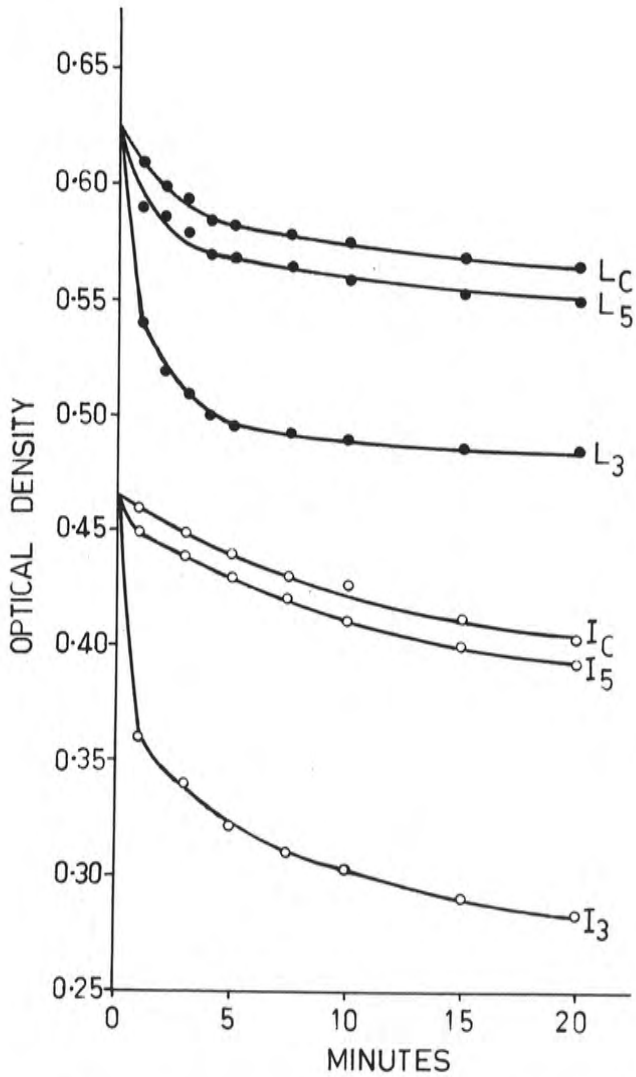

Figure 58.- The effect of thyroxine on the swelling of mitochondria isolated from the liver (L) and the intestine (I) of the toad. Subscript C = endogenous swelling; subscript 5 and 3 = swelling in the presence of 1×10^{-5} and 1×10^{-3} M thyroxine, respectively.

For experimental details see p. 57.

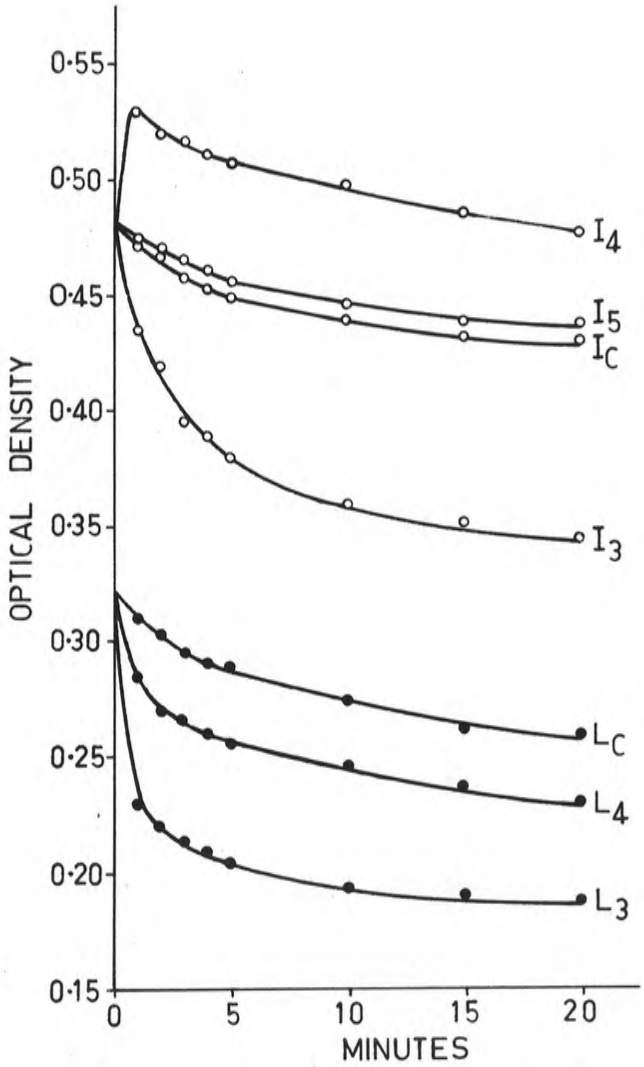

Figure 59.- The effect of thyroxine on the swelling of mitochondria isolated from the liver (L) and the intestine (I) of the carp. Subscript C = endogenous swelling; subscript 5, 4, and 3 = swelling in the presence of 1×10^{-5}, 1×10^{-4}, and 1×10^{-3} M thyroxine, respectively. For experimental details see p. 57.

thinner than usual, and white fatty-looking material collected on the surface of the suspension while it was being centrifuged. These liver mitochondria swelled less endogenously than those isolated from the liver of the rat or the guinea pig. The swelling induced by 1×10^{-5} M thyroxine was also less, although in 1×10^{-3} M thyroxine the swelling was considerable. The intestinal mitochondria of the chicken were easy to prepare. They did not swell much endogenously - about the same amount as the intestinal mitochondria of the guinea pig. However, in 1×10^{-5} and 1×10^{-4} M thyroxine the intestinal mitochondria swelled less than they did endogenously. The latter concentration gave less protection from swelling. 1×10^{-3} M thyroxine, as usual, caused considerable swelling. The results of these experiments are shown in Figure 57.

The mitochondria isolated from the liver and the intestine of the toad behaved similarly. Endogenous swelling was relatively limited. 1×10^{-5} M thyroxine caused slight swelling; but 1×10^{-3} M thyroxine had a considerable effect (see Figure 58).

Mitochondria isolated from the liver of the carp swelled endogenously to a limited extent only. 1×10^{-3} M thyroxine caused considerable swelling and 1×10^{-4} M thyroxine somewhat less. 1×10^{-5} M thyroxine sometimes caused very slight swelling, sometimes no effect at all, and sometimes appeared to give a slight protection against swelling. The intestinal mitochondria of the carp showed limited endogenous swelling and, like the liver mitochondria, swelled considerably in 1×10^{-3} M thyroxine. 1×10^{-5} M thyroxine gave some protection from swelling and 1×10^{-4} M thyroxine actually caused the mitochondria to contract. The process of contraction was rapid; after one minute the contracted mitochondria started to swell again. After 20 minutes they had swollen to about the same size as they were at the start of the experiment. These results are shown in Figure 59.

The results obtained with the mitochondria isolated from the liver of the rat are similar to those obtained previously by other biochemists (Lehninger, 1956; Dickens and Salmony, 1956; Emmelot and Bos, 1957) although no "lag period*" occurred before the mitochondria started to swell endogenously. The high temperature (approximately 30' C, see the Methods, p. 58) at which the experiments in the present investigation were carried out was probably responsible for the immediate swelling. The rate of swelling has been shown to be critically dependent on the temperature (Lehninger, Ray, and Schneider, 1959). The hypothesis that the high temperature is responsible for the absence of the lag period is supported by the earlier experiments of Lehninger and his co-workers (Tapley, Cooper, and Lehninger, 1955). They found that there was no lag period when the mitochondria were incubated at 23 - 25' C, while their later experiments, in which they did obtain a lag period, were done in a constant temperature water bath at 20' C. Emmelot and Bos (1957) who did their experiments at "room temperature" did not obtain a lag period.

The intestinal mitochondria of the rat did not swell - even in the presence of thyroxine. The reason for this is almost certainly associated with the blocking of the respiration of these mitochondria by the fatty acid inhibitor which appears when the intestinal cells of the rat are homogenized. Respiratory inhibitors such as cyanide, Antimycin A, and sodium amytal prevent mitochondrial swelling and it has been shown that swelling can not occur unless respiration is proceeding concomitantly (Chappel and Greville, 1958; Lehninger, Ray, and Schneider, 1959; Hunter, Levy, Fink, Schultz, Guerra, and Hurwitz, 1959).

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*Lehninger (1956) found that little swelling occured for the first two minutes of the experiment when mitochondria were suspended in 0.03 M sucrose containing 0.02 M tris buffer, pH 7.4. In 1×10^{-5} M thyroxine no lag period occurred.)

Lehninger (1960 a) has suggested that mitochondria have sufficient endogenous respiration to allow swelling to occur. This conclusion was bourne out in the present investigation. The addition of succinate to the mitochondria isolated from the guinea pig did not alter either the rate or amount of endogenous or of thyroxine-induced swelling. The rate of endogenous respiration, however must be quite slow, because it is difficult to measure the endogenous respiration of mitochondria by routine laboratory methods.

The mitochondria isolated from the intestine of the guinea pig swelled less than those isolated from the liver. This could be interpreted as indicating that the intestinal mitochondria suffer more damage during isolation than the liver mitochondria; the intestinal mitochondria might nearly have reached the limit of possible swelling, while the liver mitochondria still have some way to go to reach this point. This is unlikely, however, because:

(i) the addition of 1×10^{-3} M thyroxine always stimulated some swelling: if the mitochondria had reached the limit of their swelling one would not expect thyroxine to cause further swelling unless this swelling was of a different origin;

and (ii) the intestinal mitochondria isolated from some species swelled more than those isolated from others. Yet electronmicrographs of the isolated mitochondria did not support the hypothesis that the mitochondria from any one species were more swollen than from another.

Ways in which thyroxine may cause mitochondria to swell are considered in the Discussion (see pp. 154 to 158). The sequence of events occurring during the contraction of mitochondria is also considered.

IV. DISCUSSION

In a sense every organ may be said to depend upon the intestine, as it is through the intestine that the foodstuffs for the rest of the body are absorbed. But although much research has been directed at the intestine as a whole, relatively little has been done with the mitochondria of the intestine compared to the mitochondria of other tissues - such as muscle, liver, kidney, brain, and heart.

The large scale operations possible in the preparation of mitochondria from heart and muscle (Hele, 1954; Crane, Glen, and Green, 1956; Hatefi and Lester, 1958) can not be applied readily to the intestine, but there are no obvious difficulties in working on a smaller scale. It is unfortunate, though, that the rat, which is perhaps the most widely used

experimental animal, has intestinal cells which:

(i) release considerable quantities of mucus, thus making centrifugation of their contents difficult;

and (ii) upon homogenization release an inhibitor of cytochrome oxidase.

Although this may explain why the intestinal mitochondria of the rat are little mentioned in the literature, it does not explain why the intestinal mitochondria of other animals are not mentioned; and the absence of any report (before 1959) about the low oxygen consumption of the intestinal mitochondria of the rat when incubated with succinate is puzzling.

The distribution of mitochondria in intestinal cells.- As was expected all the species examined were found to have mitochondria in their intestinal cells. The mitochondria varied in shape from round to elongate and both types were distributed throughout the cell. The mitochondria in the intestinal cells of mice are distributed similarly (Zetterqvist, 1956), although earlier reports had suggested that the elongate mitochondria were found only between the nucleus and the brush border.

In the intestinal cells of the toad only a few mitochondria were seen in the basal portion of the cell. But the number of mitochondria per cell was lower in the toad than in the other species examined, perhaps because these animals had not been fed for 3 - 4 weeks.

Mitochondria rarely appeared to touch the brush border. This was especially noticeable under phase contrast illumination. A strip, approximately 0.5 μ wide, immediately adjacent to the brush border, could be seen which was apparently free of mitochondria (Figures 21 and 22, p. 66). The existence of this zone was confirmed later by electron microscopy (Figures 35 and 36, pp. 99 and 100).

Some hypotheses about the mitochondria-free zone.- The absence of mitochondria from the zone adjacent to the brush border is puzzling. If the entry of foodstuffs into the cell is controlled by the brush border, there would seem to be an advantage in having mitochondria close to the brush border to supply the necessary energy. But if the absorption of foodstuffs leads to marked changes in the concentration of salts, monosaccharides, amino acids, short chain fatty acids, etc. in the zone next to the brush border, mitochondria situated there may be less efficient at supplying energy than those situated further away, where the environment is more constant. Furthermore, if the area immediately underneath the brush border contains a protein cytoskeleton to make the apical part of the cell more rigid (Puchtler and Leblond, 1958; Palay and Karlin, 1959 a), there may be no room left in this area for mitochondria. Thus, the mitochondria-free zone seems to result from a compromise between the different requirements of the apical part of the intestinal cell.

The cells lining the tubules of the kidney also posses a brush border. Examination of some of the published micrographs of this brush border (Sjostrand and Rhodin, 1953; Sampaio, Brunner, and Filho, 1958) suggests that these cells also have a mitochondria-free zone.

The QO_2 of intestinal cells.- In the present investigation the QO_2 of whole intestinal cells of the rat was found to be 7.65 when no substrate was added. The addition of 0.33% (0.018 M) glucose increased the QO_2 by 25% to 9.55. Table 23 shows that the value obtained by Dickens and Weil-Malherbe (1941) for the QO_2 of similar cells in the presence of 0.2% (0.011 M) glucose was 8.25.

TABLE 23

A COMPARISON OF VARIOUS ESTIMATES OF THE QO_2 OF WHOLE INTESTINAL CELLS OF THE RAT

PART OF INTESTINE	QO_2 IN ABSENCE OF ADDED SUBSTRATE	QO_2 IN 0.2% GLUCOSE (Dickens and Weil-Malherbe, 1941)	QO_2 IN 0.33% GLUCOSE
Duodenum		7.6	
Jejunum		14.1	
Ileum		3.0	
ARITHMETIC MEAN	7.65	8.25	9.55

There is no way of telling whether the differences between the estimates of the QO_2 were caused by the concentration of glucose, the method of preparing the cells (separation *versus* scraping), or the species of the rat (Dickens and Weil-Malherbe may have used a different species; they did not say what species they worked with).

Table 24 shows that the QO_2 of whole intestinal cells is higher than that of many other cells of the rat. *A priori* this is understandable as:

(i) the rate of regeneration of intestinal cells is very fast (about 1 1/3 days in the rat - Leblond and Stevens, 1948), which means there is a great synthetic activity for which a supply of energy is essential;

and (ii) a considerable amount of energy must be required by the cells for the active transport of substances across the mucosa.

TABLE 24

THE QO_2 OF VARIOUS TISSUES OF THE RAT

All figures except that for the intestine were taken from "The handbook of biological data" edited by W.S. Spector, and published by W.B. Saunders Co., Philadelphia in 1956. The QO_2s were estimated in Ringer glucose.

TISSUE	QO_2
Intestinal mucosa	8.25, 9.55
Cerebral cortex	8.5 - 17.1
Liver	6.5 - 11.6
Lung	4.4 - 7.8
Medulla	2.5 - 4.9
Heart	3.8 - 10.4
Skeletal muscle	2.3 - 3.1
Gastric smooth muscle	3.5
Ovary	5.7
Pancreas	5.2
Pituitary	5.2 - 5.9
Prostate	7.6
Spleen	7.2 - 12.9
Testis	7.2 - 14.3
Thymus	5.5 - 5.8
Thyroid	12.5 - 13.0
Uterus	7.6

Some factors which may control the QO_2 of cells.- When the QO_2 of a cell is estimated experimentally, what is measured is the rate of a pacemaker reaction under the existing experimental conditions. With whole cells, both "physical" pacemaker reactions (i.e. those concerned with diffusion and permeability) as well as "chemical" pacemaker reactions (i.e. those directly involving enzymes) may be involved.

As the activation energy constants for the intestinal cells of the rat (13,100) and the lizard (13,200) were found to be similar, the pacemaker reactions controlling the QO_2 of these cells are probably the same in both animals. However, as:

(i) the enzymes in the cells of the lizard were more heat labile than those of the rat;

and (ii) glucose did not affect the oxygen uptake of the intestinal cells of both animals in the same way,

at least some of the enzymatic material must be different in the two animals.

Experimental identification of the pacemaker reaction controlling the QO_2 of intestinal cells would be difficult. If the QO_2 of a suspension of whole cells is to be compared with that of an homogenate prepared from the same suspension, it might be argued, on the one hand, that because the QO_2s were approximately equal, the overall reaction can not be limited by the rate of entry of any compound into (or exit from) the cell. On the other hand, one might argue that in attempting to overcome the problem of permeability, other, perhaps more serious, problems have been created. Whole cells are precisely integrated, whereas in homogenates some of the spatial and temporal relationships have been lost, and consequently, one would predict the QO_2 of an homogenate to be less than that of a whole cell. This argument leads to the conclusion that the diffusion of substances into or out of the cell might well be

controlling the overall rate of the reaction.

But the loss of integration when a cell is homogenized does not affect all reactions equally, because some reactions take place in just one part of the cell. Isolated mitochondria will oxidise succinate by themselves; the presence of other cellular components is not required because the enzymes responsible are normally located in the mitochondria: whereas the enzymes for the oxidation of glucose, for example, are more randomly distributed and cellular components other than mitochondria are normally required (though see p. 22).

The composition of the endogenous substrate in intestinal cells.- Only a small portion of the endogenous substrate can consist of compounds in the act of shuttling round the metabolic sequences, because these compounds would soon disappear unless they were continuously replenished by one of the "key" intermediates such as glucose-6-phosphate, triose phosphate, or acetyl Co A.

The larger portion of endogenous substrate probably consists of "storage" compounds such as glycogen and fatty acids. If intestinal cells are actively absorbing compounds when they are removed from the intestine, then some of the compounds which have just been absorbed may also form part of the endogenous substrate. So although the amount of glycogen in the kidney, lung, and mucosa of the rat is similar (0.87, 0.83, and 0.85 mg/g wet weight of tissue respectively) (Villar-Palasi and Larner, 1960), the mucosa may contain more endogenous substrate than other tissues.

The effect of the medium on the activity of mitochondria.- It was planned to isolate mitochondria in 0.44 M sucrose solution. However, homogenates of intestinal cells of the rat were found to contain mucus which made preparation of mitochondria by differential centrifugation difficult, because the components of the homogenate did not separate easily. In an attempt to lower the viscosity of the suspension, the molarity of the sucrose solution was reduced from 0.44 to 0.25 M. Although this did not solve the problem, it made the separation easier, and 0.25 M sucrose solution was adopted as the standard isolation medium.

The molarity of the medium used to isolate the intestinal mitochondria is mentioned here, before the results are discussed, because the type of medium used to isolate mitochondria often has a considerable bearing on the results obtained. If mitochondria are isolated in a medium of low osmolarity, they swell and rupture and this may affect the activity of their enzymes.

The medium can also affect the results in more subtle ways. For example, the mitochondria of the flight muscles of Locusta migratoria when isolated in 0.2 M sucrose do not show respiratory control, i.e. their respiration is not stimulated by a phosphate acceptor (Rees, 1954); whereas if they are isolated in a medium containing 0.3 M sucrose, 0.0002 M versene, and buffered at pH 7.2 with 0.01 M triethanolamine, they do show respiratory control (Klingenberg and Bucher, 1959). Similarly, the mitochondria of the flight muscles of Musca domestica show respiratory control when isolated in a complex medium (0.25 M sucrose, 0.003 M versene, 0.003 M $MgCl_2$, 0.006 M citrate, 0.0006 M succinate, 0.006 M pyruvate, 0.1 M phosphate, and buffered at pH 7.4 with 0.05 M tris buffer), but if they

are isolated in 0.25 M sucrose only they do not (Gregg, Heisler, and Remmert, 1960; Chance and Sacktor, 1958).

The source of the fatty acids which appear when the intestinal cells of the rat are homogenized.- The inhibition of the succinoxidase system in mitochondria isolated from the intestine of the rat has been attributed in this thesis and by others (Nakamura, Pichette, Broitman, Bezman, Zamcheck, and Vitale, 1959) to the presence of fatty acids. The source of these fatty acids is a matter of conjecture.

The lipide content of the whole intestine of the rat has been shown to be 78 mg/g compared with 43, 38, and 35 mg/g for the liver, kidney, and heart respectively (Marinetti, Witter, and Stotz, 1957). Van Bruggen and his co-workers (Van Bruggen, Yamada, Hutchens, and West, 1954) found that the "gut" of rats contains 36 mg of fatty acids per gram compared to 26 mg/g in liver. This figure probably refers to the whole gut; if so, the fatty acid concentration in the mucosa alone may be many times that of other tissues.

Figures for the fatty acid content of the intestine of other mammals (or even other vertebrates) are not available. If the fatty acid content of the intestinal mucosa of nearly all vertebrates is similar, the inhibition of succinoxidase in intestinal homogenates of the rat can not be explained solely on the basis of an especially high concentration of fatty acids in its mucosa, because suspensions of homogenized intestinal cells from other species exhibit succinoxidase activity.

It was shown in the present investigation that the mitochondria isolated from the intestine of the rat contained approximately three times as much fatty acid as those isolated from the intestine of the other animals examined, or of the

mitochondria isolated from the liver of rats. If it is assumed that the fatty acid content of the intestinal mucosa varies little between species, then the fatty acid content of the mucosa must increase during or after homogenization.

Recently, DiNella, Meng, and Park (1960) reported that the lipase activity of mucosal homogenates of the rat is greater than that of hog, dog, rabbit, or cow (although they did not quote any figures for comparison). If the intestinal mucosa of rats contains an especially active lipase which is released in an active form when the mucosa is homogenized, then the lipase will break down any fat present liberating fatty acids. This hypothesis explains why oxygen uptakes were more often observed in intestinal homogenates of the rat than in suspensions of mitochondria prepared from these homogenates, because the lipase would have more time in which to produce fatty acids during the preparation of mitochondria.

The lipase may also attack those parts of the mitochondrial membrane that are composed of lipid, disrupting the mitochondrial structure and reducing the activity of the enzymes. The mitochondria isolated from rats did not look different under the electron microscope from the mitochondria isolated from the other animals examined, so if such changes do occur, they must be small ones.

If the inhibition of succinoxidase in the homogenates and the mitochondria prepared from the intestine of rats is caused solely by the fatty acid content of the mucosa *per se*, one might expect the inhibition to lessen if the animal fasted and the fatty acid level fell. But neither the homogenate nor the mitochondria prepared from the intestine of rats that had been fasted up to 36 hours showed any increase in succinoxidase activity. This lends negative support to the lipase hypothesis providing the fatty acids involved are not "structural" ones

which are not affected by fasting.

Analogous inhibitions in other tissues.- There have been several other reports of the formation of fatty acids by lipase during the preparation of mitochondria, and of the inhibition of enzymes by fatty acids. When mitochondria are isolated from the larvae of the wax moth Galleria mellonella (Wojtczak and Wojtczak, 1959), or from the thorax of the blow fly Calliphora erythrocephala (Lewis and Fowler, 1960), long chain fatty acids are released enzymatically as soon as the intact tissue is homogenized or crushed. These fatty acids have been shown to inhibit some of the reactions involved in oxidative phosphorylation and to lower the P/O ratio. Similarly, the enzymatic formation of fatty acids from endogenous lipids is thought to cause the lower P/O ratios obtained with "aged" mitochondria isolated from the liver of rats (Lehninger and Remmert, 1959). The active component of mitochrome, the haeme protein from liver mitochondria of rats which uncouples oxidative phosphorylation, has been shown to be a mixture of lipids, including oleic, linoleic, palmitic, and stearic acids (Hulsmann, Elliott, and Rudney, 1958; Hulsmann, Elliott, and Slater, 1960; Wojtczak and Wojtczak, 1960).

Edwards and Ball (1954) have shown that the venom of the cobra and the toxin of Clostridium welchii inhibit succinoxidase because they contain a phospholipase which releases fatty acids.

There is some evidence that low concentrations (approximately 10^{-5} M) of fatty acids inhibit oxidative phosphorylation only, while higher concentrations inhibit succinoxidase and respiration generally. A lipoprotein can be isolated from leucocytes which at low concentrations inhibits oxidative phosphorylation, but which at higher concentrations

inhibits respiration as well. Like the inhibitor from the intestine of the rat, this inhibitor is non-dialysable and heat stable. As lipoproteins are in general rather fragile, it seems likely that the inhibitory effect is due primarily to the lipid portion and the protein has no special inhibitory effect (Fishman and Pullman, 1960).

Villar-Palasi and Larner (1960) have found that extracts of the intestinal mucosa of rats inhibit muscle phosphorylase. The nature of this inhibitor and whether or not it affects other enzymes is not known, but it is conceivable that fatty acids are responsible.

Ways in which fatty acids may inhibit succinoxidase.- The fatty acids which inhibit succinoxidase in the homogenized intestine of the rat were shown to act at the level of cytochrome oxidase (see p. 93).

The fatty acids may disrupt a lipid link between cytochrome oxidase and the preceeding member of the chain. Or perhaps they prevent oxygen reaching the active site of cytochrome oxidase.

The significance of the lipase producing the fatty acids.- The significance of the presence of a powerful lipase in the intestinal cells of the rat is difficult to discover. Although the metabolism of fats in the intestine of the rat is known to differ slightly from that in the guinea pig (for references see Spencer and Knox, 1960), no major differences have yet been discovered. However, Nakamura and his co-workers (Nakamura, Pichette, Broitman, Bezman, Zamcheck, and Vitale, 1959) have pointed out that "the rat, which is rather resistant to the production of arteriosclerosis, is unlike the guinea pig (Duff, 1935), which is particularly sensitive to atheromata". A connection between a lipase in the intestine and arteriosclerosis

seems unlikely on first considerations, but is quite possible. An active lipase might raise the level of unsaturated fatty acids sufficiently to allow most of the free cholesterol to be converted to the harmless esterified form.

The storage of lipase within the intestinal cell.- Whatever function the lipase may have it does not appear to disrupt the metabolism of the intact cell. Potentially dangerous enzymes are usually controlled in one of two ways:

(i) they may be kept in an inactive form and activated only when required;

or (ii) they may be kept in the active form but apart from the substrate.

Rupture of the cell seems more likely to release an already active enzyme from somewhere within the cell than to make an active enzyme from a previously inactive precursor.

It is possible that the lipase in the intestinal cells of the rat is non-functional, and is retained only because features genetically associated with it are advantageous.

The succinoxidase activity of the intestinal mitochondria of vertebrates other than the rat.- Of the animals examined, the toad had the lowest QO_2 N(succinate). The toad also appeared to have the fewest mitochondria per cell. This may indicate that cofactors and soluble enzymes are leached from intestinal mitochondria during their isolation. Hormones or other agents which influence the intracellular environment are unlikely to be the cause because the effect is observed with isolated mitochondria; but it could be explained if structural differences in some mitochondria increased the efficiency of enzymes in these mitochondria. No marked differences in size or internal structure were seen during the histological examination of intestinal mitochondria, but minor differences

could have easily escaped notice. For instance, a change in the ratio of the surface area of the cristae to the volume of the intercristal fluid, although difficult to see, might have a considerable bearing on the integration of the enzymes, and consequently upon the activity of the mitochondrion as a whole.

Paul and Sperling (1952) have also presented evidence to show that the activity of a mitochondrion may be related to the number of mitochondria in the cell from which it was isolated. They found that the QO_2 (succinate) of a crude mitochondrial suspension increased with mitochondrial density (see Table 25).

In view of these observations it might be interesting to estimate the number of mitochondria in the intestinal cells of various species, and so compare the number with the QO_2.

The mitochondria of analogous and of different vertebrate tissues.- The intestinal mitochondria of the rat, guinea pig, chicken, lizard, toad, and carp looked similar under the electron microscope. This was expected because few structural differences have been demonstrated between the mitochondria of analogous tissues in vertebrates.

But differences between tissues do occur. In most of the vertebrates that have been examined, the mitochondria in the liver have few cristae and a matrix which, under the electron microscope, looks much denser than the surrounding cytoplasm (Bernhard and Rouiller, 1956). Intestinal mitochondria contain more cristae, but their matrix is less dense than that of liver mitochondria. Pancreatic mitochondria have numerous densely packed cristae and a relatively clear matrix - under the electron microscope it looks about as dense as the surrounding cytoplasm (Sjostrand and Hanzon, 1954).

The enzymes responsible for supplying energy in living

TABLE 25

THE QO_2* (SUCCINATE) AND MITOCHONDRIAL DENSITY OF VARIOUS TISSUES

The values in this table have been taken from Paul and Sperling (1952).

TISSUE	ANIMAL	QO_2* (SUCCINATE)	MITOCHONDRIAL DENSITY
1. Muscles			
Back	Rabbit	1.1	0
Breast	Chicken	1.5	0
Gastronemicus	Rabbit	2.7	0
Soleus	Rabbit	4.7	+
Leg	Rat	6.7	+
Forearm	Bat	30.6	++
Diaphragm	Rabbit	25.0	++
Breast	Mallard	21.9	+++
Heart	Rabbit	56.7	++++
Breast	Pigeon	33.0	++++
2. Organ			
Kidney	Rabbit	40.3	++++

*Oxygen uptake in ul/hr/mg dry weight of suspension after correction for the salt content. The suspension was made as follows: the tissue was blendorized in a Waring blendor for one minute with 10 volumes of cold 0.9% potassium chloride. It was then passed through a double thickness of gauze to remove fibrous tissue, and centrifuged at 0° C for 8 minutes at 2,500 g. The precipitate was resuspended with 0.9% potassium chloride, and the resultant suspension was used in the experiments.

organisms are closely associated with, or actually a part of, the cristae mitochondrales (Palade, 1952; Green, 1956 b, 1961). The other enzymes known to be present in mitochondria are thought to be located in the matrix; and presumably the density of the matrix (as it appears under the electron microscope) is roughly proportional to the amount of enzymatic material present.

The liver is primarily a synthetic organ and its mitochondria contain many enzymes associated with syntheses. Energy is required for synthetic reactions, but at a steady or constant rate. One would expect the mitochondria of liver to have a dense matrix and just enough cristae for their immediate requirements. Large numbers of "dormant" cristae are not required as in pancreatic and intestinal mitochondria where some cristae, normally held in reserve, are used only during the more or less regular emergencies that arise in these organs. The intestine requires energy for active transport; the pancreas for the synthesis of digestive enzymes and hormones. Neither of these processes is continuous; they occur in cycles, rising to a maximum and then decreasing. One would expect intestinal and pancreatic mitochondria to have relatively more cristae than liver mitochondria, because there must be sufficient cristae (energy) for the periods of maximum activity. The intestine, like the liver, is a synthetic organ, but not on such a vast scale. The matrix of the intestinal mitochondria should be, therefore, less dense than that of liver mitochondria. The pancreas is also a synthetic organ, but it produces mainly proteins. As proteins are known to be synthesized outside the mitochondria, one would expect the matrix of pancreatic mitochondria to be relatively clear.

Table 26 summarizes this cytological and biochemical information. It can be seen that the cytological evidence

TABLE 26

A SUMMARY OF THE CYTOLOGICAL AND BIOCHEMICAL INFORMATION AVAILABLE ABOUT THE MITOCHONDRIA OF THE LIVER, INTESTINE, AND PANCREAS OF VERTEBRATES.

	ORGAN		
	LIVER	INTESTINE	PANCREAS
BIOCHEMICAL FUNCTION	synthetic	synthetic absorptive	specialized synthetic
REQUIREMENTS	numerous compounds; regular production of energy	many compounds; cyclical production of energy	few compounds
	few cristae; dense matrix; many cristae		
EXPECTED APPEARANCE	v. dense matrix; few cristae	dense matrix; many cristae	numerous cristae; clear matrix
ACTUAL CYTOLOGICAL APPEARANCE	v. dense matrix; few, small cristae	dense matrix; many cristae	clear matrix; numerous cristae

agrees with the biochemical prediction about the structure of the various types of mitochondria.

Is the density of the mitochondrial matrix inversely proportional to the number of cristae present?- Rouiller (1960) has suggested that the density of the matrix of mitochondria is inversely proportional to the number of cristae mitochondrales present. But whether this is a universal phenomenom, or whether it only applies to mitochondria in the same type of cell is not clear.

The universal hypothesis is attractive because of its simplicity. A priori, however, there seems to be no biochemical reason why mitochondria should not have many cristae and a dense matrix. To test the hypothesis satisfactorily the mitochondria of different tissues would have to be stained, sectioned, and electronmicrographs made with rigid control. This is necessary because the thickness of the section, the method used to fix and stain the cell, and the contrast of the photographic paper all have a bearing on the final appearance of the electron micrograph; and the use of different methods might cause mitochondrial structures of the same density to look different.

Rouiller's hypothesis may apply only to mitochondria in the same type of cell. If it does, the differences in mitochondrial structure may be observed over a period of time, or at the same time. If the differences are observed over a period of time, they may be the result of some kind of stress, such as inadequate nutrition, disease, or the general aging of the animal. If the differences are observed at the same time, they may be simply the result of small variations which occur without any especial cause. Or perhaps the newly formed mitochondria of all cells are similar to one another, and the cellular differences develop over a period of time. In this case, all tissues should contain some "baby" mitochondria of a standard pattern, and only the adult mitochondria would be characteristic of a tissue.

Some unidentified structures were seen in the intestinal cells of carp which may be immature stages of mitochondria (Figure 38, p. 101).

The dense granules in mitochondria.- The intestinal mitochondria were found to contain dense granules. Similar granules have been seen in the mitochondria of the liver (Bernhard and Rouiller, 1956), pancreas (Sjostrand and Hanzon, 1954), and the brush border and proximal convoluted tubules of the kidney (Sjostrand and Rhodin, 1953). Dense granules are seldom seen in the mitochondria in muscles.

Weiss (1955) found that the mitochondria of the duodenal absorptive cells of mice contained more dense granules when the mice were given a dilute salt solution (0.15 M KCl or NaCl) instead of water to drink. He suggested that the dense granules might be deposits of unwanted cations which the mitochondria had collected from the cell, and which were excreted bodily from the mitochondria and out of the cell as occasion demanded. Weiss suggested that all cells which transport large amounts of cations and water should contain dense granules in their mitochondria. This is difficult to reconcile with the presence of dense granules in liver mitochondria, and their absence in the mitochondria of such protozoans as Paramecium, Amoeba, and Euplotes (Powers, Ehret, Roth, and Minick, 1956; Pappas and Brandt, 1959); although it might be argued that the mitochondria of such protozoans are not comparable as their cristae have a different structure and their mitochondria may represent a side-line in the evolutionary trend - perhaps mitochondria took over the job of collecting and excreting cations fairly late in their evolution (from the cell wall and contractile vacuole?). However, Weiss' hypothesis does fit in nicely with the known situation in the cells of the intestinal mucosa, proximal convoluted tubules of the kidney and

possibly also the pancreas, on the one hand; and on the other, those of muscles.

Dense granules are rarely seen in isolated mitochondria. This is not surprising, if, as Weiss suggested, the granules are bags of cations, because one would expect a bag of cations to be delicate and easily ruptured. Alternatively, if the dense granules are able to withstand moderately rough treatment, they may be set free, either actively or passively, from the mitochondria during the isolation process. Once outside the mitochondria the dense granules would be difficult to distinguish from mitochondrial or other cellular debris, even if they precipitated with the mitochondrial fraction and did not stay in suspension. This difficulty of recognition arose during the present investigation. In the isolated mitochondria in which the matrix appeared to be in the process of being lost (Figures 47 and 48, p. 109), structures were seen which might have been dense granules - but which might equally have well been fragments of the mitochondrial membrane.

Sub-mitochondrial particles.- The damage sustained by the intestinal mitochondria upon isolation seemed to be the result of a connected series of events. The mitochondria swelled and their cristae appeared to migrate to one side. As the wall ruptured and the mitochondrial matrix was lost, circular vesicles were formed, either from the cristae or from the mitochondrial wall. The remainder of the cristae then spread randomly over what was left of the original mitochondrion.

These changes are similar to some of those seen by Green and his co-workers during the isolation of mitochondria from ox heart. That similar forms were found is not surprising. Probably all mitochondria, from whatever source, would react similarly if their osmotic equilibrium was similarly upset.

Whether or not Green's lipid-rupturing reagents split the

sub-mitochondrial particles of all mitochondria into similar fragments, however, is open to question. Moreover, the fragments which are formed may recombine in different ways, forming pieces of structural biochemistry quite different from any which exist in the intact mitochondrion.

The distribution of enzymes within the intestinal cell.- The electron micrographs of the isolated intestinal mitochondria showed that:

(i) the mitochondria were damaged;
and (ii) the mitochondrial pellet contained some microsomal material.

This should be bourne in mind when interpreting the biochemical results obtained with the isolated mitochondria. Furthermore, the site at which the enzymatic material is concentrated may not be that at which the reaction takes place in the living cell. But, as Schneider (1959) has pointed out, although studies of the distribution of enzymes in cellular fractions can not predict where in the intact cell a reaction occurs, they do predict where the reaction is most likely to occur. A more difficult problem is to decide whether the enzymes present in low concentrations in isolated mitochondria are adsorbed onto the mitochondria during their isolation.

In the present investigation it was found that, of the enzymes examined, only TPN isocitric dehydrogenase was distributed differently in the intestinal cells of the chicken and lizard. That most of the enzymes were similarly distributed in the two species was expected, because one is more surprised when a difference is found in the distribution of an enzyme belonging to such a fundamental metabolic sequence as the Krebs cycle.

No DPN isocitric dehydrogenase was found in the intestinal cells of either the chicken or the lizard despite an attempt to demonstrate it.

Prediction of the behaviour of an hormone.- If there are difficulties in extrapolating from the distribution of an enzyme in an homogenate to the site of reaction in the cell, they seem small when one is trying to extrapolate from the known effect of a hormone *in vitro* to its physiological effect *in vivo*. This is especially so when the hormone is thyroxine, which has different effects on different animals. Thyroxine increases the basal metabolic rate of mammals and birds, causes metamorphosis in amphibians, and promotes nitrogen excretion in fish. It seems scarcely possible that all these effects can be brought about by the same hormone affecting the same metabolic reaction. An uncoupling of oxidative phosphorylation might account for the higher basal metabolic rate of homiotherms, but how could it affect metamorphosis or the excretion of nitrogen?

The effect in mammals will be considered first.

Lehninger's mechano-enzyme hypothesis about thyroxine-induced mitochondrial swelling.- Low concentrations of thyroxine cause liver mitochondria to swell; slightly higher concentrations uncouple oxidative phosphorylation. Lehninger (1960 a) has suggested that thyroxine causes mitochondria to swell by the following mechanism. He visualized in the mitochondrial membrane a "mechano-enzyme" (M) which can control the permeability of the membrane and which also is associated with oxidative phosphorylation, thus:

1. $\text{carrier}_{\text{reduced}} + \text{acceptor}_{\text{oxidized}} + X \rightleftharpoons \text{carrier}_{\text{oxidized}} \sim X + \text{acceptor}_{\text{reduced}}$

2. $\text{carrier}_{\text{oxidized}} \sim X + Pi \rightleftharpoons \text{carrier}_{\text{oxidized}} + P \sim X$

3. $P \sim X + E \rightleftharpoons P \sim E + X$

$P \sim E + ADP \rightleftharpoons ATP + E$

$P \sim E + M \rightleftharpoons M \sim P + E$

Lehninger suggested that when M is phosphorylated the membrane is contracted and swelling does not occur. He suggested that thyroxine prevents the phosphorylation of M, thus allowing the mitochondria to swell.

Although this hypothesis explains how swelling occurs it does not explain how oxidative phosphorylation is uncoupled. It does not explain the action of dinitrophenol (DNP) which uncouples oxidative phosphorylation but prevents mitochondrial swelling. (DNP is thought to interfere with reaction 2 (Bronk, 1960); this explains the uncoupling, but one would expect concomitant swelling as the failure of reaction 2 would prevent phosphorylation of the mechano-enzyme.) Lehninger's hypothesis does not explain why swelling can occur only while respiration is proceeding.

A modified mechano-enzyme hypothesis.- A modification of Lehninger's hypothesis will fit the above facts. Instead of M~P preventing swelling, it must be postulated that M—X does.

1. $\text{carrier}_{\text{reduced}} + \text{aceptor}_{\text{oxidised}} + X \rightleftharpoons \text{carrier}_{\text{oxidized}} \sim X + \text{acceptor}_{\text{reduced}}$

2. $\text{carrier}_{\text{oxidized}} \sim X + Pi \rightleftharpoons \text{carrier}_{\text{oxidized}} + P \sim X$

3. $P \sim X + E \rightleftharpoons P \sim E + X \longrightarrow X - M$

4. $P \sim E + ADP \rightleftharpoons ATP + E$

M—X causes the membrane to become impermeable and therefore swelling can not occur. When respiration starts, X is released from M—X and swelling occurs as the mechano-enzyme is not in the combined form. When respiration and phosphorylation are proceeding, X is regenerated by reaction 3, X—M is reformed and swelling is inhibited. DNP inhibits swelling and uncouples oxidative phosphorylation by forming the complex, $\text{carrier}_{\text{oxidized}} \sim$ DNP, thus:

$$\text{carrier}_{\text{oxidized}} \sim X + DNP \longrightarrow \text{carrier}_{\text{oxidized}} \sim DNP + X$$

which prevents the phosphorylation reactions and also releases X, which prevents swelling. Thyroxine combines with X. Low concentrations of thyroxine cause mitochondrial swelling by preventing the formation of M—X. As the concentration of thyroxine increases, the compound $\text{carrier}_{\text{oxidized}} \sim X$ is gradually robbed of its X until no phosphorylation is possible. This gradual removal of X may explain why respiration in the presence of thyroxine changes from "tightly coupled" to "loosely coupled" respiration (Hoch and Lipman, 1954).

The reason why thyroxine does not uncouple oxidative phosphorylation in sub-mitochondrial particles remains to be explained. It may that thyroxine, as such, is not the compound which combines with X. Thyroxine may have to be converted to the active form before combination can occur. If mitochondria can catalyze this reaction but sub-mitochondrial particles can not, the situation is explained. Bronk's recent finding that tetra-iodothyroacetic acid and tri-iodothyroacetic acid caused an immediate inhibition of phosphorylation in sub-mitochondrial particles (Bronk, 1960) lends support to this hypothesis.

In the above discussion it is assumed that changes in the permeability of the inner mitochondrial membrane are responsible for the swelling phenomenom. The outer membrane is thought to be permeable to sucrose at all times (Tedeschi and Harris, 1958). Thyroxine is thought to affect only the inner mitochondrial membrane and the cristae mitochondrales (Lehninger, Ray, and Schneider, 1959; Lehninger, 1960 a). How this fits in with the electron micrographs of isolated mitochondria is not clear. Lehninger, Ray, and Schneider (1959) have shown that mitochondria isolated in low molarity sucrose (0.1 - 0.5 M) swelled much more in the presence of thyroxine than those isolated in 0.5 - 1.0 M

sucrose. Yet the above hypothesis about the way in which thyroxine acts presupposes an intact membrane which mitochondria isolated in 0.25 M sucrose do not seem to have. Perhaps parts of the ruptured mitochondria still have a space into which sucrose may penetrate and which is still surrounded by an intact membrane.

In order to explain how thyroxine uncouples oxidative phosphorylation in mitochondria which do not swell in the presence of thyroxine (e.g. the mitochondria of the heart and skeletal muscles of the rat - Tapley and Cooper, 1956 b; Vitale, Nakamura, and Hegsted, 1957; Wiswell and Braverman, 1957), it is necessary to postulate that the mechano-enzyme, M, either has different affinities, or is not present in the membranes of these mitochondria.

A simpler hypothesis about the action of thyroxine.- A simpler hypothesis about the action of thyroxine might be built on the observation of Vitale and his co-workers that the concentration of magnesium in the serum of thyrotoxic animals is lower than normal (Vitale, Hegsted, Nakamura, and Connors, 1957). Many enzymes, besides those involved in oxidative phosphorylation, have a requirement for Mg^{++}. If it is postulated that an enzyme system which prevents mitochondrial swelling is more sensitive to lack of magnesium than the reactions involved in oxidative phosphorylation, then a low concentration of thyroxine would cause mitochondrial swelling without uncoupling oxidative phosphorylation. This is in agreement with the observation that a higher concentration of thyroxine is required to uncouple oxidative phosphorylation than is required to bring about mitochondrial swelling (Lehninger, Ray, and Schneider, 1959). This hypothesis also explains why the addition of magnesium antagonizes the uncoupling of oxidative

phosphorylation by thyroxine. Magnesium is also known to protect mitochondria from thyroxine-induced swelling (Lehninger, 1960 a).

Thyroxine-induced mitochondrial contraction.- It was shown in the present investigation that thyroxine was more effective at inducing swelling in the liver mitochondria of higher than of lower vertebrates. Thyroxine caused the intestinal mitochondria of higher vertebrates to swell, but it inhibited the endogenous swelling and even caused the contraction of the mitochondria of the lower vertebrates. Yet thyroxine caused uncoupling of oxidative phosphorylation in all the species examined. These results can be explained by assuming that:

(i) liver mitochondria are more sensitive to thyroxine than intestinal mitochondria;

(ii) the enzymes associated with oxidative phosphorylation in the mitochondria of lower vertebrates are less sensitive to thyroxine than those in higher vertebrates;

and (iii) the immediate effect of thyroxine is to cause oxidative phosphorylation to become loosely coupled; complete uncoupling occurs sometime later, depending on the thyroxine concentration.

It can thus be explained why the liver mitochondria of the lower vertebrates swelled less in the same concentration of thyroxine than did the liver mitochondria of higher vertebrates. The contraction that is observed when the intestinal mitochondria of the carp are put into 1×10^{-4} M thyroxine is also explained. In these mitochondria, to which only the near minimum stimulatory concentration of thyroxine was added, oxidative phosphorylation becomes at first loosely coupled. A burst of respiration ensues with a concomitant but transient increase in ATP. Packer and Golder (1960) have suggested that an increase in ATP causes mitochondria to contract. Consequently,

the sudden increase in ATP causes a sudden contraction of the intestinal mitochondria, which then start to swell again as oxidative phosphorylation is uncoupled completely and the amount of ATP in the mitochondria decreases. The contraction of the mitochondria must be strong enough, in this instance, to over-ride the swelling caused by the action of thyroxine on the mechano-enzyme. The intestinal mitochondria must possess a mechano-enzyme because 1×10^{-3} M thyroxine stimulated swelling.

The sequence of events by which certain concentrations of thyroxine inhibit the endogenous swelling of mitochondria, but do not cause observable contraction, is not clear. Perhaps the concentration of thyroxine which allows contraction is very critical, so that rarely is enough ATP formed to cause a contraction which is easily observable. A little more, or a little less than the critical amount of thyroxine may allow enough ATP to be formed to delay the endogenous swelling for a few seconds, but not enough to cause observable contraction.

Some support for the above hypothesis is given by the recent work of Sokoloff and Kaufman (1961). They found that increasing the concentration of thyroxine from 1×10^{-7} to 6.4×10^{-4} M increased by up to 77% the rate of amino acid incorporation into protein of cell-free liver homogenates prepared from the rat. But between a concentration of 6.5×10^{-4} to 7.5×10^{-4} M thyroxine this stimulation of amino acid incorporation changed abruptly to an 80% inhibition. Evidently the concentration of thyroxine can be very critical in other systems, too.

Other known effect of thyroxine.- The way in which thyroxine brings about metamorphosis in amphibians and increases nitrogen excretion in fish is not known. It is known, however, that thyroxine increases the synthesis of enzymes in the liver of the tadpole (Paik and Cohen, 1960). In the liver of the rat thyroxine has been shown to increase the formation of fatty acids (Spirtes, Medes, and Weinhouse, 1953), cholesterol (Fletcher and Myant, 1958), and glycogen (Burton, Robbins, and Byers, 1957). It is probable that at least some of these stimulations aid metamorphosis. And the increase in the turnover of amino acids mentioned above (Sokoloff and Kaufman, 1961) may be a contributing factor to the stimulation of nitrogen excretion in fish.

Thyroxine has a large range of effects on vertebrates. Although part of this range can be demonstrated in a single animal *in vitro*, it is probable that *in vivo* the range is limited still further by the specificity of the enzymes.

V. APPENDIX

The "t" test used to find out whether there was a significant difference between the arithmetic means of the values obtained for the QO_2 of whole intestinal cells of the rat and the lizard was based on the equation:

$$t_{m+n-2} = \frac{\bar{x} - \bar{y}}{s\sqrt{1/m + 1/n}}$$

where m = number of experiments in one set of experiments;

n = number of experiments in the other set;

$\bar{x}$ = arithmetic mean of the results of the first set of experiments;

$\bar{y}$ = arithmetic mean of the results of the second set;

$$s^2 = \frac{\leq(x - \bar{x})^2 + \leq(y - \bar{y})^2}{m + n - 2}$$

and t measures the probability that a difference as large as $\bar{x} - \bar{y}$ might arise by chance when sampling from two groups.

TABLE 27

PROBABILITY CALCULATION FOR A SIGNIFICANT DIFFERENCE BETWEEN THE ARITHMETIC MEANS OF THE VALUES OBTAINED FOR THE QO_2 OF THE WHOLE INTESTINAL CELLS OF THE RAT AND LIZARD.

	QO_2s OF RAT	QO_2s OF LIZARD
	3.7	5.8
	4.7	7.2
	5.3	7.2
	6.9	7.6
	7.7	8.5
	8.2	
	8.7	
	8.8	
	9.2	
	9.8	
	11.0	
Arithmetic mean	7.65	7.3
$\leq(x - \bar{x})^2$	51.37	
$\leq(y - \bar{y})^2$	4.0	
$\leq(x - \bar{x})^2 + \leq(y - \bar{y})^2$	55.37	
$(m + n) - 2$	14.0	
s^2	3.98	
s	1.995	
$\sqrt{1/m + 1/n}$	0.539	
$s\sqrt{1/m + 1/n}$	1.076	
$\bar{x} - \bar{y}$	0.35	
t_{14}	0.326	
Probability	$0.8 > p > 0.7$	

TABLE 28

ESTIMATE WITHIN 95% CONFIDENCE LIMITS OF THE $QO_2(N)$ IN THE PRESENCE OF SUCCINATE OF THE INTESTINAL MITOCHONDRIA OF VARIOUS VERTEBRATES.

EXPERIMENT	CARP	TOAD	LIZARD	CHICKEN	GUINEA PIG	RAT
1	307	172	405	447	372	15 in 16 experiments
2	333	223	451	518	403	
3	361	240	462	522	428	
4	380	-	501	570	448	
5	398	-	518	571	465	
Arithmetic mean	356	212	467	526	420	

Variance of QO_2 in these vertebrates $= \frac{1799}{18} = 99.9$

Variance of mean of 5 observations $= \frac{1799}{18 \times 5} = 20.0$

Standard deviation of the mean of 5 observations (Standard Error) $= \sqrt{\frac{1799}{18 \times 5}} = \pm 4.47$

95% confidence limits of the mean of any 5 observations $= \bar{x} \pm t \sqrt{\frac{1799}{18 \times 5}} = \bar{x} \pm 12.6$

Variance of mean of 3 observations $= \frac{1799}{18 \times 3} = 33.3$

Standard deviation of the mean of 3 observations (Standard Error) $= \sqrt{\frac{1799}{18 \times 3}} = \pm 5.77$

95% confidence limits of the mean of any 3 observations $= \bar{x} \pm t \sqrt{\frac{1799}{18 \times 3}} = \bar{x} \pm 24.9$

ESTIMATE OF QO_2 VALUES WITHIN 95% CONFIDENCE LIMITS

Carp	343 - 369
Toad	187 - 237
Lizard	454 - 480
Chicken	513 - 539
Guinea Pig	407 - 433

TABLE 29

PROBABILITY CALCULATION FOR A SIGNIFICANT DIFFERENCE BETWEEN THE QO_2(N)s IN THE PRESENCE OF SUCCINATE OF THE INTESTINAL MITOCHONDRIA OF VARIOUS SPECIES

SUM OF SQUARES

EXPERIMENT	CARP		TOAD		LIZARD		CHICKEN		GUINEA PIG	
	x	x^2	x	x^2	x	x^2	x	x^2	x	x^2
1	307	94249	172	29584	405	164025	447	199809	372	138384
2	333	110889	223	49729	451	203401	518	268324	403	162409
3	361	130321	240	57600	462	213444	522	272484	428	183184
4	380	144400	-	-	501	251001	570	324900	448	200704
5	397	158404	-	-	518	268324	571	326041	465	216225
TOTAL	1779	638263	635	136913	2337	1100195	2628	1391558	2116	900906

ANIMAL	TOTAL	SUM OF SQUARES
CARP	1779	638263
TOAD	635	136913
LIZARD	2337	1100195
CHICKEN	2628	1391558
GUINEA PIG	2116	900906
	9495	4167835

Total sum of squares = $Sx^2 - \frac{(Sx)^2}{23} = 4167835 - \frac{90155025}{23} = 248052$.....(1)

Sum of squares between treatments = $\frac{(Sxc)^2}{5} + \frac{(Sxt)^2}{3} + \frac{(Sxl)^2}{5} + \frac{(Sxc)^2}{5} + \frac{(Sxg)^2}{5} - \frac{(Sx)^2}{23}$

= 631968 + 134408 + 1092314 + 1381277 + 895491 - 3919783

= 215675.....(2)

Sum of squares within treatments = (2) - (1) = 248052 - 215675 = 32377

SOURCES OF VARIANCE	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	VARIANCE
BETWEEN TREATMENTS	4	215675	53919	30***
WITHIN TREATMENTS	18	32377	1799	-
TOTAL	22	248052	-	-

The difference between the QO_2s of the various species is highly significant.

TABLE 30

PROBABILITY CALCULATIONS FOR SIGNIFICANT DIFFERENCES BETWEEN THE ARITHMETIC MEANS OF THE VALUES OBTAINED FOR THE DISTRIBUTION OF ENZYMES OF THE KREBS CYCLE IN THE INTESTINAL CELLS OF THE RAT AND LIZARD

	DPNH CYTOCHROME C REDUCTASE		FUMARASE		MALIC DEHYDROGENASE	
% ENZYME ACTIVITY	CHICKEN	LIZARD	CHICKEN	LIZARD	CHICKEN	LIZARD
	24	26	30	21	15	16
	29	22	22	14	18	-
	-	27	-	-	-	-
	-	26	-	-	-	-
ARITHMETIC MEAN	26.5	25.25	26	17.5	16,5	16.0
$\Sigma (x - \bar{x})^2$	12.5		32.0		4.5	
$\Sigma (y - \bar{y})^2$	14.7		24.5		0	
$\Sigma(x - \bar{x})^2 + \Sigma (y - \bar{y})^2$	27.2		56.5		4.5	
$(m + n) - 2$	4		2		1	
s^2	6.8		28.3		4.5	
s	2.6		5.3		2.1	
$\sqrt{1/m + 1/n}$	0.9		1		1.2	
$s\sqrt{1/m + 1/n}$	2.3		5.3		2.6	
$\bar{x} - \bar{y}$	1.3		8.5		0.5	
t	0.6		1.6		0.2	
PROBABILITY	$0.6 > p > 0.7$		$0.2 > p > 0.3$		$0.8 > p > 0.9$	

TABLE 31

PROBABILITY CALCULATIONS FOR SIGNIFICANT DIFFERENCES BETWEEN THE ARITHMETIC MEANS OF THE VALUES OBTAINED FOR THE DISTRIBUTION OF ENZYMES OF THE KREBS CYCLE IN THE INTESTINAL CELLS OF THE RAT AND LIZARD

	ISOCITRIC DEHYDROGENASE		ACONITASE		DIAPHORASE	
	CHICKEN	LIZARD	CHICKEN	LIZARD	CHICKEN	LIZARD
% ENZYME ACTIVITY	23	14	33	30	31	19
	20	15	34	29	-	25
	24	13	-	-	-	-
	21	13	-	-	-	-
	17	-	-	-	-	-
ARITHMETIC MEAN	21.0	13.8	33.5	29.5	31	22
$\Sigma(x - \bar{x})^2$	30.0		0.5		0	
$\Sigma(y - \bar{y})^2$	2.8		0.5		18	
$\Sigma(x - \bar{x})^2 + \Sigma(y - \bar{y})^2$	32.8		1		18	
$(m + n) - 2$	7		2		1	
s^2	4.8		0.5		18	
s	2.2		0.7		4.2	
$\sqrt{1/m + 1/n}$	0.8		1.0		1.2	
$s\sqrt{1/m + 1/n}$	1.5		0.7		5.2	
$\bar{x} - \bar{y}$	7.3		4.0		9.0	
t	5.0		5.7		1.7	
PROBABILITY	$0.001 > p > 0.01$		$0.02 > p > 0.05$		$0.3 > p > 0.4$	

VI. REFERENCES

(The pages in this section are not numbered but bear the initial letter of authors' names on the top right hand corner.)

Abercrombie, M., Hickman, C. J., and Johnson, M. L. (1951).- "A dictionary of biology," p. 246. (Penguin Books Ltd.: Harmondsworth, Middlesex.)

Abood, L. G. (1954).- Mechanism of inhibition of phosphorylation in brain mitochondria by electrical impulses. Am. J. Physiol. 176: 247 - 252.

Abood, L. G. (1955).- Effect of chlorpromazine on phosphorylation of brain mitochondria. Proc. Soc. Exp. Biol. and Med. 88: 688 - 690.

Aebi, H., and Abelin, I. (1953).- Elektrolyt-und Fermenthaushalt der hyperthyreotischen Leber. Biochem. Z. 324: 364 - 384.

Ahmed, K., and Scholefield, P. G. (1960).- Effects of fatty acids on the ^{32}P. adenosine triphosphate exchange reaction in rat liver mitochondria. Nature 186: 1046 - 1047.

Allard, C., Mathieu, R., de Lamirarande, G., and Cantero, A. (1952).- Mitochondrial population in mammalian cells. I. Description of a counting technic and preliminary results on rat liver in different physiological and pathological conditions. Cancer Research 12: 407 - 412.

Altmann, R. (1890).- "Die Elementarorganismen und ihre Beziehungen zu den Zellen." (Veit and Co.: Leipzig.)

Antopol, W., Glaubach, S., and Goldman, C. (1948).- Effects of a new tetrazolium derivative on tissue bacteria and onion root tips. Pub. Health Rep. 63: 1231 - 1238.

Arrhenius, S. (1899).- Über die Reaktionsgeschwindigeit bei der Inversion von Rohrzucker duch Sauren. Z. phys. Chem. 4: 226 - 248.

Arrhenius, S. (1907).- "Immunochemistry." (Macmillan: New York.)

Asher, L. (1908).- Das Verhalten des Darmepithelels bei verschiedened funktionellen Zustanden. Z. fur Biol. 51: 115

Basford, R. E., Tisdale, H. D., Glenn, J. L., and Green, D. E. (1957).- Studies on the terminal electron transport system. VII. Further studies on the succinic dehydrogenase complex. Biochim. et Biophys. Acta 24: 107 - 115.

Bartholomew, J. W., and Levin, R. (1955).- The structure of Saccharomyces carlsbergensis and S. cerevisiae as determined by ultra-thin sectioning methods and electron microscopy. J. Gen Microbiol. 12: 473 - 477.

Bates, H. M., and Simpson, M. V. (1959).- The net synthesis of cytochrome c in calf-heart mitochondria. Biochim. et Biophys. Acta 32: 597 - 599.

Beams, H. W., and Tahmisian, T. N. (1954).- Structure of the mitochondria in the male germ cells of Helix as revealed by the electron microscope. Exptl. Cell Research 6: 87 - 93.

Beinert, H. (1951).- The extent of artificial redistribution of cytochrome c in rat liver homogenates. J. Biol. Chem.

Beinert, H., and Crane, F. L. (1956).- The function of the electron transferring flavoprotein in the first oxidative step of the fatty acid cycle. In "Inorganic nitrogen metabolism," pp. 601 - 626. (Ed. W. D. McElroy and B. Glass.) (The John Hopkins Press: Baltimore.)

Benda, C. (1900).- Weitere Beobachtungen uber die Mitochondria und ihr verhaltnis zu sekret Granulationen. Arch. Anat. Physiol. 24: 166 - 187.

Bennet, H. S., and Porter, K. R. (1953).- An electron microscope study of sectioned breast muscle of the domestic fowl. Am. J. Anat. 93: 61 - 105.

Bensley, R. R., and Hoerr, N. L. (1934).- Studies on the cell structure by the freezing-drying method. VI. The preparation and properties of mitochondria. Anat. Rec. 60: 449 - 455.

Bernhard, W., and Rouiller, C. (1956).- Close topographical relationship between mitochondria and ergastoplasm of liver cells in a definite phase of cellular activity. J. Biophys. Biochem. Cytol. 2, No. 4, suppl.: 73 - 78.

Best, C. H., and Taylor, N. B. (1955).-"The physiological basis of medical practice." (The Williams and Wilkins Co.: Baltimore.)

Belitzer, V. A., and Tsibakowa, E. T. (1939).- Sur le mecanisme des phosphorylations couples avec la respiration. Biokimyia 4: 534 - 535.

Beyer, R. E. (1958).- Vitamin k, a component of the mitochondrial oxidative phosphorylation system. Biochim. et Biophys. Acta 28: 663 - 664.

Block, K. (1956).- Synthesis of branched chain compounds. In "Essays in biochemistry," pp. 24 - 34. (Ed. S. Graff.) (John Wiley and Sons, Inc.: New York.)

Bodansky, O. (1939).- The energy of activation of the hydrolysis of sodium beta-glycerophosphate by bone phosphatase at optimal pH. J. Biol. Chem. 129: 197 - 206.

Bourne, G. (1942).- "Cytology and cell physiology," p. 99. (Oxford University Press.)

Bradfield, J. R. G. (1950).- The localization of enzymes in cells. Biol. Rev. 25: 113 - 157.

Brady, R. O., and Gurin, S. (1952).- Biosynthesis of fatty acids by cell-free or water-soluble enzyme systems. J. Biol. Chem. 199: 421 - 431.

Brody, T. M., and Bain, J. A. (1951).- Effect of barbiturates on oxidative phosphorylation. Proc. Soc. Exp. Biol. and Med. 77: 50 - 53.

Bronk, J. R. (1960).- The influence of thyroxine and related compounds on oxidative rate and efficiency of phosphorylation in liver mitochondria and sub-mitochondrial particles. Ann. N. Y. Acad. Sci. 86: 494 - 505.

Burton, S. D., Robbins, E., and Byers, S. O. (1957).- Effect of hyperthyrodism on glycogen content of the isolated rat liver. Am. J. Physiol. 188: 509 - 513.

Cantoni, G. L., and Durrell, J. (1957).- Activation of methionine for transmethylation. II. The methionine-activating enzyme: studies on the mechanism of the reaction. J. Biol. Chem. 225: 1033 - 1048.

Causy, G., and Hoffman, H. (1955).- Cytoplasmic synthesis in nerve cells. Brit. J. Cancer 9: 666 - 673.

Champy, C (1911).- Recherches sur l'absorption intestinale et le role des mitochondries dans l'absorption et la secretion. Arch. d'anat. micr. 13: 55. (Quoted from C. C. Macklin and M. T. Macklin, (1932).- The intestinal epithelium. In "Special cytology," vol 1, pp. 231 - 332. (Ed. E. V. Cowdry.) (Paul B. Hoeber Inc.: New York.)

Chance, B. (1952).- Spectra and reaction kinetics of respiratory pigments of homogenized and intact cells. Nature 169: 215 - 221.

Chance, B. (1954).- Enzyme mechanisms in living cells. In "Mechanism of enzyme action," pp. 399 - 460. (Ed. W. D. McElroy and B. Glass.) (John Hopkins Press: Baltimore.)

Chance, B., and Sacktor, B. (1958).- Respiratory metabolism of insect flight muscle. II. Kinetics of respiratory enzymes in flight muscle sarcosomes. Arch. Biochem. Biopys. 76: 509 - 531.

Chance, B., and Williams, G. R. (1955).- Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. J. Biol. Chem. 217: 383 - 393.

Chance, B., and Williams, G. R. (1956).- The respiratory chain and oxidative phosphorylation. Advances in Enzymol. 17: 65 - 134.

Chantrenne, H. (1947).- Hetrogeneite des granules cytoplasmiques du foie de souris. Biochim. et Biophys. Acta 1: 437 - 448.

Chapell, J. B., and Greville, G. D. (1954).- Effect of silver ions on mitochondrial adenosine triphosphatase. Nature 174: 930 - 931.

Chapell, J. B., and Greville, G. D. (1958).- Dependence of mitochondrial swelling on oxidizable substrates. Nature 182 813 - 814.

Chin, C., and Bueding, E. (1954).- Occurence of oxidative phosphorylation in the muscle of Ascaris lumbricoides. Biochim. et Biophys. Acta 13: 331 - 337.

Claude, A. (1944).- The constitution of mitochondria and microsomes, and the distribution of nucleic acid in the cytoplasm of a leukemic cell. J. Exp. Med. 80: 19 - 29.

Claude, A. (1946).- Fractionation of mammalian liver cells by differential centrifugation. I. Problems, methods and preparation of extract. J. Exp. Med. 84: 51 - 59.

Conglio, J. G., and Cate, D. L. (1958).- The distribution and biosynthesis of palmitic and stearic acids in liver, intestine, and carcass of intact normal and fasted rats. J. Biol. Chem. 232: 361 - 368.

Cornforth, J. W., Cornforth, R. H., Popjack, G., and Gore, I. Y. (1958).- Studies on the biosynthesis of cholesterol. 5. Biosynthesis of squalene from DL-3-hydroxy-methyl-(2-^{14}C)pentano-5-lactone. Biochem J. 69: 146 - 155.

Cota-Robles, E. H., Marr, A. G., and Nilson, E. H. (1958).- Submicroscopic particles in extracts of Azobacter agilis. J. Bacteriol. 75: 243 - 252.

Cowdry, E. V. (1924).- "General cytology," p. 313. (The University of Chicago Press: Illinois.)

Cowdry, E. V. (1926).- Surface film theory of the function of mitochondria. Amer. Nat. 60: 157 - 165.

Crane, F. L., and Glenn, J. L. (1957).- Studies on the electron transport system with deoxycholate. Biochim. et Biophys. Acta 24: 100 - 107.

Crane, F. L., Glenn, J. L., and Green, D. E. (1956).- Studies on the electron transfer system. IV. The electron transfer particle. Biochim. et Biophys. Acta 22: 475 - 487.

Crane, F. L., Hatefi, Y., Lester, R. L., and Widmer, C. (1957).- Isolation of a quinone from beef heart mitochondria. Biochim. et Biophys. Acta 25: 220 - 221.

Crane, F. L., Hauge, J. G., and Beinert, H. (1955).- Flavoproteins involved in the first oxidative step of the fatty acid cycle. Biochim. et Biophys. Acta 17: 292 - 294.

Crane, F. L., Widmer, C., Lester, R. L., and Hatefi, Y. (1959).- Studies on the electron transport system. XV. Coenzyme Q (Q_{275}) and the succinoxidase activity of the electron transport particle. Biochim. et Biophys. Acta 31: 476 - 489.

Crane, R. K., and Sols, A. (1953).- The association of hexokinase with particulate fractions of brain and other tissue homogenates. J. Biol. Chem. 203: 273 - 292.

Cross, R. J., Taggart, J. V., Covo, G. A., and Green, D. E. (1949).- Studies on the cytophorase system. VI. The coupling of oxidation and phopsphorylation. J. Biol. Chem. 177: 655 - 678.

Dalton, A. J. (1951).- Electron microscopy of epithelial cells of the gastro-intestinal tract and pancreas. Am. J. Anat. 89: 109 - 133.

Dalton, A. J., and Felix, M. D. (1957).- Electron microscopy of mitochondria and the golgi complex. S. E. B. Symposia X: 148 - 159.

Danielli, J. F. (1946).- Establishment of cytochemical techniques. Nature 157: 755 - 757.

Davson, H., and Danielli, J. F. (1943).- "The permeability of natural membranes," p. 136. (Cambridge University Press.)

de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955).- Tissue fractionation studies. 6. Intrcellular distribution patterns of enzymes in rat-liver tissue. Biochem. J. 60: 604 - 617.

Dempsy, E. W. (1956).- Variations in the structure of mitochondria. J. Biophys. Biochem. Cytol. 2, No. 4, suppl.: 85 - 89.

Dickens, F., and Salmony, D. (1956).- Effects of thyroid hormones in vitro on tissue respiration, oxidative phosphorylation and the swelling of mitochondria. Biochem. J. 64: 645 - 651.

Dickens, F., and Weil-Malherbe, H. (1941).- Metabolism of normal and tumour tissue. 19. The metabolism of intestinal mucous membrane. Biochem. J. 35: 7 - 15.

Dickman, S. R., and Speyer, J. F. (1954).- Factors affecting the activity of mitochondrial and soluble aconitase. J. Biol. Chem. 206: 67 - 75.

DiNella, R. R., Meng, H. C., and Park, C. R. (1960).- Properties of intestinal lipase. J. Biol. Chem. 235: 3076 - 3081.

Dituri, F., Shaw, W. N., Warms, J. V. B., and Gurin, S. (1957).- Lipogenesis in particle-free extracts of rat liver. I. Substrates and cofactor requirements. J. Biol. Chem. 226: 407 - 416.

DuBuy, H. G., and Hesselbach, M. L. (1958).- Glucose utilization by washed mitochondria of normal and neoplastic mouse tissues. J. Nat. Cancer Inst. 20: 403 - 415.

Duel, H. J. (1951).- "The Lipids," vol. 1. (Interscience publishers: New York.)

Duff, G. L. (1935).- Experimental cholesterol arteriosclerosis and its relationship to human arteriosclerosis. Arch. Path. 20: 81 - 123 and 259 - 304.

Edelhoch, H., Hayaishi, O., and Teply, L. J. (1952).- The preparation and properties of a soluble diphosphopyridine nucleotide cytochrome c reductase. J. Biol. Chem. 197: 97 - 104.

Edwards, G. A., and Ruska, H. (1955).- The function and metabolism of certain insect muscles in relation to their structure. Quart. J. Microscop. Sci. 96: 151 - 159.

Edwards, S. W., and Ball, E. G. (1954).- The action of phospholipases on succinate oxidase and cytochrome oxidase. J. Biol. Chem. 209: 619 - 633.

Eichel, H. J. (1957).- Intracellular localization of enzymes in spleen. I. Reduced diphosphopyridine nucleotide cytochrome c reductase, cytochrome c oxidase, and succinic dehydrogenase in the rat and the guinea pig. J. Biophys. Biochem. Cytol. 3: 397 - 412.

Eichenberger, M. (1953).- Elektronmikroskopische Beobachtungen uber die Entstehung der Mitochondrien aus Mikrosomen. Exptl. Cell Reasearch 4: 275 - 285.

Ekholm, R., and Sjostrand, F. S. (1957).- The ultrastructural organization of the mouse thyroid gland. J. Ultrastructure Research 1 178 - 199.

Emmelot, D., and Bos, C. J. (1957).- The effect of thyroxine on the swelling of mitochondria isolated from normal and neoplastic livers. Exptl. Cell Research 12: 191 - 195.

Ernster, L. (1956).- Organization of mitochondrial DPN-linked systems. Exptl. Cell Research 10: 704 - 720.

Fawcett, D. W. (1955).- Observations on the cytology and electron microscopy of hepatic cells. J. Nat. Cancer Inst. 15: 1475 - 1511.

Ferreira, D. (1957).- L'ultrastructure des cellules du pancreas endocrine chez l'embryon et le rat noveau-ne. J. Ultrastructure Research 1: 14 - 25.

Fiske, C.H., and Subbarow, Y. (1925).- The colorimetric determination of phosphorous. J. Biol. Chem. 66: 375 - 400.

Fletcher, K., and Myant, N. B. (1958).- Influence of the thyroid on the synthesis of cholesterol by liver and skin in vitro. J. Physiol. 144: 361 - 372.

Fishman, M., and Pullman, M. E. (1960).- An uncoupler of oxidative phosphorylation from leucocyte mitochondria exhibiting bactericidal activity. Fed. Proc. 19: 39.

Gallager, C. H., Judah, J. D., and Rees, K. R. (1956).- Glucose oxidation by brain mitochondria. Biochem. J. 62: 436 - 440.

Gale, E. F. (1956).- Nucleic acids and enzyme synthesis. In "Enzymes: units of biological structure and function," pp. 49 - 66. (Ed. O. H. Gaebler.) (Academic Press Inc.: New York.)

Gale, E. F., and Folkes, J. P. (1954).- Effect of nucleic acids on protein synthesis and amino-acid incorporation in disrupted staphylococcal cells. Nature 173: 1223 - 1227.

Glauert, A. M., and Hopwood, D. A. (1959).- A membranous component of the cytoplasm in Streptomyces coelicolor. J. Biophys. Biochem. Cytol. 6: 515 - 518.

Green, D. E. (1951).- The cyclophorase system. In "Enzymes and enzyme systems," pp. 15 - 46. (Ed. J. T. Edsall.) (Harvard University Press: Cambridge, Massachusetts.)

Green, D. E. (1956 a).- The structure of the electron transport system of mitochondria. In "Proceedings of the third international congess of biochemistry," pp. 281 - 284. (Ed. C. Liebeiq.) (Academic Press Inc.: New York.)

Green, D. E. (1956 b).- The structure and enzymatic pattern of the electron transport system. In "Enzymes: units of biological structure and function," pp. 465 - 481. (Ed. O. H. Gaebler.) (Academic Press Inc.: New York.)

Green, D. E. (1958).- Studies in organized enzyme systems. Harvey Lectures 52: 177 - 227.

Green, D. E. (1959 a).- Mitochondrial structure and function. In "Subcellular particles," pp. 84 - 103. (Ed. T. Hayashi.) (American Physiological Society: Washington, D. C.)

Green, D. E. (1956 b).- Electron transport and oxidative phosphorylation. Advanc. Enzymol. 21: 73 - 129.

Green, D. E. (1959 c).- Mitochondrial structure and function. Laboratory Investigation 8: 443 - 459.

Green, D. E., and Crane, F. L. (1958).- Structure of the mitochondrial electron transport system. In "Proceedings of the international symposium on enzyme chemistry," pp. 275 - 286. (Tokyo and Kyoto, 1957.)

Green, D. E., and Hatefi, Y. (1961).- The mitochondrion and biochemical machines. Science 133: 13 - 19.

Green, D. E., Jarnfelt, J., and Tisdale, H. D. (1959).- Studies on the electron transport system. XIV. The isolation and properties of soluble cytochrome c_1. Biochim. et Biophys. Acta 31: 24 - 46.

Green, D. E., Lester, R. L., and Ziegler, D. M. (1957).- Studies on the mechanism of oxidative phosphorylation. I. Preparation and properties of a phosphorylating electron transport particle from beef heart mitochondria. Biochim. et Biophys. Acta 23: 516 - 524.

Green, D. E., Mackler, B., Repaske, R., and Mahler, H. R. (1954).- DPNH oxidase. Biochim. et Biophys. Acta 15: 435 - 437.

Green, D. E., Mii, S., and Kahout, P. M. (1955).- Studies on the terminal electron transport system. I. Succinic dehydrogenase. J. Biol. Chem. 217: 551 - 567.

Gregg, C. T., Heisler, C. R., and Remmert, L. F. (1960).- Oxidative phosphorylation and respiratory control in housefly mitochondria. Biochim. et Biophys. Acta 45: 561 - 570.

Grisolia, S., and Cohen, P. P. (1951).- Study of citrulline synthesis with soluble enzyme preparations. J. Biol. Chem. 191: 189 - 202.

Hartiala, K., and Grossman, M. I. (1952).- Studies on chemical and physical changes in duodenal mucus. J. Biol. Chem. 195: 251 - 256.

Hatefi, Y. (1959).- Activation of cytochrome oxidase by a mitochondrial lipoprotein. Biochim. et Biophys. Acta 30: 648 - 649.

Hatefi, Y., and Lester, R. L. (1958).- Studies on the mechanism of oxidative phosphorylation. III. Phosphorylating particle types from beef heart. Biochim. et Biophys. Acta 27: 83 - 88.

Hatefi, Y., Lester, R. L., Crane, F. L., and Widmer, C. (1959).- Studies on the electron transport system. XVI. Enzymic oxidoreduction reaction of coenzyme Q. Biochim. et Biophys. Acta 31: 490 - 501.

Harman, J. W. (1950).- The selective staining of mitochondria. Stain Technology 25: 69 - 72.

Harman, J. W., and Feiglson, M. (1952).- Studies on mitochondria. III. The relationship of structure and function of mitochondria from heart muscle. Exptl. Cell Research 3: 47 - 58.

Harvey, E. B. (1946).- Structure and development of the clear quarter of the Arbacia punctula egg. J. Exptl. Zool. 102: 253 - 275.

Hele, P. (1954).- The acetate activating enzyme of beef heart. J. Biol. Chem. 206: 671 - 676.

Hesselbach, M. L., and DuBuy, H. G. (1953).- Localization of glycolytic and respiratory enzyme systems on isolated mouse brain mitochondria. Proc. Soc. Exp. Biol. and Med. 83: 62 - 69.

Hoch, F. L., and Lipman, F. (1954).- The uncoupling of respiration and phosphorylation by thyroid hormones. Proc. Natl. Acad. Sci. 40: 909 - 921.

Hochstein, P. (1957).- Glycolysis by tumour mitochondria and the action of insulin. Science 125: 496 - 498.

Hoerr, N. L. (1943).- Methods of isolation of the morphological constituents of the liver cell. Biol. Symp. 10: 185 - 231.

Hoffman, H., and Grigg, G. W. (1958).- An electron microscopic study of mitochondrial formation. Exptl. Cell Research 15: 118 - 131.

Hogeboom, G. H. (1949).- Cytochemical studies of mammalian tissues. I. The distribution of diphosphopyridine nucleotide-cytochrome c reductase in rat liver fractions. J. Biol. Chem. 177: 847 - 858.

Hogeboom, G. H. (1955).- Fractionation of cell components of animal tissues. In "Methods in enzymology," vol. I, pp. 16 - 19. (Ed. S. P. Colowick and N. O. Kaplan.) (Academic Press Inc.: New York.)

Hogeboom, G. H., and Schneider, W. C. (1950 a).- Cytological studies of mammalian tissues, III. Isocitric dehydrogenase and triphosphopyridine nucleotide cytochrome c reductase of mouse liver. J. Biol. Chem. 186: 417 - 427.

Hogeboom, G. H., and Schneider, W. C. (1950 b).- Intracellular distribution of enzymes. VIII. The distribution of diphosphopyridine nucleotide-cytochrome c reductase in normal mouse liver and mouse hepatoma. J. Nat. Cancer Inst. 10: 983 - 987.

Hogeboom, G. H., Schneider, W. C., and Palade, G. E. (1948).- Cytochemical studies of mammalian tissues. I. Isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material. J. Biol. Chem. 172: 619 - 635.

Hollander, F., and Lauber, F. U. (1948).- Eugenol as a stimulus for gastric mucus secretion. Proc. Soc. Exp. Biol. and Med. 67: 34 - 37.

Horning, E. S. (1926).- Studies on the mitochondria of Paramoecium. Australian J. Exp. Biol. and Med. Science 3: 89 - 95.

Horning, E. S. (1928).- Studies on the behaviour of mitochondria within the living cell. Australian J. of Exp. Biol. and Med. Sci. 5: 143 - 148.

Howatson, A. F. (1956).- The structure of pigeon breast muscle mitochondria. J. Biophys. Biochem. Cytol. 2, No. 4, appl.: 363 - 368.

Hulsmann, W. C., Elliott, W. B., and Rudney, H. (1958).- The nature and action of mitochrome. Biochim. et Biophys. Acta 27: 663 - 664.

Hulsmann, W. C., Elliott, W. B., and Slater, E. C. (1960).- The nature and mechanism of action of uncoupling agents present in mitochrome preparations. Biochim. et Biophys. Acta 39: 267 - 276.

Hulsmann, W. C., and Slater, E. C. (1957).- Relationship between the hydrolysis of adenosine triphosphate and oxidative phosphorylation. Nature 180: 372 - 374.

Hunter, F. E., Levy, J. F. Fink, J., Schultz, B., Guerra, F., and Hurwitz, A. (1959).- Studies on the mechanism by which anaerobiosis prevents swelling of mitochondria in vitro: effect of electron transport chain inhibitors. J. Biol. Chem. 234: 2176 - 2186.

Jacobs, E. E., Jacob, M., Sanadi, D. R., and Bradley, C. B. (1956).- Uncoupling of oxidative phosphorylation by cadmium ion. J. Biol. Chem. 223: 147 - 156.

Jalling, O., Low, H., Ernster, L., and Lindberg, O. (1957).- The relation of the site of action of amytal to oxidative phosphorylation. Biochim. et Biophys. Acta 26: 231 - 232.

Jarnfelt, J., Basford, R. E., Tisdale, H. R., and Green, D. E. (1958).- Studies on the terminal electron transport system. XII. Fragmentation of the succinic dehydrogenase complex. Biochim. et Biophys. Acta 29: 123 - 132.

Jeener, R. (1952).- Studies on the evolution of nucleoprotein fractions of the cytoplasm during the growth of a culture of Polytomella caeca. II. Rate of synthesis of nucleic acid, studied by means of labelled phosphate. Biochim. et Biophys. Acta 8: 270 - 282.

Jeener, R., and Szafarz, D. (1950).- Relations between the rate of renewal and the intracellular localization of ribonucleic acid. Arch. Biochem. 26: 54 - 67.

Judah, J. D., and Williams-Ashman, H. G. (1951).- The inhibition of oxidative phosphorylation. Biochem. J. 48: 33 - 42.

Kalchar, H. (1937).- Phosphorylation in kidney tissues. Enzymologia 2: 47 - 52.

Kaplan, E. H., Still, J. L., and Mahler, H. R. (1951).- Studies on the cyclophorase system. XVIII. The oxido-reductions of glycolysis. Arch. Biochem. 34: 16 - 23.

Kaufman, H. P., and Nitsch, W. H. (1954).- Die Papier-Chromatographie auf dem Fettgebiet. XVI. Weitere Versuche zur Trennung von Fettsäuren. Fette u Siefen 56: 154 - 158.

Kaufman, S. (1955).- alpha ketoglutaric dehydrogenase system and phosphorylating enzyme from heart muscle. In "Methods in enzymology," vol. I, pp. 714 - 722. (Ed. S. P. Colowick and N. O. Kaplan.) (Academic Press Inc.: New York.)

Keilin, D. (1929).- Cytochrome and respiratory enzymes. Proc. Roy. Soc., B 104: 206 - 152.

Keilin, D. (1930).- Cytochrome and intracellular oxidase. Proc. Roy. Soc., B 106: 418 - 444.

Keilin, D. (1933).- Cytochrome and intracellular respiratory enzymes. Ergebn. Enzymforsch. 2: 239 - 271.

Keilin, D., and Hartree, E. F. (1937).- Preparation of pure cytochrome c from heart muscle and some of its properties. Proc. Roy. Soc., B 122: 298 - 308.

Keilin, D., and Hartree, E. F. (1938).- Cytochrome a and cytochrome oxidase. Nature 141: 870 - 871.

Keilin, D., and Hartree, E. F. (1939).- Cytochrome a and cytochrome oxidase. Proc. Roy. Soc., B 127: 167 - 191.

Keilin, D., and Hartree, E. F. (1940).- Succinic dehydrogenase-cytochrome system catalyzing aerobic oxidation of succinic acid. Proc. Roy. Soc., B 129: 227 - 306.

Keilin, D., and King, T. E. (1958).- Reconstitution of the succinic oxidase system from a soluble succinic dehydrogenase and a particulate cytochrome system preparation. Nature 181: 1520 - 1522.

Kennedy, E. P. (1953).- Synthesis of phosphatides in isolated mitochondria. J. Biol. Chem. 201: 399 - 412.

Kennedy, E. P., and Lehninger, A. L. (1948).- Intracellular structures and the fatty acid oxidase system of rat liver. J. Biol. Chem. 172: 847 - 848.

Kennedy, E. P., and Weiss, S. B. (1956).- The function of cytidine coenzymes in the biosynthesis of phospholipids. J. Biol. Chem. 222: 193 - 214.

Kielly, R. J. (1957).- Inhibitory effect of some amino-azo dyes and other carcinogens on glutamate oxidation in mitochondria of riboflavine-deficient rat liver. J. Nat. Cancer Inst. 19: 1077 - 1085.

Kingsbury, B. F. (1912).- Cytoplasmic fixation. Anat. Rec. 6: 39 - 52.

Klemperer, H. G. (1955).- The uncoupling of oxidative phosphorylation in rat-liver mitochondria by thyroxine, tri-iodothyronine, and related substances. Biochem. J. 60: 122 - 128.

Kline, E. S., and Warardekar, V. S. (1960).- Inhibitor of succinoxidase activity from Hydra litteralis. J. Biol. Chem. 235: 1803 - 1808.

Klingenberg, M., and Bucher, T. (1959).- Flugmuskel-mitochondrien aus Locusta migratoria mit Atmungskontrolle. Biochem. Z. 331: 312 - 333.

Kornberg, A., and Prior, M. E. (1953).- Enzymatic esterification of alpha-glycerophosphate by long chain fatty acids. J. Biol. Chem. 204: 345 - 357.

Kuff, E. L. (1954).- The distribution of fumarase activity in mouse liver homogenates. J. Biol. Chem. 207: 361 - 365.

Langdon, R. G. (1957).- The biosynthesis of fatty acids in rat liver. J. Biol. Chem. 226: 615 - 629.

Lardy, H. A., and Wellman, H. (1952).- Oxidative phosphorylations: role of inorganic phosphate and acceptor systems in control of metabolic rates. J. Biol. Chem. 195: 215 - 224.

Lazarow, A. (1943).- The chemical structure of cytoplasm investigated in Professor Bensley's laboratory during the past ten years. Biol. Symp. 10: 9 - 26.

Leblond, C. P., and Stevens, C. E. (1948).- The renewal of the intestinal epithelium in the albino rat. Anat. Rec. 100: 357 - 377.

Lehninger, A. L. (1949).- Esterification of inorganic phosphate coupled to electron transport between dihydrophosphopyridine nucleotide and oxygen. J. Biol. Chem. 178: 625 - 644.

Lehninger, A. L. (1956).- Physiology of mitochondria. In "Enzymes: units of biological structure and function," pps. 217 - 233. (Ed. O. H. Gaebler.) (Academic Press Inc.: New York.)

Lehninger, A. L. (1960 a).- Thyroxine and the swelling and contraction cycle in mitochondria. Ann. N. Y. Acad. Sci. 86: 484 - 493.

Lehninger, A. L. (1960 b).- Oxidative phosphorylation in sub-mitochondrial systems. Federation Proc. 19: 952 - 962.

Lehninger, A. L., Ray, B. L., and Schneider, M. (1959).- The swelling of rat liver mitochondria by thyroxine and its reversal. J. Biophys. Biochem. Cytol. 5: 97 - 108.

Lehninger, A. L., and Remmert, L. F. (1959).- An endogenous uncoupling and swelling agent in liver mitochondria and its enzymatic formation. J. Biol. Chem. 234: 2459 - 2464.

Lerner, A. B., and Barnum, C. P., (1946).- The ultra-violet absorption of the plasma proteins. Arch. Biochem. 10: 417 - 425.

Lewis, S. E., and Fowler, K. S. (1960).- Nature and origin of an inhibitor of oxidative phosphorylation present in freshly isolated sarcosomes from blowfly thoraces. Biochim. et Biophys. Acta 38: 564 - 565.

Lewis, S. E., and Slater, E. C. (1954).- Oxidative phosphorylation in insect sarcosomes. Biochem. J. 58: 207 - 217.

Lindberg, O., and Ernster, L. (1954).- Chemistry and physiology of mitochondria and microsomes. Protplasmatologia III (A4): 1 - 136.

Lindegren, C. C. (1949).- "The yeast cell, its genetics and cytology." (Educational Publ.: St Louis.)

Linnane, A. W. (1958).- A soluble component required for oxidative phosphorylation by a sub-mitochondrial particle from beef heart muscle. Biochim. et Biophys. Acta 30: 221 - 222.

Linnane, A. W., and Still, J. L. (1955).- The isolation of respiring mitochondria from baker's yeast. Arch. Biochem. 59: 383 - 392.

Linnane, A. W., and Ziegler, D. M. (1958).- Studies on the mechanism of oxidative phosphorylation. V. The phosphorylating electron transport particle. Biochim. et Biophys. Acta 29: 630 - 638.

Lipmann, F. (1958).- Biological sulfate activation and transfer. Science 128: 575 - 580.

Loomis, W. F., and Lipmann, F. (1948).- Reversible inhibition of the coupling between phosphorylation and oxidation. J. Biol. Chem. 173: 807 - 808.

Low, F. N. (1956).- Mitochondrial structure. J. Biophys. Biochem. Cytol. 2, No. 4, suppl.: 337 - 340.

Mackler, B., and Green, D. E. (1956).- Studies on the electron transport system. II. On the opening phenomenom. Biochim. et Biophys. Acta 21: 1 - 6.

Mackler, B., and Penn, B. (1957).- Studies on the electron transport system. IX. Fragmentation of DPNH oxidase. Biochim. et Biophys. Acta 24: 294 - 300.

Macklin, C. C., and Macklin, M. T. (1932).- The intestinal epithelium. In "Special cytology," pp. 231 - 332. (Ed. E. V. Cowdry.) (Paul B. Hoeber Inc.: New York.)

Mahler, H. R. (1957).- Biological oxidations. Ann. Rev. Biochem. 26: 17 - 62.

Mahler, H. R., Sarkar, N. K., Vernon, L. P., and Alberty, R. A. (1952).- Studies on diphosphopyridine nucleotide-cytochrome c reductase. II. Purification and properties. J. Biol. Chem. 199: 585 - 597.

Marinetti, G. V., Witter, R. F., and Stotz, E. (1957).- The incorporation in vivo of P^{32} labelled orthophosphate into individual phosphatides of rat tissues. J. Biol. Chem. 226: 475 - 484.

Mark, J. S. T. (1956).- An electron microscope study of uterine smooth muscle. Anat. Rec. 125: 473 - 493.

Marr, A. G. (1960).- Localization of enzymes in bacteria. In "The bacteria, vol. I, pp. 443 - 468. (Ed. I. C. Gunsalus and R. Y. Stanier.) (Academic Press Inc.: New York.)

Marston, H. R. (1923).- The azine and azonium compounds of the proteolytic enzymes. Biochem. J. 17: 851 - 859.

Marston, H. R. (1926).- On the acceleration of enzymatic synthesis of proteins by lipoidal emulsions. Australian J. Exp. Biol. and Med. Sci. 3: 233 - 236.

McLean, J. R., Cohn, G. L., Brandt, I. K., and Simpson, M. V. (1958).- Incorporation of labelled amino acids into the protein of muscle and liver mitochondria. J. Biol. Chem. 233: 657 - 663.

McMurray, W. C., Maley, F. G., and Lardy, H. A. (1958).- Oxidative phosphorylation by sonic extracts of mitochondria. J. Biol. Chem. 230: 219 - 229.

Meeves, F. (1908).- Die Chondriosomen als Trager erblicher Anlagen. Cytologishe Studien am Huhnerembryo. Arch. Mikrosk. Anat. u. Entw. gesch. 72: 816 - 867.

Meeves, F. (1918).- Eine neue Stutze fur die Plastosomen-theorie der Vererbung. Anat Anz. 50: 551 - 557.

Mehler, A. H., Kornberg, A., Grisolia, S., and Ochoa, S. (1948).- The enzymatic mechanism of oxidation-reductions between malate or isocitrate and pyruvate. J. Biol. Chem. 174: 961 - 977.

Michaelis, L. (1931).- Der Acetat-Veronal-Puffer. Biochem. Z. 234: 139 - 141

Moore, D. H., and Ruska, H. (1957).- Electron microscope study of mammalian cardiac muscle cells. J. Biophys. Biochem. Cytol. 3 261 - 268.

Morton, R. K. (1954).- The purification of alkaline phosphatase of animal tissues. Biochem. J. 57: 595 - 603.

Murray, R. G. E. (1960).- The internal structure of the cell. In "The bacteria," vol. I, pp. 35 - 96. (Ed. I. C. Gunsalus and R. Y. Stanier.) (Academic Press: New York.)

Myers, D. K., and Slater, E. C. (1957 a).- The enzymic hydrolysis of adenosine triphosphate by liver mitochondria. I. Activities at different pH values. Biochem. J. 67: 558 - 572.

Myers, D. K., and Slater, E. C. (1957 b).- The enzymic hydrolysis of adenosine triphosphate by liver mitochondria. 2. Effect of inhibitors and added cofactors. Biochem. J. 67: 572 - 579.

Nachlas, M. N., Morgulies, S. I., and Seligman, A. M. (1960).- Site of electron transfer to terazolium salts in the succinoxidase system. J. Biol. Chem. 235: 2739 - 1743.

Nakamura, M., Pichette, P., Broitman, S., Bezman, A. L., Zamcheck, N., and Vitale, J. J. (1959).- Studies on the inhibitory effect of intestinal mucosa on succinoxidase activity. J. Biol. Chem. 234: 206 - 210.

Neirhiser, D. H., and Wells, W. W. (1959).- The structure of methostenol and its distribution in rat tissues. Arch. Biochem. Biophys. 81: 300 - 308.

Nizamuddin, M. (1960).- "Studies on the anatomy, morphology, and taxonomy of Southern Australian Fucales and the genus Notheia." (Ph. D. Thesis: University of Adelaide.)

Novikoff, A. B. (1956).- Microscopy and differential centrifugation. In "Proceedings of the third international congess of biochemistry," pp. 315 - 317. (Ed. C. Liebecq.) (Academic Press Inc.: New York.)

Oberling, C. (1959).- The structure of cytoplasm. Intern. Rev. Cytol. 8: 1 - 32.

Ochoa, S. (1941).- "Coupling" of phosphorylation with oxidation of pyruvic acid in brain. J. Biol. Chem. 138: 751 - 773.

Ochoa, S. (1948).- Biosynthesis of tricarboxylic acids by carbon dioxide fixation. III. Enzymatic mechanisms. J. Biol. Chem. 174: 133 - 157.

Packer, L., and Golder, R. H. (1960).- Correlation of structural and metabolic changes accompanying the addition of carbohydrates to Ehrlich ascites tumor cells. J. Biol. Chem. 235: 1234 - 1240.

Le Page, G. A., and Schneider, W. C. (1948).- Centrifugal fractionation of glycolytic enzymes in tissue homogenates. J. Biol. Chem. 176: 1021 - 1027.

Paik, W. K., and Cohen, P. P. (1960).- Biochemical studies on amphibian metamorphosis. I. The effect of thyroxine on protein synthesis in the tadpole. J. Gen. Physiol. 43: 683 - 696.

Palade, G. E. (1952).- Fine structure of mitochondria. Anat. Rec. 114: 427 - 451.

Palade, G. E. (1953).- An electron microscope study of mitochondrial structure. J. Histochem. Cytochem. 1: 188 - 211.

Palay, S. L., and Karlin, L. J. (1959 a).- An electron microscope study of the intestinal villus. I. The fasting animal. J. Biophys. Biochem. Cytol. 5: 363 - 372.

Palay, S. L., and Karlin, L. J. (1959 b).- An electron microscope study of the intestinal villus. II. The pathway of fat absorption. J. Biophys. Biochem. Cytol. 5: 373 - 384.

Pappas, G. D., and Brant, P. W. (1959).- Mitochondria. I. Fine structure of the complex patterns in the mitochondria of Pelomyxa carolinensis. J. Biophys. Biochem. Cytol. 6: 85 - 90.

Paul, M. H., and Sperling, E. (1952).- Cyclophorase system. XXIII. Correlation of cyclophorase activity and mitochondrial density in striated muscle. Proc. Soc. Exp. Biol. 79: 352 - 354.

Peterson, A. R., Conn, H. J., and Melin, C. G. (1934).- Methods for the standardization of biological stains. The triphenylmethane derivatives. Stain Technology 9: 41 - 48.

Popjak, G., and Tietz, A. (1955).- Biosynthesis of fatty acids in cell-free preparations. 2. Synthesis of fatty acids from acetate by a soluble enzyme system prepared from rat mammary gland. Biochem. J. 60: 147 - 155.

Portier, P. (1919).- Reponse a la communication de C. Regaud. Comptes rendus de la Soc. de Biologie LXXXII: 247 - 250.

Puchtler, H., and Leblond, C. P. (1958).- Histochemical analysis of cell membranes and associated structures as seen in the intestinal epithelium. Amer. J. Anat. 102: 1 - 32.

Rabinowitz, M., and de Bernard, B. (1957).- Studies on the electron transport system. X. Preparation and spectral properties of a particulate DPNH and succinate cytochrome c reductase from heart muscle. Biochim. et Biophys. Acta 26: 22 - 29.

Racker, E. (1950).- Spectrophotometric measurements of the enzymatic formation of fumaric and cis-aconitic acids. Biochim. et Biophys. Acta 4: 211 - 214.

Rees, K. R. (1954).- Aerobic metabolism of the muscle of Locusta migratoria. Biochem J. 58: 196 - 202.

Regaud, C. (1909).- Etudes sur la structure des tubes seminiferes et la spermatogenese chez les mammiferes. Arch. d'Anat. Micr. 11: 291 - 431.

Reichard, P., Smith, L. H., and Hanshoff, G. (1955).- Enzymic synthesis of ureidosuccinic acid from citrulline via compound X and carbamyl phosphate. Acta. Chem. Scand. 9: 1010 - 1012.

Rhodin, J. (1954).- Correlation of ultrastructural organization and function in normal and experimentally changed proximal convoluted tubule cells of mouse kidney. (Ahkiebolaget Godvil: Stockholm.)

Robertson, J. D. (1959).- The ultrastructure of cell membranes and their derivatives. Biochem. Soc. Symposia. 16: 3 - 43.

Rodbell, M., and Hanahan, D. J. (1955).- Some aspects of the metabolism of lecithin and its derivatives in liver. J. Biol. Chem. 214: 595 - 606.

Rouiller, C. (1960).- Physical and pathological changes in mitochondrial morphology. International Rev. Cytol. 9: 227 - 292.

Rouiller, C., and Bernhard, W. (1956).- "Microbodies" and the problem of mitochondrial regeneration in liver cells. J. Biophys. Biochem. Cytol. 2, No. 4, suppl.: 355 - 360.

Sacktor, B. (1954).- Investigations on the mitochondria of the housefly, Musca domestica. III. Requirements for oxidative phosphorylation. J. Gen. Physiol. 37: 343 - 359.

Sampaio, M. M., Brunner, A., and Filho, B. O. (1958).- Aspects of the ultrastructure of the brush border of the kidney of normal mouse. J. Biophys. Biochem. Cytol. 4: 355 - 356.

Schachter, D., and Taggart, J. V. (1953).- Benzoyl coenzyme A and hippurate synthesis. J. Biol. Chem. 203: 925 - 934.

Schneider, W. C. (1948).- Intracellular distribution of enzymes. III. The oxidation of octanoic acid by rat liver fractions. J. Biol. Chem. 176: 259 - 266.

Schneider, W. C. (1959).- Mitochondrial metabolism. Advances in Enzymol. 19: 1 - 72.

Schneider, W. C., and Hogeboom, G. H. (1951).- Cytochemical studies of mammalian tissues: the isolation of cell components by differential centrifugation. Cancer Research 11: 1 - 22.

Schneider, W. C., and Potter, V. R. (1949).- Intracellular distribution of enzymes. IV. The distribution of oxaloacetic oxidase activity in rat liver and rat kidney fractions. J. Biol. Chem. 177: 893 - 903.

Scholefield, P. G. (1956).- Studies of fatty acid oxidation. 5. The effect of decanoic acid on oxidative phosphorylation. Can. J. Biochem. Physiol. 34: 1227 - 1232.

Schultz, H., Low, H., Ernster, L., and Sjostrand, F. S. (1957).- Elektronen-mikroskopische Studien an Leberschnitten von Thyroxin-behandelten Ratten. In "Electron microscopy - Proceedings of the Stockholm conference, September 1956," pp. 134 - 137. (Ed. F. S. Sjostrand and J. Rhodin.) (Almqvist and Wiksell: Stockholm.)

Sedar, A. W., and Rudzinska, M. A. (1956).- Mitochondria of protozoa. J. Biophys. Biochem. Cytol. 2, No. 4, suppl.: 331 - 336.

Seligman, A. M., and Rutenberg, A. M. (1951).- The histochemical demonstration of succinic dehydrogenase. Science 113: 317 - 320.

Shelton, E., Schneider, W. C., and Striebich, M. J. (1953).- A method for counting mitochondria in tissue homogenates. Exptl. Cell Research 4: 32 - 41.

Shepherd, J. A. (1955).- Factors infuencing the determination of the intracellular distribution of isocitric dehydrogenase in rabbit cerebral cortex. J. Histochem. Cytochem. 4: 47 - 54.

Shepherd, J. A., and Kalnitsky, G. (1954).- Intracellular distribution of fumarase, aconitase, and isocitric dehydrogenase in rabbit cerebral cortex. J. Biol. Chem. 207: 605 - 611.

Shinohara, C., Fukushi, K., and Suzuki, J. (1957).- Mitochondria-like structures in ultrathin sections of Mycobacterium avium. J. Bacteriol. 74: 413 - 415.

Siekevitz, P. (1952).- Uptake of radioactive alanine *in vitro* into the protein of rat liver fractions. *J. Biol. Chem.* **195**: 549 - 565.

Siekevitz, P., and Potter, V. R. (1953).- Intramitochondrial regulation of oxidative rate. *J. Biol. Chem.* **201**: 1 - 13.

Siekevitz, P., and Potter, V. R. (1955).- Biochemical structure of mitochondria. II. Radioactive labeling of intramitochondrial nucleotides during oxidative phosphorylation. *J. Biol. Chem.* **215**: 237 - 255.

Simpson, M. V., and McLean, J. R. (1955).- The incorporation of labeled amino acids into the cytoplasmic particles of rat muscle. *Biochim. et Biophys. Acta* **18**: 573 - 575.

Sizer, I. W. (1938).- Sucrose inversion by baker's yeast as a function of temperature. *J. Gen. Physiol.* **21**: 695 - 706.

Sizer, I. W. (1939).- Temperature activation of the urease-urea system using crude and crystalline urease. *J. Gen. Physiol.* **22**: 719 - 741.

Sizer, I. W. (1943).- Effects of temperature on enzyme kinetics. *Advances in Enzymol.* **3**: 35 - 62.

Sjostrand, F. S. (1953 a).- Electron microscopy of mitochondrial and cytoplasmic double membranes. Ultrastructure of rod shaped mitochondria. Nature 171: 30 - 31.

Sjostrand, F. S. (1953 b).- The ultrastructure of the inner segments of the retinal rods of the guinea pig eye as revealed by electron microscopy. J. Cell. and Comp. Physiol. 42: 45 - 70.

Sjostrand, F. S. (1954).- Unpublished observation quoted from H. Zetterqvist. (1956).- "The ultrastructural organization of the columnar absorbing cells of the mouse jejunum. (Ahtiebolaget Godvil: Stockholm.)

Sjostrand, F. S. (1956).- The ultrastructural organization of retinal rods and cones. J. Applied Phys. 24: 117.

Sjostrand, F. S., and Hanzon, V. (1954).- Membrane structures of cytoplasm and mitochondria in exocrine cells of mouse pancreas as revealed by high resolution electron microscopy. Exptl. Cell Research 7: 393 - 414.

Sjostrand, F. S., and Rhodin, J. (1953).- The ultrastructure of the proximal convoluted tubules of the mouse kidney as revealed by high resolution electron microscopy. Exptl. Cell Research 4: 426 - 439.

X

Slater, E. C. (1949 a).- A comparative study of the succinic dehydrogenase-cytochrome system in heart muscle and kidney. Biochem. J. 45: 1 - 8.

Slater, E. C. (1949 b).- The action of inhibitors on the system of enzymes which catalyze the aerobic oxidation of succinate. Biochem. J. 45: 8 - 13.

Slater, E. C. (1949 c).- A respiratory catalyst required for the reduction of cytochrome c by cytochrome b. Biochem. J. 45: 14 - 30.

Slater, E. C. (1950 a).- The components of the dihydrocozymase oxidase system. Biochem. J. 46: 484 - 499.

Slater, E. C. (1950 b).- The dihydrocozymase-cytochrome c reductase activity of heart muscle preparation. Biochem. J. 46: 499 - 508.

Slater, E. C. (1956).- Respiratory chain phosphorylation. In "Proceedings of the third international congress of biochemistry," pp. 264 - 277. (Ed. C. Liebecq.) (Academic Press Inc.: New York.)

Slater, E. C., and Cleland, K. W. (1953).- Respiratory granules of heart muscle. Biochem. J. 53: 547 - 556.

Sokoloff, L., and Kaufman, S. (1961).- Thyroxine stimulation of amino acid incorporation into protein. J. Biol. Chem. 236: 795 - 803.

Spencer, R. P., and Knox, W. E. (1960).- Comparative enzyme apparatus of the gut mucosa. Federation Proc. 19: 886 - 897.

Steberl, E. A., Wasson, G. W., and Porter, J. W. (1960).- The condensation of malonyl Co A with acetyl or butyryl Co A. Biochem. Biophys. Research Communs. 2: 174 - 178.

Stein, Y., Tietz, A., and Shapiro, B. (1957).- Glyceride synthesis by rat liver mitochondria. Biochim. et Biophys. Acta 26: 286 - 293.

Stern, K. G. (1939).- Respiratory catalysts in heart muscle. Cold Spring Harbour Symposia Quant. Biol. 7: 312 - 322.

Storck, R., and Wachsman, J. T. (1957).- Enzyme localization in Bacillus megaterium. J. Bacteriol. 73: 784 - 790.

Stumpf, P. K., and Barber, G. A. (1957).- Fat metabolism in higher plants. IX. Enzymic synthesis of long chain fatty acids by avocado particles. J. Biol. Chem. 227: 407 - 417.

Spirtes, M. A., Medes, G., and Weinhouse, S. (1953).- A study of acetate metabolism and fatty acid synthesis in liver slices of hyperthyroid rats. J. Biol. Chem. 204: 705 - 713.

Stotz, E., Sidwell, A. E., and Hogness, T. E. (1938).- The role of the cytochromes in the action of "indophenol oxidase." J. Biol. Chem. 124: 733 - 744.

Strittmatter, C. F., and Ball, E. G. (1954).- The intracellular distribution of cytochrome components and of oxidative enzyme activity in rat liver. J. Cell. and Comp. Physiol. 43: 57 - 78.

Szent Gyorgi, A. (1937).- "Studies on biological oxidation." (Budapest, Leipzig.)

Tapley, D. F. (1956).- The effect of thyroxine and other substances on the swelling of isolated rat liver mitochondria. J. Biol. Chem. 222: 325 - 339.

Tapley, D. F., and Cooper, C. (1956 a).- The effect of thyroxine and related compounds on oxidative phosphorylation. J. Biol. Chem. 222: 341 - 349.

Tapley, D. F., and Cooper, C. (1956 b).- Effect of thyroxine on the swelling of mitochondria isolated from various tissues of the rat. Nature 178: 1119.

Tapley, D. F., Cooper, C., and Lehninger, A. L. (1955).- The action of thyroxine on mitochondria and oxidative phosphorylation. Biochim. et Biophys. Acta 18: 597 - 598.

Tedeschi, H., and Harris, D. L. (1958).- Some observations on the photometric estimation of mitochondrial volume. Biochim. et Biophys. Acta 28: 392 - 402.

Umbreit, W. W., Burris, R. H., and Staufer, J. F. (1957).- "Manometric techniques." 3rd. Ed., p. 338. (Burgess Publishing Co.: Minnesota.)

Van Bekkum, D. W. (1955).- The disturbance of oxidative phosphorylation and the breakdown of ATP in speen tissue after irrariation. Biochim. et Biophys. Acta 16: 437 - 438.

Van Bruggen, J. T., Yamada, P., Hutchens, T. T., and West, E. S. (1954).- Lipogenesis of the intact alloxan-diabetic rat. J. Biol. Chem. 209: 635 - 640.

Villar-Palasi, C., and Larner, J. (1960).- Levels of activity of enzymes of the glycogen cycle in rat tissues. Arch. Biochem. Biophys. 86: 270 - 273.

Vitale, J. J., and Hegsted, D. M., Nakamura, M., and Conners, P. (1957).- The effect of thyroxine on magnesium requirement. J. Biol. Chem. 226: 597 - 601.

Vitale, J. J., Nakamura, M., and Hegsted, D. M. (1957).- The effect of magnesium defficiency on oxidative phosphorylation. J. Biol. Chem. 228: 573 - 576.

Wade, R., and Jones, H. W. (1956).- Effect of progesterone on mitochondrial adenosine-triphosphatase. J. Biol. Chem. 220: 547 - 551.

Wallin, I. E. (1922).- On the nature of mitochondria. Am. J. Anat. 30: 203 - 229.

Wallin, I. E. (1923).- The mitochondria problem. Amer. Nat. 57: 255 - 261.

Warburg, O. (1913).- Uber sauerstoffmende Kornchen aus Leberzellen und uber sauerstoffatmung in Berkefeldfiltrated wasseriger Leberextrakte. Pflugers. Arch. 154: 599 - 617.

Weinbach, E. C. (1956).- Pentachlorphenol and mitochondrial adenosinetriphosphatase. J. Biol. Chem. 221: 609 - 617.

Weibull, C. (1953).- Characterization of the protoplasmic constituents of Bacillus megaterium. J. Bacteriol. 66: 696- 702.

Weinstein, H. J. (1954).- An electron microscope study of cardiac muscle. Exptl. Cell Research 7: 130 - 146.

Weiss, J. M. (1953).- The ergastoplasm. Its fine structure and relation to protein synthesis as studied with the electron microscope in the pancreas of the Swiss albino mouse. J. Exp. Med. 98: 607 - 618.

Weiss, J. M. (1955).- Mitochondrial changes induced by potassium and sodium in the duodenal absorptive cell as studied with the electron microscope. J. Exp. Med. 102: 783 -787

Williams, C. M. (1953).- The giant mitochondria of insects. (A reprint from the Proceedings of the XIV International Congress of Zoology, Copenhagen, 1953.)

Wiswell, J. G., and Braverman, M. G. (1957).- The effects of thyroxine and certain metal ions upon oxidation and phosphorylation in vitro. Endocrinology 61: 153 - 159.

Wittenberg, J., and Kornberg, A (1953).- Choline phosphokinase. J. Biol. Chem. 202: 431 - 444.

Witter, R. F., Watson, M. L., and Cottone, M. A. (1955).- Morphology and ATP-ase of isolated mitochondria. J. Biophys. Biochem. Cytol. 1: 127 - 138.

Wojtczak, L., and Wojtczak, A. B. (1959).- The action of serum albumin on oxidative phosphorylation in insect mitochondria. Biochim. et Biophys. Acta 31: 297 - 299.

Zellestron, R., and Ernster, L. (1956).- Bilirubin, an uncoupler of oxidative phosphorylation in isolated mitochondria. Nature 178: 1335 - 1337.

Zetterqvist, A. (1956).- The ultrastructural organization of the columnar absorbing cells of the mouse jejunum. (Ahkebolaget Godvil: Stockholm.)

Ziegler, D. M., Lester, R. L., and Green, D. E. (1956).- Oxidative phosphorylation by an electron transport particle from beef heart. Biochim. et Biophys. Acta 21: 80 - 85.

Ziegler, D. M., and Linnane, A. W. (1958).- Studies on the electron trancport system. XIII. Mitochondrial structure and dehydrogenase activiy in isolated mitochondria. Biochim. et Biophys. Acta 30: 53 - 63.

Zollinger, H. V. (1950).- Les mitochondries. Leur etude a l'aude du microscope a contrast de phases. Revue d'Hematologie 5 695 - 745.

Zwart, A. G. (1952).- A modified method for calculation of Warburg flasks and manometrs. Biochim. et Biophys. Acta 9: 104.