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STUDIES OF SOME EFFECTS OF COLD EXPOSURE ON THE FUNCTION AND STRUCTURE OF MITOCHONDRIA OF RATS

by

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A Thesis submitted to the Faculty of
the University of Ottawa in partial
fulfillment of the requirements for
the degree of Doctor of Philosophy

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ABSTRACT

The structure and function of mitochondria of cold-acclimated rats (+2° C) in comparison with those of control rats (+23° C) have been studied in relation to (A) uncoupling of oxidative phosphorylation, (B) protein synthesis, and (C) mitochondrial ultra structure, in an attempt to find the cause of extra heat production (non-shivering thermogenesis) which occurs during cold exposure.

The dry mass, number and total protein content of mitochondria per gram of original fresh liver tissue was found to be reduced in cold-acclimated rats. Cold exposure also resulted in a decrease of the protein contents of the soluble and insoluble fractions of mitochondria. The reduction of dry mass per gram of original fresh liver tissue and the reduction of soluble and insoluble protein contents per gram of original fresh liver tissue seems to be due to the reduction of number of mitochondria since the dry mass per unit number of mitochondria was found to be more or less the same.

Cold treatment also resulted in a decrease of the P:O ratio, owing to a higher uptake of oxygen and lower uptake of inorganic phosphate.

Study on incorporation of uniformly labeled C^{14}-amino acids into mitochondrial protein indicated that cold exposure induced a reduced accumulation of radioactivity in mitochondrial protein both *in vitro* and *in vivo*, this effect being more pronounced in the soluble protein fraction.

Studies with the electron microscope revealed that a great number of mitochondria of cold-acclimated rats were larger than those of control rats, with more ruffled outer membrane and pale matrix. This enlargement of mitochondria of cold-acclimated rats could be due to swelling *in vivo*. The glycogen content of the liver cells was found to be higher in cold-acclimated rats than in control rats.

It is concluded that cold exposure exerts its effect on mitochondrial protein synthesis, particularly the soluble fraction. This impaired protein synthesis with enlargement of mitochondria of cold-acclimated rats could be the reason for the swelling and the changes in the properties of the membrane, leading to the uncoupling of oxidative phosphorylation.

RESUME

Les présents travaux ont porté sur la structure et certaines fonctions de mitochondries prélevées chez des rats acclimatés au froid (+2° C) ou à la chaleur (+23° C).

Afin d'apporter une nouvelle contribution au problème de la thermogénèse sans frisson, l'étude a porté en particulier sur la phosphorylation oxidative, la synthèse protéique et l'ultrastructure des mitochondries.

Les premières observations ont montré que, pour chaque gramme de foie frais prélevé de rats acclimatés au froid, le poids sec total des mitochondries, leur nombre et leur teneur totale en protéines étaient diminués, ainsi que les fractions soluble et insoluble de ces protéines. Il est apparu que la diminution du poids sec total des mitochondries ainsi que de la teneur en protéines étaient directement reliés à la diminution du nombre de mitochondries.

Les études "in vitro" des mitochondries prélevées chez l'animal acclimaté au froid ont montré un abaissement du rapport P/O, abaissement dû à la fois à une augmentation de la consommation d'oxygène et à une diminution de l'incorporation de phosphore inorganique.

D'autre part, l'incorporation mitochondriale d'acides aminés uniformément marqués au radio-carbone-14 était diminuée chez l'acclimaté au froid, aussi bien in vivo qu' in vitro; cette diminution était plus prononcée dans la fraction protéique soluble.

Finalement, l'examen au microscope électronique a révélé que les mitochondries de rats acclimatés au froid étaient plus grosses que celles des animaux témoins; leur membrane extérieure était aussi plus irrégulière, et la densité matricielle fortement diminuée. Il est possible que ces changements aient été provoqués par le gonflement mitochondrial chez le rat acclimaté au froid. De plus, les images ont montré une augmentation de la teneur en glycogène du foie chez les animaux acclimatés au froid.

Le fait le plus significatif semble être la diminution de la synthèse protéique, plus particulièrement celle des protéines solubles; les changements subséquents des propriétés de la membrane amèneraient le gonflement des mitochondries et le découplement de la phosphorylation oxidative.

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Abbreviations and Symbols used in the text

AMP, ADP, ATP	- adenosinemono-, di, and triphosphate
ATPase	- adenosine triphosphatase
cyt. c	- cytochrome c
CoA	- coenzyme A
CoQ	- coenzyme Q
EFA	- essential fatty acid
NAD	- nicotinamide adenine dinucleotide
$NADH_2$	- reduced nicotinamide adenine dinucleotide
NADP	- nicotinamide adenine dinucleotide phosphate
$NADPH_2$	- reduced nicotinamide adenine dinucleotide phosphate
O.D.	- optical density
O_2	- oxygen
Pi	- inorganic phosphate
P:O ratio	- ratio of µmoles of inorganic phosphate esterified to µatoms oxygen utilized
RNA	- ribonucleic acid
TCA	- trichloracetic acid
UL-amino acid	- uniformly labeled amino acid

CHAPTER I

Introduction and Literature Review

The characteristic features of the physiology of rats, when chronically exposed to lower temperatures, are the disappearance of shivering, increased food intake (Baker and Sellers, 1953; Beaton, 1963 and Klain and Vaughan, 1963), slower growth rate (Dugal and Dufour, 1954; Héroux and Hart, 1954; Cottle and Carlson, 1954; Young and Cook, 1955; Barnett and Manly, 1956; Masoro et al, 1957, and Biggers et al, 1958) and the establishment of non-shivering thermogenesis. The dominant aspect of the general metabolism is the extra heat production through non-shivering thermogenesis with a greater utilization of substrate, and a concomitantly increased consumption of oxygen (Sellers and You, 1950; Sellers et al, 1951 and 1954; Krog et al, 1955; Héroux et al, 1956; Cottle and Carlson, 1956, and Depocas, 1960).

A great deal of study has been carried out by many investigators to identify the substrate(s) from which the extra heat is derived by oxidation, and the mechanism of extra heat production in cold-acclimated rats. Special attention has been given to the fate of the major foodstuffs; carbohydrate, fat and protein, in relation to the source of extra heat production.

Carbohydrate Metabolism

Cordier and Piery (1950), Baker and Sellers (1953) and Pagé et al (1955) have reported a considerable rise in the rate of glucose absorption by the intestine of cold-acclimated rats. Furthermore, Baker and Sellers observed an increased turn over rate of glucose with increased insulin secretion following cold exposure. These authors concluded that the increased glucose absorption was due to the elevated metabolic rate of the cold-acclimated rats, with an associated increase in the utilization of carbohydrate.

Masoro et al (1955) also reported an alteration of carbohydrate metabolism in cold-acclimated rats. They observed a higher liver glycogen in fasted, cold-acclimated rats. An increased rate of glycogen accumulation was also observed by Pagé et al (1955) in cold-acclimated rats fed a high carbohydrate diet. That this accumulation was due to an increased synthesis of glycogen was demonstrated in a study of C^{14}-glucose metabolism by Depocas et al (1957), who observed that there was an increased accumulation of radioactivity in glycogen from intraperitoneally injected C^{14}-glucose (uniformly labeled) in cold-acclimated rats as compared to normal rats. During the initial 30 minute period after the injection of C^{14}-glucose, more glucose was oxidized to carbon dioxide and water in cold-acclimated rats than in non-acclimated ones. However, cold-acclimated rats were found to oxidize less C^{14}-glucose than non-acclimated rats after a

period of 2.5 hours, and a greater portion of the radioactivity was found in complex body fractions (but not fatty acids). Later, Depocas and Masironi (1960) found that only 20-22% of the total carbon dioxide produced was derived from UL-C^{14}-glucose in cold-acclimated rats. Therefore, the higher total production of carbon dioxide in the cold-acclimated rats is derived from sources other than glucose.

Fat Metabolism

On the basis of respiratory quotient studies, Kayser (1937) reported that rats exposed to a lower environmental temperature produced the extra heat required by a preferential oxidation of fats. A beneficial effect of a higher fat diet on the growth of cold-acclimated rats was reported by Dugal et al (1945). The total lipid content of cold-acclimated rats, in spite of an increased food intake in comparison with that of non-acclimated rats, was also found to be reduced (Young and Cook, 1955). The excessive deposition of fat in the liver did not occur in cold-acclimated rats (0-2° C for 2 weeks) despite an increased consumption of a severely hypolipotrophic diet (Sellers and You, 1949). Even pre-existing fatty liver could be cured by prolonged cold exposure (Treadwell et al, 1958). Furthermore, Pagé and Chénier (1953) reported not only that fat is oxidized preferentially, but also that the turn-over rate of sugar into fat is increased in the cold-acclimated rats. Evidence from these studies indicated that a high fat diet is beneficial for survival of cold-acclimated

rats. It was pointed out by Pagé and Babineau (1953) that the effect of high fat diet was not due to additional fat deposition nor to a different distribution of body fat which would provide better insulation, and that a high level of protein in a complete ration was no better than a moderate level in the diet of cold-acclimated rats. Fats, thus, are considered as a primary source of extra heat production, compared to carbohydrate and protein (Pagé, 1957).

Masoro _et al_ (1957) reported that the fatty acid content was 18% less in rats exposed to 0-2° C for one day and 32% less in rats exposed to this temperature for two days as compared to the control rats, suggesting a relatively high rate of fatty acid oxidation in the cold exposed rats. Furthermore, a greater production of $C^{14}O_2$ from the oxidation of acetate-C^{14}, as well as a reduced ability to convert acetate-C^{14} to long chain fatty acids in liver slices of rats exposed to 0-2° C for 120-160 days compared to those from non-acclimated rats, has also been demonstrated by Masoro _et al_ (1957). Kline _et al_ (1956) found decreased synthesis of phospholipid (49%) from acetate-1-C^{14} in liver slices of cold-acclimated rats, while a relatively high rate of oxidation of long chain fatty acids was reported by Felts and Masoro (1959) and Masironi and Depocas (1961), who found an increased conversion of palmitate-C^{14} into respiratory $C^{14}O_2$ in cold-acclimated rats. The increased oxidation of fatty acids rather than synthesis of fatty acids may be associated with an increased level of coenzyme A

found in the liver tissue of cold-acclimated rats (Bradly et al, 1956 and Campbell et al, 1960 a, b, and c).

Protein Metabolism

High turn-over rates of protein, which resulted in a decreased protein synthesis and an increased level of non-protein nitrogen in the urine, were found to occur in cold-acclimated rats by many investigators (Lathe and Peters, 1949; Chinn et al, 1950; Hoberman, 1950; Ingle et al, 1953; Young and Cook, 1955; Hale and Mefferd, 1958, and Hannon and Young, 1959). Seventy-seven percent more nitrogen was excreted in the urine of cold-acclimated rats than in that of non-acclimated rats. When food intake was doubled by cold-acclimated rats, urinary nitrogen was increased further to about 2.3 times its original value (You et al, 1950), thus a slower body weight gain by cold-acclimated animals was believed to result from either a decreased anabolism or an increased catabolism of protein. Hoberman (1950) and Beaton (1963) observed an increased catabolism of amino acids in rats exposed to 2-3° C for 7 days. Beaton suggested that amino acids could be utilized as a respiratory substrate to meet energy requirements. This consequently induces a reduction of protein synthesis because of the lessened availability of amino acids.

Mefferd et al (1958), and Hale and Mefferd (1958) found a greater excretion of amino acids (alanine, valine, serine, threonine, glycine and glutamic acid) in cold-acclimated rats than in non-acclimated

ones. An increased urinary excretion of arginine, uric acid, urea, creatine and taurine by cold-acclimated rats was also reported by Klain and Vaughan (1963). Rangneker and Dugal (1958) detected the disappearance of phenylalanine and tyrosine from the plasma and urine of rats and guinea pigs exposed to 2° C for 72 hours. They found that while phenylalanine disappeared from both plasma and urine, tyrosine disappeared only from urine, trace amounts being found in the plasma of cold-exposed rats. The cold-acclimated animals readily consumed an imbalanced amino acid mixture and were able to grow, while this imbalanced diet of amino acid mixture caused a considerable decrease in both food intake and the rate of growth of non-acclimated rats (Klain and Vaughan, 1963). This finding suggested that cold exposed animals could catabolize preferentially those amino acids which the imbalanced mixture contained in excess and utilize them for heat production, while the others could then be effectively utilized for tissue protein synthesis.

Cold acclimation was also shown to be accompanied by changes in protein levels. Hannon and Young (1958) showed a significant decrease in plasma protein levels in rats exposed at 5° C for a month. Trapani and Campbell (1959) reported a rapid disappearance of administered antibody in rabbits kept at -15° C for 10 weeks. The changes in the electrophoretic patterns of plasma proteins of cold exposed rabbits were studied by Waugh (1952) and Sutherland _et al_ (1958); rabbits

exposed to 3° C for 20 days exhibited a strikingly decreased level of serum β-globulin and an elevated level of γ-globulin. Contrary to these findings, Shield et al (1960) showed a marked decrease in plasma albumin and β-globulin at the end of 40 days exposure to cold, and an increase in the α-fraction. From these results it could however be concluded that cold exposure causes an increased catabolism and a decreased anabolism of proteins.

Alteration in Intermediary Metabolism

Increased activity of glycolytic enzymes such as glucokinase (Hannon, 1960a) and glucose-6-phosphatase (Hannon, 1960a and Hannon and Vaughan, 1960), and increased activity of certain oxidative enzymes such as succinate and malate dehydrogenases (You and Sellers, 1951; Clark et al, 1954; DesMarais, 1954 and 1955, and Hannon, 1960d), NADP dehydrogenase (Hannon, 1958), NAD dehydrogenase (Hannon, 1960d), NAD or NADP cytochrome c reductase (Reynafarje and Chaffee, 1962) and cytochrome oxidase (Hannon, 1960d), as well as increased levels of other components such as cytochrome c (Klain, 1961 and 1963), and coenzyme Q (Beyer et al, 1962) have been reported in various tissue homogenates from cold-acclimated rats. However, the activity of lactic dehydrogenase from liver or muscle (Hannon, 1960b), NAD-NADP transhydrogenase from liver (Smith and Fairhurst, 1958), $NADH_2$-cytochrome c reductase (Hannon, 1960d) and $NADPH_2$-cytochrome c reductase (Smith and Fairhurst, 1958) have been found to be unaffected in cold-acclimated rats.

Increased activity of enzymes concerned with intermediary amino acid metabolism have also been found to occur during cold exposure. The activity of glutamate-oxalacetate and glutamate-pyruvate transaminases increased during prolonged cold exposure (Hannon, 1963 and Klain and Vaughan, 1963) while the activity of alanine-glutamate and aspartate-glutamate transaminases and phosphate activated glutaminase have been increased in acute cold exposure (Beaton, 1963).

However, no definite pattern has yet emerged from these scattered data on enzymatic activities. In vitro studies may not reflect the situation in vivo. The observed increased enzymatic activities in tissues obtained from cold-acclimated rats may also represent either a real increase in enzyme content or an increase in availability and utilization of the enzyme (Janský, 1963) or a stimulation of latent enzyme activity. Therefore, it might be possible that these increased enzyme activities may have been associated with an increase in size and/or number of mitochondria, or with a physical alteration of the mitochondrial structure such as to make the enzyme more available to substrate.

Since the respiratory electron transport is an important process in energy exchange and heat production, it appears that the next problem would be to investigate how the respiratory electron transport system is altered in the direction of extra heat production

during chronic cold exposure. When a mole of glucose is completely oxidized by oxygen, 673 kcal. of heat are produced. However, in mitochondria, where the electron transport system is located, most of the energy originally contained in the food molecule, is released in two forms: one as direct heat, the other as chemically trapped energy. Usually approximately 60% of the energy of the glucose molecule is trapped as chemical energy in the form of ATP and the remaining 40% is released as direct heat.

There is sufficient experimental evidence to conclude that the extra heat is produced in association with an increased rate of oxygen consumption in cold exposed animals in order to maintain the body temperature (Sellers et al, 1951; You and Sellers, 1951; Cottle and Carlson, 1954, and Krog et al, 1955).

Without any alteration of the overall sequence in the respiratory electron transport system, it is very difficult to visualize how the extra heat production is brought about. The extra heat production would eventually result in a lack of available ADP and thus inhibit the oxidation of substrate, unless there was a sufficient amount of ADP present in cold-acclimated rats. The availability of either inorganic phosphate or ADP, but not of ATP, profoundly influences the rate of oxidation of a wide variety of substrates by rat liver mitochondria (Lardy and Wellman, 1952). However, a reduction of ADP availability in cold-exposed animals has never been directly studied.

Potter (1958) speculated that the extra heat production might be linked with the uncoupling of oxidative phosphorylation. Evidence of this uncoupling (a lowered P:O ratio) has been demonstrated by Smith and Fairhurst (1958) and Panagos et al (1958).

The biochemical mechanism whereby a reduced efficiency of oxidative phosphorylation is achieved in cold-acclimated rats has been the subject of a considerable number of studies. A review of the literature indicates that there are several possible working mechanisms to explain the uncoupling of oxidative phosphorylation.

(1) One possible mechanism could be that exposure to cold causes an increased hydrolysis of ATP after completion of the normal coupling of oxidative phosphorylation: cold exposure stimulates ATPase activity, resulting in apparent net increase of ADP and inorganic phosphate, and thus a lowered P:O ratio. In this manner, an increased oxidation of substrate in cold-exposed animals does not upset the overall respiratory electron transport system and also sufficient ADP is made available. Impairment of phosphorylating ability as the result of an increased activity of ATPase during aging of mitochondria has been reported by Kielley and Kielley (1951). However, very little effect of cold exposure was observed on ATPase activity (Smith and Fairhurst, 1958; Hannon, 1960c; Aldridge and Stoner, 1963, and Chaffee, 1963).

(2) Two pathways are known to exist in the respiratory electron transport system. One pathway is through $NADH_2 \longrightarrow NADH_2$-CoQ

reductase → $CoQH_2$-cytochrome c reductase → cytochrome c → reduced cytochrome oxidase → oxygen. The other pathway is through succinate dehydrogenase → succinate-CoQ reductase → $CoQH_2$-cytochrome c reductase → cytochrome c → reduced cytochrome oxidase → oxygen. Theoretically, in the $NADH_2$ pathway, three molecules of ATP are formed at three sites, so that the P:O ratio is 3. In the succinate dehydrogenase system, only two molecules of ATP are formed at two sites, thus the P:O ratio is 2. Therefore, with the same amount of oxygen, the P:O ratio through the succinate dehydrogenase system is lower than that through the $NADH_2$ system, and more direct heat is thereby made available (Figure 1).

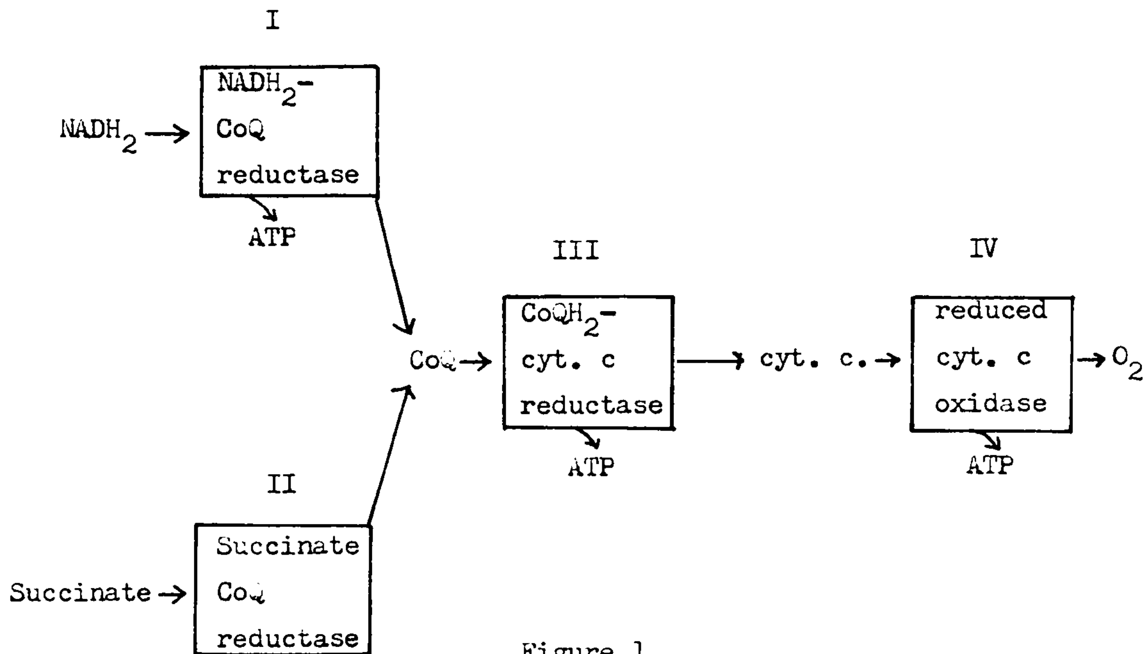

Figure 1

The arrangement of the enzyme complexes, and sites of ATP formation in the electron transfer chain (Green *et al*, 1963)

Whether or not a preferential operation of the succinate dehydrogenase system over the $NADH_2$ system takes place in cold-acclimated rats has not been directly demonstrated. This is, however, supported by indirect evidence. It has been reported by Hannon (1960d) and DesMarais (1954 and 1955) that the activity of succinate dehydrogenase in cold-acclimated rats is increased, whereas no increased activity of NAD-NADP transhydrogenase was observed by Smith and Fairhurst (1958). Beyer et al (1961) suggested that an increased oxidation of succinate was presumably coupled with the higher level of coenzyme Q in cold-acclimated rats. It has been known that a fat diet is more beneficial than a protein or carbohydrate diet in cold-acclimated animals (Dugal et al, 1945; Pagé and Chénier, 1953, and Masoro et al, 1954 and 1957). When a fatty acid is oxidized, its conversion to acetyl-CoA is mediated by the succinate dehydrogenase pathway. This finding suggests that this pathway might be a major electron transport system in cold-acclimated rats, resulting in a lowered P:O ratio.

(3) Lehninger (1949 and 1951) has demonstrated that $NADH_2$ may be oxidized by an "internal" and an "external" pathway in mitochondria. The external pathway from either $NADH_2$ or $NADPH_2$ was later shown to be insensitive to the inhibitor antimycin. Therefore, the electron flow in this pathway is probably by-passing the cytochrome b-cytochrome c complex (de Duve et al, 1955). In addition, it has been shown that the portion of reduced pyridine nucleotide oxidation which proceeds via the external route is insensitive to the inhibitor

amytal and phosphorylation is not involved in this pathway (Ernster, 1959). On the basis of the above mentioned studies, Beyer (1963) studied the effect of DT-diaphorase in the rat liver mitochondria. In rat liver mitochondria, DT-diaphorase appears to be reduced by $NADH_2$ and can transfer electrons in the presence of vitamin K_3 and amytal to an electron transport component after the amytal-sensitive site, possibly coenzyme Q or cytochrome b. $NADH_2$ oxidized via this route would thus be amytal insensitive and would bypass the first phosphorylation site, thought to be the major point of respiratory control. Since such a pathway would serve to supply electrons from $NADH_2$ to the respiratory chain at a point beyond the main region of respiratory control, Beyer investigated its activity in liver mitochondria isolated from control rats and rats acclimated to cold for 30 days. The functional quinone link between DT-diaphorase and the phosphorylating electron transport chain is not understood at present, but he found an elevated mitochondrial DT-diaphorase activity in the cold-acclimated rats. He suggested from these data that DT-diaphorase may serve as a link allowing a rapid influx of electrons to the respiratory chain for heat production, without being accompanied by phosphorylation.

(4) A considerable amount of work has shown that ascorbic acid plays a large role in the uncoupling of oxidative phosphorylation. It is suggested as a possible mechanism that ascorbic acid is able to

accept electrons from either $NADH_2$ or $NADPH_2$ and transfer it directly to cytochrome b and oxygen (Kielley and Bronk, 1957 and Lehninger et al, 1954).

Kersten et al (1956) studied the effect of ascorbic acid on the transfer of electrons with $NADH_2$ as the reducing agent, and oxygen as the electron acceptor in mitochondria. They determined the effect of ascorbic acid on the oxidation of $NADH_2$ by measuring the disappearance of $NADH_2$ from the assay system. The optical density without ascorbic acid was 0.4, with the addition of 2.5 µmoles of ascorbic acid it was 0.32, and with the addition of 50 µmoles of ascorbic acid the optical density fell down to 0.05. If both $NADH_2$ and cytochrome c were present in the reaction system, the optical density of the mixture fell in 30 minutes from 0.4 to 0.1 in the absence of ascorbic acid; with the addition of 10 µmoles of ascorbic acid, the optical density fell to 0.01 in 19 minutes.

Kersten (1956) also measured the effect of ascorbic acid upon $NADH_2$ oxidation in a mitochondrial system. In Warburg vessels, mitochondria alone consume 1 µl of oxygen in 150 minutes. When $NADH_2$ alone was added to the mitochondrial suspension the oxygen uptake was increased to 10 µl. When both $NADH_2$ and ascorbic acid were added to the assay system, the uptake of oxygen was surprisingly increased to 150 µl. A similar effect of ascorbic acid on microsomal suspension has been reported in swine adrenals. Hagen (1954) and Kanfer et al

(1959) found that in the presence of both $NADH_2$ and cytochrome b the assay system consumed 85 µl of oxygen, and with the addition of 0.5 µmoles ascorbic acid the assay system increased its uptake to 180 µl. The availability of ascorbic acid to mitochondria and microsomes is believed to be important in cellular metabolism in cold-acclimated animals. Their data indicate that ascorbic acid can increase the rate of oxidation without a concurrent increase in phosphorylation.

Green et al (1963) reported evidence of ascorbic acid participation in oxidation but not phosphorylation in the electron transport system of beef heart mitochondria. They found three factors responsible for phosphorylation. They are: Factor 1, which is a $NADH_2$ coupling factor functioning at only site 1; Factor 2, which is a $CoQH_2$ coupling factor functioning at only site 2; and Factor 3, which is a reduced cytochrome c coupling factor functioning at only site 3. No interchange can be made among these three factors since there is a specific site for each one. If Factor 3 is added to cytochrome c, the P:O ratio becomes 0.9-1.0 but if ascorbic acid is added to this system without addition of Factor 3, the P:O ratio becomes zero.

(5) Cold exposure of animals causes swelling of mitochondria. This swelling may cause a change in the permeability of the membrane to substrate or certain cofactors. For instance, NAD may leak from the membrane into the cytoplasm, and NADase in the cytoplasm could then break down NAD to nicotinamide and adenosine diphosphate ribose

(Jacobson and Kaplan, 1957). The structural change of the membrane might cause a leak of one or two of the factors needed for oxidative phosphorylation. It is also possible that the swelling of the membrane causes dislocation of the active centers of respiratory or coupling enzymes, thus lowering the rate of oxidative phosphorylation.

The objective of the present study

A strong correlation has been found between the number of cristae in the mitochondria of a tissue and the oxidative capacity of the same tissue, by comparing, for example, mitochondria from heart and flight muscle and mitochondria from liver (Palade, 1952; Edwards et al, 1958 and Green, 1961). In a review paper, Green (1961) compared mitochondria of heart and liver with respect to the number of cristae and the content of insoluble protein. Insoluble protein constitutes 80% of total protein in heart mitochondria, but only 40% in liver mitochondria. As we have seen so far, there is evidence that cold exposure increases oxygen consumption and also increases the activity of oxidative enzymes, such as succinate and malate dehydrogenases and cytochrome oxidase, or increases the concentration of cofactors such as coenzyme Q in the electron transport system. Many of the oxidative enzymes reported to increase in activity with cold-acclimation are insoluble proteins. Therefore, the cold exposure may increase the content of insoluble protein of mitochondria.

If one considers these categories of metabolic and morphological changes of mitochondria with respect to the effect of cold on

animals, one can make the following assumptions. It is known that the electron transport particle is the smallest unit of the cristae in mitochondria. Since cold exposure increases the activity of oxidative enzymes and oxygen consumption, one would expect either that the number of cristae in mitochondria would be increased, or that there would be an increase in size and/or number of mitochondria. An alternative assumption would be a change in mitochondrial structure, stimulating latent enzyme activity without any quantitative change.

Although extensive studies have been conducted to elucidate the mechanism of alteration of the pathways of intermediary metabolism in cold-acclimated animals, and although it has been known that the uncoupling of oxidative phosphorylation appears to be linked in a complex way with the electron transport system confined to the mitochondrial membrane, there is no available information on the relationship between the observed effects of cold exposure, and the function and structure of mitochondria. If the increased oxygen consumption in cold-acclimated animals were due to an increased number of cristae in the mitochondria, there should be a proportional increase in the insoluble protein fraction of mitochondria. Roodyn _et al_ (1961) have reported that in isolated mitochondria there is a negligible synthesis of soluble protein, whereas synthesis of insoluble protein is predominant. Therefore, bearing these results in mind, the present study has been initiated in an attempt to establish a link between uncoupling

of oxidative phosphorylation, protein synthesis in mitochondria, and structure of mitochondria in cold-acclimated rats in the state of non-shivering thermogenesis. To do this, the following measurements were made on isolated liver mitochondria: uptake of oxygen and of inorganic phosphate (P:O ratio), dry weight, protein content (total, insoluble and soluble), number of mitochondria per unit weight of liver tissue, and incorporation of UL-C^{14}-amino acids into mitochondrial protein of liver and other internal organs. Also the structure of liver mitochondria was investigated by means of electron microscopy.

CHAPTER II

Materials and Methods

1. Materials

Male white rats, Wistar strain, obtained from the Romain Rabidoux Animal Farms, St. Constant (La Prairie), Quebec, were used throughout the present study. The rats were 8-8.5 weeks old on arrival, and weighed 185-200 grams each. They were divided into two groups: one as control, the other as experimental. Cold exposure was started when the rats reached a body weight of 200 grams $\pm$ 5 grams. The control group was kept at an ambient room temperature of 23° C $\pm$ 2° C, the experimental group at a temperature of 2° C $\pm$ 1° C in a cold room. The body weight of both groups of rats was determined at the same time (9:00-10:00 a.m.) at each weighing period, including the final weighing before their sacrifice for experimental use. The animals were supplied with food pellets (Purina Lab. chow) and tap water *ad libitum*. The rats of both groups were healthy and active throughout the experimental period, but those in the cold were smaller and fuzzy in appearance because of their longer hair.

2. Methods

(a) Determination of weight

i. Body weight

The live rat was placed on a balance and a reading was quickly taken for the record.

ii. The weights of excised organs

After the body weight of the rats was measured, they were killed by decapitation and bled. The entire liver, heart, kidney, spleen, pancreas and brain were rapidly removed and washed in ice cold 0.44M sucrose solution and quickly rolled on filter paper to sponge off excess fluid. The fresh weights were measured with a chemical balance (Type H16 E. Mettler Zurich), after which the organs were placed in an oven and dried to constant weight at 100° C.

(b) Isolation of liver mitochondria

In a preliminary experiment, methods for the isolation of liver mitochondria reported by Schneider and Hogeboom (1950), Allard *et al* (1952) and Dounce *et al* (1955) were tried. A modified Dounce method was adopted, giving an increased purity of the isolated mitochondria.

After the fresh weight of the liver (or other organs except heart) was determined, it was quickly minced with scissors, and homogenized in 5 volumes of chilled 0.44M sucrose solution in a Dounce homogenizer with a loosely fitting plunger. The heart tissue was ground in a mortar before homogenization. The pH of the homogenate was adjusted to 6.0-6.2 by adding 0.1M citric acid during homogenization. The homogenate was then centrifuged in a refrigerated centrifuge, model PR-2, at 900 g (2500 r.p.m. in Head #811a) for 15 minutes to remove intact cells, red blood cells, and other debris. The supernatant

fluid was then decanted carefully with a pipette into a chilled tube and centrifuged at 12,000g (13,500 r.p.m. in Head #296) for 15 minutes to obtain mitochondrial pellets. The 12,000g supernatant was discarded. The mitochondrial pellets were washed by resuspension in 7 ml of 0.44M sucrose solution and centrifuged at 12,000g for 10 minutes. This procedure of washing was repeated three times, and each time the supernatant fraction was discarded. The supernatant of the final washing was clear.

(c) Mitochondrial dry weight

A 2 ml aliquot of the final mitochondrial suspension containing the mitochondria from 2 grams of fresh liver was transferred into a centrifuge tube and centrifuged at 12,000g for 30 minutes in 5 ml of 0.9% KCl solution. The mitochondrial pellet was transferred onto an aluminum weighing flask with 2 ml of distilled water, and dried to constant weight in an oven at 100° C. The dried sample was weighed and the total dry weight of mitochondria per gram of fresh liver was calculated. The weighing flasks, whose weights ranged from 380-400 mg., were prepared in our laboratory.

(d) Preparation of the homogenate for counting mitochondria

The method for the preparation of liver homogenates for counting mitochondria has been described by Allard *et al* (1952) and Shelton *et al* (1953). However, a modified method for the preparation of a sample for counting has been used in this experiment.

After the liver homogenate was prepared as described in section (b), 0.5 ml of homogenate containing mitochondria from 0.5 grams of fresh liver and 0.2 ml of 1:1,000 Janus Green B solution were made up to 25 ml with 0.44$\underline{M}$ sucrose solution in a volumetric flask. One ml of this mixed suspension was further diluted to 25 ml with 1.8$\underline{M}$ sucrose solution—a total dilution of 1,250 times.

The number of mitochondria was counted in a Petroff-Hausser bacteria counting chamber. The number of mitochondria in 5 groups of 20 small squares was counted, and the average number of mitochondria per 10^{-3} $mm.^3$ was determined. The volume over each small square is .05 x .05 x .02 or 5 x 10^{-5} $mm.^3$ (5 x 10^{-8} $cm.^3$) or the volume over 20 small squares is 10^{-6} $cm.^3$ The calculation of the number of mitochondria per 0.5 ml of whole homogenate was made with the use of the following formula:

Average number of mitochondria/20 small squares x dilution factors x 10^6 = number of mitochondria per 0.5 ml of homogenate

Counting was carried out in phase contrast with a Zeiss-Winkle binocular microscope. An oil immersion objective 100X and 1.3 numerical aperture was employed in conjunction with a 12.5 Hygens eye-piece. In actual counting, the second homogenate diluted with 1.8$\underline{M}$ sucrose solution was kept on ice and was well mixed before the removal of a given volume of suspension. An aliquot was removed with a pipette,

placed in the counting chamber, and allowed to warm to room temperature. In this way, the Brownian movement of mitochondrial particles due to expansion of liquid in the chamber was somewhat avoided. Since the particles did not sediment in the chamber they were counted from the base to the top of a prism, and vice versa. This was conveniently done by focusing the objective on the base of a prism, followed by a very slow ascending movement of the objective to the upper surface of the same prism. With such a slow movement of the objective, each mitochondrion appeared and disappeared progressively. They were counted only when perfectly focussed.

(e) Determination of protein content of mitochondria

i. Determination of total protein of mitochondria

Mitochondrial protein was precipitated from 1.0 ml of suspension containing mitochondria from 0.2 grams of fresh liver by addition of 1 ml of 10% TCA (w/v). The precipitate was collected by centrifugation and dissolved in 0.5 ml of 1 N NaOH, heated on a hot plate at 100° C for 30 minutes, and diluted to a final volume of 5 ml with distilled water. The protein content in a given volume of the final preparation was determined colorimetrically by the method of Lowry *et al* (1951). Crystalline bovine albumin dissolved in distilled water was used to prepare a standard curve for each set of experimental determinations. Optical density readings of the samples were made at 500 mμ using a Bausch and Lomb Spectronic "20". A standard curve from an average of 10 readings is presented in Figure 2.

Figure 2

A standard calibration curve for the determination of protein content (µg) from the reading of optical density at 500 mµ (an average of 10 determinations).

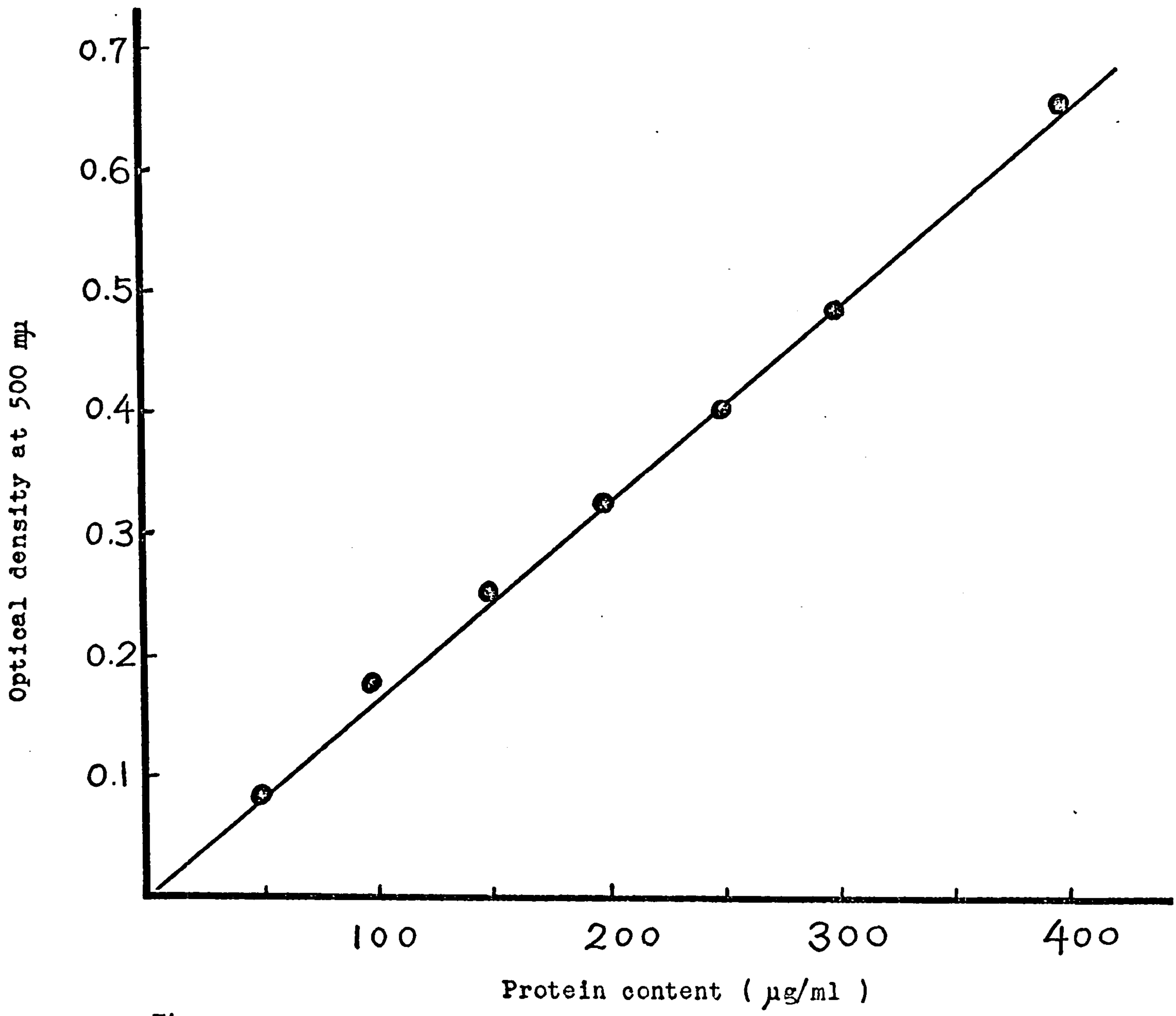

Figure 2

ii. Determination of the soluble and insoluble proteins of mitochondria

Mitochondrial protein was divided into a soluble and insoluble fraction by treatment of mitochondria with the detergent Triton X-100. Several methods have been reported for obtaining the soluble and insoluble fractions from mitochondria (Siekevitz and Watson, 1956; Green, 1959 and 1961, and Roodyn et al, 1961). The method of Roodyn et al (1961) has been adopted in this study, with the following modification. Ten ml of detergent Triton X-100 solution (0.1%, v/v of Triton X-100 in 0.44M sucrose) was added to 2 ml of a mitochondrial suspension in 0.44M sucrose in a lusteroid Spinco centrifuge tube (capacity 12 ml). The mixture was quickly mixed and the tube sealed. After centrifugation at 105,000g for 60 minutes in a Spinco ultra-centrifuge Model L, the supernatant was removed for the analysis of soluble protein, while the pellet was used for the analysis of insoluble protein after resuspension in 3 ml of 0.44M sucrose. The Lowry method (1951) previously described was used for the determination of protein in both fractions.

(f) Oxidative phosphorylation

i. Uptake of oxygen and inorganic phosphate

A standard Warburg manometric method (Umbreit et al, 1959) was employed for the measurement of oxygen uptake by mitochondria.

A Warburg flask with a capacity of 15 ml was used, with 0.2 ml of 20% KOH in the center well, 0.5 ml of 30% TCA in the single

side arm and 2.5 ml of the basic incubation medium in the main compartment. The components of the basic incubation medium were as follows:

Component	Final molarity
α-ketoglutaric acid	0.01M
$MgCl_2 6H_2O$	0.002M
$K_2HPO_4 \cdot 3H_2O$	0.015M
Glucose	0.02M
NaF	0.01M
* K_2ATP	0.0015M
* NAD	0.0002M
* Co-carboxylase	0.0002M
* Coenzyme A	0.0002M
* Yeast hexokinase	3 mg (100 Kunitz-MacDonald units/mg)

* In preparing the basic medium, a mixture of these components must be added last.

The final pH was adjusted to 7.4 with 1N KOH.

The experiment was started with the addition of 0.5 ml of the mitochondrial suspension (see section b) to the main compartment. Oxidations were carried out at 30° C with air as the gas phase.

The flasks were equilibrated for 10 minutes before the stopcocks were closed. At the termination of the incubation period (0, 15, 30, 45 and 60 minutes) TCA was added from the side arm into the main compartment.

The contents of the sample flasks were centrifuged at 25,000g. Determination of the inorganic phosphate content of the

clear supernatant and the protein content of the precipitate was made. The phosphate esterified during the experimental period was determined from the difference in inorganic phosphate content of the zero time and later samples.

ii. Determination of inorganic phosphate

Inorganic phosphate was determined by the method of Fiske and Subbarow (1925). A standard curve was prepared for each set of experimental determinations using KH_2PO_4 and an average of 10 readings, as shown in Figure 3.

iii. The P:O ratio

The P:O ratio was calculated from the results of the measurement of the uptake of inorganic phosphate and oxygen.

(g) Incorporation of C^{14}-amino acids into mitochondrial protein

i. Procurement of radioactive amino acids

A mixture of uniformly labelled L-(C^{14})-amino acids, a protein hydrolysate of the green alga Chlorella vulgaris, was obtained from the Radiochemical Center, Amersham, Becks, England, through Atomic Energy of Canada Ltd. Its specific activity was 200 µc/mg. of hydrolysate.

Although it was expected that all the amino acids of the protein hydrolysate would be labelled, the mixture was chromatogrammed and radioautographed to provide information on the kinds of amino acids, and their labelling. It was found that all the amino

Figure 3

A standard calibration curve for the determination of inorganic phosphate (mg) from the reading of optical density at 660 mμ (an average of 10 determinations).

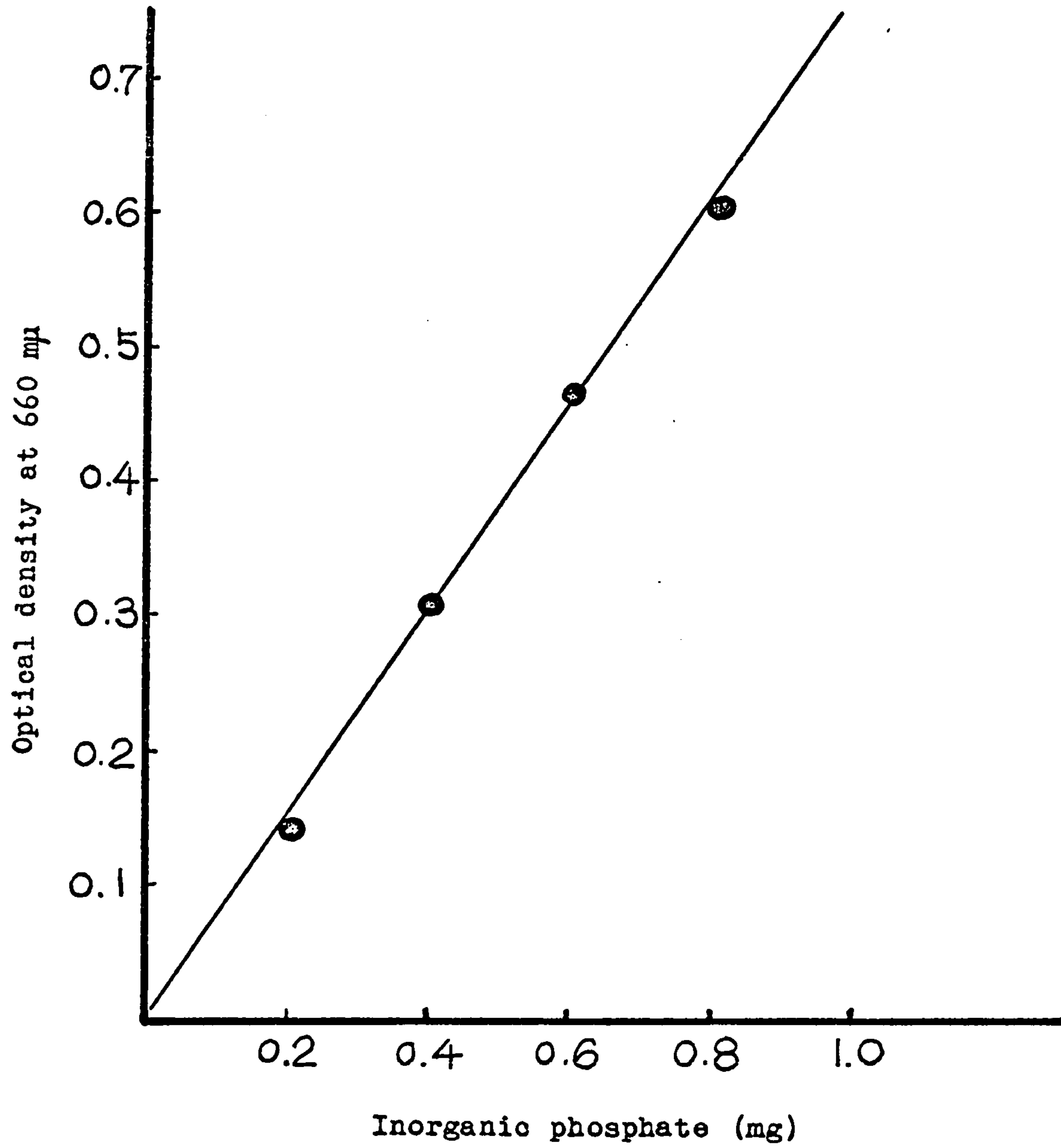

Figure 3

acids detected by a ninhydrin spray were labelled (Figure 4). The C^{14}-amino acids mixture, which arrived in the form of a powder, as dissolved in distilled water to give a solution with a specific activity of 10 µc/ml and stored in a freezer.

ii. Incorporation of C^{14}-amino acids into mitochondrial protein (in vitro)

Roodyn *et al* (1961), Sokolof and Kaufman (1961), and Truman and Korner (1962) have reported methods for the study of the incorporation of labelled compounds into mitochondrial protein. The following method was adopted for the purpose of the present study.

Reaction mixture - 3 ml of the mixture described by Roodyn *et al* (1961) was added to a 50 ml Erlenmeyer flask, along with 1.5 ml of cell sap, the preparation of which is described below, 1 ml of a 2 µc/ml solution of C^{14}-labelled amino acids, and 1.5 ml of a suspension of isolated mitochondria containing 18-20 mg of mitochondrial protein.

The mixture was incubated in a Dubnoff shaking water bath at 30° C under a gas phase of 95% O_2 + 5% CO_2 and shaken at a rate of 100 cycles per minute.

Preparation of cell sap - Rat liver was homogenized with a Dounce homogenizer in 5 volumes of a mixture of sucrose (0.3*M*), ethylenediaminetetraacetate (EDTA) (2m*M*) and nicotinamide (0.03*M*) adjusted to pH 7.1 with KOH. The homogenate was centrigued at 900*g*

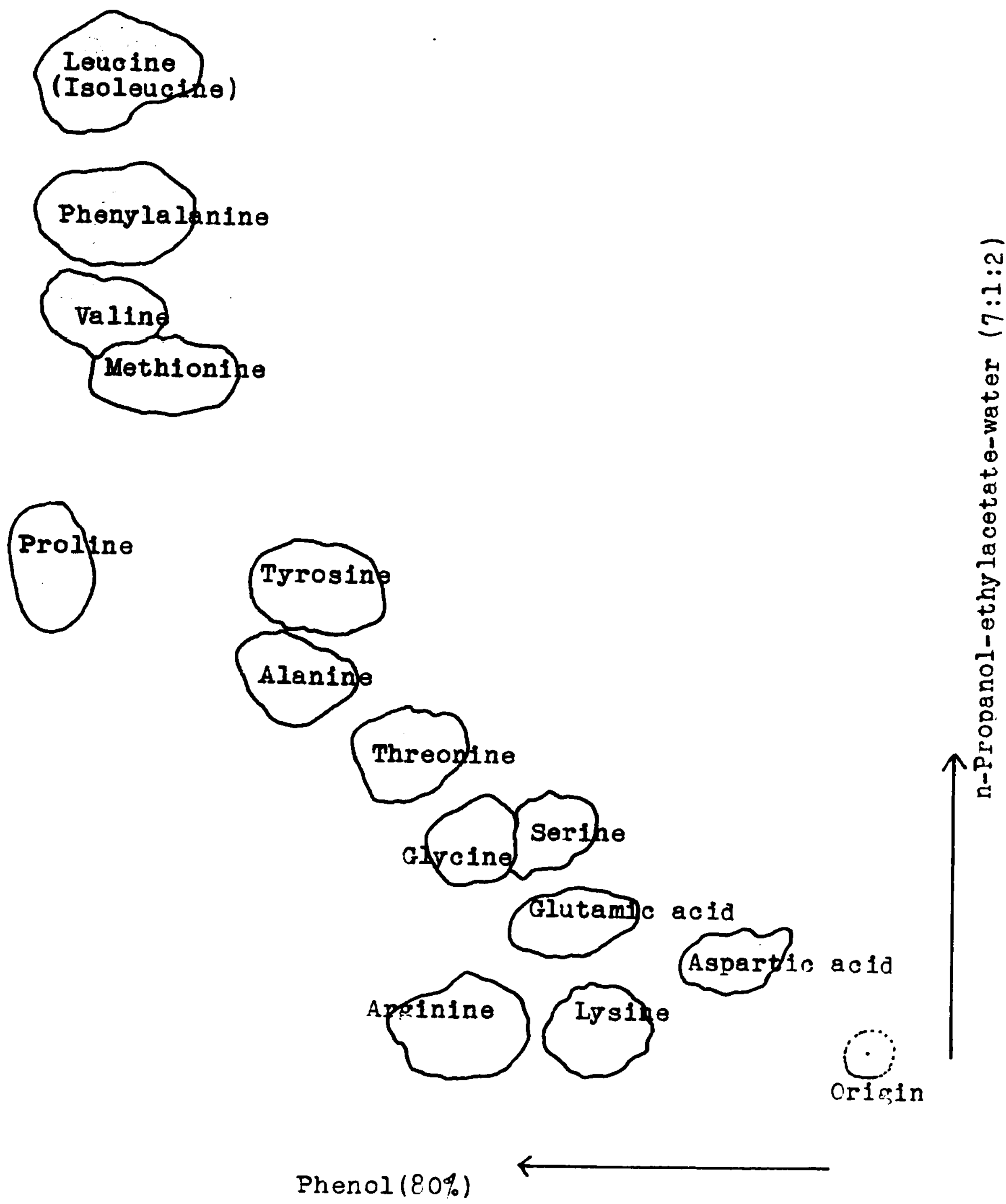

Figure 4. A radioautogram of paperchromatogram of C^{14} - amino acids (uniformly labelled) of algal protein hydrolysate (*Chlorella vulgaris*).

Fig. 4

for 30 minutes, the pellet discarded, and the supernatant recentrifuged at 105,000g in a Spinco ultra-centrifuge Model L for 3 hours. The bright red supernatant so obtained is referred to hereafter as cell sap. It was stored in a freezer (-10° C) for later use and under these conditions was stable for periods of up to one month.

<u>Preparation of the protein fraction from the samples</u> - At various time intervals, the reaction was terminated by the addition of an equal volume of 10% TCA containing carrier amino acids. Total protein of mitochondria was determined by the method described in section e-i. Soluble and insoluble protein from mitochondria was determined by the method described in section e-ii. The precipitated protein, total, soluble, or insoluble, as the case might be, was dissolved in 0.5% NH_4OH, to which a large excess of carrier amino acid mixture was added. It was treated with the following procedure in order to remove RNA and amino acids bound to, but not incorporated into the protein. Dissolved protein was reprecipitated with TCA and washed twice with 5% TCA at room temperature, resuspended in 5% TCA and heated 15 minutes at 90° C, washed twice more with 5% TCA at room temperature, twice with acetone, twice with ethanol-ether (3:1) at 60° C and finally twice with ether (Figure 5).

In the course of this washing procedure the radioactivity in the washing at each step (Figure 5) was determined. It was found that there was a negligible amount of radioactivity in the final

whole liver + 3 volumes of sucrose solution (Roodyn et al, 1961)

↓

isolated mitochondria

↓

1.5 ml of mitochondrial suspension added to 3.0 ml of reaction medium

↓

addition of C^{14}-amino acids and 1.5 ml of cell sap to above reaction mixture, and incubate for 60 minutes

washing procedures
(washed twice at each step)

addition of 3 ml of carrier amino acids and 10 ml of 10% TCA to above reaction mixture

↓ *

addition of 4 ml of 0.5% NH_4OH

↓ *

addition of 4 ml of carrier amino acids and 8 ml of 10% TCA

↓ *

addition of 1 ml of carrier amino acids and 3.5 ml of 5% TCA

↓ *

addition of 1 ml carrier amino acids and 3.5 ml of 5% TCA heated at 90° C

↓ *

addition of 1 ml of carrier amino acids and 3.5 ml of cold 5% TCA

↓ *

addition of 3 ml of acetone

↓ *

addition of 3 ml of ethanol : ether at 60° C

↓ *

addition of 3 ml of ether

↓ *

final sample

* Centrifugation at 12,000g

Figure 5 Procedures for the preparation and sampling of mitochondria isolated from the liver of normal and cold-acclimated rats before and after the incorporation of C^{14}-amino acids into mitochondrial protein

stage of washing, indicating that the radioactive amino acids adsorbed on the protein was sufficiently washed off.

The washed mitochondrial protein precipitate was dissolved in 90% formic acid, the volume of formic acid depending on the assumed protein content. An aliquot of 0.2 ml of this protein solution was pipetted into the center of a stainless steel planchet (diameter 2.3 cm.) having a concentric ridge to prevent spreading of the sample after plating. Each protein sample was plated onto a constant area of 1.32 cm^2. and dried under an infrared lamp in a fume hood for counting. After counting, the protein content of each sample was determined by the method previously described.

iii. Incorporation of C^{14}-amino acids into mitochondrial protein (in vivo)

The radioactive C^{14}-amino acid mixture was injected to each rat by syringe through a polyethylene cannula, which had been inserted into the carotid artery by the operation described by Popovic and Popovic (1960). Operated rats were kept under normal or cold conditions for 10 days and were fasted for 18 hours before the injection of radioactive amino acids. During the injection, the animal was tightly held in an Econo-restraining cage.

Since these were short term experiments, a relatively large dose of radioactivity (10 μc in one ml of 0.9% saline solution) was injected. The injected rats were kept at their respective

temperature of acclimation, and 60 minutes after injection they were quickly decapitated and the various organs removed. Mitochondria were isolated from each organ by the method described previously. The protein of the isolated mitochondria was precipitated by the addition of TCA and collected by centrifugation. The precipitate was dissolved in 90% formic acid and prepared for counting following the procedure described in the previous section.

The radioactivity in the total protein of mitochondria of each organ was measured. In the case of the liver preparation, mitochondrial protein was further fractionated into soluble and insoluble fraction.

iv. Determination of radioactivity

Radioactivity was determined with a gas-flow Geiger counter (Nuclear Chicago Model DC-47) having a micromil window, and low background automatic sample changer (Model 110A). Determinations were made to a probable error of 2% in most cases. The radioactivity measured was corrected for self-absorption with a standard curve which was prepared by serial dilution of the radioactive mitochondrial sample.

(h) Electron microscopic technique

The liver, excised as described in section a-ii, was cut with a razor blade into small strips, 1-2 mm. thick. These strips were fixed for 30 minutes in 1.5% osmium tetroxide solution buffered with veronal acetate buffer at pH 7.3, after which they were cut into still smaller

pieces, not larger than 1.0-2.0 mm. on a side. The pieces were transferred to fresh osmium tetroxide fixative for 1.5 hours, so that the total fixation time was about 2 hours. At this time, a few pieces were removed to a glass slide, cut in half, and observed under a binocular microscope. If they did not appear to be properly fixed, the tissue was left in the fixative for 2 more hours. This two step method of fixation resulted in less damage to the tissues than if they had been cut into smaller pieces at the beginning.

The well-fixed tissues were rinsed for 15 minutes in Tyrode's solution, and dehydrated in a graded series of acetone beginning with

Tyrode's solution

component	concentration
NaCl	8.0 (gram/liter)
KCl	0.2
* $CaCl_2$	0.2
$MgCl_2$	0.01
NaH_2PO_4	0.05
Dextrose	1.0
$NaHCO_3$	1.0
H_2O	1000 ml

* $CaCl_2$ must be added after a mixture of these components has been gassed with 95% O_2 + 5% CO_2.

30% for 15 minutes, 50% for 15 minutes, 75% for 15 minutes, 90% for 15 minutes, 100% for 30 minutes and 100% for 60 minutes.

After completion of dehydration, the tissues were placed in a solution of Vestopal, obtained from Martin Jaeger, Vesenaz, Geneva, Switzerland, and acetone with the following combination of ratios and incubation times; Vestopal:acetone (v/v) 1:3 (90 minutes), 1:1 (90 minutes), 3:1 (90 minutes) and finally in 100% Vestopal for 18-20 hours. The tissues were then embedded in Vestopal containing activator (1%) and initiator (1%) in a "oo" gelatine capsule. The capsule was placed in an oven for 50 hours at 50° C at which temperature the Vestopal polymerized. Embedding of the tissues in Epon was compared with that in Vestopal in preliminary experiments. It was found that the block of Vestopal was easier to section than the block of Epon.

The Vestopal blocks were cut into sections of 500 Å with a Serval Porter-Blum microtome, using a glass knife, and the sections were mounted on a 300 mesh grid, previously coated with 0.25% Formvar film (obtained from Ladd Research Industries, Inc., Vermont, U.S.A.).

They were stained with lead hydroxide for 10 minutes (Karnovosky, 1961), or 1% uranyl acetate for 5-10 minutes.

The electron microscope used in this study was a Philips E.M. 100 equipped with a noncompensible "25 Å" short focus lens. Performance was limited to an overall resolution of about 35 Å by the residual astigmatism of this lens. Micrographs were taken at 80 kv. and 60,000X initial magnification. Further enlargement was obtained optically. The beam potential was 100 kv.

(i) Treatment of data

The results obtained in all experiments were subjected to the "t" test. The standard error were calculated, and most of the data were expressed as percent of control.

The results of the number of mitochondria of rat liver were analysed with a modified Steel and Torrie Rx2 table, which is a more suitable test for these results. This "test" was carried out by Professor Reid at the School of Hygiene, University of Toronto, using an I. B. M. computor, model 7094.

CHAPTER III

Results and Discussion

Experiment I-1 Comparisons of dry weight and number of liver mitochondria in control and cold-acclimated rats

Results

A. Dry weight of mitochondria

The dry weight of liver mitochondria isolated from control and cold-acclimated rats are compared in Table 1 and Figure 6. The data were analysed statistically, and subjected to the "t" test. As shown in Table 1, the dry weight of liver mitochondria isolated from control rats did not change very much from 70 days to 230 days of the experimental period, ranging from 35.75 milligrams to 39.51 milligrams. However, in cold-acclimated rats, the dry weight per gram of original fresh liver tissue decreased steadily with the length of duration of cold exposure, from 91.17 percent at 70th day to 60.84 percent of control by 230th day.

B. Number of mitochondria

The number of liver mitochondria of control and cold-acclimated rats was counted at various intervals up to 7 months of the cold exposure. The number was expressed per gram of original fresh liver tissue and the data are presented in Table II-a and Figure 7. The results show that the number of mitochondria isolated from control

TABLE I

Dry weight of mitochondria (mg) per gram of fresh liver tissue of control rats (+23° C) and cold-acclimated rats (+2° C) at various times of the experimental period.

Days	Fresh body weight (g)		Fresh liver tissue (g)	
	Control	Cold-acclimated	Control	Cold-acclimated
70	431.00 (7)	315.33 (3)	12.69	12.92
90	455.83 (6)	321.57 (7)	14.19	11.70
165	569.25 (4)	404.75 (4)	13.80	14.68
230	554.00 (2)	380.50 (2)	15.83	11.51
Mean	502.27	355.54	14.13	12.70

Days	Mitochondrial dry weight mg/gm of fresh liver			
	Control	Cold-acclimated	"t" test	% of control
70	39.51 ± 0.65**	36.02 ± 1.44**	*	91.17
90	36.91 ± 2.30	27.52 ± 1.45	*	74.56
165	35.75 ± 2.35	28.21 ± 1.35	*	78.91
230	37.74 ± 0.01	22.96 ± 0.26	*	60.84
Mean	37.48	28.68		76.52

* "t" value is significant at 5% level

** standard error

( ) number of animals

Figure 6

Dry weight of mitochondria (mg) per gram of fresh liver tissue of control rats (+23° C) and cold-acclimated rats (+2° C) at various times of the experimental period.

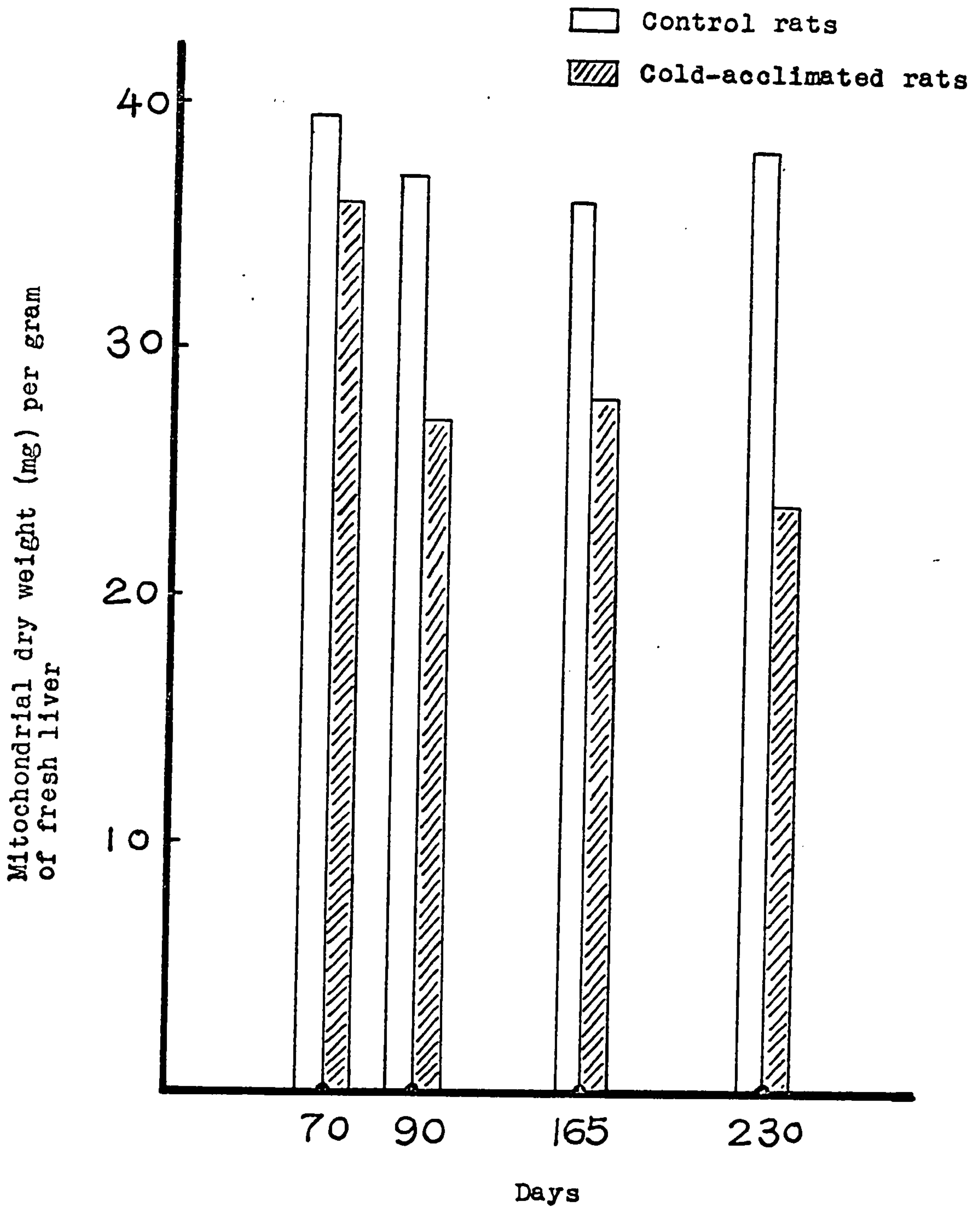

Figure 6

TABLE II-a

Number of mitochondria per gram of fresh liver tissue of control rats (+23° C) and cold-acclimated rats (+2° C) at various times of the experimental period

Month	Number of mitochondria/g of fresh liver (2×10^{10})		"t" test	% of control
	Control	Cold acclimated		
0	21.58 ± 4.17** (6)			100.00
½	22.03 ± 0.80 (4)	16.91 ± 0.70 (4)	*	76.71
1	21.86 ± 1.00 (4)	16.74 ± 1.91 (4)	*	76.58
1½	22.53 ± 1.35 (4)	18.26 ± 0.59 (4)	*	81.05
2	19.91 ± 0.42 (6)	16.37 ± 0.18 (4)	*	82.22
3	22.83 ± 1.46 (4)	15.19 ± 0.11 (4)	*	66.54
5½	20.10 ± 1.43 (4)	14.88 ± 1.19 (4)	*	74.03
7	20.92 ± 0.30 (4)	16.67 ± 0.25 (2)	*	74.90
Mean	21.45	16.29		75.94

* "t" value is significant at 5% level
** standard error
( ) number of animals

TABLE II-b

Analysis of variance for the data in Table II-a

	df	Mean square	F
Temperature	1	350.4542	90.47**
Length of experimental period	6	7.9284	2.05
Interaction	6	3.3606	0.87
Within groups	40	3.8736	

** significant at 1% level

Figure 7

Number of mitochondria per gram of fresh liver tissue of control rats (+23° C) and cold-acclimated rats (+2° C) at various times of the experimental period.

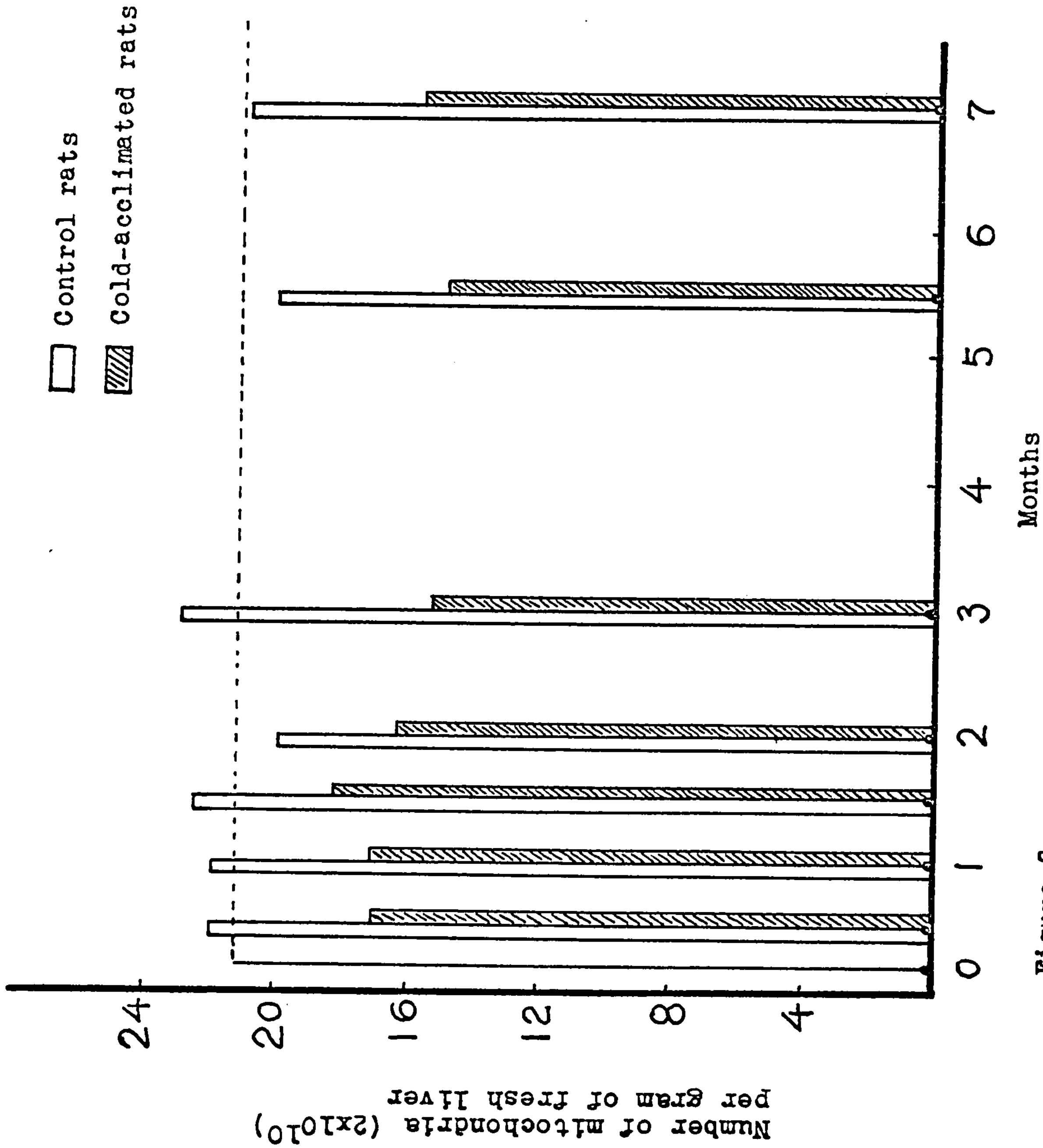

Figure 7

rats did not change very much up to 7 months of the experimental period. The values obtained from the control rats ranged from 19.91 x $(2x10^{10})$ to 22.83 x $(2x10^{10})$. However, the number of mitochondria isolated from cold-acclimated rats decreased, starting from the 14th day of the experimental period. The analysis of variance is shown in Table II-b.

Discussion

Many difficulties are encountered when the dry weight and the number of mitochondria isolated from control and cold-acclimated rats are compared, owing to the fact that it is impossible at present to isolate mitochondrial pellets free of contamination of other cell components.

In the comparisons of dry weight and number of mitochondria in the present experiment, the criteria was set in such a way that only the mitochondria sedimenting between 900g and 12,000g were considered. The mitochondria of this particular fraction were examined under the phase-contrast microscope and were found to be free from other cell components. Therefore, in this study, only the mitochondria of this particular fraction were considered for comparisons. According to Dr. Lusena (personal communication) about 20 percent of the mitochondria were lost in the course of mitochondrial isolation.

The dry weight of mitochondria might be overestimated by about 11.3 percent since no correction was made for the dry weight

of 5 milliliters of 0.9 percent KCl and 2 milliliters of 0.44M sucrose solution introduced during the preparation of the mitochondrial pellets. According to Dr. Lusena (personal communication), 30 percent of the mass of packed pellets is extramitochondrial (chiefly water, KCl and sucrose) and 70 percent of packed pellets of mitochondria is mitochondrial. This portion of mitochondrial pellets contains, according to Werkheiser and Bartley (1957), 34 percent of dry matter of mitochondria and 66 percent of water, KCl and sucrose (Figure 8). Assuming a similar situation in our experiments, calculations showed that our values were overestimated by 11.3 percent.

Considerable care was taken during counting of mitochondrial particles with the phase-contrast microscope, since there are many technical difficulties. Mitochondria suspended in 0.25M or 0.88M sucrose solutions can not be easily counted in the chamber because of the extent of the Brownian movement. In this study, a 1.8M sucrose solution was used in order to reduce the Brownian movement as recommended by Allard et al (1952). Mitochondria were counted at 1 - 3 particles per square and from the limit of visibility to approximately 2 µ in size. Fading of mitochondria might present another difficulty in counting of aged mitochondria. However, it was observed in this study that the original form and number of mitochondria remained constant in 1.8M sucrose solution at 0 - 5° C for a period of 24 hours. The number of mitochondria in control rats (Table II-a) was found to

Mitochondrial dry weight for
one milligram of packed pellets

0.3 ml is extramitochondrial (containing chiefly water, KCl, and sucrose)

$$\frac{0.3 \times (\frac{2}{7}\,15 + \frac{5}{7}\,0.9)}{100} = 0.015$$

* 0.015 g. due to KCl and sucrose.

7 ml of original suspension

2 ml 15% (0.44M) sucrose

5 ml 0.9% KCl

0.7 ml is mitochondrial (assuming 34% due to matter of mitochondria, and 66% due to water, KCl and sucrose).

0.238 ml of dry matter (assuming a mitochondrial density of 1.25)

$0.238 \times 1.25 = 0.298$

* 0.298 g. due to mitochondrial matter.

0.462 ml contains water, KCl and sucrose

$0.462 \times (\frac{2}{7}\,15 \times \frac{5}{7}\,0.9) = 0.023$

* 0.023 g. due to KCl and sucrose

$$\frac{0.015 + 0.023}{0.038 + 0.298} = \frac{0.038}{0.336} = 11.3$$ ----- 11.3 percent overestimation

Figure 8

be in good agreement with the data of other investigators (Allard _et al_, 1952; Miller _et al_, 1959 and Borrows _et al_, 1960).

It is evident from Table I and II-a that cold exposure resulted in a reduction of the dry weight and the number of mitochondria when expressed per gram of the original fresh liver weight. It is likely that the reduction of the total dry weight of mitochondria of cold-acclimated rats resulted from an actual lower count of mitochondria, indicating that the values of percent of control of dry weight and number of mitochondria in grand means are 76.52 and 75.94 respectively. However, it is still not certain that this is the only reason for the decrease of the dry weight of mitochondria. The reduction in the mass of each mitochondrion or a concomitant reduction in number with the mass of each individual mitochondrion might also be possible in cold-acclimated rats.

Since the dry weight and the number of mitochondria were expressed per gram of original fresh liver tissue, further calculations were made, using the grand means in Tables I and II-a. From the ratio between the average number of mitochondria and the average weight of mitochondria during an experimental period of about seven months, it was found that the number of mitochondria per one milligram of the dry weight of mitochondria was 11.45×10^9 in normal rats, and 11.35×10^9 (99.12% of control) in cold-acclimated rats (Table III). This indicates that the number of mitochondria per milligram of dry

TABLE III

Number of mitochondria expressed as per milligram of mitochondrial dry weight of liver from control rats (+23° C) and cold-acclimated rats (+2° C). These calculations are based on the data in Tables I and II-a.

	Control	Cold acclimated	% of control
Mitochondrial dry weight (mg/g of fresh liver)	37.48	28.68	76.52
Number of mitochondria (1×10^{10}/g of liver)	42.90	32.58	75.94
Number of mitochondria per mg of dry weight (1×10^{9})	11.45	11.35	99.12
Mitochondrial dry weight (mg/number of mitochondria (1×10^{10}))	0.87	0.88	101.15

weight of mitochondria was 11.45 x 10^9 in normal rats, and 11.35 x 10^9 (99.12% of control) in cold-acclimated rats (Table III). This indicates that the number of mitochondria per milligram of dry weight is practically the same in control as in cold-acclimated rats. The dry weight of mitochondria per unit number of mitochondria (1 x 10^{10}) was found to be 0.87 milligrams in control rats, and 0.88 milligrams in cold-acclimated rats. Thus, the dry weight of each mitochondria would appear to be similar in both control and cold-acclimated rats. Therefore, the reduction in the total dry mass of mitochondria from one gram of original fresh liver tissue from cold-acclimated rats must be solely due to a decrease in the number of mitochondria per unit weight of fresh liver. The data, furthermore, suggest indirectly that there is no cold effect on an individual mitochondrion in relation to the dry mass, but there might be a slower rate of mitochondrial replication, resulting in the reduction of mitochondria as a whole.

The number of mitochondria was reduced with time in cold-acclimated rats during a seven month experimental period, but not so in control rats; therefore, aging was not the cause of this reduction in number of mitochondria. It would rather appear to be a progressive effect of continuous cold exposure, at least for the period studied.

Experiment I-2 Measurement of total, soluble, and insoluble proteins of liver mitochondria

Results

The total protein content of mitochondria as well as the soluble and insoluble protein fractions were compared at various times in the course of the experiment. The results are presented in Table IV, and show that the total protein and soluble fraction of mitochondria from cold-acclimated rats tended to parallel one another, decreasing steadily from 25 days to 95 days. The insoluble protein remained more or less constant. In only one instance (35 - 45 days), was the insoluble protein higher in cold-acclimated rats than in control rats. In general, the protein content gradually decreased in mitochondria of cold-acclimated rats relative to those of control rats, showing average percent values of control 86.01 in total protein, 86.45 in soluble fraction and 90.37 in insoluble fraction of mitochondrial protein.

Discussion

The reliability of measurements of total, soluble, and insoluble protein of mitochondria with the standardization of bovine albumin was assessed by calculating recoveries in the preliminary experiments. These were from 98 percent to 102.5 percent. Apparently, no protein was lost by subfractionation.

The present results show that the mitochondrial protein content is decreased in cold-acclimated rats, due mainly to a decline

TABLE IV

Content of total, soluble, and insoluble protein of mitochondria per gram of fresh liver tissue of control rats (+23°) and cold-acclimated rats (+2° C) at various times of experimental period

	Days	Control	Cold acclimated	"t" test	% of control
Total protein (mg)	25	5.90 ± 0.71** (3)	5.48 ± 0.30 (3)	*	92.88
	35	6.93 ± 0.15 (3)	6.26 ± 0.26 (3)		90.33
	45	6.15 ± 0.26 (4)	5.58 ± 0.68 (3)	*	90.73
	55	6.58 ± 0.32 (3)	5.50 ± 0.38 (3)	*	83.59
	65	6.50 ± 0.30 (3)	5.05 ± 0.47 (3)		77.69
	95	6.08 (1)	4.95 (1)		81.41
	Mean	6.36	5.47		86.01
Soluble protein (mg)	25	4.23 ± 0.15	4.12 ± 0.12		97.40
	35	5.06 ± 0.13	4.16 ± 0.08	*	82.21
	45	4.58 ± 0.21	3.84 ± 0.48		85.33
	55	4.71 ± 0.24	4.07 ± 0.10	*	86.41
	65	4.80 ± 1.55	4.08 ± 0.13		85.00
	95	4.24	3.54		85.38
	Mean	4.59	3.97		86.45
Insoluble protein (mg)	25	1.60 ± 0.28	1.46 ± 0.03	*	91.25
	35	1.91 ± 0.45	2.24 ± 0.07	*	117.28
	45	1.86 ± 0.16	1.87 ± 0.32		100.54
	55	1.95 ± 0.11	1.48 ± 0.15		75.90
	65	1.92 ± 0.08	1.48 ± 0.26		77.08
	95	2.02	1.59		78.71
	Mean	1.87	1.69		90.37

* "t" value is significant at 5% level
** standard error
( ) number of animals

of the soluble fraction. Recently, Lusena and Dass (1966) found that soluble protein (glutamic dehydrogenase) is released from isolated mitochondria after freezing for 18 hours at -5° C and consequent thawing at 37° C. After this treatment, mitochondria appeared swollen and emptied, indicating a leaching of soluble protein. These authors also noted that the release of insoluble protein (3-hydroxybutyrate dehydrogenase) was associated with the breakage of the mitochondrial membranes. It is thus possible, in our study, that some mitochondrial protein was lost during the isolation of mitochondria, since it is known that mitochondria from cold-acclimated rats tend to swell and to become fragile (Fairhurst et al, 1958 and Boatman et al, 1962).

There is evidence that cold exposure induces an increase in the utilization of protein as a respiratory substrate. This evidence is based on the increased urinary excretion of nitrogen or amino acids (Rangneker and Dugal, 1958; Klain and Vaughan, 1963; Beaton, 1963 and Hannon, 1963). The relatively greater reduction of total protein of mitochondria in cold-acclimated rats observed in this experiment would also suggest a greater catabolism of mitochondrial protein in cold-acclimated rats. Judging from the wide fluctuation in the content of soluble protein, and the relatively constant levels of insoluble protein, it would appear that it is the soluble protein which is directly used as a substrate. Ultimately, however, the insoluble protein is

used as well since the content of both fractions is reduced in cold-acclimated rats.

There is no way of knowing the percentage of release of soluble and insoluble protein during the isolation procedure. At any rate, the reduction of soluble mitochondrial protein could be considered as indirect evidence that there is an effect of cold exposure on the protein content of mitochondria.

Experiment II Oxidative phosphorylation

This experiment was carried out to determine whether a direct relationship could be observed between oxidative phosphorylation, number of mitochondria and mitochondrial protein content in cold-acclimated rats.

Results

Oxidative phosphorylation was determined in liver mitochondria isolated from control and cold-acclimated rats, as a function of the time of incubation of mitochondria, as a function of the duration of the cold exposure, and as a function of the age of the experimental animals. The data are shown in Table V and VI. The uptake of oxygen and of inorganic phosphate by isolated mitochondria increased continuously up to the end of the experiment (60 minutes) in both control rats and rats acclimated to cold for approximately 3 months (Table V and Figure 9). Table V shows that oxygen uptake by mitochondria isolated from cold-acclimated rats exceeded that by those from control rats. On the other hand, the uptake of inorganic phosphate was greater in the latter than in the former. Therefore, a lowered P:O ratio resulted in liver mitochondria from cold-acclimated rats as compared to those from control rats. The P:O ratio of control rats is slightly increased from the beginning to the end of the experiment in the oxidative medium used in this experiment, whereas that of cold-acclimated rats remained more or less constant.

TABLE V

Uptake of oxygen and of inorganic phosphate and the P:O ratio at various incubation times by liver mitochondria isolated from control rats (+23° C) and cold-acclimated rats (+2° C).

	Min.	Control	Cold-acclimated	"t" test	% of control
μatom O_2/mg of protein	15	1.35 ± 0.23** (3)	1.38 ± 0.21** (3)		102.22
	30	2.63 ± 0.16	2.68 ± 0.45		101.90
	45	3.83 ± 0.54	4.19 ± 0.83		107.79
	60	4.91 ± 0.75	5.69 ± 1.10		108.77
μM Pi/mg of protein	15	2.91 ± 0.52	2.62 ± 0.37		90.34
	30	5.53 ± 0.83	4.92 ± 0.71		88.96
	45	7.16 ± 0.38	7.12 ± 1.47		99.44
	60	11.88 ± 1.75	10.64 ± 1.84		92.49
P/O ratio	15	2.12 ± 0.11	1.90 ± 0.01		89.62
	30	2.08 ± 0.36	1.80 ± 0.04		86.56
	45	2.39 ± 0.02	1.70 ± 0.01	*	71.13
	60	2.43 ± 0.10	1.90 ± 0.17	*	78.19

* "t" value is significant at 5% level
** standard error
( ) number of animals - 3 animals were used in each group

Figure 9

The rate of uptake of oxygen and of inorganic phosphate, and the P:O ratio at various incubation times by liver mitochondria isolated from control rats (+23°C) and cold-acclimated rats (+2°C).

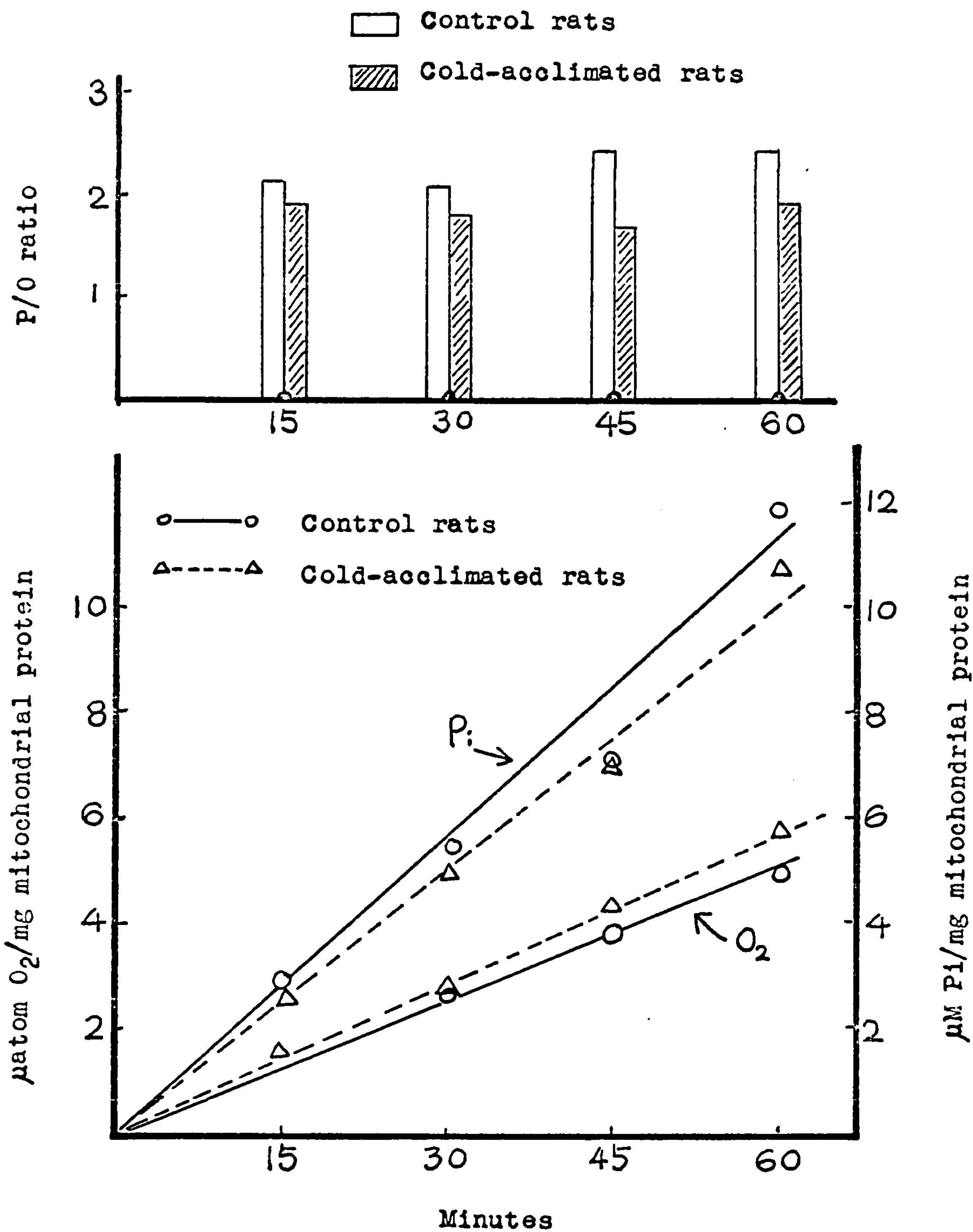

Figure 9

TABLE VI

Uptake of oxygen and of inorganic phosphate and the P:O ratio at various times of the experimental period by liver mitochondria isolated from control rats (+23° C) and cold-acclimated rats (+2° C).

	Days	Control	Cold-acclimated	"t" test	% of control
µatom O_2/mg of protein/60'	0	6.25 ± 0.47** (5)			100.00
	35	4.58 ± 0.19 (3)	7.31 ± 0.94 (3)	*	159.45
	75	5.12 ± 0.51 (4)	6.26 ± 0.72 (3)		122.38
	95	4.08 ± 0.41 (3)	5.10 ± 0.58 (3)		124.95
	125	4.53 ± 0.56 (4)	5.42 ± 0.83 (4)		119.42
	Mean	4.58	6.09		132.97
µM Pi/mg of protein/60'	0	14.34 ± 1.83			100.00
	35	11.18 ± 0.97	10.43 ± 2.63		92.35
	75	15.05 ± 3.15	10.64 ± 0.11		70.74
	95	10.60 ± 2.08	8.47 ± 0.57		79.86
	125	11.90 ± 1.20	9.87 ± 1.50		82.10
	Mean	12.18	9.85		80.87
P/O ratio	0	2.25 ± 0.24			100.00
	35	2.42 ± 0.14	2.29 ± 0.14		94.59
	75	3.01 ± 0.36	1.71 ± 0.14	*	56.90
	95	2.53 ± 0.35	1.71 ± 0.24		66.74
	125	2.65 ± 0.22	1.83 ± 0.10	*	69.21
	Mean	2.65	1.89		71.88

* "t" value is significant at 5% level
** standard error
( ) number of animals

Figure 10

Uptake of oxygen and of inorganic phosphate, and the P:O ratio at various times of the experimental period by liver mitochondria isolated from control rats (+23° C) and cold-acclimated rats (+2° C).

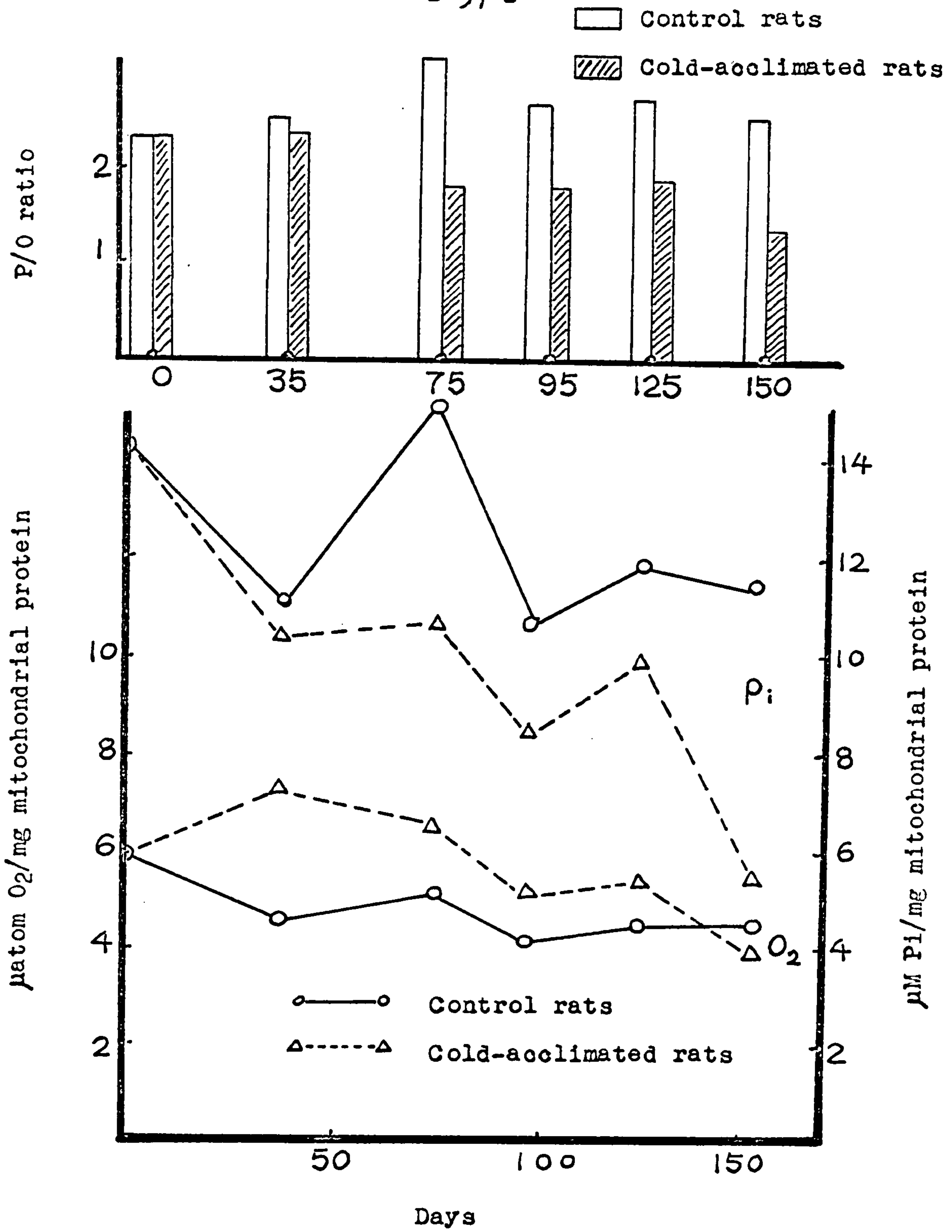

Figure 10

Table VI shows that the uptake of oxygen and of inorganic phosphate by liver mitochondria isolated from both control rats and cold-acclimated rats gradually decreased with increased age of the rats and increased length of duration of cold exposure. Although the P:O ratio of control rats was not affected by this decline in uptake of oxygen and inorganic phosphate, the P:O ratio was progressively decreased with time in cold-acclimated rats. For example, at the end of the experiment, uptakes of oxygen and of inorganic phosphate were 72.32% and 82.92% respectively of initial values (0 day) in control rats and 86.72% and 68.12% respectively in cold-acclimated rats.

Discussion

It is known that uncoupling of oxidative phosphorylation develops with "aging" of isolated mitochondria (Pardee and Potter, 1949; Lehninger, 1949, and Kielley and Kielley, 1951). Beyer et al (1955) found a significant correlation between uncoupling of oxidative phosphorylation and the swelling of mitochondria during aging. Furthermore, Lehninger (1962) found that induced swelling of mitochondria is accompanied by loss of certain cofactors such as NAD and protein components, resulting in the uncoupling of oxidative phosphorylation. Thus, it might be argued that the uncoupling of oxidative phosphorylation observed in the present study could be caused either by "aging" of isolated mitochondria or by a direct effect of cold on mitochondria.

However, there is evidence that mitochondria isolated from cold-acclimated rats swell more readily and consequently the mitochondrial membrane becomes more permeable. In this condition, certain factors would possibly be leached from mitochondria (Fairhurst et al, 1958 and Boatman et al, 1962). Lianides and Beyer (1960a) observed that mitochondria isolated from cold-acclimated rats are more susceptible to "aging". Therefore, it could be concluded that the uncoupling of oxidative phosphorylation could possibly result from the acquired behaviour of liver mitochondria from cold-acclimated rats to "age" sooner during the isolation procedure as compared to liver mitochondria from normal rats.

It was clearly shown that the P:O ratio was lowered in mitochondria isolated from cold-acclimated rats, and that the P:O ratio was gradually declining during the cold exposure of the rats, while increasing slightly in control rats during the same period (Table VI). These results fit in well with the work reported by Smith and Fairhurst, 1958; Panagos et al, 1958; Lianides and Beyer, 1960a and b. However, other workers have reported that the P:O ratio was unchanged in mitochondria isolated from cold-acclimated rats (Patkin and Masoro, 1960; Frehn and Anthony, 1962; and Aldridge and Stoner, 1964). According to Hefferd (1960), the above discrepancies may be due to variations in individual laboratories, such as temperature, air movement, humidity, length of exposure to cold, lighting schedules, kinds of diets, age of rats, and the strain or sex used.

Smith (1956) found a relationship between oxygen uptake and the number and mass of mitochondria, but suggested that the oxygen uptake of liver homogenates was linked with the total mitochondrial number per gram or original fresh liver tissue, rather than with the mitochondrial mass. Green and Hatefi (1961) reported that the increased number of mitochondrial cristae could be a factor responsible for the increase in oxygen uptake because electron transfer particles are more or less confined in mitochondrial cristae. In summary, increased oxygen uptake has been associated with an increased number of mitochondria, mitochondrial cristae or an increased mitochondrial dry mass. However, these morphological changes must reflect other and possibly more basic biochemical changes.

The possible mechanism leading to a decline of the P:O ratio has been discussed in the Literature Review (see Introduction). Lowered P:O ratios can result from an increased oxygen uptake without a corresponding increase in the uptake of inorganic phosphate. In this discussion, an attempt will be made to establish a link between oxidative phosphorylation, and the number of mitochondria or the mitochondrial mass. The grand means (for an experimental period of 3 months) of the number and protein content of mitochondria from both control and cold-acclimated rats was expressed per gram of original fresh liver tissue (Tables II-a and IV). The average oxygen uptake (for an experimental period of 3 months) was expressed as µatoms per milligram

of protein of mitochondria (Table VI), and from the number of mitochondria per milligram of protein, the uptake of oxygen and of inorganic phosphate per unit number of mitochondria was calculated. Since an analysis of protein content of mitochondria is available only for an experimental period of 3 months, the grand means of the number, and of the uptake of oxygen and of inorganic phosphate were also calculated for an experimental period of 3 months from Tables II-a and VI. The results are presented in Table VII, and show that oxygen uptake is greater in mitochondria isolated from cold-acclimated rats (1.00 µatoms: 0.67 µatoms of control rats, or 148.81% over control), when it is expressed on the basis of a unit number of mitochondria (1×10^{10}). On the other hand, the uptake of inorganic phosphate is reduced in mitochondria isolated from cold-acclimated rats (1.61 µM: 1.81 µM of control rats, or 89.50% of control), when it is expressed on the basis of a unit number of mitochondria (1×10^{10}). There is no proportionality between oxygen uptake and uptake of inorganic phosphate. It can be concluded that even with a lesser number of mitochondria than in normal rat liver the cold-acclimated rats are able to take up more oxygen. To account for this increase of oxygen uptake, cold exposure must have been induced in the mitochondria themselves, such as a change in physical behaviour or the electron transport system.

TABLE VII

Uptake of oxygen and of inorganic phosphate per unit number of mitochondria ($1x10^{10}$) of control rats (+23° C) and cold-acclimated rats (+2° C). These calculations are based on the grand means up to 3 months of the experimental period (Tables II-a, IV, and VI).

	Control	Cold-acclimated	% of control
Number of mitochondria per gram of fresh liver ($1x10^{10}$)	43.26	33.38	77.16
Mitochondrial protein per gram of fresh liver (mg)	6.36	5.47	86.01
O_2 uptake (µatom/mg protein)	4.57	6.22	136.11
O_2 uptake µatom/number of mitochondria ($1x10^{10}$)	0.67	1.00	148.21
Pi uptake (µM/mg protein)	12.28	9.85	80.21
Pi uptake µM/number of mitochondria ($1x10^{10}$)	1.81	1.61	89.50

The increased susceptibility to "aging" with the increase of the length of duration of cold exposure and the age of rats observed in this study (% of control in Table VI) could be a reason for the progressively lowered P:O ratio.

Experiment III Protein synthesis in vitro and in vivo

1. Incorporation of C^{14}-amino acids into proteins of mitochondria isolated from rat liver

Results

A. The rate of the incorporation of C^{14}-amino acids into total protein of mitochondria

Liver mitochondria isolated from both control and cold-acclimated rats after an experimental period of 90 days were incubated with uniformly labeled C^{14}-amino acids (Chlorella vulgaris hydrolysate), and the radioactivity incorporated into total protein was determined at 30 minute intervals for 2 hours. The results are presented in Table VIII and Figure 11. The total incorporation of C^{14}-amino acids into mitochondrial protein was gradually increased with time in both groups with the maximum incorporation occurring after 90 minutes of incubation; the rate of incorporation of C^{14}-amino acids was slightly higher in the control rats. After 90 minutes, the amount of C^{14}-amino acids per milligram of protein declines more rapidly in the mitochondria isolated from control rats than in mitochondria from cold-acclimated rats.

B. The distribution of incorporated radioactivity between soluble and insoluble proteins of isolated mitochondria after 60 minutes of incubation.

The radioactivity incorporated into total, soluble, and insoluble proteins of liver mitochondria isolated from both control

TABLE VIII

Incorporation of UL-C^{14}-amino acids *in vitro* into total protein of liver mitochondria isolated from control rats (+23° C) and cold-acclimated rats (+2° C) at various incubation times after an experimental period of 90 days

Temperature	Control				Cold-acclimated			
Minutes	30	60	90	120	30	60	90	120
Radioactivity cpm/mg of mitochondrial protein	62.14	104.48	115.14	70.50	52.11	76.00	89.47	63.99
	53.45	93.94	84.43	70.43	41.50	57.16	66.24	43.27
	56.30	64.28	89.73	60.34	46.67	60.96	72.78	48.33
	57.00	89.52	109.77	63.76	65.43	64.14	79.01	68.62
	-	-	-	-	63.57	86.42	93.63	-
Mean	55.72 ± 2.54	88.05 ± 8.52	99.77 ± 7.48	66.26 ± 2.53	53.87 ± 4.66	68.74 ± 5.22	80.23 ± 5.08	56.05 ± 6.09
% of control	100.00	100.00	100.00	100.00	96.60	78.07	80.41	84.59

Figure 11

Incorporation of UL-C^{14}-amino acids *in vitro* into total protein of liver mitochondria isolated from control rats (+23°C) and cold-acclimated rats (+2°C) at various incubation times after an experimental period of 90 days.

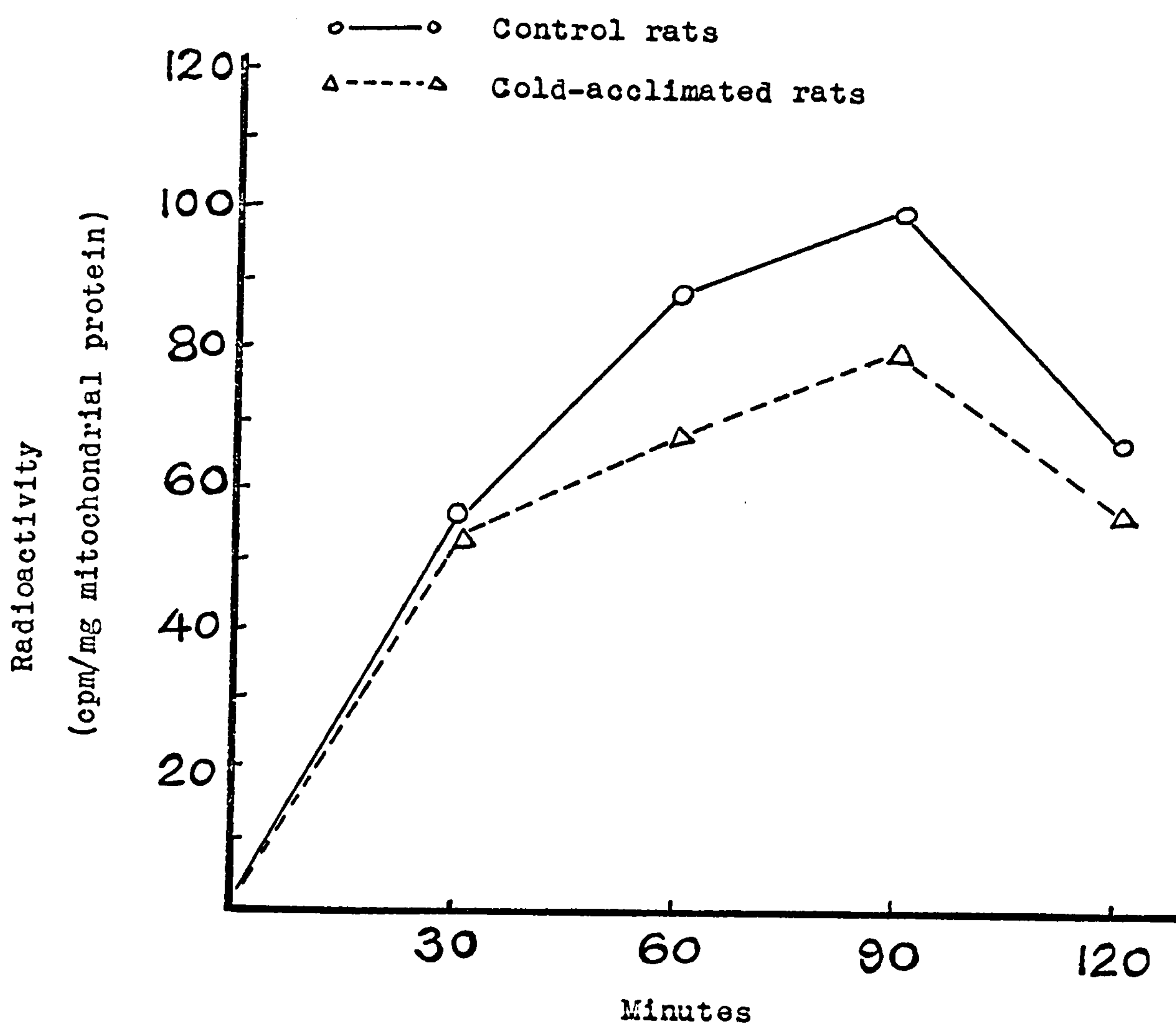

Figure 11

and cold-acclimated rats was determined and the results are presented in Table IX and Figure 12. The specific activities of total, soluble, and insoluble proteins of mitochondria isolated from cold-acclimated rats (Table IX) were only 88.14%, 79.86% and 89.23% that of control rats, respectively. These figures indicate that cold exposure induced a greater reduction in incorporation of C^{14}-amino acids into soluble protein than into insoluble protein. This indication is more clearly seen when the distribution of radioactivity of subfractions of mitochondria within each group is compared. The specific activity of soluble protein of mitochondria isolated from cold-acclimated rats is 54.78% that of total protein, compared to 60.46% for the comparable fraction from control rats. The specific activity of insoluble protein, on the other hand, in relation to that of the total, is almost the same in both groups (182.62% in control rats, 184.88% in cold-acclimated rats).

It is of interest to note that the specific activity was higher in insoluble protein than in soluble protein in both groups (Table IX).

2. <u>Incorporation of C^{14}-amino acids in vivo into protein of mitochondria of control and cold-acclimated rats</u>

It was felt necessary to investigate the incorporation of C^{14}-amino acids into mitochondrial protein *in vivo* in order to visualize protein synthesis in the intact system. It was shown in the preceding investigation that protein synthesis *in vitro* was reduced

TABLE IX

Incorporation of UL-C^{14}-amino acids *in vitro* for 60 minutes into total, soluble, and insoluble proteins of liver mitochondria isolated from control rats (+23° C) and cold-acclimated rats (+2° C) at 90 days experimental period

Fractions analysed	Total protein		Soluble protein		Insoluble protein	
Temperature	Control	Cold - acclimated	Control	Cold - acclimated	Control	Cold - acclimated
Radioactivity	73.41	60.07	34.38	31.38	119.27	115.70
cpm/mg of protein	74.29	61.95	44.75	29.83	135.90	114.88
of each fraction	69.49	67.03	46.09	37.74	114.57	117.68
per 60'	64.61	70.70	49.50	30.19	140.00	113.60
	74.74	64.88	44.60	41.35	148.77	127.74
	86.50	65.86	48.52	43.42	150.59	132.33
Mean	73.84 ± 7.92	65.08 ± 1.54	44.64 ± 2.18	35.65 ± 2.44	134.85 ± 19.35	120.32 ± 1.00
% of control	100.00	88.14	100.00	79.86	100.00	89.23
% of total mitochondrial protein	100.00	100.00	60.46	54.78	182.62	184.88

Figure 12

Incorporation of UL-C^{14}-amino acids *in vitro* for 60 minutes into total, soluble, and insoluble proteins of liver mitochondria isolated from control rats (+23°C) and cold-acclimated rats (+2°C) at 90 days experimental period.

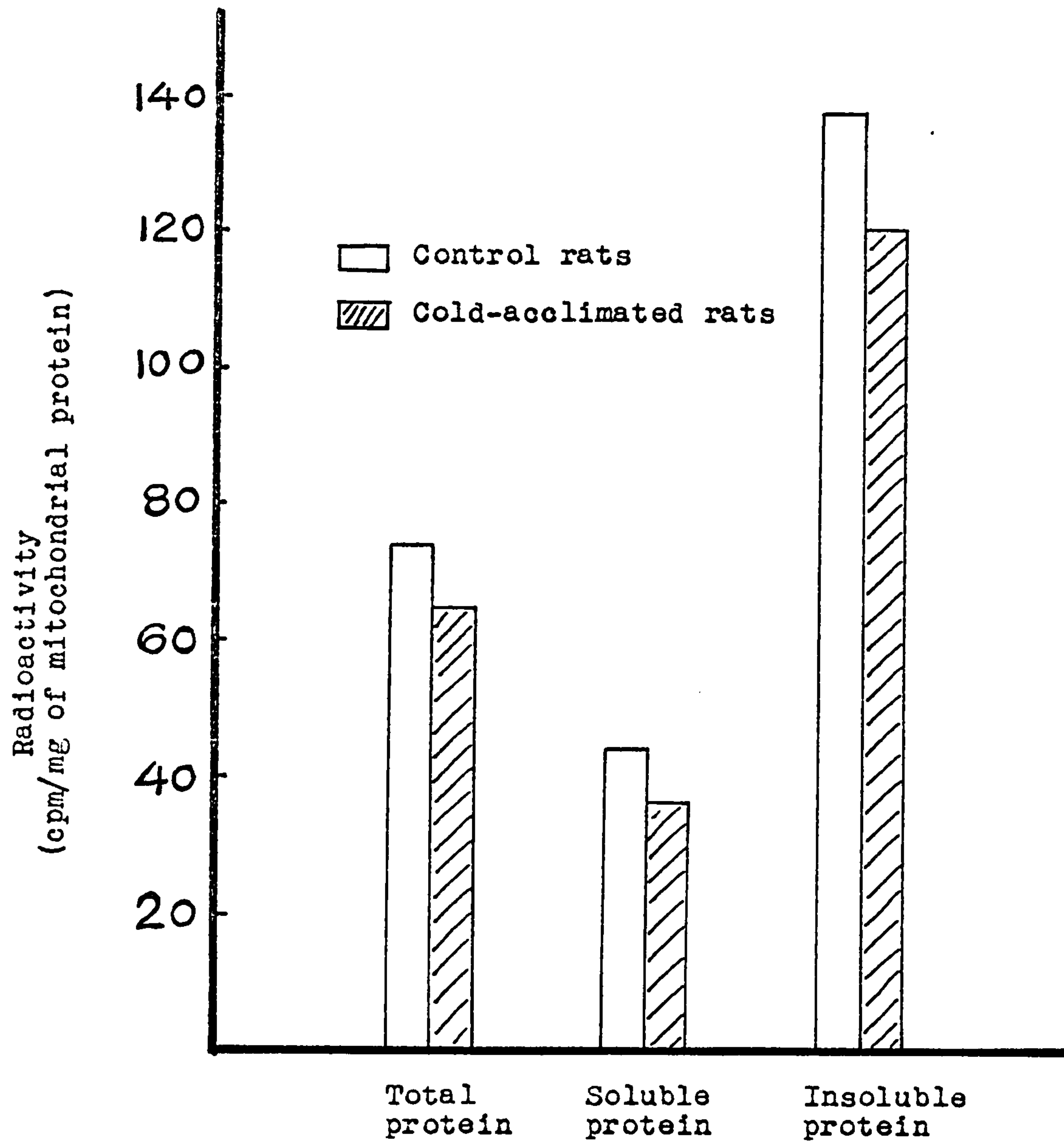

Figure 12

in cold-acclimated rats, more specifically so in the soluble protein fraction. Roodyn (1962) has shown that protein synthesis is dependent on a cytoplasmic factor, which is lacking in an *in vitro* system. Therefore, this *in vivo* study would provide information as to whether cold exposure would affect protein synthesis in all the internal organs equally or in only one or two specific organs; the general physiology of rats in relation to the utilization of C^{14}-amino acids administered; and finally, the distribution of radioactivity in soluble and insoluble proteins of mitochondria in the intact system.

Results

A. The distribution of incorporated radioactivity between soluble and insoluble protein of liver mitochondria (in vivo)

The incorporation of C^{14}-amino acids into total, soluble, and insoluble proteins of liver mitochondria of control and cold-acclimated rats was determined, and the results are presented in Table X and Figure 13. Since the same amount (10 μc) of C^{14}-amino acids was injected into each rat, the observed specific activity was converted to "standard specific" activity ((1) observed specific activity x body weight of rat/body weight of rat at zero day, and (2) observed specific activity x organ weight of rat/organ weight of rat at zero day). In a preliminary experiment on the growth rate of rats, the cold-acclimated rats gained less weight than the control rats, having

TABLE X

Radioactivity (cpm/mg mitochondrial protein) found in total, soluble, liver mitochondria, after the incorporation of UL-C^{14}-amino acids in v control rats (+23° C) and cold-acclimated rats (+2° C) at various time

	Treatment (days)	Observed specific activity		Standard specific acti	
		Control	Cold-acclimated	Control	Cold-acclim
Total protein	0	311.85 ± 8.43** (8)			311.85
	30	194.16 ± 6.08 (8)	198.23 ± 18.08 (8)	330.07	259.68
	75	225.06 ± 6.78 (8)	163.14 ± 17.12 (8)	504.13	275.71
	170	196.44 ± 12.25 (8)	180.03 ± 21.63 (10)	493.24	343.86
	Mean	205.22 ± 9.94	180.47 ± 9.90	446.15	293.08
	% of total	-	-	-	-
Soluble protein	0	467.67 ± 21.24 (8)			467.67
	30	274.71 ± 10.34 (8)	256.54 ± 19.80 (8)	467.01	335.07
	75	231.69 ± 6.25 (8)	160.64 ± 7.75 (8)	519.98	282.30
	170	220.24 ± 15.50 (8)	166.16 ± 18.94 (8)	541.79	317.37
	Mean	242.21 ± 16.58	196.58 ± 29.98	509.59	311.91
	% of total	-	-	-	-
Insoluble protein	0	236.75 ± 7.28 (8)			236.75
	30	131.46 ± 3.32 (8)	141.61 ± 12.12 (8)	223.48	195.51
	75	162.76 ± 5.29 (8)	130.64 ± 10.15 (8)	364.58	220.78
	170	160.07 ± 9.38 (8)	152.91 ± 17.85 (8)	393.77	292.06
	Mean	151.43 ± 10.02	141.72 ± 6.43	327.28	236.12
	% of total	-	-	-	-

* figures of % of control are based on standard specific activity (1)
** standard error
( ) number of animals

Standard specific activity (1) ——— Observed specific activity x -

Standard specific activity (2) ——— Observed specific activity x -

TABLE X

n) found in total, soluble, and insoluble proteins of
n of UL-C^{14}-amino acids *in vivo* for 60 minutes, of
rats (+2° C) at various times of the experimental period

	Standard specific activity (1)		Standard specific activity (2)		% of* control
ed	Control	Cold-acclimated	Control	Cold-acclimated	
	311.85		311.85		100.00
(8)	330.07	259.68	264.06	263.65	78.64
(8)	504.13	275.71	367.85	246.34	57.11
(10)	493.24	343.86	343.77	301.65	71.41
	446.15	293.08	325.23	270.55	69.05
	-	-	100.00	100.00	-
	467.67		467.67		100.00
(8)	467.01	335.07	373.61	341.20	71.94
(8)	519.98	282.30	377.65	252.23	56.15
(8)	541.79	317.37	385.32	277.48	58.80
	509.59	311.91	378.89	290.30	62.29
	-	-	116.50	107.30	-
	236.75		236.75		100.00
(8)	223.48	195.51	178.79	188.34	83.50
(8)	364.58	220.78	265.31	197.27	62.49
(8)	393.77	292.06	280.12	245.36	77.65
	327.28	236.12	241.41	210.32	74.55
	-	-	74.23	77.74	-

specific activity (1)

served specific activity x $\frac{\text{body weight}}{\text{body weight at zero day}}$

served specific activity x $\frac{\text{Organ weight}}{\text{Organ weight at zero day}}$

Figure 13

Radioactivity (cpm/mg mitochondrial protein) found in total, soluble, and insoluble proteins of liver mitochondria, after the incorporation of UL-C^{14}-amino acids *in vivo* for 60 minutes, of control rats (+23°C) and cold-acclimated rats (+2°C) at various times of the experimental period.

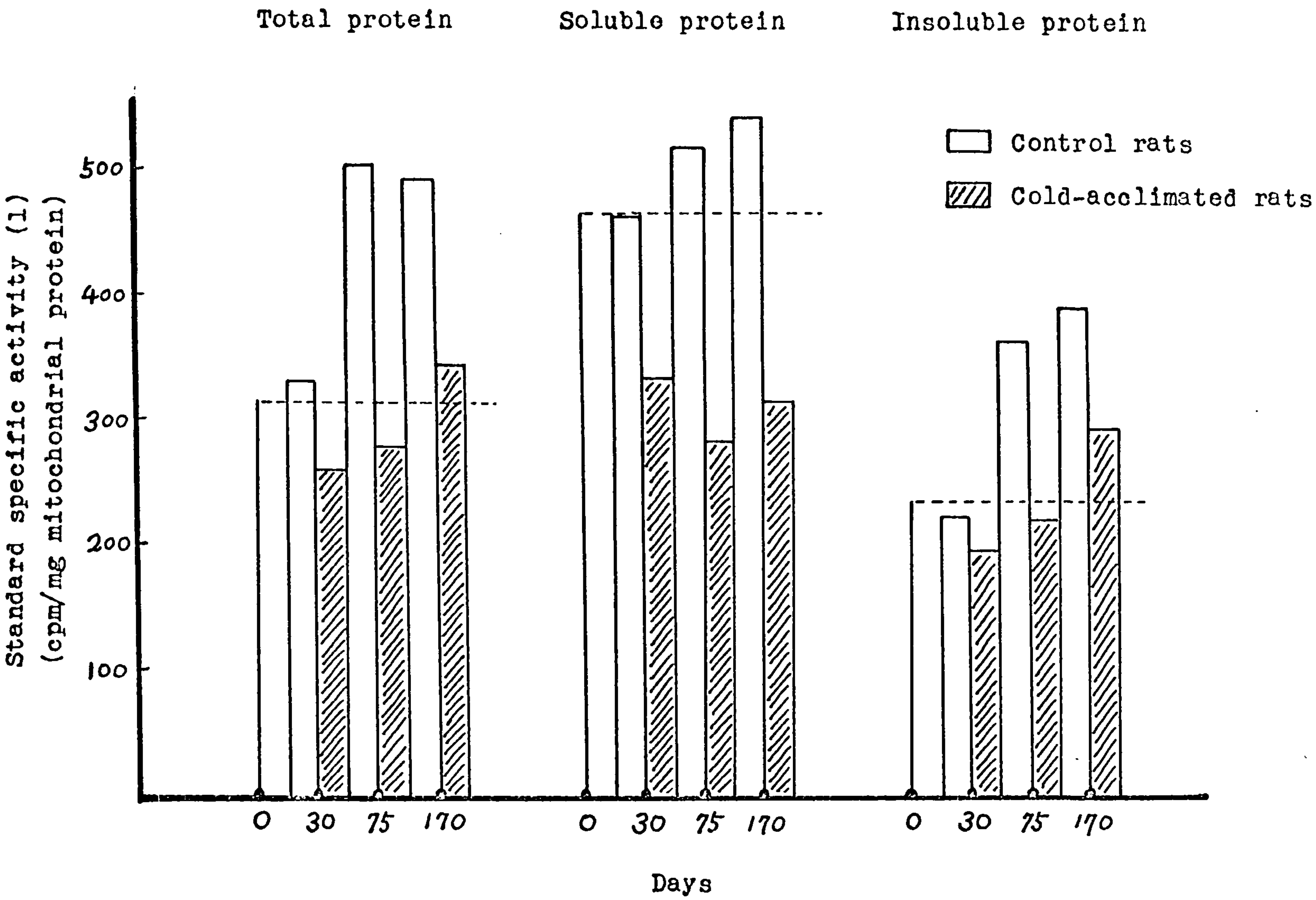

Figure 13

a lesser muscular mass and a relatively larger liver. The effect of the duration of cold exposure on cold-acclimated rats, as well as the effect of age on control rats, on the distribution of incorporated C^{14}-amino acids into subfractions of mitochondria were examined. The data were compared between control and cold-acclimated rats at a given age. In order to find out a more generalized tendency in incorporation of C^{14}-amino acids, the data were also treated in such a way that the average of the data (regardless of age) from control rats was compared with that from cold-acclimated rats.

The cold exposure resulted in less incorporation of C^{14}-amino acids into the various fractions analyzed (mean in Table X). There was 69.05% of the activity in total protein, 62.29% in soluble protein, and 74.55% in insoluble protein of mitochondria of cold-acclimated rats as compared to those of control rats. Reduced incorporation in cold-acclimated rats was especially evident in the soluble protein fraction. This effect of cold exposure is shown more clearly when the distribution of the specific activity of the subfractions of mitochondria is compared within each group between control and cold-acclimated rats. It was smaller (107.30% of total) in cold-acclimated rats than in control rats (116.50% of total). However, the insoluble protein pool of cold-acclimated rats (77.74% of total) was larger than that of control rats (74.23% of total).

The specific activity of the soluble protein was higher than that of the insoluble protein in both groups. This differs from

the results of the in vitro study, where the insoluble protein had a higher specific activity than the soluble protein. This difference in the distribution of incorporation C^{14}-amino acids seems to be due to a different behaviour of mitochondria in vitro and in vivo.

B. Incorporation of C^{14}-amino acids into total protein of mitochondria of liver, kidney, brain, heart, spleen, and pancreas

The incorporation of C^{14}-amino acids into the total protein of mitochondria of several organs was determined 60 minutes after injection of C^{14}-amino acids into intact control and cold-acclimated rats. The effect of age of rats of both groups on the incorporation of C^{14}-amino acids into these organs was also studied. The results are presented in Table XI and Figure 14. The observed specific activity is also converted to "standard specific" activity. As can be seen from the data, cold exposure reduced the incorporation of C^{14}-amino acids into mitochondrial proteins of liver, kidney, brain, heart, spleen and pancreas. This reduction became more pronounced towards the end of the experimental period.

It is of interest to compare the order of standard specific activity of the mitochondrial protein of various organs: in both groups the order of standard specific activity in organs was found to be pancreas > spleen > kidney > liver > heart > brain. It is evident that cold treatment did not have a specific effect on any particular

TABLE XI

Radioactivity (cpm/mg mitochondrial protein) found in total protein of m
internal organs; liver, kidney, brain, heart, spleen, and pancreas, 60 m
injection of UL-C^{14}-amino acids into intact, control rats (+23° C) and cc
rats (+2° C) at various times of the experimental period

Organ	Treatment (days)	Observed specific activity		Standard specific activi	
		Control	Cold-acclimated	Control	Cold-acclima
Liver	0	311.85 ± 8.43** (8)		311.85	
	30	194.16 ± 6.08 (8)	198.23 ± 18.03 (8)	330.07	259.68
	75	225.06 ± 6.78 (8)	163.16 ± 17.15 (8)	504.13	275.71
	170	186.44 ± 12.25 (8)	180.02 ± 21.63 (8)	493.24	343.86
	Mean	205.23	180.47	442.48	293.08
Kidney	0	378.43 ± 33.39		378.43	
	30	242.04 ± 8.19	227.02 ± 18.06	411.47	297.46
	75	230.81 ± 11.58	160.12 ± 14.80	517.01	270.60
	170	206.80 ± 11.79	154.92 ± 24.33	508.73	295.90
	Mean	226.55	180.68	478.07	287.97
Brain	0	94.63 ± 6.24		94.63	
	30	46.96 ± 4.12	50.14 ± 4.12	79.83	65.68
	75	60.92 ± 3.32	55.67 ± 8.89	136.46	94.08
	170	58.83 ± 4.47	38.42 ± 4.36	144.72	73.38
	Mean	55.57	48.08	120.34	77.71

* figures of % of control are based on standard specific activity (1)
** standard error
( ) number of animals

Standard specific activity (1) ———— Observed specific activity x $\frac{Lo}{Lo}$

Standard specific activity (2) ———— Observed specific activity x $\frac{Or}{Or}$

ABLE XI

n) found in total protein of mitochondria of each of six
rt, spleen, and pancreas, 60 minutes after
t, control rats (+23° C) and cold-acclimated
imental period

d	Standard specific activity(1)		Standard specific activity(2)		% of* control
	Control	Cold-acclimated	Control	Cold-acclimated	
	311.85		311.85		100.00
(8)	330.07	259.68	264.06	263.65	78.64
(8)	504.13	275.71	367.85	246.34	57.11
(8)	493.24	343.86	343.77	301.65	71.41
	442.48	293.08	325.23	270.55	69.05
	378.43		378.43		100.00
	411.47	297.46	317.07	301.94	72.63
	517.01	270.60	362.41	259.39	54.13
	508.73	295.90	341.22	294.35	58.33
	478.07	287.97	340.23	285.23	61.70
	94.63		94.63		100.00
	79.83	65.68	51.13	48.64	83.20
	136.46	94.08	69.45	65.60	71.15
	144.72	73.38	68.24	42.26	50.89
	120.34	77.71	63.94	52.17	75.08

specific activity (1)

erved specific activity x $\frac{\text{Body weight}}{\text{Body weight at zero day}}$

erved specific activity x $\frac{\text{Organ weight}}{\text{Organ weight at zero day}}$

TABLE XI cont'd

	Treatment (days)	Observed specific activity		Standard specific acti	
		Control	Cold-acclimated	Control	Cold-accli
Heart	0	184.26 ± 38.69**		184.26	
	30	109.36 ± 13.82	105.13 ± 21.35	185.91	137.72
	75	107.43 ± 12.88	81.16 ± 14.28	240.64	137.16
	170	96.30 ± 15.00	72.06 ± 13.56	236.70	137.63
	Mean	104.37	86.11	231.18	137.30
Spleen	0	441.39 ± 34.39		441.39	
	30	252.21 ± 12.69	262.06 ± 35.65	398.76	343.30
	75	252.36 ± 41.13	194.29 ± 24.15	565.27	328.35
	170	232.79 ± 4.58	226.96 ± 32.83	552.66	432.49
	Mean	245.78	227.77	505.36	368.05
Pancreas	0	1076.15 ± 92.25		1076.15	
	30	992.21 ± 34.54	1013.20 ± 44.51	1686.76	1327.29
	75	695.33 ± 77.65	693.90 ± 19.82	2005.54	1172.69
	170	879.80 ± 65.04	591.16 ± 9.93	2164.31	1129.12
	Mean	922.44	766.08	1952.20	1209.70

ty mated	Standard specific activity(1) Control	Cold-acclimated	Standard specific activity(2) Control	Cold-acclimated	% of control
	134.26		184.26		100.00
1.35	185.91	137.72	131.23	133.41	74.10
4.28	240.64	137.16	162.22	142.74	58.90
3.56	236.70	137.63	189.71	128.99	58.31
	231.18	137.30	161.09	135.05	63.77
	441.39		441.39		100.00
5.65	398.76	343.30	315.26	277.78	82.71
4.15	565.27	328.35	370.97	225.37	59.95
2.83	552.66	432.49	349.19	285.77	74.17
	505.36	368.05	345.14	263.04	72.28
	1076.15		1076.15		100.00
4.51	1686.76	1327.29	1339.48	1104.39	79.34
9.82	2005.54	1172.69	1656.36	1490.65	70.02
9.93	2164.31	1129.12	1425.28	1052.26	52.89
	1952.20	1209.70	1373.71	1215.77	67.42

Figure 14

Radioactivity (cpm/mg mitochondrial protein) found in total protein of mitochondria of each of six internal organs; liver, kidney, brain, heart, spleen, and pancreas, 60 minutes after injection of UL-C^{14}-amino acids into intact, control rats (+23°C) and cold-acclimated rats (+2°C) at various times of the experimental period.

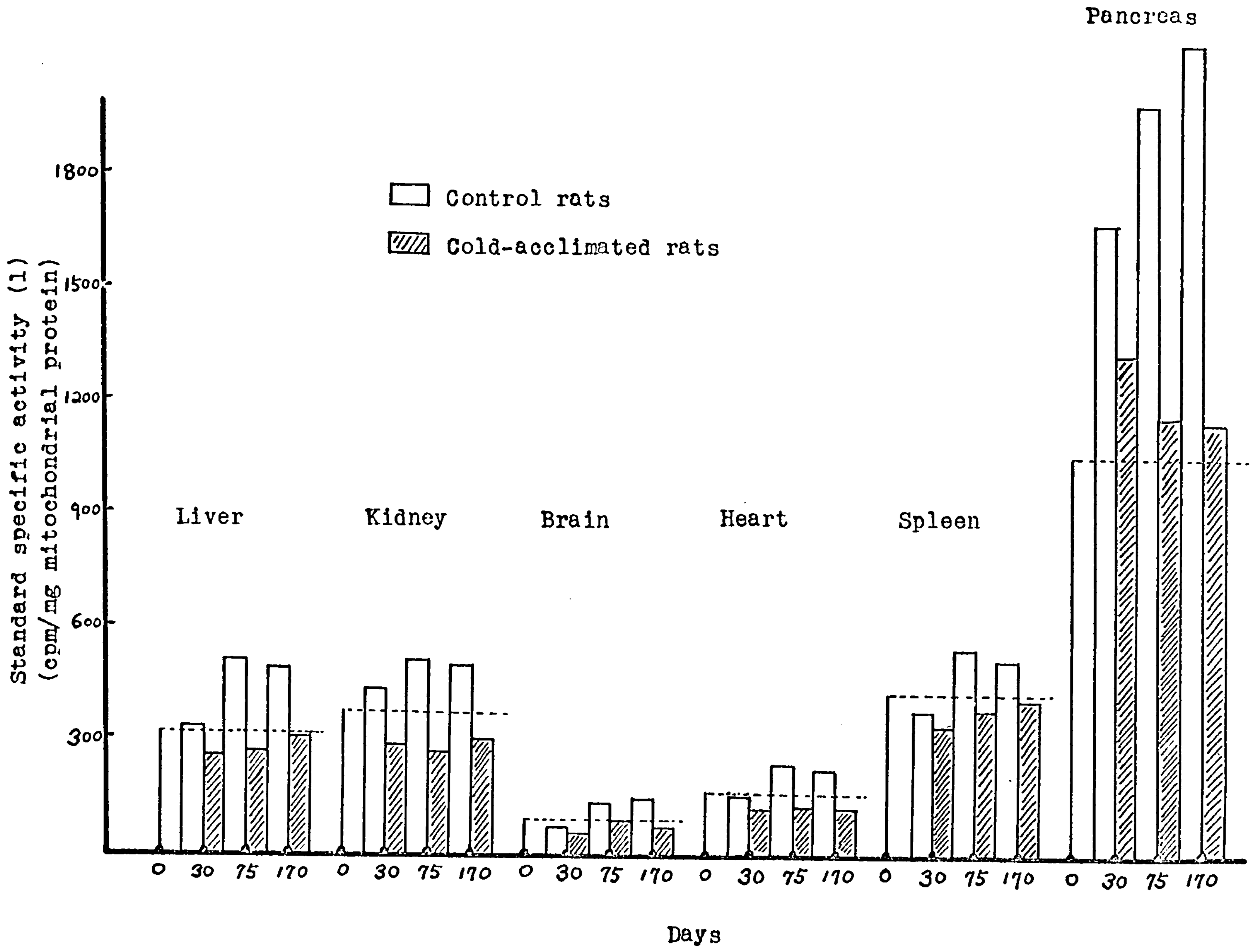

Figure 14

organ, since the level of C^{14}-amino acids incorporation in each organ was reduced to the same extent by cold exposure.

Discussion

The incorporation of labeled amino acids into mitochondrial protein *in vivo* has been reported by several investigators (Simpson and McLean, 1955; Marsh and Drabkin, 1957; McLean *et al*, 1958; Fletcher *et al*, 1961, and Michels *et al*, 1963). Incorporation of C^{14}-amino acids into the protein of isolated mitochondria has also been reported (Reis *et al*, 1959; Roodyn *et al*, 1961 and 1965; Truman and Korner, 1962a and b; Kalf, 1963 and Kroon, 1965). Further experiments of Roodyn (1962) and Truman (1961 and 1964) have led to the conclusion that the major portion of amino acids incorporation was into the insoluble protein *in vitro*, which is probably derived from the mitochondrial membrane. However, comparable studies had not been carried out in rats acclimated to cold.

The total incorporation of C^{14}-amino acids into mitochondrial protein (Figure **11**) was gradually increased with time in both groups with the maximum incorporation occurring at 90 minutes incubation. The fact that the incorporation of C^{14}-amino acids into mitochondrial protein *in vitro* was decreased after 90 minutes incubation in both groups, although slightly less in cold-acclimated rats, could have resulted from several causes: (a) mitochondria failed to function; or (b) an essential factor normally available from the cytoplasm became

exhausted in the *in vitro* system; or (c) protein synthesized (or free amino acids) leached into the medium; or (d) loss of mitochondrial protein owing to the breakdown of fragile mitochondria. From these results, a 60 minute period was selected for the comparison studies of incorporation of C^{14}-amino acids into protein of subfractions of mitochondria *in vitro* and *in vivo*.

The incorporation of C^{14}-amino acids into protein by isolated mitochondria might be an artefact due to contamination by microsomes or bacteria. However, the mitochondria used in these experiments had been well washed, and probably had a low microsomal contamination. It was observed by Roodyn (1961) and Truman and Korner (1962a) that the incorporation of C^{14}-amino acids was actually increased with repeated washings. These treatments also probably reduced the amount of RNA, as would be expected from the removal of microsomal material. Another difference between microsomes and mitochondria is that incorporation in isolated mitochondria proceeds over a period of at least one and one half hour, whereas in isolated microsomes maximum incorporation is reached at about half an hour of incubation (Kroon, 1963 and 1965). Absence of bacterial contamination is difficult to prove. It is not surprising that bacteria would flourish on a non-sterile suspension of mitochondria. However, the time-curve of C^{14}-amino acids incorporation (Figure 11) suggested that bacteria are probably not involved, since bacterial growth under the

incubation conditions used would lead to linear C^{14}-amino acids incorporation. Furthermore, if only bacterial contamination showed the incorporation of C^{14}-amino acids into protein, no difference in results of C^{14}-amino acids incorporation between normal and cold-acclimated rats would be expected.

It was found in the present studies (Tables IX and X), that cold exposure reduced the overall incorporation of C^{14}-amino acids into mitochondrial protein, and that it affected the incorporation into soluble protein more than into insoluble protein. This is consistent with the results obtained in Experiment II, in which protein contents of mitochondria were compared (Table IV) and found lower in cold-acclimated rats.

Although an increase in insoluble protein after 35-45 days of cold exposure was observed (Table IV), the incorporation of C^{14}-amino acids into this protein fraction was reduced to 83.50% of control (Table X). This indicates that amino acids could be utilized as respiratory substrate before they are available at the site of protein synthesis, suggesting a higher rate of amino acid metabolism.

Roodyn (1962) formulated a scheme to represent the assembly of mitochondria of rat liver at the subcellular level.

"After the RNA-protein complex is formed (probably derived from the nucleolus), RNA-lipoprotein complex is formed, containing attached respiratory enzymes arranged in their correct relative position, and possibly corresponding to the mitochondrial subunit described by Green (1959). The subunits then polymerize to form the mitochondrial membrane, thus enclosing the soluble mitochondrial

protein. The soluble protein and the bound respiratory enzymes of the membrane are synthesized on the ribosomes in a region of the endoplasmic reticulum close to the site of assembly of the mitochondria. The mitochondrial RNA controls only the synthesis of the insoluble structural protein of the membrane. In vivo, therefore, simultaneous labelling of all components could occur. In vitro, incubation of mitochondria would result only in labelling of the insoluble structural material. Such a view should be regarded only as a preliminary working hypothesis, since the synthesis of a well characterized structural protein of high purity has not yet been demonstrated."

If one considered Roodyn's scheme as a working mechanism for the synthesis of soluble protein in the assembly of mitochondria, then the reduction in the synthesis of soluble protein in cold-acclimated rats could be considered to have resulted from the influence of cold exposure on some cytoplasmic factor which normally contributes to the synthesis of soluble protein. Conversely, cold exposure might induce the formation of an inhibitor of protein synthesis. If this was the case, something must happen in the soluble protein synthesis through one or more of the following reactions:

(a) cold exposure reduces the supply of energy, thus inhibiting the activation of amino acids

(b) cold exposure stimulates amino acid metabolism in another direction, e.g. catabolism

(c) cold exposure stimulates the rate of turnover of protein in the direction of its reduction

(d) cold exposure may reduce the level of an essential cofactor for the synthesis of soluble protein

It has been reported that ATP is required for protein synthesis in mitochondria (Kalf and Simpson, 1959; Reis et al, 1959; Roodyn et al, 1961, and 1965; Roodyn, 1962; Truman and Korner, 1962, and Kroon, 1963 and 1964). The observed reduction in oxidative phosphorylation in mitochondria of acclimated rats in this study could therefore result in a lower rate of protein synthesis.

The increased metabolism of amino acids and protein (Hoberman, 1950) and the decreased protein synthesis due to faster protein turnover rates (Beaton, 1963) have been reported in cold-acclimated rats. Williams et al (1950) noted a marked decrease in proline, methionine, threonine, arginine and lysine, and increased levels of leucine, phenylalanine and valine in plasma of cold-acclimated rats. Furthermore, an increased urinary excretion of alanine, valine, serine, threonine, glycine and glutamine from cold-acclimated rats has also been reported (Mefferd et al, 1955 and Hale and Mefferd, 1958).

As evidenced from Experiment II and from above, cold exposure causes metabolic changes. Therefore, it can be concluded that the increased catabolism with the reduced anabolism of protein could be a reason for the reduced synthesis of protein of mitochondria particularly in the soluble fraction, through a direct effect of cold exposure on a cytoplasmic factor; the participation of soluble protein in the assembly of mitochondria would therefore be reduced.

Similarily, the gradual decrease in the level of incorporation of C^{14}-amino acids into total protein, as well as in soluble and insoluble protein of mitochondria, with duration of cold exposure might have resulted from the progressive fashion and accumulative effect of cold on the presumed cytoplasmic factor (% control in Table X) affected.

Such an effect of cold exposure on protein synthesis is not limited to any one specific organ of rats, since protein synthesis of mitochondria was reduced to the same extent in several internal organs studied in cold-acclimated rats.

Experiment IV <u>An electron microscopic study of liver mitochondria of control and cold-acclimated rats</u>

Since there has been increasing evidence of a relation between physiological and structural changes in the mitochondria of animals (see Literature Review), it was felt desirable to determine whether cold exposure induced a structural change in mitochondria. Therefore, an electron microscopic study on mitochondria of intact liver tissue was initiated.

<u>Observations</u>

The cytological alterations revealed by the electron micrographs are presented in tabulated form (Table XII) in order to permit an easy comparison between control and cold-acclimated rats. There was a considerable variation in size of mitochondria from cell to cell within the same liver. This variation could be minimized by scanning several hundred cells (1 or 2 cells per picture) from each of the rats. Because of the heterogeneity of the mitochondrial size in a single cell, it is not safe to compare all mitochondria in liver cells of control and cold-acclimated rats. Therefore, comparisons were restricted to the largest mitochondria from both groups. The measurements of "mitochondrial size" were taken on the largest mitochondria per unit area of the photograph prepared at the same magnification in samples from both control and from cold-acclimated rats. "Mitochondrial length" was defined as the longest straight distance across

TABLE XII

Comparison of electron microscopic structures of liver mitochondria of control rats (+23° C) and cold-acclimated rats (+2° C) at 90 days of the experimental period

Criteria compared	Control	Cold-acclimated
Size of mitochondria		A great number of mitochondria are larger than those of control rats
Shape of mitochondria	Most are spherical	Many are long and sausage-shaped
Number of mitochondria	(see text)	(see text)
Mitochondrial outer membrane	More or less smooth outer membrane	More ruffled outer membrane than those of control rats
Cristae	clear, well defined	appear loose
Matrix of mitochondria	dense	pale
Length of longest mitochondria	1.47 µ	2.37 µ (161% over control)
Width of widest mitochondria	0.76 µ	0.87 µ (114% over control)
Glycogen as electron dense spots in cytoplasm		More electron dense spots are detected than in control rats

the mitochondrial matrix. "Mitochondrial width" was then defined as the greatest mitochondrial diameter perpendicular to the longer axis.

Comparison of electron micrographs indicated that a great number of mitochondria in the liver cell of cold-acclimated rats were longer than those observed in samples from control rats. In liver cells of control rats (Figures 15 and 16), mitochondria were mostly spherical with a more or less smooth outer membrane. Mitochondria of liver cells of cold-acclimated rats were larger with more ruffled outer membranes than those of control rats. The mitochondria of liver cells from cold-acclimated rats were 161% longer and 114% wider than those of control rats.

The mitochondrial matrix in control rats was dense with well defined cristae, whereas that in cold-acclimated rats looked pale, and the cristae appeared loose.

Because of the difficulties in obtaining comparable surface sections and of a shift from three dimensional to two dimensional observations, it was very difficult to tell from electron micrographs whether cold exposure reduces the number of mitochondria per unit area of cell. Therefore, no attempt was made to determine the number of mitochondria per cell or per unit area.

Apparently, there was no significant difference in the number of cristae per mitochondrion between the two groups. However, the glycogen content, as shown by electron dense spots throughout

the cytoplasm, was apparently high in cold-acclimated rats (Figures 17-b and 18-c).

Discussion

These observations on mitochondria of intact liver cells with the electron microscope confirm the premise that the physiological changes observed in mitochondria of cold-acclimated rat liver cells are associated with structural changes. The uncoupling of oxidative phosphorylation is reported to be due to a defect of the mitochondrial membrane, since the entire mechanism of intracellular oxidative phosphorylation is confined to this membrane (Porter and Bruni, 1959; Wilson and Leduc, 1963; Douglas, 1964, and Smith and DeLuca, 1964). Although it is clear that the uncoupling of oxidative phosphorylation is one of the characteristics of the physiological effect of cold exposure, there has been no evidence that this uncoupling is associated with structural changes of the mitochondria.

The present observations with the electron microscope show a structural change of mitochondria in cold-acclimated rats; enlarged mitochondria with more ruffled outer membrane, pale matrix and loose cristae.

Enlargement of mitochondria in intact liver cells has been reported in several different experimental conditions; in fasting rats (Fawcett, 1955); in fasting mice (Wilson and Leduc, 1963); in thyrotoxic rats (Douglas, 1964 and Peachy and Greif, 1965); in EFA-deficient

mice (Wilson and Leduc, 1963), and azo-dye (3-Me-DAB) fed rats (Porter and Bruni, 1959). In fasting rats, the mitochondria were enlarged with a decreased number of cristae. In azo-dye fed rats, the mitochondria were enlarged, but no change of the submitochondrial structure was apparent. In the cases of thyrotoxic rats and EFA-deficient mice, the mitochondria were enlarged with an increased number of cristae. However, the matrix of enlarged mitochondria in EFA-deficient rats was often denser than that of control rats, whereas the matrix of the thyrotoxic rats was pale compared to that of the control rats. The observed enlargement of mitochondria of rat liver cells produced by cold exposure in this study was found to be unique, because enlarged mitochondria, which possessed the same number of cristae as that of control rats, possessed a pale matrix.

Smith and DeLuca (1964) reported that uncoupling of oxidative phosphorylation resulted from an increased ATPase activity and extensive structural damage of mitochondria in EFA-deficient rats. Wilson and Leduc (1963) observed a decrease in ATP production in enlarged mitochondria of EFA-deficient mice. Water taken into the mitochondria by osmosis is normally removed by the action of ATP. In the absence of ATP production, water accumulates and causes the swelling of mitochondria. This may lead to a structural change of mitochondria, if only by stretching. Since it is known that the isolated mitochondria of cold-acclimated rats tend to swell faster than those of control rats

(Fairhurst et al, 1958 and Boatman et al, 1962) it is possible that the same occurs in vivo leading to enlarged mitochondria in cold-acclimated rats. This swelling of mitochondria could be responsible for the fragility of the mitochondrial membrane and for the uncoupling of oxidative phosphorylation. Although a great number of mitochondria of cold-acclimated rats are longer and larger than those of control rats, their soluble and insoluble protein contents were significantly reduced (Experiment I-2). As has been discussed, this decrease might have resulted from the reduction in the number of mitochondria per unit weight of original fresh liver tissue of cold-acclimated rats. However, in addition to this it might also be argued that the decreased soluble protein levels could have resulted from the swelling of mitochondria which is known to accompany a release of soluble protein. This would be a reason why soluble protein was reduced proportionally more than insoluble protein. The reduction of insoluble protein in isolated mitochondria by freezing was found to be associated with the breakage of mitochondrial membranes. This may not be the case in this study because no mitochondrial membrane was shown to be broken (Figures 17 and 18). The explanation must be in the reduced number of mitochondria in cold-acclimated rats.

The higher glycogen content seems to play a role in cold-acclimated rats because less glycogen could be detected in control rats (Figures 17-b and 18-c). There is extensive evidence that

glycogenesis is greater in cold-acclimated rats than in control rats (Pagé *et al*, 1955; Masoro *et al*, 1955; Depocas *et al*, 1957, and Hannon, 1958).

Figure 15-a, b, and c

Electron micrographs of liver mitochondria from control rats (+23°C) at 3 months of an experimental period. Magnification: 9,400X

Figure 16-a, b, c, and d

Electron micrographs of liver mitochondria from control rats (+23°C) at 3 months of an experimental period. Magnification: 37,000X

Figure 17-a, b, and c

Electron micrographs of liver mitochondria from cold-acclimated rats (+2°C) at 3 months of an experimental period.

Magnification: 9,400X

Figure-18-a, b, c, and d

Electron micrographs of liver mitochondria from cold-acclimated rats (+2°C) at 3 months of an experimental period.

Magnification: 37,000X

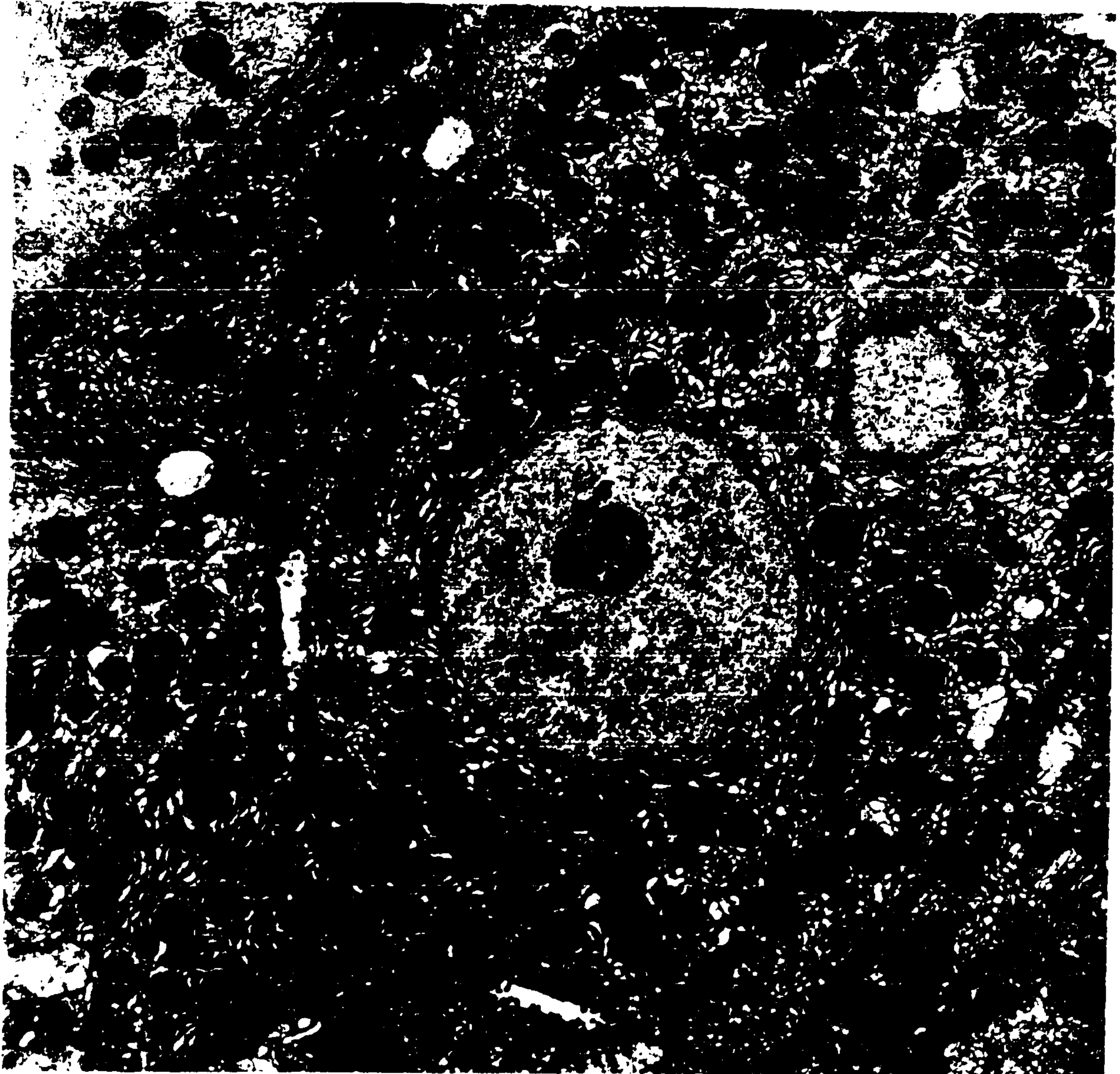

Figure 15-a

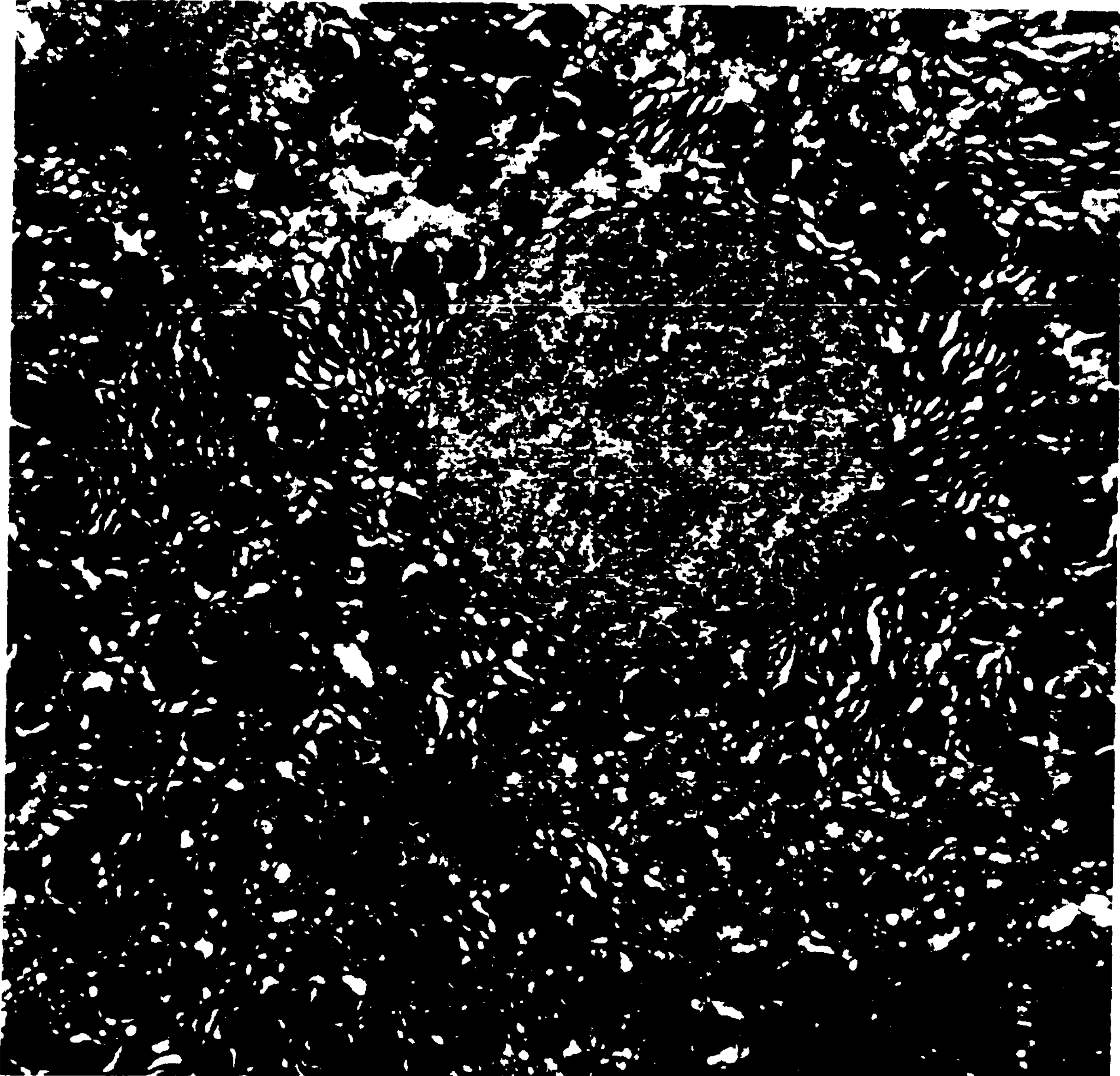

Figure 15-b

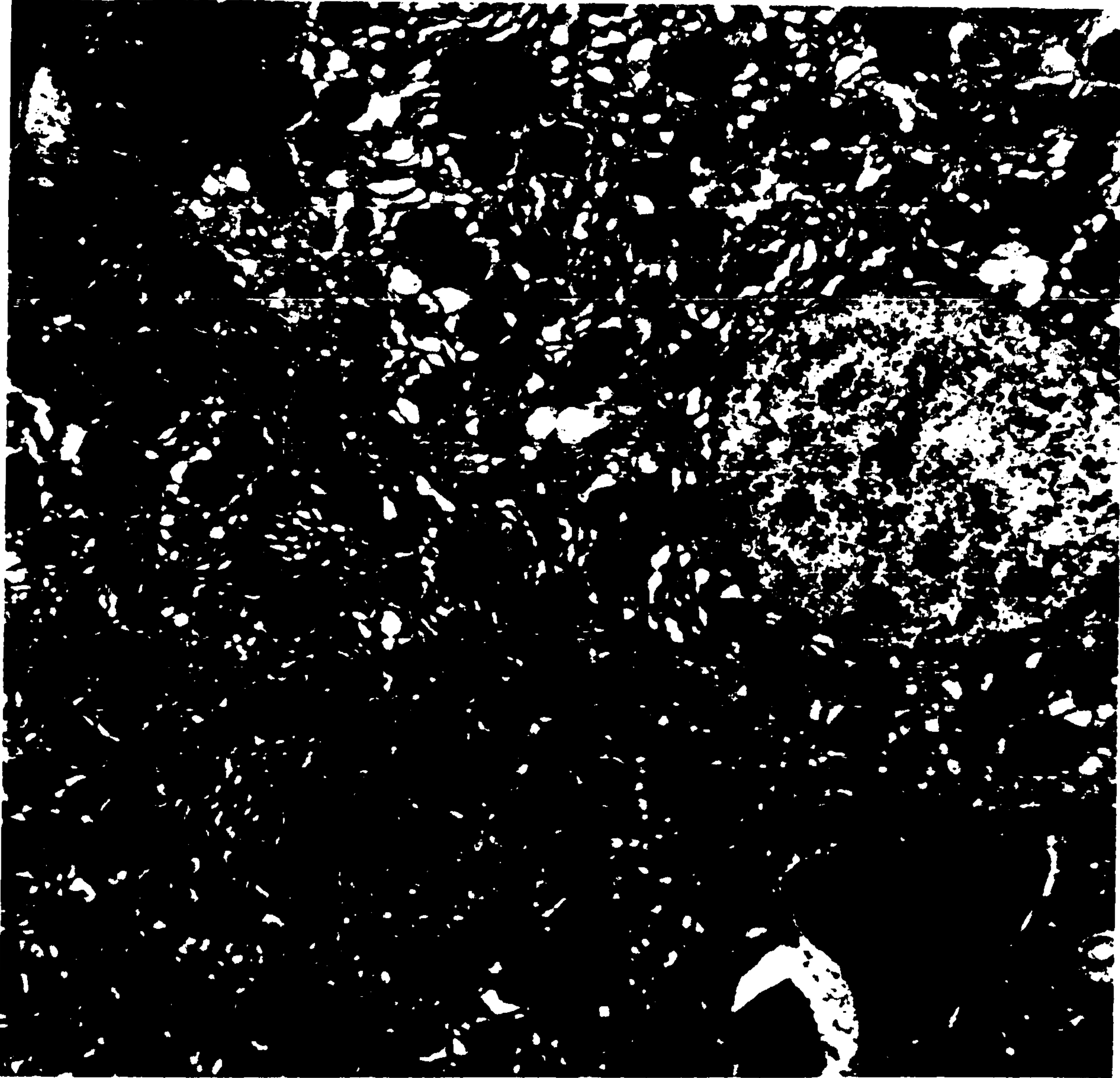

Figure 15-c

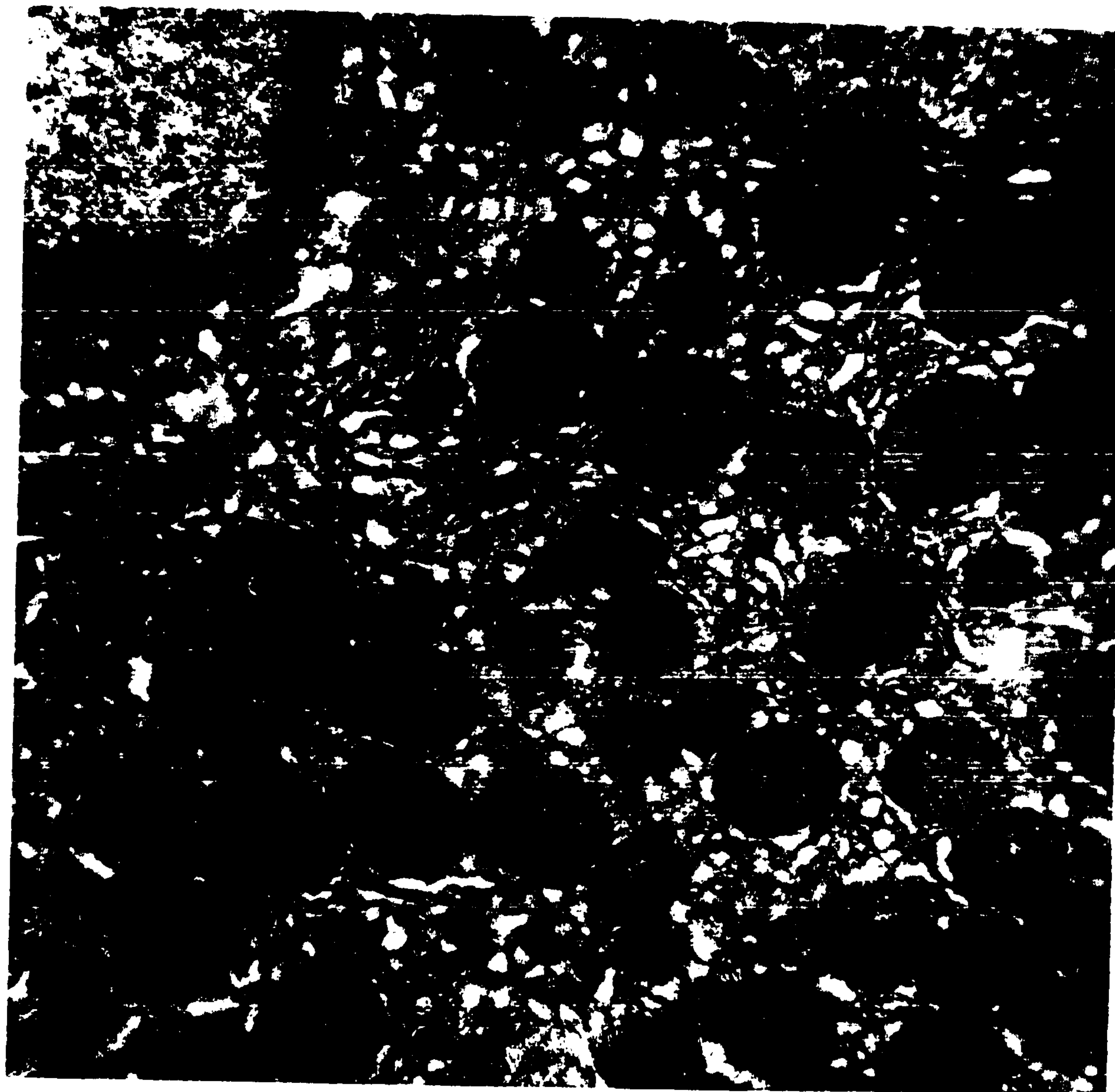

Figure 16-a

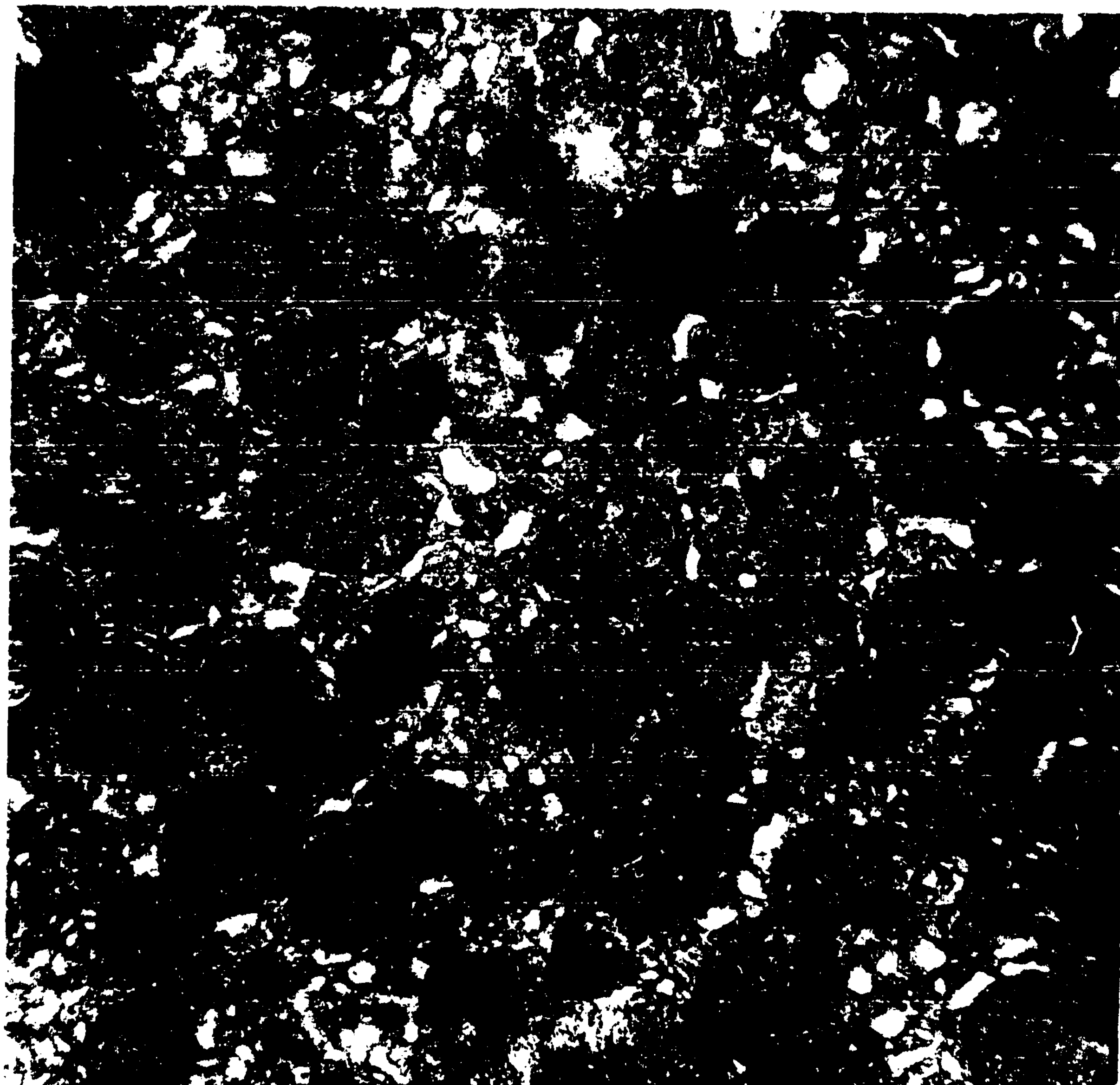

Figure 16-b

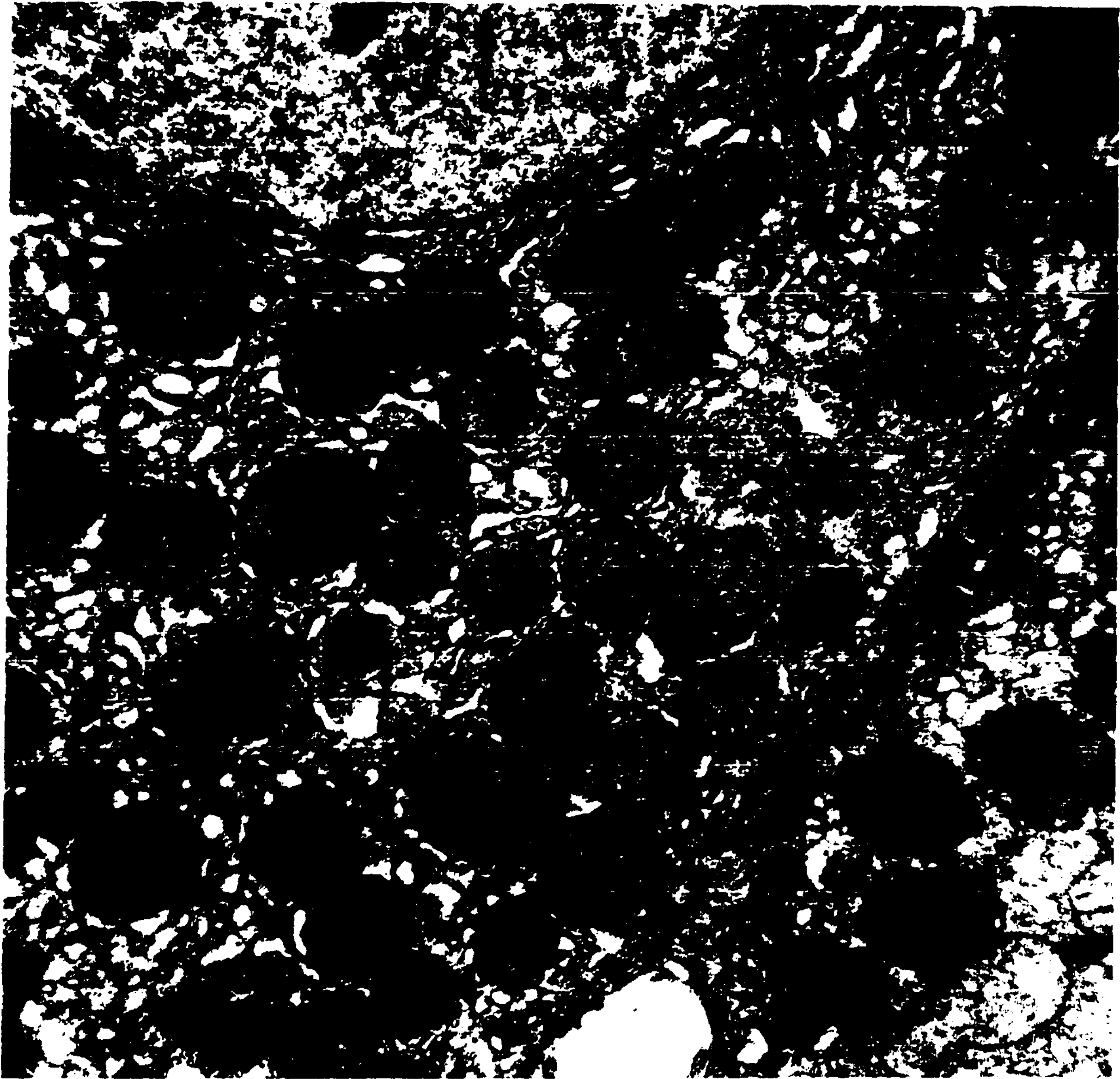

Figure 1[illegible]-c

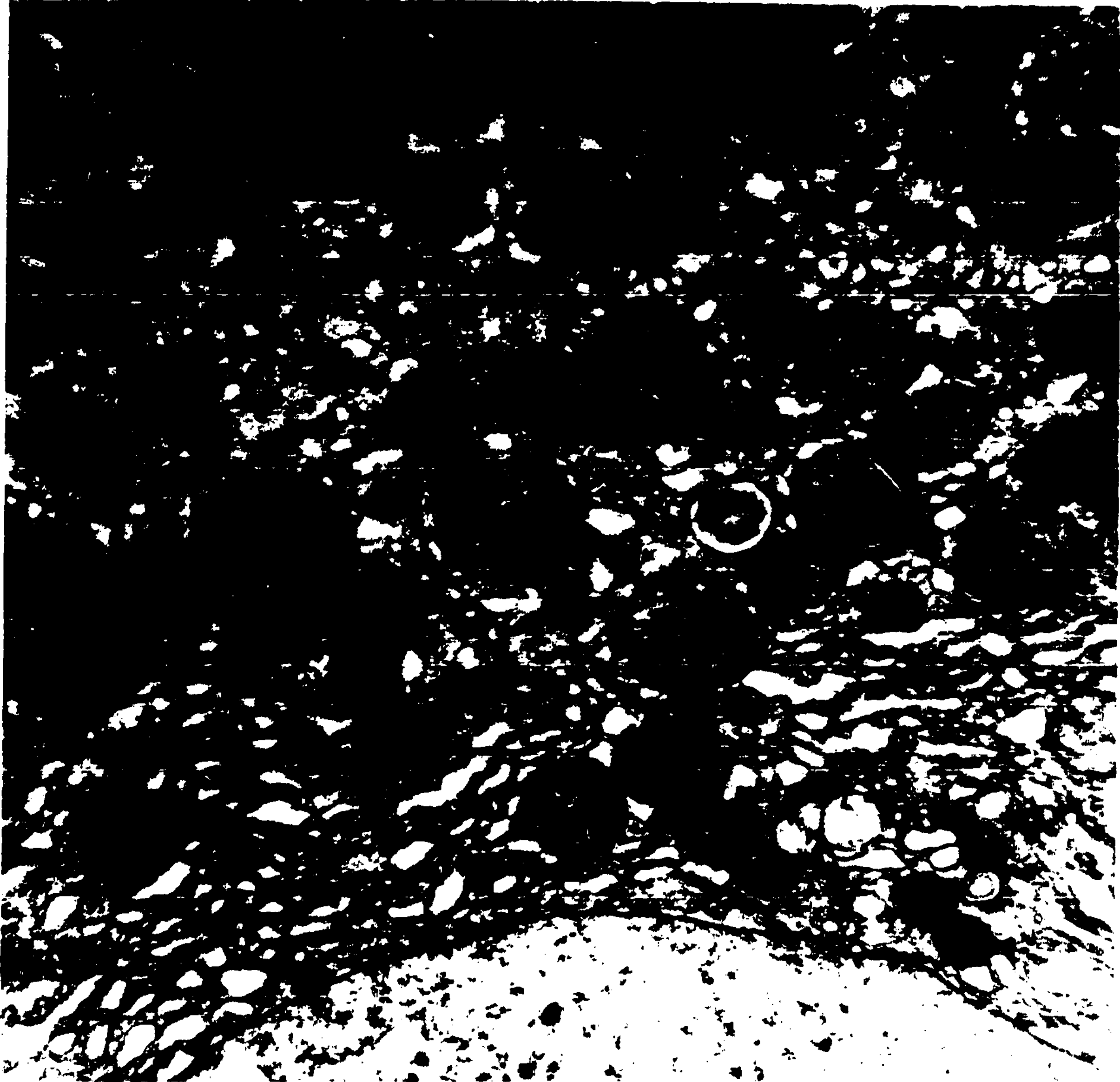

Figure 16-d

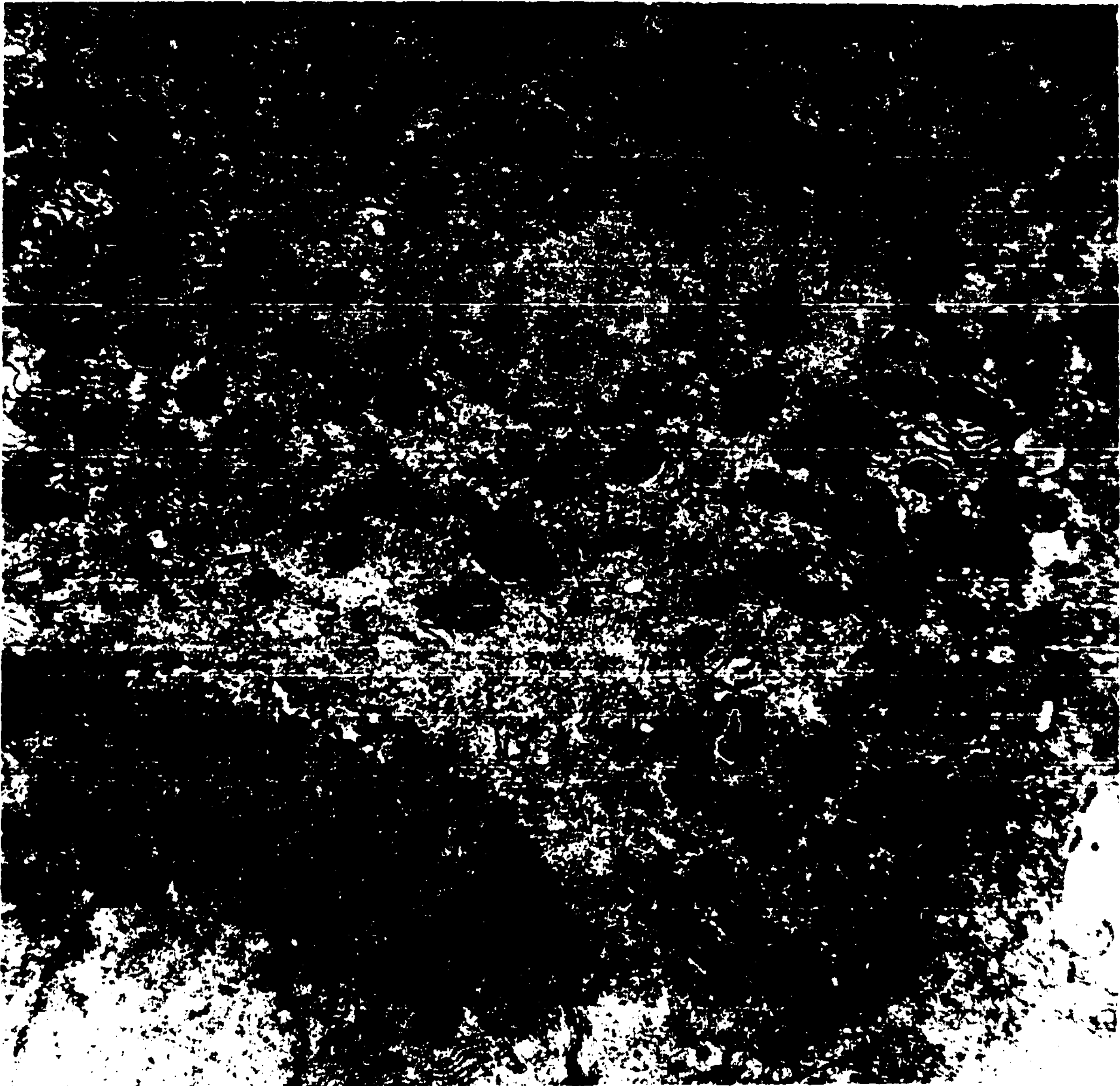

Figure 17-a

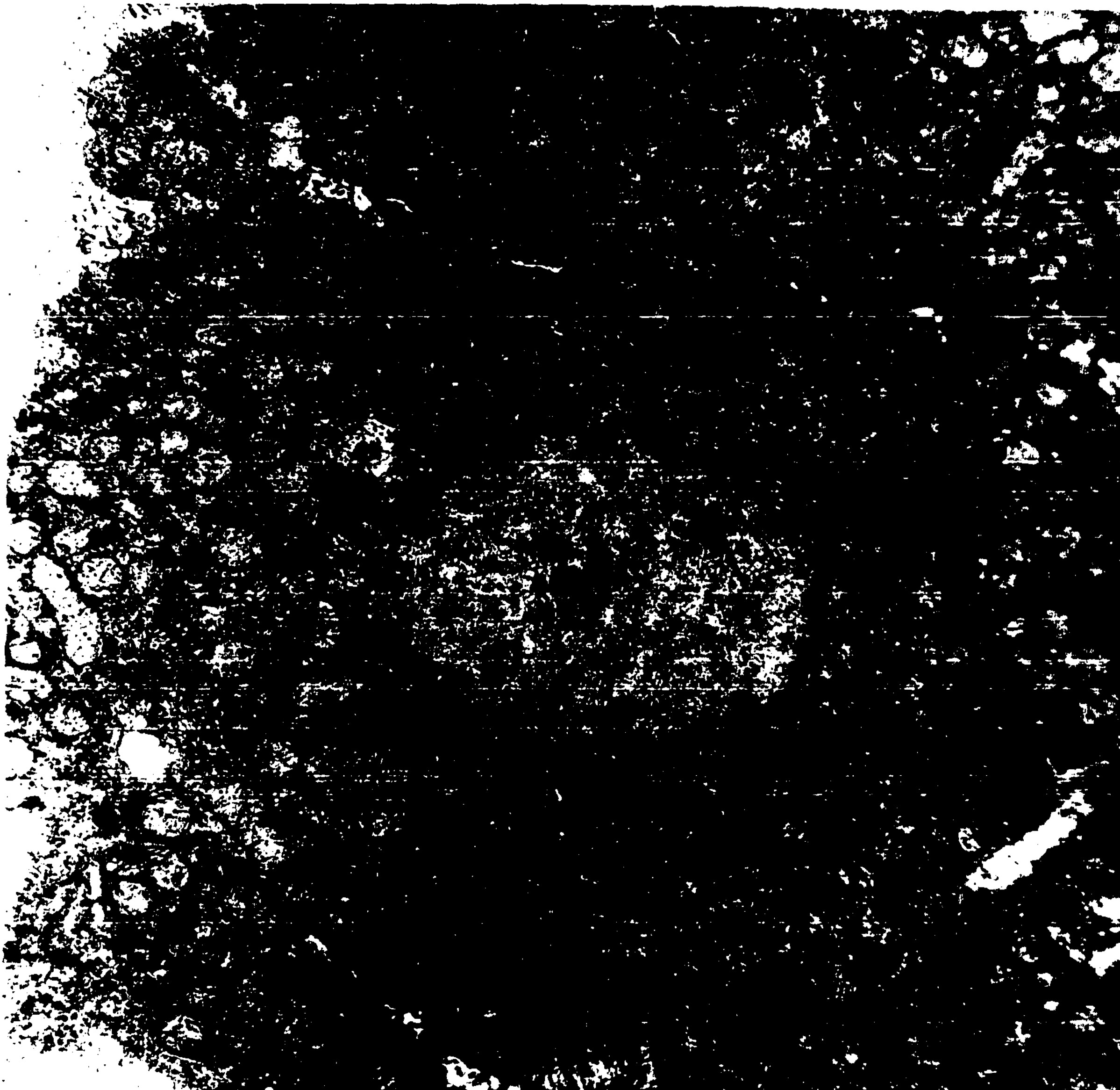

Figure 17-b

Figure 17-c

Fi--re 1--

Figure 1 -b

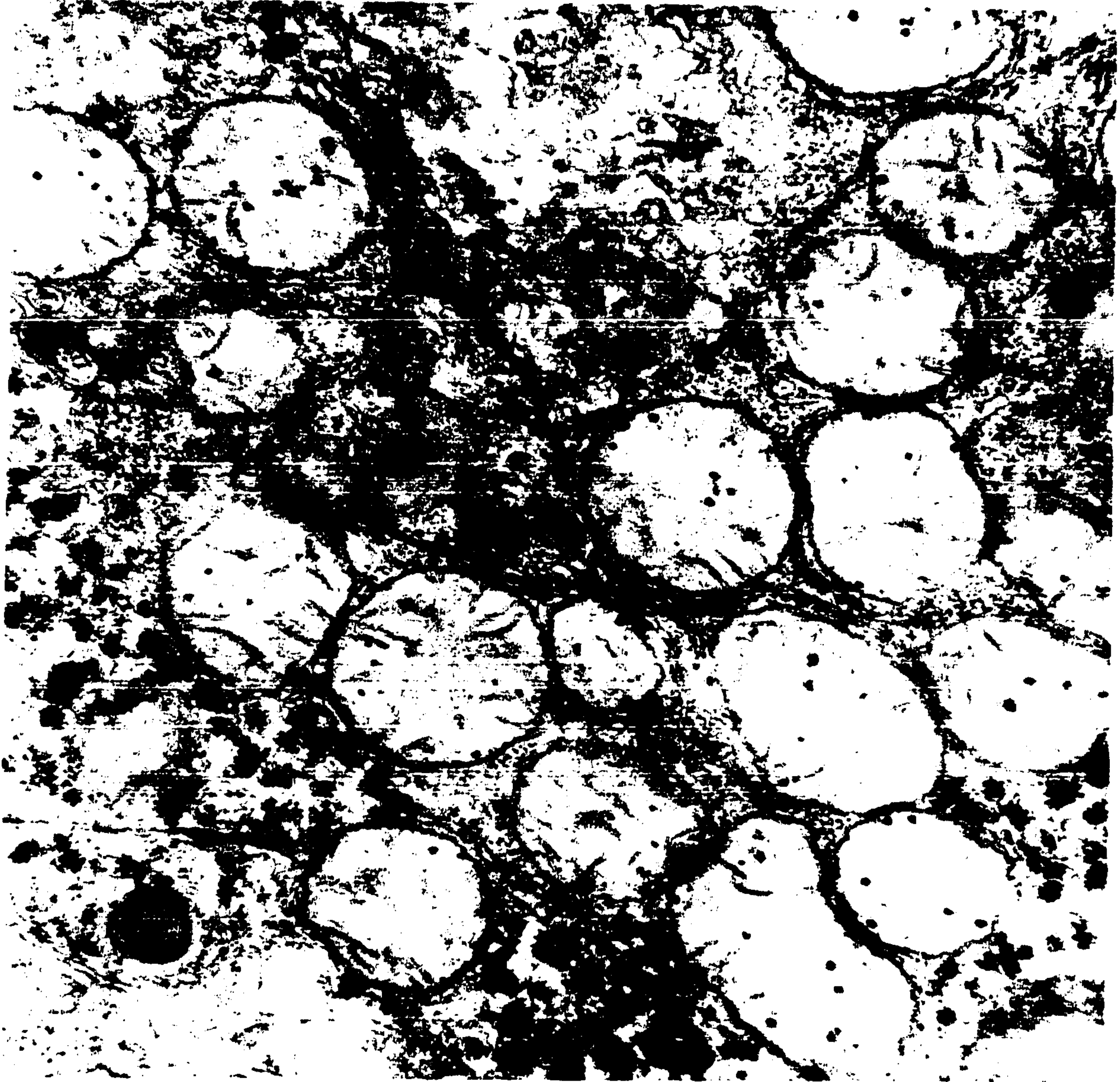

Figure 1[illegible]-c

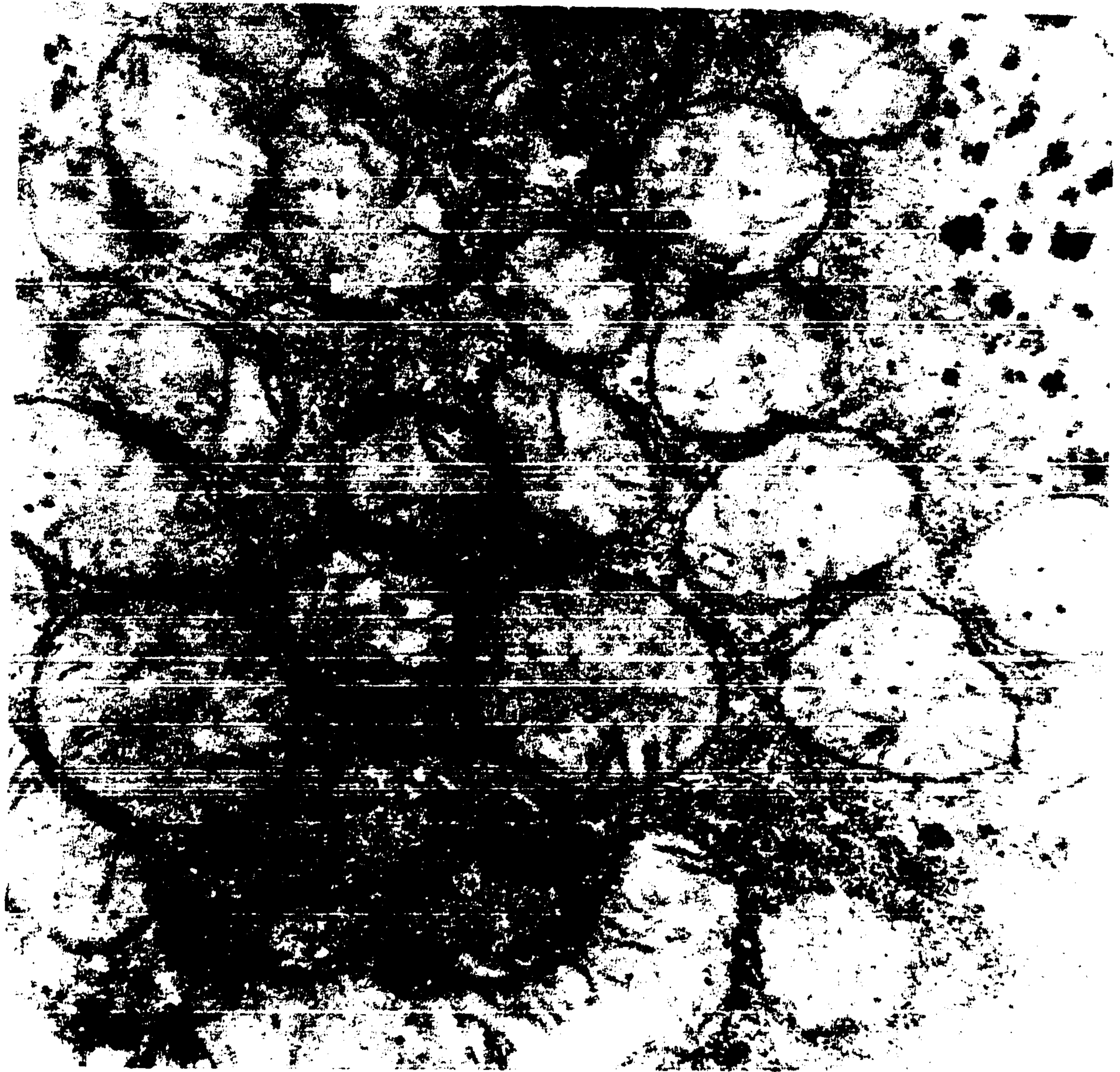

Figure 1[illegible]-d

CHAPTER IV

General Discussion

When rats are exposed to cold, they produce extra heat through non-shivering thermogenesis. In this study, the effect of cold exposure on the mitochondria of such rats has been investigated from several directions; namely, (a) dry mass, number, and protein content (b) oxygen uptake and the P:O ratio, (c) protein synthesis, as measured by the incorporation of C^{14}-amino acids, and (d) mitochondrial ultra structure. Individual experimental results have already been discussed under the appropriate headings. The object of this final discussion is to try and draw some general conclusion about the interrelations of these various aspects of mitochondrial structure and function, and their relation to the metabolism of cold-acclimated rats.

It was demonstrated in Experiment I that the dry mass, number and protein content of mitochondria were decreased in the liver cells of cold-acclimated rats. This reduction of the dry mass seems to be due to the reduction of the number of mitochondria since the dry mass per unit number of mitochondria is more or less the same between control and cold-acclimated rats. The reduced content of soluble and insoluble proteins of mitochondria in cold-acclimated rats might result from the reduction in the number of mitochondria.

As was demonstrated in Experiment II, oxygen uptake by mitochondria isolated from cold-acclimated rats was higher than that of mitochondria isolated from control rats. This increased uptake occurred with a corresponding decrease in inorganic phosphate uptake so that the P:O ratio was reduced. These observations lead one to conclude that the physical structure of mitochondria was altered as result of cold exposure.

The question here would be whether such a change in the physical behaviour of mitochondria resulted primarily from an indirect effect mediated through a cytoplasmic factor, or from a direct effect on the mitochondria themselves.

The results of the study of C^{14}-amino acids incorporation in Experiment III indicated that cold exposure induced a reduction of protein synthesis, particularly that of soluble protein. Since the synthesis of soluble protein is known to be controlled by cytoplasmic RNA and by other cytoplasmic factors (Roodyn, 1962), these results thus imply that cold exposure had its effect on mitochondria through the cytoplasm, i.e., an indirect effect. This reduction of protein synthesis of mitochondria suggests that protein (or amino acids) might be utilized to a larger extent as a respiratory substrate in cold-acclimated rats.

Cold exposure reduced protein synthesis in several internal organs by the same factor (Experiment III) without any specific effect

on any specific organ or organs. However, the synthesis of soluble protein was specifically reduced.

The electron microscopic study in Experiment IV revealed that cold exposure induced enlargement of mitochondria. This enlargement seems to be due to swelling. In addition, an increased glycogen content in cells of cold-acclimated rats was observed in the electron micrographs. The increased glycogen content in cells of cold-acclimated rats suggests the possibility of an altered intermediary metabolism, in which glycogenesis is involved.

CHAPTER V

Summary and Conclusions

1. The dry mass, number, and protein content of mitochondria in liver cells of cold-acclimated rats were found to be lower than those of control rats. This reduction of the dry mass seems to be due to the reduction of the number of mitochondria since the dry mass per unit number of mitochondria is more or less the same between control and cold-acclimated rats. The reduction of soluble and insoluble protein content might result from the reduced number of mitochondria in cold-acclimated rats.

2. In mitochondria isolated from cold-acclimated rats, oxygen uptake was increased, and the uptake of inorganic phosphate was reduced. Hence oxidative phosphorylation was reduced as a result of cold exposure.

3. A study of the incorporation of C^{14}-amino acids into protein of mitochondria isolated from liver cells of cold-acclimated rats showed that in general, the incorporation of C^{14}-amino acids into protein was reduced by cold exposure, more so for the soluble protein than for the insoluble protein.

4. It was demonstrated that there was no specific effect of cold treatment on the incorporation of C^{14}-amino acids into mitochondrial proteins of liver, kidney, brain, heart, spleen, and pancreas

in vivo, although the radioactivity accumulated in each of these organs was reduced by cold exposure. *In vivo* study on the incorporation of C^{14}-amino acids into subfractions of intact liver mitochondria again showed that the reduction in protein synthesis occurred mainly in the soluble fraction. It has been argued that the impaired protein synthesis *in vivo* in cold-acclimated rats might have been due to an energy deficiency resulting from a decline of the P:O ratio, or an increased utilization of protein (or amino acids) as a respiratory substrate, particularly of the soluble protein fraction.

5. An electron microscopic study of intact livers of cold-acclimated rats revealed that a great number of mitochondria were larger than those of control rats. The majority of mitochondria of control rats were spherical with more or less smooth outer membranes and with a dense matrix, but those of cold-acclimated rats were larger with ruffled outer membranes and a pale matrix. The number of mitochondrial cristae is more or less the same in both groups. The livers of cold-acclimated rats also showed numerous electron dense spots in the cytoplasm, suggesting that the glycogen content is increased during cold exposure. These electron dense spots were less numerous in sections from livers of control rats.

6. If a general conclusion can be drawn from the results of this study, it is that cold exposure exerts its effect indirectly on mitochondria through its effect on cytoplasmic factors. The influence causes particularly a reduction in mitochondrial soluble

protein synthesis. This reduced protein synthesis with enlargement of mitochondria of cold-acclimated rats could be the reason for the swelling and the change in the configuration of the membrane. Finally, these changes result in an increased oxygen uptake which could serve as the basic mechanism of non-shivering thermogenesis.

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