propyl phosphorodiamidic fluoride it has been established that phenyl phenylacetate and phenyl 3-phenylpropionate are hydrolysed by two or more esterases.

4. These esterases are present in brain, spinal cord and sciatic nerve.

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*Biochem. J.* (1964) **90**, 189

# Changes in Glucose Utilization during Development of the Lamb

BY I. G. JARRETT, G. B. JONES AND B. J. POTTER
*C.S.I.R.O. Division of Biochemistry and General Nutrition, University of Adelaide, South Australia*

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The young lamb relies to a great extent for its energy requirements on exogenous carbohydrate and higher fatty acids, substances which are constituents of milk. The adult sheep, however, obtains the major proportion of its energy requirements from lower fatty acids which are absorbed from the rumen where they are produced by microbial fermentation of cellulose and related carbohydrates.

Annison & Lewis (1959) have discussed the importance of acetate in the metabolism of adult sheep, and Lindsay (1959) has reviewed various aspects of the role of glucose in ruminants. In adult sheep injected glucose is removed from the blood stream at a much slower rate than in non-ruminants or in very young lambs (McCandless & Dye, 1950; Jarrett & Potter, 1952). Lambs differ from adult

sheep and more closely resemble non-ruminants in many other aspects of carbohydrate metabolism. Jarrett & Filsell (1958) have reported that the ability of intestinal mucosa from adult sheep to phosphorylate glucose is much less than that of the young lamb. In a study of the activities of enzymes associated with carbohydrate metabolism in livers from sheep and lambs, Filsell, Jarrett, Atkinson, Caiger & Morton (1963) found much higher rates of reduction of NADP in the younger animals. Under the experimental conditions these were interpreted as higher activities of glucokinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the young lambs.

The utilization of glucose in adult sheep has been investigated by Annison & White (1961), Kronfeld & Simeson (1961) and Bergman (1963). Isotope-dilution techniques were employed and values for glucose pool, glucose space and turnover rates were determined. From these studies it is suggested that the glucose metabolism of sheep in the post-absorptive or non-fed state may be less than that of non-ruminants.

Isotope dilution with [$^{14}$C]glucose has been applied in this current study to determine glucose-utilization rates, glucose-pool size and glucose space in young lambs when they are dependent on constituents of milk for their metabolic requirements, and in older lambs and adult sheep which normally have a limited supply of glucose available from the gastrointestinal tract.

## EXPERIMENTAL

*Animals.* Merino sheep that had been accustomed to being handled and had been trained to remain placid in metabolism cages were used. They were given 1000 g. of a mixture of 2 parts of chaffed wheaten hay and 1 part of chaffed lucerne hay each morning except on the day of the experiment. The lambs, which had been running with their dams on an improved mixed pasture, were separated from the ewes and confined in a small cage just before the experiment. They were kept as quiet as possible in close proximity to their dams throughout the experimental period.

*Procedure.* A polythene tube filled with a heparin solution was secured into each external jugular vein at least 1 hr. before the initial injection. Uniformly labelled D-[$^{14}$C]-glucose was administered into the left vein and blood samples were withdrawn from the right vein. In some of the experiments (see Table 1) eight wether lambs (aged 2–8 weeks), two adult wethers and 1 adult ewe (aged 1–3 years) were each given a single injection of 0·9% NaCl solution containing [$^{14}$C]glucose (50 $\mu$c for the lambs and 150 $\mu$c for the adults), and blood samples were taken at intervals of 15 min. for 1 hr. With two of the lambs (nos. 26 and 28) the experimental period was extended to 3 hr.

In a subsequent series of experiments (see Table 2) two wether lambs (aged 2 weeks), two ewe lambs (aged 3 weeks and again at 6 weeks of age) and two adult wethers (aged 1–2 years) were each given a single priming injection of 10 ml. of 0·9% NaCl solution containing 20–40 $\mu$c of [$^{14}$C]glucose into the left jugular vein. This injection was immediately followed by a continuous infusion of 0·9% NaCl solution containing 25–60 $\mu$c of [$^{14}$C]glucose/100 ml. at a rate of 0·5 ml./min. for 3 hr. Blood samples were taken at 15 min. intervals for the first hour and at 30 min. intervals for the next 2 hr. This procedure was similar to that of Steele, Wall, de Bodo & Altszuler (1956).

Since the [$^{14}$C]glucose, obtained from The Radiochemical Centre, Amersham, Bucks., had a specific activity of either 20 or 410 $\mu$c/mg., the total amount of glucose injected was insignificant relative to the quantity normally present in the animals.

*Determination of specific activity of blood glucose.* The blood samples were treated with $Ba(OH)_2$ and $ZnSO_4$ (Somogyi, 1952) and the precipitated proteins removed by filtration. The filtrate was used for the determination of the concentration of reducing sugar in the blood (Nelson, 1944; Somogyi, 1952) and for the specific activity of the blood glucose. To 25 ml. of the filtrate (equivalent to 2·5 ml. of blood) 90 mg. of unlabelled carrier glucose was added and the specific activity of the labelled glucose determined. Glucose was isolated as the phenylglucosazone which was then converted into phenylglucosotriazole (Steele, Bernstein & Bjerknes, 1957). Care was taken to prevent thermal decomposition of the product by reducing the loss of water to a minimum and by maintaining the level of the oil bath always below the level of the solution in the test tube. Yields of up to 45 mg. of the purified triazole were obtained.

A phosphor solution was modified from Bray's (1960) mixture and consisted of: naphthalene, 60 g.; 2,5-diphenyloxazole, 4 g.; *p*-bis-(5-phenyloxazol-2-yl)benzene, 0·2 g.; methanol, 100 ml.; ethylene glycol, 20 ml.; 1,4-dioxan, 880 ml.; boric acid, 25 g. (Jones & Henschke, 1963). This scintillation mixture is a better solvent for the glucosotriazole and provides improved counting efficiency and lower background than the ethanol–xylene solvent mixture used by Steele *et al.* (1957). To 5 ml. of this mixture 20–25 mg. of triazole was added and the activity of the triazole was measured in duplicate in an Ekco liquid-scintillation counter at room temperature. A known quantity of the same [$^{14}$C]glucose as that injected was converted into the triazole and used as a standard. From the specific activity of the glucosotriazole, the carrier-glucose dilution factor and the blood glucose concentration, the specific activity of the original blood glucose was calculated as $\mu$c/mg. of blood glucose.

In some of the initial experiments with the single-injection procedure (see Table 1) 25 ml. of protein-free filtrate was evaporated to dryness under vacuum and the organic residue oxidized by the Van Slyke, Plazin & Weisiger (1951) method to $^{14}CO_2$, which was absorbed in NaOH and precipitated as $Ba^{14}CO_3$. This was counted as layers of 'infinite thickness' under an end-window Geiger–Müller tube. The activity obtained in this manner was related to the glucose content of the blood filtrate and expressed as $\mu$c/mg. of blood glucose on the premise that the $^{14}$C activity in the filtrate was present as [$^{14}$C]glucose during the first hour after a single injection.

*Calculation of glucose pool, space and utilization rate.* In determination of these parameters by using isotope-dilution techniques it is usual to consider the glucose pool as the amount of glucose which dilutes the [$^{14}$C]glucose

Table 1. *Glucose pool, glucose space and glucose-utilization rates in lambs and sheep measured by the single-injection technique*

Details are given in the text.

| Animal no. | Age (weeks) | Body wt. (kg.) | Mean concn. of blood sugar (mg./100 ml.) | [$^{14}$C]-Glucose injected ($\mu$c) | Sp. activity at zero time ($\mu$mc/mg. of blood glucose) | Glucose pool (mg./kg.) | Glucose-utilization rate (mg./min./kg.) | Glucose space (% of body wt.) |
|---|---|---|---|---|---|---|---|---|
| 68* | 2 | 8·0 | 82 | 50 | 13·0 | 480 | 8·6 | 59 |
| 36* | 2 | 7·0 | 80 | 50 | 14·9 | 480 | 8·7 | 60 |
| 19* | 2 | 7·0 | 74 | 50 | 14·8 | 480 | 7·6 | 65 |
| 26 | 3 | 10·5 | 95 | 40 | 70·0 | 540 | 8·3 | 57 |
| 28 | 3 | 9·0 | 100 | 40 | 78·0 | 570 | 8·1 | 57 |
| 95* | 4 | 9·1 | 67 | 50 | 16·0 | 343 | 6·6 | 51 |
| 87* | 6 | 15·1 | 71 | 75 | 15·3 | 327 | 5·6 | 46 |
| 26 | 6 | 22·2 | 72 | 40 | 60·0 | 300 | 3·0 | 42 |
| 28 | 6 | 20·4 | 67 | 40 | 47·0 | 417 | 4·4 | 62 |
| 92* | 8 | 9·5 | 51 | 50 | 15·3 | 344 | 3·7 | 67 |
| 73* | 1 year | 21·0 | 57 | 50 | 12·0 | 198 | 2·2 | 35 |
| 52* | 2 years | 29·8 | 52 | 160 | 34·0 | 158 | 2·0 | 31 |
| 47* | 3 years | 38·7 | 51 | 150 | 19·4 | 200 | 1·7 | 39 |

* Animals in which the activities were determined by end-window counting of $Ba^{14}CO_3$.

injected, and which is present principally in the extracellular fluid. The glucose space is that volume of fluid of the same glucose concentration as the plasma and which contains the amount of glucose present in the glucose pool.

In the single-injection experiments the specific activity of the blood glucose was plotted against time on a semi-logarithmic scale. Extrapolation of the line to zero time gave the specific activity of the blood glucose at zero time, and thus the determination of the initial dilution of the specific activity enabled the blood glucose pool size to be calculated. By determining the logarithmic drop in specific activity with time the utilization rate was calculated from the first-order reaction rate by using the formula:

$$U = 2{\cdot}3\,(b/t)\,\log(a_0/a_t)$$

where $U$ is the utilization rate (mg./min./kg.), $b$ is the blood glucose concentration (mg./100 ml.) which remains constant throughout the experimental period, and $a_0$ and $a_t$ are the specific activities of the glucose at the initial and final times (Dunn, Friedmann, Maass, Reichard & Weinhouse, 1957).

In the continuous-infusion experiments the specific activities were plotted against time. Provided that a straight horizontal line was the best fit for values obtained between 60 and 180 min. after the priming injection (see Fig. 2) and the blood glucose concentration did not change during the experimental period, the glucose pool and utilization rate could be calculated. Steele *et al.* (1956) have discussed the validity of using a simplified relationship when these requirements are fulfilled, and the relationship:

$$\text{Sp.act.} = P/G = F/U$$

where sp.act. is the specific activity at time $t$, $P$ is the priming dose in $\mu$c of $^{14}$C, $G$ is the body glucose-pool size in mg. of glucose, $F$ is the infusion rate in $\mu$c of $^{14}$C/min. and $U$ is the utilization rate of glucose (mg./min./kg.), has been used in these calculations.

The glucose space was calculated by dividing the glucose pool by the blood glucose concentration and expressing this value as a percentage of the body weight.

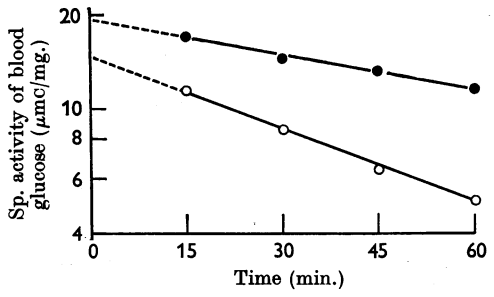


Fig. 1. Specific activity of blood glucose in a typical experiment with a single injection of [$^{14}$C]glucose. ●, Values obtained for adult sheep (no. 47); ○, values obtained for lamb (no. 36) aged 2 weeks.

## RESULTS

The specific activities of the blood glucose plotted on a semi-logarithmic scale at various times after a single injection of [$^{14}$C]glucose into a lamb aged 2 weeks and a sheep aged 3 years are shown in Fig. 1. The line of best fit, extrapolated back to zero time, is shown for both animals and is representative of the pattern obtained for all other lambs and sheep. Estimations of the size of the glucose pool, of the glucose space and of glucose-utilization rates in lambs and adult sheep are shown in Table 1. The size of the glucose pool was much larger in young lambs (approx. 500 mg./kg.) than in adult sheep (approx. 200 mg./kg.). In the young lamb the glucose space was much greater than in adults, and the utilization rate diminished from about 8 mg./min./kg. in 2–3-week-old lambs to about 2 mg./min./kg. in adult sheep.

Table 2. *Glucose pool, glucose space and glucose-utilization rates in lambs and sheep measured by the continuous-infusion technique*

Details are given in the text.

| Animal no. | Age (weeks) | Body wt. (kg.) | Mean concn. of blood sugar (mg./100 ml.) | [$^{14}$C]-Glucose in priming injection ($\mu$c) | [$^{14}$C]Glucose infusion rate ($\mu$c/min.) | Sp. activity ($\mu$mc/mg. of blood glucose) | Glucose pool (mg./kg.) | Glucose-utilization rate (mg./min./kg.) | Glucose space (% of body wt.) |
|---|---|---|---|---|---|---|---|---|---|
| 21 | 2 | 8·7 | 93 | 20 | 0·15 | 3·30 | 690 | 5·0 | 74 |
| 25 | 2 | 9·8 | 92 | 20 | 0·15 | 3·45 | 582 | 4·2 | 63 |
| 43 | 3 | 11·7 | 87 | 30 | 0·25 | 3·70 | 692 | 5·8 | 79 |
| 44 | 3 | 10·0 | 87 | 30 | 0·25 | 5·00 | 600 | 5·0 | 69 |
| 43 | 6 | 19·0 | 80 | 30 | 0·25 | 3·50 | 450 | 3·8 | 56 |
| 44 | 6 | 16·5 | 79 | 30 | 0·25 | 4·30 | 424 | 3·5 | 53 |
| 30 | 2 years | 33·3 | 52 | 40 | 0·33 | 9·25 | 130 | 1·1 | 25 |
| 33 | 2 years | 27·8 | 50 | 40 | 0·33 | 9·25 | 156 | 1·3 | 31 |

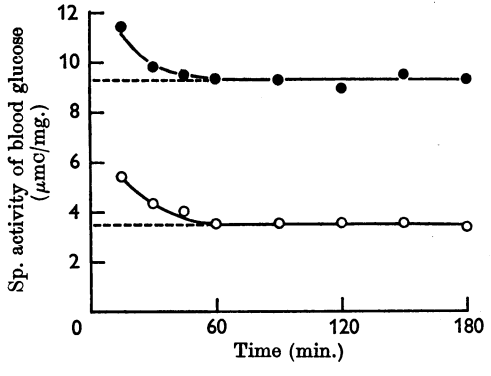


Fig. 2. Specific activity of blood glucose in a typical experiment with a priming dose of [$^{14}$C]glucose followed by a continuous infusion. ●, Values obtained for adult sheep (no. 30); ○, values obtained for lamb (no. 25) aged 2 weeks.

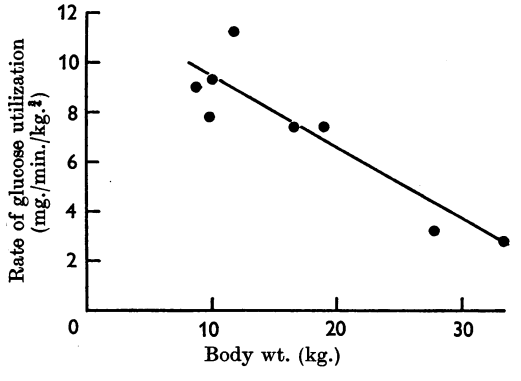


Fig. 3. Relationship between glucose-utilization rates, expressed as mg./min./kg.$^{\frac{3}{4}}$, and body weight, for lambs and sheep receiving a continuous infusion of [$^{14}$C]glucose.

Fig. 2 shows the specific activities of the blood glucose plotted against time after a priming injection of [$^{14}$C]glucose immediately followed by a continuous infusion at a constant rate into a lamb aged 3 weeks and a sheep aged 1 year. Straight horizontal lines were obtained over the period 60–180 min. after the injection, and when extrapolated back to zero time gave the values for specific activities which were used to calculate the glucose pools and utilization rates (see the Experimental section). The plotted values and lines shown are representative of those obtained for the other sheep and lambs, each of which gave a straight horizontal line as the best fit to the values obtained during this time-interval. Estimations of the size of the glucose pool, of the glucose space and of the glucose-utilization rate determined by this procedure are shown in Table 2. Similar differences to those observed with the single-injection technique were found to exist between lambs and sheep: the continuous-infusion method gave a pool size of approx. 640 mg./kg. for the lambs and of 140 mg./kg. for the adults, with a utilization rate of 5·0 mg./min./kg. for lambs and of 1·2 mg./min./kg. for adults.

When the utilization rates were calculated on the basis of the $\frac{3}{4}$ power of the body weight the same general trend was observed. Fig. 3 shows the regression line (regression coefficient $-0·29$; $P < 0·01$) obtained when the values from the infusion method were calculated as mg. of glucose utilized/min./kg.$^{\frac{3}{4}}$ and plotted against body weight (kg.).

The mean blood sugar concentrations are shown in both Tables 1 and 2, and simple linear regressions of blood sugar concentrations against time gave horizontal straight lines for most of the animals, indicating no significant differences of any of the values from the mean. With animals nos. 25, 26, 47 and 28, however, statistically-significant linear trends were evident, but the deviation from the mean was small and blood sugar concentrations were considered as a homogeneous group and the mean values were used for estimation of the glucose space in all cases.

## DISCUSSION

The estimations of the rate of turnover of glucose reported above for adult sheep agree with those reported by Annison & White (1961). Values obtained by the continuous-infusion procedure (1·1–1·3 mg./min./kg.) were lower than those obtained with the single injection of [$^{14}$C]glucose (1·7–2·2 mg./min./kg.): because of the limitations inherent in the latter method the lower values probably represent a more accurate estimate (Steele *et al.* 1956).

Despite the difficulties of interpreting the movement of glucose into and out of the various fluid 'compartments' of the whole animal, estimates based on isotope-dilution techniques can give a close approximation to the real values for glucose pool and glucose space (Wrenshall, Hetenyi & Best, 1961).

In the present study where a comparison was made between lambs of various ages and adult sheep, the glucose pool and space were calculated from determinations of glucose concentrations and specific activities in whole blood rather than in plasma. In the adult sheep the erythrocytes are almost devoid of glucose, whereas in the lamb during the first week after birth glucose is present in the erythrocytes in appreciable amounts (Reid, 1953; Goodwin, 1956; Leng & Annison, 1962), and this should be considered in any comparison of glucose space.

However, although the values obtained for glucose pool, space and utilization rates in either the sheep or the lambs may be subject to some correction owing to the use of whole blood, the magnitude of the changes in these parameters during development of the lambs was so great that any minor alterations would not affect the general conclusions.

The values for glucose space in adult sheep reported in the present study (mean 32 % of body weight, range 25–39 % for five animals) are slightly higher than the range of 16–30 % of body weight observed by Annison & White (1961) and of 23–33 % of body weight found by Kronfeld & Simesen (1961), based on both the single-injection and continuous-infusion techniques. If allowance is made for the erythrocyte volume in the whole blood, and the space is calculated on plasma values, a mean value of 24 % of body weight is obtained: this value is consistent with the concept that glucose space is equivalent to the volume of extracellular fluid (Steele *et al.* 1956).

Values of about 70 % of body weight for glucose space (based on determinations on whole blood) in the young lamb suggest that a greater entry of glucose into the intracellular space had taken place, a possibility consistent with the recognized presence of glucose within the erythrocytes of the young lamb. When the values of glucose space for the young lambs were calculated with allowance for the erythrocyte volume and glucose content, values 10–15 % lower than those shown in Tables 1 and 2 were obtained.

Kronfeld, Tombropoulos & Kleiber (1959) reported that the glucose space in ketotic cows was between 54 % and 80 % of body weight, and considered that this could be attributed to an expansion of the glucose space into the intracellular 'compartment'.

The higher values in the young lambs reported in the present work compared with those for adult sheep could, however, be partly accounted for by the relatively larger volume of extracellular fluid characteristic of young animals. Elkinton & Danowski (1955) have discussed the relatively larger extracellular 'compartment'/unit wt. in infants compared with adult humans and quote values of 42–44 % for extracellular space in the young and 22–27 % in adults.

The greater glucose-utilization rate observed in the young lamb (about 5 mg./min./kg.) falling to about 1·3 mg./min./kg. in the adult indicates the greater turnover of glucose in lambs compared with that in adult sheep. Kleiber (1947) has reported that for warm-blooded animals varying in body size the daily metabolic rate divided by the $\frac{3}{4}$ power of the body weight is nearly the same. As the utilization rates observed in this present study, when calculated on the basis of either body weight (kg.) or metabolic body size (kg.$^{\frac{3}{4}}$), decreased with increasing body weight, it is unlikely that the greater utilization of glucose by young lambs could be associated solely with metabolic activity. The present study therefore provides further evidence of the differences in carbohydrate metabolism between lambs and adult sheep in which absorption of exogenous glucose normally does not occur.

## SUMMARY

1. The glucose pool, glucose space and glucose-utilization rate in young lambs and adult sheep have been determined by isotope-dilution techniques with [$^{14}$C]glucose.

2. Both a continuous-infusion procedure and a single-injection technique were used: lower values for glucose-utilization rate were obtained by the former method.

3. The glucose-utilization rate for young lambs (about 5 mg./min./kg. body wt.) was much higher than that for adult sheep (1·3 mg./min./kg.).

4. In the young lamb glucose pool (about 500 mg./kg. body wt.) was larger than that of adult sheep (200 mg./kg.), and the space which this occupied decreased from values of about 60–70 %

of body weight in the very young lamb to about 24% of body weight in the adult.

5. These results are discussed in relation to differences in carbohydrate metabolism between young lambs and mature sheep.

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# The Metabolism of Phenolic Antioxidants

## 4. THE METABOLITES OF GENTISIC ACID IN THE DOG*

BY B. D. ASTILL, D. W. FASSETT AND R. L. ROUDABUSH
*Laboratory of Industrial Medicine, Eastman Kodak Co., Rochester 4, N.Y., U.S.A.*

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Gentisic acid and its sodium salt are potentially useful antioxidants in the stabilization of edible fat (Wishnetzky & Stuckey, 1961). Many studies of the fate of gentisic acid after absorption by mice, rats, rabbits and man have been reported (Williams, 1959*a*, p. 369), but its metabolic fate is obscure. As a part of a study to test the suitability for use in foodstuffs of gentisic acid and sodium gentisate, a detailed account of their disposal in a large mammal such as the dog was needed, with particular emphasis being placed on elucidating the nature of the metabolites.

Gentisic acid is known to be rapidly excreted, often largely unchanged, in the urine after oral ingestion, and increases in urinary *O*-sulphate and *O*-glucuronide concentrations may occur. No metabolites were isolated, although chromatographic evidence has been presented for *O*-glucuronide formation with the 5-hydroxyl group (Haberland, Madenwald & Köster, 1957), for dehydroxylation to salicylic acid in fevered humans (Consden & Stanier, 1951) and for methylation of the 5-hydroxyl group in humans (Sakamoto, Inamori & Nasu, 1959). The only previous study in the dog (Likhatscheff, 1895) found an increased urinary *O*-sulphate output to account for 18% of the dose of gentisic acid.

We now report the isolation and characterization of the products of the metabolism of gentisic acid in the dog, which appear to be formed exclusively by conjugation at the 5-hydroxyl group. Small amounts of the principal metabolites are also excreted as conjugates of gentisic acid after the ingestion of acetylsalicylic acid by the dog.

* Part 3: Astill, Mills, Fassett, Roudabush & Terhaar (1962).