DEVELOPMENT OF BROWN ADIPOSE TISSUE IN THE RAT

by

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ABSTRACT

Interscapular brown adipose tissue (BAT) was studied during perinatal and early postnatal life of the rat. Several parameters of apparent functional importance were analysed, particularly respiratory enzymes which are related to the main function of BAT, heat generation.

I. Functional development of brown adipose tissue in the rat.

The ratio fresh weight of BAT/body weight peaked at day 1 after birth and declined subsequently; cold exposure reversed the decline. Protein content of the tissue increased sharply during perinatal development. Specific activities of several respiratory enzymes in the tissue increased rapidly during late fetal and early postnatal life, reached a peak by day 17 and declined thereafter. Cold acclimation of older animals returned the activities up to the same level as observed on day 17. A similar bi-phasic pattern was observed when the total BAT activities of the respiratory enzymes were related to body weight. These data together with some additional parameters, such as DNA, RNA, glycogen and norepinephrine content of the tissue, activities of monoamine oxidase and some enzymes involved in glycolysis, fatty acid and triglyceride synthesis, as well as morphological observations were interpreted in the following way:

The tissue starts a very rapid rate of proliferation and differentiation prior to birth, reaches a high degree of maturation and activity during the second postnatal week and involution commences during the fourth week after birth. Involution of BAT continues indefinitely at neutral ambient temperature and can be reversed by cold exposure. This developmental pattern

relates well to changing functional demands for non-shivering thermogenesis, pronounced mostly during the earliest postnatal stage and also during prolonged cold exposure of more mature animals.

II. Development of mitochondria in brown adipose tissue.

Isolated BAT mitochondria were studied and developmental changes observed in activities as well as amounts of respiratory chain enzymes and phospholipids. The biochemical results corresponded to morphological changes in the inner mitochondrial membrane. Hence the observed developmental changes of the tissue respiratory capacity seem to be a reflection of changes in the mitochondria. In addition BAT mitochondria proved to be a convenient model for studies of mitochondriogenesis in a mammalian tissue.

III. Hormones and regulation of brown adipose tissue development.

After the developmental pattern of BAT was established the problem of regulatory mechanism(s) governing the developmental alterations was approached. The in vivo effects of norepinephrine, epinephrine, thyroxine and cortisone as well as the effect of ambient temperature were studied during both the proliferative and the involutive phases of BAT development. Norepinephrine and cyclic AMP seem to be involved in the initiation and control of BAT differentiation. Cortisone exerted a detrimental effect upon the tissue and the possibility that corticoids are somehow involved in the second, involutive phase of BAT development was suggested.

IV. The hormonal receptor system in brown adipose tissue and its development.

An adenyl cyclase, selectively sensitive to catecholamines,

increased in activity up to the 20th postnatal day. Cyclic AMP levels in the tissue reached a peak just prior to birth and decreased postnatally. Cyclic AMP - dependent protein kinase activity increased perinatally and decreased after day 20. An inhibitor of protein kinase was most active perinatally. Phosphorylase and phosphorylase kinase were both present primarily in the activated form before birth and both increased immediately prior to parturition; then declined rapidly. The inactive form prevailed during postnatal life. Cold exposure caused increases in total phosphorylase, protein kinase, adenyl cyclase and cyclic AMP levels.

V. Interpretation.

Developmental patterns of enzymes involved in the hormonal stimulation pathway together with the in vivo experiments of Part III seem to support an involvement of a hormonal regulatory system in BAT development. A speculative scheme was proposed for the mechanism by which catecholamines may exert a trophic effect upon the tissue in addition to their short-term functional stimulatory action.

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First of all I would like to express my sincere appreciation and gratitude for the support and encouragement of an outstanding researcher and teacher, Dr. Peter Hahn, given to me with his friendship, and extending over a period of twelve years and over two continents.

The work presented here has been done within the period from 1968 to 1973, during which time I have been fortunate in working in close cooperation with several outstanding scientists and friends. The results presented in Part I and II of this communication were mostly obtained in the laboratory of Dr. Olov Lindberg, of the Wenner-Gren Institute, during my one-year stay in Stockholm. His help, advice and friendship will never be forgotten. The friendship and cooperation of Dr. Tudor Barnard were most helpful during that year, which was spent working on two aspects of the same problem, within our 'common four walls'. The results of Part III and IV were obtained during my stay in Dr. Hahn's laboratory, (Departments of Pediatrics and of Obstetrics & Gynecology, U.B.C.), which commenced in September 1969. I would like also to thank Dr. George Drummond of the Department of Pharmacology, U.B.C. for his most helpful criticism, discussions and advice, as well as the use of his facilities for some of the assays presented in Part IV. Some of the adenyl cyclase experiments were performed during my visits to Dr. O. Hechter's laboratory in the A.M.A. Research Institute in Chicago, where the cooperation, advice and friendship of Dr. Theodor Braun were most appreciated. In addition, I would like to

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FREQUENTLY USED ABBREVIATIONS

AMP	adenosine monophosphate
a.p.	ante partum - before birth
ATP	adenosine triphosphate
BAT	brown adipose tissue
CA	cold acclimation
CoA	coenzyme A
COX	cytochrome c oxidase
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetate
FFA	free fatty acids
GPDH	mitochondrial alpha-glycerophosphate dehydrogenase
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride
ISBAT	interscapular brown adipose tissue
MAO	monoamine oxidase
NAD	nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NE	norepinephrine
p.p.	post partum - after birth
P	phosphorus
PL	phospholipids
PMS	phenazine methosulphate
RNA	ribonucleic acid
SDH	succinate dehydrogenase
TCA	trichloracetic acid
TMPD	N, N, N', N', - tetramethyl - p - phenylenediamine dihydrochloride
TRIS	tris (hydroxymethyl) aminomethane

FOREWORD

Developmental physiology is a relatively new area of biological and medical science. The physiology of the perinatal period in mammals, as a part of developmental physiology, comprises very important stages that climaxes with parturition of the foetus from its protective intrauterine environment.

Developmental physiology differs from adult physiology in important ways. In adults, the range of functional capacity is more or less fixed, except for some adaptive mechanisms, whereas the functional capacities of developing organisms change dramatically with ontogenesis. As a consequence, studies of development are more complicated, bringing into the picture a new parameter - time. On the other hand, the developmental approach to any physiological or biochemical problem has many advantages. The two most important, at least according to the author's opinion, are:

1. Growth and development are terms synonymous with the disciplines of pediatrics. Hence, the current resurgence of interest in developmental physiology and biochemistry has been stimulated to a large extent by pediatric problems related e.g., to an understanding of inborn errors of metabolism. Moreover, it has become increasingly evident in recent years that the prenatal and early postnatal pattern of development, and influences exerted upon it, may have far-reaching effects on the future life of the individual. Since it seems inevitable that a preventive approach to medicine will eventually take over the current

therapeutically oriented approach, a detailed knowledge of the stage of proliferation and differentiation in an individual's life will be basic even for other disciplines of medicine than pediatrics.

2. Today's physiologists study chiefly two kinds of phenomena: Processes (i.e. what occurs in living units and by what means) and regulations (i.e. when and how much is done - quando et quanto). The two kinds are very closely linked in that every process contains not only a conjunction of working parts and materials, but also a control of speed and intensity. If we define regulation simply as the automatic control over an organism's processes or state, the development of the young individual has to provide not only new structures and processes but also the tools of control; the tools of physiological regulations. As the developing organism unfolds, visible events appear in an orderly sequence. Each event actually marks a new process or combination of processes. Hence the extremely complicated regulatory mechanisms found in a mature organism develop sequentially, level after level, during the course of ontogenesis. Remarkable features of development are the stereotyped sequences and concordant timing of the differentiations which activate the onset of physiological regulations. Each regulation depends on specific responses, some of which appear to develop independently of others. When they materialize, they work, in conjunction with arrangements already present, to form a regulatory system that lasts a lifetime. For example, birth activates more regulatory systems than any other event in life: some systems are

already in action but shift their set - points, others come out of inhibition and still others are built anew. Hence particularly in studies of control and regulation, the developmental approach (i.e. following the gradual process of building, level after level, of the final complicated regulatory machinery) seems more meaningful and even conveninent than the currently used analytical approach (i.e. artificially separating the components of a fully developed system). We can therefore expect that developmental physiology and biochemistry will inevitably bring more understanding even into the knowledge of basic physiological and biochemical mechanisms, operating in the mature organism.

GENERAL INTRODUCTION

Thermoregulation in mammals is the sum of a number of different processes, (e.g.) shivering thermogenesis, regulation of basal metabolism, vascular regulation of heat flow, insulation and so-called 'non-shivering thermogenesis'. The postnatal development of thermoregulation has been described repeatedly (for review see 72). It appears that total heat production in the rat is fully developed by day 18 postnatally and that control of heat loss develops gradually with day 18, and day 30 after birth being of particular importance; on day 18 fur and piloerection function well and on day 30 there is a further improvement, probably due to the development of some vascular mechanism (72). Non-shivering heat generation seems to be extremely important prior to the completion of the gradual postnatal development of homeothermy; for the early postnatal stage of development it is the only, or at least one of the very few, mechanisms by which the newborn can cope with the sudden drop in environmental temperature. Brown adipose tissue is the only organ known, the main and probably the only physiological function of which is considered to be non-shivering thermogenesis (for review see 197). Hence this tissue is apparently most important during the early neonatal stages of mammalian development.

The progress of biology has frequently been intimately connected with the discovery of a suitable model or method for investigation. Pure bacterial strains, tissue cultures, the sea urchin egg, and the electric organ are all examples of materials on which fundamental studies have been made. It may sound paradoxical that it is the very specialized nature of

these systems that makes them so suitable for studies of generalized importance, but the complex nature of biology causes the vast number of reactions that take place in less specialized cells to overshadow the process which may be of particular interest. Even a brief glance at sections of brown adipose tissue in the electron microscope suggests that the metabolism of the mitochondria dominates the biochemistry of the adipocytes. As it emerges that the role of the adipocyte in brown fat is to transduce the energy stored in fatty acids into heat, this process occurring in the mitochondria, (see 157), brown adipose tissue seems to be a very specialized organ. As such it may be a very suitable model for a developmental study. Moreover, since the requirements for non-shivering thermogenesis appear very suddenly after parturition and diminish rather early in life (as the other means of temperature regulation gradually take over), one can expect a pronounced and possible bi-phasic developmental pattern in brown fat. The tissue gains its importance as a site of non-shivering thermogenesis under two other conditions in addition to the early neonatal stage - during arousal from hibernation and during cold acclimation. When an adult rat is exposed to low ambient temperature for prolonged periods of time, the need for heat generation probably exceeds the limits of other means of thermoregulation and the functional activity of brown adipose tissue is in demand. Thus in the case of brown adipose tissue we have the rare possibility to alter the developmental pattern simply by exposing the animal to a cold environment. We can employ several different environmental temperatures and institute the treatment at different stages of development, and study the tissue under

these conditions. In summary, brown adipose tissue seemed, and eventually proved to be, a very convenient model tissue to be approached for a developmental study.

One additional reason for a developmental study of brown fat has to be stressed. Brown adipose tissue and its function - non-shivering thermogenesis - seem to play an important role even in the newborn human baby. As more and more babies now survive the premature delivery and immaturity, the problem of thermoregulation in such infants gains in importance in clinical neonatology.

Our developmental studies of brown adipose tissue in the rat were divided into four experimental stages according to the questions asked.

I. What is the developmental pattern of the tissue under normal circumstances? Does it indeed correlate with the changing functional demands on non-shivering heat production? Can cold exposure alter the pattern? The study concentrated mainly on functional development even though simultaneous morphological studies were undertaken. (These were performed by Dr. Barnard of the Wenner-Gren Institute, University of Stockholm).

II. Since the mitochondria are responsible for the production of heat by oxidation of substrates, do these organelles undergo developmental changes? Does the mitochondrion change as such or does the number of these organelles change during development? A detailed developmental study of isolated mitochondria was undertaken in order to answer the above questions and to establish the developmental pattern at the subcellular level.

III. What is the nature of stimulus which is responsible for the developmental changes? Is it the environmental temperature or is it a genetically coded information or a possible combination of both? Are hormones involved? Several series of experiments were performed in order to answer, at least partially, these questions and to develop a working hypothesis for further, more detailed inquiries into the molecular mechanism of the regulatory processes.

IV. Since the previous stage warranted a tentative conclusion that a hormonal regulatory mechanism may indeed be at least partially involved, a study of the hormonal receptor system in the tissue and its development was initiated.

In the following presentation the study will be divided into the four parts according to the above description. An attempt will be made to treat each part separately and a speculation as to their interrelations will be presented as Part V.

It is a common experience that each series of experiments opens some other problems. Hence also in our study several related projects have been undertaken. In order to keep this presentation clear, these will not be included in full and since the majority of the data were already published elsewhere, only references will be made for the interested reader.

PART I

FUNCTIONAL DEVELOPMENT OF BROWN ADIPOSE TISSUE

(Based on the following publications:

16, 73, 76, 80, 82, 83, 182, 185, 186, 191)

INTRODUCTION

The development of brown adipose tissue (BAT) was an uncharted area. Early investigators, using the light microscope, were only concerned to gather ontogenic evidence illuminating the relationship of brown to white adipose tissue (84, 21). The conclusion, in the early 1960's, that brown fat is involved in thermogenesis (196) greatly stimulated interest in the nature of the thermogenic reactions and their control (for review see 197). Investigators used rather indiscriminately cold acclimated, hibernating or neonatal animals of different species, but until recently only a few contributions were made to the ontogenesis of this tissue (e.g. see 177). In order to study regulatory mechanisms governing the developmental changes, the developmental pattern has first to be firmly established. Since no methodical ontogenic studies on rat BAT were available in the literature, we had to undertake rather wide studies of many parameters, which were considered important from the functional standpoint. These parameters, together with the discussion of their relevance to the BAT function, will be presented in this section.

As the morphological ontogenesis of BAT in the rat was extensively reviewed by Barnard & Skala (17), a short summary will be presented here in order to allow for comparisons with our functional results.

The colour of BAT ranges from pale buff to dark reddish brown depending on age of the animal. The colour derives largely from the blood hemoglobin as the tissue is highly vascularized and from a high level of heme porphyrins (largely cytochromes) and flavin compounds (103, 197).

As such it is distinguishable from the overlying white fat, particularly in younger animals. The typical distribution may be described as middorsal (interscapular), subscapullar and axillary, dorsal cervical, thoracic, suprasternal, inferior cervical, superior mediastinal, perirenal and retrolumbar. Hence these very 'strategic' locations ensure preferential application of the produced heat to the thoracocervical region of the spinal cord and all vital organs in the thoracic area as well as the kidneys and adrenals (for more detailed discussion see 1, 197).

Microscopically, BAT is made up of adipocytes, capillaries, nerves and connective tissue. The tissue is organized into lobes, which consist of a number of lobules. Within each lobule there is a gradient of organization (177). The large majority of cells are adipocytes, which are polygonal or roundish cells of fairly large diameter. The cytoplasm is abundant and contains a variable number of triglyceride droplets; hence such adipocytes are termed multilocular. The mitochondria are large, numerous and packed with cristae. Moderate amounts of smooth, but very little of rough, endoplasmic reticulum are present.

The interscapular brown fat pad comprises up to 50% of the total amount of BAT and it is the largest single location of this tissue. The following morphological observations were made on this interscapular tissue:

6 days before birth the tissue is very loosely organized and there are no cells mature enough to be described as adipocytes, although a number of small, undifferentiated cells with abundant endoplasmic reticulum containing a few small triglyceride droplets, is found. The

mitochondria have only few cristae and are relatively large. Some intramitochondrial granules are seen.

3 days before birth the tissue is filled with clearly differentiating adipocytes. They frequently contain large fields of glycogen granules. Mitochondria appear to be more numerous, number of cristae per mitochondrion is increased and the cristae are more regularly arranged. The intramitochondrial dense granules are bigger and more numerous than at the previous stage. They seem to have an internal structure consisting of osmiophilic material arranged as clusters of small tubules and lamellae.

1 day before birth smooth endoplasmic reticulum is abundant and the size and number of the triglyceride droplets are increased, as is the extent of the glycogen fields. Mitochondria contain more cristae. The intramitochondrial dense granules are abundant and large, with an average diameter over 1000 A. The inner structure of the large granules shows on some occasions a unit membrane-like structure and seems sometimes to be confluent with the inner membrane.

By some hours after birth substrate stores are largely depleted. Autophagic vacuoles are common. Mitochondria appear large and contain more cristae than at the previous stage.

1 day after birth the size of triglyceride droplets increases somewhat. The intramitochondrial large granules are no longer seen, instead a large number of smaller granules appears.

In the interval 1 - 20 days after birth the volume of adipocytes

increases and so does the number and size of triglyceride droplets. The amount of mitochondria and the surface area of the inner membrane seem to increase.

By the 30th postnatal day the lipid droplets are even larger, but the mitochondria appear to be less numerous and they contain fewer cristae.

In the adult rat reared at room temperature most adipocytes contain several large triglyceride droplets. There are also unilocular cells, some of which have an appearance typical for adipocytes of white fat. There is very little rough endoplasmic reticulum and variable amounts of glycogen. Mitochondria are large, much less packed with cristae and occupy a large percentage of the scarce cytoplasm.

After 8 weeks of cold acclimation, the most marked changes are within mitochondria. The size of the organelles is increased and they are packed with cristae. Lipid droplets seem to be smaller and more numerous.

METHODS

Presentation of the methodological experiments performed for all the assays used in this communication would take much space which can be more effectively used for presentation of developmental results. Hence only modifications of the assays will be described in detail and the optimal conditions stated only if they differ from those described in the literature. Otherwise only reference to published methods will be made, even though all the conditions were tested with BAT enzyme preparations in each instance.

Animals and tissue preparation.

Sprague-Dawley rats of known ages (± several hours for the postnatal animals and timed pregnancies for the fetal stages) were used. Each litter was reduced to 8 animals on day 1 after birth. The morphological considerations, summarized in the introduction, together with the available information on development of homeothermy in the rat, were used to decide upon the stages of development at which BAT was examined.

Cold acclimation, as used in the experiments presented here, was achieved by subjecting rats of an initial age of 30 - 40 days, individually housed, to an ambient temperature of 4 ± 1 °C for 8 weeks.

Interscapular BAT was removed immediately after decapitation. Care was taken to remove the complete pad. The tissue was carefully trimmed of extraneous tissues on a filter paper moistened with ice-cold 0.25 M sucrose. BAT from all animals in the same age group was placed in a tared beaker containing ice-cold 0.25 M sucrose. The pooled sample, sucrose and beaker were then weighed. The average number of animals

comprising a group for each experimental stage is shown in Table I. After weighing, the pooled tissue was chopped into small pieces with scissors and washed several times with chilled 0.25 M sucrose. Tissue was then homogenized in about 8 volumes of the same medium using a glass homogenizer with a Teflon pestle rotating at approximately 800 rev./min. The homogenate was filtered through nylon cloth in order to remove fat and to obtain more homogenous samples for enzymic assays. Filtration was, however, omitted during preparation of the samples used only to determine protein concentration in the tissue.

Alpha-glycerophosphate dehydrogenase (mitochondrial alpha-glycerophosphate: PMS oxidoreductase, E.C. 1.1.95.5) and succinate dehydrogenase (succinate: PMS oxidoreductase, E.C. 1.3.99.1)

activities were estimated colorimetrically according to Nachlas et al. (140), Singer & Lusty (179), Pennington (151) and Lee & Lardy (125) with some modifications described below. INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride) was used as the final electron acceptor, and PMS (phenazine methosulphate) as the artificial electron carrier. The enzyme preparations (tissue homogenates in this part of the presentation and isolated mitochondria in Part II) were incubated for 15 minutes at 37°C in the incubation medium (total volume 1.0 ml) containing potassium phosphate buffer, 50 mM pH 7.5; PMS, 0.025%; INT, 0.1%; KCN, 1 mM; sucrose, 50 mM and substrate (DL-alpha-glycerophosphate as a disodium salt, 0.1 M; or sodium succinate, 0.05M). Efficiency of the above medium was tested in a series of experiments in order to make sure that V_{max} values are obtained for each enzyme.

Fig. 1 shows the time dependency of the assays for both enzymes in the presence of both INT and PMS or in the presence of only INT. The velocity levelled off somewhat, but 15 minutes was considered as a satisfactory incubation time. In Fig. 2 increasing amounts of INT were tested and 1.0 mg INT/0.1 mg of mitoch.protein found to be a saturation dose. In order to determine the optimal ratio PMS/INT we have tested increasing amounts of PMS in the presence of stable INT amounts (Fig. 3) and found a certain degree of self-reduction of INT in the presence of PMS. 1:4 ratio for PMS: INT was considered as optimal. The effect of KCN was rather surprising (Fig. 4 and Fig. 5) since this cytochrome oxidase inhibitor showed an activation. This has been described by Nachlas et al. (140) for succinate dehydrogenase in liver mitochondria. We found this activation to apply for both dehydrogenases. The lack of effect of Azide which acts at the same site of the electron respiratory chain (see Fig. 8) seems to support a possibility that KCN acts directly on the flavoprotein molecule of the dehydrogenases and the activation effect is distinct from its inhibition of cytochrome oxidase site. The kinetics of the reactions with respect to substrates is shown in Fig. 6. High substrate levels (about 50-fold in excess of the amounts actually oxidized during the time of reaction) were required in order to obtain an apparent V_{max}. Two-fold amounts of alpha glycerophosphate than succinate were necessary, presumably due to the use of DL-glycerophosphate.

Reactions were stopped by addition of 1.0 ml of 10% TCA (trichloroacetic acid) and the formed formazan (reduced INT) extracted in 4.0 ml ethylacetate. Absorption of an aliquot was measured at 490 ηm,

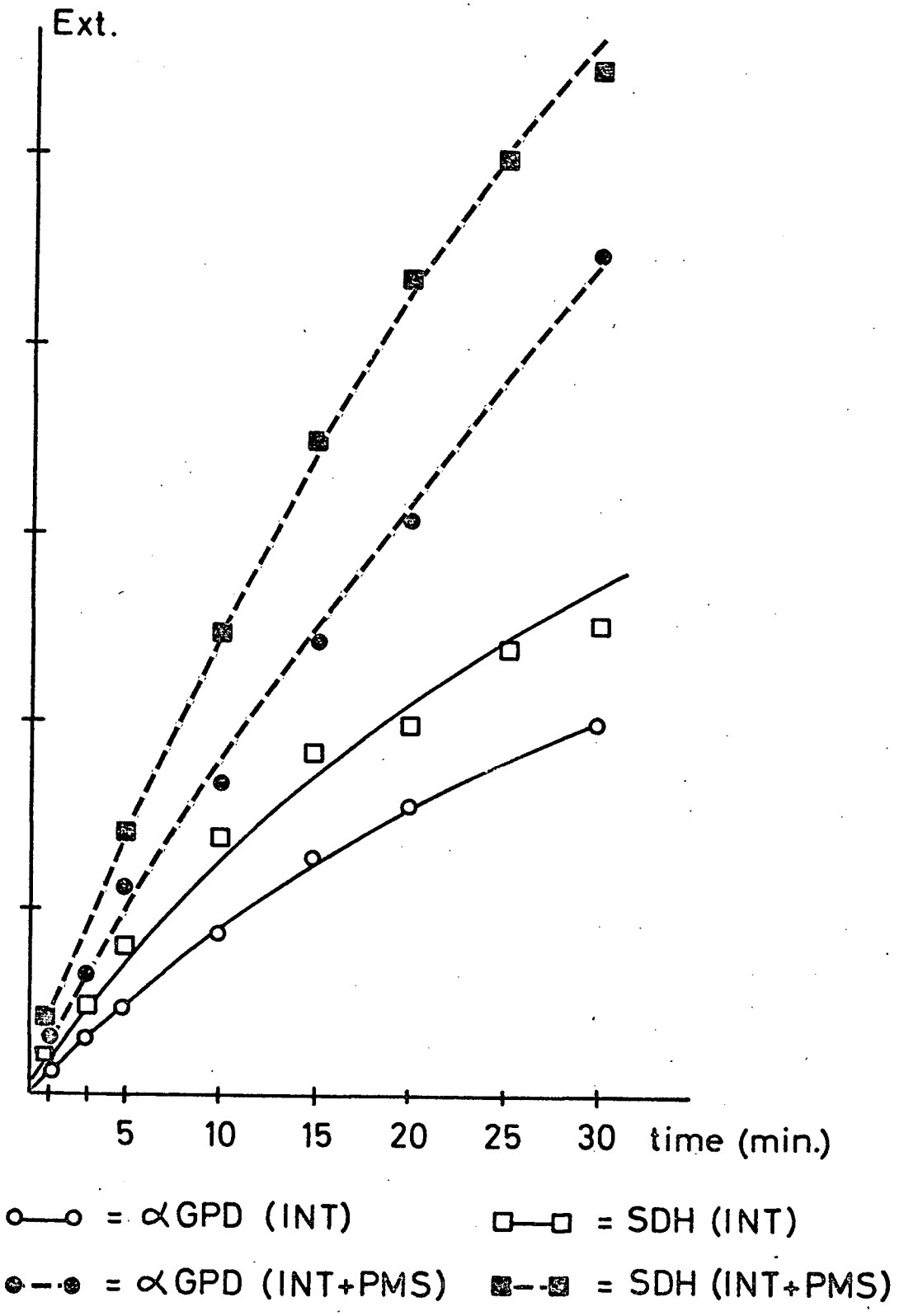


Fig.]. The Effect of Incubation Time on the Activities of succinate Dehydrogenase and alpha-glycerophosphate Dehydrogenase in Isolated BAT Mitochondria.Both assays were performed in the absence and in the presence of PMS.

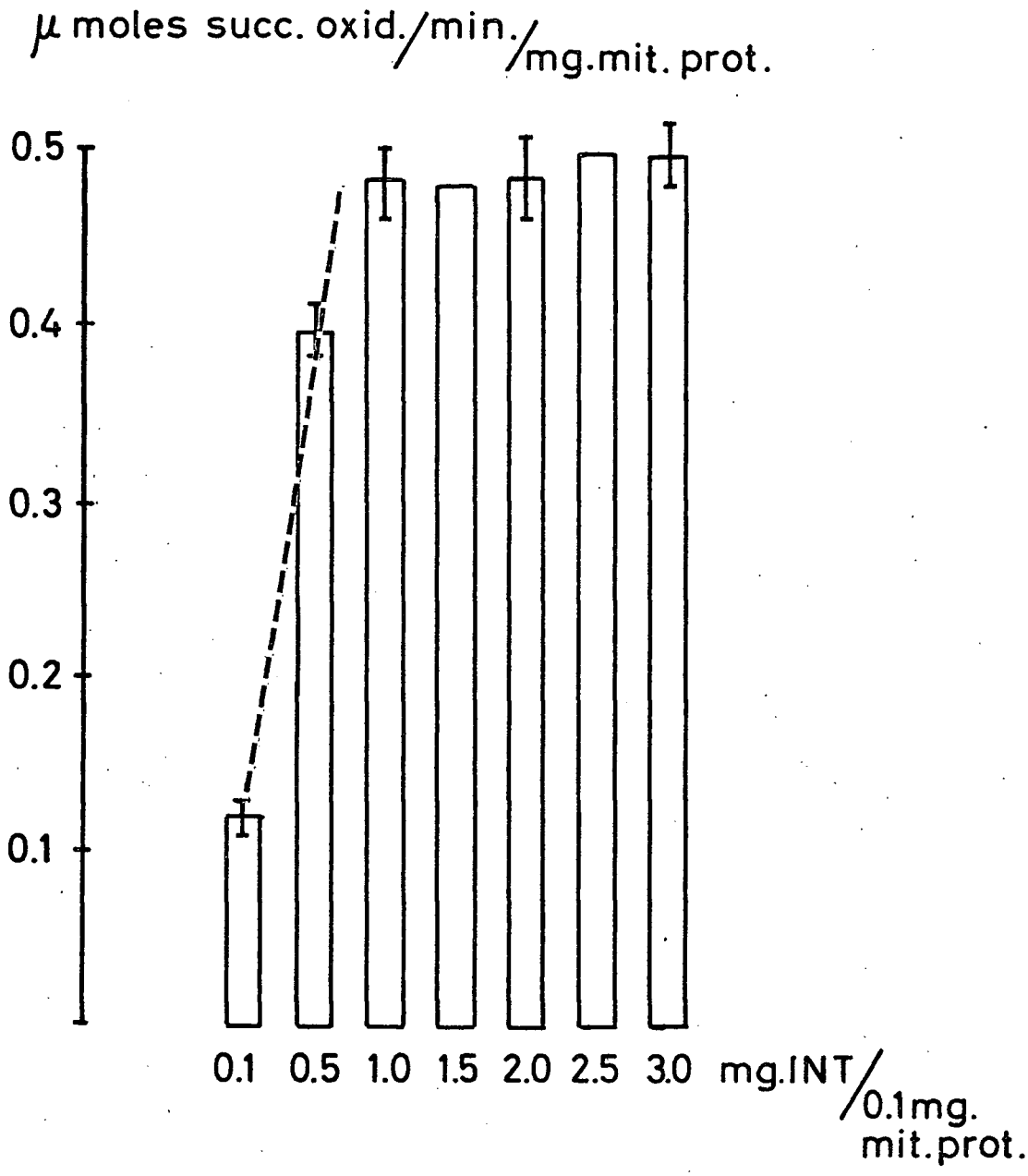


Fig.2. The Effect of Increasing INT Concentrations on Succinate Dehydrogenase Activity in Isolated Brown Fat Mitochondria.

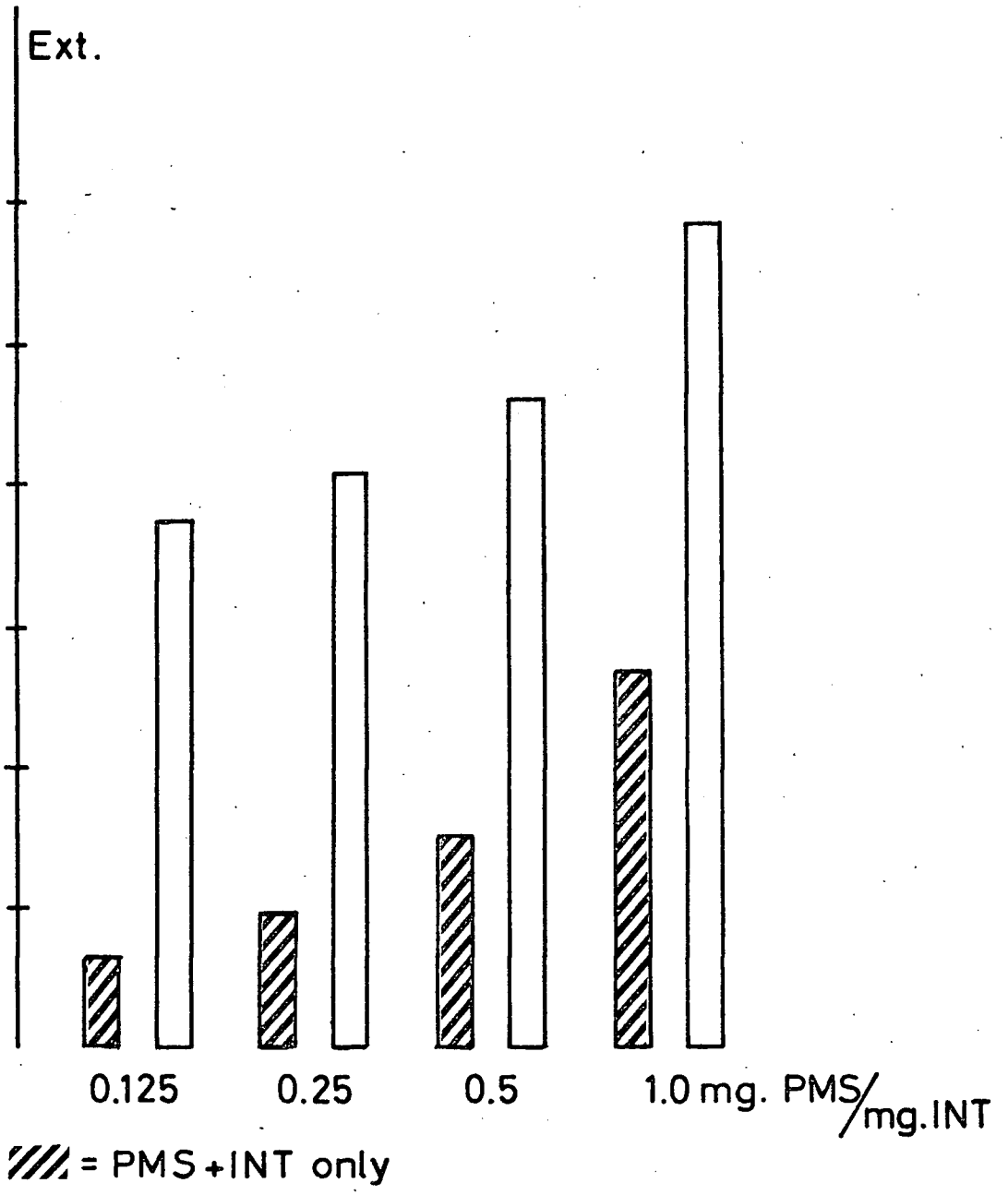


Fig. 3. The Effect of Increasing PMS Concentrations on Succinate Dehydrogenase Activity in Isolated Brown Fat Mitochondria. The amount of INT was unchanged. The dark columnes represent the reduced INT formed in the presence of the respective amount of PMS with no enzyme present.

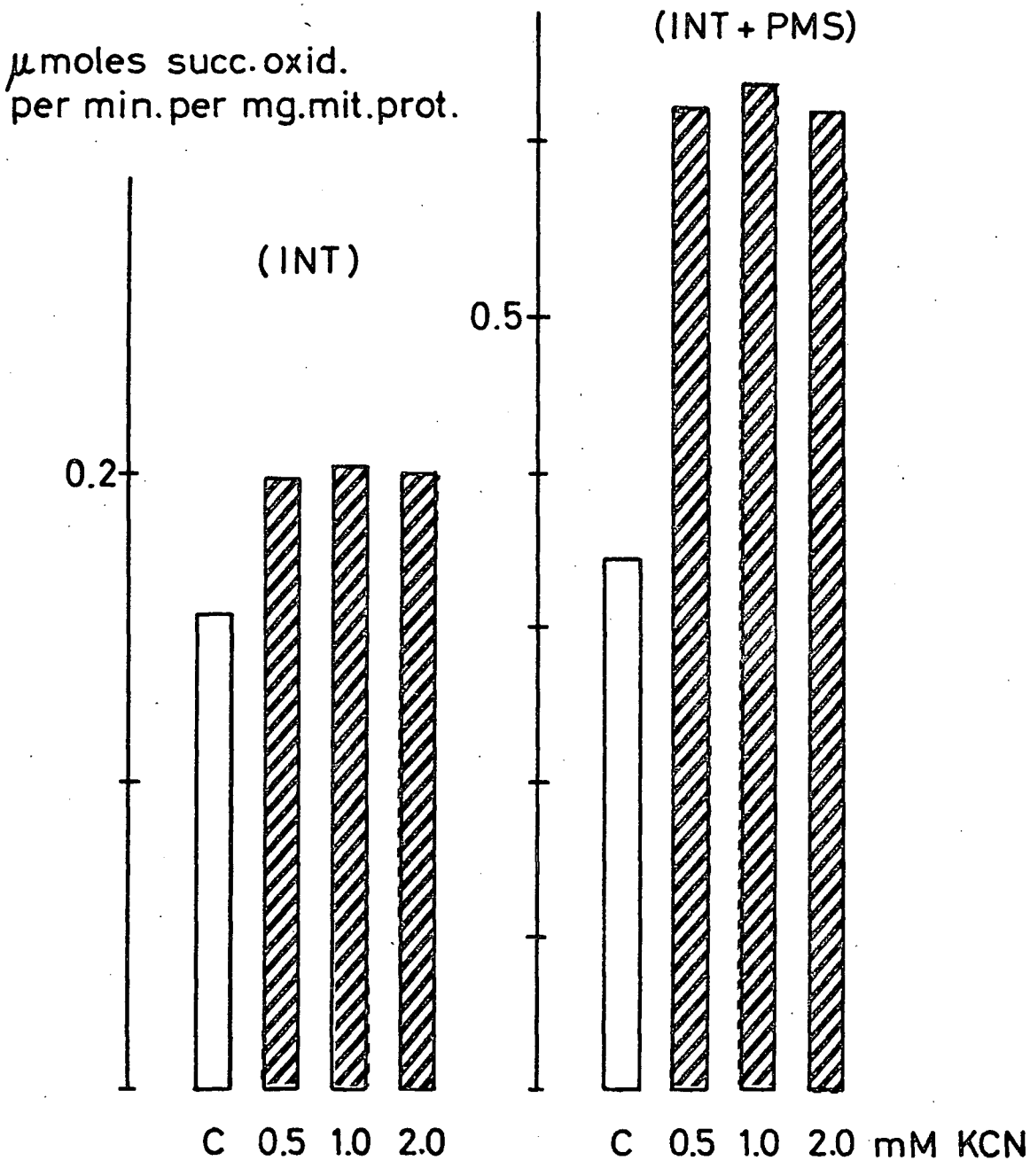


Fig. 4. The Effect of KCN on Succinate Dehydrogenase Activity in Isolated Brown Fat Mitochondria. Activity was determined both in the absence and in the presence of PMS. C = no KCN added.

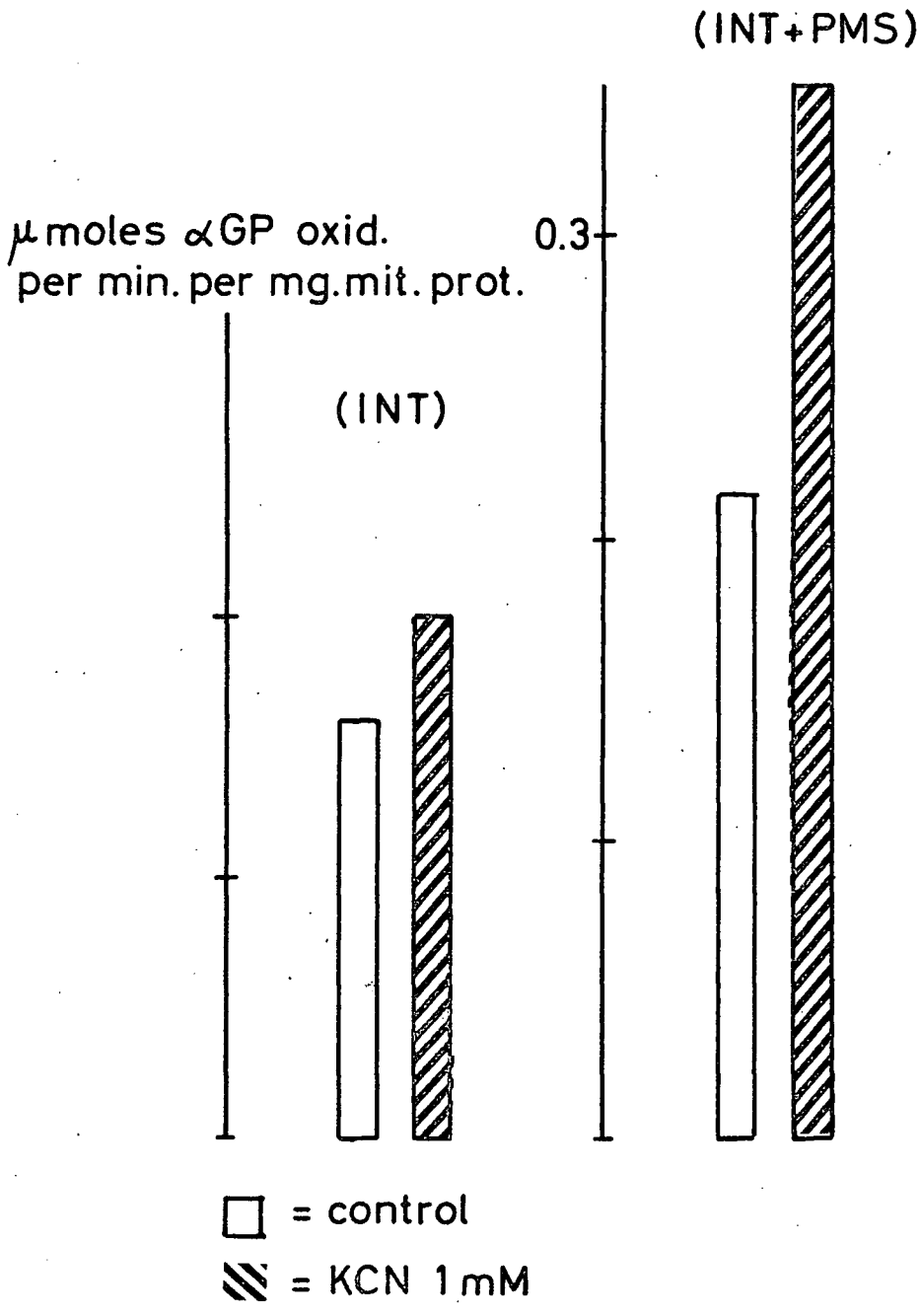


Fig. 5. The Effect of KCN on alpha-glycerophosphate dehydrogenase Activity in Isolated Brown Fat Mitochondria. Activity was determined both in the absence and in the presence of PMS.

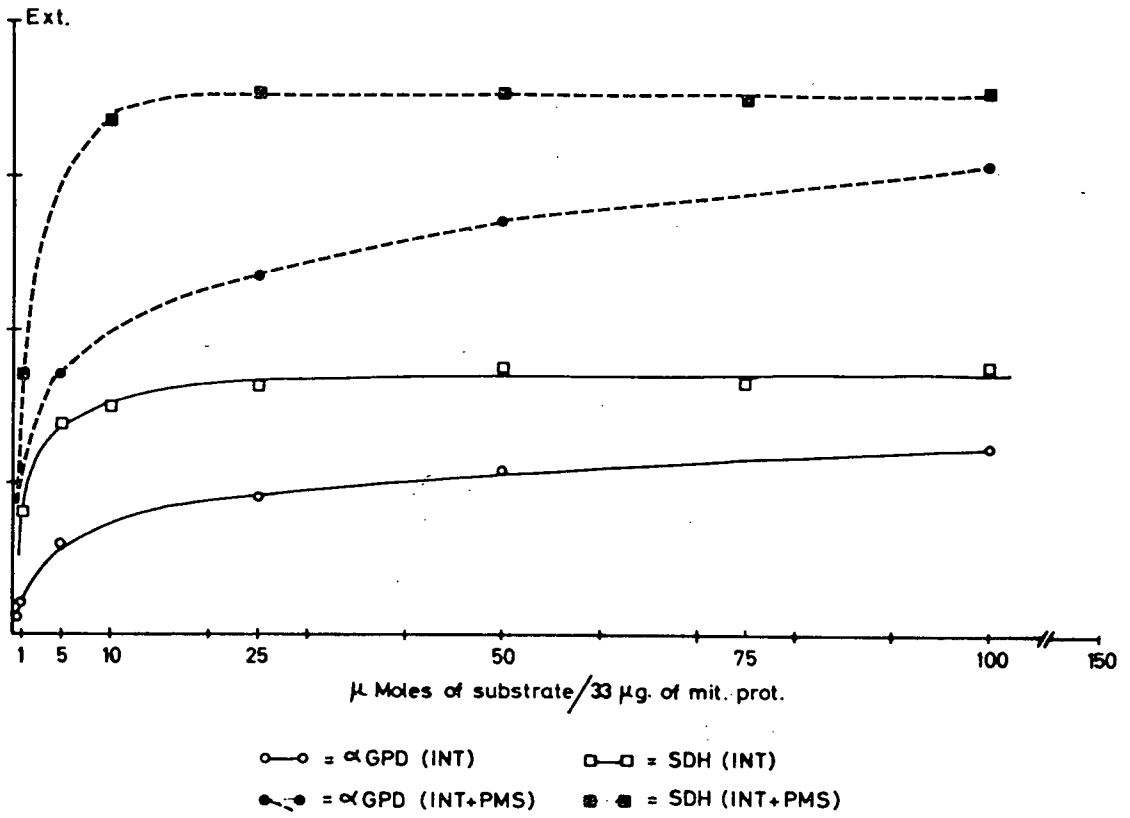


Fig. 6. The Effect of Substrate Concentrations on Reaction Velocities of Succinate Dehydrogenase and alpha-glycerophosphate Dehydrogenase in Isolated Brown Fat Mitochondria. Activities were determined both in the absence and in the presence of PMS.

which is the absorbance peak observed for formazane both in the absence and presence of PMS (Fig. 7). The extinction coefficient used for activity calculations was 20.1×10^3 for 1 M formazan.

Sonication (80 kH/6 x 20 seconds) of the enzyme preparation or addition of 9 mM Ca^{++} to the incubation medium were not routinely used since a permeability barrier of BAT mitochondria to PMS (similar to that described by Singer & Lusty - 179 and Arrigoni & Singer - 6) has not been observed. Singer (180) described the pre-incubation of mitochondria in the presence of substrate and absence of the electron acceptor as a requirement for obtaining V_{max} of SDH in adult rat liver mitochondria. However, we were not able to confirm this for BAT mitochondria, so pre-incubation was not routinely used. The enzymic specifities of both reactions were examined using some inhibitors acting at different sites of the respiratory chain (Fig. 8). With exception of KCN (see above) no effect was found thus indicating that no other parts of the respiratory chain interferred. The inhibitory effect of oxaloacetate on succinate dehydrogenase activity is generally recognized. To remove oxaloacetate from crude enzyme preparations, Singer & Lusty (179) used cysteine sulphinate in the reaction medium. In our experiments the presence of cysteine sulphinate had no effect on alpha-glycerophosphate dehydrogenase activity and resulted in 20 - 25% increases in succinate dehydrogenase activity. Preliminary experiments showed this increase to be standard and the same at all ages studied, thus the removal of oxaloacetate was not used in experiments presented in this communication. The linearity of both the assays with the amount of protein is shown in Fig. 9 and Fig. 10, respectively.

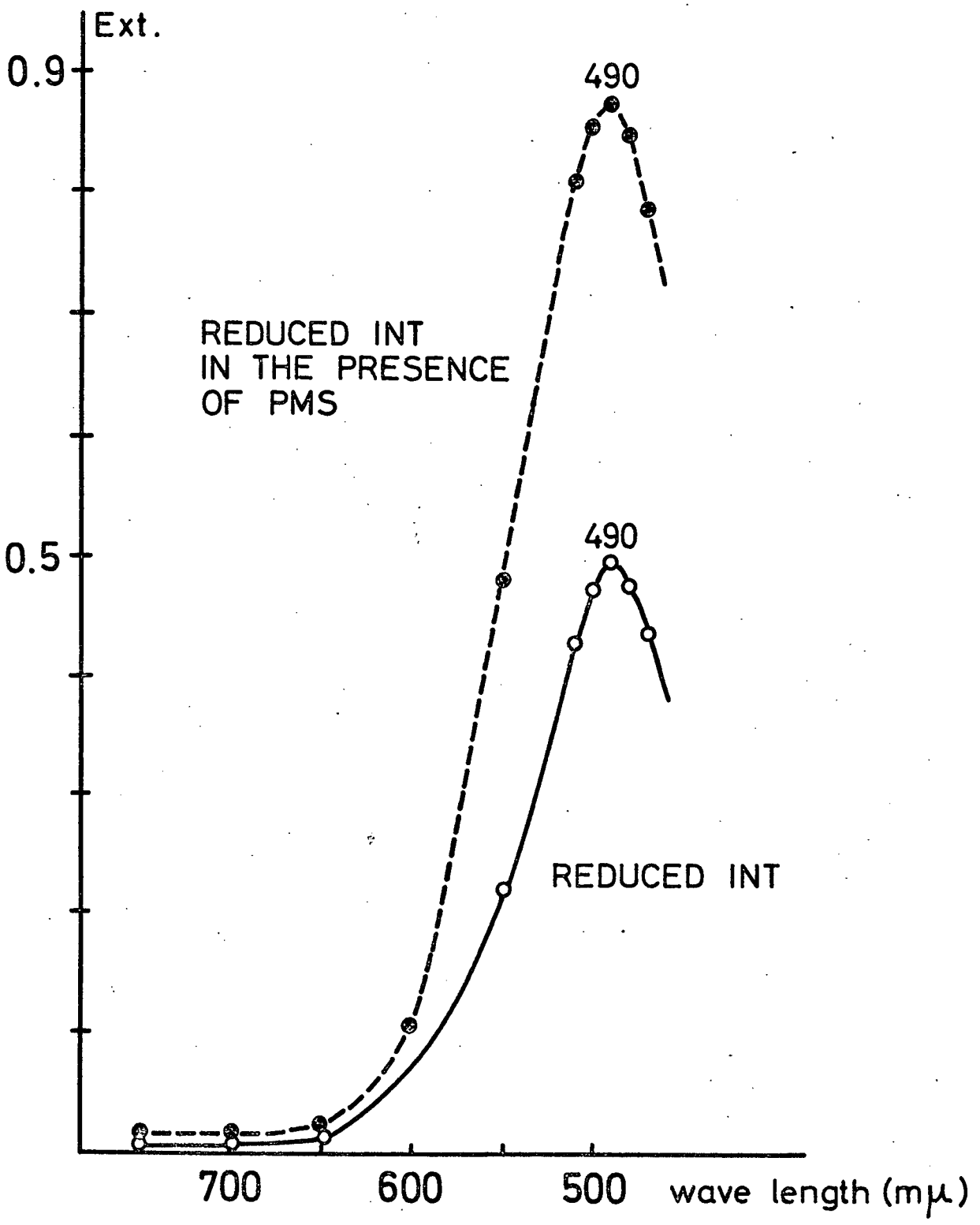


Fig. 7. The Absorption Spectrum of Reduced INT . Recordings were made both in the absence and in the presence of PMS.

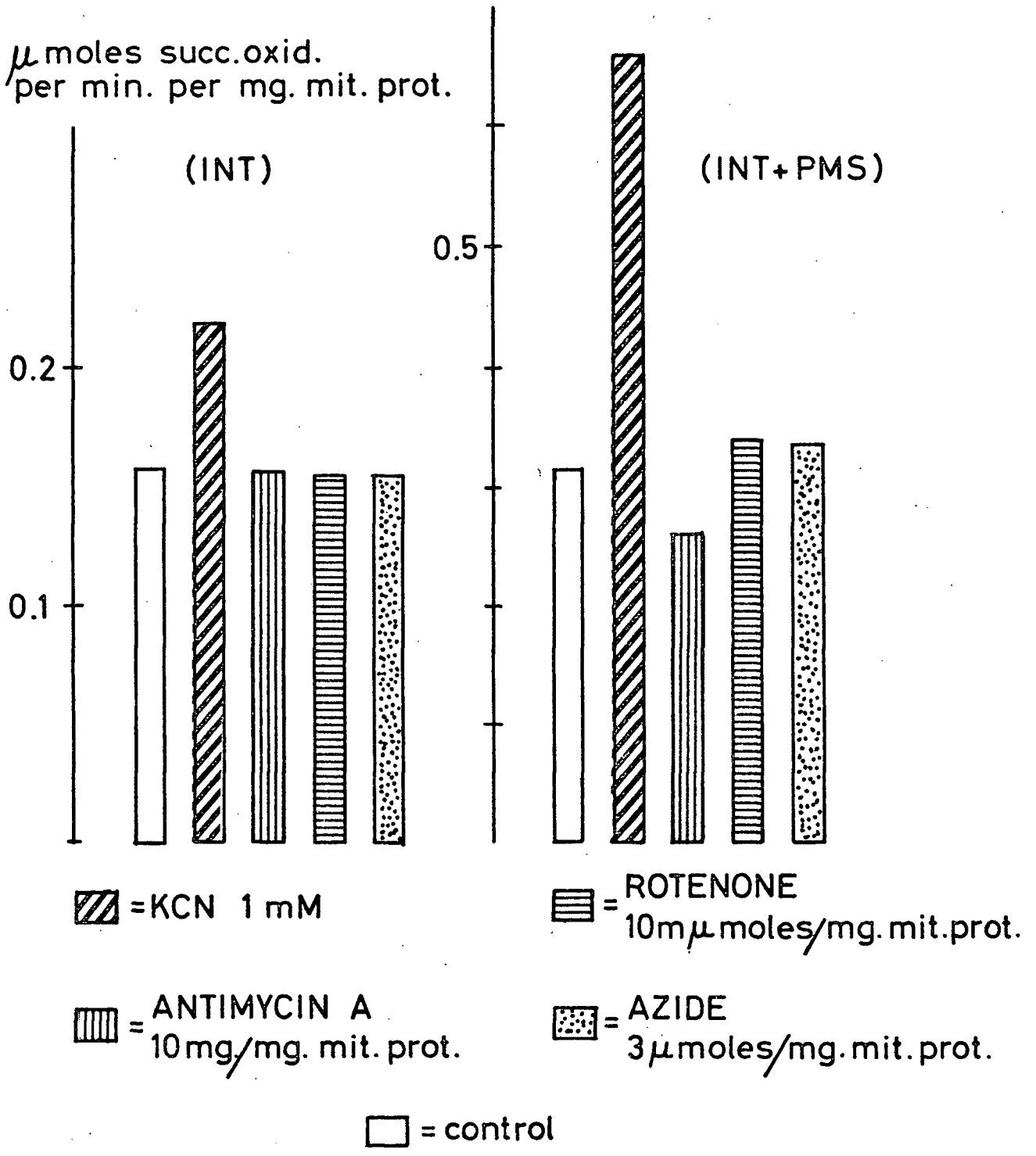


Fig. 8. The Effect of Some Respiratory Chain Inhibitors on Succinate Dehydrogenase Activity in Isolated Brown Fat Mitochondria. Activity was determined both in the absence and in the presence of PMS.

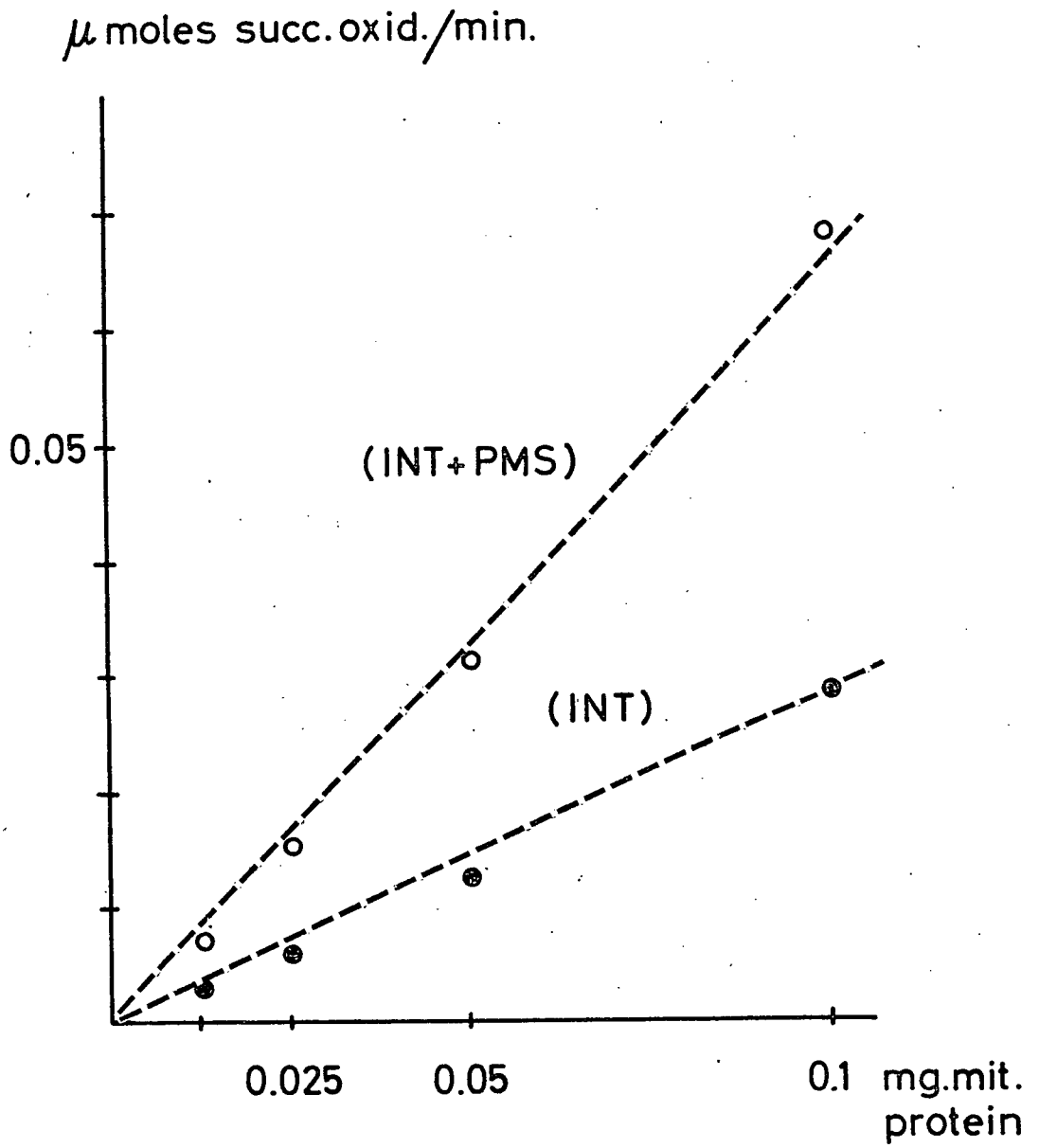


Fig. 9. The Effect of Increasing Amounts of Brown Fat Mitochondrial Protein on Succinate Dehydrogenase Activity. Measurements were performed both in the absence and in the presence of PMS.

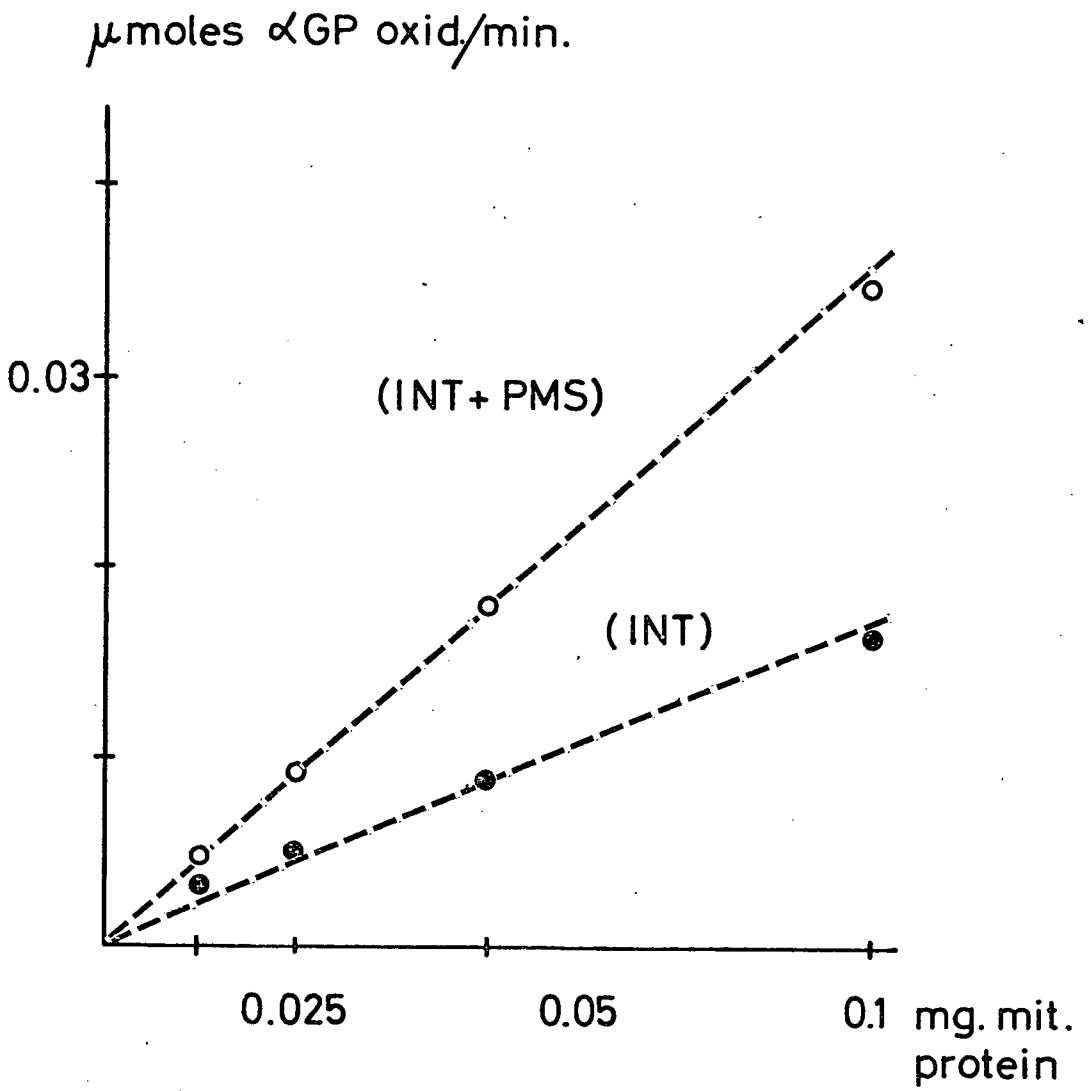


Fig. 10. The Effect of Increasing Amounts of Brown Fat Mitochondrial Protein on alpha-glycerophosphate Dehydrogenase Activity. Determinations were made both in the absence and in the presence of PMS.

Cytochrome c oxidase

(ferrocytochrome c : oxygen oxidoreductase, E.C. 1.9.3.1) activity was estimated as the uptake of oxygen measured polarographically at 30°C using a Clark-type oxygen electrode according to Schnaitman et al. (171). Schnaitman & Greenawalt (172) have described the use of a nonionic detergent Lubrol as a useful alternative to sonication of mitochondria. In contrast to their results with mitochondria from adult rat liver, no additional 'released' activity of cytochrome oxidase after Lubrol pretreatment (0.1 - 0.6 mg/mg protein) was observed in brown fat mitochondria. Since the quality of the mitochondrial preparations in these experiments was checked by electron microscopy (see Part II) and no damaged membranes were found, we can assume that the mitochondrial membrane in brown fat is permeable to the TMPD (N, N, N', N' - tetramethyl - p - phenylenediamine dihydrochloride) - ascorbate system used in our assay. Hence no pretreatment was routinely used in our experiments. The dependency of the assay on the amount of protein is shown in Fig. 11.

Monoamine oxidase

(monoamine:oxygen oxidoreductase, E.C. 1.4.3.4) activity was measured as the rate of production of ^{14}C- indolacetic acid from ^{14}C-tryptamine as described by Wurtman & Axelrod (225). The formed indolacetic acid was selectively extracted from the reaction medium using toluene. Less than 0.2% of the ^{14}C-tryptamine originally present was extracted as a contaminant. The assay was linear with time up to 15 minutes (Fig. 12). The method was found to be very sensitive; the activity

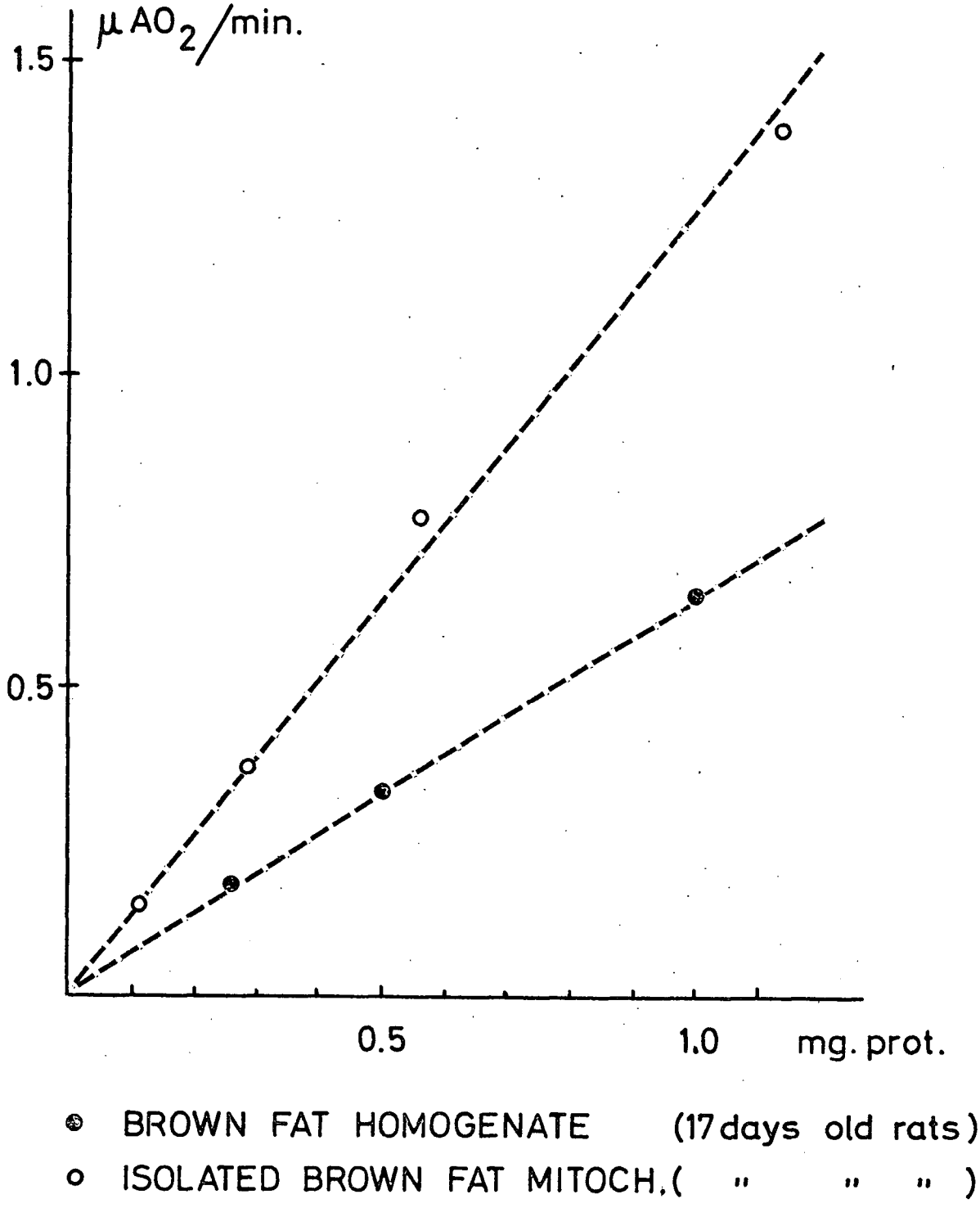


Fig. 11. The Effect of Increasing Amounts of Brown Fat Protein on Cytochrome c Oxidase Activity Both in Tissue Homogenates and Isolated Mitochondria.

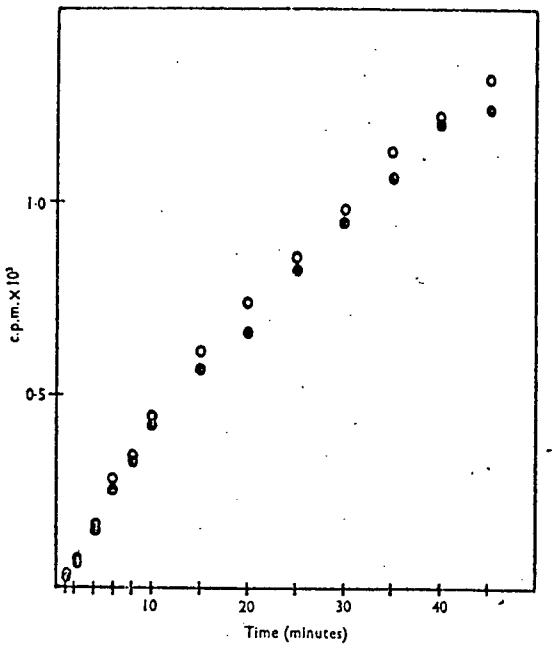


FIG. 12 –Time course of monoamine oxidase reaction (2 experiments); 0·2 mg. of ISBAT mitochondrial protein was used in each experiment.

of as little as 10 µg of mitochondrial protein and 20 µg of homogenate protein could be reproducibly detected (Fig. 13). This corresponds to the production of about 10^{-12} moles of indolacetic acid. As such the method was considered adequate for detection of the monoamine oxidase activity in a tissue with relatively very low level of the enzyme activity, such as BAT. Wurtman & Axelrod (225) reported a pH optimum of 7.5 for the enzyme in liver homogenate but no information for isolated mitochondria was available. We found the same pH to be optimal for both homogenate and isolated mitochondria of BAT (Fig. 14). Sonication had no pronounced effect on monoamine oxidase activity of isolated BAT mitochondria. If intact mitochondria were assigned an activity index of 100%, sonication (80 kH/6 x 20 seconds) resulted in a small decrease to 92.7 $\pm$ 2.9%, no activation, as might be expected, was found. This is in good agreement with observations on liver

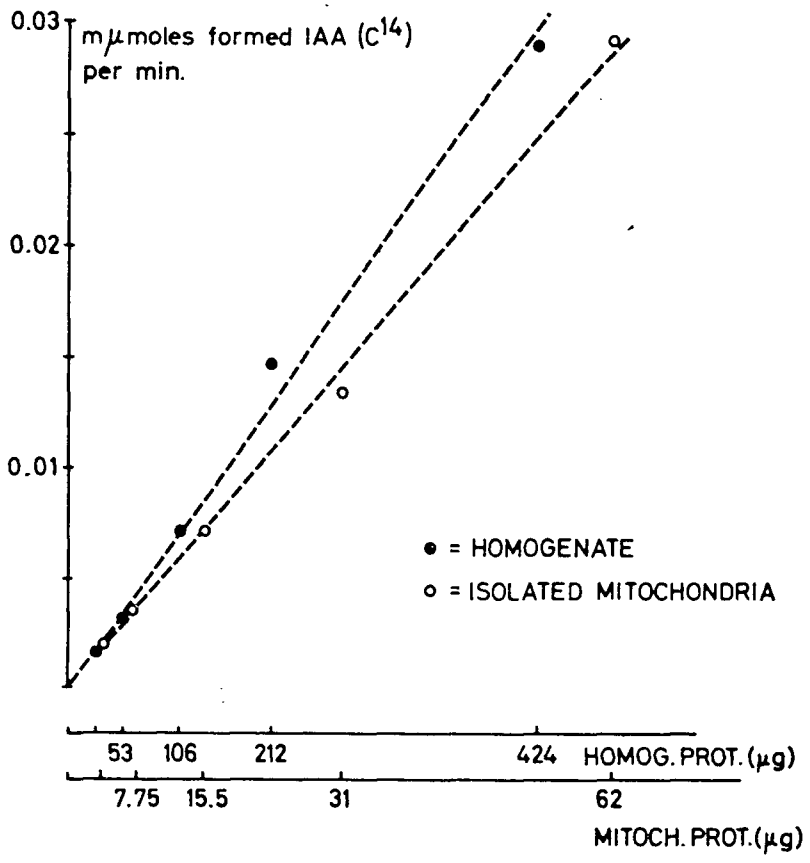


Fig. 13. The Effect of Increasing Amounts of Brown Fat Protein on Monoamine Oxidase Activity Both in Tissue Homogenates and in Isolated Mitochondria.

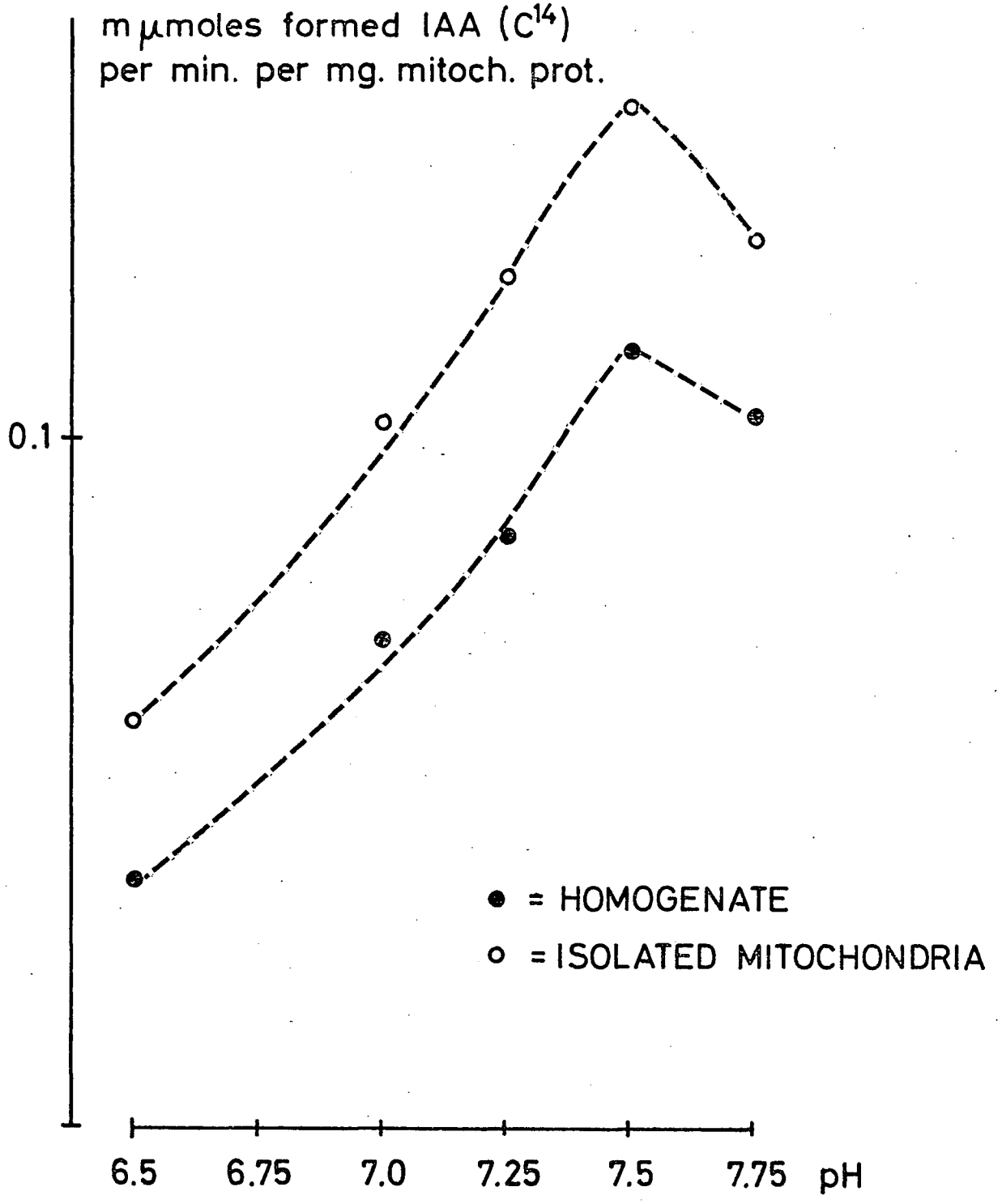


Fig. 14. The Effect of pH on Monoamine Oxidase Activity in Both Brown Fat Homogenates and in Isolated Mitochondria.

mitochondria (Kuylenstierna, personal communication). Thus no sonication was routinely used.

Glycogen

was determined by the enzymic method of Johnson et al. (106). Tissues of the same age-group were combined after rapid freezing in liquid nitrogen (see the tissue preparation for c-AMP assays in Part IV) to yield samples of approximately 50 - 100 mg each and were kept at -60° C until use.

DNA and RNA

content of the tissue were determined according to Burton (31).

Protein

was determined by the method of Lowry et al. (130).

Materials: All the chemicals used in the assays were purchased from Sigma or Calbiochem and were of highest commercially available purity.

RESULTS AND DISCUSSION

(a) Weight of the tissue

It has previously been shown that the interscapular pad represents up to 50% of all the brown fat tissue in the rat during neonatal development and after cold acclimation (100, 32). Furthermore, BAT from this site is easiest to remove, a factor of some importance when up to 150 animals were used per experiment. Therefore, all our results are based only upon analysis of BAT interscapularly located, a point to be noted especially when comparing the results on brown fat weight presented here with those in the literature. However, ultrastructural observations on development of the dorsal cervical and interscapular brown fat (14, 15) have not shown any differences, so it seems very probable that our results are representative for rat BAT as a whole.

During development BAT changed not only in size but also in colour and therefore, the ease with which it could be distinguished from the surrounding white adipose tissue also changed. In newborn and in cold acclimated animals, the difference between the two types of adipose tissue was greatest and isolation of uncontamined pads of BAT was performed without any difficulty. In prenatal animals the tissue around the pad had not yet assumed the appearance of mature white fat and it was not easy to distinguish exactly the boundary between BAT and its surroundings. This difficulty was reflected in a relatively wide spread for the weight of the tissue at this stage. Between 3 days after birth and at least 30 days post-natally, there were increasing amounts of lipids accumulating within BAT.

As shown in Table I, the increase in weight of BAT was most rapid during the early stages investigated, being linear up to day 5 postnatally and then levelling off somewhat by day 30. The variation between the individual values for any postnatal stage were within 5% of the mean.

TABLE I—GENERAL CHANGES OF ISBAT DURING DEVELOPMENT AND AFTER COLD ACCLIMATION OF RATS, AND SAMPLE SIZES USED

Age (days)	Average body wt. (g)	Average ISBAT wt. (mg)	Ratio ISBAT wt./body wt. × 10^3	Protein in wet weight of ISBAT* (%)	No. of observations	Average No. of animals for 1 pooled sample
1 a.p.	5·2	30·0	5·77	9·55 ± 0·82	5–8	80
1 p.p.	6·0	47·1	7·85	10·5 ± 0·41	5–8	50
5 p.p.	11·7	74·3	6·35	11·2	5–8	30
17 p.p.	36·6	134·0	3·67	13·0 ± 0·31	5–8	20
30 p.p.	85·5	164·0	1·92	17·3 ± 2·48	4	12
Cold acclimated	242·0	697·0	2·88	20·3 ± 2·1	5	2

* $\bar{X} \pm$ S.E. ($s/\sqrt{n}$).

The mean body weights during development of the strain of rats used are also given in Table I as well as the ratios of BAT fresh weight/body weight. (This ratio has frequently been used as an indicator of the physiological importance of BAT - e.g. 162, 90). Unlike the growth of BAT, total body weight increased more rapidly between 17 and 30 days after birth; the ratio of these two parameters peaked at day 1 after birth and declined thereafter. In cold acclimated animals there was, however, a reversal of this trend.

From these results alone, BAT would appear to be physiologically most important during the earliest neonatal period and during prolonged exposure to cold, but such a conclusion would ignore possible alterations in tissue composition and activity during development.

(b) Gross composition.

We investigated changes in the gross composition of rat BAT some years ago (182, 73). The dry weight of the tissue increased most rapidly during the first ten days after birth and did not change thereafter. Water content decreased steadily during the first 30 postnatal days. The content of esterified fatty acids continued to increase during postnatal development, the most rapid increase occurring during the first ten days. The total tissue protein as the percentage of tissue fresh weight increased from about 10% at birth to about 20% by day 30 (Table I).

The glycogen content of BAT, immediately before birth and during the postnatal period is shown in Table II. The extremely rapid fall immediately after parturition is comparable to the findings in liver tissue (41). However, no increase was found in BAT during the first month of life, as was found in liver. Some increase occurred between the 30th and 50th postnatal day. The glycogen content of BAT in animals kept at 4 ± 1 °C for 20 days did not differ significantly from that in animals raised at room temperature. This finding is in agreement with that of Thompson et al. (213, 214) who observed some disappearance of glycogen granules only during the initial phase of cold acclimation in 6 - 9 week old rats.

Table II—Glycogen Content of Brown Fat during Development

Age (days)	−2	1	4	10	20	30	50
Glycogen (percentage of tissue wet weight)	1·880	0·092	0·150	0·140	0·100	0·120	0·280
S.E.	0·230	0·006	0·019	0·019	0·005	0·052	0·079

Each value represents the mean (±S.E.) of 5–10 samples. The negative value refers to days before birth.

DNA content of a tissue may be used as a simple criterion of tissue cellularity. The DNA content of BAT (Fig. 15) decreased throughout the whole period examined if expressed per unit of fresh weight. Bearing in mind the overall increase in protein content, this result may be interpreted as an indication of a constant number of adipocytes that accumulate mitochondrial and cytoplasmic protein and triglycerides during development. The RNA content of the BAT was examined (Fig. 15) and was found to be highest in the late fetal period and to decrease progressively thereafter. This may reflect a high protein synthetic activity in the late prenatal and perinatal period. The DNA and RNA content of BAT from fully cold acclimated animals (9 weeks old after 6 weeks of cold exposure) did not differ significantly from that of controls.

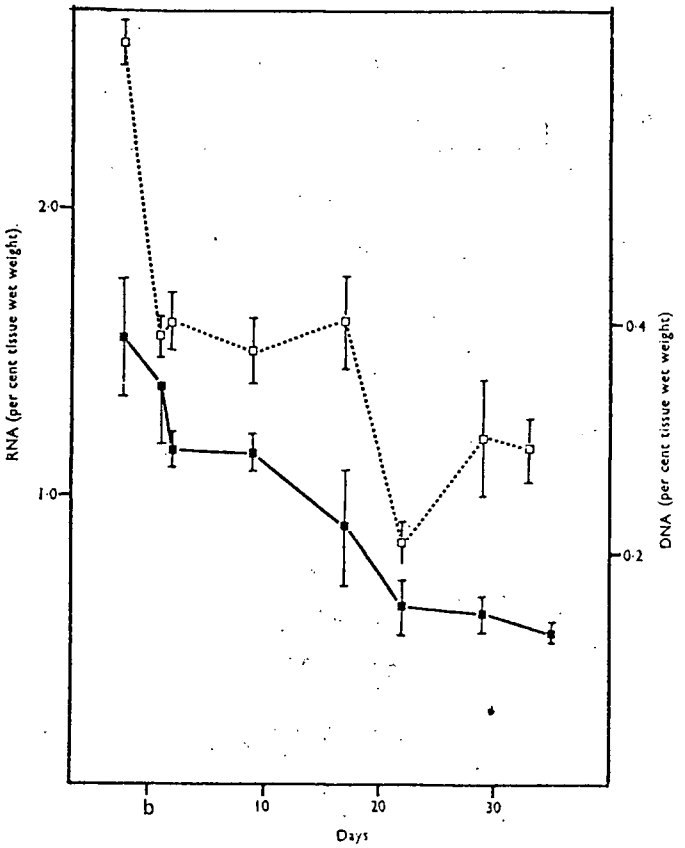


FIG. 15—RNA and DNA content of brown adipose tissue in rats of various ages. Values are expressed as percentages of tissue wet weight; ■, DNA; □, RNA. Vertical bars represent the S.E.M. of 6–8 samples.

(c) The significance of succinate dehydrogenase, alpha-glycerophosphate dehydrogenase and cytochrome oxidase activities for tissue respiratory activity.

Any study of ontogenic changes in a tissue is not complete until consideration has been given to its principal metabolic function. From thermodynamic data, it is clear that the bioenergetic basis of heat production is respiration. In heat production, the need is to bring about the maximum conversion of chemical energy into heat, whereas in most other circumstances, chemical energy is utilized for work with the highest efficiency possible (155, 156). From the studies of Prusiner et al. (157, 158), it is well established that the basis of catecholamine - induced thermogenesis in brown fat is mitochondrial oxidation of fatty acids. This process proceeds via a normal respiratory chain, not via specialized oxygenases or hydroxylases. Electron transport along the mitochondrial respiratory chain during maximal activity of BAT is not rate-limited by coupled phosphorylation (158). Hence, for this organ it seems valid to use the maximal activities of electron transport enzymes as indices of the theoretical maximal metabolic capacity of the tissue, as originally proposed by Jansky (98, 99). Therefore, the changes in the maximal activities of respiratory chain enzymes in brown adipose tissue during ontogenesis and after cold acclimation were used to indicate the period(s) during which the tissue can be functionally most active under stimulation.

Both succinate and alpha-glycerophosphate have been shown to be excellent substrates for respiration of isolated BAT mitochondria (91 and Z. Drahota, personal communication); the dehydrogenases of these compounds were chosen for assay. By a measurement of the enzymes as tetrazolium reductases using an artificial electron carrier PMS, the involvement of a

rate-limiting factor other than the amount and activity of the enzyme concerned was most probably eliminated (180). Cytochrome c oxidase (COX) has previously been used for calculations of the theoretical maximal contribution of BAT to total non-shivering thermogenesis (100). As with the succinate dehydrogenase (SDH) and alpha-glycerophosphate dehydrogenase (GPDH), the most active artificial electron carrier described yet for cytochrome c, i.e. TMPD, was used for the assay. The possibility of misinterpreting the results, for instance because of an extraneous rate-limiting step in the assay system, should be reduced by comparing the patterns of activities of the three different parts of the respiratory chain. If metabolic control of, say SDH, occurred, one might expect to see a noticeable difference between this enzyme and the other two. However, since the patterns of specific activity changes for all three enzymes were very similar, (see Table III), it seems that no such extraneous rate-limiting steps were present. Therefore, the results may be considered to represent the maximal possible respiratory activity of the tissue at each developmental stage. Of course, in an integrated system, it may be expected that other factors participate in the control of in vivo respiration; nevertheless the apparent maximal velocities of the three enzymes concerned might serve here as an indicator of the possible maximal activity that can be attained under maximal stimulation and as such, it is considered to be a good parameter for principal metabolic function of BAT.

In order to allow for comparisons with data from the literature on the three enzymes concerned, we have also measured the activities in

TABLE III--SPECIFIC ACTIVITIES OF SDH, α-GPD AND COX IN ISBAT HOMOGENATES

Age (days)	SDH (μmoles succinate oxidized/min per mg protein)	α-GPD (μmoles α-GP oxidized/min per mg protein)	COX (μatoms O_2/min per mg protein)
1 a.p.	0·123 ± 0·0059	0·072 ± 0·0016	0·374 ± 0·002
1 p.p.	0·142 ± 0·083	0·092 ± 0·0193	0·424 ± 0·010
5 p.p.	0·195 ± 0·0114	0·167 ± 0·0141	0·533 ± 0·008
17 p.p.	0·237 ± 0·0301	0·203 ± 0·0072	0·659 ± 0·012
30 p.p.	0·186	0·134	0·545 ± 0·003
Cold acclimated*	0·220 ± 0·0143	0·186 ± 0·0278	0·733 ± 0·027
Adult rat liver	0·155	0·020	0·222

*$\bar{X} \pm$ S.E. ($s/\sqrt{n}$).

homogenates of adult rat liver by our techniques. As can be seen in Table III, the specific activities of SDH, GPDH and COX were all considerably higher in homogenates of BAT from 17-day-old rats than in those from adult rat liver. During development of BAT, the patterns of changes in specific activities of each enzyme were very similar. A rapid increase between 1 day before birth and day 5 after birth was followed by a further slower increase up to the 17th day postnatally. By day 30, all activities had declined considerably. Some additional experiments on 40- and 90-day-old rats revealed even lower activities, thus indicating that the observed fall in activities between day 17 and day 30 was not a temporary one. After cold acclimation, the specific activities of all three enzymes were increased to about the same levels as were attained on the 17th postnatal day. Table IV shows the enzyme activities expressed per g of tissue fresh weight. On this basis, increases in enzyme activities between the late fetal stage and day 17 after birth were more pronounced and no decline in activity appeared by the 30th postnatal day. These differences are due to the continually increasing concentration of tissue protein over the whole period. From these results,

TABLE IV—ACTIVITIES OF SDH, α-GPD AND COX PER g ISBAT

Age (days)	SDH (μmoles succinate oxidized/min per g)	α-GPD (μmoles α-GP oxidized/min per g)	COX (μatoms O_2/min per g)
1 a.p.	11·8 ± 0·56	6·90 ± 0·15	35·7 ± 0·21
1 p.p.	14·9 ± 0·87	9·62 ± 2·03	44·5 ± 1·03
5 p.p.	21·8 ± 1·28	18·8 ± 1·58	59·7 ± 0·87
17 p.p.	32·0	23·1	94·1 ± 0·53
Cold acclimated	44·8 ± 2·91	37·9 ± 5·65	149 ± 5·50

$\bar{X} \pm$ S.E. ($s/\sqrt{n}$).

estimates of the maximal total activities in interscapular BAT of the three enzymes were calculated. This is shown in Table V.

TABLE V—CALCULATED TOTAL ACTIVITIES OF SDH, α-GPD AND COX IN ISBAT

Age (days)	SDH (μmoles succinate oxidized/min per ISBAT)	α-GPD (μmoles α-GP oxidized/min per ISBAT)	COX (μatoms O_2/min per ISBAT)
1 a.p.	0·353	0·205	1·07
1 p.p.	0·702	0·453	2·10
5 p.p.	1·62	1·39	4·44
17 p.p.	4·16	3·56	11·6
30 p.p.	5·24	3·78	15·4
Cold acclimated	31·0	26·4	104·0

Finally, we have related these total activities to body weights at each developmental stage (Fig. 16). The changes in these parameters, which can be called total maximal activities per unit of body weight, resembled once again the pattern of alterations of enzyme specific activities. In this case, the peak occurred at 5 days after birth, i.e. somewhat earlier than the peak in specific activities. Again, the values attained after cold acclimation resemble closely those at 5 days after birth.

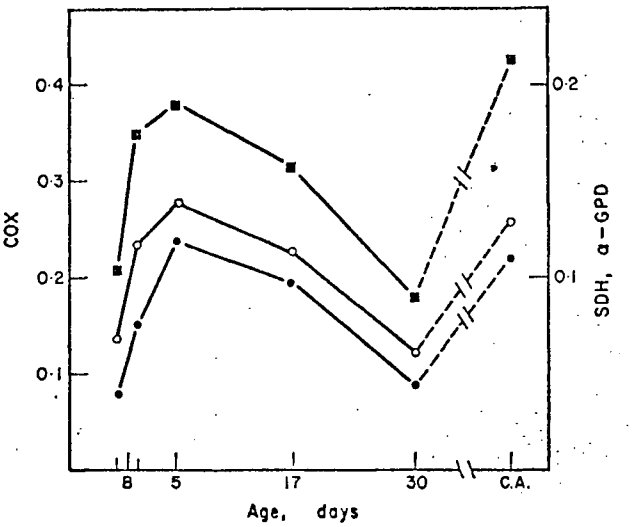


FIG. 16. Total respiratory capacity per body weight in ISBAT of some electron transport enzymes during development (B = birth) and after cold acclimation (C.A.). ■, COX (μatoms O_2/min per ISBAT per g body wt.); ○ SDH (μmoles succinate oxidized/min per ISBAT per g body wt.); ●, α-GPD (μmoles α-GP oxidized/min per ISBAT per g body wt.).

From these results it can be concluded that development of BAT involves not only an increase in tissue mass and alterations in tissue composition, but also changes in activity of some functionally important enzymes.

(d) Some aspects of catecholamine metabolism.

BAT has a profuse adrenergic innervation (for review see 38). It was suggested that the thermoregulatory function of BAT is controlled through its sympathetic innervation by release of norepinephrine (NE) (173). Himms-Hagen (88) reviewed the literature documenting the stimulation of lipolysis in BAT by catecholamines. Numerous experiments (for review see 158) support the conclusion that NE initiates thermogenesis in BAT. In addition, the sympathetic system seems to exert a trophic effect upon BAT (for detailed and thorough discussion see 173). Hence, catecholamines, more specifically NE are closely associated with the functional activity of

BAT. Since the functional activity of the tissue changes during development, catecholamine metabolism might also be expected to undergo developmental changes. We have observed (76) that the NE content of BAT drops dramatically during delivery and then increases rapidly. The capacity of BAT to bind ^{3}H-NE in vitro increased rapidly during the first ten days of postnatal life and decreased again by the 30th day. According to B. Cannon (personal communication) the in vitro stimulatory effect of NE on respiration of tissue slices from BAT was relatively low in newborn rats, reached 75% by day 10 and 100% by the 15th postnatal day. Hirvonen (89) studied histochemically monoamine oxidase activity in BAT in fetal, young and adult guinea pigs and found a very good correlation between the activity and the level of thermogenic activity. Monoamine oxidase (MAO) is involved in the metabolism of catecholamines (227, 63); moreover, it is most probably located in the outer mitochondrial membrane (171) and as such, it seemed to be an attractive parameter to be studied during development of BAT.

Fig. 17 summarizes our results on MAO activities in tissue homogenates and isolated mitochondria (for details of isolation procedure see Part II) of BAT during perinatal and early postnatal development and after cold acclimation. The values are about one-tenth of those observed in adult rat liver (2.19 ± 0.18 and 3.74 ± 0.13 μmoles iodoacetic acid formed per min. per mg of protein of homogenate and isolated mitochondria, respectively). Developmental changes were very similar to those observed for the respiratory enzymes. An exception was that the activity achieved after cold acclimation was 1.5 - 1.8 times as high as the maximum activities

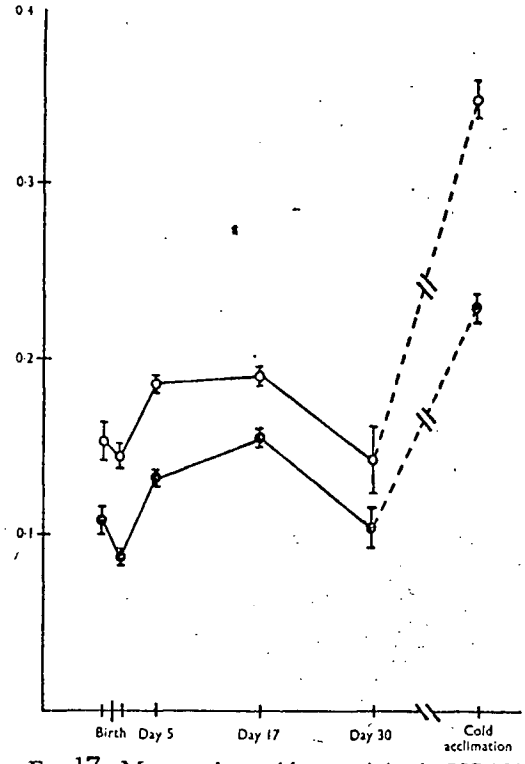


FIG. 17—Monoamine oxidase activity in ISBAT homogenate (O) and isolated mitochondria (O) during early development and after cold acclimation of rats. Activity is expressed as μmoles IAA (^{14}C) formed per minute per mg. of homogenate or mitochondrial protein. Vertical lines represent $\pm$S.E. ($s/\sqrt{n}$).

reached during early postnatal development. This seems to be related to the findings of Cottle et al. (37) of a much greater NE content and dramatically increased rate of turnover of NE in BAT from cold acclimated rats as compared with rats reared at room temperature.

(e) Some additional data.

In order to obtain developmental information on the glycolytic, gluconeogenetic, hexoso-monophosphate and fatty acid synthetizing pathways, additional studies on some enzymes involved in these pathways were undertaken. Only a summary will be presented here, details may be found in Hahn & Skala (83).

Malic enzyme (E. C. 1.1.1.40) and glucose-6-phosphate

dehydrogenase (E.C. 1.1.1.49) are considered to be important for NADPH supply for fatty acid synthesis. No change in malic enzyme activity was observed between the 1st and 30th postnatal day and a gradual decrease in glucose-6-phosphate dehydrogenase activity was found in BAT between day 1 and 40 postnatally.

Since glycerol kinase activity is low in brown fat (75), the main source of alpha-glycerophosphate for triglyceride formation is dihydroxyacetone phosphate which is converted into alpha glycerophosphate by cytoplasmic glycerophosphate dehydrogenase (E.C. 1.1.1.8). Activity of this enzyme was highest in the late fetal and neonatal periods and it decreased rapidly, reaching nearly adult low values by day 10. Activity was increased by cold acclimation.

Glyceraldehyde phosphate dehydrogenase (E.C. 1.2.1.12) activity showed a very similar but less pronounced postnatal decrease.

Phosphofructokinase (E.C. 2.7.1.11) as a key glycolytic enzyme increased in activity up to day 10 postnatally and decreased thereafter.

Pyruvate kinase (E.C. 2.7.1.40) increased in activity after birth, reached a peak by day 15 and declined considerably between the 20th and 50th postnatal day.

We can summarize that the key enzymes of glycolysis show a similar developmental pattern as the mitochondrial respiratory enzymes. Fatty acid synthesis seems to be most pronounced at early stages (as also revealed by direct measurements - 78) similarly to the rate of triglyceride

formation. The lower rate of fatty acid synthesis at the developmental stages when these compounds are assumed to be oxidized at a very high rate for heat generation seem to indicate that fatty acids utilized originate also from other sites than BAT itself (83). In accordance with this assumption, a high fat diet instituted for 24 hours in young rats resulted in significantly increased triglyceride content of BAT (Hahn, unpublished results).

The rate at which fatty acids are oxidized by BAT mitochondria is almost completely dependent on the presence of carnitine and ATP (47). In order to cross the inner mitochondrial membrane, fatty acids must form carnitine esters (60). At least two enzymes are involved in this process: carnitine acetyltransferase (E. C. 2. 3. 1. 7) and carnitine acyltransferase (E. C. 2. 3. 1. -) even though their exact function is not yet clear (82). In BAT, carnitine content increased rapidly after birth, reached a peak by day 10 and decreased to adulthood; similar pattern was observed for acylcarnitine, free carnitine and total acid soluble carnitine content (82). Age changes in the activity of carnitine acetyltransferase closely followed those of carnitine content (82). Hence also these parameters of fatty acid transport system show a striking similarity to the developmental changes of the tissue respiratory capacity.

CONCLUDING REMARKS

Jansky et al. (100) suggested that considerable functional changes occur in BAT and that the functional importance of this tissue varies during development. We have attempted to rationalize this assumption by analyzing a wide variety of parameters pertinent to the function of this tissue, i.e. heat production. Moreover, we have been able to compare these functional data with a detailed morphological study of the tissue. All the available evidence seems to form a clear ontogenic pattern which can be represented by Fig. 16: BAT proliferates and differentiates rapidly during perinatal development in order to achieve a very high degree of respiratory capacity. The tissue seems to be at a peak level of its ability to carry out heat production already by the 5th postnatal day and continues to keep this highest capacity until the third week of postnatal life. After this stage, all the parameters seem to decline, indicating a gradual loss of the tissue functional capacity simultaneous with morphological deterioration. This second phase of BAT development is reversed if the animal is exposed to low ambient temperature. It seems from Fig. 16 that the same functional capacity of BAT as related to body weight is evoked after cold acclimation and during early postnatal life, in spite of obviously large differences between the thermoregulatory status of rats under these two conditions. This fact, together with the observation of similar specific activities of the respiratory enzymes, may indicate that there is a limit up to which the respiratory capacity of the tissue may be induced by functional demands.

Hence, we have established the developmental pattern of BAT

and can now investigate whether this pattern correlates with the overall development of homeothermy in the rat. To keep a system in a steady state at a constant temperature, the changes in heat loss must be balanced by a corresponding change in the heat production and vice versa. Obviously, the heat loss is greatest prior to the maturation of heat insulation, i.e. during the first two weeks of life in the rat. Vascular regulation of heat flow is poorly developed in the first week and movement as well as shivering thermogenesis are limited. Nevertheless, in spite of early suggestions that newborn homeotherms are like poikilotherms in their response to lower ambient temperature, it seems that they are able to control their body temperature with increasing effectiveness during the first few postnatal days (72). This fact must be related in some way to their ability to produce heat very efficiently by non-shivering thermogenesis since a considerable number of other processes involved in thermoregulation are not yet developed. The mother is an additional heat source for the newborn rat but the intervals of her absence in the nest increase in a few days after delivery, thus leaving the infant animals to cope with the drop in ambient temperature. Their ability to resist a fall in the environmental temperature seems to develop rapidly during the first days of life (72), which bears a striking similarity to the observed development of BAT and its functional capacity, as opposed by a much later maturation of other means of heat production.

We can conclude, that the development of BAT and its functional capacity corresponds exactly with the stages of the greatest demand for non-shivering heat production in the rat. By the same token,

once the whole thermoregulatory mechanism matures and the need for non-shivering thermogenesis gradually wanes, BAT responds by a decline in its thermogenetic capacity. It is interesting to note that this developmental pattern of BAT does not apply for all mammalian species. Again, in each species analyzed, BAT development seems to follow closely the changing demands for non-shivering generation of heat (for more detailed discussion see Barnard & Skala - 17).

SUMMARY

1. The ratio of fresh BAT weight per body weight peaked 1 day after birth and declined thereafter. Cold acclimation reversed this decline.
2. The protein content of BAT increased sharply at early stages of development and continued to rise slowly up to day 30. The highest protein content found was in BAT of 90-day-old rats exposed to cold for 8 weeks.
3. The glycogen content of the tissue fell off sharply immediately after birth and stayed very low for the first month of life. No change after cold acclimation was noted.
4. The DNA and RNA content of BAT per fresh tissue weight was highest during the fetal life and declined steadily postnatally. No change after several weeks of cold exposure was observed.
5. Specific activities of SDH, GPDH and COX showed similar ontogenic patterns. A rapid increase between late fetal stage and day 5 postnatally was followed by a slower rise up to the 17th day; then activities declined. After cold acclimation, the activities increased up to the same values as were maximal during early postnatal development.
6. Total respiratory capacity of each enzyme in interscapular BAT as related to body weight was calculated and it showed a rapid increase up to the 5th postnatal day. Pronounced decline was observed between day 17 and 30. Cold acclimation returned these parameters to the values reached at day 5.
7. MAO activity increased between day 1 and 17 postnatally and later

declined. Activity reached after cold acclimation was nearly twice as high as that on day 17.

8. Some glycolytic enzymes and some parameters of fatty acid transport system showed similar ontogenic pattern as the respiratory enzymes described above.

9. Some indirect evidence presented here supports previous observations that the fatty acid and triglyceride synthetizing activity of the tissue is highest perinatally and declines during postnatal life.

The following developmental pattern for BAT in the rat was derived from the above data as well as from morphological observations: The tissue starts a very rapid rate of proliferation and differentiation perinatally and reaches the peak of its functional capacity as soon as day 5 after birth. Between day 5 and day 17, it is fully active and already by the fourth week of life, both morphological and functional involution commences. This involution process continuing indefinitely at neutral ambient temperature, can be reversed by exposing the animals to cold for prolonged periods of time (6 - 8 weeks seems to be adequate to restore once again the full activity of BAT). Both the functional and morphological developmental patterns seem to fit precisely into the overall development of thermoregulation in the rat.

PART II

DEVELOPMENT OF MITOCHONDRIA IN BROWN ADIPOSE TISSUE

(Based on the following publications:

185, 186, 187)

INTRODUCTION

One of the best recognized structural characteristics of BAT is its abundance of mitochondria, tightly packed with inner membranes (141). The enzymes (SDH, GPDH and COX) used as parameters of tissue functional capacity in Part I are known to be localized entirely within the mitochondria (for review see 166). In fact they are found only within the inner membrane (199, 146). The specific activities of COX and SDH in isolated mitochondria have been used as a measure of developmental changes in mitochondrial inner membrane concentration (66, 70, 183, 184). Thus it seemed possible that the changes of specific activities of BAT respiratory enzymes, as reported in Part I, might represent an increase in the amount of the mitochondrial inner membrane mass as opposed to altered enzyme activities. Such a possibility was strengthened by ultrastructural observations on BAT mitochondria during prenatal and postnatal development of the rat (see Introduction in Part I). There was clearly an increase in the area of inner membrane per mitochondrion as well as an increase in the total number of mitochondria in the tissue during the last three days of prenatal life. Other changes observed were an increase in the mean mitochondrial volume perinatally and also the appearance and disappearance of numerous, unusually large intramitochondrial dense granules (15, 204, 205). In order to interpret more fully the changes of mitochondrial enzyme activities and some of the observed ultrastructural alterations, we investigated mitochondrial fractions isolated from BAT of rats of different well-defined ages and also of rats fully cold-acclimated.

There was another reason behind undertaking a detailed study of mitochondrial development in BAT. The interest in mitochondriogenesis has increased rapidly in the past decade. Most of the work has been done on protozoa and fungi (for review see 159). The available data on the ontogenic development of mitochondria in animal tissues are sparse (for review see 81), yet it may be safely concluded that mitochondria undergo differentiation in their biochemical activities accompanied by structural maturation. The process of maturation consists of an increase in the number and size of fully developed mitochondria and of an increase in the concentration of the inner membrane and of structural respiratory chain enzymes within the mitochondrial cristae. In mammals, the rate of mitochondrial differentiation and maturation and the period of development at which this occurs seem to depend not only on the species but also on the tissue. In the liver, for example, the rate of maturation is slow and occurs prenatally and only shortly after birth (203, 45). In heart muscle, more pronounced changes are spread over a longer period of time (184, 118). Since the respiratory activity of BAT showed relatively very rapid changes, there was a possibility that mitochondria in this tissue may undergo a rapid and pronounced maturation and as such to be a good and flexible model for study of mitochondriogenesis in a mammalian tissue.

METHODS

Isolation of mitochondria.

Two methods for isolation of BAT mitochondria are in general use. One procedure uses a preliminary high-speed centrifugation of tissue homogenate to separate fat as a cake overlying the supernatant, with most of the particulate material as a pellet. After removal of the fat layer, the pellet and supernatant are rehomogenized and recentrifuged at increasing g-force in order to separate the subcellular fractions (162). Another method is to filter the nuclear fraction supernatant through medical gauze and also to remove carefully fat adhering to the walls of the centrifuge tubes after each centrifugation (Drahota, personal communication). We have compared both the methods for purity of the rewashed mitochondrial fractions and for the yield of isolated mitochondria and found the yield to be higher in the latter procedure (presumably due to the repeated homogenization required in the former method) and the purity of the fractions to be the same. Therefore, we used the filtration removal of fat routinely. The nuclear supernatant was prepared by centrifugation of the tissue homogenate in 0.25 M sucrose (1:10, w/v) at 650 x g for 10 minutes and rewashing the nuclear pellet by rehomogenization and recentrifugation. The combined supernatants were filtered and centrifuged at 8 500 x g for 10 minutes. The mitochondrial pellet was then re-suspended in the same medium and re-centrifuged several times. The final pellet was suspended in 0.25 M sucrose at appropriate dilutions for the various enzyme assays. The entire isolation procedure was performed at 1 - 4°C.

The purity of mitochondrial fractions was checked by measurement of recoveries in the mitochondrial pellets of acid phosphatase and glucose-6-phosphatase activities. Mitochondrial pellets from all age groups were also examined by electron microscopy.

SDH, GPDH and COX activities

were assayed as described in Part I.

Acid phosphatase

(orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.2) was measured by the method of Appelmans & DeDuve (4).

Glucose-6-phosphatase

(D-glucose-6-phosphate phosphohydrolase, E.C. 3.1.3.9) was assayed according to DeDuve et al. (43).

Cytochrome and flavoprotein concentrations

were determined from the difference spectra of oxidized against dithionite-reduced mitochondrial suspensions, at concentrations between 2 and 3 mg of mitochondrial protein per ml (33). The mitochondria were isolated in 0.25 M sucrose containing 5 mM EDTA. The oxidized mitochondrial suspension was prepared by adding malonate (50 mM) and rotenone (1 mM) and perfusing the suspension with oxygen for 3 - 5 minutes. The amount of dithionite added in order to prepare the reduced mitochondrial suspension was at least 25 times in excess of the required reduction dose. Suspensions were then solubilized by addition of sodium deoxycholate as a 10% solution to concentrations not exceeding 2 mg of deoxycholate per mg of mitochondrial protein. The absorption spectrum was recorded using a

Hitachi-Perkin-Elmer model 124 spectrophotometer equipped with a model 165 recorder. A linear relationship between the amount of protein and concentrations of all the cytochromes was found in the range of 1 - 5 mg of mitochondrial protein/ml of suspension. Wavelengths and extinction coefficients used in the calculations are shown in Table VI.

TABLE VI–WAVELENGTHS AND EXTINCTION COEFFICIENTS USED TO CALCULATE CONCENTRATIONS OF CYTOCHROMES AND FLAVOPROTEINS

Enzyme	Wavelength (mμ)	Ext. coeff. (cm^2/mol.)
Cytochromes-$a+a_3$	605–625	$24{\cdot}0 \times 10^6$*
Cytochrome-b	562–575	$20{\cdot}0 \times 10^6$
Cytochrome-$c+c_1$	551–540	$19{\cdot}1 \times 10^6$
Flavoproteins	500–460	$11{\cdot}5 \times 10^6$

* According to Van Gelder (1966); others according to Estabrook & Holowinsky (1961).

Phosphorus-containing compounds were separated into three fractions.

(a) Acid-soluble phosphorus, by extracting 2.0 ml of mitochondrial suspension (4 - 6 mg of mitochondrial protein) with 2 volumes of 20%, ice-cold trichloracetic acid. After centrifugation to remove the precipitate, the P content was determined.

(b) Phospholipid phosphorus. The pellet from (a) was first washed once with 2.0 ml of 5% TCA and then extracted for 3 hours at room temperature using 4.0 ml of chloroform-methanol (2:1, v/v). The organic phase was purified by thorough mixing with an equal volume of 0.05 M $MgCl_2$. After centrifugation

of the mixture, the aqueous phase was discarded and the remainder washed twice with 'saturated upper phase' (168). Care was taken not to remove material from the interphase pellet. The P content of the organic phase was then determined.

(c) Residual phosphorus was measured as the P content of the interphase pellet obtained from the last wash. The P lost in the washing fluids was negligible.

Phospholipid composition

of mitochondria was determined on phospholipid extracts made and purified as described by Rouser & Fleisher (168). In preliminary experiments, phospholipids (PL) were separated by two-dimensional chromatography on silica gel "H". These results did not demonstrate any PL unusual for mammalian mitochondria. Hence PL were routinely separated by one-dimensional chromatography on silica gel "H" using chloroform-methanol-acetic acid-water (25:15:4:2 v/v) as a developing mixture (195). For separation of phosphatidic acid from diphosphatidylglycerol extracts of mitochondria were run on silica gel "H" made up with 0.25 M oxalic acid using chloroform-methanol-hydrochloric acid (87:13:0.5 v/v) as the developing mixture (154). Spots were visualized with iodine vapour and identified by R_f values, presented in Table IX. Phosphorus content of the spots was expressed as percentage of the total recovered P (168).

Phosphorus was determined, after combustion of all samples with 70% perchloric acid according to Fiske & Subbarow (55).

Protein

was determined by the method of Lowry et al. (130).

RESULTS AND DISCUSSION

(a) The purity of mitochondrial fractions.

Glucose-phosphatese and acid phosphatase activities as markers of, respectively, endoplasmic reticulum and lysozymes in preparations from several age-groups were used. Specific activity of glucose-6-phosphatase in BAT homogenates was found to be less than one-tenth of that in adult rat liver homogenates (at such a low level of activity a possibility of nonspecific phosphatases as a part of the measured activity has to be considered). Nevertheless, less than 10% of the total activity was recovered in the mitochondrial fraction and repeated washing of the pellet lowered the value even more. Specific activities of acid phosphatase in BAT homogenates were 65 - 80% lower than those of adult rat liver and approximately 30% of the total activity was recovered in the mitochondrial pellet. Lysozomal contamination is considered to be most difficult to avoid in the mitochondrial fraction and this fact is reflected in the relatively high percentage of total lysozymes recovered in our mitochondrial fractions. On the other hand, Barnard (14) showed that the absolute amount of lysozymes in BAT is very low and this was reflected by the very low total activities of acid phosphatase observed in BAT as compared with liver tissue. In such a situation, the 30% recovery actually means a very low total amount of lysozymes contaminating the mitochondrial pellet from BAT. No difference in contamination of the mitochondrial pellets by the two enzymes was observed between the different age groups studied.

Mitochondrial preparations from all the developmental stages

were examined by electron microscopy. These results showed that only insignificant level of contamination by other structures was present in all age groups. Higher magnification confirmed that the mitochondria were not damaged during isolation. The intramitochondrial dense granules seen in tissue slices (15, 204, 205) were also clearly visible and unchanged in the isolated mitochondrial fractions. The prominence of the granules clearly decreased during the later stages of development.

We may therefore, conclude that the isolation procedure routinely employed was satisfactory for both purity and yield of undamaged mitochondria and that the quality of mitochondrial fractions did not change with age, a fact of importance if any meaningful comparisons between differently aged preparations are to be made.

(b) Amount of mitochondrial protein.

The percentage of total homogenate protein recovered in the mitochondrial fractions was determined in every experiment. We checked the yield of mitochondria for variations from stage to stage by calculating the ratios between total COX activities in the whole homogenates and total COX activities recovered in the mitochondrial fractions (Table VII). The results show that the yield decreased with age. In addition, from the values of total homogenate protein, protein recovered in the mitochondrial fractions and the yield of COX activity, we were able to calculate the amount of mitochondrial protein (as the percentage of the total tissue protein) in tissue homogenates before the isolation procedure (Table VII). The only comparable data in the literature are from cold acclimated animals; our result of 34%

Table VII - Mitochondrial protein during ontogenesis of ISBAT

Age (days)	Average yield of mitochondria %	Calculated mitochondrial protein in total homogenate protein %
1 a.p.	49	21·6
1 p.p.	47	27·2
17 p.p.	42	32·5
30 p.p.	40	35·0
Cold acclimated	40	34·0

of mitochondrial protein is in good agreement with findings of Roberts & Smith (162). The total mitochondrial protein as the percentage of the total tissue protein increases markedly during perinatal development. This may be taken as an indication of rapid de novo synthesis of mitochondrial protein since the possibility that other tissue proteins reduce in amount during this period is contradictory to all our observations.

(c) Activities of SDH, GPDH and COX.

The specific activities of SDH, GPDH and COX in isolated mitochondrial fractions during BAT development are shown in Fig. 18. For reference also, the activities in isolated liver mitochondria are given. All three activities were lower in liver mitochondria with the greatest difference in GPDH activity, which is in good agreement with results reported by Ohkawa et al. (147). The developmental patterns for all three enzymes in BAT were strikingly similar. A rapid increase up to day 5 after birth was followed by a decrease, mostly occurring between day 17 and day 30. SDH activity was even lower at 90 days of age, thus indicating a continuous decrease with age. After cold acclimation, the specific activities of all three enzymes were increased to approximately the same levels as observed

on the 5th day after birth.

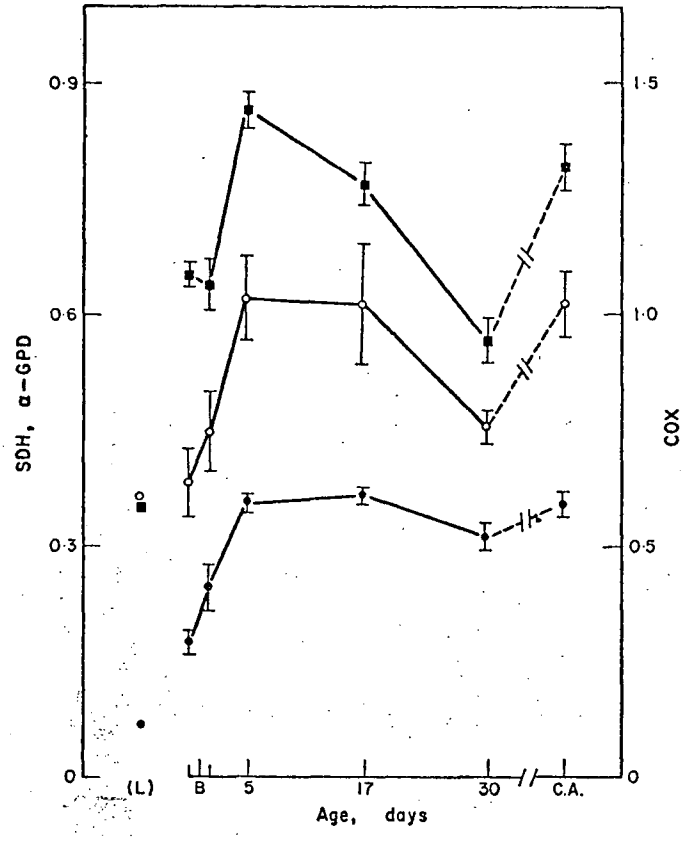


FIG. 18. Specific activities of some electron transport enzymes in isolated mitochondria of ISBAT during ontogenesis (B= birth) and after cold acclimation (C.A.) of rats. Average values (three experiments) for mitochondria isolated from adult rat liver are also shown (L). ◻, Cytochrome-*c* oxidase (μatoms O_2/min per mg protein); ○, succinate dehydrogenase (μmoles succinate oxidized/min per mg protein); ●, mitochondrial α-GPD (μmoles α-GP oxidized/min per mg protein). Vertical lines represent ± S.E. ($s/\sqrt{n}$).

(d) Concentrations of some respiratory chain components.

The concentrations of cytochromes a + a_3, b and c + c_1 as well as of total flavoproteins were determined spectrophotometrically. The patterns of concentration changes (Fig. 19) showed remarkable similarity to those observed in the enzyme specific activities. Again, increases were found during early development and peaks of the concentrations occurred on day 5 after birth. Then they decreased somewhat by the 30th day. Cold

acclimation stimulated a recovery of enzyme concentrations to similar high values as observed on day 5. For comparison, the concentrations observed on adult rat liver mitochondria are also given and it can be seen that they were considerably lower than those in BAT mitochondria.

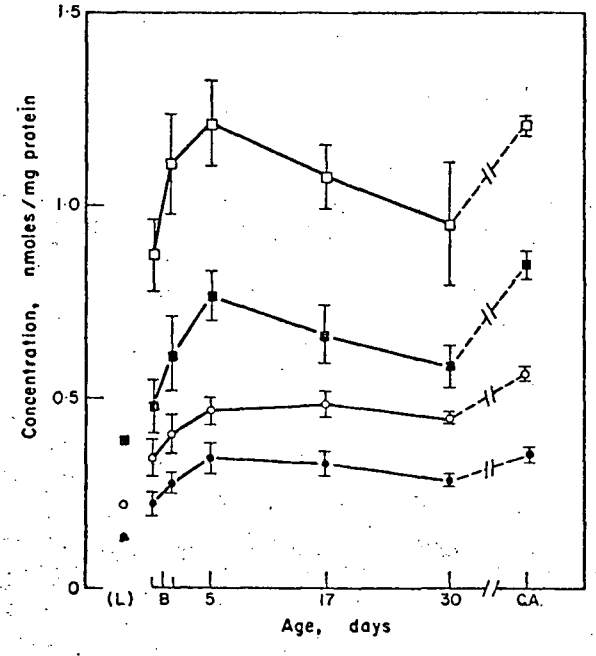


FIG. 19. Concentrations of cytochromes and flavoproteins in isolated mitochondria of interscapular brown adipose tissue during ontogenesis (B= birth) and after cold acclimation (C.A.) of rats. Concentrations are expressed as nmoles/mg protein. Average values (three experiments) for mitochondria isolated from adult rat liver are also shown (L). ●, Cytochromes-$a+a_3$; ○, cytochrome-b; ■, cytochromes-$c+c_1$; □, flavoproteins. Vertical lines represent ± S.E. ($s/\sqrt{n}$).

(e) Phospholipids.

Phospholipids are another component of mitochondrial membranes and a positive correlation between concentrations of electron transport enzymes and PL has been noted (42, 56). As shown in Table VIII, the total P concentration in BAT mitochondria was found to be about 1.5 times higher than in adult rat liver mitochondria. From 1 day before birth up to 17 days postnatally, no changes were observed in the total P/mg protein.

Then there was a 30% decrease by day 30. After cold acclimation, the total P concentration returned to the same level as during early ontogenesis.

Table VIII—Concentrations of phosphorus in isolated mitochondria of ISBAT during ontogenesis and after cold acclimation of rats

Age (Days)	Fraction (μg P/mg mitochondrial protein)			
	Total	Acid-soluble	Chloroform–methanol-soluble	Residual
1 a.p.	14·8 ± 0·65	3·15 ± 0·30	8·63 ± 0·39	2·21 ± 0·16
1 p.p.	13·2 ± 1·09	2·73 ± 0·38	8·34 ± 0·54	2·31 ± 0·45
5 p.p.	14·5 ± 1·06	3·56 ± 0·37	7·97 ± 0·41	1·94 ± 0·45
17 p.p.	13·1 ± 0·60	3·05 ± 0·31	8·43 ± 0·75	1·59
30 p.p.	10·4 ± 0·79	2·64 ± 0·42	5·66 ± 0·46	1·91 ± 0·17
Cold acclimation	14·2 ± 0·67	2·95 ± 0·37	9·72 ± 0·70	1·83 ± 0·25
Adult liver mitochondria	9·28	3·38	4·58	1·06

Results (means ± S.E.) are expressed as μg phosphorus/mg protein. Values for mitochondria isolated from adult rat liver are also given.

Table VIII also contains the distribution of P between acid-soluble, chloroform-methanol-soluble (i.e. PL) and residual fractions. The difference between BAT mitochondria and liver mitochondria is localized mainly in the PL fraction. Moreover, the PL are also responsible for the decrease in the total P noted after day 17 in BAT.

As can be seen from Table IX, BAT mitochondria contain the same classes of PL in the same proportions as beef heart mitochondria (most of the PL studies were done with beef heart mitochondria and therefore, these were chosen for comparison in this case). We have observed some small changes in PL composition between BAT mitochondria from rats 1 day before delivery and 1 day after delivery. While diphosphatidyl glycerol decreased in this period by 16%, phosphatidyl choline increased by the same

percentage ($p < 0.05$). On the other hand, no changes were detected in phosphatidyl ethanolamin, phosphatidyl inositol and lysophosphatidyl choline.

TABLE IX–R_f VALUES OF, AND PERCENTAGE OF TOTAL PHOSPHORUS RECOVERED IN, PHOSPHOLIPID SPOTS SEPARATED BY ONE-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY (METHOD OF SKIPSKI *et al.*, 1964)

Age (Days)		Spot 1	2	3	4	5	Remainder
1 a.p.	R_f	0·96	0·66	0·47	0·28	0·09	—
	%P	19·2 ± 0·54	33·1 ± 1·65	7·13 ± 0·49	30·5 ± 0·36	3·33 ± 0·65	5·96 ± 1·31
1 p.p.	R_f	0·96	0·59	0·42	0·24	0·06	—
	%P	16·1 ± 0·70	33·2 ± 0·84	6·34 ± 0·77	35·3 ± 0·68	4·78 ± 1·06	7·70 ± 1·97
HBHM	R_f	0·90	0·62	0·40	0·26	—	—
	%P	16	33	6	33	—	13

Means ± S.E. ($s/\sqrt{n}$). HBHM = heavy beef heart mitochondria (two experiments). The spots were identified as follows: 1, diphosphatidyl glycerol; 2, phosphatidyl ethanolamine; 3, phosphatidyl inositol; 4, phosphatidyl choline; 5, lysophosphatidyl choline.

(f) Interpretation.

1. Correlation between specific activities and concentrations of mitochondrial electron transport enzymes.

We measured electron transport enzymes both as specific activities and as concentrations. The relationship between these two groups of measurements can be defined with varying degrees of clarity. On the one hand, cytochrome c oxidase activity is essentially the activity of cytochrome a + a_3 (126); thus, in this case we followed both activity and amount of the same enzymic complex. On the other hand, succinate and alpha-glycerophosphate dehydrogenases are only two of several mitochondrial flavoproteins (181); therefore, specific activities of these two enzymes need not be closely related to the total flavoprotein concentration. Yet, in spite of this, specific

activities and flavoprotein concentrations showed similar differences between BAT and liver mitochondria. Furthermore, the developmental patterns of specific activities and of concentrations of total flavoproteins in BAT mitochondria showed close resemblances. Therefore, we feel justified in concluding that changes in specific activities of SDH, GPDH and COX (as measured using the very active artificial electron carriers PMS and TMPD under optimal conditions) predominantly reflected altered amounts rather than altered activities of these enzymes.

2. Correlation between changes in electron transport enzyme concentrations and changes in inner mitochondrial membrane area.

An approximate positive correlation has been observed to exist between concentrations of electron transport enzymes and content of inner membrane of mitochondria from different tissues (e.g. 111). If the developmental changes of enzyme concentrations are closely proportional to the changes of inner membrane area, then the concentration of these enzymes per unit membrane area should be stable during ontogenesis. Only in this case, for example, will an increase in concentration of electron transport enzymes indicate an enrichment of the mitochondria with inner membrane relative to other protein-containing mitochondrial components, i.e. outer membrane and matrix. There are some data in the literature supporting this proposition. Skala et al. (184) found that in the mitochondria of developing rat heart, the specific activity of SDH increased proportionally with the increase of the ratio of mitochondrial membrane protein to matrix protein. Greenfield and Boell (66) have found similar relationships for both

COX and SDH in developing chick heart, liver and skeletal muscle. The ratios between specific activities of these two enzymes and the particulate mitochondrial protein were constant for the ontogenic period studied. Furthermore, the ratio between COX and SDH specific activities was constant in all three tissues. About 90% of the particulate mitochondrial protein is derived from the inner mitochondrial membrane (186) - therefore, these results indicate that the amounts of COX and SDH are constant per unit area of inner mitochondrial membrane during development.

Our results have shown an increase of both specific activities and concentrations of some electron transport enzymes during early development of BAT. This finding has a structural parallel in the considerable enrichment of mitochondrial inner membrane during the same period as observed by Barnard & Lindberg (15) and calculated morphometrically (129). We may therefore, conclude that the increase in the amounts of electron transport enzymes are closely related to an increase in the inner mitochondrial membrane area.

3. Correlation between PL concentration and the membrane content of mitochondria.

PL are structural components of both mitochondrial membranes (e.g. 42). It has also been noted that the lipid content, more than 90% of which are PL, approximately parallels the concentrations of electron transport enzymes in mitochondria of beef liver, kidney and heart (56). However, in contrast to electron transport enzymes, PL are found not only in the inner, but also in the outer mitochondrial membrane, where, at least in

liver, they are more concentrated (127). Hence, a correlation between PL and electron transport enzymes should be close only in mitochondria where inner membrane is abundant relative to outer membrane. This is the case in BAT.

4. Comparison of BAT and liver mitochondria.

Electron microscopy has clearly shown that mitochondria from BAT of neonatal rats (e.g. 141) and of cold acclimated rats (e.g. 206) have much more abundant inner membrane than liver mitochondria (e.g. 148). We have shown that specific activities and concentrations of some electron transport enzymes as well as concentrations of PL were considerably higher in BAT mitochondria than those in liver mitochondria. In view of the argument advanced above (2 and 3), the observed differences were not surprising and they support the proposed relationship between concentrations of electron transport enzymes and the amount of inner mitochondrial membrane.

5. An interpretation of our results in the context of mitochondrial development in BAT.

It appears that mitochondrial development can be divided into two phases, similar to those described for the physiological development of interscapular BAT of the rat. The first extends from the late prenatal period and is completed by the 5th day after delivery; the second begins at around the third week of life and continues with increasing age.

(i) During the <u>first phase</u>, specific activities and concentrations of the enzymes studied rose markedly to maximal levels on day 5 after birth. From the arguments in (1) and (2) and from the morphological

studies (129) performed simultaneously with our experiments, these findings indicate a considerable rate of production of the inner membrane components during this period. However, the concentration of PL was already maximal at day 1 before birth and did not change up to 5 days of age. There is thus a discrepancy between the enrichment of mitochondria with inner membrane and unchanged PL concentration. This discrepancy may be used as an argument against the assumption of a stable ratio between concentration of PL and electron transport enzymes during mitochondrial development as advanced in (3). On the other hand, it is tempting to associate the relative abundance of PL over electron transport enzymes in prenatal mitochondria with the presence of numerous, unusually large intramitochondrial dense granules found in BAT at this stage of development. If these granules were made up of a unit membrane, abundant with PL but temporarily lacking the electron transport enzymes, then this may account for the discrepancy found for the whole mitochondria. Since the isolation of the granules and their chemical analysis was not feasible at the time of these studies, some indirect measurements were made in order to prove that the granules indeed lack the electron transport enzymes. Barnard et al. (18) stained the mitochondria from prenatal BAT for COX activity and found markedly lowered, if any, positive reaction in the dense granules. Since the membrane-like structure of these granules had been clearly demonstrated, one can then assume that PL are present in a large excess over electron transport enzymes (at least the active enzymes). This assumption is supported by findings of Haddock et al. (see 81) who showed relatively high PL/cytochrome

oxidase ratio in fetal liver mitochondria as compared with adult values.

The ultrastructural appearance of, and the changes in, the large intramitochondrial dense granules during a well defined proliferative period of BAT development suggested a possible function of these structures in mitochondriogenesis. Similar conclusions were made by Yotsuyanagi (226) for intramitochondrial granules, structurally the same as in BAT, found in Sacharomyces cereviciae during glucose derepression when a rapid proliferation of mitochondria occurs. Similar large intramitochondrial granules have been described in the literature in a number of situations; differentiating spermatocytes of H. pomantia (3), developing indirect flight muscle of honey bee (87), developing BAT of prenatal guinea pig (Rafael, personal communication), developing brown fat of prenatal rabbits (17), cancer (102, 124). A common feature of these situations seems to be a rapid increase in the amount of mitochondrial inner membrane at the time of and immediately after the appearance of granules. On the other hand, increases in amount of inner membrane have also been observed in the absence of abundant large granules, for example during cold acclimation (206), and during the development of heart mitochondria (169). The most obvious difference between these two groups of situations is that the amount of inner membrane increases more rapidly in the mitochondria possessing the granules. Therefore, we have suggested (see 17) that the large granules appear when the rate of synthesis of some inner membrane components exceeds their rate of incorporation into fully formed inner membrane. The granules are considered to consist of incompletely differentiated inner membrane, the

composition of which is at least quantitatively different from the fully formed cristae; for instance, the phospholipid bilayer has, as yet, not incorporated the structural components of the electron transport chain, resulting in the observed disproportion in the amounts of PL and respiratory enzymes.

(ii) During the second phase of the development, the specific activities and concentrations of some electron transport enzymes as well as of PL, decreased. In terms of arguments in (2) and (3), these changes may be caused by a decrease in the amount of inner membrane within the mitochondria. A second interpretation may be advanced, that these decreases result from a "dilution" of enzymatic proteins and PL by increased amounts of other mitochondrial proteins. This argumentation was impossible to disprove until complete morphometric measurements were made (129). These provided unequivocal evidence that an absolute decrease occurs in the area of inner mitochondrial membrane per mean mitochondrial volume at this period and thus confirmed our original interpretation.

(iii) Effect of cold acclimation on BAT mitochondria. Using the arguments from (2) and (3), our results may be interpreted as a considerable enrichment of inner mitochondrial membrane area in the BAT mitochondria during cold acclimation. Ultrastructural changes in mitochondria during cold adaptation have been described by other authors (206, 214). Suter made estimates (205) of the number of cristae per micron of mitochondrial diameter and found that this increased by 10% after 8 days and by 30% after 8 weeks of cold exposure. The mean mitochondrial diameter was increased from the 4th day through the 6th week by 20% over the value in the

controls (214). The morphometrical results additional to our study (129) confirmed that indeed the area of inner mitochondrial membrane is increased during cold acclimation. Thus cold acclimation, when commenced during the period of mitochondrial "involution" seems to reverse this process. Furthermore, our results of similar activities and concentrations attained after cold acclimation and at 5 days after birth, seem to indicate that this process restores inner membrane content to the same level as found during early postnatal development at room temperature.

On the other hand, Stratman & Hohorst (202) have used another model for studies of cold exposure of infant animals. They compared changes in mitochondrial structure and COX activity in BAT mitochondria of guinea pigs developing from birth either at 20°C or at 3°C. In newborn animals, mitochondria contained abundant closely packed cristae and had very high COX specific activity; this was also the case in the mitochondria from the experimental group reared for 42 postnatal days at 3°C. However, both the amount of cristae and COX activity had decreased considerably after 20 days of development at 20°C. From these experiments it seems that cold exposure commencing at the time of full activity and peak level of mitochondrial maturation (which in guinea pigs occurs at birth), does not increase the activity any further but protects this high level of differentiation against involution, which takes place during postnatal development at neutral environmental temperature. (see Part III).

(g) Monoamine oxidase activity and its significance for outer mitochondrial membrane changes.

Up to this point, only components of the inner mitochondrial

membrane were considered. MAO is an enzyme localized primarily in the mitochondria (39), more specifically, in the outer mitochondrial membrane (171). As such, it was studied during development of BAT in the isolated mitochondrial fractions (Fig. 17) as well as in the whole tissue homogenates. It was found that a considerable part of the activity found in tissue homogenates originates from other tissue components than adipocytes (see 185). Nevertheless, there was a considerable portion of the total tissue activity recovered in the rewashed mitochondrial fraction. The increases in the inner membrane protein may be expected to greatly exceed those of outer membrane protein because the inner membrane of BAT mitochondria forms more than 90% of the total membrane protein at all developmental stages (186, 17). One may therefore, expect the activity of outer membrane 'marker' to be 'diluted' by the increasing inner membrane protein during the first phase of development as described above. But our observations show that the specific activity of MAO increased during early development simultaneously with the increases of total mitochondrial protein and concentration of inner membrane. There are two possible interpretations - either MAO is not entirely localized within the outer mitochondrial membrane (which would support the generally rejected finding of Green et al. -65), or its activity rather than amount is changed. In any case, it indicates that in contrast to SDH, GPDH and COX, the specific activity of MAO cannot be used as measure for changes in outer mitochondrial membrane area. This conclusion was confirmed morphometrically (129). Similar arguments apply to our finding of considerably higher MAO specific activity in cold acclimated rats.

CONCLUDING REMARKS

An increase of inner membrane within the mitochondria in a variety of tissues and organisms has been described (e.g. 22, 28, 66, 70, 71, 81, 87, 183, 184). We found a similar phenomenon in BAT mitochondria during both early development and cold acclimation. The fact that the specific activities and concentrations of electron transport enzymes and concentrations of PL after complete cold acclimation attained the same levels as the maximum values observed during early postnatal development seems particularly interesting. From this it would appear that there is a limit to which the individual mitochondria can increase their content of inner membrane. In other words, the normal maturation and differentiation of mitochondria is not an unlimited process.

From our own data, we can only speculate about the mechanisms which could result in alterations in amount of inner membrane per mitochondrion. At least two possibilities can be considered. The first is that the individual mitochondrion remains the same between the different stages studied and that the amount of inner membrane increases by accentuated synthesis of the membrane material. Such an interpretation seems particularly probable in, for instance, skeletal muscle; there mitochondria are localized exactly in the Z lines and a significant part of the observed developmental enlargement of mitochondrial total volume results from an elongation of the individual mitochondrion (71). The second possibility is that the observed changes might be produced by an altered proportion between distinct mitochondrial populations, each having a constant, but

different amount of inner membrane. Obviously in tissues consisting of more than one predominant cell type, such an alteration could be achieved by a change in the proportion of cell populations. This explanation seems to be preferred by Rafael et al. (160). On the other hand our studies, including the observations of the dense granules and their changes, seem to favour the first possibility. Also, the radioautographic results of Hellman & Hellerstrom (86) showing relatively very slow cellular turnover in BAT of young rats seem to fit more into the first explanation. In any case, it can be concluded that BAT mitochondria and their developmental changes seem to provide a flexible model for studies of mitochondriogenesis in a mammalian tissue.

SUMMARY

1. The percentage of mitochondrial protein in total tissue protein increased in BAT with age up to day 30 with the most rapid increase during the first 5 days of life. The yield of isolated mitochondria decreased with age.
2. The rapid increase of specific activities of SDH, GPDH and COX and concentrations of cytochromes and flavoproteins up to day 5 postnatally was followed by subsequent decreases, pronounced between day 17 and 30.
3. PL concentration was already maximal before delivery and did not change until day 17, thereafter a decrease occurred by day 30. Phospholipid composition showed similarity to that of beef heart mitochondria; some proportional changes were noted perinatally.
4. The presence of large intramitochondrial dense granules in the perinatal period was confirmed and their appearance and changes were discussed in the context of mitochondrial compositional changes at that time.
5. After 8 weeks of cold exposure, the levels of mitochondrial parameters measured reached once again the maximal values as observed on day 5 postnatally.
6. The correspondence between compositional changes and ultrastructural observations on alterations of inner mitochondrial membrane is discussed in terms of mitochondrial maturation. BAT proved to be a convenient model for studies of mitochondriogenesis in a mammalian tissue.

7. MAO was studied in isolated mitochondria and was disregarded as a marker parameter for changes of outer mitochondrial membrane.
8. The study has shown that the previously described ontogenic pattern of BAT respiratory capacity can be related to changes in the mitochondria.

Part III

HORMONES AND REGULATION OF BROWN ADIPOSE TISSUE DEVELOPMENT

(Based on the following publications: 76, 187, 193)

INTRODUCTION

The results presented in Part I and II established the developmental pattern of BAT in the rat. What are the stimuli and what is the mechanism responsible for the observed developmental changes?

The examination of a system (in our case developing BAT) can be simplified by taking the available information as a function of the system in terms of its outputs and inputs. In our case, the 'output' is the developmental pattern of the tissue and the 'input' can be defined as the stimuli causing these changes. The system can then be defined as a mechanism by which the tissue responds to the 'input' to rearrange its structure and function. Obviously, the input, or the stimuli triggering the proliferative and differentiation processes in BAT, can be environmental temperature and its changes, or the genetic code acting directly or indirectly (via hormones, for example) during the course of development, or a combination of both. In the first part of our studies of the regulatory mechanism(s) governing development of BAT, we have restricted ourselves to the question of the nature of the 'input'. This will be presented in Part III, whereas the studies pertinent to the actual mechanism by which the tissue responds will be discussed in Part IV.

Regardless of whether external termperature or the genetic code act as the first stimuli, some 'messenger', connecting the stimuli with the adipocyte, might be expected. There were several indications in the literature that hormones may possess this role:

(a) Catecholamines, more specifically, norepinephrine, are res-

ponsible for functional stimulation of BAT (for reviews see 158, 161, 173).

(b) Suter (204) studied the ultrastructural development of BAT mitochondria and from the effect of NE and cyclic-AMP on the appearance of the intramitochondrial dense granules, suggested that catecholamines are somehow involved in the regulation of BAT mitochondrial development.

(c) Daily injections of NE can simulate the effect of cold exposure by increasing the potential for non-shivering thermogenesis in the rat (94).

(d) The literature on the effect of thyroid hormones on BAT is somewhat controversial. Some studies seem to favour only a minor participation of thyroid hormones in cold resistance (for review see 123). Even though there was an observation of BAT hypertrophy in experimental hyperthyroidism and after thyroxine administration in adult animals, this response is distinctly different from hypertrophy caused by cold acclimation (for detailed discussion see 197). On the other hand, it was observed that combined treatment with NE and thyroxine was more effective than NE alone in improving thermogenic capacity and resistance to cold; the beneficial effect of both these treatments was directly proportional to the size of interscapular BAT (122).

(e) Adrenal corticoids and ACTH were analyzed on the basis of an

earlier interest in the relationship between adrenal cortex and BAT in acute cold stress. Smith & Horwitz reviewed the literature (197) and concluded that there is no experimental evidence for a direct relationship except a possibility that corticoids increase the availability of substrate for thermogenesis. On the other hand, a single large dose of cortisone clearly affects BAT (76) and its mitochondria (187) in young rats.

(f) Clinically, there are some hormonal disorders that cause growth and de novo appearance of brown fat in between subcutaneous white adipose tissue in humans. Thus, pheochromocytoma may cause such a condition (53, 137, 176). A similar overgrowth of BAT was obtained when rabbits were injected with epinephrine for 21 days (176) clearly relating the growth of BAT to increased levels of serum catecholamines in pheochromocytoma. Addison's disease (insufficient production of adrenocortical hormones) has also been claimed to be associated with growth of brown fat (57, 95).

We are aware of the limitations of the in vivo approach, involving as it does a combination of many unknown and uncontrolled factors, which makes it difficult to specify exactly the role of any single stimulus. Nevertheless, before approaching the questions of the sequence and mechanism(s) of control of BAT ontogeny by other means, we attempted to demonstrate that a hormonal mechanism may indeed be involved. Hence, we studied the

effects of environmental temperature, together with the administration of several hormones, on BAT in developing rats.

METHODS

Prenatal administration of hormones.

Albino Wistar rats with timed (± 12 hours) pregnancies were used. Nevertheless, the only reliable parameter of the age of fetuses was their body weight, obviously not available at the time of injections. Thus, it was sometimes necessary to repeat the experiment several times before the desired final age of fetuses was obtained. The pregnant rat was anaesthetized with ether and laparotomy was performed under sterile conditions. Fetuses were injected through the uterine wall (67, 68). Care was taken to inject them intraperitoneally through the lateral part of their backs in order to avoid outflow of the hormone from the peritoneal cavity after syringe removal. If visible hemorrhage occurred, the experiment was terminated. After the injections, the abdominal wall of the mother animal was sutured in three layers and care taken to prevent infection. The mortality of the fetuses was approximately 10% if pregnancy was ended by section. If spontaneous delivery was allowed, a relatively high percentage of newborns were killed by the mother animals.

Warm and cold exposure of newborn animals.

Warm exposed animals were delivered at 30 - 32°C in a ventilated baby incubator with controlled humidity and kept in the incubator together with the mother animal for the desired period of time. It was impossible to use any higher temperature since 30 - 32°C was the highest temperature the mother animals could cope with in an apparently healthy state. The weight gains of infant animals at the above temperature did not

differ significantly during the first five postnatal days from those of animals kept at room temperature.

Cold exposure of infant rats was performed in the following way (113): Immediately after delivery, the cage in which the newborns and the mother were housed was transferred into 5 - 6°C for 30 minutes, followed by 30 minutes at room temperature. Later, the intervals of cold and room temperature were increased to 60 minutes each. Five sixty-minute intervals in the cold were routinely used per day for the desired number of days.

Cold exposure of older rats.

In contrast to the technique used for cold adaptation in Part I and II, the cage housing 8 fifteen-day-old animals, together with their mother, was placed into a cold room (4 - 6°C) for the desired number of days (generally 2 - 3 weeks, i.e. much less than used in Part I and II where rats of more advanced initial age were used).

Postnatal administration of hormones.

Hormones were injected intraperitoneally in approx. 0.05 ml of sterile saline. Care was taken to prevent outflow of the hormone by injecting through a solid muscle mass. The injections were performed at exactly 24 hour intervals.

Removal of the tissue, material preparation and enzyme assays.

All the procedures and assays used were described in Parts I and II.

RESULTS AND DISCUSSION

Our studies were divided into two parts. In the first, we attempted to influence the first phase of BAT development, i.e. the stage of proliferation and differentiation which commences prenatally and extends through the second week of life. In the second part, we aimed our experiments at the second phase of BAT development, i.e. the 'involutive' period commencing in the third week and continuing at least up to day 30.

(a) The effect of hormones and of temperature on the first phase of BAT development.

In the first group of experiments presented in Table X, we injected fetuses in one uterine horn with a hormone (see 67, 68, 69) and used the fetuses from the other horn as controls in order to minimize biological variation. This approach obviously resulted in scarcity of the material and it was therefore impossible to analyze all the parameters as described in Part I and II; no isolation of mitochondria was attempted and only some homogenate activities were measured. As shown in part A of Table X, the injection of saline resulted in no significant difference of the studied parameters 56 hours later, when pregnancy was terminated by section. Injection of epinephrine on the 19th gestational day had no appreciable effect 28 hours later. Similar injections of NE showed rather inconsistent effects; in some experiments (as shown in part C) it seemed to increase SDH and GPDH activities, in other experiments (as illustrated in Part D), the effect was even the opposite. Hence, epinephrine and NE administration in dosages used had no appreciable effect one day later if the

TABLE X.

The Effect of Prenatal Catecholamine Injections on Brown Adipose Tissue 20 to 28 Hours Later

No. of Mothers	A		B		C		D		E		
	1		1		2		2		2		
	No Injec.	Saline 0.05 ml	No Injec.	Epin. 4µg/anim.	No Injec.	NE 4µg/anim.	Saline 0.05 ml	NE 5µg/anim.	No Injec.	Saline 0.05 ml	Epin. 4µg/anim.
Fetuses: Died		1		2							
Survived	2	5	4	5	4;6	6;7	7;6	6;7	7	3	7
Interval(Hrs.)*	56	56	28	28	20	20	23	23	22	22	22
Body Weight(g)	6.15	5.32±0.15	4.32±0.26	4.28±0.14	4.11±0.09 3.67±0.16	4.16±0.18 4.05±0.15	4.43±0.12 3.32±0.14	4.43±0.19 3.45±0.18	4.28±0.15	5.55	4.44±0.17
Protein in BAT (% Fresh Wt.)	9.3	11.6	8.3	8.5					10.6	10.4	12.2
Homogenate Activities											
SDH**	117±6	100±4	85±4	95±6	68±3	73±3	53±3	45±3	54±4	66±6	72±7
GPDH**	66±3	61±4	73±3	64±5	43±3	54±2	39±2	34±2			

Each value represents the mean ± S.E. In some cases only the mean of 2 to 3 determinations on pooled samples is given.

*Time elapsed between hormone injection and the termination of pregnancy by section.

**mµmoles/min./mg. prot.

T A B L E XI

The Effect of Prenatal Injection of Hormones on Brown Adipose Tissue One Day After Spontaneous Delivery

	Saline (10) 0.05 ml	Thyroxine (10) 3.0µg/anim.	Cortisone (10) 0.1mg/anim.	NE (11) 3.0µg/anim.
Body Weight(g)	6.98±0.31	5.44±0.12	5.51±0.23	6.02±0.22
BAT Weight(mg)	-	39.1 ±2.55	32.0 ±2.85	46.82±2.27
Weight Ratio BAT/Body x 10^3	-	7.13±0.42	5.80±0.36	7.75±0.25
BAT Protein (%fresh weight)	10.7	11.2	11.7	10.7
Yield of Isolated Mitochondria	36%	38%	26%	-
Homogenate Activities				
GPDH*	0.087±0.004	0.053±0.003 (1)	0.066±0.004 (2)	0.130±0.008 (1)
COX**	0.408±0.025	0.259±0.046 (2)	0.399±0.055	0.518±0.018 (1)
Mitochondrial Activities				
GPDH*	0.256±0.006	0.163±0.011 (1)	0.143±0.004 (1)	0.360±0.015 (1)
COX**	1.21 ±0.08	1.28 ±0.04	1.11 ±0.10	1.69 ±0.07 (1)

Number of animals used in each group is given in brackets. Each individual value represents the mean + S.E. Some values given only as the mean of 2 to 3 determinations on pooled samples. All measurements were made 24 to 32 hours after delivery. Hormones were injected 3 days prior to delivery with the exception of NE which was injected 24 hours before delivery.

*µmoles/min./mg. prot. **µatoms O_2/min./mg. prot.

(1) $p < 0.01$ (2) $p < 0.02$

hormones were applied on approximately the 19th day of gestation. One of the possible explanations was, that 24 hours is not a long enough interval for the changes to be detectable by our techniques.

In order to confirm or exclude this possibility, we performed a series of experiments shown in Table XI. All the fetuses of one mother animal were injected, spontaneous delivery was allowed to occur and the analyses were performed 24 - 32 hours after birth. Administration of 3.0 μg of thyroxine in one dose on the 19th gestational day resulted in significantly lowered dehydrogenase activities 1 day after birth. Homogenate activity of GPDH was lowered by 39% and that of SDH by 36.5%, while the specific activity of GPDH in isolated mitochondria showed a 36% decrease. Cortisone administration (0.1 mg/animal) resulted in much lower mitochondrial yield which is in agreement with our findings in 9-day-old rats (187) and was presumably due to mitochondrial swelling and increased fragility. The specific activity of GPDH in tissue homogenate was decreased by 24% and that in isolated mitochondria by 44%. On the other hand, NE administration (3.0 μg/animal) resulted in an increase of GPDH (49%) and SDH (27%) in the homogenate; reflected also in isolated mitochondria where SDH was increased by 40% and GPDH by 40%. All the data were compared with saline-injected controls.

From these results, it seems that NE administered prenatally can increase activities of mitochondrial respiratory chain enzymes one day after delivery. Two factors in this series of experiments have to be emphasized: NE was administered rather late in the course of prenatal life as opposed to thyroxine and cortisone administration and the animals in all

these experiments were allowed to experience the postnatal drop in environmental temperature from 37 to 23°C. Hence, the combination of late prenatal NE administration and an ambient temperature drop after birth may have participated in the observed BAT changes. In addition, in all three experimental groups, the BAT/body weight ratios were rather high if compared with the control group presented in Table XII.

NE acts most probably via the membrane-localized adenyl cyclase system which generates adenosine 3', 5' monophosphate intracellularly (208, 209). In order to determine whether this is the case also in BAT, we administered prenatally dibutyryl-cyclic AMP (69) and analyzed BAT one day after delivery (Table XII). In this case, the injection (0.5 mg/animal) was performed on the 18th day of gestation. The amount of BAT was increased and so was the ratio BAT/body weight (by 47%). The average activities of SDH and GPDH in tissue homogenates were higher than those in sham-operated animals, even though the data were insufficient to allow for statistical treatment. Nevertheless, it seems that cyclic AMP is capable of simulating the effect of NE even if administered at an earlier stage of development. The involvement of the adenyl cyclase system was indicated and confirmed later by direct measurements (see Part IV).

The most pronounced changes occur in BAT during the first 5 postnatal days. Is it possible to influence this developmental pattern by altered environmental temperature or administration of hormones immediately following delivery? As shown in Table XIII, we divided 8 litters of newborns, which were interchanged in such a way that each mother had one

TABLE XII

The Effect of Dibutyryl-cyclic-AMP Injected 4 Days Before Birth on Brown Adipose Tissue One Day After Spontaneous Delivery

	Controls(15)	Sham-operated Mother (9)	DB-c-AMP(10) 0.5 mg/anim.
Body Weight(g)	5.63 ± 0.30	5.10 ± 0.49	6.34 ± 0.61
BAT Weight(mg)	22.2 ± 5.6	22.0 ± 4.4	40.1 ± 8.4 (2)
Weight Ratio BAT/Body x 10^3	3.95 ± 0.27	4.31 ± 0.24	6.32 ± 0.81 (1)
Homogenate Activities			
GPDH*	101.0	102.0	141.0
SDH**	136.0	133.0	182.0

Number of animals used in each group is given in brackets. Individual values represent the mean ± S.E. In some cases only the mean of 2 to 3 determinations on pooled samples is given.

* mμmoles/min./mg. prot.

(1) $p < 0.05$

(2) $p < 0.1$

offspring of her own and one from all other mother's, into three groups. Two litters were kept at room temperature, three litters were exposed to 30 - 32°C immediately after delivery and three litters were intermittently exposed to 4 - 6°C as described under Methods. One litter from both heat and cold-exposed groups was injected daily with NE (5.0 µg/animal) and one litter with cortisone (0.1 mg/animal). The experiment lasted for 5 days. Animals kept for 5 days at 30 - 32°C did not show any significant alterations in BAT and body weight as compared with animals kept at room temperature. Animals exposed to cold had lower body weights and their BAT/body weight ratio was 47% higher than in controls. The average protein content of interscapular BAT was higher than in animals at room temperature, and the mitochondrial GPDH activity was increased.

Cortisone administration to warm-exposed animals resulted in lowered body weights, dramatically lower BAT weight and BAT/body weight ratio. The mean protein content was also lower as were the homogenate and mitochondrial enzyme activities. Four animals in this group died during the treatment. No such effect of cortisone was found in cold-exposed animals. NE administration resulted in hardly any changes; the only exception being an increased average protein content of the tissue in warm-exposed animals. The combination of NE treatment and cold exposure resulted in higher BAT weight as well as in a higher BAT/body weight ratio. Homogenate GPDH activity and all three enzymes in isolated mitochondria were higher than those in controls. In order to test the NE effect at room temperature, infant rats were injected with 100 µg NE/100 g body weight daily for the

TABLE XIII.

The Effect of Ambient Temperature and Hormone Administration for the First Five Postnatal Days on Brown Adipose Tissue in 6-day-old Rats

	Controls Kept at Room Temperature	Kept at 30-32°C			Cold-exposed*		
		No Injections	+ NE Daily 5µg/anim.	+ Cortis. Daily 0.1mg/anim.	No Injections	+ NE Daily 5µg/anim.	+ Cortis. Daily 0.1mg/anim.
No. of Animals	16	9	6	4	8	8	7
Body Weight (g)	11.47 ± 0.17	10.58 ± 0.45	8.40 ± 0.38	6.03 ± 0.36 (1)	8.04 ± 0.21 (2)	9.36 ± 0.53	7.89 ± 0.43
BAT Weight (mg)	66.13 ± 2.22	65.66 ± 4.54	53.00 ± 4.82	13.45 ± 2.52 (1)	67.62 ± 4.42	78.87 ± 2.78 (2)	51.14 ± 3.85
Weight Ratio BAT/Body x 10^3	5.76 ± 0.22	6.15 ± 0.23	6.37 ± 0.57	2.20 ± 0.31 (1)	8.50 ± 0.51 (1)	8.42 ± 0.18 (1)	6.47 ± 0.35
BAT Protein (% Fresh Wt.)	13.45	13.0	15.70	12.80	15.10	14.80	13.20
Homogenate Activities							
GPDH**	165 ± 13	150 ± 6	150 ± 9	69 ± 7 (1)	165 ± 12	204 ± 9 (2)	180 ± 11
SDH**	233 ± 11	-	217 ± 9	160 ±12 (1)	197 ± 23	250 ±43	224 ± 16
COX***	540	470	560	305	540	540	510
Mitochondrial Activities							
GPDH**	321 ± 22	358 ± 17	-	148 ± 20 (1)	474 ± 39 (1)	504 ± 60 (1)	349 ± 11
SDH**	432 ± 51	-	-	267 ± 18 (1)	468 ± 42	555 ± 20 (2)	457 ± 45
COX***	1,460 ±100	-	-	850	1,717	1,980 ±100 (2)	1,670 ±180

Each value represents the mean ± S.E. In some cases only the mean of 2 to 3 determinations on pooled samples is given.

*The whole litter with the mother exposed 5 to 6 times daily to 4-6°C for 60 minutes each time.

**mµmoles/min./mg. prot. ***mµatoms O_2/min./mg. prot.

(1) $p < 0.01$ (2) $p < 0.02$

TABLE XIV

The Effect of Norepinephrine Administration (10μg/100g body weight in 1 dose daily for the first nine postnatal days) on Brown Adipose Tissue in 10-day-old Rats

	Controls(8) Saline Injected	NE(8)
Body Weight (g)	13.63 ± 1.30	15.60 ± 0.30
BAT Weight (mg)	76.5 ± 5.2	99.8 ± 2.4(1)
Weight Ratio BAT/Body x 10^3	5.61 ± 0.41	6.40 ± 0.18(2)
Homogenate Activity		
GPDH*	229 ± 16	224 ± 12
Mitochondrial Activity		
GPDH*	438 ± 19	434 ± 25

Individual values represent the mean ± S.E. Number of animals in brackets.

* mμmoles/min./mg/ prot.

(1) $p < 0.01$

(2) $p < 0.02$

first 9 postnatal days (Table XIV). The only difference found was a larger amount of interscapular BAT in the NE-injected group as compared with that of saline-injected animals.

From these experiments it seems that if, indeed, the environmental temperature is required to accelerate BAT functional maturation, then even the less pronounced postnatal drop (from 38 to 30°C) is adequate to do so. Cold exposure of newborn animals has a general tendency to support BAT functional development but not to any significant extent beyond that achieved already at 30°C. NE administration during the immediate postnatal period failed to produce any appreciable improvement in the parameters tested regardless of environmental temperature. On the other hand, a pronounced detrimental effect of cortisone administration observed at 30°C seemed to be prevented by low ambient temperature.

(b) The effect of hormones and of temperature on the second phase of BAT development.

As shown in Part I and II, BAT starts to involute beginning with the third week of postnatal life. Is it possible to prevent this decrease in BAT activity by exposing the animals to cold? As shown in Table XV, we exposed 3 litters of 17-day-old animals to 4 - 6°C for the next 20 days and compared the BAT/body weight ratios of these animals to those of rats of the same age kept at room temperature. Interchanged litters were used in order to minimize biological variation. In all three experiments, the ratios were higher in cold-exposed animals (in average by 37%, $p < 0.02$). In another experiment (Table XVI) BAT was analyzed in more detail in rats exposed to cold at 16 days of age and kept in the cold until the 30th day.

TABLE XV

The Effect of Cold Exposure Commencing on Day 17 and Continuing Until Day 37 Postnatally on the Ratio of BAT Weight/Body Weight x 10^3

	Controls(Room Temp.)	Cold-exposed (4-6°C)
Experiment 1	$1.43 \pm 0.11(6)$	$1.79 \pm 0.14(6)^{(2)}$
Experiment 2	$1.42 \pm 0.10(5)$	$1.94 \pm 0.15(6)^{(1)}$
Experiment 3	$1.49 \pm 0.12(6)$	$2.22 \pm 0.23(6)^{(1)}$

Individual values represent the mean ± S.E.
Number of animals in each group given in brackets.

$^{(1)}p < 0.02$
$^{(2)}p < 0.05$

TABLE XVI

The Effect of Cold Exposure Commencing on Day 16 Postnatally on Brown Adipose Tissue of 30-day-old Rats

	Controls(Room Temp.) (5)	Cold-exposed (4-6°C) (8)
Body Weight(g)	90.6 ± 2.2	47.7 ± 2.2 (1)
BAT Weight(mg)	165.0 ± 17.2	106.9 ± 4.8 (2)
Weight Ratio BAT/Body x 10^3	1.81 ± 0.17	2.29 ± 0.16 (3)
BAT Protein (% Fresh Wt.)	11.9 ± 0.6	17.5 ± 0.7 (1)
Homogenate Activities		
GPDH*	142 ± 14	181 ± 10 (3)
SDH*	178 ± 15	202 ± 7

Each value represents the mean ± S.E. Number of animals given in brackets.

* mμmoles/min./mg. prot.

(1) $p < 0.01$

(2) $p < 0.02$

(3) $p < 0.05$

TABLE XVII

The Effect of Daily Injections of Norepinephrine (100μg/100g body weight) Commencing on Day 15 on Brown Adipose Tissue in 30-day-old Rats

	Controls(8) Saline-injected	NE(8)
Body Weight(g)	68.16 ± 1.55	65.27 ± 2.91
BAT Weight(mg)	161.38 ± 5.80	199.00 ± 8.70[1]
Weight Ratio BAT/Body x 10^3	2.36 ± 0.07	3.11 ± 0.22[1]
Total BAT Protein(mg)	18.20 ± 0.97	25.10 ± 2.30[2]
BAT Protein (% Fresh Wt.)	11.40 ± 0.50	12.35 ± 0.80
Homogenate Activities		
GPDH*	145 ± 6	219 ± 10[1]
SDH*	180 ± 18	260 ± 30
COX**	586 ± 61	798 ± 82[3]
Mitochondrial Activities		
GPDH*	292 ± 16	366 ± 30
SDH*	463 ± 36	510 ± 25

Number of animals in each group given in brackets. Each value represents the mean ± S.E.

* μmoles/min./mg. prot. [1] $p < 0.01$ [2] $p < 0.02$

** μatoms O_2/min./mg.prot. [3] $p < 0.05$

The increase in BAT/body weight ratio proved to be due to the much lower body weight of cold-exposed animals. On the other hand, there was a much higher (by 43%) protein content of the tissue and higher homogenate GPDH activity in the cold-exposed group. Hence in agreement with findings of Rafael et al. (160) on newborn guinea pigs, it seems that cold exposure, if applied at the peak level of BAT activity, prevents the involution occurring at room temperature.

Repeated administration of NE was shown to simulate the changes occurring during cold acclimation of adult rats (94, 122). Does the same phenomenon occur during the 17 - 30 days age period? NE was administered daily for 15 days to rats, starting on day 15. Control animals received saline. The two litters were interchanged at 15 days of age in such a manner that each mother animal had 4 of her own and 4 of the other mother's offsprings. The NE-injected group showed (Table XVII) increased BAT weight (by 25%), increased BAT/body weight ratios (by 36%), increased total BAT protein (by 38%), and increased homogenate and mitochondrial GPDH activities. We can therefore, conclude that NE treatment has a similar effect to cold exposure in rats aged 15 - 30 days.

(c) Some additional data.

A series of experiments on the effect of a single large dose of cortisone (5 mg /100 g body weight) on BAT in 9-day-old rats was performed (76, 187). Only the summary of the results will be presented here. 24 hours after the injection, the following changes were observed:

(a) the fresh weight of interscapular BAT was increased due to fat accumulation;

(b) the DNA content of the tissue stayed unaltered while the RNA content increased;

(c) specific activities of cytoplasmic alpha-glycerophosphate drhydrogenase and malic enzyme were increased;

(d) the percentage of mitochondrial protein in total tissue protein was not changed, but mitochondria seemed to be more fragile, fewer were recovered by a standard isolation procedure and more COX contaminated the microsomal fraction;

(e) mitochondrial GPDH and SDH activities were decreased per mg of homogenate protein and (in isolated mitochondria) per mg of mitochondrial protein;

(f) the endogenous respiration of BAT mitochondria was activated much less by carnitine and CoA;

(g) CO_2 formation from ^{14}C-palmitate by isolated BAT mitochondria was considerably lower. A similar injection to 30-day-old rats had no significant effect.

It was suggested that cortisone in the dose used affected the mitochondrial structure in BAT and the activity of FA utilizing enzymes on day 10 but not in older rats; this might be related to an age-difference in mitochondrial sensitivity to this hormone, which in turn, might be associated with the stage of mitochondrial differentiation.

CONCLUDING REMARKS

We have shown that a single injection of NE one day before birth increases several functional parameters of BAT in the rat as determined one day after spontaneous delivery. Cyclic AMP showed a similar effect even when applied 4 days prior to delivery. Thyroxine and cortisone in the dosage used (similar to that used by Greengard and Dewey - 68) showed no such effects; in fact their effects were opposite to those of NE. On the other hand, catecholamines failed to show any consistent effects if applied at days 18 to 19 of gestation and if the tissue was analyzed 24 hours later. The discrepancy between the catecholamine effects at these 2 different developmental stages may be due to several factors. Twenty four hours may be too short an interval for the changes to be detectable by our techniques or, alternatively, at 4 days before delivery the hormonal receptor may not yet be fully developed. The latter possibility is also supported by our finding that adenyl cyclase in BAT is not activated by NE at this stage of development (see Part IV). Again, the drop in environmental temperature experienced after delivery may be a prerequisite for NE to reveal its effect. In any case, NE proved to be the only hormone of those tested which exerted a positive effect on BAT of the rat during perinatal development.

In the earliest postnatal phase of brown fat development, the effects of different environmental temperatures and of NE were minor. This fact is not surprising if the already extremely rapid rate of differentiation occurring in the tissue at room temperature (see Barnard and Skala, 17) is taken into consideration. The fact that 30 - 32^{o}C failed to retard brown

fat development and that cold exposure failed to accelerate it to any considerable degree, may indicate that even the 7°C drop in ambient temperature is an adequate stimulus. On the other hand, the environmental temperature may not be the only stimulus; additional, possibly hormonal, factors may participate. Such a combination seems to be indicated also by the observed difference in cortisone effects at the 2 ambient temperatures. The pronounced detrimental effect of cortisone on warm-exposed animals is similar to that observed in 9-day-old rats at room temperature (76, 187). Since the thermoregulatory response to cold in 10-day-old rats depends on the presence of an intact adrenal gland (72), it is also possible that there is a greater requirement for adrenal steroids in cold-exposed infant rats.

Intrauterine injections not only of cortisone, but also of thyroxine, have a deleterious effect on BAT mitochondrial development. Nevertheless, both hormones cause an increase in the relative weight of BAT, suggesting an increase in the fat content of the tissue. This has been demonstrated for cortisone (76, 187). Thyroxine is known to increase the activity of malic enzyme in BAT (228) and hence it is possible that it, too, causes an increase in fat content.

After 15 days of age, both cold exposure and NE administration seem to prevent the involutive changes occurring at room temperature between days 17 and 30. This indicates that as physical thermoregulatory mechanisms mature, the temperature stimulus for NE release has to become stronger. In other words, lower ambient temperatures are required for NE to be released. Theoretically, corticoids may also be involved in

the initiation of the second phase of brown fat development. Their administration was shown to result in lower respiratory activity of the tissue accompanied by triglyceride accumulation. These changes are similar to those that occur spontaneously in the rat during the fourth postnatal week. Moreover, cortico-steroid secretion accelerates in the rat during the third and fourth weeks of life (e.g. 152, 229), increasing almost two-fold between days 18 and 22 (230), and it is during this period that brown fat involution also commences.

SUMMARY

1. Norepinephrine and epinephrine (4-5 μg/animal in one dose) administration to rat fetuses on days 18 to 19 of gestation did not result in any significant changes in brown adipose tissue one day later.
2. Norepinephrine (3.0 μg/animal) administration one day prior to birth resulted in an increase of alpha-glycerophosphate dehydrogenase and cytochrome c oxidase activities in tissue homogenates and isolated mitochondria one day after spontaneous delivery.
3. A similar effect was observed one day after delivery when dibutyryl cyclic AMP (0.5 mg/animal) was administered on gestational day 18.
4. Administration of cortisone (0.1 mg/animal) or thyroxine (3.0 μg/animal) three days before birth had the opposite effect, i.e. a decrease in the above activities in one-day-old animals.
5. Cold exposure and warm exposure of newborn animals for five days failed to produce any pronounced alterations in brown adipose tissue. Repeated administration of cortisone (0.1 mg/animal daily for five days) had a pronounced detrimental effect on brown fat of warm-exposed animals; cold exposure seemed to prevent this effect.
6. Norepinephrine administration (100 μg/100 g body weight) during the first ten postnatal days at room temperature resulted in hypertrophy of brown fat; no other changes were noted.
7. Cold exposure of 16-day-old rats for 14 days resulted in higher brown fat/body weight ratios, higher protein content of the tissue and higher dehydrogenase activities.

8. Daily administration of norepinephrine (100 μg/100 g body weight) between days 16 and 30 postnatally had an effect similar to that of cold exposure.

PART IV

THE HORMONAL RECEPTOR SYSTEM IN BROWN ADIPOSE TISSUE AND ITS DEVELOPMENT

(Based on the following publications:

188, 190, 191, 194)

INTRODUCTION

Several hormones, including catecholamines, ACTH, and glucagon have been shown to stimulate free fatty acid release, oxygen consumption and heat generation in BAT (for review see 197). Our data presented in Part III revealed a possible involvement of catecholamines in the processes of tissue development. Thus, the hormonal receptor system in BAT seems to be of considerable importance, both for the function of the tissue and for its development. In spite of this, there was an absolute lack of information on the subject in the literature and therefore, we had to undertake detailed studies in order to establish and characterize the system and its developmental pattern.

Any hormone acting via cyclic AMP comes in contact with a cell at the discriminatory subunit of the adenyl cyclase system (165). This unit is responsible for the hormonal specificity of a tissue. It can be visualized as a protein molecule (or a complex of protein with phospholipid) oriented to the outside of cellular membrane and it possesses a binding site specific for a particular hormone molecule. The contact between a hormone and its receptor is probably a binding process and it initiates an activation of the catalytic subunit of the adenyl cyclase complex. A hypothetical third unit was included in the complex - the transducing subunit connecting the discriminator with the catalytic unit. This does not necessarily mean that it is a separate morphological unit, it can be, for example, visualized as a conformational change of the catalytic subunit brought about by the discriminator-hormone complex. As a consequence, the reaction

$ATP\text{-}Mg^{+}$ ------> 3', 5' AMP is catalyzed and the cyclic nucleotide accumulates intracellularly. There is another way of activation of the catalytic subunit beside hormonal stimulation. Fluoride ion (NaF) has been shown to activate either directly the catalytic subunit or to activate the transducing subunit; in neither case, does this activation include the discriminator subunit (50). Using both, hormones and NaF, one can therefore distinguish between the catalytic activity of the cyclase and its hormonal sensitivity.

Cyclic AMP is broken down by the action of 3', 5'-cyclic nucleotide phosphodiesterase (e.g. 209) and this reaction has been shown to be an important regulatory mechanism under several conditions; its regulatory function has been most recently strengthened by the discovery of an activator of the enzyme (35, 212). Hence, the intracellular level of the cyclic nucleotide is the result of the ratio of activity of the adenyl cyclase system on the one hand and the reaction velocity of the phosphodiesterase on the other.

The intracellular 'receptor' for cyclic AMP (at least the one presently known) is the protein kinase system (for review see 163). This system has been discovered by Walsh et al. (218) and shown to be widely distributed (114) among species, tissues and even intracellular components (61, 134). It seems, therefore, that the mechanism by which cyclic AMP accumulation is 'translated' into a final effect of the hormonal stimulation is provided by protein kinase. This enzyme system consists of a binding subunit (which is highly specific for the cyclic nucleotide and there are

several nucleotide-specific kinase systems described in the literature - (115, 117, 153), the catalytic subunit and an inhibitor protein recently discovered (7, 220). Excluding the inhibitor (the function of which is yet completely unknown), the reaction sequence of protein kinase seems already established. The binding subunit, which covers the catalytic centre of the enzyme and thus suppresses its activity, binds the cyclic nucleotide and the complex cyclic AMP-binding protein is released from the catalytic unit. The free catalytic site then proceeds with the transfer of a phosphoryl group from ATP to a protein substrate (29). Protein kinase(s) is(are) rather unspecific in respect to the protein substrate and can phosphorylate a wide variety of proteins. It depends on the nature of the protein phosphorylated what the final effect of a hormonal stimulation will be.

To simplify the schematic picture of the enzymatic chain of reactions occurring during hormonal stimulation of a cell, the whole chain can be divided into two parts. The first can be called the 'common pathway' and it extends from the discriminator subunit of adenyl cyclase as far as the de-inhibition of the catalytic subunit of protein kinase. At this point, there are several further possibilities which can occur in respect to the phosphorylated protein. It is possible to visualize these as 'branches' and from the available data, several such 'branches' of the final part of a hormonal stimulation pathway can be established:

(a) It has been well documented (for review see 163), that protein kinase catalyses the phosphorylation of phosphorylase b kinase, thus converting the enzyme from the inactive to the active form. The

activated enzyme then catalyses the conversion of inactive phosphorylase b into the active phosphorylase a. Glycogen synthetase also undergoes phosphorylation of its molecule catalysed by protein kinase, and as such becomes inactive. Thus by the same reaction - a transfer of a phosphoryl group to (mainly) serine residues of the protein molecule - protein kinase activates glycogenolysis and inactivates synthesis of this compound.

(b) It has been shown that protein kinase catalyses phosphorylation of a hormone-sensitive lipase and by this means activates the enzyme (96, 109).

(c) Finally, protein kinase is known to phosphorylate histones (121) and ribosomal proteins (51) and via these reactions can probably influence processes of proliferation and differentiation. This may be particularly relevant during perinatal and early postnatal development of BAT, when the rate of tissue proliferation and differentiation seems to be extremely high. In order to evaluate the possible connection between the hormonal receptor and the processes of tissue maturation (see also 211), we have searched the literature and came up with the following supporting evidence. In spite of the fact that probably the largest amount of research into this problem has been done in unicellular organisms (e.g. see 216, 149, 150), we will restrict the presented data on mammalian systems. In renewal tissues such as thymus, which contains an actively cycling population of lymphoblasts, increases in cyclic AMP immediately preceding the initiation of DNA

synthesis have been reported after treatment with epinephrine (132), parathyroid hormone (223) and prostaglandins (58, 224). It was suggested that also in non-cycling cells such as hepatocytes (133), cyclic AMP may initiate and control the complicated chain of derepressive and synthetic events that precede the start of DNA synthesis. Several laboratories recently demonstrated that mammalian cells in culture undergo morphological transformation when dibutyryl-cyclic AMP is added (93, 105, 174). Lim & Mitsunobu (128) suggested a possible involvement of protein synthesis in the dibutyryl-cyclic AMP - induced morphological differentiation. Cyclic AMP may in fact not only regulate differentiation, but also keep the proliferation under control at the same time. It has been shown, for example, to have an inhibitory effect on cellular proliferation in fibroblast cultures (128) and some cells of malignant origin have been shown to contain very low or no membrane adenyl cyclase as compared with the same cells of non-malignant state (64, 135). A possible stimulation of RNA polymerase activity by cyclic AMP-dependent protein kinase was recently also indicated (136, 108).

What is the possible mechanism by which cyclic AMP exerts these effects? The proteins phosphorylated by protein kinase(s) include basic proteins such as histones (119). Phosphorylation of f_1 histones, demonstrated both in vitro and in vivo (120, 121, 8), suggest that cyclic AMP via protein kinase might affect the transcriptional level by affecting the types and amount of RNA

produced. At a given ratio of histone to DNA, phosphorylated histone is markedly less effective in altering the circular dichroic spectrum of DNA (121), indicating that phosphorylation reduces the ability of histone to produce changes in DNA structure. Measurements in fact, indicate that the introduction of approximately one phosphate group per molecule of lysine-rich histone, most of which is located on a specific serine residue, can effectively alter the interaction of the histone with DNA. In respect to the observed specificity of cyclic AMP effect in terms of stimulation of some synthetic processes and repression of others, an hypothesis has been advanced (59) that cyclic AMP-dependent histone phosphorylation results in a decline in the relative template activity of chromatin specific for rRNA while increasing template activities of other chromatins. There is one result which brings doubts into the above advanced transcriptional control mechanism of cyclic AMP. The acidic nuclear proteins have been shown to restore the histone-inhibited DNA-dependent RNA synthetic activity of chromatin isolated from uterine tissue (216) and these proteins are generally thought to be regulators of the transcriptional events in mammalian cells.

At the translational level, kinase activated by cyclic AMP has been shown to be involved in phosphorylation of ribosomal subunits (51).

All three pathways of hormonal stimulation may be of particular interest in BAT. In order to establish the activities and the

developmental patterns of all individual members of the pathway, we have undertaken a study of the adenyl cyclase system, the levels of cyclic AMP, the protein kinase system and a study of activities of phosphorylase b kinase and phosphorylase. Since there were some data on development of hormone-sensitive lipase (74) and 3'5'-cyclic nucleotide phosphodiesterase (77) activities in BAT, we did not include these into our study.

METHODS

Isolation of brown fat cells.

Isolation by collagenase treatment according to Rodbell (164) was used. Minced interscapular BAT from 3 litters of 12-day-old rats was incubated for 60 minutes at 37°C in Krebs-Ringer-phosphate buffer ($CaCl_2$ was omitted and replaced by NaCl according to a personal communication by Dr. T. Braun) in the presence of collagenase (Sigma) 3 mg/ml and 2.5% bovine serum albumin (fraction V, Armour) purified according to Chen (34). After filtration and low-speed centrifugation, the cells were re-washed five times with the same buffer containing 1% albumin. For the assay of adenyl cyclase, 1.0 ml of the final cell suspension was homogenized in the same volume of 0.25M sucrose with 7.5 mM EDTA, pH 7.4. Since the fat content of brown adipocytes from young rats is low, flotation and hence the yield of isolated cells were extremely low.

Adenyl cyclase assay.

Tissue was homogenized in a glass homogenizer with a teflon pestle in 20 volumes of ice-cold 0.25 M sucrose containing 7.5 mM EDTA, pH 7.4. After taking an aliquot for determination of cyclase activity in the whole homogenate, subcellular fractions were prepared as shown in Table XVIII. Samples were assayed either immediately after preparation, or after rapid freezing and storage at -60°C in the sucrose medium containing 0.1% albumin. Under these conditions, full activity was retained for 5 months of storage.

Activity was measured by assaying the rate of conversion of

alpha-^{32}P-ATP to cyclic ^{32}P-3'5'AMP. The incubation procedure and subsequent chromatographic separation of the cyclic nucleotide on polyethyleneimine impregnated cellulose thin layer sheets was performed according to Bär & Hechter (10, 11). The incubation mixture contained in a total volume of 50 µl: TRIS-HCl buffer 40 mM, pH 8.0; $MgCl_2$ 5 mM; unlabelled cyclic 3'5'AMP 0.5 mM; bovine serum albumin 0.1%; phosphoenulpyruvate 8 mM; pyruvate kinase 0.1 mg/ml; EDTA 0.1 mM; alpha-^{32}P-ATP 0.1 mM and enzyme preparation equivalent to 0.2 -0.6 mg protein/ml.

Materials: alpha-^{32}P-ATP (300-500 Ci/mole) was purchased from ICN, Irvine, Calif., polyethyleneimine impregnated cellulose thin layer sheets from Macherey-Nagel & Co./Brinkman, L-norepinephrine, L-epinephrine, serotonin, histamine, dichloroisoproterenol from Sigma, St. Louis. Phentolamine was a gift from CIBA, Summit, N.J., synthetic beta $^{1-24}$ACTH from Dr. W. Rittl, CIBA, Basel, glucagon from Eli Lilly & Co., secretin from SQUIBB.

5'-nucleotidase activity (5'-ribonucleotide phosphohydrolase, E.C. 3.1.3.5) was assayed according to Bär et al. (9).

Cyclic AMP concentration.

For cyclic AMP determinations, animals of all ages were lightly anaesthetized with ether, the interscapular BAT pad was carefully surgically freed from overlying tissues without disturbing the blood or nerve supply and the tissue was then quickly removed and immersed in liquid nitrogen. The frozen tissue was weighed and extracted according to Wastila et al. (222) and cyclic AMP was determined by the binding protein assay of Gilman (62) except that binding protein from bovine heart was used. Bovine heart binding

protein (protein kinase) was purified by the method of Kuo et al. (116). The preparation was taken as far as the DEAE-cellulose step. Protein kinase inhibitory factor was purified from skeletal muscle by the method of Appleman et al. (5). ^{3}H-cyclic AMP was purchased from NEN, Dorval, Que.

Protein kinase assay (ATP: protein phosphotransferase, E.C. 2.7.1.37).

For protein kinase determinations, samples were homogenized in 5 volumes of 4 mM EDTA, pH 7.2 in a Potter-Elvehjem glass homogenizer with a teflon pestle. The homogenates were centrifuged at 20,000 x g for 20 minutes and the supernatant fractions were stored after rapid freezing at -60°C for several days while all the samples from various age-groups could be accumulated. The assay used was that described by Kuo et al. (116) except that 200 µg of histone was used per assay. One unit of activity was defined as that amount of enzyme which catalyzed the transfer of 1 picomole of ^{32}P from gamma-^{32}P-ATP to histone in 5 minutes under the conditions of the assay.

Materials: gamma-^{32}P-ATP was purchased from ICN, Irvine, Calif. Histone purified from salmon testes was a gift from Dr. G.H. Dixon.

Phosphorylase assay (alpha-1,4-glucan:orthophosphate glucosyltransferase E.C. 2.4.1.1).

Phosphorylase activity was determined in the same supernatant fractions as the protein kinase. The assay was that of Cori, essentially as described by Krebs et al. (112). Prior to the assay, supernatants were diluted in 40 mM TRIS-beta glycerol phosphate, pH 6.8 containing 30 mM mercaptoethanol. In samples to be assayed for phosphorylase a (the active form), 0.2 ml aliquots were treated with 10 µl (40 µg) of adenylate deaminase

for 2 minutes. Aliquots were then added to 0.2 ml of the phosphorylase assay mix containing 32 mM glucose-1-phosphate, 2% glycogen and 300 µg/ml bovine serum albumin. The mixture was incubated at 30°C for 5 minutes. Control reactions with extract alone and with the assay mix alone (each brought to volume with water) were also run each time. The reaction was terminated by the addition of 0.1 ml of 12% TCA, the tubes were centrifuged at 4°C and a 0.4 ml aliquot of the clear supernatant assayed for inorganic phosphate using the procedure of Krebs et al. (112). For total phosphorylase activity, the assay mix contained 3 mM 5'AMP (adenylate deaminase omitted).

The assay in the absence of 5'AMP gave the levels of phosphorylase a activity. The assay in its presence gave the total enzyme activity, the difference then obviously represented the phosphorylase b activity. Activities were expressed as Cori units (36).

Materials: glycogen and glucose-1-phosphate were purchased from Sigma and were purified by the methods of Drummond et al. (48).

Phosphorylase b kinase assay (ATP:phosphorylase phosphotransferase, E.C. 2.7.1.38).

The enzyme was assayed in the same supernatant fractions as the phosphorylase. The assay was that of Krebs et al. (112) as modified by Drummond & Duncan (49). After 5 minutes of incubation, the reaction was stopped by diluting a 0.1 ml aliquot into 1.9 ml of 40 mM beta-glycerol phosphate with 30 mM mercaptoethanol, pH 6.8 containing 100 µg (20 µl) of adenylate deaminase. The tubes were kept at room temperature for 1 minute and were then immersed in ice and assayed immediately for

phosphorylase a. Treatment with adenylate deaminase destroyed effectively any 5'AMP produced from ATP during the kinase assay, which if carried into the phosphorylase assay, could give erroneous results (49). Kinase assays were performed at pH 6.8 and pH 8.2; the ratio of activities at these two pH values was used as an index of the portion of the enzyme present in the activated form (112). One unit of phosphorylase kinase was defined as that activity which catalyzed the formation of 100 Cori units of phosphorylase a in 5 minutes under the conditions of the assay (49).

Materials: phosphorylase b was crystallized from frozen rabbit muscle by the procedure of Fisher & Krebs (54) using the modification outlined by DeLange et al. (44).

RESULTS AND DISCUSSION

(a) Adenyl cyclase system.

Adenyl cyclase activity was found in the whole homogenate and in all subcellular fractions of BAT in 12-day-old rats, as well as in the homogenate of isolated brown fat cells (Table XVIII). Cyclase activity in all fractions is stimulated to a considerable extent by NE and NaF. ACTH is without any pronounced effect. The specific activities are highest in the 100,000 x g x 60 minutes pellet. The bulk of activity is associated with the same fraction. A considerable amount of adenyl cyclase activity is still present in the 100,000 x g x 60 minutes supernatant and it is hormone-sensitive. Further centrifugation at 200,000 x g for 60 minutes was required to sediment all the remaining hormone-sensitive activity, but some NaF-sensitive adenyl cyclase was still present in the supernatant. If we accept the hypothesis that the adenyl cyclase system consists of the hormonal receptor (the discriminatory unit), the transducing unit and the catalytic unit, our results might be due to the fact that the hormonal receptor unit is rather large as compared to the catalytic subunit. The presence of the discriminator is required for hormonal sensitivity; this is apparently gradually lost with increasing centrifugation force. On the other hand, the catalytic subunit, possibly together with the transducing unit, could be recovered in the supernatant even after very high-speed centrifugation, (they may be in partially solubilized form) and these are the site at which NaF exerts its effect.

TABLE XVIII

Specific activity and relative distribution of adenyl cyclase in subcellular fractions and isolated cells from brown adipose tissue of young rats.

Preparation	Adenyl Cyclase Activity picomol.c-AMP/min./mg prot.				% of Total Hom.Activity				%Prot.
	BAS	ACTH	NE	NaF	BAS	ACTH	NE	NaF	
Whole homogenate	3.68	4.02	17.3	41.5	100	100	100	100	100
650 x g x 10 min. pellet	2.67	2.30	8.5	26.0	20	15	11	14	13
8,500 x g x 10 min. pellet	1.90	2.27	9.5	20.2	7	7	7	6	14
100,000 x g x 60 min. pellet	8.72	9.15	34.2	128.0	31	30	31	42	13
100,000 x g x 60 min. supernatant	2.67	2.67	6.3	15.4	16	11	10	10	33
Isol.cells homog.	3.00	3.90	35.7	33.3					
Isol. cells homog. (fat removed by filtration)	7.70	8.20	64.0	55.7					

Each value (except for those for isolated cells homogenates) represents the mean of four determinations (40 rats used).
Final concentrations of ACTH, norepinephrine (NE) and NaF were 3.3×10^{-6}M, 5×10^{-6}M and 10^{-2}M, respectively.

An attempt was made to define the 'microsomal fraction' in BAT of young rats more clearly by determining the distribution of some 'marker' enzymes. The highest activities of acid phosphatase (lysozomal marker) and glucose-6-phosphatase (microsomal marker) were recovered in the microsomal fraction. Surprisingly, 5'-nucleotidase (assumed to be a plasma membrane marker), showed the highest specific activity in both the 100,000 x g x 60 min. pellet and supernatant. In other words, it had a distribution similar to that of adenyl cyclase. Most authors find adenyl cyclase activity from other organs of adult animals in membrane fractions sedimenting at low g-force. Dousa & Rychlik (46) found adenyl cyclase activity in mammary gland to be distributed similarly as BAT cyclase.

Vaughan & Murad (217) discarded their microsomal fractions and found the activity to be lowest in the nuclear fraction of white fat. From our experiments, it may be concluded that the bulk of activity is associated with fine particulate material. Since 5'-nucleotidase activity was associated with the same fractions as cyclase activity and since the former enzyme is thought to be derived from the cell membrane, our results indicate that also cyclase is probably localized in the cell membrane which apparently has a different sedimentation pattern than in other organs of adult animals. It seems probable that during homogenization, the cellular membrane of infant BAT adipocytes is broken into very fine particulate material which sediments mainly with the so-called 'microsomal' fraction.

Is the adenyl cyclase activity we measure derived from the adipocytes or from other tissue elements? Cells were isolated from BAT, homogenized, and the activity assayed. The results (Table XVIII) clearly show the presence of adenyl cyclase activity in the adipocytes. If the fat content of the cell homogenate was lowered by filtration across cheese-cloth, the specific activities were higher. Moreover, the degree of activation of the cyclase preparation from isolated adipocytes by NE was higher than that observed with adenyl cyclase derived from whole tissue homogenate, whereas NaF activation was not higher. If one assumes that the adenyl cyclase measured in the whole tissue homogenate is derived not only from the adipocytes themselves, but also probably from nerve endings and vascular walls, then the more pronounced catecholamine sensitivity in homogenates from isolated cells is the property of the adipocyte cyclase as

opposed to the contaminating cyclases. In conclusion, the brown adipocyte cyclase is highly NE - sensitive.

The properties of adenyl cyclase in the 100,000 x g pellet were further investigated. Basal, NE- and NaF- stimulated activities were directly proportional to the amount of protein used over a range from 0.16 to 0.65 mg protein/ml. The rate of cyclic AMP formation either in the absence or in the presence of catecholamines and NaF is linear with time for 20 - 30 minutes at 25, 30 and 37°C (Fig. 20). Increasing the temperature from 25 to 37°C resulted in slight increase of basal and catecholamine-stimulated activity and in pronounced increase of NaF - stimulated activity. pH changes between 7.0 and 9.0 were found to have no pronounced effect on the activity, with highest activity recorded between pH 8.0 and 8.5. The pH optimum in the presence of catecholamines was 8.0 and hence this pH was adopted for routine assays. Both the basal and NE-stimulated adenyl cyclase activities were found to be stable for at least 45 minutes if preparations were kept at 0°C. After 3 hours, a decay of about 20% was noted.

In white adipose tissue $CaCl_2$ 0.085 mM inhibits adenyl cyclase activity (217). In the microsomal fraction of BAT, on the other hand, concentrations up to 0.6 mM were ineffective. A concentration of 1 mM inhibited the activity by 60%.

High levels of biogenic amines have been demonstrated in BAT (e.g. 201, 76). Fig. 21 shows that only NE and epinephrine activated adenyl cyclase in our preparations from 12-day-old rats. Serotonin and histamine were ineffective even if added to cyclase preparations pre-

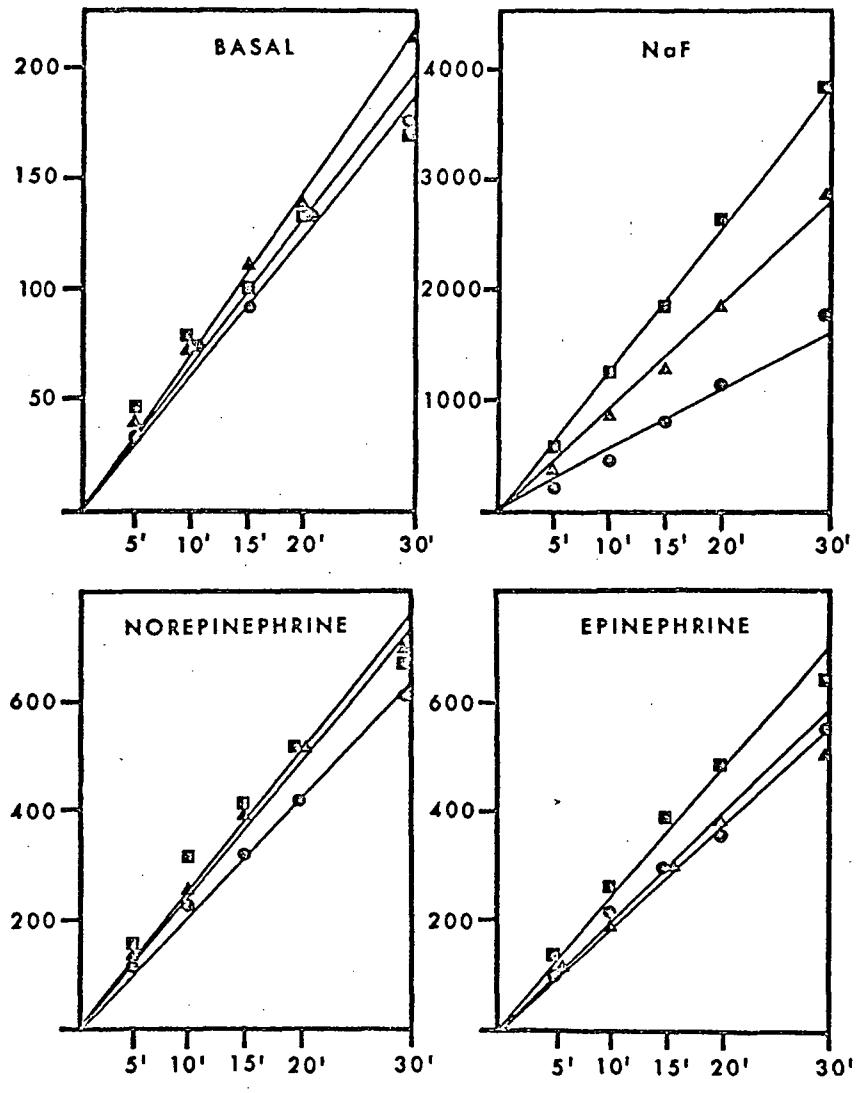


Fig. 20. The effect of temperature (●—● 25°C; ▲—▲ 30°C; ■—■ 37°C) on basal, NaF (10^{-2}M), norepinephrine and epinephrine (5 x 10^{-5}M) - stimulated adenyl cyclase activity in the 100,000 x g x 60 min. pellet from brown adipose tissue of 12-day-old rats. Activity is expressed as picomoles of formed c-3',5'-AMP per mg of protein.

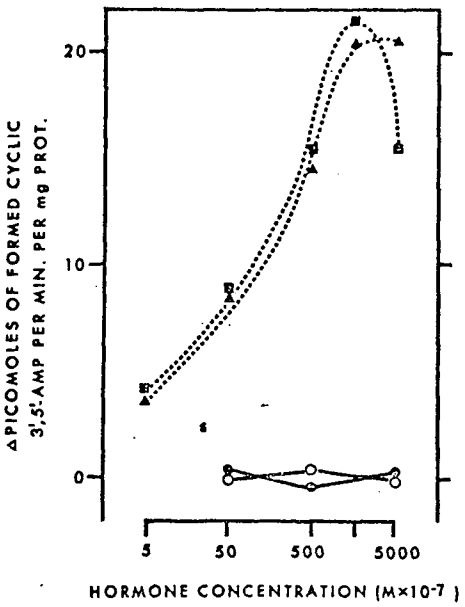


Fig. 21. The effect of various concentrations of norepinephrine (■—■), epinephrine (▲—▲), serotonin (●—●), and histamine (○—○) on adenyl cyclase activity in 100,000 x g x 60 min. pellet from brown adipose tissue of 12-day-old rats. Each point represents the mean of two determinations which agreed closely.

incubated for 10 minutes in the presence of a monoamine oxidase inhibitor (Pargyline, 5×10^{-4}M). A variety of polypeptide hormones and other substances known to affect lipolysis in either brown or white adipose tissue was also tested for their ability to stimulate adenyl cyclase in BAT in 12-day-old animals. A very slight and variable stimulation (by 15 - 20%) was observed with ACTH (10 - 100 μg/ml), beta-MSH (100 μg/ml), bovine growth hormone (10 μg/ml) and secretin (0.1-100 μg/ml). Glucagon (10 - 100 μg/ml), TSH (10 - 100 μg/ml), beta-LTH (100 μg/ml), ICSH (100 μg/ml), alpha-MSH (100 μg/ml), arginin-vasopressin (30 μg/ml), thyroxine (1-100 μg/ml), insulin (1mU/ml), prostaglandin E_1 (1-31.5 μg/ml), oxytocin (30 μg/ml) and dexamethazone (1-10 μg/ml) all were without any detectable effect. Hence, it appears that adenyl cyclase in BAT of infant rats is selectively stimulated by catecholamines.

Although serotonin and some polypeptide hormones (TSH,

ACTH and glucagon) have been reported to increase the rate of lipolysis in adult BAT (for review see 197) in our study the above hormones were unable to stimulate adenyl cyclase activity in broken cells preparations. In the light of previous observations of Beviz et al. (23) that the beta-blocking agents inhibited not only the metabolic effect of NE but also those of serotonin and ACTH, it seems possible that the metabolic effects of the above hormones in intact tissue are exerted through catecholamine release from storage sites. Alternatively, it can be speculated that the ineffectiveness of serotonin and polypeptide hormones to stimulate adenyl cyclase in our broken cell preparations is due to alterations or destruction of the hormone-selective receptor sites for these agents during tissue preparations. It must also be stressed that our preparations were taken from suckling animals and that it is possible that the sensitivity of adenyl cyclase to the above hormones might depend upon the stage of development. It has been shown, for example, that ACTH and glucagon responses of BAT change during the first 30 days of life (79). In order to clarify this possibility, which seems very attractive from the developmental standpoint, we have examined in some pilot experiments the sensitivity of adenyl cyclase from 1-, 10- and 45-day old animals to glucagon, ACTH, NE and NaF. As shown in Fig. 24 C, the results were not conclusive. There was clearly no stimulation by glucagon or ACTH in 10-day-old animals, even though the activation by NE and NaF as well as the basal activity was greater than in the other two age groups. On the other hand, there was a certain degree of stimulation observed with both glucagon and ACTH in 1-day-old and 45-day-

old animals, to a greater extent in the older animals. The same was observed by Hahn (79) in respect to lipolysis. In interpretation of these results, it is necessary to point out that in the 45-day-old rats the isolation of BAT is difficult and some contamination by white adipose tissue is possible. Therefore, the observed stimulation by glucagon and ACTH in this age group need not necessarily belong to the brown fat enzyme but may be due to contaminating while adipose tissue enzyme. In any case, these results warrant further detailed investigation since a possible developmental change in the discriminatory unit of the adenyl cyclase system would be of great importance for any hormonal regulatory mechanism under study.

In further experiments, it was found that dichloroisoproterenol (a beta-adrenergic blocking agent) clearly abolished the stimulatory effect of catecholamines but not that of NaF (Fig. 22). The inhibitory effect is overcome by increased concentrations of catecholamines, thus suggesting a competitive type of inhibition. Phentolamine (an alpha-adrenergic blocking agent) on the other hand, had no effect on either catecholamine- or NaF-stimulation of BAT adenyl cyclase activity. This indicates that the adenyl cyclase system of BAT is associated with a beta-adrenergic type of catecholamine receptor and it is in good agreement with several reports that the cellular effects of catecholamines in BAT are abolished by beta-blocking agents (e.g. 23, 155, 204). The lowest concentration of catecholamines which significantly stimulates cyclase in our preparations (5×10^{-7}M) is comparable to the dose of epinephrine which already significantly increases the metabolism of BAT (2×10^{-7}) as reported by Joel (104). A concentration

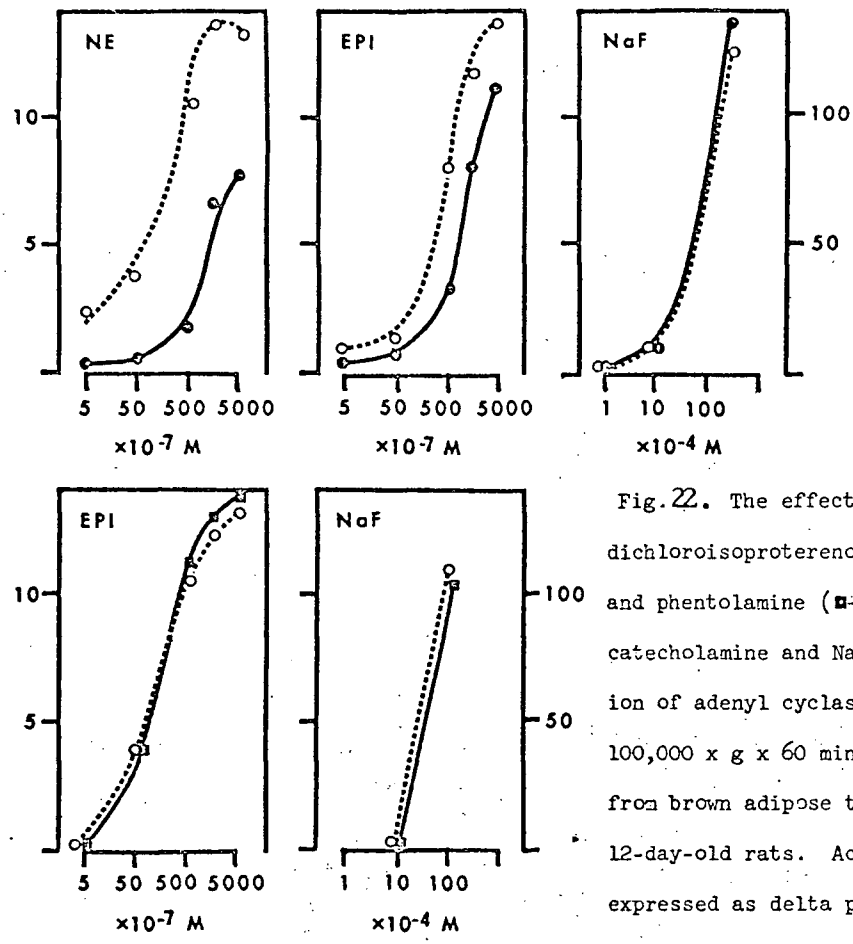


Fig. 22. The effect of dichloroisoproterenol (●——●) and phentolamine (■——■) on catecholamine and NaF-stimulation of adenyl cyclase in 100,000 x g x 60 min. pellet from brown adipose tissue of 12-day-old rats. Activity is expressed as delta picomoles of formed c-3',5'-AMP per min. per mg prot. Both DCI and phentolamine were 5×10^{-5}M when the effect on catecholamines was tested and 5×10^{-4}M when added to NaF.

of about 10^{-5}M of catecholamines is required to evoke a half-maximal stimulation of adenyl cyclase; stimulation is maximal at 5×10^{-5} - 1.5×10^{-4} M (Fig. 21).

After characterization of adenyl cyclase in BAT of 12-day-old animals, we studied the developmental pattern for the basal, NE- and NaF-stimulated enzyme (Fig. 23). In order to preserve the total activity

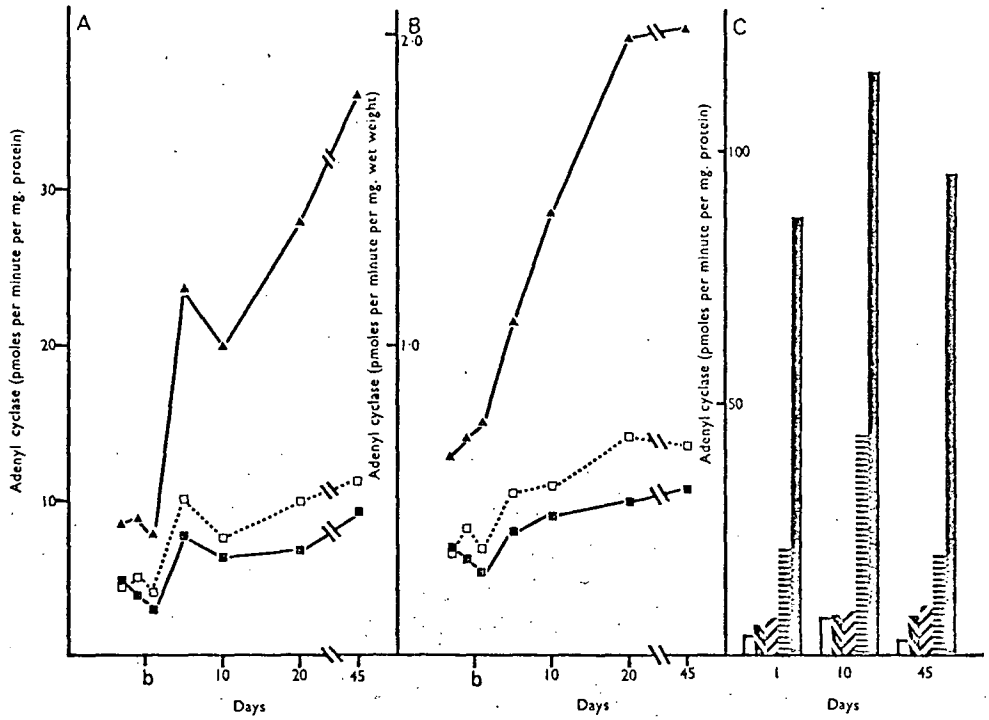


FIG. 23—Adenyl cyclase activity in brown fat from rats of different ages. Basal (▣), norepinephrine-sensitive (□), and fluoride-sensitive (▲) activities are expressed on a protein basis (A), and on a tissue-weight basis (B). Whole homogenates were used; each value is the mean of 3 samples. C, The effect of glucagon, 10 μg. per ml. (▨); ACTH, 10 μg. per ml. (▧); norepinephrine, 5 μ*M* (▤); and NaF, 10 m*M* (▯) on basal (▯) adenyl cyclase activity in microsomal fractions from brown adipose tissue of animals of the ages indicated. Each value is the mean of 3 samples.

and thus allow for developmental comparisons, we used the whole tissue homogenates for this series of experiments, even though the hormonal activation was much less pronounced in such crude preparations (which, of course, might also be due to freezing of samples required to accumulate material from all the age groups). Nevertheless, we feel safe to assume that even though the maximal values might be affected, developmental comparisons could be made since all the materials were treated in the same manner. NaF-sensitive activity increased considerably after birth and seemed to level off after the 20th postnatal day when expressed on a tissue fresh weight basis (Fig. 23 B), while specific activity appeared to increase over the whole period studied (Fig. 23 A). Similar, but less pronounced

changes were observed in basal and NE-stimulated activity. No NE stimulation of cyclase activity was observed in tissue removed from animals 3-4 days before birth. This finding was of particular interest since basal and NaF-activated enzyme was demonstrable in the tissue well before this stage. It may, in fact, indicate that the hormonal receptor unit develops independently of the catalytic unit as also suggested by results of Schmidt & Robison (170) on developing rat brain. Further, detailed analysis is required before a definite answer can be reached. The postnatal increase of adenyl cyclase activity observed in our experiments is in agreement with findings of Counis & Raulin (40) who described a perinatal increase of the NaF-activated enzyme. It is similar to the findings of increasing cyclase activities in some other tissues (12, 92).

Muirhead & Himms-Hagen (139) reported a transient increase in NaF-stimulated adenyl cyclase of BAT during the initial phase of cold acclimation in adult rats. Our results (Table XIX) revealed no changes in the specific activities of basal, NE- and NaF-activated enzyme in 40-day-old animals acclimated to cold for twenty days. However, when expressed on a tissue fresh weight basis, an increase was apparent. Since the protein

Table XIX–Adenyl Cyclase Activity in Brown Fat of Cold-exposed Rats

Experimental Group	Adenyl Cyclase Activity					
	Picomoles per minute per mg. wet weight			Picomoles per minute per mg. protein		
	Basal	+NE	+NaF	Basal	+NE	+NaF
Control	0·53	0·71	1·95	9·05	13·40	34·50
Cold-acclimated	0·75	1·15	3·99	7·45	11·20	39·70

Each value represents the mean of 2–3 samples. The final concentration of norepinephrine (NE) in the assay was 5 μM; of NaF, 10 mM.

content of brown adipocytes from cold acclimated animals is higher, while the cell population (as judged from the DNA content - see Part I) remains the same, it is probably justified to consider expression of the activity on the tissue fresh weight basis as more physiologically meaningful. Hence, perhaps the hormonal receptor system is more active in BAT of rats reared at low ambient temperature, as indicated also by significantly elevated tissue levels of cyclic AMP as will be shown below.

(b) Tissue levels of cyclic AMP.

Intracellular levels of cyclic AMP are known to undergo rapid and drastic changes, especially in response to hormones and tissue trauma. Elaborate precautions, particularly instant freezing, are frequently necessary to prevent changes occurring during tissue removal and preparations of extracts. In the present experiments, animals were anaesthetized with ether and the interscapular brown fat pad was isolated surgically. Although such techniques might stimulate cyclic AMP formation, they were essential in order to separate BAT from the overlying fascia and from white adipose tissue in older age groups. The tissue was effectively isolated with care taken not to disturb nerve and blood supply, then rapidly removed and frozen in liquid nitrogen. Low variations in the levels in tissues of animals in each age group indicated that this procedure did not cause trauma sufficient to alter intracellular level of cyclic AMP spuriously.

Developmental changes in cyclic AMP levels in interscapular BAT of the rat are presented in Fig. 24. A sharp rise in the level was observed in the late stage of intrauterine life. Levels decreased rapidly

after delivery and continued to fall slowly up to the 50th postnatal day. In 40-day-old rats acclimated for 20 days to 4-6°C cyclic AMP in BAT was increased significantly, reaching levels equivalent to those occurring perinatally. Hahn (77) has shown that the activity of 3'5' cyclic nucleotide phosphodiesterase does not change between the late fetal period and day 10 after birth in BAT of the rat. It decreases by about 60% by day 20 and by additional 50% by the adulthood. Hence, it seems that the observed developmental changes in cyclic AMP level reflect more the adenyl cyclase activation than the phosphodiesterase developmental changes.

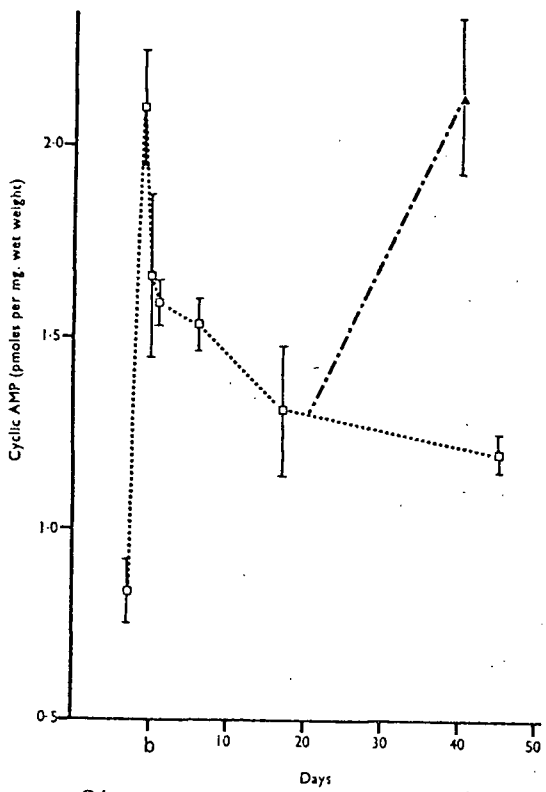


FIG. 24.—Cyclic AMP content of interscapular brown fat during development. Levels are expressed (□) on the basis of tissue wet weight. ▲, Represents levels after 20 days of cold acclimation. Vertical lines are the S.E.M. of 8–12 samples.

(c) Phosphorylase.

The assay of this enzyme in the presence of 5'AMP provides a measure of total activity. Phosphorylase b is inactive in the absence of this nucleotide and is stimulated maximally in its presence. Phosphorylase a, on the other hand, is fully active in the absence of 5'AMP. Therefore, we have assayed the enzyme both in the presence and in the absence of this nucleotide and the results are given in Fig. 25 A. Both phosphorylase a and the total enzyme showed similar developmental changes; low activity before birth increased with approaching parturition and a maximum was reached immediately after delivery. This peak appeared concomittantly with the dramatic fall in tissue glycogen content (see Table II in part I). Starting shortly after birth, activities decreased steadily through the period (50 days) of observation. It can be noted that except in the prenatal period, the enzyme existed primarily in the inactive form. This provides an indication that little or no activation of the enzyme occurred during removal and extraction of the tissue, even though this procedure could not be effected with such an extreme rapidity as, for example, preparation of samples for cyclic AMP measurements.

Cold exposure did not change phosphorylase a activity significantly, but an increase was observed in the total phosphorylase activity.

(d) Phosphorylase b kinase.

This enzyme can be extracted from several tissues, particularly skeletal muscle and heart, in a form which has very low activity if assayed at pH 7.0 and below. Krebs et al. (112) has referred to the

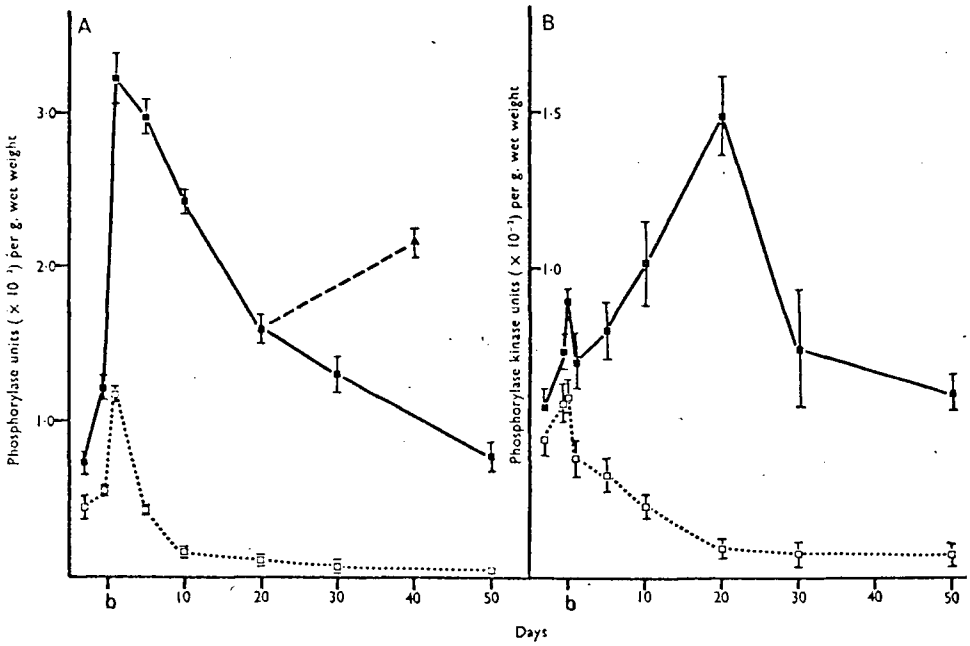


FIG. 25.—A, Phosphorylase activity in brown fat during development. Enzyme activity was assayed in the absence of 5′-AMP (□) (phosphorylase *a*) and in the presence of the nucleotide (■) (total phosphorylase); ▲, total phosphorylase after 20 days' cold exposure. Vertical bars represent the S.E.M. of 4–8 samples. b indicates birth. B, Phosphorylase kinase activity in brown fat during development. Activity was assayed at *p*H 6·8 (□) and at *p*H 8·2 (■). Vertical bars represent the S.E.M. of 4–8 samples.

enzyme with this pH profile as non-activated phosphorylase kinase. At pH values higher than 7.5 , the activity rises sharply to a maximum at pH 8.2. The enzyme can be converted to an activated form through the mediation of cyclic AMP (44, 49, 112, 219) and by Ca^{++} (138). Activation is especially marked when the enzyme is assayed at pH values less than 6.8 and is less apparent when the assays are conducted at pH 8.2. The ratio of activity at pH 6.8 to that at pH 8.2 has therefore been used as an index of the amount of enzyme in the activated form (112). The activity of phosphorylase kinase in BAT during development assayed at both pH 6.8 and pH 8.2 is given in Fig. 25 B. It can be seen that a dissimilar developmental pattern was observed at these two pH values. Before and immediately after birth, the ratio of activities at 6.8/8.2 was very high, suggesting that most of the

enzyme existed in an activated form. Thereafter, the activity assayed at pH 6.8 dropped rapidly to reach a minimum at day 50 while the activity assayed at pH 8.2 continued to rise to a maximum on day 20; thereafter, it declined as well. During the latter period, therefore, the enzyme was predominantly in a non-activated form. It may be significant that in the period immediately preceding birth, phosphorylase was also largely in the activated form. In fact, the developmental pattern of phosphorylase kinase, assayed at pH 6.8, closely parallels that of phosphorylase a with a prenatal increase, a peak at parturition, followed by a decrease up to the 50th postnatal day. In contrast, phosphorylase kinase activity assayed at pH 8.2 (total activity) increased progressively up to the 20th postnatal day. It seems that this pattern is similar to that established for the physiological activity of the tissue (see Part I). It may in fact, suggest some role for this enzyme in the BAT in addition to phosphorylase b to phosphorylase a conversion. This is also supported by findings of Khoo et al. (110) who suggested that phosphorylase b kinase in adipose tissue might not only have another role, but also it might not be the only enzymic pathway activating phosphorylase.

Activities of phosphorylase and phosphorylase kinase were determined in the 20,000 x g x 20 minutes supernatant fractions. No attempt was made to purify these crude preparations further before assay because of the danger of losses of activity. Even with such crude preparations, the possibility should be considered that enzyme compartmentation changes during development (see 143, 192). Phosphorylase and phosphorylase kinase are known to be at least partially associated with glycogen

particles in liver (13). If this was the case in BAT, such activities might not be recovered in our supernatant fractions. Even though such a possibility might exist during the fetal period, peaks in activity were associated with the perinatal period and activity decreased during postnatal development, when there was almost no glycogen left in the cells. This would tend to rule out any alterations in the sedimentation characteristics of phosphorylase and phosphorylase kinase, at least during postnatal development.

(e) Protein content of the 20,000 x g supernatant.

Our results for phosphorylase and phosphorylase kinase activities are expressed per fresh weight of the tissue. In order to allow for comparisons of specific activities, the protein content of the 20,000 x g x 20 minutes supernatant fractions are presented in Table XX. The prenatal increase in protein content of this fraction coincides with the increase in protein content of the whole tissue (Table I in Part I). While the total tissue protein content continued to increase after birth, protein in the 20,000 x g supernatant fraction showed a decrease. This is probably a consequence of the fact that the total tissue protein postnatally becomes more and more a reflection of mitochondrial protein. The development of phosphorylase and phosphorylase kinase specific activities would show a somewhat

Table XX –Protein Content of 20,000 *g* Supernatant Fractions from Brown Adipose Tissue during Development

Age (days)	−3	−2	1	5	10	20	30	50
Protein content (percentage of wet weight)	8·1	11·5	10·0	8·2	6·6	4·9	6·2	3·9

Each value represents the mean of 3 samples which did not differ by more than 10 per cent. Negative values refer to days before birth.

less pronounced prenatal increase and postnatal decrease. The question of whether specific activities or activities per unit of tissue fresh weight are more meaningful physiologically remains unanswered but we prefer the latter.

(f) Protein kinase.

Assay of this enzyme in extracts of BAT presented several problems. The effect of protein on reaction velocity in the absence and presence of cyclic AMP in 20,000 x g supernatant fractions from tissue removed one day before birth and 30 days after birth is given in Fig. 26. Velocity was a linear function of protein only up to 30 µg; thereafter, it fell

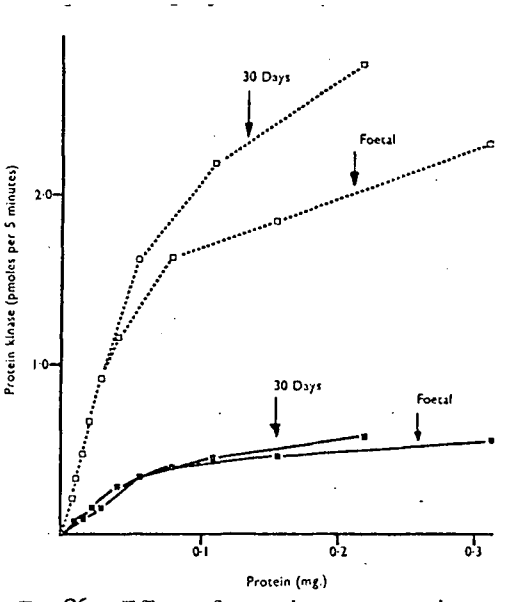


FIG. 26.—Effect of protein concentration on protein kinase assay in extracts of brown fat. Activity was assayed in the absence (□) and presence (□) of 5 µ*M* cyclic AMP. Extracts from late foetal and 30-day-old rats were used as indicated.

off sharply. A similar experiment was performed with purified beef heart protein kinase, which revealed that the reaction rate was proportional to

protein content up to velocities at least eight times higher than those achieved in Fig. 26. A possible reason for this was that higher concentrations of histone were required to saturate the brown fat enzyme. Saturation of the assay with histone with two concentrations of protein, 17.5 and 280 μg in the assay, is shown in Fig. 27 A and B, respectively. In both cases,

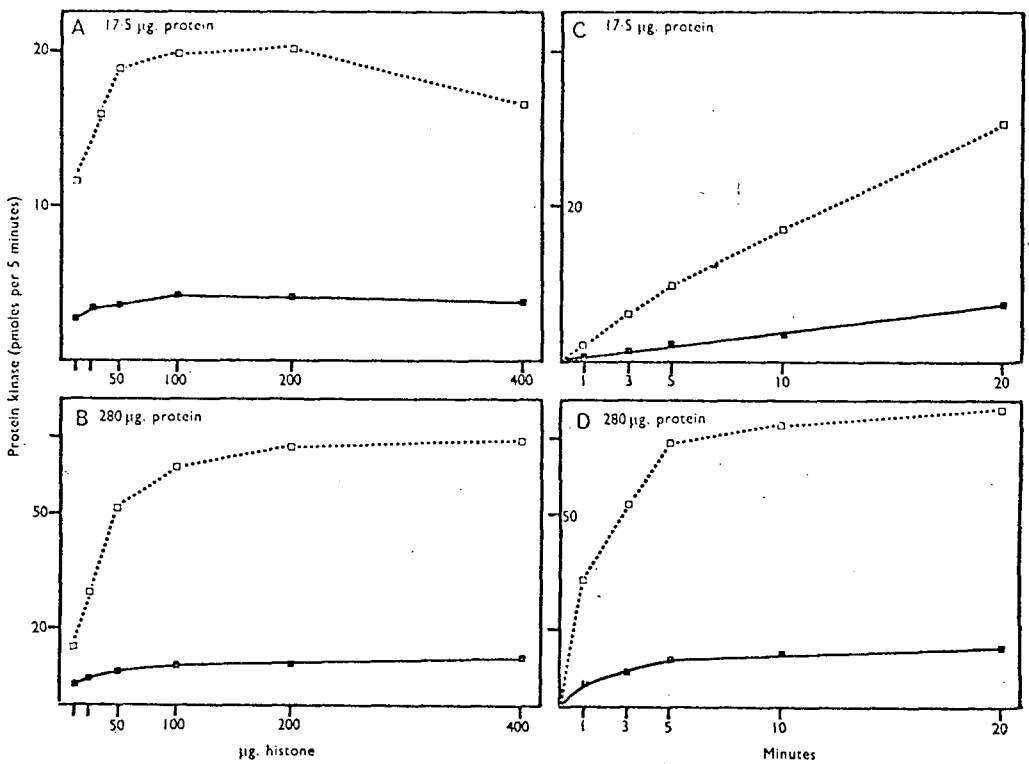


FIG. 27—Assay of protein kinase in brown adipose tissue. A and B, Effect of histone concentration on reaction velocity using 17·5 μg. and 280 μg. protein respectively in the absence (■) and presence (□) of 5 μ*M* cyclic AMP. C and D, Effect of time of incubation on reaction velocity, using 17·5 and 280 μg. protein respectively in the absence (■) and presence (□) of 5 μ*M* cyclic AMP. Each assay contained 200 μg. histone.

200 μg of histone per assay produced maximal velocities. It can be seen that increasing the protein from 17.5 μg to 280 μg (a fifteen-fold increase) caused only a threefold increase in maximum velocity. Since the non-linearity with respect to protein was not due to insufficient histone, the reaction was examined with respect to time. This is shown for the same two protein

concentrations in Fig. 27 C and D, respectively. Reaction velocity was proportional to time up to 20 minutes with low protein concentration, but fell off drastically after only 1 minute when 280 μg of protein were used. A possible explanation for the lack of proportionality of velocity to protein concentration was the presence of a protein kinase inhibitor in tissue extracts, similar to that described in skeletal muscle by Walsh et al (218). As will be shown below, we have confirmed this possibility by a series of subsequent experiments. Hence, it seems that the dilution characteristics of the enzyme and its inhibitor differ in such a way that in very high dilutions (under 30 μg protein per assay), the inhibitor looses its effect, whereas the enzyme is still detectable.

With these limitations, special precautions were necessary in order to ensure a valid assay of protein kinase in BAT. In particular, the enzyme was assayed with small amounts of protein, so that the velocity did not exceed 10 picomoles of phosphate transferred during the 5-minute incubation period, and 200 μg of histone were used in the assay. Fig. 28 represents the developmental pattern of the enzyme in BAT assayed under these conditions. No pronounced changes in cyclic AMP-independent activity were found when activity was expressed on protein basis (Fig. 28 A). When expressed on a tissue fresh-weight basis (Fig. 28 B), cyclic AMP-independent activity rose to a peak 1 day after birth, followed by a continuous decrease to about 50% in rats older than 1 month. Cyclic AMP-dependent protein kinase showed a rapid rise in the perinatal period when expressed on a tissue weight basis (Fig. 28 B), while the specific activity (Fig. 28 A)

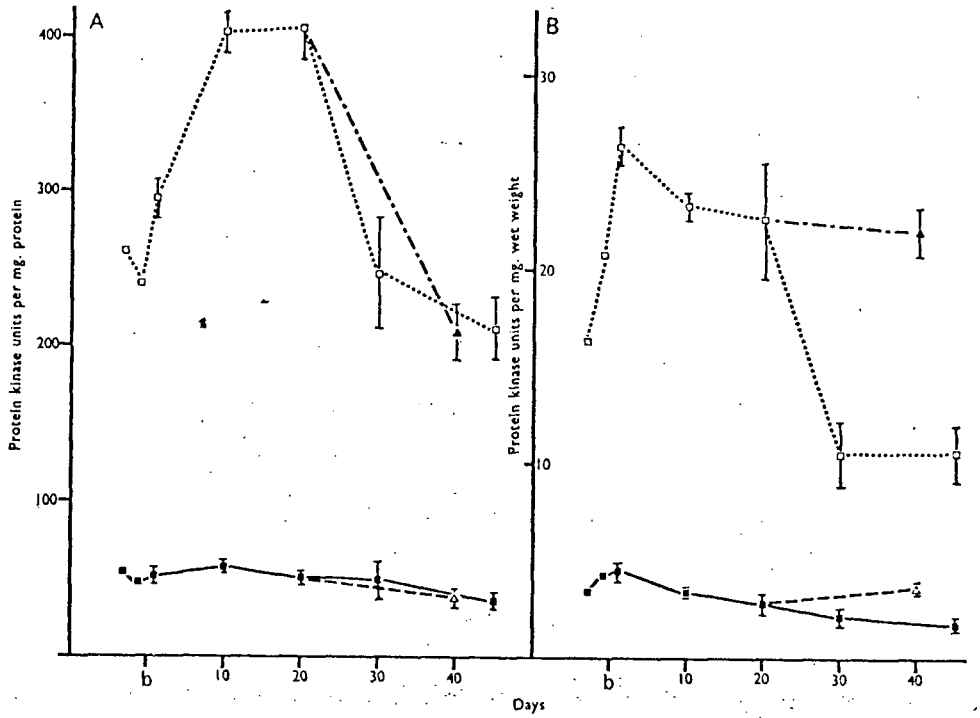


FIG. 28—Developmental changes in protein kinase in interscapular brown fat. Activity was measured in the absence (■) and presence (□) of 5 μ*M* cyclic AMP. ▲, (△), Represent cyclic AMP-dependent and cyclic AMP-independent activities respectively in rats exposed to cold (4° C.) for 20 days. Activity is expressed on a tissue-protein basis (A), and on a tissue-wet-weight basis (B). Vertical bars represent the S.E.M. of 6–10 samples.

continued to rise up to the 10th postnatal day. In both cases, a significant decrease in activity occurred between the 20th and 40th day after birth. In cold acclimated rats, this decrease did not occur when the activity was expressed on a tissue fresh-weight-basis (Fig. 28 B).

Subcellular distribution of the protein kinase activity was studied in BAT of newborn rats (Fig. 29). Cyclic AMP-independent kinase showed similar specific activities in all fractions studied, whereas the cyclic AMP-dependent activity was clearly highest in the 100,000 x g supernatant fraction. The bulk of both activities was associated with the same supernatant fraction, more remarkably so in the cyclic AMP-dependent

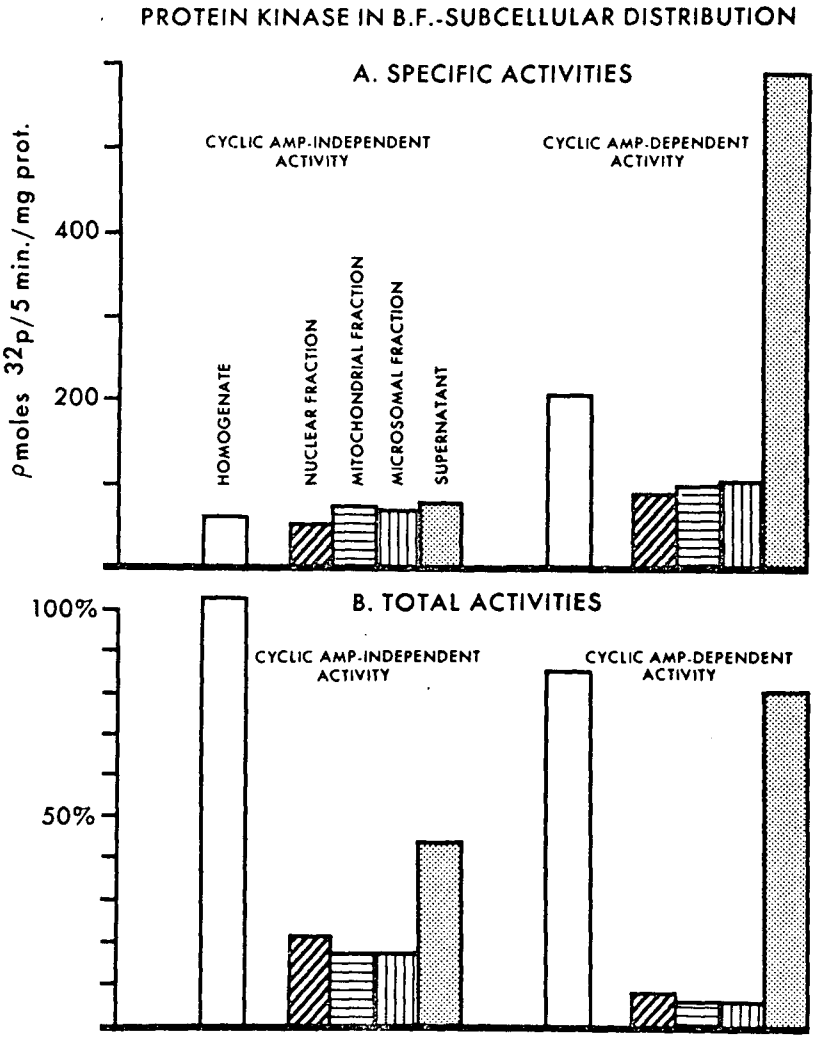


Fig. 29. The Subcellular Distribution of Protein Kinase Activity in Brown Adipose Tissue. 1-day-old animals were used. Activity was determined both in the absence and in the presence of 5 μM cyclic AMP. White columnes in B represent the recovery of the activity (tissue homogenate = 100%).

activity.

The cyclic AMP-dependent protein kinase activities measured in the present study are much higher than those reported by Kuo & Greengard (114) for BAT as well as other tissues of adult rat. Our assays were performed under conditions of low initial velocity (small amount of protein added) to avoid complications due to the presence of the apparent inhibitor described below. Perhaps this accounts for the difference between our values and theirs. On the other hand, since we have noted a dramatic decrease with age after day 20, it might equally be possible that activities decrease to the very low values in older rats used in studies of Kuo & Greengard (114).

(g) Protein kinase inhibitor.

The inhibitor in skeletal muscle can be freed of protein kinase activity by heating tissue extracts at 60°C; the inhibitory activity is stable under these conditions while the protein kinase enzyme is largely destroyed (220). We have tested the same properties of the BAT system. Fig. 30 A shows that indeed protein kinase activity is almost completely abolished by heating the 20,000 x g supernatants at 60°C for 5 minutes. Such heat-treated preparations were then tested for their ability to inhibit purified beef heart protein kinase both in the presence (Figs. 30 B and 31 A) and absence (Fig. 31 B) of cyclic AMP. Concentration-dependent inhibition was observed in both cases. Moreover, it seems that the inhibition is more pronounced in the absence of cyclic AMP (Fig. 31 A). It must be stressed, however, that 4 times more of enzymic protein was used in the absence of cyclic nucleotide

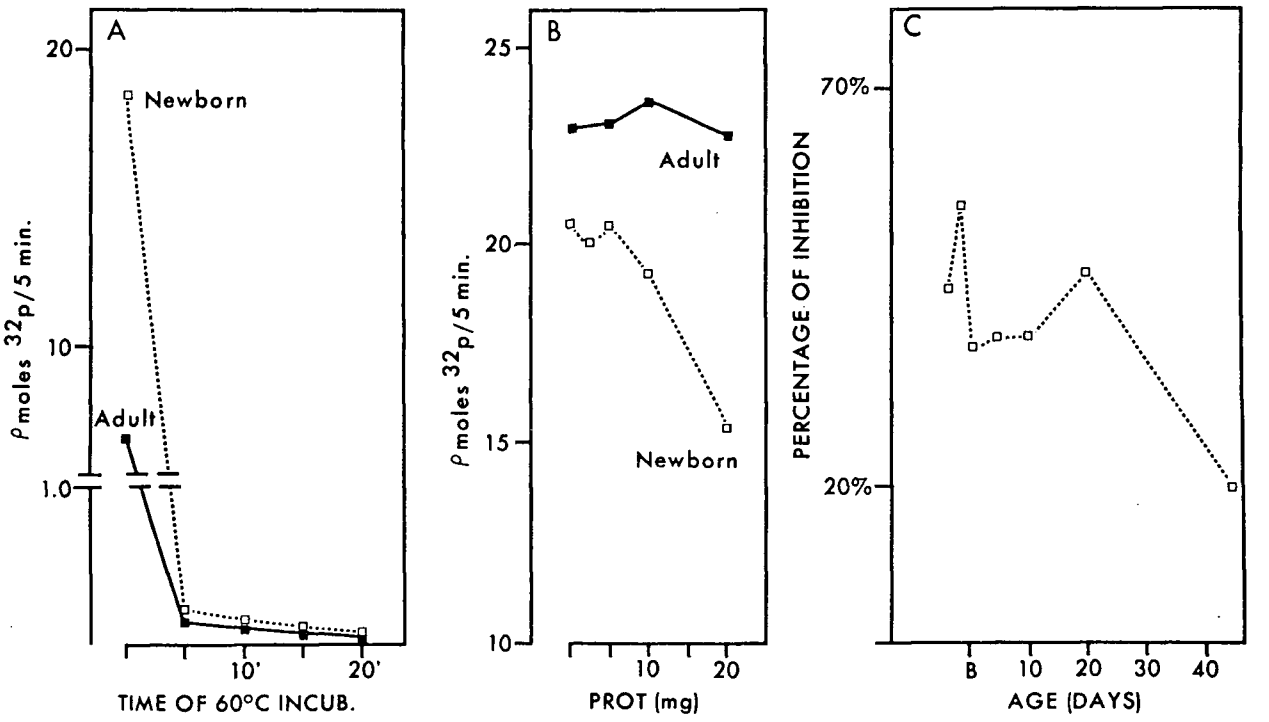


Fig. 30. A. The Effect of Heat-treatment on Protein Kinase Activity in Brown Adipose Tissue from Newborn and Adult Rats. B. The Effect of Heat-treated Brown Fat Preparations from Newborn and Adult Rats on the Activity of Purified Beef Heart Protein Kinase. C. The Percentage of Inhibition of the Activity of Purified Beef Heart Protein Kinase by the same Amount of Heat-treated Brown Fat Preparations from Rats of Different Ages. 100 μg of BAT preparations and 1.3 μg of beef heart enzyme were used. 5 μM cyclic AMP was added in all three experiments.

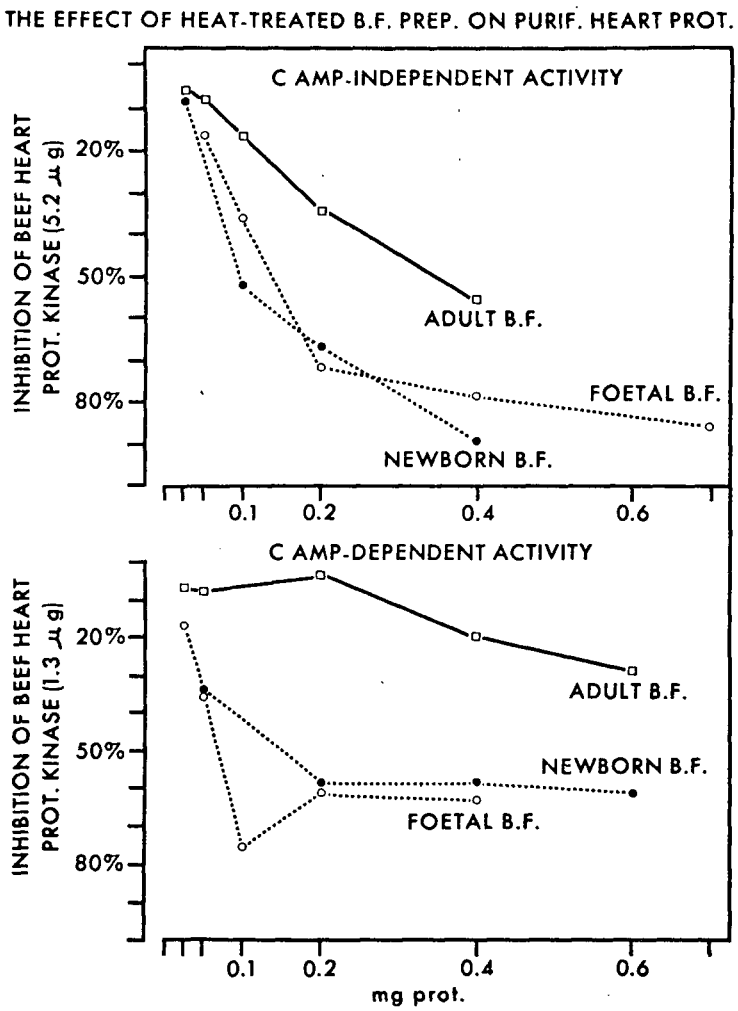


Fig. 31. The Effect of Heat-treated Brown Fat Preparations from Fetal, Newborn, and Adult Rats on the Activity of Purified Beef Heart Protein Kinase. Activity was determined both in the absence and in the presence of cyclic AMP (5 μM).

in order to obtain the same order of velocity and this might in fact, be the reason of the higher degree of inhibition under this condition; more of the enzymatic protein was available for the inhibitory action. This explanation is also suggested by Ashby & Walsh (7) in their studies of the skeletal muscle system. It is important to point out that the inhibitory activity of heat-treated brown fat preparations differed according to age of the animals: it was much more pronounced in preparations from late fetal and newborn animals than in preparations from 45-day-old rats.

As shown in Fig. 32, the kinetics of inhibition was studied. Since both V_{max} and K_M in respect to ATP were clearly affected, a combination between competitive and non-competitive types of inhibition seem to be present. Even though these experiments did not clarify the precise means of inhibition, they ruled out the possibility that competition for substrate (ATP), such as could be expected if the inhibition were due to ATPase activity, was present to any large extent.

Is the inhibitory activity of BAT preparations due to a large molecule, such as protein, or is it caused by some ionic or metabolite action? The heat-treated preparations were dialysed against 50 volumes of 4 mM EDTA for 12 hours with frequent change of the medium and the inhibitory activity of such dialysed preparations was tested. It can be seen from Fig. 33 A that no appreciable difference was observed between dialysed and non-dialysed preparations and the dialysed preparation again inhibited both the cyclic AMP-dependent (Fig. 33 A) and cyclic AMP-independent activity (Fig. 33 B) of purified beef heart protein kinase. Hence, a large

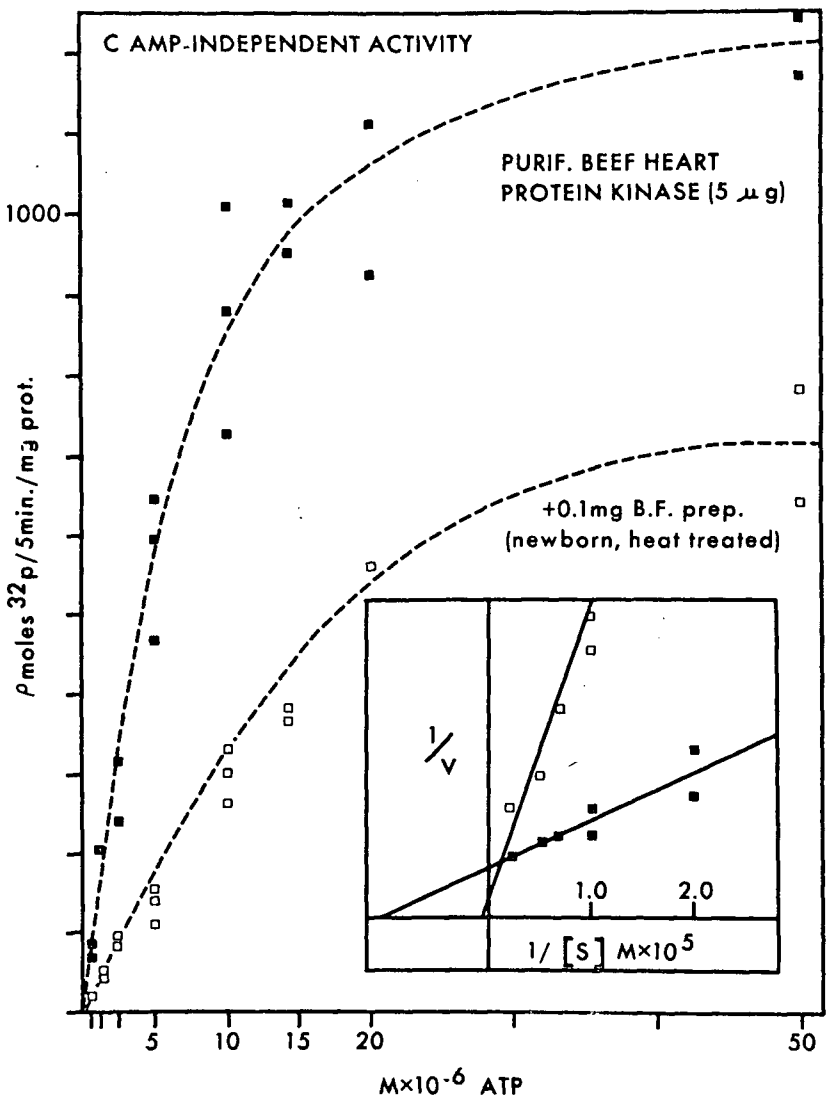


Fig. 32. The Effect of ATP Concentration on the Reaction Velocity of the Purified Beef Heart Protein Kinase in the Absence and in the Presence of the Heat-treated Brown Fat Preparation from Newborn Rats. Cyclic AMP (5 μM) was present.

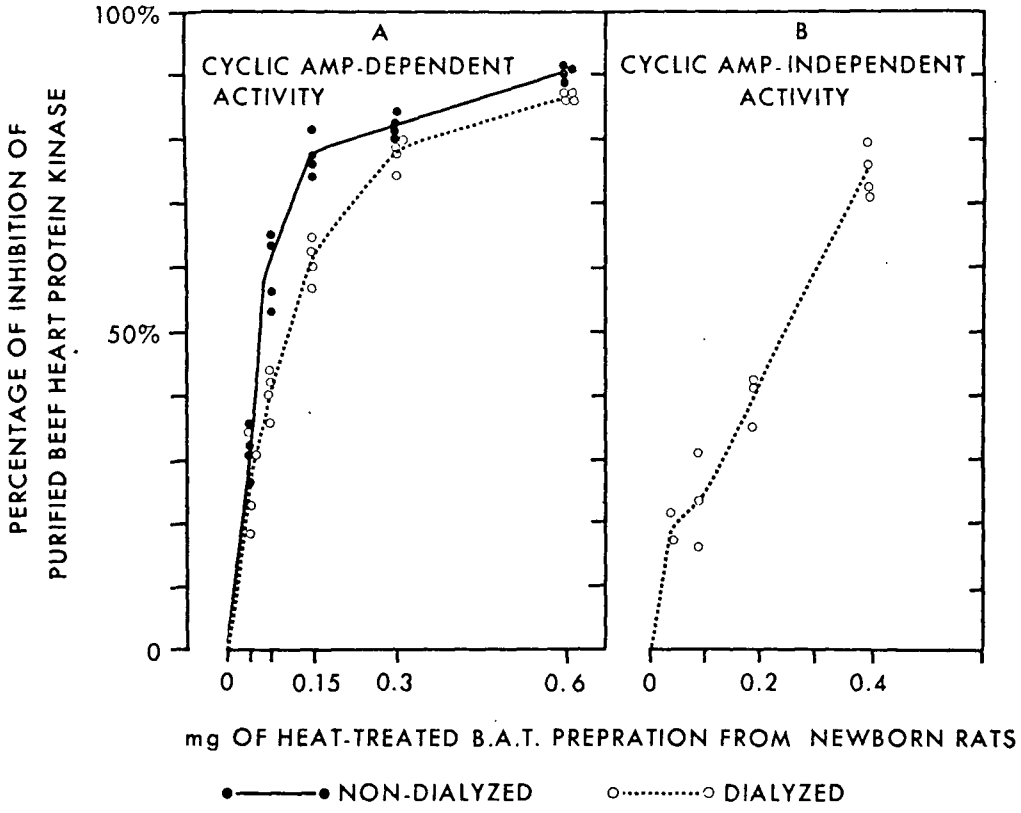


Fig. 33. The Effect of the Heat-treated and Dialysed Brown Fat Preparation from Newborn Rats on the Purified Beef Heart Protein Kinase. A. The activity of 1.3 μg of the enzyme was assayed in the presence of 5 μM cyclic AMP. B. The activity of 10.0 μg of the enzyme was assayed in the absence of cyclic AMP.

molecule, presumably a protein is the inhibitor.

The possibility that the inhibitory action of the brown fat preparations was due to a proteolytic action exerted upon the protein kinase molecule was tested in the following experiment. Purified beef heart enzyme was preincubated in the presence of the brown fat heat-treated preparation for 5 - 45 minutes in the absence of substrate (ATP). Then the substrate was added and assay incubation run for an additional 5 minutes in each case. A half-maximum inhibitory dose of the brown fat preparation was used in each assay. If the inhibition was due to proteolytic action, one could expect that the longer the period of preincubation, the higher the degree of inhibition. As can be seen in Fig. 34, this was not the case with the brown fat preparations. Protein kinase activity in the presence of the inhibitory preparation stayed unaltered regardless of the time of preincubation. Protein kinase in the absence of the inhibitor, on the other hand, showed a slight decrease in activity with time of preincubation. This difference may be due to a protective effect of the inhibitor on the kinase.

The subcellular distribution of the inhibitory activity in BAT of newborn rats was then tested. As shown in Fig. 35, the bulk of activity was associated with the 100,000 x g x 60 minutes supernatant fraction. Activity observed in the nuclear and mitochondrial fractions could be due to contamination of these fractions since no rewashing of the pellets was performed in this experiment.

The preparation procedure for the brown fat inhibitory protein was then modified in the following way. The 100,000 x g x 60 minutes supernatant of BAT homogenate in 4 mM EDTA, pH 7.2 was incubated for 5

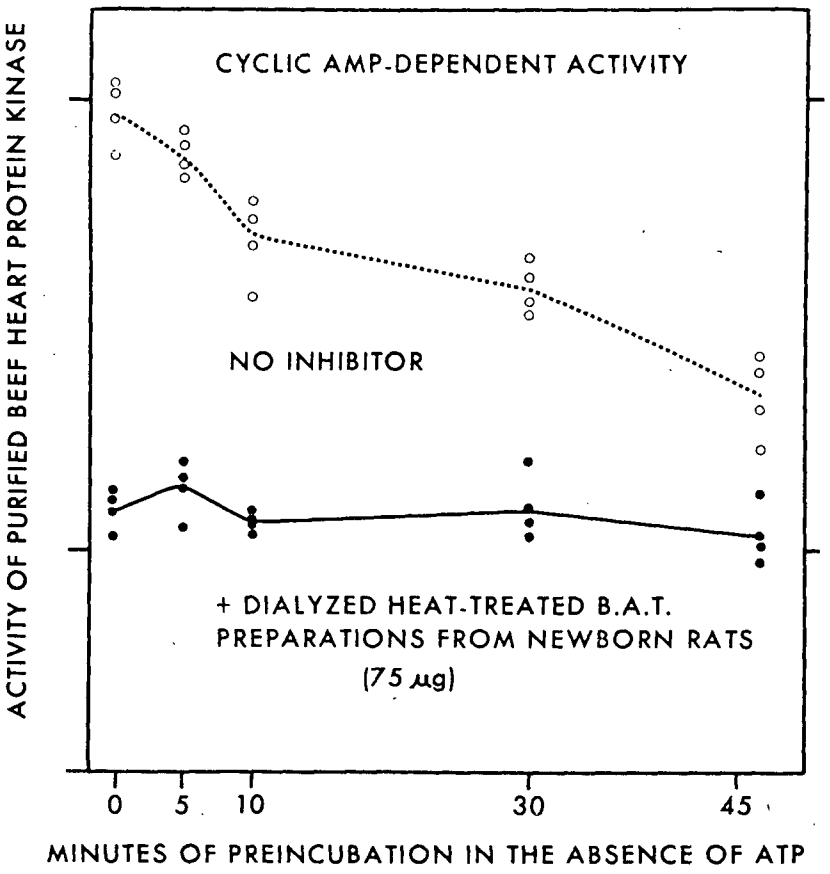


Fig. 34. The Effect of Preincubation of the Purified Beef Heart Protein Kinase with the Brown Fat Preparation in the Absence of Substrate (ATP) on the Degree of Inhibition. 1.3 µg of the enzyme was incubated with 75 µg of the heat-treated and dialysed brown fat praparation from newborn rats in the presence of 5 µM cyclic AMP for 5 - 46 minutes at 37° C. The assay incubation with ATP was then carried out for 5 minutes in each group.

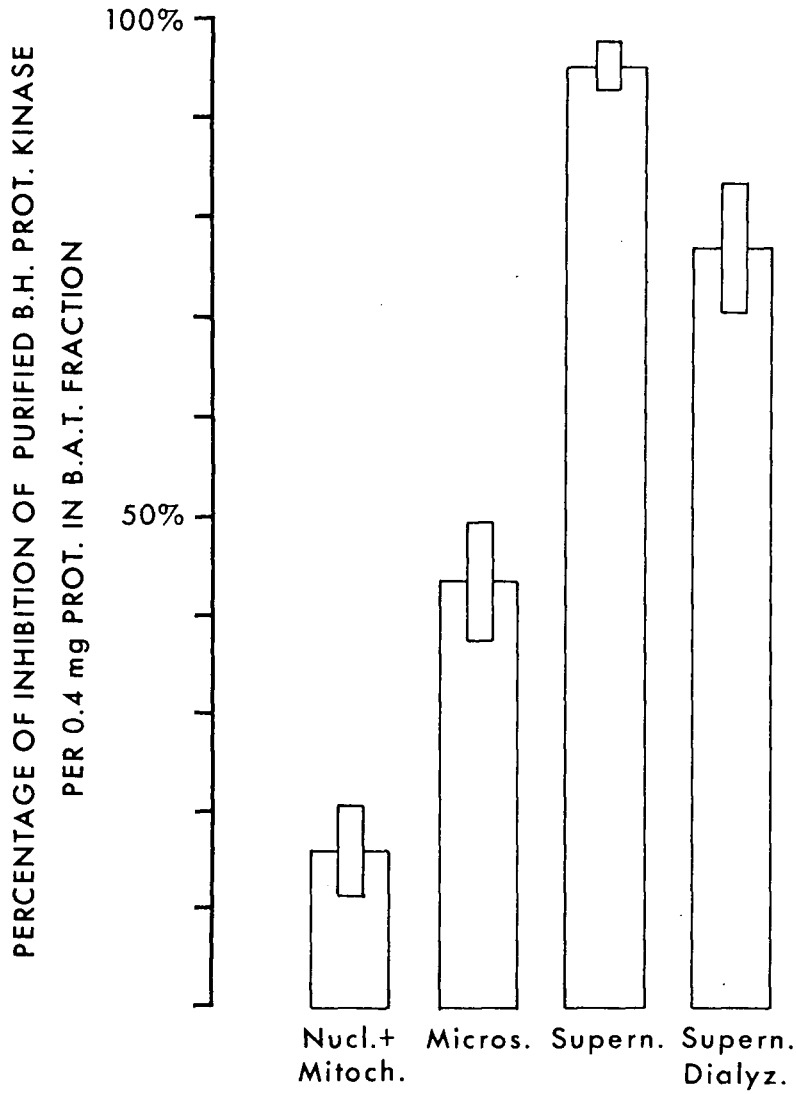


Fig. 35. The Subcellular Distribution of the Inhibitor of Protein Kinase in Brown Adipose Tissue of Newborn Rats. 200.0 and 400.0 µg of protein from each fraction were tested with 5.0 µg of the purified beef heart protein kinase in the presence of 5 µM cyclic AMP.

minutes at 60°C. The precipitated protein was spun down and the supernatant was then dialysed against 50 volumes of the same medium (the medium was changed at least 5 times) for 12 hours at 2-4°C. The dialysed preparation was rapidly frozen and kept at -60°C until use.

The final question was whether the BAT inhibitory protein exerts its effect upon brown fat protein kinase as well as upon the purified beef heart enzyme. Since we could not possibly attempt purification of the protein kinase from BAT because of small amount of material, another approach was required to ensure that the endogenous inhibitory protein in the crude kinase preparation would not interfere with the inhibitor assay. As shown in Fig. 31, BAT of 45-day-old rats contains relatively minor inhibitory activity as compared with the tissue of perinatal rats. Hence, we have prepared the inhibitor from newborn rats and used a crude enzyme preparation from 45-day-old rats. As shown in Fig. 36, both the cyclic AMP - dependent and cyclic AMP - independent protein kinase activities were clearly inhibited. Hence, we have confirmed that the inhibitor from BAT is capable of inhibiting the BAT enzyme.

Simultaneously, with our studies, Ashby & Walsh (7) proceeded with studies on the skeletal muscle protein kinase inhibitor and most recently published the results. They were able to confirm by some additional means that the inhibitor does not interfere with protein kinase activity by acting as an ATPase, demonstrated that the inhibitor is a distinct unit in addition to the cyclic AMP - binding subunit of the enzyme and that it is not affected by the nucleotide. Similar inhibitory activity was ob-

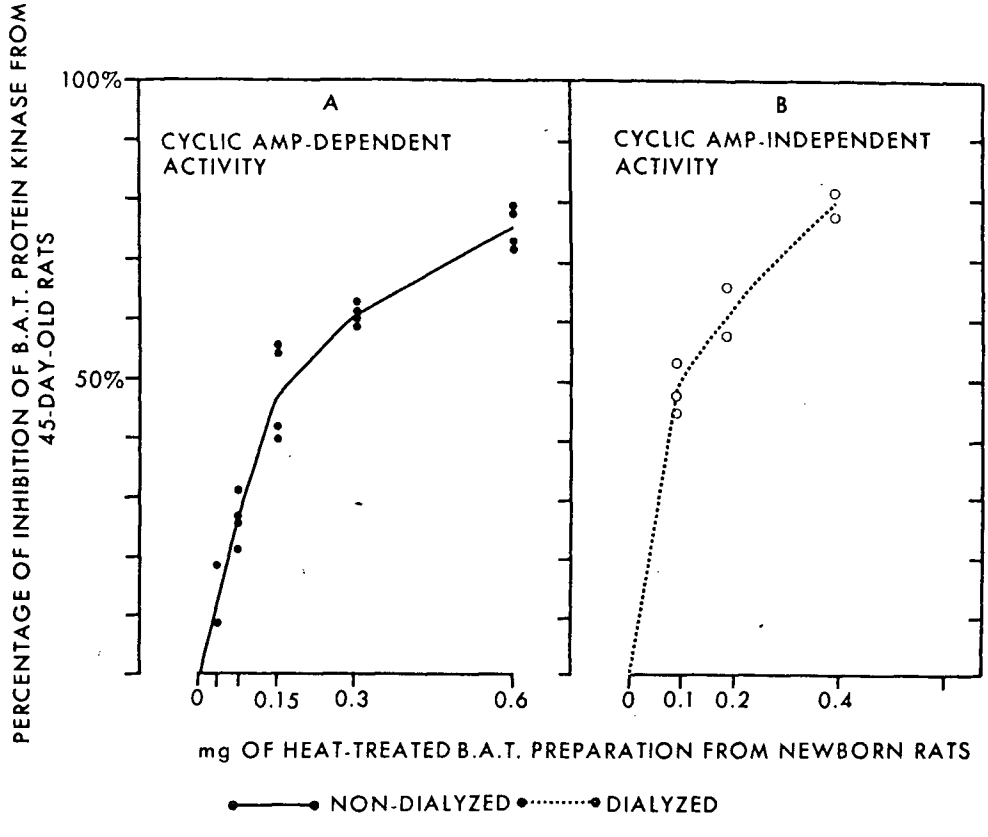


Fig. 36. The Effect of the Heat-treated and Dialysed Brown Fat Preparation from Newborn Rats on the Protein Kinase Activity in Brown Adipose Tissue of 45-day-old Rats. The 20,000 x g x 20 min. supernatant (70 µg protein) was used as enzyme preparation. A. Cyclic AMP (5 µM) present. B. No cyclic AMP added.

served in several tissues and a partial purification from rabbit skeletal muscle was achieved (220).

One very important finding in our experiments deserves to be pointed out. The inhibitory activity is 2 - 4 times as high in the perinatal period than it is later in life as demonstrated in Fig. 31. We have also attempted to correlate at least tentatively, the inhibitory activity with age in a few experiments as shown in Fig. 30 C and again the highest degree of inhibition was observed in preparations from perinatal rats. This fact may be of help in assessing the role of the inhibitor, which presumably has some general function as indicated by its wide-spread distribution.

CONCLUDING REMARKS

The prenatal increase in tissue glycogen levels in several organs is followed by a rapid fall immediately after parturition (41). This seems to be a general adaptation mechanism by which the newborn copes with the sudden termination of maternal energy supply. BAT is no exception in this respect. The glycogen content dropped by more than 90% simultaneously with the occurrence of highest phosphorylase a and activated phosphorylase kinase activities. Thus, it seems that conversion of phosphorylase kinase to the activated form (by protein kinase) with subsequent activation of phosphorylase is responsible for the glycogen break-down in BAT. There seems to be no information available on the activity of glycogen synthetase in BAT. Since cyclic AMP via protein kinase has been shown to convert glycogen synthetase to its less active form by phosphorylation occurring simultaneously with activation of phosphorylase b kinase in some other tissues (198), one might assume that a similar mechanism exists in BAT which may participate in the observed decline of glycogen level at parturition. The close temporal relationship between the drop in glycogen level, peak activity of phosphorylase a, peak activity of phosphorylase b kinase in the activated form can be extended to the high level of protein kinase activity and a peak level of cyclic AMP observed in BAT immediately before delivery, thus indicating an activation of the whole pathway. It remains an open question as to which hormone is responsible for adenyl cyclase stimulation immediately before delivery. NE produced more stimulation of adenyl cyclase in 1-day-old animals than did glucagon, even though there was

some degree of activation observed with the latter hormone as opposed by no activation observed in 10-day-old animals. It seems to us that NE is the most likely triggering agent in BAT. Its role in stimulation of glycogenolysis may also be inferred from the fact that glycogen accumulates in BAT after severance of the adrenergic nerve supply (175) and from the fact that a dramatic perinatal drop occurs in the NE content of BAT (76).

Thus far we have related the whole chain of enzymes involved in the 'hormonal stimulation pathway' only to glycogenolysis and the glycogen content of the tissue. Even though we observed a very good correlation in the developmental changes of all parts of the pathway to this particular target function, this does not mean that all members of the pathway possess only one function. Particularly,the 'cyclase-cyclic AMP-protein kinase' part of the chain seems to be a common pathway for some other target functions in addition to glycogenolysis as discussed in the Introduction.

Fatty acids are the main substrate for BAT mitochondria during heat generation (155, 156, 157, 158). In addition to their role as the supply of substrate, they are probably also responsible for a very important characteristic of BAT mitochondria - uncoupling of the respiration from production of ATP (91). By this mechanism, the extremely rapid rate of respiration occurring in BAT mitochondria of infant or cold acclimated animals is not accompanied by the production of large amounts of ATP, but rather by production of heat, which is certainly more meaningful in a tissue with heat generation as the main function. Thus, hydrolysis of triglycerides can be considered as a very important process in BAT, providing free fatty

acids both as a substrate and as an uncoupling agent. In agreement with this assumption is the fact that the activity of a hormone sensitive lipase in BAT increases after birth (74). Hormone-sensitive lipase is activated by protein kinase which, in turn, is activated by cyclic AMP, provided by adenyl cyclase. The perinatal increase in adenyl cyclase observed in BAT, together with the perinatal peak in cyclic AMP content and the perinatal increase of protein kinase activity seem, therefore, also to fit into the development of the lipolytic pathway, in addition to their participation in glycogenolysis.

The results in Part III indicated a possible hormonal involvement in tissue proliferation and differentiation processes. As discussed in the Introduction, there is enough evidence in the literature to support this assumption and also to suggest the mechanism(s) by which such a hormonal control can be exerted upon the transcriptional and translational processes of a cell. If, indeed, the hormonal receptor pathway participates in the tissue maturation, then from the developmental patterns presented in this section, we could expect that the rate of tissue maturation would have to be highest at the perinatal stage of development also. This certainly seems to be the case and thus, our data on development of the hormonal stimulation chain of events can be related to BAT differentiation and proliferation as well as to glycogenolysis and lipolysis.

SUMMARY

1. Adenyl cyclase was studied in BAT homogenate, subcellular fractions and homogenates of isolated adipocytes. In 12-day-old rats, it was found to be localized mainly in the microsomal fraction, containing probably fine cell membrane fragments, and to be selectively stimulated by catecholamines, via a beta-adrenergic catecholamine receptor. In 1-day-old animals, some activation by glucagon and ACTH was observed similar to that observed in 45-day-old animals. No NE-sensitivity was observed 4 days prior to parturition, even though the basal and NaF-stimulated enzyme was already developed. The activity increased postnatally, most rapidly up to the 20th day. Cold exposure increased the activity per fresh weight but not the specific activity.
2. A sharp rise in the cyclic AMP content of BAT was observed in the latest stage of intrauterine life, followed by a drop after delivery and continuing decrease up to the 50th postnatal day. Cold exposure resulted in a significant increase.
3. Phosphorylase and phosphorylase kinase were both present,primarily in the activated form prenatally and both activities increased significantly at parturition; then declined, with more enzymes present in the inactive form. Non-activated form of phosphorylase kinase continued to increase for 20 postnatal days. Cold exposure resulted in an increased total phosphorylase activity.
4. Cyclic AMP - dependent protein kinase activity increased perinatally

and decreased after day 20. Cold exposure increased the activity if expressed per fresh weight. The enzyme was found in all subcellular fractions, the bulk being associated with the high-speed supernatant.

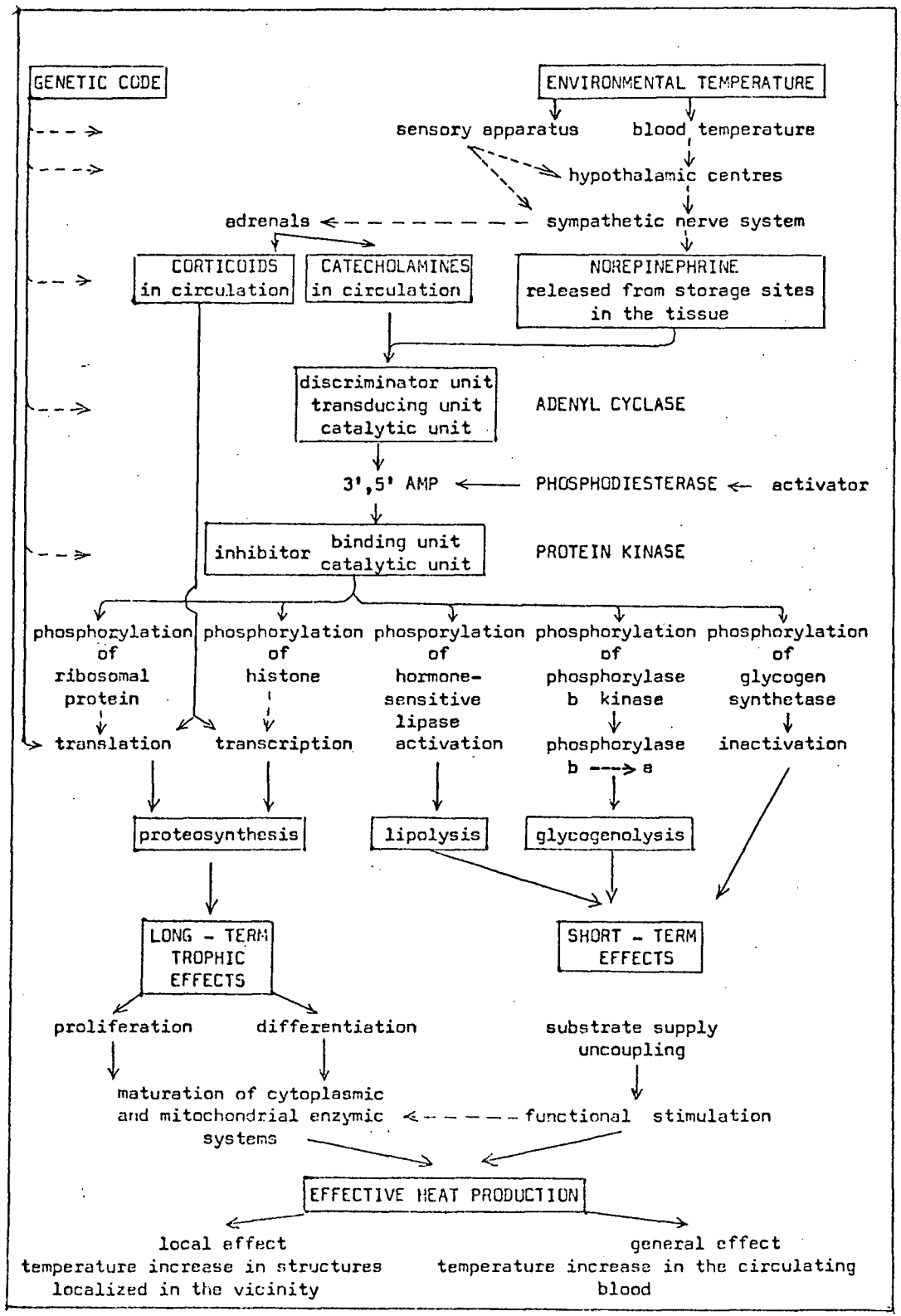
5. An inhibitor of protein kinase was found in the same supernatant fraction. It was heat-stable (in contrast to protein kinase), non-dialysable and it inhibited significantly both the cyclic-AMP-independent and cyclic-AMP-dependent activities of purified beef heart enzyme and non-purified BAT enzyme. Kinetic studies showed a combination of competitive and non-competitive inhibition and ruled out proteolytic action of the inhibitor. Inhibitory activity was highest in BAT from late fetal and neonatal rats and had decreased considerably by the 45th postnatal day.

PART V

INTERPRETATION

On the basis of the results presented in this communication and on the basis of literary data, we can now attempt to build a scheme of the regulatory mechanisms responsible for both the immediate hormonal stimulation of BAT and for its maturation in the rat. In some parts, the scheme will contain data proved beyond any doubt. In some parts, only speculative connections will be proposed. We are aware that any such scheme at the present time will be grossly incomplete, presumably lacking many functionally important connections and factors. In some parts of the scheme, we will have to omit known pathways and details in order to keep the scheme as clear as possible. The scheme is presented in page 159.

The environmental temperature has been demonstrated to be a stimulus for functional activation of BAT (197). We have shown that the ambient temperature is a stimulus also for tissue proliferation, at least in animals of three weeks of initial age. Moreover, we have obtained some evidence that the lower ambient temperature suddenly encountered after birth may also be a stimulus. The connection between environmental temperature and brown fat adipocytes remains an unsolved problem. Some evidence seems to point to a possible connection via the hypothalamus and the sympathetic nervous system leading to activation of postganglionic endings in the vicinity of the adipocyte (for review see 30). Also, the temperature of circulating blood seems to play some role since some temperature-sensitive cells have been described in the hypothalamus (30). The adrenals are also included in our scheme as an organ responding to any stress situation and, therefore, also to the acute cold stress. It seems


GENETIC CODE
ENVIRONMENTAL TEMPERATURE
sensory apparatus
blood temperature
hypothalamic centres
adrenals
sympathetic nerve system
CORTICOIDS in circulation
CATECHOLAMINES in circulation
NOREPINEPHRINE released from storage sites in the tissue
discriminator unit
transducing unit
catalytic unit
ADENYL CYCLASE
3',5' AMP
PHOSPHODIESTERASE
activator
inhibitor
binding unit
catalytic unit
PROTEIN KINASE
phosphorylation of ribosomal protein
phosphorylation of histone
phosporylation of hormone-sensitive lipase
phosphorylation of phosphorylase b kinase
phosphorylation of glycogen synthetase
translation
transcription
activation
phosphorylase b ---> a
inactivation
proteosynthesis
lipolysis
glycogenolysis
LONG - TERM TROPHIC EFFECTS
SHORT - TERM EFFECTS
proliferation
differentiation
substrate supply uncoupling
maturation of cytoplasmic and mitochondrial enzymic systems
functional stimulation
EFFECTIVE HEAT PRODUCTION
local effect
temperature increase in structures localized in the vicinity
general effect
temperature increase in the circulating blood

that not only catecholamines, but also corticoids are implicated in BAT regulation (see Part III). Corticoids, as well as all steroids, do not act via the adenyl cyclase system; they exert their effect directly intracellularly either on the translational or the transcriptional level, the mechanism of which is not known in either case. Nevertheless, they are included in our scheme. On the other hand, we did not include thyroid hormones in spite of the possibility that they exert a 'permissive' effect upon BAT (e.g. 122, 123, 197, 200). The evidence for this is incomplete at the present time and we ourselves did not study the effect of thyroxine in enough detail as to allow for any meaningful conclusion. Nevertheless, it seems from our data (Part IV) that they do not exert their effect (if indeed there is any direct effect) via adenyl cyclase. The activation of the sympathetic nervous system, as well as the activation of the adrenals result in NE and possibly also epinephrine stimulation of brown fat adipocytes. Hence, it seems that the original stimulus of the environmental temperature has been 'translated' into a hormonal stimulation at the adipocyte level.

We have established in Part IV the enzymic pathways which compose together the chain of events connecting the initial hormonal contact with a cell with the final intracellular effect. These are all presented in our scheme since our results indicate that all of them are functional in BAT of the rat. Our results on adenyl cyclase support the concept of at least two building units of this system, and they even indicate that these may be synthetized independently at different stages of fetal development. Our observation of an inhibitor of protein kinase supports the concept of a complex

regulatory nature of this enzymic system. Moreover, the fact that the inhibitory activity undergoes developmental changes may be of importance in search for its function.

Finally, our observations showed a temporal positive correlation between the individual enzyme components of the whole pathway and the final results such as the level of glycogen and the rate of lipolysis as well as the rate of tissue maturation.

Mitochondrial maturation as documented in Part II seems to dominate the developmental pattern of BAT. We have presented literary evidence in support of the connection between protein kinase and the processes of tissue proliferation and differentiation. Is it possible to visualize any connection between protein kinase activation and the synthesis of mitochondrial respiratory chain components in BAT? Mitochondria have been proved to be capable of autonomous synthesis of nucleic acids (19, 131, 178), glycolipids (24, 26), proteins (20, 26, 107, 142, 167) and glycoproteins (25, 210). The effects of cyclic AMP were studied on protein, glycoprotein, RNA and DNA synthesis in isolated liver mitochondria in vitro (27). It was found that cyclic AMP at high concentrations (1-10mM) increased the rate of protein and RNA synthesis and inhibited or had no effect on DNA synthesis by isolated mitochondria. Even though the high doses used warrant caution in the interpretation, they underline the possibility that proteosynthetic processes can be affected by a hormonal mechanism also at the mitochondrial level. Another support for this possibility comes from the results on the adrenals, where ACTH was observed to stimulate mitochondrial RNA syn-

thesis (144, 145). Hence, we have proposed a connection between hormonal stimulation and the synthesis of mitochondrial components in our scheme.

All the above events can finally lead to so-called 'short-term effects' and 'long-term effects'. The former term represents such immediate hormonal effects as glycogenolysis, lipolysis and stimulation of tissue respiration. The latter term can be thought of as a preparation of the tissue for its function, e.g. a rapid synthesis of mitochondrial components in order to reach the very high content of cytochromes and flavoproteins within the well organized and closely packed cristae. It can be noted that a te ntative connection has been inserted in between these two groups of effects. It acknowledges a theoretical possibility that continuous functional stimulation of a tissue may, in fact, be somehow responsible for its further development; it may stimulate an 'adaptation' to the high functional demands.

There is a good deal of evidence that cyclic AMP is an intracellular mediator of the rapid action of ACTH on corticoidogenesis (see 145). In addition, recent evidence indicates that cyclic AMP is also a mediator of the long-term trophic action of ACTH on both the maintenance and stimulation of adrenal growth associated with stimulation of both nuclear and mitochondrial RNA synthesis (144). Hence, in the adrenal gland ACTH seems to have both types of effects. This supports our hypothesis that in BAT of the rat, NE is capable of regulating both, the immediate functional response and the long-term, trophic tissue response.

It can be noted that besides the environmental temperature as a stimulus, the genetically coded information was included in our scheme.

Since the tissue commences its proliferative processes well before delivery, the environmental temperature cannot possibly be the only initial stimulus. A coded genetic information has to be considered, even though it may act indirectly, perhaps via developmental changes in hormonal secretion. The developmental difference, in respect to the age and degree of maturation at birth, observed in BAT developmental pattern among several mammalian species (for review see 17) seem to be in support of such a genetically coded regulation. At which level and by what means is the genetical code expressed during development? The lack of any evidence is indicated in our scheme by possible connections at several levels: it can be the genetically coded maturation of the sensory organs, maturation of the hypothalamic centres, maturation of the endocrine system, maturation of the hormonal receptor unit, developmental appearance and disappearance of the protein kinase system components and obviously the information encoded directly in the nucleus of an adipocyte.

It will probably take years before all the blank spots in our scheme will be filled in and before all the interrelations will be clarified. Nevertheless, we feel that up to this stage of our research project, an important first step towards the final understanding of developmental regulatory mechanisms was completed: we have proved that BAT is a very suitable model organ and we have layed down the foundations on which future, more sophisticated experiments can be based.

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