DEVELOPMENT OF PYRUVATE DEHYDROGENASE IN WHITE FAT, BROWN FAT AND LIVER OF THE RAT

by

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I ABSTRACT

The total activity and the fraction of the enzyme in the active form of pyruvate dehydrogenase was assayed in white fat, brown fat and liver throughout the development of the rat.

In white adipose tissue, the total activity of pyruvate dehydrogenase and the fraction of the enzyme in the active form did not change significantly during development.

In brown adipose tissue, the total activity increased until the late suckling period. After weaning, a decrease was noted. The fraction of the enzyme in the active form did not increase until after ten days of age, reached its highest level in the late suckling period and remained at this level after weaning.

Pyruvate dehydrogenase in liver decreased in both total activity and percentage activity in the early neonatal period. Both parameters increased after this period, reaching their highest levels in the late suckling period.

In both fetal liver and fetal brown fat, the total activity of pyruvate dehydrogenase was increased by *in vitro* incubation with insulin.

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Acetyl-CoA	Acetyl Coenzyme 'A'
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
cAMP	Cyclic Adenosine Monophosphate
EGTA	Ethylene Glycol Tetracetate
FAD	Flavin Adenine Dinucleotide
KRB	Krebs-Ringer Bicarbonate
NAD^+	Nicotine Adenine Dinucleotide (oxidized)
$NADH,H^+$	Nicotine Adenine Dinucleotide (reduced)
PDH	Pyruvate Dehydrogenase
PDHa	Pyruvate Dehydrogenase (active)
PDHb	Pyruvate Dehydrogenase (inactive)
PDHt	Pyruvate Dehydrogenase (total)
PEPCK	Phosphoenolpyruvate Carboxykinase
TAT	Tyrosine Aminotransferase
TPP	Thiamine Pyrophosphate
n	nano-
u	micro-
m	milli-
g	gram
l	litre
M	molar

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VII INTRODUCTION

Until recently, animal development has been investigated largely from the morphological point of view. In 1931, Joseph Needham wrote Chemical Embryology and concluded in his chapter, "Enzymes in Ontogenesis", that it would remain for some time the most unsatisfactory part of the book. Improved biochemical techniques have, however, allowed major advances in this field of research. Patterns of development are known for many enzymes as well as some of the stimuli which control these patterns although the action of these stimuli remain obscure.

The changing environment during fetal, neonatal and adult life provides some of the stimuli which control development. Although changes in enzyme activity may precede an environmental change, they often follow one and an unnatural environment can sometimes alter an enzyme's development. The response of an enzyme to an environmental stimulus is, however, limited by the maturity of the tissue which is controlled by the genome.

An example which illustrates the relationship between environment and genome is the development of the enzyme tyrosine amino transferase (TAT). This has been described by Greengard (1969). In the rat, this enzyme increases significantly in activity at birth. This increase can be prevented by delaying birth of by injecting glucose. The hypoglycemia which develops at birth stimulates the secretion of glucagon which increases enzyme activity through the intracellular messenger, cyclic adenosine monophosphate (cAMP). This particular response is

lost in older rats; the enzyme becoming, instead, sensitive to stress, adrenocorticotrophic hormone and cortisone.

If birth is one day premature, enzyme activity is still increased by the consequent hypoglycemia, but two days prior to term only glucagon or cAMP can stimulate activity. Three to four days before the natural end of gestation, only cAMP can increase the activity of TAT.

One can see that stimuli such as cAMP, glucagon or birth can effectively alter the time of appearance of increased enzyme activity but that their effect is limited by the ultimate control of the genome.

Another enzyme which responds to environmental stimuli is the mitochondrial enzyme, pyruvate dehydrogenase (PDH). It responds to a variety of stimuli including the hormone, insulin (Jungas and Taylor, 1972). Since PDH is also a key enzyme in carbohydrate metabolism, it was considered of interest to study the development of this enzyme and to determine the effect of insulin on this enzyme at various times during the maturation of tissues. PDH is one enzyme which may control the conversion of carbohydrates to fats. The tissues studied in this research, liver, brown fat and white fat; all have high rates of fatty acid synthesis.

A. The Environment of the Developing Rat

The emergence of enzymes or enzyme clusters resulting in new metabolic potentials should correlate with new physiological needs within the developing animal. To effectively study the development of a particular enzyme, it is necessary

to understand the changing physiological status as maturation progresses. Since the rat was the experimental animal used in this research, the following describes the physiological maturation of this animal.

The fetus is adequately supplied with glucose and amino acids during gestation. Enzymes involved in the synthesis of glucose and amino acids are not present or are at low levels until the end of gestation. Although fatty acids cross the placenta to some extent, fatty acid synthesis occurs at a high rate in the fetus (Hahn, 1970; Roux and Yoshika, 1970). In late fetal life, the enzymes involved in glycogen synthesis emerge (Ballard and Oliver, 1963; Busch et al., 1963; Jacquot and Kretchner, 1964). Glycogen is laid down extensively during the last days of gestation and provides the first source of energy for the neonatal animal. The animal is, thus, born with a limited source of energy.

Birth, in spite of glycogen stores, is followed by hypoglycemia. Nutrients now are ingested in the form of milk which is taken intermittently as opposed to the steady circulation of nutrients during fetal life. Milk is high in fat and protein and fat now becomes the major source of energy. Since tissues such as brain require a supply of glucose, the synthesis of glucose (gluconeogenesis) from glycolytic products and amino acids becomes necessary and the required enzymes emerge soon after birth (Ballard, 1970).

The next critical change in the life of the rat occurs at the time of weaning. This period begins at about fourteen days of age and is complete by thirty days. The normal solid

laboratory diet, unlike milk, is high in carbohydrates and low in fat and protein. Weaned animals require greater capability in handling exogenous carbohydrates as well as more interconversion of amino acids. Lipid synthesis again becomes an important functional necessity with the change in diet from high fat to low fat. The activities of glucokinase, aminotransferases and of enzymes providing lipid precursors increase significantly at this time (Greengard, 1971).

The development of an enzyme may not be uniform in the animal as a whole. Different organs mature at different rates and a specific enzyme may be required in one organ long before it is necessary in another. The changing physiological status of the rat, just described, correlates well with the functional maturation of the liver. Other organs may show different developmental patterns because of reaction to different environmental stimuli than has been described or because their function is necessary at a different stage in development. Brown adipose tissue, for example, responds to cold by producing heat. This function is unnecessary prior to birth and brown fat begins to develop only in the last days of gestation. White adipose tissue begins to develop after birth in the rat. Apparently the insulation and storage properties of this organ are not immediately necessary.

B. Pyruvate Metabolism in Development

Pyruvate can be metabolized in several ways (Figure 1). It can enter the gluconeogenic pathway through carboxylation by pyruvate carboxylase. Pyruvate carboxylase catalyzes the formation of oxaloacetate, the substrate for phosphoenolpyruvate carboxykinase (PEPCK). PEPCK catalyzes the formation of phosphoenol pyruvate, a precursor of glucose.

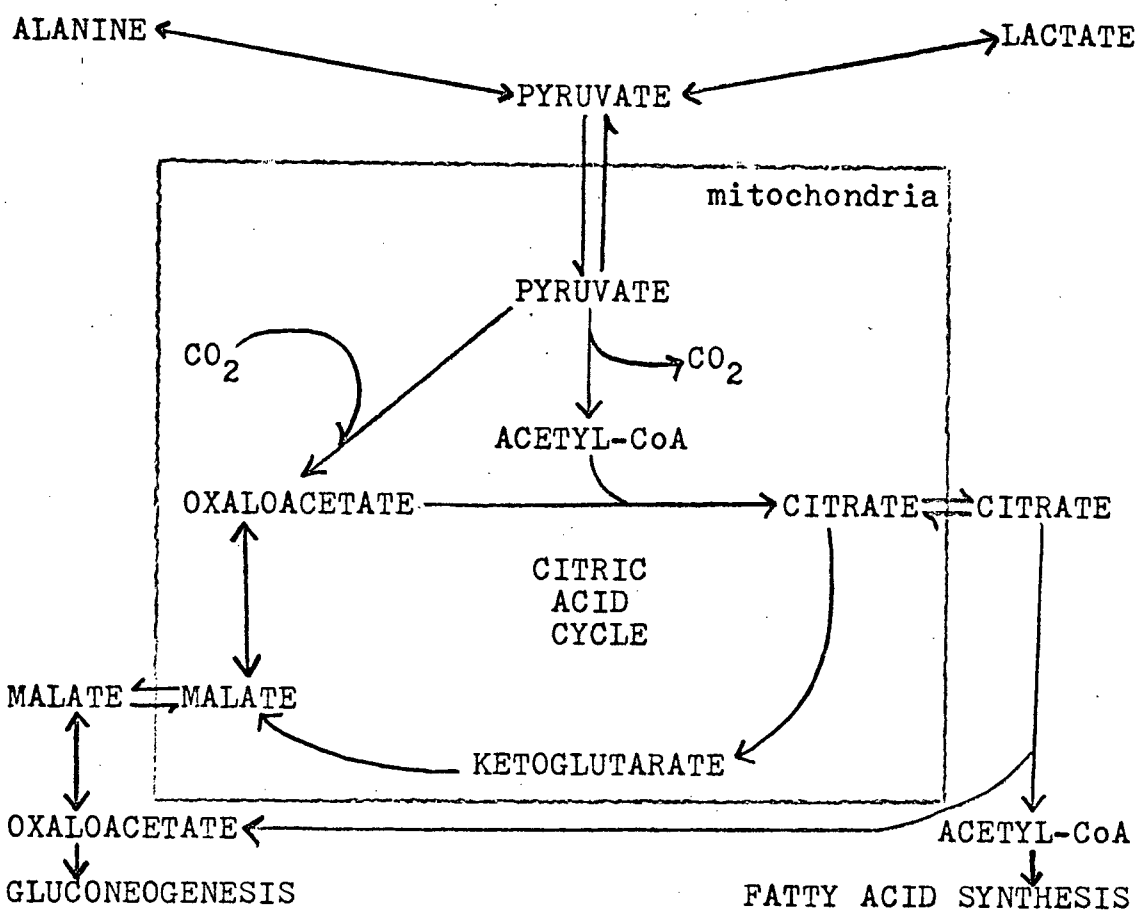
PDH catalyzes the oxidation of pyruvate, forming acetyl-CoA. Acetyl-CoA is a substrate for the citric acid cycle but in tissues such as liver and fat most of the acetyl-CoA leaves the mitochondria in the form of citrate. After cleavage of the citrate to acetyl-CoA and oxaloacetate in the cytoplasm, the acetyl-CoA can enter fatty acid synthesis.

Pyruvate can be aminated to form the amino acid, alanine. It can also be converted to lactate and, thus, provide a source of oxidized nicotine adenine dinucleotide (NAD^+).

As already mentioned, fatty acid synthesis, in contrast to gluconeogenesis and transamination, occurs at a high rate in fetal liver. After birth, there is a precipitous decline in the rate of fatty acid synthesis and an increase in the rates of gluconeogenesis and transamination reflecting both the necessity for replacing the nutrients formerly supplied by the mother and the change to a high fat diet of milk.

Pyruvate should, therefore, be oxidized to a greater extent in fetal liver and carboxylated to a greater extent after birth. Using the observation that the position of isotopic labelling in glutamic acid by pyruvate-2-C^{14} depends on

Figure 1. Metabolism of pyruvate.



the route of entry of pyruvate into the citric acid cycle, this hypothesis has been confirmed (Freedman and Nemeth, 1961). A specific assay for the enzyme, pyruvate carboxylase, which carboxylates pyruvate, forming oxaloacetate, has been used to show that this enzyme is, in fact, low in activity before birth and increases sharply postnatally (Yeung et al., 1967). PDH activity, which would represent the amount of pyruvate oxidized, has been shown to drop sharply after birth (Knowles and Ballard, 1974).

As the content of carbohydrate in the diet increases, one would expect more pyruvate to be oxidized and this has

been shown to be true by Knowles and Ballard (1974) in liver. Neither white or brown fat have been investigated.

C. Pyruvate Dehydrogenase in Development

Why study the development of PDH? Until the publication of the work of Linn et al. (1969) there was little in the literature about PDH. Since then, numerous papers have appeared but only two have dealt with the development of this enzyme (Hommes et al., 1973; Knowles and Ballard, 1974). Since PDH catalyzes the reaction which connects glycolysis with the citric acid cycle, its development is of major importance in understanding the maturation of carbohydrate metabolism.

PDH is regulated by a phosphorylation-dephosphorylation mechanism (Linn et al., 1969a, b). PDH, thus, limits the amount of carbohydrate to enter oxidative metabolism. Since acetyl-CoA is a precursor of fatty acids and cholesterol, PDH may be a rate-limiting enzyme in the conversion of carbohydrates to fat. Enzymes which are rate-limiting have been considered of key importance in measuring the maturation of a metabolic pathway. The formation of phosphoenolpyruvate from oxaloacetate, for example, is a rate-limiting step in gluconeogenesis and the emergence of the enzyme catalyzing this reaction, PEPCK, correlates exactly with the start of gluconeogenesis in the first day of life (Ballard and Hanson, 1967b).

PDH has also been shown to respond to insulin (Jungas, 1970, 1971; Denton et al., 1971; Coore et al., 1971; Weiss et al., 1971). This response is an increase in the fraction of the enzyme in the active form. Insulin may, in fact, increase the

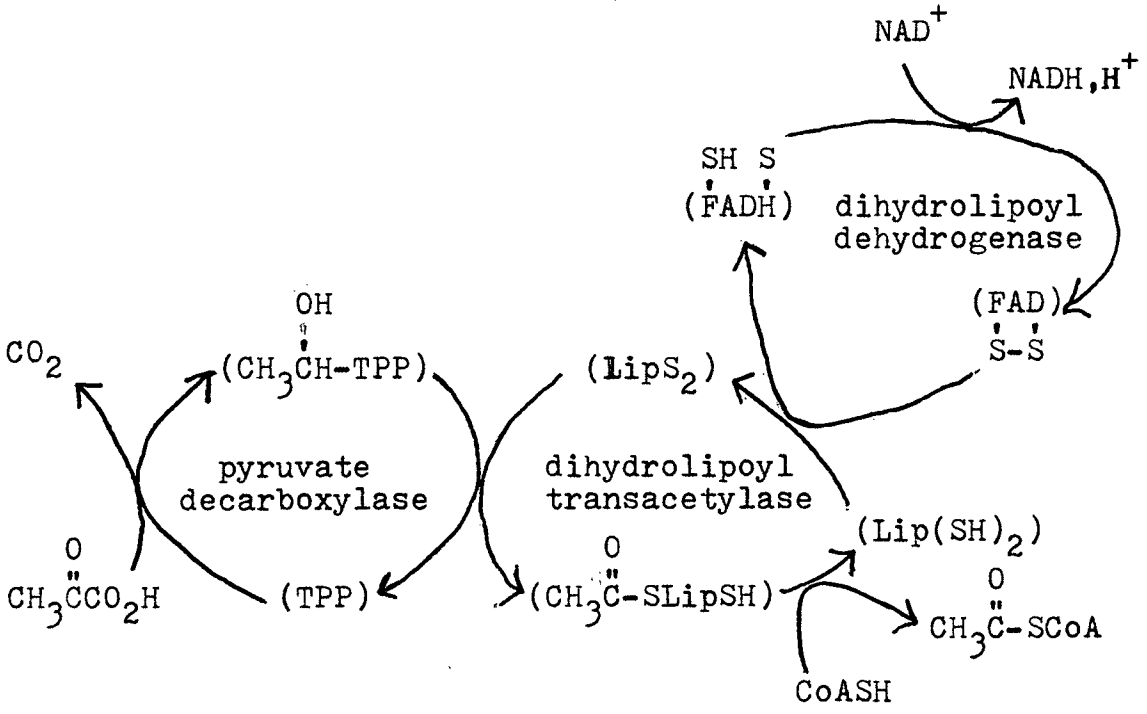
total amount of PDH as well as the amount of active PDH (Sica and Cuatracasas, 1973). An enzyme which may be responding to a hormone at the translational or transcriptional level is interesting to study during its development. Its development, if altered by the hormone, gives clues to the sequence of the enzyme's development as well as providing a basis for measuring the maturation of hormone receptors. If development of an enzyme is altered by a hormone, it is possible that an over-supply of the hormone at a critical stage of development may have unfavourable consequences.

Increased synthesis of acetyl-CoA prior to birth would be likely to result in increased fatty acid synthesis with obesity possibly resulting. Fatty acid synthesis is already functioning at a high rate prior to birth whereas the enzymes of the citric acid cycle, at least in rats, are low in activity until after birth (Dawkins, 1959).

D. Biochemistry of Pyruvate Dehydrogenase

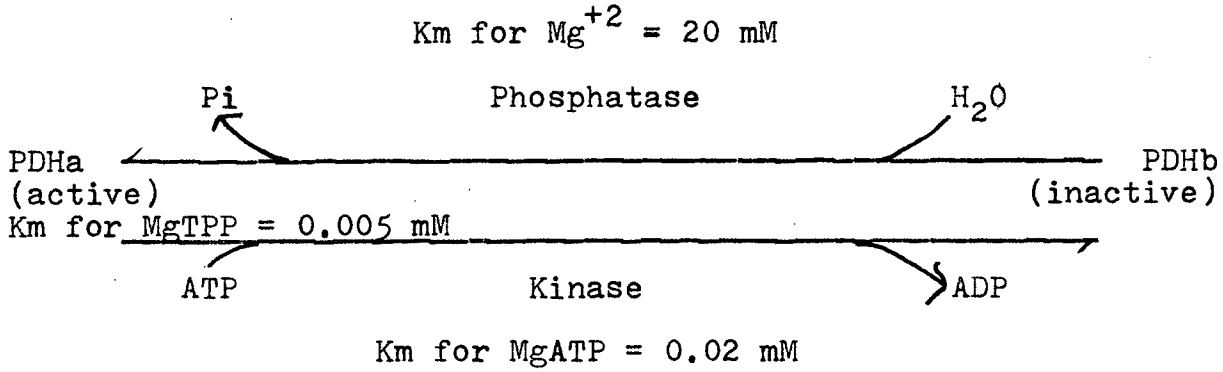
PDH catalyzes the decarboxylation of pyruvate, forming acetyl-CoA and releasing carbon dioxide. It is an enzyme complex, consisting of three catalytic enzymes and two regulatory enzymes (Figure 2). Five coenzymes are required. Three of these, thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD) and lipoic acid, are contained within the enzyme complex (Reed and Cox, 1966).

Figure 2. Reaction sequence in pyruvate oxidation.



The enzyme is regulated by a phosphorylation-dephosphorylation mechanism (Figure 3) (Linn et al., 1969a, b).

Figure 3. Regulation of PDH



Both the phosphatase and the kinase are contained within the enzyme complex but the phosphatase is bound less tightly. It is the enzyme, pyruvate decarboxylase, which undergoes phosphorylation and dephosphorylation. This enzyme has two subunits. The first catalyzes the decarboxylation of pyruvate and is regulated by phosphorylation-dephosphorylation. The second subunit catalyzes the reductive acetylation of the lipoyl moieties of the transacetylase using hydroxyethyl-TPP as a substrate and is not regulated.

The enzyme complex is considered to be totally activated by incubation for thirty minutes with 10 mM Mg^{+2}. This process is used to determine the total activity of the enzyme and will be referred to as PDHt. The activity of the enzyme (PDHa) is expressed as a percentage of total activity (PDHt).

E. Infants of Diabetic Women

One situation where insulin levels may be higher than normal is in infants of diabetic women. These infants tend to be both hyperinsulinemic and obese at birth (Francois et al., 1974). The obesity tends to continue postnatally. Children of diabetic parents have been assessed for weight. Fifty percent of males and twenty percent of females were more than thirty pounds overweight when the mother was diabetic. Only four percent of the males and two percent of the females were similarly obese when the father was the diabetic parent (Joslin's Diabetes Mellitus). This suggests that the uterine environment provided by the diabetic may predispose her offspring to obesity. One factor that could contribute to obesity

may be the prenatal hyperinsulinemia. Increased minsulin may increase the activity or even total amount of PDH, increasing the conversion of carbohydrates to fat.

The type of diabetes referred to as 'maturity-onset' is frequently associated with obesity. It is not clear whether diabetes causes, or results from, the obesity (Steiner, 1973). If obesity can cause diabetes, infants of diabetic women may develop diabetes because of the uterine environment as well as their genotype.

VIII METHODS AND MATERIALS

A. Rats

Wistar rats from Woodland Farms, Guelph, Ontario were used. Purina Chow was fed ad libitum. Rats were removed from the mother at thirty days of age. Rats less than thirty days of age were killed by cervical dislocation. Older rats and pregnant rats were killed by a blow to the head.

B. Treatment of Tissue

1. Developmental Experiments

Brown fat, gluteal white fat and liver were used in the developmental experiments. Tissue was rapidly excised and weighed. For each gram of tissue, 10 ml. of a 25 mM Sucrose:3.4 mM Tris:1 mM EGTA buffer (pH 7.4) was added. Only 3 ml. of buffer per gram was added to gluteal white fat. The tissue was homogenized in a Virtis "23" homogenizer with a teflon pestle and centrifuged in the cold for 15 minutes at 1200 rpm in an IEC Model PR-J centrifuge. The supernatant was removed by Pasteur pipette and frozen. Enzyme activity was assayed within four days.

2. Mitochondria

Mitochondria from liver and brown fat were separated by centrifuging the supernatant obtained above in the cold at 12,000 rpm for 20 minutes in an IEC Model B-20 centrifuge. The supernatant from this spin was discarded and the mitochon-

drial pellet resuspended in a 25 mM Sucrose:3.4 mM Tris:1 mM EGTA buffer (pH7.4). All samples were then frozen and assayed within four days.

3. Insulin Experiments

Tissue to be used in in vitro incubation experiments with and without insulin was removed rapidly and put into cold Krebs-Ringer-Bicarbonate (KRB) buffer (Krebs and Henseleit, 1932) containing half the recommended calcium concentration, no magnesium, 0.2% (w/v) albumin and 5.5 mM glucose. Before use the buffer was oxygenated with an O_2:CO_2 (95:5) gas mixture for thirty minutes and the pH was adjusted to 7.4 with 1 N NaOH.

The tissue was cut into small pieces and divided between two plastic vials. Two ml. of fresh KRB buffer was then added to each vial and O_2:CO_2 (95:5) was bubbled through for one minute.

After thirty minutes of preincubation at 37°C in a Dubnoff Metabolic Shaking Incubator, the buffer was replaced with fresh KRB and insulin was added to one of the two vials. Incubation was continued for one hour. The tissue was then rinsed in cold 25 mM Sucrose:3.4 mM Tris:1 mM EGTA buffer (pH 7.4) and treated as described in the section on Developmental Experiments.

C. Measurement of PDH Activity and Percentage Activity

1. Activation by Magnesium

Prior to assaying enzyme activity, half of each sample was incubated at 37°C for thirty minutes with 10 mM $MgCl_2$ to determine total activity of the sample (PDHt). This was assumed to totally activate the enzyme. Activity of the sample without magnesium was taken to indicate the active part of the enzyme.

Each sample was, therefore, assayed for total enzyme activity (PDHt) as well as for the amount of the enzyme in the active form (PDHa). PDHa was calculated as a percentage of PDHt activity.

2. PDH Assay

PDH activity was determined by measuring the rate of formation of $^{14}CO_2$ from (1-^{14}C)-pyruvate (Jungas, 1970). Each assay was performed in duplicate or triplicate. Samples of 50-200 ul. each were added to an incubation mixture (kept at 4°C) which consisted of 30 mM potassium phosphate, 50 mM NaCl, 0.5 mM dithiothreitol, 0.6 mM pyruvate, 0.5 mM NAD^+, 0.1 mM CoA, 1 mM TPP and 0.25 uCi of (1-^{14}C)-pyruvate, pH 7.0. The vials were capped with rubber stoppers holding glass centre wells fitted with gelatin capsules. Phenethylamine (0.25 ml.) was injected into each gelatin capsule. Each sample was incubated for five minutes in a Dubnoff Metabolic Shaking Incubator at 37°C. The reaction was stopped by injecting 0.5 ml. of 2.5 M H_2SO_4 into the buffer. After

thirty minutes of further incubation, the gelatin capsule and its contents were removed by forceps and placed in a scintillation vial containing 10 ml. of scintillation fluid. The scintillation fluid consisted of 12 gm. of 98% PPO/2% BisMSB dissolved in 1 litre of Triton X-100 and 2 litres of Toluene. Radioactivity was measured in a Beckman scintillation counter. Results were corrected for blank values obtained in vessals in which a 50 mM NaCl/30 mM potassium phosphate buffer (pH 7.0) replaced the tissue homogenate.

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Results were expressed as nanomoles of CO_2 released/minute of incubation time/milligram of protein.

Chemicals were from Sigma Company, St. Louis, Missouri except for sodium (1-^{14}C)-pyruvate (specific activity 13.1 mCi/mMol) which was supplied by Amersham-Searle, Don Mills, Ontario.

D. Experiments

1. Assay

The dependence of activity on enzyme concentration, CoA concentration and incubation time was determined using liver homogenate.

Samples were assayed in the presence of avidin (.0015 gm./sample) to determine the effect on the evolution of CO_2. Pyruvate carboxylase which catalyzes the carboxylation of pyruvate, is inhibited by avidin (Scrutton et al., 1969).

2. Developmental Experiments

At least two different age groups were assayed in each experiment. Liver, brown fat and gluteal white fat were used. Both PDHa activity and total activity (PDHt) were assayed. Initially both homogenate and mitochondria were used from liver and brown fat. The variability between experiments was very high with mitochondria although in individual experiments significant differences between different age groups were usually detected. Because of this problem, the results obtained from mitochondria were not used in determining developmental patterns and later experiments were done only on tissue homogenate.

3. Incubation with Insulin

Three experiments using 120 uU of insulin per ml. were made. This was the least amount of insulin which had been found effective on PDH (Sica and Cuatracasas, 1973). This concentration failed to affect PDH activity so concentration studies using liver and epididymal white fat were performed to find the best concentration. The effect of excluding magnesium and including glucose in the KRB buffer were evaluated.

4. Effect of Insulin on Liver, White Fat and Brown Fat

Liver, gluteal white fat and brown fat from various age groups were incubated with or without insulin (1 mU/ml.). The tissue was then assayed for PDHa and PDHt activity.

5. In Vivo Experiment

A single in vivo experiment was performed in which three 10 day old rats were injected with normal saline, three with insulin, and one received no injection. After one hour, the rats were sacrificed and liver and brown fat were assayed for PDHa and PDH t activity.

6. Magnesium Content of Brown Fat

Dr. V. Palaty of the Department of Anatomy measured the content of magnesium in dry fat-free samples of brown adipose tissue from fetal, 8 day old and 25 day old rats using atomic absorption spectrophotometry.

7. PDH Activity in Human Fetal Liver

The liver was removed from six human fetuses which had been obtained by hysterotomy. PDH activity and total activity were assayed. The fetuses ranged in crown-rump length from 9.7 to 19.7 centimeters.

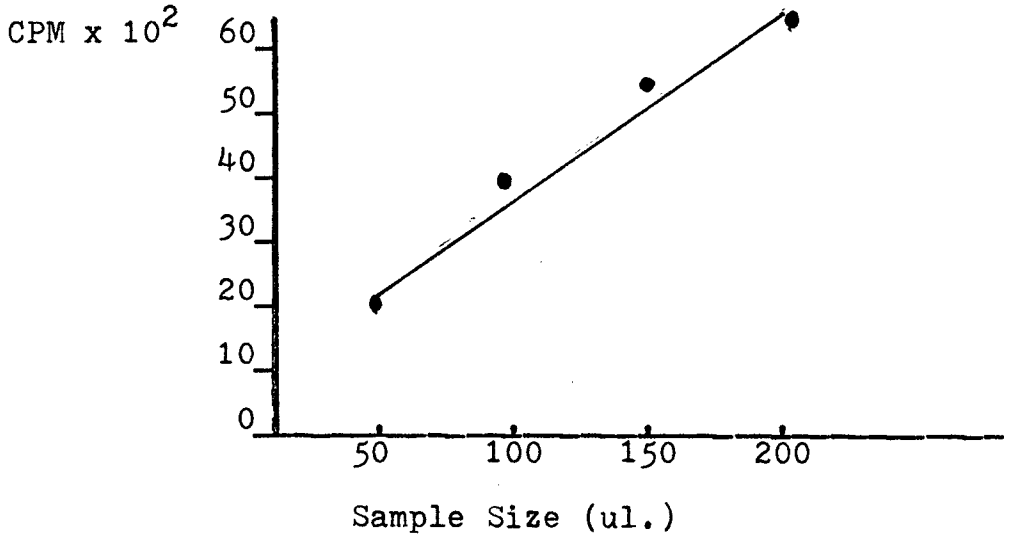
IX RESULTS

A. PDH Assay

1. Sample Size

The activity of PDH in liver homogenate varied directly with the sample size (Figure 4).

Figure 4. PDH activity as a function of sample size.

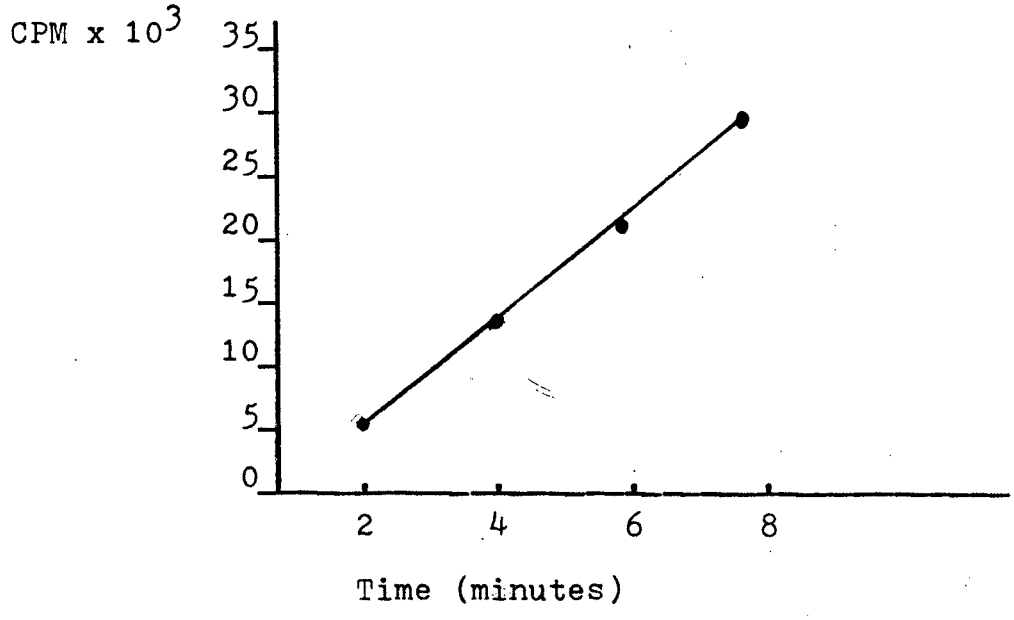


Liver homogenate had the lowest enzyme activity. For assaying activity, 200 ul. of liver homogenate and white fat homogenate were used. When assaying mitochondria and brown fat, only 50 ul. were used because of the smaller amount of material and because of the much greater enzyme activities found in these materials.

2. Time of Incubation

The activity of PDH in liver homogenate varied directly with the time of incubation (Figure 5).

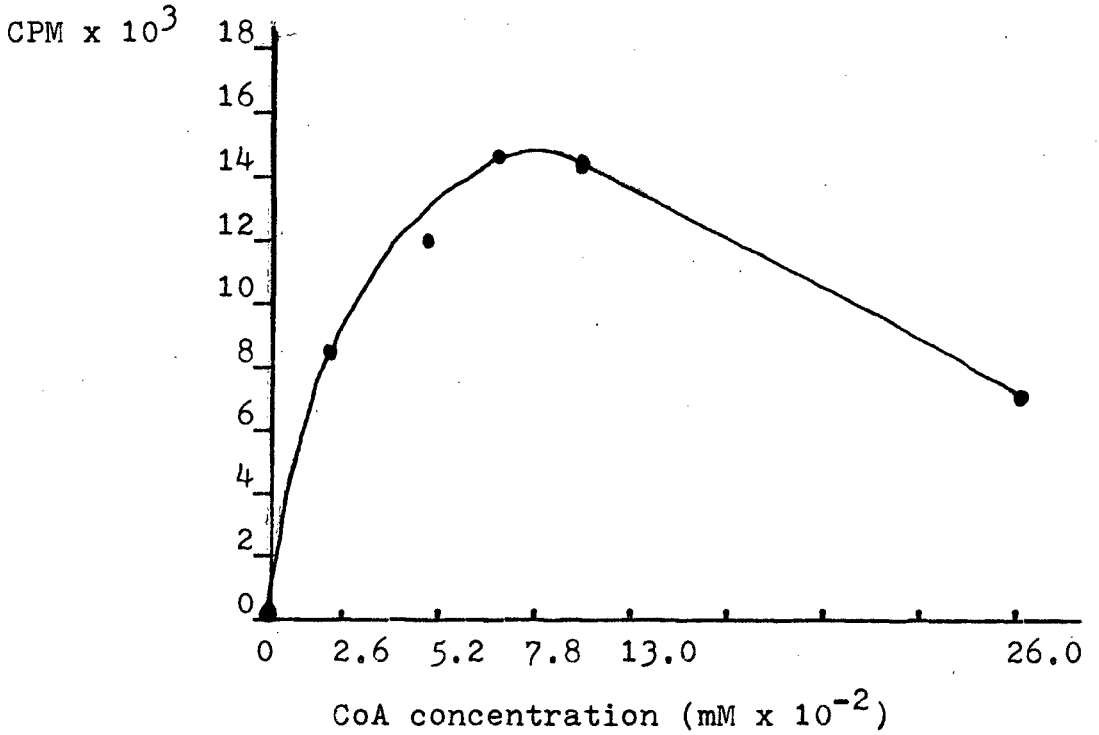
Figure 5. PDH activity as a function of incubation time.



3. CoA Concentration

In liver homogenate, activity was maximal at concentrations of CoA between 0.078 mM and 0.130 mM. Excess CoA was inhibitory (Figure 6). The concentration of CoA which has been used in this assay is 0.1 mM (Jungas, 1970). Excess CoA has previously been shown to be inhibitory (Tsai et al., 1973). CoA concnetration curves for brown fat and liver mitochondria were also done. Although enzyme activity is much higher in these tissues, a similar function of activity with CoA concentration was found.

Figure 6. PDH activity as a function of CoA concentration.



4. Inhibition of Pyruvate Carboxylase

The effect of inhibiting pyruvate carboxylase which catalyzes the carboxylation of pyruvate, forming oxaloacetate, was evaluated. Although CO_2 is a substrate for this enzyme and acetyl-CoA is a positive modifier, no effect on the PDH assay was found (Table I). The optimal pH for assaying pyruvate carboxylase is 7.8; considerably higher than that of the PDH assay, 7.0. Pyruvate carboxylase also requires ATP which may not be generated in sufficient quantities under the conditions of the PDH assay.

Table I. The effect of inhibiting pyruvate carboxylase with avidin on the evolution of CO_2. Activity is expressed as nm. CO_2/mg. protein/minute. BF represents brown fat.

Experiment	Activity		Activity in presence of Avidin	
	PDHa	PDHt	PDHa	PDHt
1) Fetal BF	0.00	2.32	0.25	1.89
2) 8 day BF	1.74	20.18	1.86	20.85
3) Fetal Liver	0.35	0.50	0.26	0.48
4) 8 day liver	0.48	0.55	0.42	0.46
5) Fetal Liver	0.15	0.62	0.16	0.62
6) 8 day liver	0.23	0.59	0.23	0.38
7) 22 day liver	0.89	1.69	0.70	1.66
8) 22 day liver mitochondria	1.21	2.55	1.06	1.67
9) 22 day BF	1.35	6.33	1.36	6.42
10) 22 day BF mitochondria	1.27	12.36	1.46	6.72
11) 35 day liver	0.35	1.05	0.39	0.68
12) 35 day BF	1.81	17.68	1.06	14.02
13) 23 day liver	0.56	0.65	0.63	1.08
14) 23 day BF	0.50	8.81	1.08	10.86
15) 23 day liver mitochondria	1.44	2.56	1.83	2.32
16) 23 day BF mitochondria	3.63	24.87	3.32	18.13

B. Liver

1. The Development of PDH in Liver

PDH activity was determined without prior incubation with magnesium and this was assumed to represent that part of the enzyme which was normally active (PDHa). The changes in PDHa activity as maturation progresses are shown in Table II and Figure 7.

Table II. PDHa activity during development of the liver. Activity is expressed as nm. CO_2/mg. protein/minute. The activity is expressed a ± the standard error. The number of samples in each group is indicated by 'n'. The statistical significance of the difference between values is also shown. N.S. represents no significant difference.

Age Group	Activity	Statistical Difference		
A Fetus n=6	0.14±0.0388			
B 1-3 days n=6	0.02±0.0127	A-B	t=2.90	p<0.02
C 7-10 days n=8	0.34±0.0675	A-C	t=2.60	p<0.05
		B-C	t=4.62	p<0.005
D 16-23 days n=9	0.59±0.0617	A-D	t=6.17	p<0.005
		B-D	t=9.02	p<0.0005
		C-D	t=2.73	p<0.025
E >28 days n=9	0.59±0.0607	A-B	t=4.85	p<0.005
		B-E	t=9.13	p<0.0005
		C-E	t=2.74	p<0.025
		D-E	t=0.00	N.S.

The activity of PDHa decreased after birth and remained low for a few days. By 7-10 days, a significant increase in activity had occurred and by the late suckling period activity

was maximal. No further increase in activity was noted after 28 days of age. Although the activity of PDHa in fetal rats was significantly higher than that of neonatal animals, it was lower than the activity seen in later suckling or adult animals.

PDH activity was determined after thirty minutes of incubation with magnesium. This was assumed to activate the enzyme totally (PDHt). The changes in PDHt activity are shown in Table III and Figure 7.

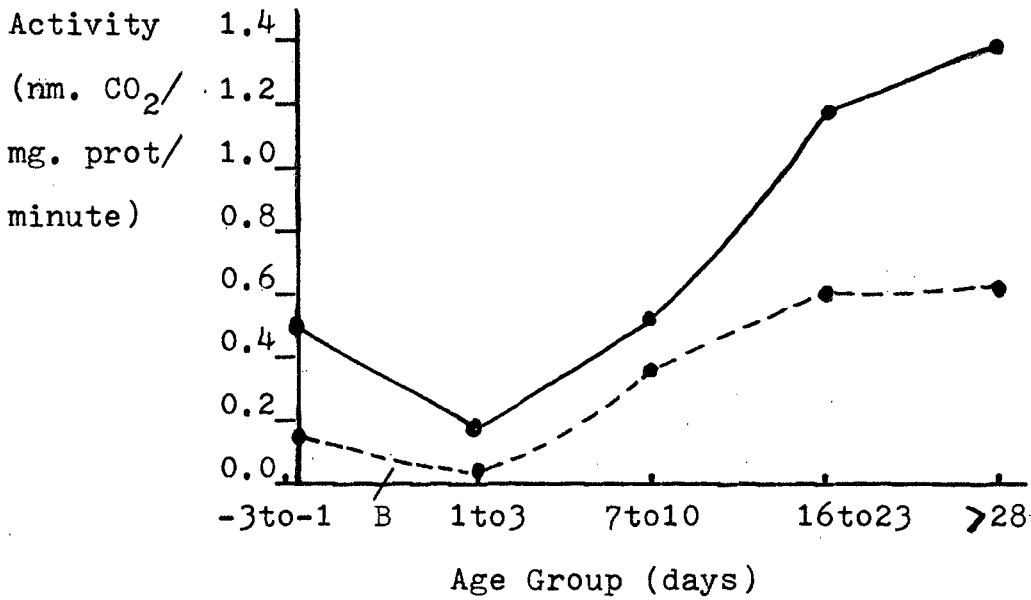
Table III. PDHt activity during development of the liver. Activity is expressed as nm. CO_2/mg. protein/minute. The activity is expressed as ± the standard error. The numbers of samples in each group is indicated by 'n'. The statistical significance of the difference between values is also shown. N.S. represents no significance.

Age Group	Activity	Statistical Difference		
A Fetus n=6	0.48±0.1207			
B 1-3 days n=6	0.18±0.0609	A-B	t=2.13	p<0.05
C 7-10 days n=8	0.52±0.0925	A-C	t=0.33	N.S.
		B-C	t=3.07	p<0.025
D 16-23 days n=9	1.17±0.1705	A-D	t=3.35	p<0.025
		B-D	t=5.47	p<0.0025
		C-D	t=3.35	p<0.01
E >28 days n=9	1.31±0.1223	A-E	t=4.88	p<0.001
		B-E	t=8.27	p<0.0005
		C-E	t=5.14	p<0.001
		D-E	t=0.66	N.S.

The development of PDHt resembled that of PDHa. A decrease in activity was noted after birth followed by a continuous rise until the late suckling period. There was no significant increase in activity after 28 days of age. Although

the activity of PDHa in 7-10 day old rats was significantly higher than in fetal rats, there was no difference between these two age groups in PDHt activity.

Figure 7. The development of PDH in liver. Results are depicted as the mean. PDHa activity is indicated by ----, PDHt activity by ___. B refers to birth.



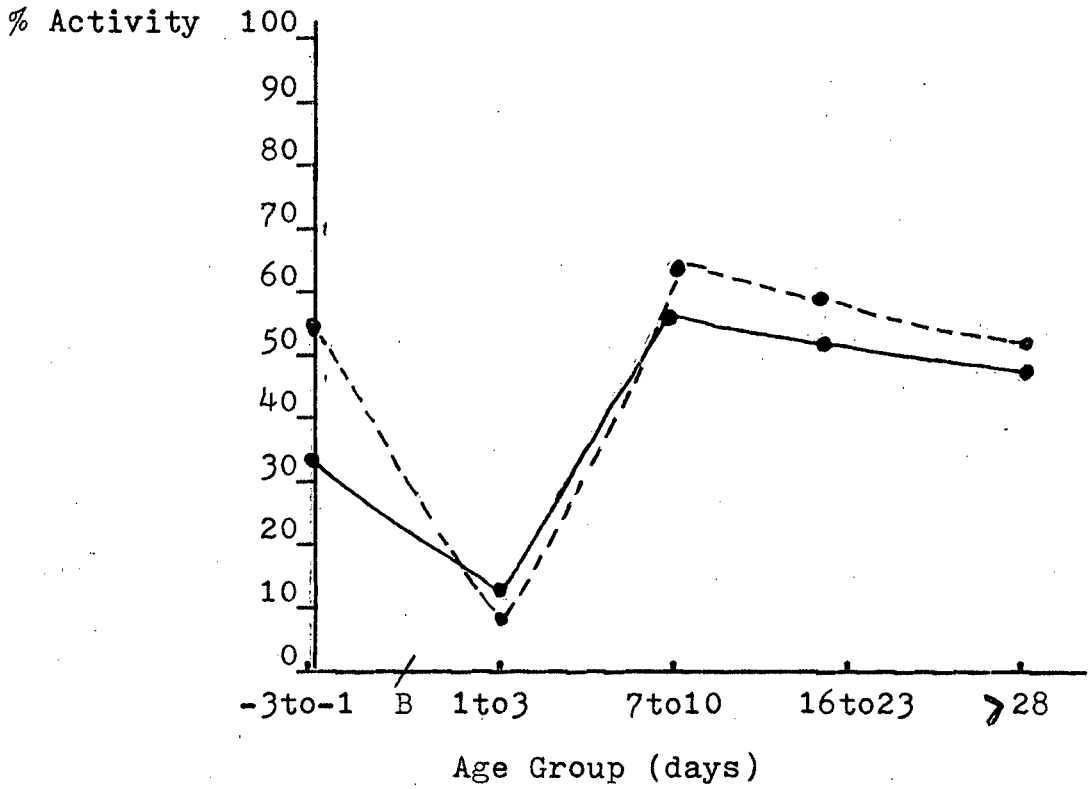
The activity of PDHa was expressed as a percentage of PDHt. The changes in this percentage during development are shown in Table IV and Figure 8. Percentage data forms a binomial, rather than a normal distribution, the deviation from normality being greater for small or large percentages (0-30% and 70-100%). If the square root of each percentage is transformed to its arcsine, then the resultant data will have an underlying distribution that is nearly normal (Biostatistical Analysis). The transformed data is shown and it is this data which were used for statistical analysis.

Table IV. The percentage of PDH in the active form during development of the liver. Results are shown as ± the standard error. The number of samples is indicated by 'n'. The data were transformed so that %' = arcsin$\sqrt{\%}$. Statistical differences between means were calculated on transformed data. N.S. represents no significance.

Age Group	%PDHa Activity	Transformed	Statistical Difference	
A Fetus n=6	56.06±6.9915	36.90±6.6185		
B 1-3 days n=6	8.00±3.6788	11.02±5.0841	A-B	t=3.12 p<0.025
C 7-10 days n=8	64.42±10.7322	53.94±7.3766	A-C	t=1.72 N.S.
			B-C	t=4.82 p<0.0025
D 16-23 days n=9	56.22±5.8589	48.80±13.0798	A-D	t=0.81 N.S.
			B-D	t=2.69 p<0.025
			C-D	t=0.63 N.S.
E >28 days n=9	47.55±6.1172	43.58±3.5795	A-E	t=0.89 N.S.
			B-E	t=5.31 p<0.0025
			C-E	t=1.26 N.S.
			D-E	t=1.03 N.S.

The percentage of PDH in the active form was significantly lower in the first few days of life. No other significant differences were noted.

Figure 8. The changes in the percentage of PDH in the active form during development of the liver. Original data are indicated by ----, transformed data by ___. B refers to birth.



2. Liver Mitochondria

Although the variability between experiments was too high in mitochondria to combine the data, individual experiments always showed differences between different age groups. Some of these data are shown in Table V to prove that changes in activity with age not entirely due to increasing numbers of mitochondria.

Experiment 1 illustrates the increasing activity of PDHt with age. Experiments 2 and 3 illustrate the low activity in the neonatal period as compared to fetal adn older rats.

Table V. The activity of PDH in isolated liver mitochondria as a function of age. Activity is expressed as nm. CO_2/mg. protein/minute.

Experiment	PDHa Activity	PDHt Activity	%PDHa
1. Fetus	0.00	1.07	0.00
17 days old	2.07	2.75	75.22
50 days old	2.58	3.14	82.17
2. Fetus	3.13	3.44	90.95
1 day old	0.52	0.52	100.00
3. 1 day old	0.00	1.09	0.00
7 days old	0.44	1.35	32.59

B. Brown Fat

1. The Development of PDH in Brown Fat

PDH activity was determined without prior incubation with magnesium and this was assumed to represent that part of the enzyme which was normally active (PDHa). The changes in PDHa activity as maturation progresses are shown in Table VI and Figure 9.

The activity of PDHa did not decrease in the neonatal period. After 10 days of age ther was an increase in activity until the late weaning period. No further increase in activity occurred after thirty days of age.

Table VI. PDHa activity during development of brown fat. Activity is expressed as nm. CO_2 evolved/mg. protein/minute. The activity is expressed as ± the standard error. The number of samples in each group is indicated by 'n'. The statistical significance of the difference between values is also shown. N.S. represents no significance.

Age Group	Activity	Statistical Difference		
Fetus n=5	0.12±0.0400			
B 1-10 days n=9	0.11±0,0340	A-B	t=0.19	N.S.
C 11-20 days n=6	0.55±0.0789	A-C	t=4.85	p<0.01
		B-C	t=4.99	p<0.0025
D 21-20 days n=13	1.57±0.3277	A-D	t=4.39	p<0.025
		B-D	t=4.42	p<0.0025
		C-D	t=3.03	p<0.025
E >30 days n=6	1.21±0.1705	A-E	t=6.22	p<0.005
		B-E	t=6.33	p<0.005
		C-E	t=3.51	p<0.01
		D-E	t=0.97	N.S.

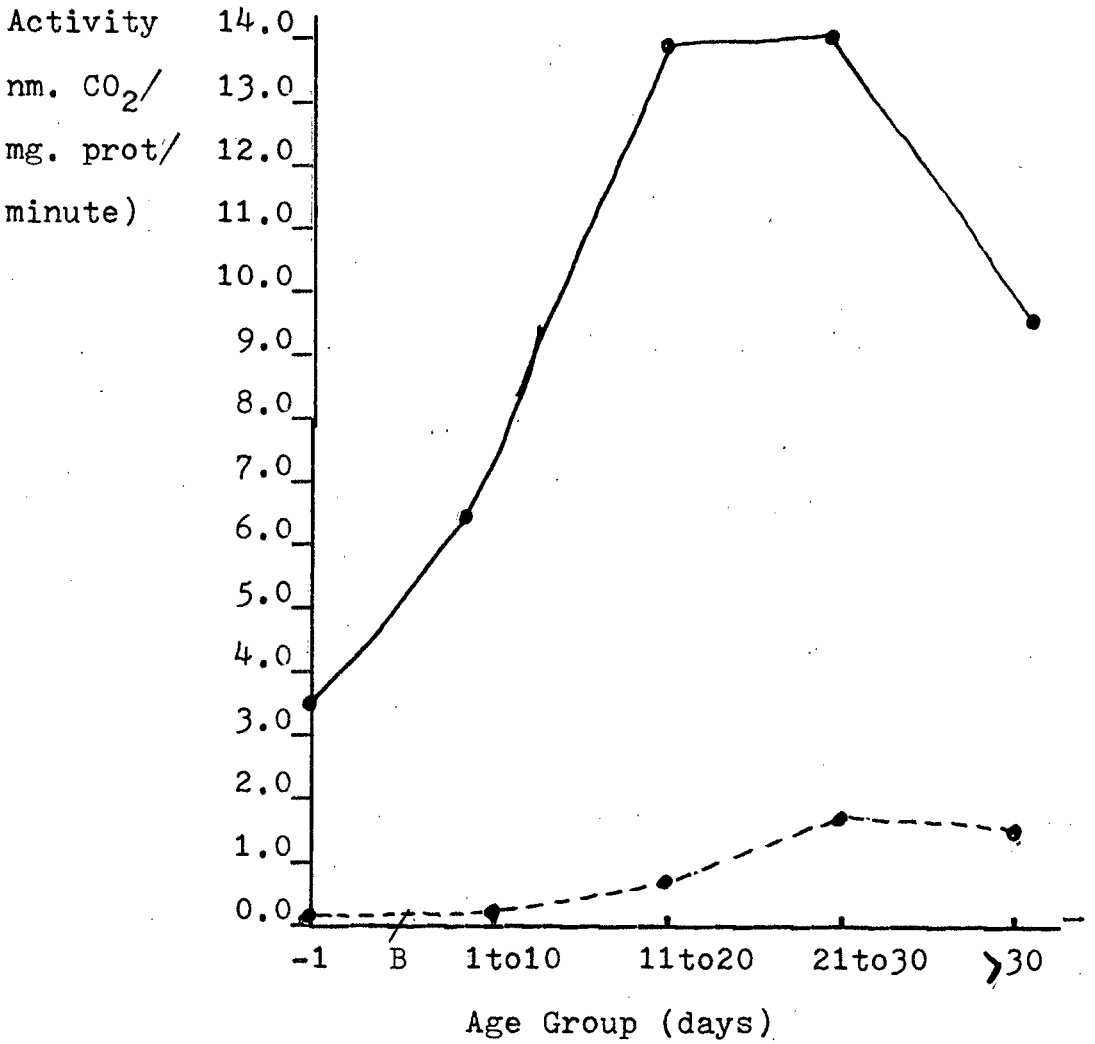
PDH activity was determined after thirty minutes of incubation with magnesium. This was assumed to activate the enzyme totally (PDHt). The changes in PDHt activity are shown in Table VII and Figure 9.

PDHt activity was much higher in brown fat than in liver. A steady increase in PDHt activity from prior to birth to after 20 days of age was noted. No further increase occurred and after 30 days of age, there was a decrease in activity. Because of the large standard errors, the decrease after thrity days of age was not significant. The activity after thirty days was, however, not significantly different from that of the early suckling period.

Table VII. Total PDH activity during development of brown fat. Activity is expressed as nm. CO_2/mg. protein/ minute. The activity is expressed as ± the standard error. The number of samples in each group is indicated by 'n'. The statistical significance of the difference between values is also shown. N.S. represents no significance.

Age Group	Activity	Statistical Difference		
A Fetus n=5	3.44±0.8218			
B 1-10 days n=9	6.41±0.8840	A-B	t=2.43	p<0.05
C 11-20 days n=6	13.69±1.5722	A-C	t=5.78	p<0.0025
		B-C	t=4.04	p<0.005
D 21-30 days n=13	13.83±1.7704	A-D	t=5.32	p<0.005
		B-D	t=3.75	p<0.005
		C-D	t=0.05	N.S.
E >30 days n=6	9.09±1.9355	A-E	t=2.69	p<0.05
		B-E	t=1.26	N.S.
		C-E	t=1.84	N.S.
		D-E	t=1.81	N.S.

Figure 9. The development of PDH in brown fat. Results are depicted as the mean. PDHa activity is indicated by ---, PDHt activity by ____. B refers to birth.



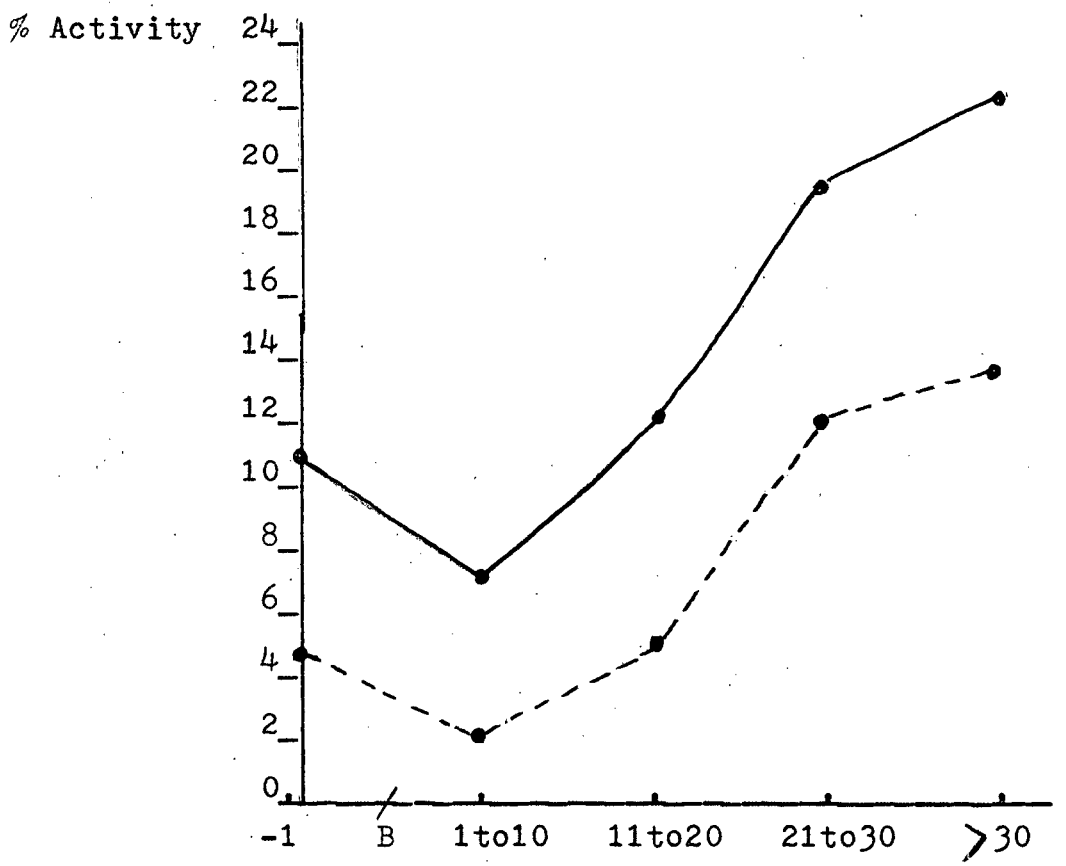
The activity of PDHa was expressed as a percentage of PDHt. The changes in this percentage during development are shown in Table VIII and Figure 10. The data have been transformed using the arcsine of the square root of the percentage as described previously. The transformed data was used for statistical analysis

Table VIII. The percentage of PDH in the active form during the development of brown fat. Results are shown as ± the standard error. The number of samples is indicated by 'n'. The data was transformed so that %' = arcsin$\sqrt{\%}$. Statistical differences between values were calculated on transformed data. N.S. represents no significance.

Age Group	%PDHa Activity	Transformed	Statistical Difference
A Fetus n=5	4.73±2.3383	11.06±3.1173	
B 1-10 days n=9	2.01±0.6953	6.79±1.8826	A-B t=1.17 N.S.
C 11-20 days n=6	4.53±0.9484	11.92±1.3905	A-C t=0.24 N.S. B-C t=2.20 p<0.05
D 21-30 days n=13	11.84±1.8969	19.26±1.8229	A-D t=2.27 p<0.05 B-D t=4.76 p<0.001 C-D t=3.21 p<0.025
E >30 days n=6	12.76±1.5017	22.28±1.9155	A-E t=3.06 p<0.025 B-E t=5.77 p<0.0025 C-E t=4.39 p<0.005 D-E t=1.14 N.S.

After birth, there was a slight decline in the percentage of PDH in the active form although this was not significant. After ten days of age the percentage activity increased until thirty days of age when no further changes were noted.

Figure 10. The changes in the percentage of PDH in the active form during development of brown fat. Original data are indicated by ----, transformed data by ___. B refers to birth.



2. Brown Fat Mitochondria

Although the variability between experiments was too high in mitochondria to combine the data, individuals experiments usually showed differences between different age groups. Some of these data are shown in Table IX.

Table IX. The activity of PDH in isolated mitochondria from brown fat as a function of age. Activity is expressed as nm. CO_2/mg. protein/minute.

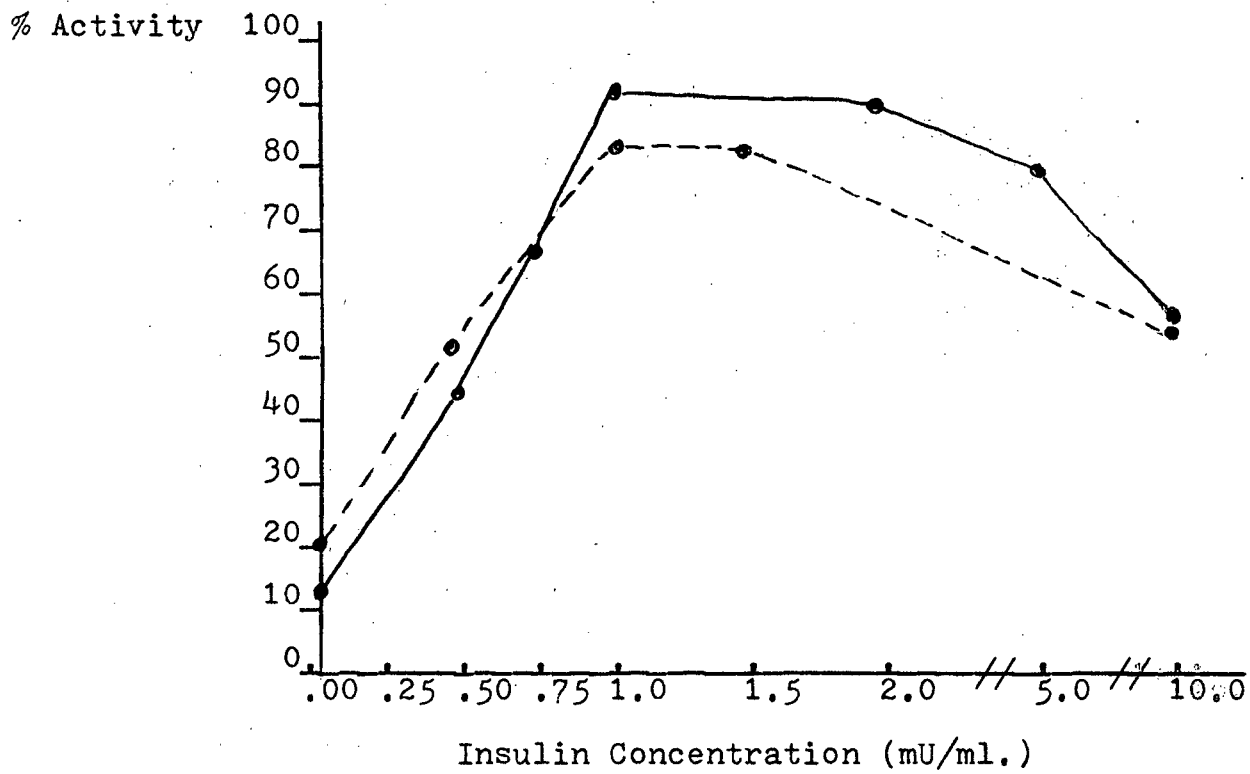
Experiment	PDHa Activity	PDHt Activity	%PDHa
1. Fetus	0.56	15.28	4.00
17 days old	3.68	13.00	28.00
50 days old	3.44	19.52	18.00
2. Fetus	0.00	8.80	0.00
1 day old	0.00	38.51	0.00
2 days old	1.51	26.41	5.60
23 days old	5.57	20.87	26.70
24 days old	1.77	19.64	9.10
39 days old	2.66	37.67	7.10
3. 1 day old	0.00	13.50	0.00
3 days old	0.40	4.11	0.90
3 days old	0.69	42.70	1.60
7 days old	1.86	35.66	5.50
7 days old	0.00	13.25	0.00
10 days old	0.00	31.38	0.00

The only conclusion which can be drawn from these data is that the activity of PDH increases in older suckling rats. These results with brown fat show clearly the amount of variability found in assaying PDH activity in mitochondria.

D. Incubation with Insulin

To determine the optimal concentration of insulin, a concentration study was made using liver and epididymal white fat (Figure 11). The results with liver are probably inaccurate because of the large size of the pieces which were incubated. Incubation of liver with a hormone is normally performed with liver slices but liver slices which were made had lost all activity. The optimum concentration of insulin was found to be 1 mU/ml.

Figure 11. The percentage of PDH in the active form as a function of insulin concentration. Samples were incubated for one hour in the presence of insulin. The results with liver are indicated by ------, those with epididymal fat by _____.



Magnesium was excluded from the incubating buffer because of its ability to activate the enzyme. Inclusion of magnesium in the buffer resulted in nearly total activation of the enzyme in the absence of insulin. Glucose was included in the incubating medium as its presence improved the amount of activity retained after incubation.

E. Effect of Insulin on PDH in Liver and Brown Fat

Insulin increased the percentage of PDH in the active form in the liver postnatally. These results, however, were not considered as reliable because of the large pieces of liver

incubated. Liver slices, which have been used for incubating liver with hormone, had no activity following incubation. This was probably due to the length of time required to make these slices. PDH activity decreases rapidly in tissues which are not frozen. Postnatally, insulin appeared to have no effect on the activity of PDH in brown fat.

In both fetal brown fat and fetal liver, there was a significant increase in the total PDH activity (PDHt) (Table X, Table XI).

Table X. The effect of insulin on total PDH activity in liver. Activity was measured after thirty minutes of incubation with magnesium and is expressed as nm. CO_2/mg. protein/minute. The statistical difference between activity without insulin and that with insulin was measured by a paired t test. d=0.551 S.E.=0.0165 t=33.03 p<0.005

Experiment	Activity without Insulin	with Insulin
1	0.000	0.076
2	0.285	0.440
3	0.043	0.059
4	0.084	0.203
5	0.115	0.157
6	0.031	0.079
7	0.059	0.122
8	0.135	0.167

Although there was no correlation of activity with average fetal weight, the 0.000 nm. CO_2/mg. protein/minute measured in liver was from the smallest fetuses; average weight, 1.82 grams.

Table XI. The effect of insulin of total PDH activity in brown fat. Activity was measured after thirty minutes of incubation with magnesium and is expressed as nm. CO_2/mg. protein/minute. The statistical difference between activity without insulin and that with insulin was measured by a paired t test.
d=1.665 S.E.=0.3613 t=3.72 p<0.005

Experiment	Activity without Insulin	with Insulin
1	3.422	4.866
2	2.684	3.857
3	0.051	2.876
4	2.570	4.360
5	0.596	0.929
6	0.513	2.876

F. Gluteal White Fat.

This tissue showed no change in activity with age (Figure XII).

Table XII. PDH activity at different ages in gluteal white fat. Activity is expressed as nm. CO_2/mg. protein/minute.

Age	PDHa	PDHt	% PDHa	Transformed (arcsin$\sqrt{\%}$)
8	0.29	1.19	24.2	29.6
15	1.40	1.96	71.4	57.7
17	0.54	0.86	62.7	52.4
18	1.52	3.51	43.3	41.2
23	1.04	1.04	100.0	90.0
25	2.52	2.70	93.3	75.0
29	1.71	1.73	98.8	83.7
30	0.82	1.35	60.7	51.2
30	0.87	1.37	63.5	52.8
50	0.49	2.14	22.8	28.5
73	0.95	1.01	94.1	75.9
$\bar{x}\pm$ S.E.	1.07± 0.18	1.68± 0.23		57.02± 5.83

Gluteal white fat lost all activity when incubated in KRB buffer with or without insulin so it was impossible to determine if insulin was affecting this tissue.

G. In Vivo Experiment

A single in vivo experiment was performed in which three 10 day old rats received an injection of normal saline, three an injection of insulin and one received no injection. The results are shown in Table XIII.

Table XIII. The in vivo effects of insulin on PDH activity in brown fat and liver of 10 day old rats. Results are expressed as nm. CO_2/mg. protein/minute.

Tissue	PDHa Activity	PDHt Activity	%PDHa
Liver			
no injection	0.097	0.340	28
normal saline	0.020	0.154	13
insulin	0.040	0.159	26
Brown Fat	0.652	6.720	10
normal saline	0.652	6.720	10
insulin	0.987	6.118	16

Brown fat and liver from rats which received insulin had a higher percentage of PDH in the active form than that found in animals which received normal saline. These results are not conclusive but they do suggest an in vivo response to insulin may be possible in PDH of liver and brown fat. An in vivo response to insulin has been shown by Wieland et al. (1972). They showed an increase in the percentage of PDH in the active form in the liver following insulin injection.

H. Magnesium Content of Brown Fat

The magnesium content of dried fat-free samples of brown fat was measured by atomic absorption spectrophotometry. This was done by Dr. V. Palaty. The results are shown in Table XIV.

There was a significant decrease in mganesium levels after birth which was significant at $p<0.005$. There was no significant difference between the magnesium content of 8 day old and 25 day old rats.

Table XIV. The magnesium content of brown fat. Results are shown as ± the standard error.

Age	Magnesium (mg./kg. fat-free dry weight)
Fetus	74.6±7.4
8 days old	54.4±6.2
25 days old	50.2±6.1

I. PDH Activity in Human Fetal Liver

The PDH activity was measured in human fetal liver from fetuses that had been removed by hysterotomy. Fetuses ranging from 9.7 to 19.7 cm. in crown-rump length were used. A linear regression analysis showed no significant relationship between crown-rump length and activity of PDH but there were only 6 samples and the variability of results found when assaying PDH activity would require many more samples. The activity of PDH in human fetuses was sginificantly lower than in 16-23 day old rats. The results obtained are shown in Table XV.

Table XV. The PDH activity of human fetal liver. Activity is expressed as nm. CO_2/mg. protein/minute.

Crown-Rump Length (cm.)	PDHa Activity	PDHt Activity	%PDHa
9.7	0.01	0.32	3.2
10.0	0.22	0.64	31.2
12.0	0.00	0.68	0.0
13.2	0.11	0.36	30.6
19.0	0.46	0.83	55.4
19.7	0.32	0.51	62.7
$\bar{x}\pm$S.E.	0.19±0.07	0.557±0.08	30.5±10.5
Statistical Difference from 16-23 day old rats	t=4.3 p<0.005	t=5.4 p<0.005	t=3.3 p<0.025

X DISCUSSION

A. Assay

1. Problems with the Assay

A good assay is a prerequisite for obtaining any meaningful results on an enzyme's activity. The assay used in this research was somewhat unsatisfactory due to the high variability in the results between experiments. Even within a given experiment, results were quite variable and it was necessary to perform all assays in duplicate or triplicate. Several experiments failed altogether; no enzyme activity being found at all. The method of collecting radioactive carbon dioxide to assay PDH activity has been found similarly unsatisfactory by others (Taylor *et al.*, 1973). The only other method suitable for assaying this enzyme in crude homogenate measures acetyl-CoA production. The decrease in absorbance of *p*-nitoaniline during acetylation to *p*-nitroacetanilide is measured (Wieland *et al.*, 1972). This particular assay requires the purification of arylamine transacetylase from pigeon liver; an elaborate procedure which produces results with similarly high standard errors.

PDH is a complex enzyme and requires very delicate technique. It was noted that significant activity was lost when there was any delay between excision of the tissue and freezing of the homogenate. Incubation with or without insulin resulted in a great loss of activity and in gluteal white fat, the loss was total; no enzyme being detectable. The process

of making liver slices for incubation with and without insulin also resulted in loss of all PDH activity, probably because of the length of time involved in this procedure. Although freezing and thawing increased activity by disrupting the mitochondria, prolonged freezing destroyed activity. After four days of freezing, PDHa activity began to decline. After one week, all the active part of the enzyme had become inactive and after two weeks of freezing, total enzyme activity had decreased significantly.

The technique for assaying this enzyme was developed using epididymal white fat (Jungas, 1970). This tissue does not appear until late in the suckling period in rats and hence is unsuitable for studying during development. Some of the problems associated with assaying this enzyme in liver, brown fat and gluteal white fat may be associated with the different characteristics of these tissues. Crude homogenate of liver has only been assayed in two laboratories (Wieland, et al., 1972; Knowles and Ballard, 1974). In both, the accumulation of acetyl-CoA was the method of assay. PDH activity in brown fat and gluteal white fat has not been reported. The dearth of data for tissues other than epididymal fat may reflect difficulties with the assay.

The numerous reports on epididymal white fat have all employed rats that were starved and refed. Since much of this involved suckling rats, the starving-refeeding technique could not be used and this may have caused variability in the results.

The method used in totally activating the enzyme by

incubation with magnesium may have been inadequate and may have produced some variability. The results obtained in measuring the percentage of PDH in the active form in the liver were strikingly different to other reports (Wieland et al., 1972; Knowles and Ballard, 1974). The percentage of PDH in the active form in liver has previously been found to be very low (below 15%) whereas we found much higher percentages (above 50%). This may be due to a failure to totally activate the enzyme.

Incubation with 10 mM magnesium has been the accepted method of totally activating PDH (Wieland and Jagow-Westermann, 1969; Denton et al., 1972; Martin et al., 1972; Wieland et al., 1972) and this method was used throughout this research. Knowles and Ballard (1974) used 15 mM magnesium to fully activate the enzyme in liver and their results for total enzyme activity were higher than those reported in this work. The phosphatase responsible for dephosphorylation of PDH has been purified and used concommitantly with the magnesium in activating the enzyme. Extra phosphatase has been used because some results have shown loss of the enzyme's own phosphatase during preparation of the tissue (Linn et al., 1969b). In the presence of excess phosphatase and magnesium, the enzyme was shown to be activated after only fifteen minutes of incubation. No extra phosphatase was used in this research which may have prevented total activation of the enzyme. However, Walajtys et al. (1974), in liver mitochondria, and Sica and Cuatracasas (1973), in epididymal white fat, have observed that extra phosphatase

is unnecessary providing that incubation with magnesium proceeds for thirty minutes. Thirty minutes of incubation were used during this research.

Variability may have resulted from escape of carbon dioxide from the vials. Both the phenethylamine used to collect the carbon dioxide and the sulphuric acid used to stop the reaction were injected and the rubber stoppers may have been sufficiently damaged to allow some seepage of gas. The stoppers were all replaced half way through this research and no significant difference was noted with the new ones.

2. Mitochondria

Attempts at assaying PDH activity in mitochondria produced particularly bad results. Isolation of the mitochondria submits the enzyme to more rigorous procedures than simple homogenization. The enzymes within the PDH complex are more likely to dissociate under these conditions, particularly the phosphatase which is loosely bound. Some activity was lost in the supernatant of the mitochondria and this loss was highly variable.

B. Liver

1. The Development of PDH in Liver

The fetus synthesizes fatty acids at a high rate (Carroll, 1964). Since glucose is the major source of calories for the fetus, one would expect it to be the major precursor of fatty acids, prenatally. The conversion of glucose to fatty

acids requires the enzyme PDH. Our data and those of Knowles and Ballard (1974) show that PDH is more active in the fetus than in the newborn animal.

The production of acetyl-CoA by PDH is important for both fatty acid synthesis and the citric acid cycle. Prior to birth, the anabolic activity of fatty acid synthesis appears to predominate over the catabolic activity of the citric acid cycle, in liver mitochondria. The turnover of the citric acid cycle, prenatally, is low due to the low activity of such enzymes as fumarase, aconitase (Hommes et al., 1971) and succinic dehydrogenase (De Vos et al., 1968). The activity of citrate synthase, another enzyme of the citric acid cycle, is, by contrast, as high in activity, prenatally, as in adult animals (Hommes et al., 1971). The synthesis of citrate is essential for transporting acetyl-CoA to the cytoplasm where fatty acid synthesis occurs. Another enzyme of the citric acid cycle which has high activity prenatally, is NADP specific isocitrate dehydrogenase (Hommes et al., 1971). The high activity of this enzyme increases the rate of supply of NADPH, necessary for lipogenesis. Close to term, fetal rat liver contains much higher amounts of cholesterol and triglycerides than adult rat liver (Ballard and Hanson, 1967a). Prenatally, it is probable that the high activity of PDH is linked to fatty acid synthesis and not to the citric acid cycle.

The activity of PDH per unit protein is lower in fetal rats than in later suckling or adult animals. The number of mitochondria is fewer prenatally and this would limit the

activity of PDH. The fetus, also, does not have to cope with intermittent ingestion of large amounts of carbohydrates as do late suckling and adult rats.

After birth, our data and those of Knowles and Ballard (1974) show a decrease in the activity of PDH. This decrease correlates with the sharp decline in the rate of fatty acid synthesis seen at this time (Carroll, 1964).

The neonatal decrease in activity is probably related to the concomitant increase in pyruvate carboxylase activity and the start of gluconeogenesis which occursshortly after birth (Ballard and Hanson, 1967b; Yeung et al., 1967). Pyruvate carboxylase, an obligatory enzyme in gluconeogenesis, would compete with PDH for substrate. The ATP:ADP ratio increases from 1.5 in the fetus to 7.0 in the newborn (Knowles and Ballard, 1974) and this would tend to repress activity. Both Soling and Bernhard (1971) and Walajtys et al, (1974) report a decrease in PDH activity as the ATP:ADP ratio increases.

After birth, carbohydrates supply only about 10% of the caloric intake. Fat becomes the major source of energy and the major source of acetyl-CoA. Acetyl-CoA is an obligatory cofactor for pyruvate carboxylase (Utter and Keech, 1963) and is also an inhibitor of PDHa activity (Garland and Randle, 1964). The oxidation of fatty acids, which leads to acetyl-CoA formation, would decrease PDH activity by increasing the activity of pyruvate carboxylase which competes for substrate and by inhibiting the activity of the active form of PDH. In the liver, ketones are end-products of fatty acid oxidation.

B-hydroxybutyrate will inhibit PDH (Taylor et al., 1973).

After the initial postnatal decline, PDH activity increases. By 7-10 days the rate of gluconeogenesis has begun to fall (Yeung et al., 1967). The citric acid cycle is increasingly functional. Isocitrate dehydrogenase and NAD specific malate dehydrogenase reach adult levels by birth but the activity of fumarase continues to increase and does not reach adult levels until after 15 days of age (Vernon and Walker, 1968). Except for the immediate postnatal drop, the change in PDHa activity parallels that of fumarase. By about 7 days of age, both enzymes are half as active as in adults. After 15 days of age, both enzymes are functioning at adult rates.

In the late suckling period, there is a further increase in PDH Activity. This increase is particularly high in PDHt activity. The late suckling period is the beginning of weaning. Gradually a diet containing 60-70% carbohydrates begins to supplement and then replace the high fat diet of milk. Greater capacity for handling exogenous carbohydrates becomes necessary. As carbohydrates replace fat as the major source of energy, the metabolic pathways which convert carbohydrates to fat become increasingly important. The rate of fatty acid synthesis increases during the weaning period (Carroll, 1964). The increase in PDH activity during the late suckling period reflects an increased capability of the liver to use and store carbohydrates.

The constant alimentation of the suckling period is replaced by meal-feeding in adult rats. Meal-feeding requires that the animal be able to respond metabolically to periods of

high caloric intake followed by periods of starvation. PDH is an enzyme whose activity can be regulated. Its activity is increased by substrates such as glucose and fructose (Martin et al., 1972) which are more available after a meal. Activity is decreased during time of starvation (Wieland et al., 1972). The increase in the activity of PDH at weaning increases the liver's capability to respond to periods of high carbohydrate intake followed by starvation. During periods of high intake, more acetyl-CoA will be produced which can be stored as fat. During periods of starvation, the lower activity of PDH saves carbohydrates from oxidative metabolism. Instead, the stored fats can be used as a source of energy. Pyruvate can be diverted to gluconeogenesis to ensure a constant supply of glucose to organs like the brain which require it.

2. Effect of Insulin on Liver

Insulin increases the activity of PDHt in fetal liver. Apparently, only the percentage of PDH in the active form is increased by insulin after birth. The increase in the percentage activity in older rats is also shown by Wieland et al. (1972).

Insulin may induce PDH by some direct effect on protein synthesis. Sica and Cuatracasas (1973) showed an increase in the total activity of PDH in epididymal white fat following incubation with insulin. This increase was prevented by the inclusion of cyclohexamide or puromycin in the incubating media. These results are unique in the literature; all others indicate only an increase in the percentage activity.

The increase in the activity of PDH, found in this work, may be due to substrate induction. Insulin may only increase the amount of glucose entering the cell and glucose may induce PDH. This work shows that, in the late suckling period when carbohydrates contribute more to the diet, the activity of PDH increases substantially. In epididymal white fat, there is an effect of insulin on PDH activity even in the absence of substrate in the incubating media (Coore et al., 1971).

Whatever the mode of action of insulin, its ability to increase the amount of enzyme in fetal tissues is of interest. It suggests a role for this hormone in the maturation of carbohydrate metabolism. It also suggests one effect of an oversupply of insulin during fetal life; that of increasing the amount of an enzyme with a major role in regulating the use of carbohydrates.

Infants of diabetic women tend to be hyperinsulinemic prior to birth because of the hyperglycemia of the mother (François et al., 1974). This constant exposure to excess insulin, if it induced PDH, would increase the conversion of carbohydrate to fat. The obesity seen in infants of diabetics may be due to increased activity of PDH.

C. Brown Fat

1. The Development of PDH in Brown Fat

PDH activity is much higher in brown fat than in liver. Brown fat has numerous, large mitochondria and in infant rats, in vitro oxygen consumption in brown fat is higher than in any

other tissue (Barnard and Skala, 1970). The total activity of PDH increases steadily until after weaning when there is a decline. This correlates well with the physical development of brown fat as described by Barnard and Skala (1970). The number of mitochondria in this tissue increases during infancy and reaches a maximum at fourteen days of age. After day thirty, the activity of PDHt decreases. This decline in the adult correlates with the involution of this tissue which occurs after weaning.

Our results show that a significant increase in the fraction of PDH which is active does not occur until the late suckling period when carbohydrate ingestion has begun. Although brown fat in the fetus has significantly higher magnesium levels than that of 8 or 25 day old rats, the activity of PDHa is still very low. Increased intracellular magnesium increases the percentage of PDH in the active form by increasing the activity of the phosphatase subunit which catalyzes the dephosphorylation of PDH (Hucho, 1974). Prior to birth, the number of mitochondria is fewer and the activity of the electron transport enzymes is lower. The low activity of PDH in the presence of high concentration of magnesium appears to be related to this immaturity of the mitochondria. A decreased ability of the mitochondria to metabolize acetyl-CoA or to transport it to the cytosol would tend to inhibit PDH. Prior to birth, substrate for PDH may be limiting due to the low activity of pyruvate kinase (Hahn and Skala, 1972). Pyruvate kinase catalyzes the formation of pyruvate and pyruvate increases the percentage of PDH in the active form (Linn et al., 1969a, b).

The enzyme itself does respond to *in vitro* incubation with magnesium and presumably would do so *in vivo* if no other factors were limiting.

After birth, the percentage of the enzyme in the active form continues to be low in spite of the increased functional capacity of the mitochondria. This is probably related to the high fat diet as fatty acid synthesis from pyruvate is inhibited by high concentrations of free fatty acids inibrown fat. (Steiner and Hall, 1968, unpublished data). PDHa activity does not start to increase until the late suckling period when the high carbohydrate solid diet begins to supplement the high fat diet of milk. PDHa activity is maximal in the late suckling eriod when carbohydrates have become an increasingly important part of the diet.

The decrease in PDHa activity after thirty days of age is slight despite the involution of brown fat. Again, the high percentage of carbohydrate in the diet probably keeps the activity of PDH high.

2. Effect of Insulin on Brown Fat

Although brown fat appears to be unresponsive to insulin postnatally, fetal brown fat shows increased PDHt activity after incubation with insulin. As in liver, one can only speculate as to the mode of action of insulin but it is interesting to note that the same enzyme in a different tissue shows a similar response.

D. Gluteal White Fat

There is no change in the activity of PDH in white fat during development. This is not unusual as many enzymes in white fat show no change in activity with age (Hahn and Skala, 1972).

It was not possible to measure the effect of insulin on gluteal white fat due to the complete loss of PDH activity during incubation. White fat from different parts of the body show different metabolic parameters and we did show a response to insulin by PDH from epididymal white fat which was similar to the literature (Jungas, 1970, 1970; Denton et al. 1971; Coore et al., 1971; Weiss et al., 1971). Since epididymal white fat develops much later than gluteal white fat, we could not determine the effect of age on the response to insulin in this tissue.

E. Human Fetal Liver

Only six fetuses were examined. No developmental trends were apparent. Activity was about the same as in fetal and suckling rat liver and significantly lower than in adult rat liver, suggesting, but not proving, that developmental changes in man may parallel those found in the rat.

XI SUMMARY

This research traces the development of PDH in white fat, brown fat and liver of the rat. PDH, a mitochondrial enzyme, is important in carbohydrate metabolism as it connects the glycolytic pathway with the citric acid cycle. It catalyzes the formation of acetyl-CoA, a precursor of fatty acids and hence is important in the conversion of carbohydrates to fat. Its development is strongly related to the availability of carbohydrates for metabolism and the rates of fatty acid synthesis and the citric acid cycle.

PDH is active in fetal liver where fatty acid synthesis occurs at a high rate. Activity decreases in the neonatal period when the animal is hypoglycemic and the introduction of a high fat diet of milk occurs. Activity increases slightly after the neonatal period which correlates with the increasing activity of the citric acid cycle. Greater increase in activity is found in the late suckling period when a high carbohydrate diet is introduced.

The total activity of PDH in brown fat increases steadily from before birth until the mid-suckling period. This correlates with the increase in the number of mitochondria during this period. Total activity decreases in the adult animal when brown fat undergoes involution. The percentage of PDH in the active form remains low until later in the suckling period when a high carbohydrate diet is introduced. Percentage activity remains high after weaning.

The activity of PDH in white fat did not change during

development.

In fetal brown fat and fetal liver, the total activity of PDH was increased by insulin. This suggests a role for insulin in the maturation of carbohydrate metabolism.

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