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Adipose tissue
β-hydroxyacyl-CoA dehydrogenase
fatty acid catabolism
glucose metabolism
neonates
phosphofructokinase

The Metabolism of Subcutaneous Adipose Tissue in the Immediate Postnatal Period of Human Newborns. 2. Developmental Changes in the Metabolism of ^{14}C-(U)-D-Glucose and in Enzyme Activities of Phosphofructokinase (PFK; EC. 2.7.1.11) and β-Hydroxyacyl-CoA Dehydrogenase (HAD; EC. 1.1.1.35)

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Extract

Changes in the *in vitro* metabolism of subcutaneous adipose tissue have been compared in normal human newborns from 2 hr to 2 weeks of age. A group of healthy adult volunteers was also included. Samples were obtained by using a needle biopsy technique. More of the isotope from ^{14}C-(U)-D-glucose was incorporated into triglycerides ($P < 0.05$) and also oxidized by suspensions of adipose cells from infants 2–3 hr of age than in older infants ($P < 0.01$). The ratio of radioactivity in carbon dioxide to radioactivity in triglyceride was also significantly greater in 2- to 3-hr-old infants than in older neonates ($P < 0.05$). Thin layer chromatography of the total lipid extract showed the greatest amount of radioactivity in the triglycerides, a small amount in 1,3-diglycerides and 1,2-diglycerides, and a trace in fatty acids and monoglycerides. These findings were compared with the developmental changes in two key enzymes: phosphofructokinase (PFK), which represents the glycolytic pathway, and β-hydroxyacyl-coenzyme A (CoA) dehydrogenase (HAD), which is involved in the β oxidation of fatty acids. The activity ratio of these enzymes decreased with age. In a preparation of isolated mitochondria, HAD activity increased with age ($P < 0.001$). These changes in substrate utilization and enzyme activity are consistent with an active metabolism of glucose in the subcutaneous adipose tissue in the first hours of life and relatively greater catabolism of fatty acids in older newborns.

Speculation

Subcutaneous adipose tissue of human newborns is in many respects more active metabolically than the same tissue in adults. For example, lipolysis and reesterification seem

to be more active, at least shortly after birth, and oxygen consumption is increased. This tissue is easily and safely accessible for examination, It responds sensitively to the metabolic situation of the newborn and could be effectively used as a source of information regarding peculiarities of newborns in whom fetal development was abnormal, such as premature delivery, intrauterine growth retardation, and maternal diabetes. Such studies might provide important information for the management of high risk infants.

Introduction

In the first hours of life the human newborn is very dependent on energy derived from its own prenatally stored deposits. Carbohydrate reserves (glycogen) are exhausted in a short time after birth and the significance of adipose tissue as an energy source increases [5, 23]. Free fatty acids formed by lipolysis of triglycerides in adipose tissue are supplied as fuel to other tissues of the body. Previous findings suggest that age-related changes in the *in vitro* metabolism of the subcutaneous adipose tissue [13, 14] parallel changes in total metabolism [15].

The reason for the active lipolysis in the newborn period, as evidenced by high levels of blood glycerol and increasing fatty acid concentrations, has often been discussed. Increased lipolysis secondary to activation of hormone-sensitive lipase seems to be one of the basic mechanisms involved [19, 21]. The carbohydrate content of the adipose tissue is important since glycolytic reactions furnish adenosine triphosphate both as an energy source and also as precursor of cyclic adenosine monophosphate [6]; however, the glycogen stores are exhausted in the adipose tissue between 2 and 6 hr of life [14]. Studies of glycerol release from subcutaneous adipose tissue of human newborns also showed increased lipolysis before 24 hr of age [13, 14]. Utilization of carbohydrate is important for reesterification of fatty acids since it produces the α-glycerophosphate required for this process. Once the content of glycogen in the adipose cells and in the extracellular glucose, including blood glucose, have decreased, the adipose tissue releases proportionately more fatty acids, since α-glycerophosphate is in short supply. These mechanisms may of course be modified by many other influences such as changes in oxygen supply, acid-base balance, hormonal stimulation [8], and the like.

In these studies, changes in *in vitro* metabolism of subcutaneous adipose tissue in groups of normal human neonates in the first hours and days of life have been compared. A group of healthy adult volunteers was also included. The metabolism of ^{14}C-(U)-D-glucose by suspensions of isolated subcutaneous adipose cells was studied by measurement of the incorporation of the radioactivity into isolated triglycerides and measurement of the production of labeled carbon dioxide. Thin layer chromatography was employed to separate the labeled lipids and to determine their chemical nature. These findings were compared with the developmental changes in two key enzyme activities, phosphofructokinase (PFK) and β-hydroxyacyl-CoA dehydrogenase (HAD); the activity ratio PFK/HAD provides some clue about the overall balance between the catabolism of glucose and fatty acids [1, 24].

Materials and Methods

Samples of 10–40 mg subcutaneous adipose tissue were obtained from normal human newborns [31] and adult volunteers by using a needle biopsy technique [11]. Criteria for the selection of normal neonates have been previously described [14]. The adult volunteers were healthy men and women of average body build who were between 25 and 55 years of age.

Incorporation of ^{14}C-(U)-D-Glucose into Triglycerides and Carbon Dioxide

Four age groups were selected for this study: 2–3 hr, 12–24 hr, 2–3 days, and adult. A sample of subcutaneous adipose tissue was obtained from six subjects in each group.

Suspensions of free adipose cells were prepared in Krebs-Ringer bicarbonate buffer (pH 7.4), containing half the usual amount of calcium, 4% albumin [27], and 0.08% collagenase [28], by a modification of the method of Rodbell [12, 20]. Buffer was freshly made daily. Five-tenths milliliter of this digestion medium was used for each approximately 10 mg tissue. Prior to incubation, the cells were washed with Krebs-Ringer bicarbonate medium (pH 7.4) containing 4% albumin. Incubation was performed at 37° in 2-ml siliconized ampules under an atmosphere of 95% oxygen-5% carbon dioxide for 40 min in a metabolic shaker at 120

cycles/min. The incubation medium was 0.5 ml Krebs-Ringer bicarbonate buffer containing 4% albumin and 50 mg/100 ml cold glucose with 2 μCi ^{14}C-(U)-D-glucose [29]. This amount of labeled glucose did not substantially increase the glucose concentration of the incubation medium; *i.e.*, the final concentration of glucose after addition of the labeled material was about 55 mg/100 ml. The total volume of cell suspension added to the incubation medium was approximately 10 μliters, which represents only a small (about 1:50) dilution of the suspension medium: the cell suspension was highly concentrated [12], so that the actual dilution of the incubation medium itself should have been minimal.

The collection of radioactive CO_2 from such small amounts of tissue and incubation medium creates certain technical difficulties which were surmounted by use of a specially constructed vessel (Fig. 1). This vessel was convenient for several reasons. The small volume of space for the gas phase improves the diffusion of carbon dioxide into the absorbant. The absorbant for the carbon dioxide (hyamine hydroxide) was placed in one-half of a small gelatine capsule, the usefulness and advantages of which have already been described [10]. The capsule was placed in a small inner vessel to protect it from contamination which may occur with shaking during the incubation of the sample. Both the incubation vessel and this inner vessel were constructed of nitrocellulose, which does not tend to destroy isolated adipose cells (glass vessels must be siliconized). The cell suspension derived from 10 to 40 mg adipose tissue was added to the medium and the vessel was closed with a rubber stopper lightly coated with silicone grease. After 60-min incubation at 37° in a metabolic shaker at 200 cycles/min, 100 μliters hyamine hydroxide in absolute methanol (1:1) were injected through the rubber stopper into the gelatine capsule and 0.25 ml 6 N H_2SO_4 was injected into the cell suspension. Shaking was continued for 60 min, during which carbon dioxide was collected. The gelatine capsule with its contents was placed as a unit into the counting vial.

Lipids were extracted from the cell suspension quantitatively, using a modified Dole's extraction mixture (isopropanol-*n*-hexane-1 N H_2SO_4, 40:10:1) [3]. The hexane phase was collected after repeated extraction and the hexane was evaporated under a stream of nitrogen. The dry residue was redissolved in 5 ml *n*-hexane and aliquots were taken for: (*1*) enzymatic estimation of glycerol content [26], (*2*) radioactivity of the total lipid fraction, and (*3*) separation of triglycerides

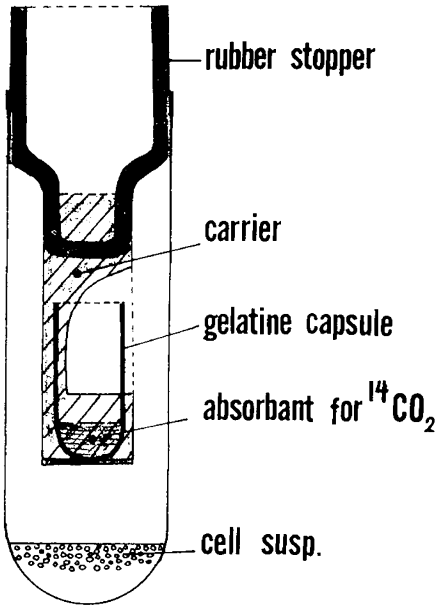

Fig. 1. Vessel for the collection of radioactive carbon dioxide from suspensions of isolated adipose cells with added labeled substrate. Cell suspension was prepared from 10 to 40 mg tissue.

by thin layer chromatography with determination of radioactivity of the triglyceride fraction.

For thin layer chromatography the lipid extract was transferred to glass fiber sheets impregnated with silicic acid [30]; chromatograms were developed ascending in a mixture of ethyl ether-ethyl acetate-*n*-hexane, 20:1:80. The individual fractions were visualized by short exposure to iodine vapors and were marked. The iodine was evaporated and the triglyceride spot was extracted directly into a counting vial with chloroform-*n*-hexane, 1:1.

After evaporation of the respective organic solvents with a stream of nitrogen, Bray's scintillation liquid [2] was added to the vial for counting. Radioactivity was measured in disintegrations per minute, using a Nuclear-Chicago Mark I scintillation counter. Disintegrations per minute were related to glycerol content after saponification of an aliquot of the extract of the adipocyte suspension (disintegrations per minute per 10^{-3} mM glyceride glycerol).

Some of the chromatograms were subjected to autoradiography to estimate the distribution of the radioactivity in lipids other than triglycerides; in this case, the amount of lipid applied at the origin was such that the radioactivity of all samples was equal.

Enzyme Activities

The activities of two enzymes, phosphofructokinase (PFK; EC. 2.7.1.11) and β-hydroxyacyl-CoA dehydro-

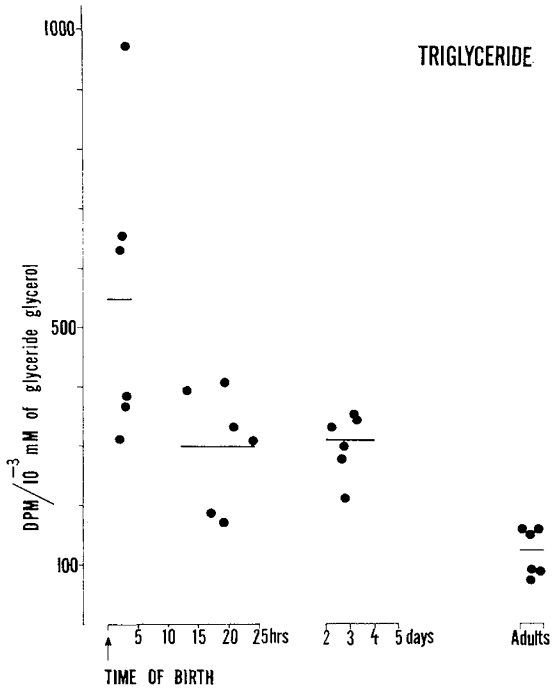

Fig. 2. Age-dependent changes in labeled triglyceride production from ^{14}C-(U)-D-glucose by suspensions of isolated human subcutaneous adipose cells. DPM: disintegrations per minute.

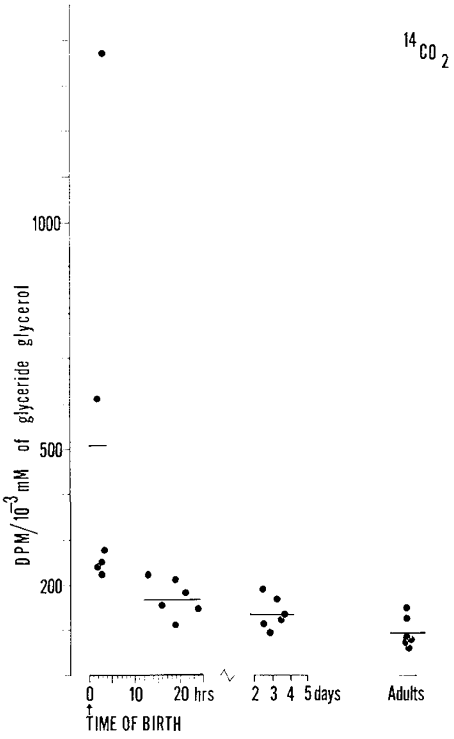

Fig. 3. Age-dependent changes in radioactive carbon dioxide production from ^{14}C-(U)-D-glucose by suspensions of isolated subcutaneous adipose cells. DPM: disintegrations per minute.

genase (HAD; EC. 1.1.1.35), were measured in the subcutaneous adipose tissue from three groups of neonates of different ages: less than 24 hr (10 infants), 24–48 hr (10 infants), 49–66 hr (9 infants), and 4–13 days (9 infants).

The samples of subcutaneous adipose tissue were placed immediately into a small vial on Dry Ice and kept frozen until analysis. The tissue was weighed, placed in 0.3 ml triethanolamine buffer (pH 7.6), and homogenized by using sonification. The lipids were separated by centrifugation (8500 $\times$ *g* for 15 min) and aliquots of the soluble protein fractions were used for enzyme assays and for protein analysis by the method of Lowry *et al.* [9]. Activities of both enzymes were measured as described by Bass *et al.* [1]. Enzyme activity was expressed in units per gram tissue protein (micromoles substrate converted per minute per gram soluble protein). Enzyme activity (HAD) was also measured in isolated mitochondria prepared from the adipose tissue biopsy samples by a microtechnique [17]. In this case, enzyme activity was related to mitochondrial protein (micromoles substrate converted per minute per gram mitochondrial protein).

Results

The incorporation of isotope from ^{14}C-(U)-D-glucose into triglycerides (disintegrations per minute per 10^{-3} mM glyceride glycerol) by suspensions of adipose cells is presented in Figure 2. Statistical analysis (Student's *t* test) revealed significantly greater incorporation of isotope into the triglyceride fraction in infants 2–3 hr of age in comparison with 12–24 hr ($P < 0.05$) and with adults ($P < 0.01$). There was also significantly more isotope incorporated into triglycerides in infants 12–24 hr of age ($P < 0.001$) and 2–4 days ($P < 0.01$) than in adults. The statistical interpretation of the findings regarding radioactive carbon dioxide production (Fig. 3) was influenced by the considerable variability of the results in infants 2–3 hr of age. The variance of this group was significantly different from that of any other group (*F* test, $P < 0.05$), yet several of the very high values of radioactive carbon dioxide release of the whole study were seen in the 2- to 3-hr group. This observation is strengthened by use of nonparametric statistics (the Mann-Whitney test, two-tailed). This test indicated that the radioactive carbon dioxide production was significantly greater at 2–3 hr than in older infants ($P < 0.01$). The ratio of radioactivity in carbon dioxide to radioactivity in triglyceride was also significantly greater in 2- to 3-hr-old infants than in older neonates ($P < 0.05$) (Table I).

Table I. Metabolism of ^{14}C-(U)-D-glucose by suspensions of adipose cells from the subcutaneous adipose tissue[1]

No.	2–3 hr	12–24 hr	2–4 days	Adult
1	0.64	0.55	0.45	0.91
2	0.97	0.55	0.42	0.48
3	0.76	0.50	0.46	0.67
4	0.77	0.84	0.34	1.22
5	1.62	0.48	0.58	1.39
6	0.39	0.65	0.45	0.50

[1] Age-dependent changes in the ratio of radioactive carbon dioxide produced (disintegrations per minute per 10^{-3} mM glyceride glycerol) to the incorporation of radioactivity into triglycerides (disintegrations per minute per 10^{-3} mM glyceride glycerol). By the Mann-Whitney test, two-tailed, ratio between 2 and 3 hr is significantly greater than in older infants ($P < 0.05$).

Radioactivity in the total lipid extract was also estimated in disintegrations per minute per 10^{-3} mM glyceride glycerol. Radioactivity in this fraction was significantly greater (Student's t test, $P < 0.05$) at 2–3 hr of age than in older infants or adults. Iodine staining of the thin layer chromatogram of the total lipid extract (Fig. 4, *left*) revealed, in descending order of R_F values: cholesterol ester, triglycerides, several unidentified fractions, 1,3-diglycerides, 1,2-diglycerides, fatty acids, monoglycerides, and possibly phospholipids. The autoradiogram of this chromatogram (Fig. 4, *right*) showed the greatest amount of radioactivity in the triglycerides, a smaller amount in 1,3-diglycerides and 1,2-diglycerides, and a trace in fatty acids and monoglycerides. There was also a radioactive spot at or near the origin. Because the phospholipids remain nearly at the origin in this solvent system, they cannot be differentiated with certainty from glucose, which might be contaminating the lipid extract and would also remain at the origin. There were no marked differences qualitatively between neonates of different ages and adults either in the fractions revealed by the iodine staining or in those containing radioactivity.

Changes in activity of PFK and HAD in the soluble protein of subcutaneous adipose tissue samples are presented in Table II. Comparisons of the means of the groups showed no significant differences, which clearly may have been due to the wide range of values in each group. However, the ratio of activities of PFK/HAD was higher in the first 24 hr of life than subsequently. There was a significant positive correlation between the activity of PFK and the ratio of PFK/HAD ($P < 0.001$). The HAD activity measured in isolated mito-

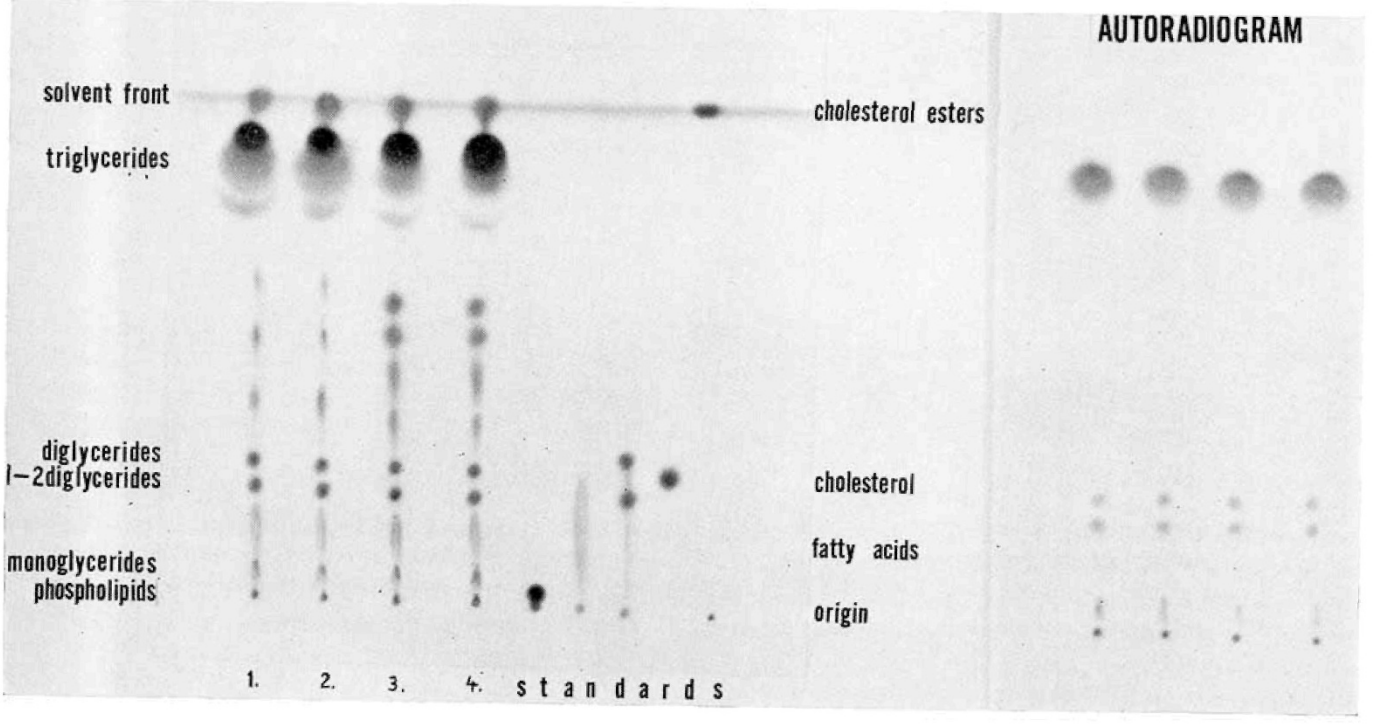

Fig. 4. Thin layer chromatogram and localization of radioactivity in different fractions of the total lipid extract. *1:* 2–3 hr of age. *2:* 12–24 hr of age. *3:* 2–4 days of age. *4:* Adult. Solvent system: ethyl ether-ethyl acetate-*n*-hexane, 20:1:80, ascending. *Left:* iodine staining (vapors). *Right:* radioautogram, exposure 21 days.

Table II. Age-dependent changes in the activities of phosphofructokinase (PFK) and β-hydroxyacyl-CoA dehydrogenase (HAD) in human subcutaneous adipose tissue

Age	PFK[1]	HAD[1]	PFK/HAD[2]
<24 hr	299 ± 105 (10)[3]	124 ± 9 (10)	2.4
24–48 hr	258 ± 105 (7)	147 ± 15 (12)	1.8
49–66 hr	173 ± 69 (8)	120 ± 21 (6)	1.4
4–13 days	201 ± 45 (8)	220 ± 63 (6)	0.9

[1] Units per gram extracted protein ± standard error.
[2] Ratio of means.
[3] Numbers in parentheses: number of samples analyzed.

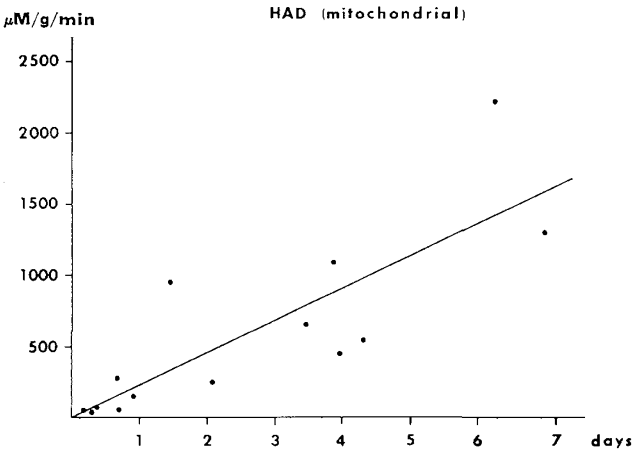

Fig. 5. Age-dependent changes in mitochondrial β-hydroxyacyl-CoA dehydrogenase (HAD) in subcutaneous adipose tissue from human newborns. $y = 9.437x + 7.43$; $r = 0.822$; $P < 0.001$.

chondrial fraction (Fig. 5) was positively correlated with age ($y = 9.437x + 7.43$; $r = 0.822$; $P < 0.001$).

Discussion

In this study more of the isotope from universally labeled glucose was incorporated into triglycerides and also oxidized by suspensions of adipose cells from infants 2–3 hr of age than from older infants and adults. This increased incorporation of isotope into triglycerides in the 2- to 3-hr group suggests an elevated involvement of the carbon skeleton of glucose in the process of reesterification and deposition of glycerides in the youngest group. It has been described that the metabolites of glucose are used, in short *in vitro* incubations, primarily as a source of α-glycerophosphate for reesterification rather than of acetyl-CoA for fatty acid synthesis *de novo* [4]. The increased production of radioactive carbon dioxide in the 2- to 3-hr-old group points to increased glucose utilization by oxidative pathways. This represents the carbon dioxide produced in the Krebs cycle. In the catabolism of glucose, carbon dioxide can also be produced by the pentose shunt. This pathway is known to be relatively active in adipose tissue of the adult rat [25]. Its activity in human subcutaneous adipose tissue has not been measured, so the possible contribution of the pentose shunt pathway to the production of radioactive carbon dioxide under the conditions of this study cannot be evaluated without additional information.

Stored glycogen disappears from the adipose tissue during the first hours of life [14]. Because glucose 6-phosphatase activity is low or absent in adipose tissue cells [25], glucose 6-phosphate formed by glycogenolysis should be retained in the adipose cells and metabolized on location. Thus the relatively high glycogen content in adipose tissue after birth ensures α-glycerophosphate formation and esterification of fatty acids [22]. Therefore, fatty acid release may be low even under conditions of elevated lipolysis. This may also explain the decreased effect of glucose on free fatty acid release from the subcutaneous adipose tissue shortly after birth [16].

The increased ratio of labeled carbon dioxide to labeled triglycerides in the youngest group indicates increased glucose oxidation and suggests that glucose is used more for energy production than for glyceride formation. The decreasing levels of blood glucose, changes in the glycogen content in tissues [23], including adipose tissue [14], and the decrease in respiratory quotient [7] indicate that carbohydrates may serve as a predominant substrate for energy production only for a short period and that free fatty acids are then increasingly utilized.

We investigated the activity of two enzymes which represent key functions for the utilization of glucose and fatty acids. Phosphofructokinase (PFK) catalyzes the conversion of fructose 6-phosphate to fructose-1,6-diphosphate in the glycolytic pathway. β-Hydroxyacyl-CoA dehydrogenase (HAD) catalyzes the conversion of β-hydroxacyl-CoA to acetoacetyl-CoA and is involved in the β oxidation of fatty acids [18]. Both are key unidirectional enzymes in their respective metabolic chains and both can be used to represent glycolysis or β oxidation of fatty acids, respectively. Changes in PFK/HAD ratio were used to evaluate the shifts in the relative importance of the two pathways [1]. The decrease in this ratio in older neonates is indicative of a decreased utilization of glucose with increasing age. The increase seen in mitochondrial HAD with increasing age is consistent with an increased utilization of fatty acids in older infants. These findings provide

further evidence that human neonates substitute fatty acid for carbohydrate catabolism in the subcutaneous adipose tissue after the first day of life.

Summary

1. Radioactivity from ^{14}C-(U)-D-glucose was incorporated into triglycerides and total lipids by adipose tissue cells of all age groups, but the 2- to 3-hr-old newborns disclosed the highest rates of incorporation. Oxidative breakdown of glucose was measured by trapping the radioactive carbon dioxide formed, and this pathway was also found to be the most active in the youngest infants. The ratio of labeled carbon dioxide to labeled triglycerides was highest in the youngest infants.

2. Isotope from universally labeled glucose was incorporated mostly into triglycerides. A small amount of radioactivity was also found in 1,3-diglycerides and 1,2-diglycerides and traces were found in fatty acids and monoglycerides.

3. The activity ratio of two key enzymes, PFK, which represents the glycolytic pathway, and HAD, which is involved in the β oxidation of fatty acids, was measured in relation to cell protein and was found to decrease with age. The activity of HAD, measured in isolated mitochondrial fraction, was positively correlated with age in human neonates ($y = 9.437x + 7.43$; $r = 0.822$; $P < 0.001$).

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30. Chrom-AR sheet 1000, 20- by 20-cm. Mallinckrodt Chemical Works, St. Louis, Mo.
31. Consent to study the infants was given by the mother prior to obtaining the fat biopsies.
32. Presented in part at the Annual Meeting of the Southern Society for Pediatric Research, New Orleans, La., November, 1970.
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