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Coenzyme A in the Livers of Young Lambs, Adult Sheep and Rats

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Adult sheep derive the greater part of their energy requirements from the lower fatty acids which are absorbed from the rumen where they are produced by microbial degradation of cellulose and related carbohydrates. Young lambs, however, rely largely on constituents of milk as the ultimate source of energy, and during the immediate post-natal period many aspects of carbohydrate and fatty acid metabolism differ from those characteristic of adult sheep and more closely resemble those of monogastric animals.

The size of the glucose pool and the rate of utilization of glucose is much greater in the young lamb than in the adult (Jarrett, Jones & Potter, 1964), and when either glucose or acetate is injected into the circulating blood stream the rate of removal of these substances is faster in lambs than in adult sheep (McCandless & Dye, 1950; Jarrett & Potter, 1952; Jarrett & Filsell, 1960).

Jarrett & Filsell (1958), McLean (1958), Gallagher & Buttery (1959) and Filsell, Jarrett, Atkinson, Caiger & Morton (1963) have reported that quantitative differences occur in the concentrations of certain enzymes associated with carbohydrate metabolism between tissues of lambs, adult sheep and rats. Caiger, Morton, Jarrett & Filsell (1962) have shown that higher concentrations of NAD are present in the livers of adult sheep than in the livers of lambs and rats. They have suggested that this relatively higher activity may be related to the metabolism of the lower fatty acids, important in sheep nutrition, via the tri-carboxylic acid cycle which is largely NAD-dependent.

CoA also plays an important role in the metabolism of intermediaries through the tricarboxylic acid cycle and in the synthesis of higher fatty acids. In view of known differences in other enzymes and

coenzymes in relation to metabolic patterns, a study of the concentrations of CoA in the livers of lambs, adult sheep and rats has been undertaken to elucidate further aspects of metabolic pathways in these animals.

EXPERIMENTAL

Animals. Merino sheep (one ewe aged 14 months, seven ewes aged 3–4 years and four wethers aged 3–4 years) were used in these experiments. The lambs (17 wethers aged 1–83 days) were born during May–June and had been running with their dams on an improved mixed pasture. The adult sheep fed on the same pasture, except for two of the ewes that were fed in pens on a daily ration of 600 g. of chaffed wheaten hay and 400 g. of lucerne chaff.

The rats (Wistar strain, male, aged 3–5 months) were fed on a commercial preparation containing wheat (45%), oats (40%), fish meal (8%), dried yeast (1%), dried skim-milk (5%) and NaCl (1%). Each ton of the cubes contained a supplement of vitamin A (8×10^8 i.u.), vitamin D₃ (2×10^6 i.u.), vitamin E (2500 i.u.), riboflavin (3 g.), thiamine hydrochloride (1 g.), vitamin K (1 g.), pantothenic acid (1 g.), nicotinic acid (5 g.), choline chloride (50 g.) and vitamin B₁₂ (6.8 mg.).

The sheep and the lambs were removed from the source of their food about 2 hr. and the rats about 30 min. before slaughter.

Procedure. The animals were killed by severing the neck and cervical cord and the livers were removed within 30 sec. With the sheep and lambs a number of slices were taken from each lobe; with the rats the whole liver was sliced. The slices were rapidly chopped into small pieces and the chopped material was thoroughly mixed. Weighed portions were taken for the determination of CoA, DNA and dry weight.

Extraction and determination of coenzyme A. Approx. 4 g. of tissue was weighed and homogenized at 2000 rev./min. for 2 min. in 20 ml. of glass-distilled water in a glass Potter–Elvehjem homogenizer with a Teflon pestle (A. H. Thomas and Co., Baltimore, U.S.A.) in a boiling-water bath. The homogenizer containing the water was preheated for 5 min. The homogenate was then heated in the boiling-water bath for a further 8 min., cooled in ice and the volume of homogenate made up to 30 ml. A clear extract was obtained by centrifuging at 60000g for 30 min. at 2°.

The CoA extracted from the liver was determined by the enzymic acetylation of 4-aminoazobenzene by pigeon-liver enzyme according to the method of Handschumacher, Mueller & Strong (1951). The enzyme from acetone-dried pigeon-liver powder was prepared according to the method of Kaplan & Lipmann (1948). A blank, standard tubes containing 4, 8 and 12 µg. of CoA, and tubes containing 0.1, 0.2 and 0.3 ml. of extract respectively were used in each determination. In each case the determination was completed on the day of extraction.

The CoA, acetone-dried pigeon-liver power and GSH were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. The disodium salt of ATP was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. The 4-aminoazobenzene (Eastman–Kodak) was recrystallized twice from hot benzene, several times from methanol and finally from hexane. The purified material formed clear-orange needles with a glistening sheen and had m.p. 125°.

Extraction and determination of deoxyribonucleic acid phosphorus. Approx. 0.4 g. was extracted according to the procedure described by Schneider (1945) except that 5% (w/v) perchloric acid replaced the 5% (w/v) trichloroacetic acid. After the ethanol–ether extraction the residue was washed with ether and then dried in a desiccator over CaCl₂ and chips of paraffin wax.

The DNA was extracted from the residue by heating with 5 ml. of 5% perchloric acid at 90° for 15 min., centrifuging and re-extracting with a further 5 ml. of 5% perchloric acid at 90° for 10 min. The extracts were combined and the final volume was adjusted to 10 ml. The DNA was determined by Burton's (1956) method; 0.2, 0.4 and 0.6 ml. of the extract and the standard solution respectively, each made up to a volume of 1.0 ml. with 5% perchloric acid, were used for each determination. To avoid any possible deterioration of the DNA solution on storage the standard was made up fresh each day. Approx. 1.5 mg. of DNA in 5 ml. of 5% perchloric acid was heated at 90° for 15 min. and then cooled, and the volume was made up to 10 ml. Owing to the difficulty of accurately weighing such a small amount of DNA the actual amount used was checked by determination of the phosphorus content by Allen's (1940) method. In each case determinations were completed within 24 hr. of sampling.

DNA (sodium salt; type I; highly polymerized; from calf thymus) was obtained from the Sigma Chemical Co. The diphenylamine and acetic acid were AnalaR grade from British Drug Houses Ltd.

Dry weight. Chopped tissue (approx. 2 g.) was weighed before and after being dried at 96° for 48 hr.

RESULTS

Reference bases. So that changes in amounts of CoA could be adequately assessed in the liver which is undergoing rapid development in the lambs, a number of parameters other than calendar age were determined. Table 1 shows the values for age, body wt., liver fresh wt., liver dry wt. and the amount of DNA phosphorus/g. fresh wt.

The percentage dry wt. in the livers of lambs up to 83 days old was 25.7% (range 25–28.6%), and in adult sheep increased to a mean value of 28.6% (range 27.8–30.2%). The amount of DNA phosphorus/g. fresh wt. was 229 µg./g. (range 137–319 µg./g.) in the lambs, and 180 µg./g. (range 114–255 µg./g.) in the adult sheep. The mean value for six rats was 179 µg./g. (range 140–212 µg./g.).

Fig. 1 shows that when liver dry wt. was plotted against body wt. the majority of the lambs and sheep fitted the regression:

$$\log[\text{liver dry wt. (g.)}] = 0.8593 + 0.8959 \log[\text{body wt. (kg.)}].$$

It is apparent that, during growth of the lambs, the increase in liver wt. in relation to the increase in body wt. was fairly constant. Liver dry wt. has been used as a reliable index to growth in assessing amounts of coenzyme in the livers of developing lambs.

Table 1. *Reference bases and the concentration of coenzyme A and deoxyribonucleic acid phosphorus in the livers of lambs, sheep and rats*

Animal	Age (days)	Body wt. (kg.)	Liver fresh wt. (g.)	Liver dry wt. (g.)	CoA ($\mu\text{g./g.}$ fresh wt.)	DNA P ($\mu\text{g./g.}$ fresh wt.)	CoA:DNA P ratio
Lambs	1	3.4	71	17.8	139	226	0.62
	1	4.8	92	24.0	118	266	0.44
	2	3.7	73	18.4	161	175	0.92
	2	3.8	84	21.0	193	282	0.68
	3	5.1	125	33.4	147	137	1.07
	4	3.5	83	23.6	133	182	0.72
	4	4.5	91	24.4	140	263	0.53
	7	6.3	170	47.0	201	259	0.78
	7	6.5	163	45.5	211	221	0.95
	12	7.4	168	44.4	215	257	0.84
	17	8.6	204	56.5	195	196	1.00
	17	10.6	284	81.3	213	148	1.44
	25	9.3	220	61.3	194	244	0.80
	45	16.3	331	93.2	259	319	0.81
	68	18.2	409	115	281	248	1.14
	75	21.7	453	128	300	262	1.15
	83	18.0	401	113	311	230	1.35
Sheep	(years)						
	1.3*	26.5	522	151	261	159	1.64
	2	31.5	557	161	246	244	1.01
	2	33.9	632	176	226	190	1.19
	3*	40.5	878	256	254	184	1.38
	3*	39.0	815	238	300	255	1.18
	3*†	38.4	478	140	207	161	1.29
	3*†	41.3	542	159	231	162	1.42
	4	44.3	704	209	218	201	1.07
	4	54.3	751	228	210	170	1.24
	4*	43.5	517	156	167	140	1.19
	4*	38.0	642	194	180	114	1.58
Rats	(months)	(g.)					
	3	283	10.9	3.4	401	208	1.93
	3	295	10.8	3.5	459	212	2.17
	3	328	14.0	4.4	459	187	2.45
	3	361	14.2	4.6	316	140	2.26
	5	455	13.7	4.3	431	160	2.70
	5	500	15.7	4.9	372	168	2.21

* Female adults.

† Pen-fed.

Concentration of coenzyme A. Table 1 shows the concentration of CoA in the livers of lambs of various ages, of adult sheep and of rats. A wide variation was observed in the values expressed on a fresh-wt. basis in the livers of adult sheep, but the general trend was for the concentration of CoA/g. fresh wt. to be higher in adult sheep than in young lambs. However, less variation was observed between adult wethers (range 210–246 $\mu\text{g./g.}$ fresh wt.) than between adult ewes (range 167–300 $\mu\text{g./g.}$ fresh wt.).

Despite the wide variation observed in the concentration of CoA in the livers of adult sheep a highly significant difference ($P < 0.001$) was found between values for sheep (mean 220 $\mu\text{g./g.}$ fresh wt.) and for adult rats (mean 304 $\mu\text{g./g.}$ fresh wt.).

When the concentration of CoA was expressed as $\mu\text{g./}\mu\text{g.}$ of DNA phosphorus, a rise from the concentrations characteristic of young lambs to those of adult sheep was definitely established, and

Fig. 2 shows the regression of the CoA ($\mu\text{g./g.}$):DNA phosphorus ($\mu\text{g./g.}$) ratio with liver dry wt. (g.). The line was plotted from the equation:

$$\log[\text{CoA } (\mu\text{g./g.}): \text{DNA phosphorus}] = -0.5510 + 0.2894 \log[\text{liver dry wt. (g.)}].$$

A highly significant difference ($P < 0.001$) was found between the CoA ($\mu\text{g./g.}$):DNA phosphorus ($\mu\text{g./g.}$) ratio in lambs up to 2 weeks old (mean 0.6), of adult sheep (mean 1.3) and of rats (mean 2.3).

DISCUSSION

In a study of nicotinamide nucleotide coenzymes and enzymes connected with carbohydrate metabolism in the livers of growing lambs and rats, Caiger *et al.* (1962) and Filsell *et al.* (1963) have discussed the validity of liver dry wt. and DNA phosphorus as acceptable parameters for assessing the concentration of coenzymes and enzymes in

Fig. 1. Changes in liver dry wt. during development of the sheep. ▲, Values for adult ewes.

Fig. 2. Change in the CoA:DNA phosphorus ratio in the liver during development of the sheep. ▲, Values for adult ewes

rapidly growing tissue. Filsell *et al.* (1963) have shown that the mean value of DNA phosphorus content of the nuclei in the livers of sheep and lambs is $11.2 \mu\text{g.}$ of DNA phosphorus/nucleus and that there was no significant trend in this value during development. The expression of coenzyme concentrations as a function of DNA phosphorus was valid as an assessment of the amount of coenzyme 'per cell'.

On this basis, the liver cells of adult sheep contain about twice the amount of CoA as those of post-natal lambs. Even by allowing for a proportion of tetraploid nuclei in the liver cells of adult rats (see Filsell *et al.* 1963), the results indicate that the higher concentration of CoA found in the liver of adult rats is associated with a greater amount of CoA per cell in rat liver than in adult sheep.

The variability between sheep in the amount of CoA expressed on a fresh wt. basis was not due to large differences in the water content of the liver, as the percentage dry wt. in the livers of the adults was relatively constant. A similar conclusion could probably be arrived at from the results of Thompson & Mayer (1962), who showed that with mice the hepatic CoA concentration, when based on protein content, was identical in an obese-hyperglycaemic strain and their lean litter mates, whereas when expressed on either a fresh-wt. or dry-wt. basis a large difference was indicated.

A much greater variation in CoA concentration on a wet-wt. basis was observed between ewes (range $167\text{--}300 \mu\text{g./g.}$) than between wethers (range $210\text{--}246 \mu\text{g./g.}$). In view of the reports of hormonal influences on the acetylation of sulphanilamide (Franz & Lata, 1957; Fishkin & Lata, 1958), it is possible that the greater variability in the ewes may have been associated with some hormonal interaction. However, it is more likely that the differences were associated with liver growth and number of cells present, perhaps as a consequence of hormonal influence, rather than as a direct effect of female sex hormones on CoA concentrations. In the animals with the highest ($300 \mu\text{g./g.}$ fresh wt.) and lowest ($167 \mu\text{g./g.}$ fresh wt.) concentrations on a fresh-wt. basis the amount of CoA per cell as assessed on the CoA:DNA phosphorus ratio was about the same (1.18 and 1.19).

In a study of tissue CoA in normal and induced state of growth in rats, Bartlett, Grimmett, Beers & Shelata (1956) showed that induction of nitrogen storage in the normal adult rat as a result of stimulation with anterior-pituitary growth hormone was accompanied by elevation of the concentration of CoA in the liver. They concluded that there appeared to be a direct relationship between CoA and growth in the rat.

When the total CoA/liver was derived from the values for the concentration of CoA in the livers of lambs and sheep and plotted against liver dry wt., the regression:

$$\log[\text{CoA (mg./liver)}] = -0.4106 + 1.1397 \log[\text{liver dry wt. (g.)}]$$

was obtained (Fig. 3). As the regression was almost linear there was virtually a constant increase in amount of CoA with increasing size of liver. A similar situation was found in livers of lambs and sheep for $([\text{NADP}] + [\text{NADPH}_2])$, but with $([\text{NAD}] + [\text{NADH}_2])$ and with total nicotinamide nucleotide coenzymes there was a greater increase in the amount of coenzyme/unit increase in wt. of liver in the larger livers (Caiger *et al.* 1962). As with the nicotinamide nucleotide coenzymes the smaller concentrations of CoA present in the liver cells of the very young lamb rising to adult con-

centrations probably reflect a fundamental aspect of growth. It is suggested, however, that the concentration of CoA in the liver cells of normal adults on an adequate diet is related to the metabolic characteristics of that particular tissue in the animal.

Novelli (1953) has discussed many aspects of the metabolic functions of pantothenic acid and CoA, and has referred to decreased utilization of pyruvate when a low CoA concentration was present in liver slices from pantothenate-deficient animals (Olson & Kaplan, 1948). The synthesis of cholesterol and fatty acids has also been shown to be impaired in liver slices in which the concentration of CoA was low (Klein & Lipmann, 1953). Gallagher (1959) has shown that on a basis of fresh wt. the CoA concentration in livers of sheep with signs of pregnancy toxæmia was about half that of normal. A metabolic impairment in the oxidation of palmitate, octanoate and propionate was observed in these animals. However, Gallagher (1959) considered that although values were lower than normal there was probably not an absolute deficiency.

In adult ruminants the synthesis of higher fatty acids is largely dependent on the supply of lower fatty acids, as there is a very limited supply of exogenous glucose and the animal relies on gluconeogenic processes for a supply of endogenous glucose. Caiger *et al.* (1962) have discussed the possible implications of the high concentration of NAD found in the livers of adult sheep in relation to the metabolism of lower fatty acids via the tri-carboxylic acid cycle, and have suggested that the higher $([NAD] + [NADH_2]) : ([NADP] + [NADPH_2])$ ratio in the livers of sheep compared with that in the livers of rats may reflect a comparative emphasis on oxidative metabolism in the liver of the sheep and on reductive syntheses in the liver of the rat.

Fig. 3. Changes in amounts of CoA in the liver during development of the sheep. ▲, Values for adult ewes.

Although heart muscle and adipose tissue of sheep actively oxidize acetate, the extent to which acetate is utilized by liver is still controversial (McCarthy, Shaw & Lakshmanan, 1958; Holdsworth, Neville, Nader, Jarrett & Filsell, 1963). It would appear from the observations of Holdsworth *et al.* (1963) that fatty acid synthesis from acetate in the liver of the sheep is not as extensive as in some other tissues, and that the extent to which acetate is used for oxidative purposes may be limited.

With the available evidence it is not possible to interpret accurately the differences in CoA concentrations observed between adult sheep and rats. If there is great synthetic, acylating or oxidative activity proceeding the amount of actual free CoA may be low in relation to CoA bound as acyl compounds. Krebs & Kornberg (1957) have discussed the utilization of energy for chemical syntheses and have pointed out that the acetyl-CoA:CoA, reduced flavoprotein:oxidized flavoprotein and $NADH_2:NAD$ ratios are important factors in determining whether the reaction:

proceeds in the direction of synthesis or degradation. If the values for the three ratios are relatively low the reaction proceeds towards the degradation of fatty acids rather than towards synthesis.

The observed $([NADH_2]:[NAD])$ ratio in sheep liver (1:7) was lower than in rat liver (1:4) (Caiger *et al.* 1962), and in this respect, with certain reservations in interpretation, could be considered consistent with the relatively low fatty acid synthesis from acetate observed in sheep liver. However, until turnover rates of CoA or values for the acetyl-CoA:CoA ratio are determined it is not known whether the lower concentration of CoA in the liver of sheep compared with that in the liver of rats can be related to quantitative or qualitative differences in metabolic pathways.

SUMMARY

1. Concentrations of CoA have been determined in the livers of lambs, adult sheep and rats, and have been expressed as amounts/unit wt. of tissue and amounts/DNA phosphorus.

2. The concentration of CoA, either on a fresh-wt. basis or on amount/cell, assessed by the CoA:DNA phosphorus ratio, was higher in the liver of the adult sheep than in the young lamb.

3. The concentration of CoA in the liver of the adult rat was higher than in the liver of the adult sheep.

4. These findings have been discussed in relation to the intermediary metabolism of lambs, sheep and rats.

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Estimation of the Molecular Weights of Proteins by Sephadex Gel-Filtration

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When a mixture of molecules is passed through a column of porous gel granules, the molecules may appear in the effluent in order of decreasing size. Fractionation is believed to occur when diffusion of the molecules into the gel pores is restricted but not prevented because of their size, and because they pass through the column at rates that are related inversely to the fluid volume accessible to them within the column. Lathe & Ruthven (1956) suggested that the dimensions of protein and polysaccharide molecules might be estimated by this technique, now widely known as gel-filtration. However, evaluation of their suggestion is difficult because of uncertainty about the size and shape of macromolecules in solution (Schachman, 1960). Although molecular weight is unlikely to be a good approximation for size in comparing such dissimilar molecules as proteins and polysaccharides, the correlation between molecular weights and gel-filtration behaviour of dextrans (Granath & Flodin,

1961) indicates that, for a homogeneous series of macromolecules, size and molecular weight are closely related. Andrews (1962) obtained evidence from experiments with agar-gel columns that this was also true for a number of proteins, and showed that gel-filtration can be used as a comparative method to give useful estimates of the molecular weights of proteins.

Agar gels can be used to separate proteins over very wide molecular-weight ranges (Polson, 1961; Andrews, 1962; Steere & Ackers, 1962), whereas over smaller ranges of molecular weight much better separations can be obtained with gel-filtration media composed of cross-linked dextrans (Sephadex, manufactured by Pharmacia, Uppsala, Sweden) (Flodin, 1962) or polyacrylamide (Hjertén & Mosbach, 1962). Therefore a more critical appraisal of the relationship between the molecular weights of proteins and their gel-filtration behaviour is possible with these media than with agar gels.