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A Comparative Study of Nicotinamide Nucleotide Coenzymes during Growth of the Sheep and Rat

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Although observations have been made on the concentrations of nicotinamide nucleotide coenzymes in the tissues of a number of laboratory animals, there appears to be no information on the concentration of the oxidized and reduced forms of these substances in ruminant tissue.

Many aspects of carbohydrate metabolism in the adult sheep differ from those in monogastric animals (see Lindsay, 1959), and differences in enzyme patterns have been shown to exist between lamb, adult-sheep and rat tissues (Jarrett & Filsell, 1958; McLean, 1958*a*; Gallagher & Buttery, 1959). Moreover, the metabolism of a young lamb more closely resembles that of a non-ruminating animal than that of an adult sheep, which relies largely on lower fatty acids for its energy requirements (Jarrett & Potter, 1952, 1953).

The quantitative significance of glucose in the metabolism of mature ruminants and the interrelationships between glucose and lower fatty acids has still to be elucidated (Lindsay, 1959; Jarrett & Filsell, 1961). Since nicotinamide nucleotide coenzymes are required for both oxidative metabolism and reductive syntheses, further information is required on the supply and demand for these coenzymes in ruminants that use acetate both for oxidative metabolism and for lipogenesis (see Lindsay, 1959; Annison & Lindsay, 1961).

The rate of synthesis and the cellular concentrations of nicotinamide nucleotide coenzymes in the liver increase considerably during growth of the mouse and rat (Branster & Morton, 1956; Morton, 1958; Dawkins, 1959), and appear to be closely related to the rate of cell division (Morton, 1961). Similar information with respect to ruminants has not been available.

The concentrations of both oxidized and reduced nicotinamide nucleotide coenzymes in the livers of lambs and sheep, and of young and mature rats, have now been measured. The results are discussed in relation to the growth and changes in metabolism of these animals.

EXPERIMENTAL

Enzyme preparations

Isocitrate dehydrogenase. This was prepared from pig heart (Graflin & Ochoa, 1950); the ammonium sulphate fraction obtained between 50 and 60% saturation was dissolved in 0·04M-sodium potassium phosphate buffer, pH 7·4, dialysed against the same buffer for about 8 hr. at 0° and then stored at −15°.

Gluthathione reductase. This was prepared from dried peas (Kaplan, Colowick & Neufeld, 1953); the ammonium sulphate fraction obtained between 40 and 60% saturation was dissolved in 0·1M-tris–HCl buffer, pH 7·5, and stored at −15°.

Alcohol dehydrogenase. A 1% (w/v) solution of the crystalline enzyme (C. F. Boehringer und Soehne, Mannheim, Germany) was used.

Enzyme specificity. Under the conditions of assay used

(see below), these dehydrogenase preparations were shown to be sufficiently specific for reliable estimation of each coenzyme in a mixture of NAD, $NADH_2$, NADP and $NADPH_2$.

Other materials. Oxidized gluthathione and sodium isocitrate (containing 42% of the D-isomer) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Animals

Four Merino ewes, age 3 years, and four groups of wether lambs, ranging in age from 4 days to 8 weeks, were obtained during the months of August to October. The sheep had been grazing on an improved mixed pasture and the lambs had been running with their dams in a separate area on the same type of pasture.

Albino rats ranging in age from 4 to 28 days and a group of 7-month-old rats (500–600 g. body wt.) were used: these had been 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 (8×10^6 i.u.), vitamin D_3 (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 B_{12} (6·8 mg.).

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

Sampling procedure. The animals were killed by severing the neck and cervical cord and the livers were removed within 30 sec.; livers from the young rats were pooled from groups of animals at the various ages. With sheep and lambs, a number of slices (approx. 5 mm. thick) were taken from each lobe; with rats the whole liver was sliced. The final sampling was done by rapidly chopping the slices into small pieces (2 mm. cubes) and thoroughly mixing the chopped material. Weighed portions were then taken for the estimation of nicotinamide nucleotide coenzymes, nuclear count, dry weight and total nitrogen. A standardized time-schedule was used and the extraction of both oxidized and reduced nicotinamide nucleotide coenzymes was commenced within 3 min. of the death of the animal, except with the pooled livers when the time was extended by 2–3 min.

Extraction and assay of coenzymes

Extraction procedures were based on those of Jacobson & Kaplan (1957); the oxidized and reduced coenzymes were extracted simultaneously from separate samples of the tissue. The concentrations of each of the coenzymes in the appropriate extract were estimated from the changes in extinction at 340 mμ after specific enzymic oxidation or reduction (see Ciotti & Kaplan, 1957). Extinction changes were measured at regular intervals and the reaction was considered to be complete when no further change was observed during a 5 min. period; the amount of enzyme added was sufficient to ensure complete oxidation or reduction in about 10 min. at 25°. All assays were completed within 5 hr. of the death of the animal.

Oxidized nicotinamide nucleotide coenzymes. Approx. 1 g. of tissue was weighed and homogenized at 2000 rev./min. for 2 min. in 15 ml. of ice-cold 0·5M-trichloroacetic acid with a Potter–Elvehjem glass homogenizer and Teflon pestle (A. H. Thomas and Co., Baltimore, U.S.A.). The homogenizer was rinsed with 5 ml. of 0·5M-trichloroacetic acid and the volume of the combined suspension was measured. The clear extract obtained by centrifuging at 2500*g* for 5 min. at 2° was used for assays.

For NAD, each cuvette (4 cm. light-path) contained 2 ml. of tissue extract and 3 ml. of a solution containing 94 mg. of glycine, 50 mg. of NaOH and 0·15 ml. of ethanol; the final pH was about 9·5. The coenzyme was reduced by the addition of 0·025 ml. of alcohol-dehydrogenase solution. For NADP, each cuvette (4 cm. light-path) contained 2 ml. of tissue extract, 3·5 ml. of 0·3M-tris, 0·1 ml. of 50 mM-D-isocitrate and 0·1 ml. of 0·1M-$MgCl_2$; the final pH was 7·5. The coenzyme was reduced by the addition of 0·02 ml. of isocitrate-dehydrogenase solution.

Reduced nicotinamide nucleotide coenzymes. Approx. 1 g. of tissue was weighed and homogenized at 2000 rev./min. for 2 min. in 15 ml. of 0·1M-Na_2CO_3 in a Potter–Elvehjem homogenizer in a bath of boiling water. The homogenizer containing the carbonate solution was preheated in the same bath for 10 min. The Teflon pestle of the homogenizer was machined to give a clearance when hot equal to that of the homogenizer used in the extraction of oxidized coenzymes.

The extract was immediately poured into a polythene bottle in liquid nitrogen, the homogenizer was rinsed with 5 ml. of 0·1M-Na_2CO_3, and the rinsings were combined with the homogenate. The extract was thawed, adjusted to pH 7·5 with 1·0M-maleic acid (approx. 0·6 ml.) and the volume measured. The homogenate was shaken with 2 ml. of liquid paraffin (British Drug Houses Ltd.) to assist in the clarification of the extract, and then centrifuged at 60000*g* for 30 min. at 2°. The clear aqueous layer was used for assays.

For $NADH_2$, each cuvette (1 cm. light-path) contained 2 ml. of the tissue extract, 1 ml. of 0·2M-tris–HCl buffer, pH 7·5, and 0·025 ml. of 0·5M-acetaldehyde. The coenzyme was oxidized by the addition of 0·025 ml. of alcohol-dehydrogenase solution.

For $NADPH_2$, each cuvette (1 cm. light-path) contained 2 ml. of the tissue extract, 1 ml. of 0·2M-tris–HCl buffer, pH 7·5, and 0·2 ml. of 0·1M-oxidized glutathione. The coenzyme was oxidized by the addition of 0·02 ml. of glutathione-reductase solution.

Other procedures

Counting of nuclei. Approx. 1 g. of tissue was weighed and homogenized in 9 ml. of ice-cold 0·85% NaCl (adjusted to pH 7·2 with 0·2M-Na_2HPO_4), and the nuclei in a suitably diluted portion of the homogenate were stained and counted in a haemocytometer as described by Yokoyama, Wilson, Tsuboi & Stowell (1953); at least 3000 nuclei were counted in each sample. DNA was extracted by Schneider's (1945) method and estimated by a modification of Webb & Levy's (1955) method in which N-NaOH saturated at 20° with trisodium phosphate was used to develop the colour. Phosphorus was determined by the method of Allen (1940). With pooled livers from two groups of 28-day-old rats the values were 219 and 304 μg. of DNA phosphorus/g. fresh wt. These samples contained 313×10^6 and 359×10^6 nuclei/g., corresponding to values of $0{\cdot}70 \times 10^{-12}$ and $0{\cdot}85 \times 10^{-12}$ g. of DNA phosphorus/nucleus respectively, which is in good agreement with published values (see Davidson, 1960). This indicates that reliable counts of nuclei were obtained.

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

Nitrogen. Material used in the determination of dry weight was analysed by a micro-Kjeldahl procedure.

RESULTS

Estimation of nicotinamide nucleotide coenzymes in liver tissue. The methods of extraction and estimation of coenzymes used in this work were based on studies by a number of previous workers (see the Experimental section). The use of sodium carbonate solution gave a less-coloured extract, more suitable for spectrophotometry, than one obtained with sodium hydroxide. The specific enzymic reactions used in conjunction with spectrophotometric procedures were of adequate sensitivity for the determination of each of NAD, $NADH_2$, NADP and $NADPH_2$ in the livers of individual animals, except for young rats for which it was necessary to pool livers. Table 1 compares the values for livers of adult rats with pertinent values obtained by other workers. The concentrations of the four coenzymes in the present study fall within the range of values reported by other workers.

Concentrations of nicotinamide nucleotide coenzymes in livers of sheep and rats. Table 2 shows the concentrations of nicotinamide nucleotide coenzymes in the livers of sheep and rats of various ages, together with the corresponding values for body wt., liver fresh wt., liver dry wt., nuclei/g. fresh wt. of liver and nitrogen/g. fresh wt. of liver. An analysis of variance was carried out on these results and showed little significant difference in the concentrations of coenzymes between any of the groups of lambs. However, there was a twofold rise in the concentration of total coenzymes from the lower levels characteristic of the young lambs to those found in adult sheep (mean value 1031 μm-moles/g. fresh wt.).

Differences were found in the other parameters determined. The percentage dry wt. of the livers and total nitrogen/g. of liver of both lambs and rats increased during development. The number of nuclei/g. fresh wt. differed little in any of the groups of lambs but the number was greater than that found in the livers of adult sheep. In young rats there were almost twice as many nuclei/unit wt. of liver as in the adult.

Total amounts of nicotinamide nucleotide coenzymes in livers of sheep and rats. From the results in Table 2 values for number of nuclei and amounts of coenzymes in the whole liver of each animal were derived. Regression analyses were carried out on those variates deemed to be relevant to this investigation. It was necessary to take logarithms of both the dependent and determining variates to produce

Table 1. *Concentrations of oxidized and reduced nicotinamide nucleotide coenzymes in adult-rat liver*

Mean values are given as μm-moles/g. fresh wt.

Reference	No. of animals	[NAD]	[$NADH_2$]	[NAD] + [$NADH_2$]	[NADP]	[$NADPH_2$]	[NADP] + [$NADPH_2$]	Concn. of total coenzymes	Ratio [NAD]: [$NADH_2$]	Ratio [$NADPH_2$]: [NADP]
Glock & McLean (1955)	6	555	306	861	8	266	274	1135	1·8	33
Jacobson & Kaplan (1957)	—	446	166	612	44	425	469	1081	2·7	10
Bassham *et al.* (1959)	8	485	152	638	25	251	276	914	3·2	11
Lowry *et al.* (1961)	4	628	252	880	115	502	617	1497	2·5	4·4
This study	4	424	108	532	29	380	409	941	3·9	13

Table 2. *Reference bases and concentrations of nicotinamide nucleotide coenzymes in livers of sheep and rats*

Species	Age (days)	Body wt. (kg.)	Liver fresh wt. (g.)	Liver dry wt. (g.)	Nitrogen (mg./g. fresh wt.)	10^{-6} × Nuclei/g. fresh wt.	Coenzyme content (μm-moles/g. fresh wt.) [NAD]	[$NADH_2$]	[NAD] + [$NADH_2$]	[NADP]	[$NADPH_2$]	[NADP] + [$NADPH_2$]	Total
Sheep	4	4·2	127	33·4	31	267	365	68	433	20	234	254	687
		4·6	141	35·7	30	242	442	48	490	10	85	95	585
		4·8	114	29·1	32	294	330	33	363	20	143	163	526
		5·0	124	31·5	33	293	322	101	423	27	154	181	604
	14	6·2	134	36·4	32	299	381	17	398	10	235	245	643
		7·8	190	50·1	31	231	220	15	235	27	230	257	492
		8·2	193	52·1	29	257	247	54	301	10	176	186	487
		8·8	215	59·4	31	250	279	18	297	10	109	119	416
	29	7·3	164	45·9	31	287	353	46	399	52	277	329	728
		10·5	245	68·6	30	292	149	58	207	40	201	241	448
		10·7	222	60·8	32	299	109	51	160	42	257	299	459
	56	11·7	264	73·0	33	282	508	56	564	34	160	194	758
		16·0	346	99·0	31	258	349	36	385	33	160	193	578
		18·8	361	104·0	33	260	507	102	609	38	182	220	829
		19·8	357	103·0	34	254	400	48	448	47	219	266	714
		19·9	324	91·2	32	309	417	62	479	50	263	313	792
	Adult*	48·0	780	235	35	203	736	111	847	15	195	210	1057
		50·2	836	251	36	191	670	116	786	28	258	286	1072
		51·9	930	277	35	232	679	107	786	26	297	323	1109
		53·2	815	244	35	240	608	36	644	33	212	245	889
		(g.)											
Rat	4 (17)†	8·3	0·28	0·07	30	271	321	71	392	5	151	156	548
	7 (13)†	13·4	0·44	0·12	29	424	413	120	533	21	234	255	788
	28 (5)†	39·8	1·62	0·47	34	323	470	113	583	20	296	316	899
	(3)†	56·5	2·69	0·78	34	313	473	83	556	5	274	279	835
	(3)†	68·0	3·40	1·00	30	359	455	96	551	15	305	320	871
	Adult‡	520	14·8	4·61	34	188	435	162	597	30	364	394	991
		560	17·3	5·33	35	185	433	98	531	25	394	419	950
		595	17·9	5·45	35	181	328	80	408	25	276	301	709
		623	17·3	5·58	34	181	500	93	593	37	487	524	1117

* 3 years old.
† Samples were taken from the pooled livers of the number of animals shown in parentheses.
‡ 7 months old.

homoscedasticity and linearity. Fig. 1 shows the regression of the amount of coenzymes/liver with liver dry wt. (Fig. 1 *a*) and with number of nuclei/liver (Fig. 1 *b*) for the sheep. The similar regressions for the rat are shown in Figs. 2 (*a*) and (*b*) respectively.

For the sheep, the regression of ([NADP]+[$NADPH_2$]) on liver dry wt. was almost linear, and thus the concentration of this coenzyme remained unchanged with increasing size of liver. However, with ([NAD]+[$NADH_2$]), and thus also with total coenzymes, there was a greater increase in the amount of coenzymes/unit increase in wt. of liver in the larger livers, i.e. in the more mature animals. Similar trends were shown by the regressions on number of nuclei/liver (Fig. 1 *b*).

For the rat, the regressions of ([NAD]+[$NADH_2$]), of ([NADP]+[$NADPH_2$]), and thus of [total coenzymes], on liver dry wt. were each linear (Fig. 2 *a*). However, the gradients of the regression curves of each of ([NAD]+[$NADH_2$]), ([NAD]+[$NADPH_2$]) and of [total coenzymes] on the number of nuclei/liver increased during development, i.e. animals with larger numbers of liver nuclei contained proportionally a greater amount of coenzymes in the liver.

To assess the rate of change of the number of nuclei/unit change of coenzymes, the regression of the log(10^{-8} × nuclei/liver) on log(total coenzymes/liver) was derived and the resulting equation was differentiated after conversion to the original units; for sheep, $N = 27{\cdot}04C^{0{\cdot}6278}$, whence $dN/dC = 16{\cdot}98C^{-0{\cdot}3722}$; for rats, $N = 3{\cdot}98C^{0{\cdot}7687}$, whence $dN/dC = 3{\cdot}06C^{-0{\cdot}2313}$, where N is the number of nuclei/liver and C is the total amount of coenzymes (μmoles/liver). Figs. 3 (*a*) and 3 (*b*) illustrate these relationships and show the change in the number of nuclei/unit change of total coenzymes relative to the amounts of coenzymes.

From the values of ([NAD]+[$NADH_2$]) and of ([NADP]+[$NADPH_2$]) obtained from the regression equations shown in Fig. 1 (*a*) (curves *B* and *C*) and Fig. 2 (*a*) (curves *B* and *C*), the coenzyme ratio ([NAD]+[$NADH_2$]):([NADP]+[$NADPH_2$]) was calculated for selected liver dry weights. The plots of these values against liver dry wt. gave the curves shown in Fig. 4 (*a*) for sheep and Fig. 4 (*b*) for rats.

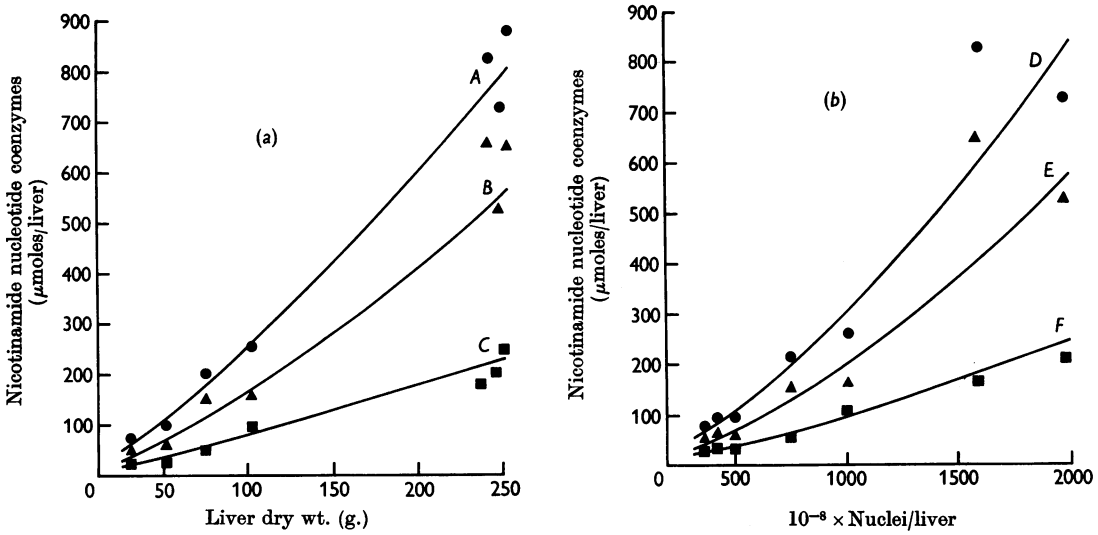

Fig. 1. Changes in amounts of nicotinamide nucleotide coenzymes of liver during development of the sheep. Curves were drawn from the regression equations:

A, log[total coenzymes] = $-0{\cdot}0462 + 1{\cdot}2265 \log W$;
B, log([NAD]+[$NADH_2$]) = $-0{\cdot}3795 + 1{\cdot}3001 \log W$;
C, log([NADP]+[$NADPH_2$]) = $-0{\cdot}2941 + 1{\cdot}1037 \log W$;
D, log[total coenzymes] = $-1{\cdot}9335 + 1{\cdot}4708 \log N$;
E, log([NAD]+[$NADH_2$]) = $-2{\cdot}3317 + 1{\cdot}5421 \log N$;
F, log([NADP]+[$NADPH_2$]) = $-2{\cdot}0816 + 1{\cdot}3548 \log N$;

where W is the liver dry wt. (g.) and N the number of nuclei × 10^{-8}. In each case $P < 0{\cdot}001$. To illustrate the fit of observations to the regression curves, some estimates of nicotinamide nucleotide coenzymes are shown for various values of liver dry wt. (curve *A*) and of number of nuclei (curve *B*) selected from the range of values shown in Table 2; for clarity, not all observations are illustrated. ●, [Total coenzymes]; ▲, ([NAD]+[$NADH_2$]); ■, ([NADP]+[$NADPH_2$]).

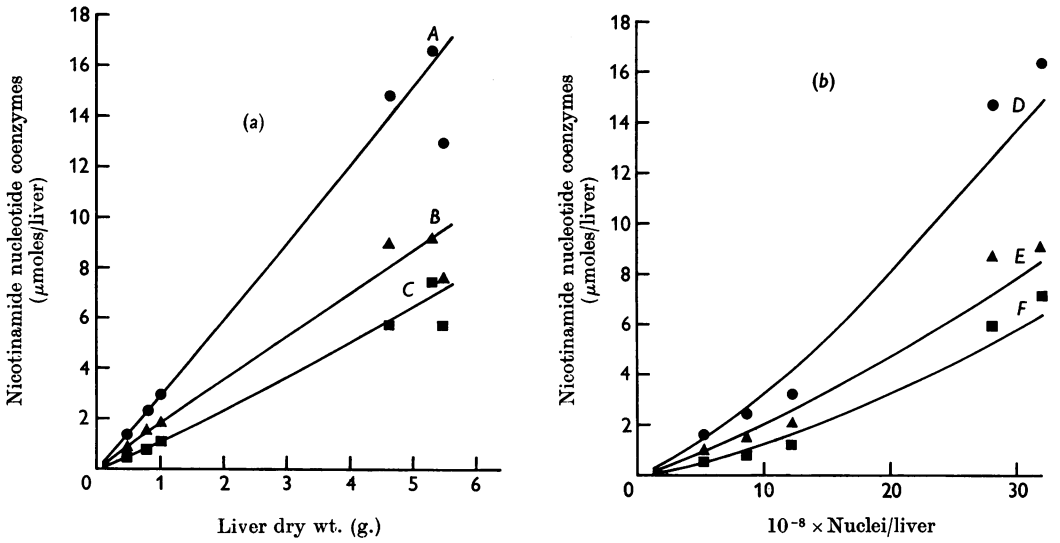

Fig. 2. Changes in amounts of nicotinamide nucleotide coenzymes of liver during development of the rat. The lettering and symbols are as for Fig. 1. Curves were drawn from the regression equations:

A, log[total coenzymes] = $0{\cdot}4587 + 1{\cdot}0330 \log W$;
B, log([NAD] + [$NADH_2$]) = $0{\cdot}2541 + 0{\cdot}9802 \log W$;
C, log([NADP] + [$NADPH_2$]) = $0{\cdot}0255 + 1{\cdot}1235 \log W$;
D, log[total coenzymes] = $-0{\cdot}7604 + 1{\cdot}2809 \log N$;
E, log([NAD] + [$NADH_2$]) = $-0{\cdot}9037 + 1{\cdot}2163 \log N$;
F, log([NADP] + [$NADPH_2$]) = $-1{\cdot}3002 + 1{\cdot}3929 \log N$.

In each case $P < 0{\cdot}001$.

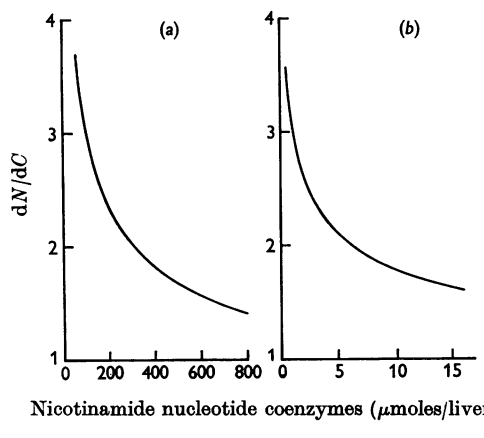

Fig. 3. Rate of change of number of nuclei, N, per unit change of total coenzymes, C, in liver during development of (*a*) the sheep and (*b*) the rat. The curves were derived from the appropriate regression equations (see Figs. 1 and 2, curves D). For sheep, $dN/dC = 16{\cdot}98C^{-0{\cdot}3722}$; for the rat, $dN/dC = 3{\cdot}06C^{-0{\cdot}2313}$.

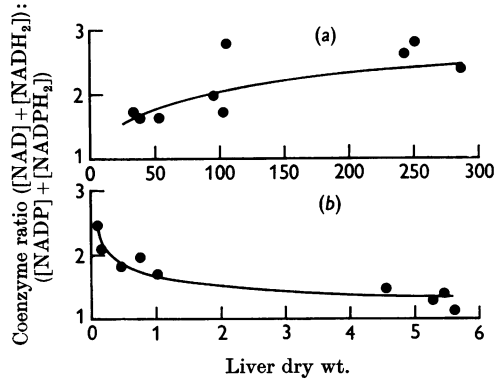

Fig. 4. Change in coenzyme ratio ([NAD] + [$NADH_2$]):([NADP] + [$NADPH_2$]) in the liver during development of (*a*) the sheep and (*b*) the rat. The curves were derived from the regression equations of Fig. 1 (curves B and C) and of Fig. 2 (curves B and C) for the sheep and rat respectively. For the sheep, the values shown are for various liver dry wt. values selected from the range of observed values (cf. Fig. 1 and Table 2).

When the coenzyme ratio was derived from Fig. 1(*b*) (curves E and F) and Fig. 2(*b*) (curves E and F), and the values plotted against the number of nuclei, essentially the same curves were obtained. Fig. 4(*a*) shows that, during development of the sheep, ([NAD] + [$NADH_2$]) increased more rapidly than did ([NADP] + [$NADPH_2$]); during development of the rat, however, ([NADP] +

[$NADPH_2$]) increased more rapidly than did ([NAD]+[$NADH_2$]) (Fig. 4*b*).

The following regressions of animal body wt. and liver dry wt. on age can be used to determine either of these parameters in terms of the other.

For sheep:

$$\log(\text{body wt., kg.}) = 0{\cdot}4039 + 0{\cdot}4340 \log(\text{age, days})$$

$$\log(\text{liver dry wt., g.}) = 1{\cdot}2749 + 0{\cdot}3725 \log(\text{age, days}).$$

For rats:

$$\log(\text{body wt., g.}) = 0{\cdot}1889 + 1{\cdot}0986 \log(\text{age, days})$$

$$\log(\text{liver dry wt., g.}) = -1{\cdot}7733 + 1{\cdot}0817 \log(\text{age, days}).$$

DISCUSSION

Reference bases in liver tissue. A number of studies (see Kosterlitz, 1958) have clearly indicated that, in any evaluation of analysis done on liver tissue, the choice of a reference base depends on the type of experiment. In the first instance the data in Table 2 were grouped under ages and an analysis of variance between and within age classes was performed. However, it was thought that liver wt. or total number of nuclei/liver gave a better indication of the stage of development of the liver than age, which makes no allowance for different growth rates within an animal group. The information gained from the regression analysis of the data in Table 2 was considered to give a better assessment of the comparative changes in amounts of coenzymes on the basis of liver dry wt. or total number of nuclei, which have a more direct bearing on liver metabolism. However, regression analysis on the basis of age, although not quite as precise, still gave a highly significant relationship resulting in the same general conclusions.

In the comparison of coenzyme concentrations in tissues, the amount/g. fresh wt. has been the most commonly used reference base (see Table 1). However, an erroneous impression may be gained, particularly when comparing tissues of different species or tissues of one species at different stages of development, if this is the only parameter used. Leslie, Fulton & Sinclair (1957) showed that, in a comparison of glycolysis rates in embryonic and malignant tissues, results expressed on the basis of unit dry wt. showed no difference between the two types of tissue; however, when the metabolic quotients were expressed on a 'per cell' basis clear differences appeared.

A similar phenomenon was noted in the present study. Jedeikin, Thomas & Weinhouse (1956) and Dawkins (1959) have reported that the concentration of NAD (μm-moles/g. fresh wt.) in the liver of the young rat reached the adult level by 7 days after birth. A similar conclusion would be reached in this study if results were compared on this basis. When compared as concentration 'per average cell' (with the nuclear count as the reference base) the concentration of NAD at 7 days was less than half that obtained for the adult rat (23×10^{-7} μm-mole/cell) and even after one month had only reached two-thirds of the adult level. It is clear that multiple reference bases are needed for comparative studies.

Nicotinamide nucleotide coenzymes and growth. Low concentrations of nicotinamide nucleotide coenzymes are a characteristic of rapidly dividing tissues (see Morton, 1958, 1961). Such widely varying tissues as mouse mammary carcinoma (Jedeikin & Weinhouse, 1955), dye-induced hepatoma and Krebs ascites tumour in the rat (Glock & McLean, 1957) and foetal-rat liver (Dawkins, 1959) have much lower concentrations of nicotinamide nucleotide coenzymes than the comparable tissues in the normal adult animal. Increasing the coenzyme concentration by injection or implantation of nicotinamide is accompanied by a decreasing rate of cell division in methylcholanthrene-treated tissue in the mouse (Fujii & Mizuno, 1958) and in regenerating rat liver (Oide, 1958).

During normal growth of the liver in both sheep (Fig. 3*a*) and rats (Fig. 3*b*) the concentration of total nicotinamide nucleotide coenzymes has reached a maximum value when growth has ceased. The concentration of total coenzymes/average cell was of the same order in the adult-liver tissue of both rat and sheep. In young animals of both species the concentration was about half this value. It would therefore appear that the concentration of total nicotinamide nucleotide coenzymes reflects fundamental growth relationships in tissues. This coenzyme concentration is related to the concentration of the nuclear enzyme, nicotinamide mononucleotide adenylyltransferase, which is lower in embryonic, regenerating and tumour tissues than in the corresponding normal adult tissue (Branster & Morton, 1956; Waravdekar, Powers & Leiter, 1956; Dawkins, 1959; Stirpe & Aldridge, 1961; Morton, 1961).

Nicotinamide nucleotide coenzymes and metabolism. The importance of nicotinamide nucleotide coenzymes in metabolism is apparent from the evidence, which indicates that the major metabolic sources of energy are channelled into a few mechanisms in which these coenzymes play a determining role (see Dickens, 1961). NAD appears to be largely concerned with biological oxidations and $NADPH_2$ with reductive syntheses (see Lowenstein, 1961). In certain instances the proportions of these coenzymes have been shown to be a limiting factor in the utilization of particular metabolic pathways,

e.g. the oxidative pentose phosphate pathway and in lipid synthesis. Although the information available from this study does not differentiate the role of coenzymes in the developing animal in relation to its changing metabolic pattern, some implications can be considered.

Various workers have shown that metabolic differences exist between lambs and sheep and between sheep and monogastric animals, particularly in relation to carbohydrate and fatty acid metabolism. The removal of injected glucose from the systemic circulation is considerably delayed in adult sheep compared with the rate of the lamb, dog or human (Reid, 1950; Jarrett & Potter, 1952). Annison & White (1961), using a constant-infusion technique with ^{14}C-labelled glucose, obtained a mean value for the glucose-utilization rate in starved sheep of 0·37 g./hr./(kg. body wt.)$^{0·6}$. This represents about 75% of the value reported for starved dogs (Steele, Wall, de Bodo & Altszuler, 1956). I. G. Jarrett, B. L. Jones & B. J. Potter (unpublished work), using a similar technique, have shown a greater utilization rate of glucose in young lambs than in adult sheep. The young lambs can also remove injected acetate from the circulation at a faster rate than can the adult sheep (Jarrett & Filsell, 1960*a*), and the ability of the adult sheep to utilize injected acetate can be altered by diet (Reid, 1958; Jarrett & Filsell, 1960*b*) or by the prior administration of glucose (Jarrett & Filsell, 1961; Annison & Lindsay, 1961).

Differences in enzyme patterns have also been observed when comparing lambs, sheep and rats. As the lamb develops towards maturity the hexokinase activity of the intestinal mucosa decreases towards the adult level, and the activity in both brain and intestinal mucosa is lower in adult sheep than in adult rat (Jarrett & Filsell, 1958). Coenzyme A concentrations in the liver of the growing lamb rise towards the adult level (150 μg./g. fresh wt.) by the time the lamb has reached 5–6 weeks of age (I. G. Jarrett & O. H. Filsell, unpublished work). Gallagher & Buttery (1959) studied a number of enzyme systems in sheep liver, brain and kidney, and reported activities which are about one-third to one-half the values given in the literature for rat tissues.

McLean (1958*a*) has compared the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the livers of lactating rats and sheep. The activities found in rat liver were on all occasions higher than those in sheep liver. In both rat and sheep mammary tissue the activity of these enzymes increased during lactation. Coenzyme concentrations were measured in the rat tissue (McLean, 1958*b*), and the marked change in NAD and $NADPH_2$ concentrations which occurred paralleled the change in enzyme activity.

Of the total nicotinamide nucleotide coenzymes found in the livers of sheep and rats in the present study, the concentration of NAD was always greater than that of $NADH_2$, and the concentration of $NADPH_2$ was greater than that of NADP. This confirms observations made on liver tissue by other workers (Glock & McLean, 1955; Jacobson & Kaplan, 1957; Bassham, Birt, Hems & Loening, 1959; Lowry, Passonneau, Schulz & Rock, 1961).

Although the total coenzyme concentration/average cell was of the same order in the adult-liver tissue of both rat and sheep, the coenzyme ratio $([NAD]+[NADH_2]):([NADP]+[NADPH_2])$ was different (see Figs. 4*a* and 4*b*). In the young animal the coenzyme ratio for the rat was about 2·4 and for the lamb 1·6; by maturity these ratios had changed to 1·3 and 2·5 respectively. This was due to a relatively greater increase in concentration of NAD during development of the sheep and of $NADPH_2$ during development of the rat (Figs. 1 and 2).

For the rat it appears likely that this change in ratio, with an increasing proportion of $NADPH_2$ as the animal matures, can be related to the greater importance of the oxidative pentose phosphate pathway in adult-liver tissue compared with rapidly proliferating tissue (see Axelrod, 1960). It is more difficult to suggest an interpretation of the higher coenzyme ratio in the livers of adult sheep. In the metabolism of adult sheep as compared with the rat there is an emphasis on the oxidation of acetate. However, the quantitative importance of the utilization of exogenous acetate by the liver of the sheep is still a controversial issue (see Lindsay, 1959). Acetate oxidation will require the operation of the tricarboxylic acid cycle, which is largely NAD-dependent; propionate and butyrate, also important in the ruminant, enter metabolic sequences via this cycle (Black, Kleiber & Brown, 1961). It may be that the relatively higher amount of NAD in sheep liver is related to this activity.

The influence of these relationships of nicotinamide nucleotide coenzymes on intermediary metabolism needs further investigation. However, it is suggested that the difference in proportions of NAD and $NADPH_2$ shown above reflect a comparative emphasis on oxidative metabolism in the liver of the sheep and on reductive syntheses in the liver of the rat.

SUMMARY

1. Specific enzymic reactions have been used to determine the concentration of both oxidized and reduced nicotinamide nucleotide coenzymes in the livers of lambs, sheep and rats.

2. Concentrations of coenzymes were expressed as amount/unit wt. of tissue and as amount/nucleus (i.e. amount/average cell); total amounts of co-

enzymes were compared on the basis of the dry wt. and of the number of nuclei of the liver.

3. During the normal growth of the liver in both sheep and rats, the concentrations of the total nicotinamide nucleotide coenzymes increased and reached a maximum value as development ceased. In the adult liver of both rats and sheep, the concentration of total coenzymes/nucleus was of the same order (approx. 490 μm-moles/10^8 nuclei), and about twice the concentration found in livers of the young animals.

4. The concentration of NAD was higher in the adult-sheep liver than in the rat, and the concentration of $NADPH_2$ was much higher in the rat liver. The coenzyme ratio ([NAD]+$NADH_2$]): ([NADP]+[$NADPH_2$]) fell from about 2·4 in the young to about 1·3 in the mature rat, and increased from about 1·6 in the lamb to about 2·5 in the adult sheep.

5. These findings have been discussed with reference to the growth and intermediary metabolism of lambs, sheep and rats.

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