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CONTROL OF PYRIDINE NUCLEOTIDES IN TISSUES

by

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Being a thesis submitted in fulfilment of
the requirements for the degree
of Doctor of Philosophy

from

The Department of Agricultural Chemistry,
Waite Agricultural Research Institute,
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ABBREVIATIONS USED

ADP	Adenosine 5'-pyrophosphate
AMP	Adenosine 5'-phosphate
ATP	Adenosine 5'-triphosphate
DNA	Deoxyribonucleic acid
NAD	Nicotinamide-adenine dinucleotide
$NADH_2$	Dihydronicotinamide-adenine dinucleotide
NADP	Nicotinamide-adenine dinucleotide phosphate
$NADPH_2$	Dihydronicotinamide-adenine dinucleotide phosphate
NMN	Nicotinamide mononucleotide
PP_i	Inorganic pyrophosphate
RNA	Ribonucleic acid
tris	2-Amino-2-hydroxymethylpropane-1,3-diol

Summary

1. Specific enzymic assays by direct spectrophotometry for both the oxidized and reduced forms of nicotinamide-adenine dinucleotide (NAD) and of nicotinamide-adenine dinucleotide phosphate (NADP) are described. Methods for extracting these coenzymes from animal tissues were investigated and the use of trichloroacetic acid at 0° for the oxidized coenzymes and of 0.1 M-sodium carbonate at 100° for the reduced coenzymes was found to be the most satisfactory procedure.

2. The concentrations of these coenzymes were determined in the livers of prenatal and postnatal lambs and adult sheep and of postnatal and adult rats. During the normal growth of the liver in both species, the concentration of total nicotinamide nucleotide coenzymes increased and reached a maximum value as development ceased. The total amounts of these coenzymes in the liver were compared by using a regression analysis with different parameters as measures of the relative stage of maturity and of the growth rate of the liver.

3. The coenzyme ratio, $([NAD] + [NADH_2]) : ([NADP] + [NADPH_2])$, fell from about 6-10 in the livers of prenatal and newborn lambs to 2-4 in the older lambs and adult sheep; in the liver of the rat, this ratio fell from 2.4 in the young to 1.3 in the mature animal. It is suggested that a high coenzyme ratio is characteristic of mammalian liver in the peri-natal animal.

4. The concentration of NAD was higher in the adult-sheep liver than in the rat, and the concentration of $NADPH_2$ was higher in the rat liver. This difference in proportions of NAD and of $NADPH_2$ may reflect a comparative emphasis on oxidative metabolism in the liver of the sheep and on reductive syntheses in the liver of the rat.

5. The rates of reduction of NADP in the presence of glucose 6-phosphate, of 6-phosphogluconate and of glucose plus adenosine triphosphate were determined in liver homogenates from foetal, newborn and young lambs and from adult sheep and rats. In each case the rates decreased during development of the lambs. The higher enzymic activities in the livers of the young lambs are consistent with the hypothesis that many aspects of the carbohydrate metabolism of the young lamb differ from those of adult sheep and more closely resemble those of non-ruminants.

6. Methods of preparation and some enzymic reactions of nicotinic acid nucleotide and of nicotinic acid-adenine dinucleotide were studied. The products from a method of combined chemical and enzymic preparation were compared with the naturally occurring compounds. Nicotinic acid nucleotide and ATP were converted quantitatively into nicotinic acid-adenine dinucleotide and pyrophosphate using ATP:NMN-adenylyltransferase. This dinucleotide and that prepared from NAD and ethyl nicotinate by an exchange reaction using NAD(P) glycohydrolase, had the same R_F in two solvent systems and were converted into NAD by NAD synthetase.

7. The preparation of pure nicotinic acid-hypoxanthine dinucleotide is described and the quantitative cleavage of the pyrophosphate bond of nicotinic acid-adenine dinucleotide is shown.

8. The possibility that nicotinic acid-adenine dinucleotide is a substrate of NAD kinase, or inhibits this enzyme was investigated. No inhibition was found, nor was any compound with the expected properties of nicotinic acid-adenine dinucleotide phosphate (an analogue of NADP) observed on electrophoresis or chromatography.

ACKNOWLEDGEMENTS

I wish to acknowledge the help and advice of my supervisors, the late Professor R. K. Morton and Dr. M. R. Atkinson. I also gratefully acknowledge the help of Dr. H. R. Marston, F.R.S., Chief of the C.S.I.R.O., Division of Biochemistry and General Nutrition, Adelaide, in providing facilities in the Division and the assistance of Mr. I. G. Jarrett and of Mr. O. H. Filsell of this Division. Mr. N. S. Stenhouse of the C.S.I.R.O. Division of Mathematical Statistics, University of Adelaide, kindly conducted the statistical analysis of the data.

I would like to thank Professor D. J. D. Nicholas for his interest and advice during the completion of this thesis. A research grant from the Anti-Cancer Foundation of the University of Adelaide is also gratefully acknowledged.

DECLARATION

I hereby declare that the work presented in this thesis has been carried out by myself, except where otherwise stated, and that it has not been submitted in any previous application for a degree.

PREFACE

Some aspects of the work reported herein have been published as follows :

"Synthesis of Nicotinic Acid Nucleotide and of Nicotinamide Nucleotide by Liver and by Maize Roots" M. R. Atkinson, P. Caiger, R. K. Morton & C. V. Ramakrishnan, (1961). Aust. J. Sci., 24, 138.

"A Comparative Study of Nicotinamide Nucleotide Coenzymes during Growth of the Sheep and Rat" P. Caiger, R. K. Morton, O. H. Filsell & I. G. Jarrett, (1962). Biochem. J. 85, 351.

"Nicotinamide Nucleotide Coenzymes and Glucose Metabolism in the Livers of Foetal and Newborn Lambs" O. H. Filsell, I. G. Jarrett, M. R. Atkinson, P. Caiger & R. K. Morton, (1963). Biochem. J. 89, 92.

GENERAL

INTRODUCTION

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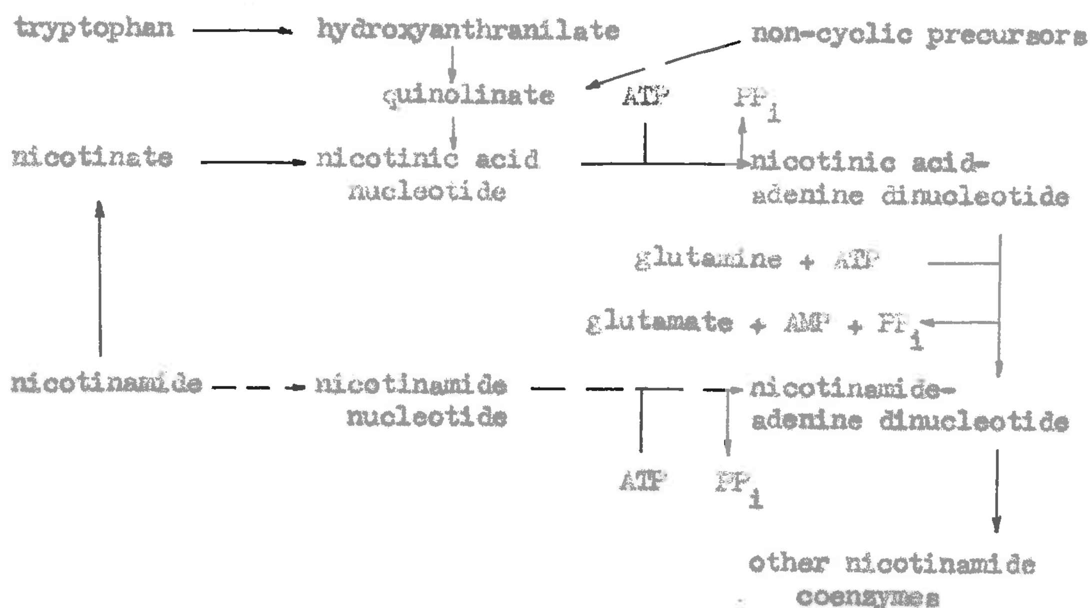
The Relationship of Nicotinamide Nucleotide Coenzyme Concentrations to Rates of Cell Division

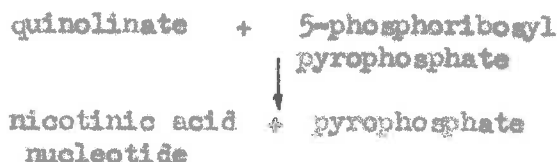
The work described in this thesis was part of an investigation of pyridine nucleotide metabolism and cell division that was initiated by the late Professor R. K. Morton.

Branster & Morton (1956) isolated nuclei from livers of mice at various stages of development and from mammary glands and tumours. They measured the rate of formation of nicotinamide adenine dinucleotide [NAD] from nicotinamide nucleotide [NMN] and adenosine triphosphate [ATP] in the presence of these nuclei, and found lower rates with nuclei from foetal livers than with those from the livers of adult mice; nuclei from tumours had less activity than those from comparable normal adult tissue (for a detailed discussion see Section II).

Kornberg (1950) had shown that formation of NAD from ATP and NMN was catalysed by an adenylyltransferase ('pyrophosphorylase') in liver and in yeast, and Hogeboom and Schneider (1952) found that the activity of this enzyme (ATP:NMN adenylyltransferase, EC 2.7.7.1) was almost entirely in the nuclear fraction of mouse liver. It now seems likely that nicotinic acid nucleotide is the essential intermediate in the biosynthesis of nicotinamide nucleotide coenzymes.

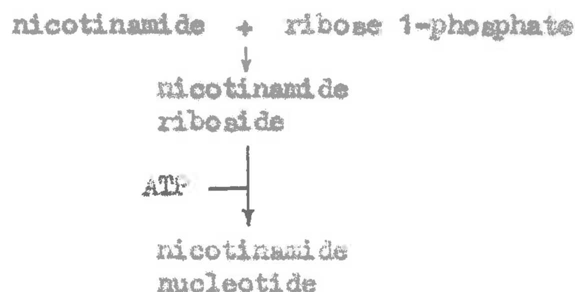


A major source of nicotinic acid nucleotide is probably the reaction:



(Gholson, Ueda, Ogasawara & Henderson, 1964), though this reaction could not be shown in a number of animal tissues by Nakamura, Ikeda, Tusji, Nishizuka & Hayaishi (1963). Quinolinate is formed from tryptophan in reactions involving hydroxyanthranilate (Mitchell & Nyc, 1948; Decker, Kang, Leach & Henderson, 1961). Nicotinic acid nucleotide is also formed directly from nicotinate and phosphoribosyl pyrophosphate (Preiss & Handler, 1958a, 1958b; Imsande & Handler, 1961) although the activity of the corresponding enzyme in an extract of acetone powder of pig liver is only a twentieth of the activity of

the enzyme that converts quinolinate into nicotinic acid nucleotide (Gholson *et al.* 1964). The reaction measured by Branster & Morton (1956) is catalysed by the enzyme that converts nicotinic acid nucleotide into nicotinic acid-adenine dinucleotide (Preiss & Handler, 1958b). Although the Michaelis constants for nicotinic acid nucleotide and nicotinamide nucleotide are similar and the maximum velocity of nicotinamide-adenine dinucleotide formation is slightly greater than that of nicotinic acid-adenine dinucleotide formation (Atkinson, Jackson & Morton, 1961; Jackson, 1962) there is no evidence that the former reaction takes place in physiological conditions. Formation of nicotinamide nucleotide from nicotinamide and phosphoribosyl pyrophosphate has been shown in erythrocytes, but the high Michaelis constant makes this reaction of doubtful physiological significance (Preiss & Handler, 1957). There is also little information on the physiological significance of the reaction sequence:



described by Rowen & Kornberg (1951) and Kornberg (1951).

Many early suggestions that nicotinamide derivatives were intermediates in the biosynthesis of nicotinamide-adenine dinucleotide followed from the observation that nicotinamide was rapidly converted into this coenzyme in liver (for a review see Kaplan, 1961). It now

seems likely that hydrolysis of nicotinamide to nicotinate by nicotinamidase and conversion of nicotinate into nicotinic acid nucleotide accounts for these observations (Petrack, Greengard, Cranston & Kalinsky, 1963). Although subsequent discoveries make it unlikely that Branster & Morton (1956) were using physiological substrates there is no reason to suppose that their assays did not indicate the relative activities of the nuclear adenylyltransferase which is essential for the biosynthesis of nicotinic acid adenine dinucleotide in the tissue that they studied.

Morton (1958) related his observations of low activities of adenylyltransferase in embryonic and tumour tissues to previous observations that tumours contained lower concentrations of nicotinamide adenine dinucleotide than did comparable normal tissues. He proposed that the activity of the transferase in the nucleus limited the supply of nicotinamide-adenine dinucleotide to the cytoplasm and thus limited the extent of growth of the cytoplasm. It was suggested that lack of coenzyme in the cytoplasm initiated cell division, leading to a correlation of high division rate, low coenzyme concentration and low activity of the nuclear adenylyltransferase. Dawkins (1959) found that the adenylyltransferase activity and the nicotinamide-adenine dinucleotide content of adult rat liver were about three times as great as those of foetal liver.

These results indicated the need for a more complete study of the concentrations of the four nucleotide coenzymes in normal tissues at various stages of growth and development. The most suitable

animals available to us for this study were lambs, sheep and rats. The livers of foetal lambs are large enough to provide enough tissue for the assay of the coenzyme from a single liver, and the larger sample permitted the measurement of a number of other parameters (dry weight, nitrogen, nucleic acids, and number of nuclei). With large numbers of animals the rate of growth at any stage of development could be calculated from the rate of change of the selected parameter.

A major objection to Morton's (1958, 1961) hypothesis was the lack of evidence that the adenylyltransferase is the rate-limiting enzyme, (cf. Section II) and Revel & Mandel's (1962) observation that a high concentration of nicotinamide-adenine dinucleotide is correlated with a low rate of synthesis of nuclear ribonucleic acid may be more relevant to processes of control of growth. The possibility that the nuclear transferase has other catalytic activities must also be considered, particularly as nicotinamide mononucleotide at a concentration of 20μM causes a 140-fold stimulation of conversion of adenosine triphosphate into polyadenylate in the presence of a preparation from liver nuclei, while 2mM-NMN causes a 1000-fold stimulation (Chambon, Weill & Mandel, 1963).

Accurate measurement of the concentrations of the four nicotinamide nucleotide coenzymes in tissues has presented many difficulties, and when the work described in this thesis was commenced it was necessary to find satisfactory methods for the extraction and assay of these coenzymes. The first section of the thesis describes specific enzymic assays by direct spectrophotometry in conditions

suitable for use with the concentrations of these compounds in animal tissues. It also describes experiments to find the best extraction procedure. Extraction of oxidized coenzymes with trichloroacetic acid at 0° and of reduced coenzymes with 0.1 M-sodium carbonate at 100° was found to be the most satisfactory procedure.

The second section of the thesis describes the application of these assay methods to samples of liver from about 75 lambs and sheep and from about 50 rats. This section also describes the methods used and the results obtained in measurement of deoxyribonucleic acid, ribonucleic acid, total nitrogen, and number of nuclei/liver, together with other measures of growth (dry and wet weight of liver, body weight of animal and 'crown-rump length' of foetal and newborn lambs). The total amounts of the nicotinamide nucleotide coenzymes in livers of sheep and rats during development were compared using a regression analysis with different parameters (e.g. liver dry weight, total number of nuclei/liver, calender age, and body weight) as measures of the relative stage of maturity of the animal.

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 content of total coenzymes/nucleus was of the same order (approx. 490 µm-moles/10^8 nuclei), and about twice the value found in the livers of the young animals.

The concentration of NAD was higher in the adult sheep liver than in the rat, whereas the concentration of reduced nicotinamide-

adenine dinucleotide phosphate ($NADPH_2$) was higher in the rat liver. The coenzyme ratio,

$$([NAD] + [NADH_2]) : ([NADP] + [NADPH_2]),$$

fell from values of 6-10 in the livers of pre-natal and newborn lambs to values of 2-4 in older lambs and adult sheep. In the rat this coenzyme ratio in the liver during development fell from about 2.4 in the young to about 1.3 in the mature animal.

Nicotinamide Nucleotide Coenzymes and Carbohydrate Metabolism in the Liver of the Developing Lamb.

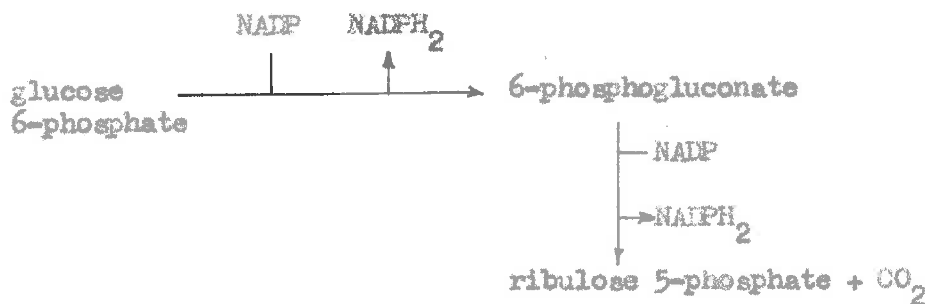
While the results in Section II were obtained in an attempt to test some aspects of Morton's (1958, 1961) suggestions about coenzymes and growth , they are also relevant to studies of the metabolism of ruminants.

Whereas adult sheep derive most of their energy requirements from the lower fatty acids, young lambs rely to a great extent on exogenous carbohydrates and higher fatty acids that are constituents of the ewes' milk (cf. Annison & Lewis, 1959; Lindsay, 1959). In the young lamb, during the immediate post-natal period, many aspects of carbohydrate and fatty acid metabolism differ from those of adult sheep, and more closely resemble those of non-ruminants (Jarrett & Potter, 1952; Jarrett & Filsell, 1960, 1964; Jarrett, Jones & Potter, 1964).

In the young lamb the blood-glucose level is comparatively high (90 mg./100 ml.) and falls to about half this value in the adult; a similar fall occurs in the rates of utilization of glucose and

acetate by the whole animal. The glucose pool and the sensitivity to insulin of the animal and the hexokinase activity of intestinal mucosa also decrease during growth of the lamb (Jarrett & Filsell, 1958). In contrast to this the concentration of volatile fatty acids in the blood increases during development (Reid, 1953), as does the capacity of ruminal epithelium and of liver to produce ketone bodies (Weidemann, 1962). The concentration of coenzyme A in the liver of the growing lamb also increases, reaching the adult level at about 6 weeks after birth (Jarrett & Filsell, 1964). The relationship of metabolic pathways to the need for oxidized or reduced NAD and NADP are discussed in Section III.

Since $NADPH_2$ is required for a number of reductive synthetic pathways (Dickens, 1961; Lowenstein, 1961) the formation of this compound by enzymic reduction is of great importance in the control of metabolic processes. One of the main sources of $NADPH_2$ in tissues of non-ruminants is the oxidation of glucose 6-phosphate and 6-phosphogluconate :



and the corresponding reactions have been shown in sheep liver (McLean, 1958; Raggi, Hansson, Simesen, Kronfeld & Luick, 1961).

The utilization of glucose by liver requires its prior phosphorylation but Gallagher & Buttery (1959) were unable to demonstrate glucokinase activity in liver homogenates from newborn lambs and adult sheep. In the studies reported in Section III, rates of reduction of NADP with glucose 6-phosphate, with 6-phosphogluconate, and with glucose and ATP were measured in the presence of extracts of livers from foetal and post-natal lambs and adult sheep. In all cases the activity of 6-phosphogluconate dehydrogenase was greater than the activity of glucose 6-phosphate dehydrogenase, and this was greater than the activity of glucokinase.

The glucokinase activity with extracts of foetal and newborn lamb livers were of the same order as those found in adult rats and much higher than those found in extracts of livers from adult sheep. The activities of both dehydrogenases also decreased during development of the livers of these animals.

Preliminary experiments were carried out to find if continuous intravenous infusion of glucose, acetate or propionate for periods up to 10 days would change the concentrations of the nicotinamide nucleotide coenzymes or of the enzymic activities associated with reduction of NADP in livers of adult sheep. It has not been possible to use large enough groups for statistical comparison, but the animals that were infused with glucose showed a decrease in NAD concentration and some increase in apparent glucokinase activity with respect to animals infused with saline. Animals infused with acetate had much lower apparent glucokinase activity than those in the other groups.

Synthesis and Properties of Nicotinic Acid Nucleotides.

With Preiss & Handler's (1957, 1958) observation that nicotinic acid nucleotide and nicotinic acid-adenine dinucleotide were intermediates in the biosynthesis of NAD, a need arose for supplies of these compounds in sufficient quantities for kinetic studies and for the development of analytical methods. Synthesis and examination of these compounds is described in Section IV.

Serlupi-Crescenzi & Ballio (1957) isolated nicotinic acid-adenine dinucleotide from *Penicillium chrysogenum* and from erythrocytes. They made the same compound in an exchange reaction with nicotinate, NAD and nicotinamide-adenine dinucleotidase from spleen (a reaction with a very low yield). The dinucleotide was identical with 'Compound II', which Preiss & Handler (1957) isolated in a radioactive form as a metabolite of $[^{14}C]$-nicotinate in extracts of erythrocytes and yeast. Lamborg, Stolzenbach & Kaplan (1958) obtained the dinucleotide by the exchange reaction of ethyl nicotinate and NAD, followed by mild alkaline hydrolysis of ethylnicotinate-adenine dinucleotide.

Nicotinic acid nucleotide has been extracted from yeast (Wheat, 1959) and from *Fusarium* (Ballio & Russi, 1959); it has also been prepared from nicotinic acid-adenine dinucleotide of enzymic origin by hydrolysis with potato pyrophosphatase (Ballio & Russi, 1959).

A more direct preparation of both nucleotides was developed in this Department, and the final section of this thesis reports

evidence that the products obtained by the new method (Atkinson & Morton, 1960) are identical with the intermediates of the biosynthesis of NAD.

Nitrous acid or HCl in amyl nitrite convert NAD into nicotinamide-hypoxanthine dinucleotide but do not convert the amide group into a carboxyl group. Nitrous anhydride converted nicotinamide nucleotide into nicotinic acid nucleotide and NAD into nicotinic acid-hypoxanthine dinucleotide. The dinucleotide was hydrolysed by potato pyrophosphatase to nicotinic acid nucleotide and inosine 5-phosphate. Nicotinic acid nucleotide and ATP are converted quantitatively into nicotinic acid adenine dinucleotide and pyrophosphate. Nicotinic acid-adenine dinucleotide was also prepared by a modification of the method described by Lamborg _et al_. (1958).

Nicotinic acid-adenine dinucleotide prepared by the two methods had the same R_F in two solvent systems and was converted into NAD by NAD synthetase in the presence of glutamine and ATP.

To investigate the possibility that nicotinic acid-adenine dinucleotide is a substrate of NAD kinase or inhibits this enzyme, formation of NADP from NAD and ATP was measured at two levels of NAD and four levels of nicotinic acid-adenine dinucleotide. There was no inhibition, nor was any compound with the expected properties of nicotinic acid-adenine dinucleotide phosphate (the nicotinic acid analogue of NADP) observed on electrophoresis.

In a preliminary attempt to protect the amino group of NAD during conversion into a nicotinic acid derivative the NAD was treated with acetic anhydride in pyridine until free of coenzyme

activity. Subsequent treatment with nitrous anhydride followed by removal of acetyl groups with dry ammonia in methanol gave nicotinic acid nucleotide and AMP. This quantitative cleavage of the pyrophosphate bond by acetic anhydride in pyridine prevented synthesis of the nicotinic acid analogue of NADP, but provides a useful non-enzymic cleavage of pyrophosphate coenzymes.

SECTION I

Measurement of the Nicotinamide Nucleotide Coenzyme Content of Animal Tissues

(a) Methods of Determination

Introduction

With the realization of the importance of these coenzymes in metabolism, many methods for their detection and assay have been described in the literature during the past thirty years. In attempts to fulfil two important criteria on which the value of an analytical biochemical procedure can be assessed - that the method be specific for the compound being determined and sufficiently sensitive to enable assays to be carried out on small amounts of tissue - methods have been developed which involve microbial assay, polarography, manometry, spectrophotometry and fluorimetry.

Although an adequate extraction procedure must be used before the nucleotide coenzymes in a tissue are determined, it is convenient to discuss methods of measurement of concentrations of coenzymes before considering methods of extraction, since the former are needed to assess the adequacy of the extraction procedures.

The practical limit of direct spectrophotometry, when determinations of nicotinamide nucleotide coenzymes are carried out using a light-path of 1 cm. and a wavelength of 340 mμ, is of the order of 10^{-5}M. Fluorimetry can extend this limit to about 10^{-8}M. and manometric techniques have been used to measure as little as 2×10^{-12} mole of NAD. Enzymic cycling combined with fluorimetry permits determination of these coenzymes at concentrations as low

as 10^{-9}M (i.e. 10^{-15}mole of coenzyme) and could possibly be extended to the determination 10^{-18} mole of coenzyme. This would bring the determination of the coenzyme content of individual cells within the scope of assay systems.

The chemistry of these coenzymes and many of the assay systems available have been reviewed by Schlenk (1945, 1952), Singer & Kearney (1954) and Kaplan (1961) and Fawcett (1963). Ciotti & Kaplan (1957) and Lowry & Passonneau (1963) have given useful summaries of many assay systems in which fluorimetry and spectrophotometry are used.

The development of a microbial assay with Haemophilus influenzae followed from the discovery by Lwoff & Lwoff (1936, 1937) that nicotinamide nucleotide coenzymes were a growth factor for this organism (see Kohn, 1938, 1940; Pittman & Frazer, 1940; Hoagland & Ward, 1942; Handler & Kohn, 1943). Gingrich & Schlenk (1944) measured the rate of change of turbidity of cultures and showed that this was proportional to the amount of coenzyme present. Although concentrations of 5 x 10^{-9}M-NAD or 10^{-8}M-NADP could be determined, the assay system is non-specific and requires only the β-nicotinamide-ribosyl moiety (see also Bachur & Kaplan, 1955). A polarographic assay for nicotinamide nucleotide coenzymes was developed by Carruthers & Suntzeff (1953), but has not been widely used.

Assay systems currently favoured depend on the use of specific dehydrogenases for the enzymic oxidation or reduction of the coenzyme moiety being determined. Since the oxidized forms of the nicotinamide nucleotide coenzymes are destroyed at high pH, and the

reduced forms are destroyed at low pH (see Schlenk, 1952; Lowry, Passonneau & Rock, 1961), use of samples treated with alkali or acid and then with a dehydrogenase that is specific for NAD or with one specific for NADP will result in completely specific assays for both of the oxidized and reduced moieties.

The methods fall into two groups : those that depend on limiting the rate of enzymic reaction with a coenzyme and those in which the absolute amount of enzymic reaction is limited by the complete oxidation or reduction of the coenzyme.

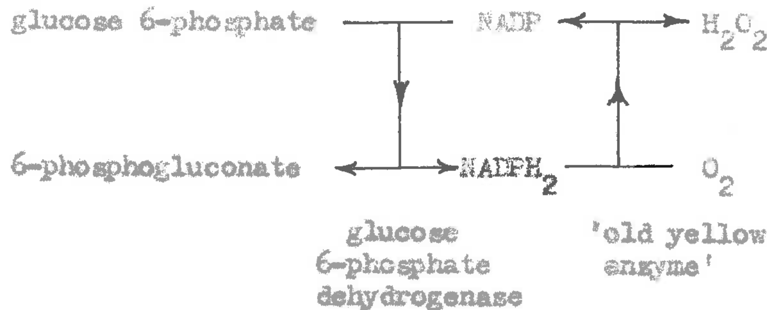
(i) Methods based on rates of enzymic reactions

These assays depend on a system of coupled enzymes by which the coenzyme is alternately oxidized and reduced and the rate of accumulation of product depends on the rate of electron transfer to and from the coenzymes ('cycling'), and this in turn depends on the amount of coenzyme present. In earlier methods, rate of production of carbon dioxide or uptake of oxygen was measured.

The earliest of these methods (Myrback, 1933) depended on the ability of added NAD to restore the fermentation of washed brewer's yeast. The coenzyme could be measured in concentrations from 2-10μM, (Schlenk & Schlenk, 1947). Jandorf, Klemperer & Hastings (1941) used a preparation from skeletal muscle, treated with charcoal, and measured the rate of production of phosphoglyceric and glycerophosphoric acids from fructose diphosphate in bicarbonate buffer in the presence of arsenate. Evolution of CO_2 was proportional to the amount of added NAD and the system was used to measure

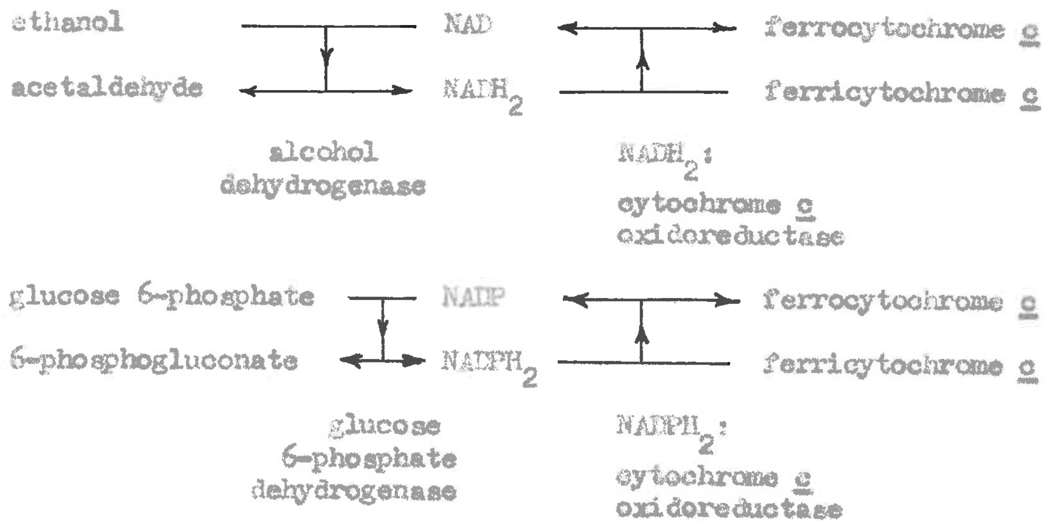
concentrations in the range 1-10µM. Anfinsen (1944) used this system with a Cartesian diver and was able to measure as little as 2×10^{-12} mole of NAD.

The coupled enzyme assay with purified enzymes was first used by Warburg, Christian & Griese (1935) in a system containing glucose 6-phosphate, glucose 6-phosphate dehydrogenase and "old yellow enzyme".



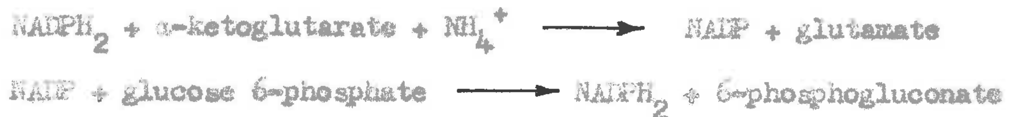
The rate of oxygen uptake was proportional to the concentration of NADP, which could be determined at concentrations of 0.1µM.

Glock & McLean (1955a) used systems which coupled an alcohol dehydrogenase specific for NAD, with $NADH_2$ cytochrome c oxidoreductase and a glucose 6-phosphate dehydrogenase which is specific for NADP with $NADPH_2$: cytochrome c oxidoreductase together with cytochrome c under conditions where the rates of reduction of the cytochrome c (followed by change in extinction at 550 mµ) were dependent on the amounts of NAD and NADP. Use of acid and alkaline extracts enabled as little as 0.1 µm-mole of oxidized and reduced forms of the coenzymes to be measured



Although this technique gave the first reliable figures for NADP and $NADPH_2$ concentrations in animal tissues (Glock & McLean, 1955 a,b,d; 1956; 1957), endogenous reduction of cytochrome c and difficulties in the preparation of stable NAD: cytochrome c oxidoreductase have hindered the application of this method.

Lowry, Passonneau, Schulz & Rock (1961) have described very sensitive coupled enzymic systems for the assay of these coenzymes. For NADP, the system utilized glutamate dehydrogenase and glucose 6-phosphate dehydrogenase.



$$NADPH_2 + \alpha\text{-ketoglutarate} + NH_4^+ \longrightarrow NADP + \text{glutamate}$$

$$NADP + \text{glucose 6-phosphate} \longrightarrow NADPH_2 + \text{6-phosphogluconate}$$

Under the conditions used, each molecule of NADP catalysed the formation of about 8 000 molecules of 6-phosphogluconate in 30 min.

The 6-phosphogluconate was then measured in a second incubation with 6-phosphogluconate dehydrogenase and added NADP. The $NADPH_2$ thus produced was determined fluorimetrically by the method of Lowry, Roberts & Kapphahn (1957).

For NAD, the system utilized glutamate dehydrogenase and lactate dehydrogenase:

$NADH_2$ + α-ketoglutarate + NH_4^+ ⟶ NAD + glutamate

NAD + lactate ⟶ $NADH_2$ + pyruvate

Pyruvate was produced in about 2 500-fold yield in 30 min. and measured in the second step with lactate dehydrogenase and added $NADH_2$ by fluorimetry (Lowry et al., 1957).

With both systems the procedures can be repeated to give an over-all multiplication of 10^7 to 10^8. If the first cycling was carried out in very small volume, then a double cycling with a multiplication factor of 10^8 could extend the sensitivity to 10^{-19} mole of coenzyme since 10^{-11} mole/ml. can be measured directly by fluorimetry. The NAD system used is not ideal since it lacks an irreversible step and the lactate/pyruvate equilibrium is not favourable. This method also has the usual disadvantages of fluorimetry (see p.33).

Several assay systems have been developed in which oxidation of the coenzymes was coupled to reduction of various dyes e.g. methylene blue, phenazine methosulphate, 2,6-dichlorophenol-indophenol and nitro-blue tetrazolium.

Sumner, Krishnan & Sisler (1947) and Sumner & Krishnan (1948) described a system similar to that reported by Warburg <u>et al.</u> (1935), and used purified lactate dehydrogenase, diaphorase and methylene blue under anaerobic conditions where the rate of decolourisation of the dye was proportional to the NAD concentration. Whatley (1951) used enzymes which are specific for NADP (iso-citrate dehydrogenase, glucose 6-phosphate dehydrogenase) in a similar system to determine this coenzyme in plant tissues.

These coupled reactions are preferably used with coenzyme concentrations less than the Michaelis constant for the oxidoreductases, so that overall rates are approximately proportional to coenzyme concentrations. In all cases, rates measured with unknowns must be compared to those with standards.

Villee (1961, 1962) has modified the method described by Glock & McLean (1955a) so that the assays can be carried out with commercially available enzyme preparations, coupling electron transfer from the reduced coenzymes to 2,6-dichlorophenol-indophenol with a diaphorase preparation from <u>Clostridium kluyveri</u> which oxidizes $NADH_2$ or $NADPH_2$. The rate of reduction of the dye was measured by the rate of decrease in extinction at 600 mμ. Phenazine methosulphate could be used to replace the diaphorase. Slater and Sawyer (1962) have described assay systems of this type which could be coupled to a tetrazolium as the final oxidant.

The principal investigations of nicotinamide nucleotide concentration by measurement of rates are listed in Table I.

Table 1. Methods of determining nicotinamide nucleotide coenzymes

(i) Methods based on rates of enzymic reactions

Method	Coenzyme measured	Reference
Manometry:		
Restoration of fermentation of brewers' yeast	NAD	Myrbäck (1933) von Euler (1936) Axelrod & Elvehjem (1939) Kensler, Suguira & Rhodes (1940) Govier (1944) Schlenk & Schlenk (1947)
Enzymic conversion of fructose diphosphate into glycerol phosphate and phosphoglycerate in $NaHCO_3$ buffer	NAD	Jandorf, Klemperer & Hastings (1941)
As above: with Cartesian diver technique	NAD	Anfinsen (1944)
Glucose 6-phosphate & dehydrogenase/ diaphorase/oxygen uptake	NADP	Warburg, Christian & Griese (1935)

Table 1. (continued)

Method	Coenzyme measured	Reference
Decolorisation of methylene blue:		
Lactate & dehydrogenase/diaphorase	NAD	Sumner, Krishnan & Sisler (1947) Sumner & Krishnan (1948) Whatley (1951)
Glucose 6-phosphate & dehydrogenase/ diaphorase	NADP	Whatley (1951)
Isocitrate & dehydrogenase/diaphorase	NADP	
Reduction of cytochrome c:		
Alcohol dehydrogenase or glucose 6-phosphate dehydrogenase/diaphorase	NAD, $NADH_2$ NADP, $NADPH_2$	Glock & McLean (1955a)
Reduction of 2,6-dichlorophenol-indophenol:		
Alcohol dehydrogenase or glucose 6-phosphate dehydrogenase/diaphorase (or phenazine methosulphate)	NAD, $NADH_2$ NADP, $NADPH_2$	Villee (1961, 1962)
Fluorimetric assay with 'cycling':		
for details see text	NAD, $NADH_2$ NADP, $NADPH_2$	Lowry, Passonneau, Schulz & Rock (1961)

(ii) Methods based on complete oxidation or reduction of the coenzyme

(a) Spectrophotometric assay

Warburg, Christian & Griese (1935) were the first to use the increase of extinction at 340 mμ on reduction of NAD as a method of assay. Determination of the molecular extinction coefficient of $NADH_2$ at 340 mμ have given values close to 6.2 x 10^3 (see Table 2).

The NAD may be reduced with sodium hydrosulphite (Warburg & Christian, 1936 ; Le Page, 1947; Gutcho & Stewart, 1948) or with ethanol and alcohol dehydrogenase (Negelein & Wulff, 1937; Horecker & Kornberg, 1948; Racker, 1950; Bonnichsen, 1950). The use of ethanol and yeast alcohol dehydrogenase makes the assay specific for NAD; several analogues of NAD are also reduced under these conditions, but at a much lower rate (Kaplan, Ciotti & Stolzenbach, 1956). Reduction of NAD with lactate and lactate dehydrogenase (Strength, Ringler & Nelson, 1954) has not been widely used since yeast alcohol dehydrogenase is readily available commercially.

The oxidation of $NADH_2$ using acetaldehyde and yeast alcohol dehydrogenase, measured by the decrease in extinction at 340 mμ, has proved to be a satisfactory assay system (Negelein & Wulff, 1937; Racker, 1950; Spirtes & Eichel, 1954; Holzer, Goldschmidt, Lamprecht & Helmreich, 1954; Jedeikin & Weinhouse, 1955). From Racker's (1950) data it is evident that in the presence of an excess of substrate (ethanol or acetaldehyde), there is almost complete

Table 2. Extinction coefficient of reduced nicotinamide-adenine dinucleotide at 340 mμ

Molecular extinction coefficient $\times 10^{-3}$	Reference
4.78	Warburg & Christian (1936)
5.90	Haas (1937)
5.12	Negelein & Wulff (1937)
6.28	Ohlmeyer (1938)
5.47	Negelein & Bromel (1939)
5.50	Schlenk (1942)
5.83	Drabkin (1945)
6.21 (mean of 6.11, 6.11, 6.25, 6.28, 6.31)	Horecker & Kornberg (1948)
6.25	Wallenfels & Christian (1957)

reduction of NAD at pH 9.5 and almost complete oxidation of $NADH_2$ at pH 7.5, with alcohol dehydrogenase.

Similar assays for NADP involving reduction with isocitrate and purified isocitrate dehydrogenase from pig heart (Ochoa, 1948; Ciotti & Kaplan, 1957) and with glucose 6-phosphate and glucose 6-phosphate dehydrogenase (Anderson & Vennesland, 1954) have been used; the reactions are carried out at about pH 7.5 and give almost complete reduction of the coenzyme.

A specific enzymic assay for $NADPH_2$ using $NADPH_2$: cytochrome c reductase from liver, coupled with cytochrome c was used by Kaplan, Colowick & Neufeld (1952) but the reaction required about 30 min. for complete oxidation of the coenzyme. A more satisfactory system using oxidized glutathione and glutathione reductase from peas was later developed (Kaplan, Colowick & Neufeld, 1953; Ciotti & Kaplan, 1957; Holzer, Busch & Kroger, 1958).

Determination of total reduced coenzymes can be carried out by oxidation with the diaphorase from Clostridium kluyveri (Weber & Kaplan, 1954; Ciotti & Kaplan, 1957) or with phenazine methosulphate using manometry (Smith, 1961) or fluorimetry (Stollar, 1960).

A method for estimation of total coenzymes was used by Haas (1944). This was based on reduction of 2:6-dichlorophenolindophenol by the reduced coenzymes.

In 1938, Meyerhof, Ohlmeyer & Möhle, found that the nicotinamide nucleotide coenzymes form addition compounds with cyanide at high pH. Colowick, Kaplan & Ciotti (1951) showed that the addition compounds had an ultraviolet absorption spectrum similar

to that of the reduced coenzymes, with a molar extinction of 6.3 x 10^3 at 325 mμ. Although similar compounds are formed by many quaternary pyridinium compounds, total NAD plus NADP can be measured by correcting for other compounds present after hydrolysis of the coenzymes with Neurospora NADase (see Ciotti & Kaplan, 1957). The reduced coenzymes do not react with cyanide. Morton (1955) has shown that alkaline phosphatase hydrolyses the phosphoryl group at the 2'-position in NADP, so that by the use of alcohol dehydrogenase followed by the phosphatase, both NAD and NADP could be estimated spectrophotometrically in the same solution.

The use of microscopic techniques applied to living tissues or suspended droplets and the use of monochromatic spectrophotometers of high resolution, makes possible the direct measurement of as little as 10^{-14} mole of nicotinamide nucleotide coenzyme (see Klingenberg & Slenczka, 1959; Ornstein & Lehrer, 1960).

Table 3 lists the main investigations of nicotinamide coenzyme assay by methods depending on complete oxidation or reduction or formation of adducts.

(b) Fluorimetric assay

(i) Condensation with ketones. The discovery by Huff (1947), that N-substituted derivatives of nicotinamide condense with acetone under alkaline conditions to give a product with a green-blue fluorescence, which intensifies and gives a stable blue fluorescence on treatment with acid, has become the basis for a number of assay methods. Huff & Perlzweig (1947) used this method for the determination of N-methyl nicotinamide and it was then

Table 3. Methods for determining nicotinamide nucleotide coenzymes, depending on complete oxidation or reduction or formation of adducts

Method	Coenzyme measured	Reference
Spectrophotometry at 340 mμ:		
Reduction with sodium hydrosulphite	NAD	Warburg, Christian & Griese (1935) Le Page (1947) Gutcho & Stewart (1948)
Reduction with ethanol/alcohol dehydrogenase	NAD	Negelein & Wulff (1937) Horecker & Kornberg (1948) Racker (1950) Bonnichsen (1950)
Reduction with lactate/lactate dehydrogenase	NAD	Warburg & Christian (1941) Strength, Ringler & Nelson (1954)
Oxidation with acetaldehyde/alcohol dehydrogenase	$NADH_2$	Negelein & Wulff (1937) Racker (1950) Jedeikin & Weinhouse (1953) Spirtes & Eichel (1954) Holzer *et al.* (1954)
Reduction with isocitrate/isocitrate dehydrogenase	NADP	Ochoa (1948) Ciotti & Kaplan (1957)

Table 3. (continued)

Method	Coenzyme measured	Reference
Spectrophotometry at 340 mμ:		
Reduction with glucose 6-phosphate & dehydrogenase	NADP	Anderson & Vennesland (1954)
Oxidation with oxidized glutathione & reductase	$NADPH_2$	Kaplan, Colowick & Neufeld (1953) Ciotti & Kaplan (1957)
Oxidation by diaphorase from *Clostridium kluyveri*	$NADH_2$ + $NADPH_2$	Weber & Kaplan (1954) Ciotti & Kaplan (1957)
Reduction using alcohol dehydrogenase and glucose 6-phosphate dehydrogenase	NAD, NADP	Holzer, Busch & Kroger (1958)
Oxidation using glycerol-phosphate dehydrogenase followed by glutamate dehydrogenase	$NADH_2$, $NADPH_2$	
Spectrophotometry at 325 mμ:		
Cyanide addition reaction: use of NAD-ase	NAD, NADP	Colowick, Kaplan & Ciotti (1951)

Table 3. (continued)

Method	Coenzyme measured	Reference
Manometry: oxygen uptake		
Oxidation coupled to phenazine methosulphate	$NADH_2$, $NADPH_2$	Smith (1961)

adapted for the measurement of nicotinamide nucleotide coenzymes in blood (Levitas, Rosen & Perlzweig, 1947) and animal tissues (Robinson, Levitas, Rosen & Perlzweig, 1947). Concentrations as low as 1 µM could be measured satisfactorily.

Carpenter & Kodicek (1950) modified this procedure and used methyl ethyl ketone for the fluorimetric estimation of N-methyl nicotinamide and this modification has been used for determination of the coenzymes (Kaplan, Goldin, Humphreys, Ciotti & Stoltzenbach, 1956). The use of enzymic oxidation or reduction, or destruction of one form of the coenzymes was combined with this assay method by Jacobson & Astrachan (1957) for the determination of each of the oxidized and reduced nicotinamide nucleotide coenzymes down to concentrations of about 0.01 µM. Modifications of this method, which require fewer enzymes and retain specificity and sensitivity have been used for determination of these coenzymes in bacteria (Weber & Schwartz, 1960) and in plant tissue (Graham & Hawker, 1962; Gayler, 1962).

(ii) Reaction with alkali. When NAD and other nicotinamide ribosides are treated with strong alkali, a fluorescent product is produced which is sufficiently stable for this reaction to be used as an assay method for concentrations as low as 1 µM (Kaplan, Colowick & Barnes, 1951). This method was modified by Lowry, Roberts, Kapphahn & Lewis (1956) using alkali and hydrogen peroxide so that NAD and NADP could be measured at concentrations of 0.01µM. The reduced coenzymes were also determined by measuring their native fluorescence at about 470 mµ

(see also Lowry, Roberts & Kapphahn, 1957). Bassham, Birt, Hems & Loening (1959) have made a careful study of the application of this method to determination of these coenzymes in animal tissues. Reduced coenzymes in alkaline extracts were oxidized enzymically before treatment with alkaline hydrogen peroxide.

(iii) Direct observation of tissues. Direct observation of the changes in oxidation and reduction of the nicotinamide nucleotide coenzymes has been made possible by the development of micro-spectrofluorimeters so that less than 10^5 molecules of reduced coenzyme can be detected. Absorption and excitation maxima for the free and bound coenzymes are at 335 mμ and 340 mμ, giving fluorescence emission peaks at 463 mμ and 480 mμ, respectively. Systems studied have ranged from suspensions of mitochondria, yeast, bacteria and ascites tumour cells to animal tissues *in vivo* (see Chance, Cohen, Jobsis & Schoener, 1962; Chance, Schoener & Ferguson, 1962; Estabrook, Maitra & Scott, 1962).

Table 4 lists the main investigations of nicotinamide coenzyme assay by methods depending on conversion into fluorescent derivatives.

Assessment of the various assay methods. It is clear that a wide range of methods is available for specific and accurate determination of both the oxidized and reduced forms of these coenzymes. It was therefore necessary to check methods and adapt them for routine use with enzymes readily available commercially or readily prepared and having sufficient specificity to allow

Table 4. Methods of determining nicotinamide nucleotide coenzymes: assay by conversion into fluorescent derivatives.

Method of formation of fluorescent derivatives	Coenzyme measured	Reference
Condensation with acetone in alkali, followed by acid	NAD, NADP	Huff & Perlzweig (1947) Levitas, Rosen, Huff & Perlzweig (1947)
Condensation with methyl ethyl ketone in alkali followed by acid	NAD, NADP	Robinson, Levitas, Rosen & Perlzweig (1947) Carpenter & Kodicek (1950) Kaplan, Goldin, Humphreys, Ciotti & Stoltzenbach (1956)
Condensation with ketone in alkali after enzymic reduction and treatment with acid	NAD, $NADH_2$ NAD, $NADPH_2$	Jacobson & Astrachan (1957) Weber & Schwartz (1960) Graham & Hawker (1962)
Treatment with strong alkali after enzymic treatments	NAD, NADP	Kaplan, Colowick & Barnes (1951)
Treatment with alkaline peroxide after enzymic reduction and treatment with acid or alkali	NAD, $NADH_2$ NADP, $NADPH_2$	Lowry, Roberts, Kapphahn & Lewis (1956) Lowry, Roberts & Kapphahn (1957) Bassham, Birt, Hems & Loening (1959)
Oxidation of coenzymes by phenazine methosulphate: following Kaplan *et al*. (1956).	$NADH_2$, $NADPH_2$	Stollar (1960)

Table 4. (continued)

Method of formation of fluorescent derivatives	Coenzyme measured	Reference
Use of native fluorescence of reduced coenzymes	$NADH_2$ + $NADPH_2$	Chance *et al.* (1962) Estabrook *et al.* (1962)

determination of one of the coenzymes in the presence of the other. Direct fluorimetry using the methyl ethyl ketone condensation or the alkali-H_2O_2 reaction has the advantage of high sensitivity. The presence of other materials which may either fluoresce or quench fluorescence requires the use of internal standards and a number of blank determinations which must be carried out concurrently. The enzymic cycling method of Lowry, Passonneau, Schulz & Rock (1961) makes full use of this high sensitivity but its use with tissue extracts requires complete destruction of NAD-ases before incubation with the substrates.

Manometric methods are of value with turbid solutions or tissue extracts that contain compounds that absorb or fluoresce strongly in the regions of measurement.

Spectrophotometric methods dependent on rates of coupled enzymic reactions are attractive because of high sensitivity, permitting the assay of 0.1 µm-mole of coenzyme. However, results must be corrected for the rate of non-enzymic reduction of the final electron acceptor and also for the activity of the coupled system in the absence of the tissue extract. Assays with standard amounts of coenzymes must be carried out, preferably as a range of internal standards. If it is necessary to use small amounts of tissue, say 200 mg. or less, or tissues in which coenzyme content is low, e.g. assay for NADP in tumour tissues (commonly as low as 3 µm-moles/g. fresh wt.; cf. Table 10) then methods of this type or fluorimetric methods with or without cycling would be required to give the necessary sensitivity.

The use of direct spectrophotometry at 340 mμ, in conjunction with specific enzymatic oxidation or reduction of the coenzymes, ensures complete specificity for the assay, removes the need for standards and does not require a series of blank determinations (see Helmreich, Holzer, Lamprecht & Goldschmidt, 1954; Holzer, Busch & Kröger, 1958; Klingenberg & Slenczka, 1959; Klingenberg & Bücher, 1960). The limit of accurate measurement is about 1-10 μM coenzyme. This was, therefore, the assay method chosen and this section describes the preparation of enzymes and substrates and the specificity of the assay systems. Methods for the extraction of these coenzymes from animal tissues are considered and results obtained for normal adult rat-liver tissue are compared with those reported by other workers.

Experimental

Preparation of enzymes

*L_s-isocitrate: NADP oxidoreductase (decarboxylating) (isocitrate dehydrogenase; EC 1.1.1.42). The enzyme from pig heart was prepared as described by Ochoa (1955). The dialysed ammonium sulphate fraction was kept at -15°. For details of the specificity of this enzyme see below.

$NADPH_2$: glutathione oxidoreductase (glutathione reductase; EC 1.6.42). The reductase from dried peas (Greenfeast variety) was prepared as described by Kaplan, Colowick & Neufeld (1953). Material precipitating between 0.4 and 0.6 saturation of ammonium sulphate was dissolved in 0.1 M tris (Cl^-, pH 7.4) and kept at -15°. The enzyme oxidized 81 µm-moles of $NADPH_2$/min./mg. of protein at 25° in an assay system containing 0.18 µmoles of $NADPH_2$, 10 µmoles of oxidized glutathione and 0.57 m-mole of tris chloride (pH 7.4) in a final volume of 3 ml. Protein was measured as described by Warburg & Christian (1942).

Glutathione reductase was also prepared from wheat germ as described by Barnett, Stafford, Conn & Vennesland (1953).

* However, Meister & Strassburger (1963) have evidence that the substrate is D_s-isocitrate.

Material precipitating between 0.25 and 0.275 saturation of ammonium sulphate had a specific activity of 150 μm-moles of $NADPH_2$ oxidized/min./mg. of protein but was less stable than the enzyme from peas and was only used for preliminary work.

L-glycerol-3-phosphate: NAD oxidoreductase (glycerolphosphate dehydrogenase; EC 1.1.1.8.). A preparation of the dehydrogenase from Sigma Chemical Co. was used. The suspension was diluted twenty-five fold in 0.1 M-tris (Cl^-, pH 7.4) before use.

Ethanol: NAD oxidoreductase (alcohol dehydrogenase; EC 1.1.1.1.). Preparations from Sigma Chemical Co. and from C. F. Boehringer & Soehne were used as solutions containing 1% (w/v) and 0.1% (w/v) of protein, respectively.

Ketose-1-phosphate aldehyde-lyase (aldolase; EC 4.1.2.7). An ammonium sulphate fraction from rabbit muscle (Atkinson, Burton & Morton, 1961) was used.

Other reagents

Oxidized glutathione, sodium isocitrate and fructose 1,6-diphosphate (barium or sodium salts) were obtained from the Sigma Chemical Co.

Dihydroxyacetone phosphate. A solution containing approx. 50 mM-dihydroxyacetone phosphate was prepared from fructose

1,6-diphosphate by treatment with aldolase, followed by removal of protein with perchloric acid and of perchloric acid as potassium perchlorate (cf. Meyerhof, 1938).

Nicotinamide-adenine dinucleotide. With the coenzyme from Sigma Chemical Co., 96% of the extinction at 259 mμ was due to β-NAD, as measured by reduction with ethanol and alcohol dehydrogenase at pH 9.5 (for details see below). In later work coenzyme from Boehringer & Soehne (stated to contain 85% of β-NAD, 5% of α-NAD and 1% of adenosine diphosphate ribose) was used.

Reduced nicotinamide-adenine dinucleotide. In the disodium salt (Sigma Chemical Co.) used in the earlier experiments 81% of the extinction at 260 mμ was due to β-$NADH_2$, as measured with dihydroxyacetone phosphate and glycerolphosphate dehydrogenase (for details see p. 40). In later work the reduced coenzyme was prepared as described by Atkinson, Burton & Morton (1961), but without precipitation as the barium salt.

Nicotinamide-adenine dinucleotide phosphate. This was obtained as the sodium salt from C. F. Boehringer & Soehne. About 81% of the extinction at 260 mμ was due to the β-isomer, estimated with isocitrate dehydrogenase.

Reduced nicotinamide-adenine dinucleotide phosphate. This was prepared from the oxidized coenzyme as described by Evans & Nason (1953).

Methods of determination: tests with individual coenzymes.

Assay for nicotinamide-adenine dinucleotide. To ensure almost complete reduction of NAD by ethanol in the presence of alcohol dehydrogenase it has previously been found necessary to establish the equilibrium:

$$NAD + CH_3CH_2OH \longrightarrow NADH + H^+ + CH_3CHO$$

at a pH greater than 8.8, or to remove acetaldehyde with trapping reagents such as semicarbazide. Both methods were tested initially, with solutions of the purified coenzymes. The method using a high pH (ii, below) was selected for the studies reported here.

(i) Assay with ethanol, semicarbazide, and alcohol dehydrogenase at pH 7.4.

Alcohol dehydrogenase (0.025 ml. of the solution described on p. 36) was added to a 1 cm. cuvette containing 2.80 ml. of 0.2 M-tris (Cl^-, pH 7.4), 0.05 ml. of 0.5 M-semicarbazide hydrochloride, 0.1 ml. of ethanol, and 0.010 ml. of 10 mM-NAD. The increase in extinction at 340 mµ measured at 25° against a blank without enzyme, in an Optica CF 4 spectrophotometer, was complete in 3 min. Increases in extinction were corrected for the absorption due to the enzyme; this was measured in a similar system without NAD.

There was no increase in extinction when 0.025 ml. of 10 mM-NADP was added after the reduction of NAD was complete, nor did the NADP inhibit the reduction of NAD added subsequently. Replicate analyses agreed within 1%. This system, based on that described by Negelein & Wulff (1937), was less suitable than (ii) for the measurement of coenzymes in tissues because the system came to equilibrium more rapidly at the higher pH.

(ii) Assay with ethanol and alcohol dehydrogenase at pH 9.5. 0.5M-Trichloroacetic acid (0.8 ml.), 10 mM-NAD (0.025 ml.) and 2.0 ml. of a solution containing 62 mg. of glycine, 33 mg. of NaOH and 0.10 ml. of ethanol were placed in a 1 cm. cuvette (final pH, 9.5). The extinction change on addition of 0.025 ml. of alcohol dehydrogenase was measured as in (i). Reduction was complete in less than 1 min. and the extent of reduction was 99% of that observed in the method with semicarbazide. From Racker's (1950) measurement of the equilibrium constant the residual NAD at equilibrium in (ii) would be 0.3% of the total NAD + $NADH_2$. This system permits the use of extracts of tissue in trichloroacetic acid; the concentrations of NaOH and glycine are calculated to neutralise the acid and bring the final pH to 9.5. This pH seems to offer the best compromise between activity of the dehydrogenase, completeness of reduction of the NAD and buffering capacity of the glycine. A molar extinction increment of 6.2×10^3 at 340 mμ on reduction of NAD was used in calculation of the concentration of this coenzyme (cf. Table 2).

Assay for reduced nicotinamide-adenine dinucleotide.

Because of reports that alcohol dehydrogenase preparations react rapidly enough with $NADPH_2$ to interfere with the analysis of $NADH_2$ (Holzer et al. 1958) this system was compared with an L-glycerol-3-phosphate system which these authors used for determination of $NADH_2$.

(i) Assay with dihydroxyacetone phosphate and glycerol-phosphate dehydrogenase. 0.13 μmole of $NADH_2$ was oxidized in less than 1 min. by 0.05 ml. of the dehydrogenase solution described above, in a solution containing 2.8 ml. of 0.1M-tris (Cl^-, pH 7.4) and 0.1 ml. of 50 mM-dihydroxyacetone phosphate. 0.1 μmole of $NADPH_2$ was added to the system after oxidation of $NADH_2$ was complete; the subsequent decrease in extinction at 340 mμ in a 1 cm. cuvette was less than 0.002 in 3 min. A further 0.125 μmole of $NADH_2$ was completely oxidized in 2 min.

This system was thus specific enough for the analytical studies described later, but since the more readily available acetaldehyde-alcohol dehydrogenase system (ii) proved to be specific for $NADH_2$, the latter system was used for the main investigations.

(ii) Assay with acetaldehyde and alcohol dehydrogenase.
$NADH_2$ (up to 0.16 mM) in 2.95 ml. of 0.2M-tris (Cl^-, pH 7.4) and 0.025 ml. of 0.5M-acetaldehyde was oxidized within 1 min. on addition of 0.025 ml. of a solution of alcohol dehydrogenase.

0.1 μmole of $NADPH_2$ was not oxidized in this system, nor did it interfere with the oxidation of $NADH_2$. Less than 0.2% of the $NADH_2$ remained in a reduced state at equilibrium (Racker, 1950).

Assay for nicotinamide-adenine dinucleotide phosphate. 0.15 μmole of NADP was reduced in less than 3 min. on addition of 0.02 ml. of a solution of isocitrate dehydrogenase to the coenzyme in 2.7 ml. of 0.1M-phosphate (K^+, pH 7.5), 0.1 ml. of 0.1M-DL-isocitrate and 0.1 ml. of 0.1M-$MgCl_2$ in a 1 cm. cuvette. There was no change in extinction at 340 mμ with 0.25 μmole of NAD in this system, nor did this coenzyme interfere with subsequent reduction of NADP. Concentrations of NADP were calculated from a molar extinction increment of 6.2×10^3 at 340 mμ on reduction (cf. Table 2). In these conditions at least 99.9% of the NADP was reduced (see Ochoa, 1955).

Assay for reduced nicotinamide-adenine dinucleotide phosphate. $NADPH_2$ (up to 0.2 μmole) was oxidized in less than 3 min. on addition of 0.020 ml. of a solution of glutathione reductase to a solution of the coenzyme in 2.85 ml. of 0.2M-tris (Cl^-, pH 7.4) and 0.1 ml. of 0.1M-oxidized glutathione in a 1 cm. cuvette. 0.25 μmole of $NADH_2$ was not oxidized in this system by any of the batches of reductase used for analysis of coenzymes in tissues and did not interfere with the analysis of $NADPH_2$. In these conditions at least 99.9% of the $NADPH_2$ was oxidized (see Conn & Vennesland, 1951).

Extraction of nicotinamide nucleotide coenzymes from animal tissues

Since the aim in determining any tissue constituent is to arrive at a value which represents its *in vivo* concentration, it is essential to consider the factors which will alter coenzymes either by oxidation or reduction or by cleavage of the nucleotides. Since the tissue structure must be destroyed in order to obtain an extract suitable for use in the assay system, destruction or change in the coenzyme molecule brought about by pH changes, temperature and enzyme action must be considered.

(a) Enzymic breakdown of nicotinamide nucleotide coenzymes

The enzymic inactivation of NAD by animal tissues was first shown by the work of Lennerstrand (1936) and of von Euler, Heiwinkel & Schlenk (1937). Subsequent studies have shown that the coenzymes may be attacked at several sites. Cleavage at the nicotinamide ribose linkage, the pyrophosphate linkage, deamination of the adenosyl moiety, hydrolysis of the 2'-phosphoryl of NADP, pyrophosphorolysis and interconversion by oxidation and reduction have been studied (Fig. 1).

(i) Nucleosidases. Mann & Quastel (1941) showed that nicotinamide inhibited the inactivation of NAD by brain. Handler & Klein (1942) demonstrated that in animal tissues, cleavage at the nicotinamide ribosyl linkage occurs and is inhibited by nicotinamide. NADP was also split by this enzyme (Handler & Klein, 1942) but $NADH_2$, $NADPH_2$, NMN and nicotinamide riboside were not (Zatman, Kaplan & Colowick, 1953).

Figure 1

Enzymic destruction and interconversion of nicotinamide nucleotide coenzymes

1 Nucleotidase

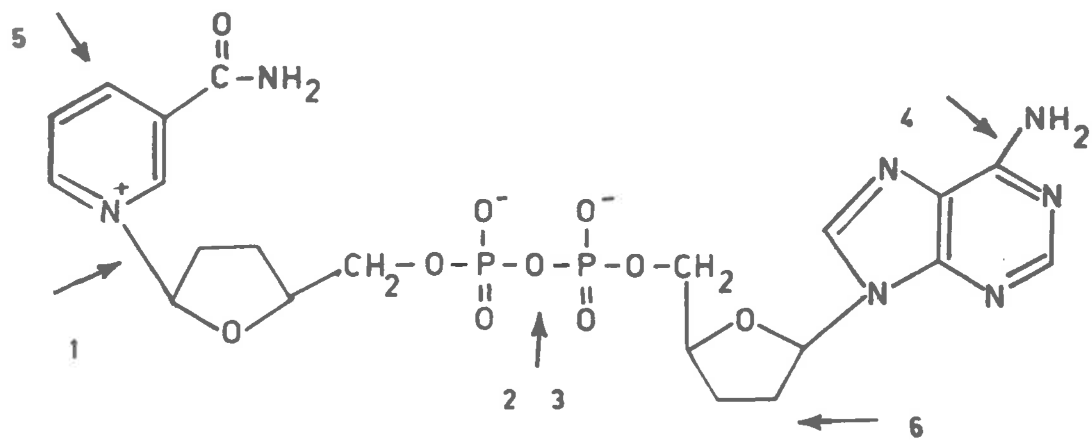
2 Pyrophosphatase

3 Adenylyltransferase

4 Adenosine deaminase

5 Oxidoreductase; transhydrogenase

6 Phosphatase; phosphokinase


5
O
C
NH2
N+
1
4
NH2
N
N
N
N
CH2
O
P
O
P
O
CH2
O-
O-
O
O
O
O
2
3
6

The NADase activity is largely found in the microsomal fraction of the cell. Sung & Williams (1952) showed that more than half the activity was in this fraction and that the remainder was divided between the nucleus and the supernatant in rat liver. A similar distribution occurs in kidney and brain (see also Jacobson & Kaplan, 1957a). Burton (1957) found very low levels of the enzyme in rat brain at birth, with a marked increase occurring in the first few weeks of post-natal life. Nemeth & Dickerman (1960) have shown similar increases in NADase activity in the liver, spleen and cerebral cortex of the guinea pig; however, adult levels are reached just before the time of birth.

An enzyme which splits NAD at the nicotinamide riboside linkage has been isolated from *Neurospora crassa* (Kaplan, Colowick & Nason, 1951). This enzyme appears to be localised in the conidia (Zalokar & Cochrane, 1956) and is greatly increased in concentration when the mould is grown in a zinc-deficient medium (Nason, Kaplan & Colowick, 1951).

The NADases of animal tissue are comparatively resistant to high temperatures (see Lowry, Passonneau, Schulz & Rock, 1961), so that inactivation of this enzyme is of major importance in making the tissue extract.

(ii) *Pyrophosphatases.* A number of enzymes have been described which will split the pyrophosphate linkage in NAD and NADP but they are generally non-specific and will hydrolyse other nucleotides as well. In the case of NAD, NMN and AMP are formed; NADP gives NMN and adenosine 2',5'-diphosphate. This cleavage is

brought about by preparations from rabbit kidney and liver (Kornberg, 1948; Kornberg & Lindberg, 1948), from potatoes (Kornberg & Pricer, 1950) and from pigeon liver (Jacobson & Kaplan, 1957<u>a</u>). Most animal tissue pyrophosphatases hydrolyse $NADH_2$ faster than NAD, however with the potato enzyme, the reverse is true. The pigeon liver pyrophosphatase hydrolyses only the reduced forms and not the oxidized forms of the coenzymes. This enzyme is found in the soluble fraction of the liver, whereas the other animal tissue pyrophosphatases are largely associated with the microsomal fraction (Jacobson & Kaplan, 1957<u>a</u>).

(iii) <u>Adenosine deaminase</u>. The enzyme from takadiastase was shown by Kaplan, Colowick & Ciotti (1952) to also deaminate NAD but not NADP.

(iv) <u>NAD-pyrophosphorylase</u> (ATP:NMN-adenylyltransferase). Both oxidized and reduced NAD are cleaved at the pyrophosphoryl link by inorganic pyrophosphate in the presence of this enzyme (Kornberg, 1950). Since inorganic pyrophosphatases occur widely in animal tissues, this reaction is probably of little quantitative significance in the breakdown of NAD (see Lasande & Handler, 1961). However, the synthesis of NAD is readily catalysed by elimination of pyrophosphate from ATP with NMN or nicotinic acid mononucleotide (see Atkinson, Jackson & Morton, 1961).

(v) <u>Oxidoreductases</u>. During extraction of coenzymes from cells these compounds come in contact with dehydrogenases and substrates so that rapid changes of the steady-state ratios of

oxidized and reduced coenzymes take place. This may be a major source of error in determining the coenzymes and is avoided as far as possible by rapid inactivation of the dehydrogenases.

(b) Destruction of nicotinamide nucleotide coenzymes by pH and temperature changes

The generalisations made by the early workers in this field still hold true, i.e. that the oxidized forms of the coenzymes are relatively stable in acid solution but are rapidly destroyed at high pH while the reduced forms are stable at high pH, but rapidly destroyed in acid solution (for reviews, see Singer & Kearney, 1954; and Kaplan, 1961).

Colowick, Kaplan & Ciotti (1951) measured the rate of destruction of NAD in the presence of various buffers and showed that citrate and phosphate increased the rate of destruction, whereas in tris buffer, the rate of destruction was much lower. The nicotinamide-ribosyl linkage appears to be the most labile bond in the NAD molecule.

Lowry, Passonneau & Rock (1961) carried out a thorough investigation on the rates of destruction of the oxidized and reduced forms of the coenzymes, varying both temperature and pH and the mechanisms of these reactions have been studied by Burton & Kaplan, (1963a,b). The stability of NAD decreases rapidly above pH 8; however there is destruction at all pH values at 100°. Both $NADH_2$ and $NADPH_2$ are quite stable when heated for 30 and 60 minutes at 100° in 0.1 N NaOH. However, in the presence of blood, there

is considerable destruction of $NADH_2$ in sodium hydroxide solution, but not in sodium carbonate solution. Methods used for the extraction of nicotinamide nucleotide coenzymes from liver tissue are listed in Table 5, together with the values obtained. These results are confined to adult rat-liver tissue, in order to compare the various techniques used.

The problem, therefore, is to extract the coenzymes from the tissue as completely as possible, under conditions where there is rapid inactivation of the enzymes that can cause alterations to these coenzymes and where destruction of the coenzymes by pH or temperature changes is minimised. Low temperature treatment of the tissue, addition of inhibitors and heating have been the methods used to minimise enzymic changes. In general, an acid extraction for the oxidized coenzymes and an alkaline extraction for the reduced forms have been the preferred methods. The use of a single extraction, buffered at a pH near 8, in an attempt to obtain both oxidized and reduced forms with minimum destruction of either has also been investigated.

In this investigation a single-extraction method with tris-maleate at pH 8.4 was compared with a double-extraction method (trichloroacetic acid at 0°; NaOH or Na_2CO_3 at 100°). Results with fresh or with frozen tissue were also compared.

Table 5. Extraction methods and nicotinamide

	Reference	Assay method
(1)	Axelrod & Elvehjem (1939)	Manometric: restoration of yeast fermentation
(2)	Kensler, Suguira & Rhodes (1940)	As above
(3)	Bernheim & Felsovanyi (1940)	V factor of *H. parainfluenzae*
(4)	Jandorf (1943)	Manometric: CO_2 production following Jandorf *et al.* (1941)
(5)	Robinson, Levitas, Rosen & Perlzweig (1947)	Fluorimetric: condensation with acetone
(6)	Fisher & Schlenk (1948)	Manometric: following Axelrod & Elvehjem (1939)
(7)	Fiegelson, Williams & Elvehjem (1950).	Reduction with $Na_2S_2O_4$: spectrophotometry
(8)	Carruthers & Suntzeff (1954)	Polarography
(9)	Strength, Ringler & Nelson (1954)	Spectrophotometric, with dehydrogenase and diaphorase
(10)	Holzer, Goldschmidt, Lamprecht & Helmreich (1954)	Spectrophotometric: oxidation or reduction using alcohol dehydrogenase

Nucleotide coenzyme concentration in adult rat liver

	Extraction procedure	No. of animals	Age (body wt. in parenthesis)	Sex
(1)	Tissue frozen at -80°, ground and extracted with water for 2 min. at 100°	6		
(2)	cf. Myrback (1933)	8		
(3)	Tissue ground with trichloroacetic acid and ferricyanide	15		
(4)	Tissue extracted in water, 3 min. at 100°; homogenized, centrifuged; residue re-extracted	15	adult	
(5)	Tissue homogenized in 2% nicotinamide: coenzymes oxidized with $Ce(SO_4)_2$	105	(100-250g.)	male & female
(6)	Tissue homogenized in water (100°/3 min.): cooled, adjusted to pH 6.5	-	-	-
(7)	Tissue frozen (acetone/dry ice, -80°); homogenized in 2% trichloroacetic acid/5% H_2O_2: absorbed on charcoal, eluted with 10% aqueous pyridine	10		male
(8)	As above: use of 4% metaphosphoric acid for extraction	-		
(9)	Tissue homogenized in 0.5M phosphate -0.5% nicotinamide pH 7.4); trichloroacetic acid added	16 16 8		male male male
(10)	Tissue homogenized in 5% trichloroacetic acid (15°) or in N NaOH (100°)	8		

(See following page for details)

Table 5 (continued)

Coenzyme content (µm-moles/g. fresh wt.)

Values given are means ± S.E.M. Range given in parenthesis

	[NAD]	[NADH$_2$]	[NAD] + [NADH$_2$]	[NADP]	[NADPH$_2$]	[NADP] + [NADPH$_2$]	Total
(1)	-	-	1670	-	-	-	-
(2)	-	-	2085	-	-	-	-
(3)	-	-	-	-	-	-	813*
(4)	-	-	1575	-	-	-	-
(5)	-	-	-	-	-	-	1330* (735 to 1935)
(6)	-	-	952	-	-	-	-
(7)	-	-	-	-	-	-	1236*
(8)	-	-	-	-	-	-	823*
(9)	1230 1255 1216	(1068 to 1390) (1141 to 1389) (1029 to 1350)					
(10)	475	180	655	-	-	-	-

* expressed as NAD

Table 5 (continued)

	Reference	Assay method
(11)	Strength, Mondy & Daniel (1954)	Lactate dehydrogenase-diaphorase: methylene blue reduction.
(12)	Spirtes & Eichel (1954)	Alcohol dehydrogenase: ethanol or acetaldehyde, spectrophotometry.
(13)	Greengard, Brink & Colowick (1954)	Methyl ethyl ketone condensation/fluorimetry. (NADase and lactate dehydrogenase).
(14)	Jedeikin & Weinhouse (1955)	Alcohol dehydrogenase with ethanol or acetaldehyde: spectrophotometry.
(15)	Glock & McLean (1955b)	$NADH_2$ or $NADPH_2$-cytochrome c-reductase/cytochrome c; spectrophotometry.
(16)	Glock & McLean (1955c)	As above.
(17)	Glock & McLean (1956)	As above
(18)	Glock & McLean (1957)	As above.
(19)	Lowry, Roberts & Kapphahn (1957).	Alcohol dehydrogenase and glucose 6-phosphate dehydrogenase with fluorimetry (8N-NaOH).
(20)	Greenbaum & Graymore (1956)	cf. Spirtes & Eichel (1954).

Table 5 (continued)

	Extraction procedure	No. of animals	Age	Sex
(11)	Tissue homogenized in 0.1M-phosphate -0.1% nicotinamide, pH 7.8 then heated (85°/3 min.) cooled and rehomogenised.	4	19 months	female
(12)	Tissue homogenized (100°/1.5 min.) in phosphate-bicarbonate, pH 7.4	8		male
(13)	Tissue heated (100°/15 sec.) in 0.1 M tris, pH 8.2	-		
(14)	Tissue heated (100°/1 min.) then homogenized 30 sec. in 0.05M-phosphate at either pH 5.4 or 8.7	6 8		
(15)	Tissue in 0.1N-HCl or 0.1N-NaOH; heated (30 sec./100°, homogenized 1.5 min; cooled.	6		
(16)	As above.	5	young adult	
(17)	As above	5	adult	female
(18)	As above	11 23	adult	female
(19)	Tissue heated in 0.05M-tris buffer, pH 8.2, (5 min./85°); homogenized, centrifuged 12 000g, 1 hr; portions treated with acid and alkali	-	adult	
(20)	cf. Spirtes & Eichel (1954)	6	4 months (170-200g.)	female

(see following page for details)

Table 5 (continued)

Coenzyme content (μm-moles/g. fresh wt.)

Values given are means ± S.E.M. Range given in parenthesis

	[NAD]	[$NADH_2$]	[NAD] + [$NADH_2$]	[NADP]	[$NADPH_2$]	[NADP] + [$NADPH_2$]	Total
(11)	822 (732 to 870)						
(12)	777 (732 to 828)	517 (381 to 627)	1294 (1186 to 1368)				
(13)	492	442	934				
(14)	559 (507 to 616)	472 (381 to 558)	1031				
(15)	555±19	306±13	861	8±1	266±8	274	1135
(16)	610±19	241±25	851	13±1	192±4	205	1056
(17)	495±13	199±12	694	13±1	240±21	253	947
	433±25	204±18	637	4±1	251±13	255	892
(18)	597±15	273±12	870	9±1	282±6	291	1161
(19)	542	218	760	71	63	134	894
(20)	600±24	320±18	920±38	-	-	-	-

Table 5 (continued)

	Reference	Assay method
(21)	Jacobson & Kaplan (1957b)	Fluorescence of methyl ethyl ketone complex; specific enzymic oxidation or reduction of coenzymes. (See Jacobson & Astrachan, 1957).
(22)	Holzer, Busch & Kroger (1958).	Specific enzymic oxidation or reduction of coenzymes: spectrophotometry.
(23)	Bucher & Klingenberg (1958)	(cf. Jedeikin & Weinhouse, 1955)
(24)	Bassham, Birt, Hems & Loening (1959)	Modification of Lowry, Roberts & Kapphahn (1957).
(25)	Lowry, Passonneau, Schulz & Rock (1961)	Enzymic cycling followed by fluorimetry (Lowry *et al.* 1957).
(26)	Klein, Mandel & Mandel (1961)	Methods of Holzer, Busch & Kroger (1958).
(27)	Slater & Sawyer (1962)	Coupled reduction of 2, 6-dichlorophenol-indophenol (Villee, 1962).
(28)	Christie & LePage (1962)	Methods of Bassham *et al.* (1959).
(29)	Caiger, Morton, Filsell & Jarrett (1962)	Specific enzymic oxidation or reduction of coenzymes: spectrophotometry. For details see text.

Table 5 (continued)

	Extraction procedure	No. of animals	Age	Sex
(21)	Tissue extracted by homogenizing either in 5% trichloroacetic acid at 15° or in 0.1M-Na_2CO_3 (pH 10) at 100° for 2.5 min.	-	adult	
(22)	Tissue samples extracted with either N-NaOH or 6% $HClO_4$; brought to pH 7.4: centrifuged	2	adult (70-180 g.)	
(23)	(cf. Jedeikin & Weinhouse, 1955)	-		
(24)	Extraction as for Glock & McLean (1955) Extracts frozen -190°	8		
(25)	Tissue homogenized in 0.02N H_2SO_4-0.1M-Na_2SO_4 or 0.02N-NaOH - 0.5mM-cysteine. Extracts heated (60°, acid sample 45 min., alkaline sample 10 min.)	4	5 months	male
(26)	(cf. Holzer, Busch & Kroger, 1958)	8	adult	
(27)	cf. Villee, 1962)	From Patterson (1963)		
(28)	homogenate (cf. Bassham et al. 1959).	19	adult (270-330 g.)	male
(29)	Tissue extracted by homogenizing for 2 min. in either 0.5M.-trichloroacetic acid at 0° or in 0.1M-Na_2CO_3 at 100°	4	7 months (520-623 g.)	male

Table 5 (continued)

Coenzyme content (μm-moles/g. fresh wt.)

Values given are means ± S.E.M. Range given in parenthesis

	[NAD]	[NADH$_2$]	[NAD] + [NADH$_2$]	[NADP]	[NADPH$_2$]	[NADP] + [NADPH$_2$]	Total
(21)	446	166	612	44	425	469	1081
(22)	765	288	1053	9	295	304	1357
(23)	622	638	1260	-	-	-	-
(24)	485±33	153±24	638	25±6	251±24	276	912±62
(25)	628 (575 to 674)	252 (230 to 279)	880 (805 to 953)	115 (108 to 128)	502 (401 to 567)	617 (513 to 695)	1497 (1391 to 1648)
(26)	637	240	877	-	-	266	1143
(27)	600	127	727	60	267	327	1054
(28)	738	238	976	141	447	588	1564
(29)	424 (328 to 500)	108 (80 to 162)	532 (408 to 597)	29 (25 to 37)	380 (276 to 487)	409 (301 to 524)	941 (709 to 1117)

Table 5 (continued)

	Reference	Assay method
(30)	Hannan & Rosenthal (1963)	Spectrophotometric: (cf. Ciotti & Kaplan, 1957).
(31)	Burch, Lowry & Von Dippe (1963).	(cf. Lowry *et al*. 1961)
(32)	Spirtes & Milstein (1963)	Spectrophotometric: (cf. Spirtes & Eichel, 1954).
(33)	Lindall & Lazarow (1964)	Fluorimetric: cf. Bassham *et al*. (1959).

Table 5 (continued)

	Extraction procedure	No. of animals	Age	Sex
(30)	Tissue frozen: homogenized in 2% $HClO_4$ (0°) or 0.1M-Na_2CO_3 (100°) for 2 min: neutralised to pH 7.0	7	adult (300-350g.)	female
(31)	Tissue frozen *in situ* with freon at -150°: extraction as for Lowry *et al.* (1961).	4		
(32)	Tissue extracted in 0.1M-potassium phosphate, pH 7.4 at 100°.	12	(150-250g.)	male
(33)	cf. Bassham *et al.* (1959)	8	young	male

(see following page for details)

Table 5 (continued)

Coenzyme content (µm-moles/g. fresh wt.)

Values given are means ± S.E.M. Range given in parenthesis

	[NAD]	[NADH$_2$]	[NAD] + [NADH$_2$]	[NADP]	[NADPH$_2$]	[NADP] + [NADPH$_2$]	Total
(30)	476±42	13±3	489±45	7±1	145±26	152±27	641
(31)	741	41	782	374	386	760	1542
(32)	663±24	438±18	1001±28	-	-	-	-
(33)	490±48	208±19	698	46±6	578±51	624	1322

Experimental

Animals. Merino ewes, age 2-4 years, that had been grazing on an improved mixed pasture, were used.

Albino rats, about 7 months old (300-600 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%), NaCl (1%). Each ton of the cubes contained a supplement of vitamins A (8×10^6 international units), D_3 (2×10^6 international units), E (2,500 international units), 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 were removed from their source of food about 2 hr. and the rats about 30 min. before slaughter.

Since most rats in animal houses are infected with a virus which causes respiratory disease (see Godwin, 1964), the animals used in this study were raised from disease-free parents. Many of the rats used were examined by Dr. K. O. Godwin (C.S.I.R.O., Division of Biochemistry and General Nutrition, Adelaide), for freedom from disease. The lungs were fixed in formol-saline and examined histologically. In no case was there evidence of infection.

Sampling procedure. The animals were killed by severing the neck and cervical cord and the livers were removed within 30 sec. With sheep, a number of slices (approx. 10 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 (4 mm. cubes) and thoroughly mixing the chopped materials. Weighed portions were then taken.

For frozen tissue, the liver sample was placed in a polythene bag which was immersed in liquid nitrogen. The time taken from the death of the animal to the tissue reaching -190° was about 2 min. The frozen liver was crushed between stainless steel plates at -15°. The tissue sample (approx. 2 g.) was weighed in a polythene scoop on a balance in a room at -15° and then transferred to the homogenizing vessel which contained the extraction medium.

Extraction and assay methods used in preliminary investigations of coenzyme levels.

(1) Extraction with hot tris-maleate buffer, pH 8.4. Approx. 2 g. of tissue was homogenized in 15 ml. of hot tris-maleate buffer (0.2 M, pH 8.4) by alternately heating for 30 sec. in a boiling water bath and disintegrating in a power-driven homogenizer at 2 000 r.p.m. The total time of heating and homogenizing was 2 min. The tube was chilled in ice and the volume of the contents was made up to 25 ml. with cold 0.2 M-tris buffer (pH 7.4). The homogenate was shaken with 3 ml. of paraffin oil and centrifuged for 30 min. at 50 000 g. at 2°. The clear aqueous layer was removed with a hypodermic syringe and used for assays.

For NAD, each cuvette (2 cm. light-path) contained 2 ml. of

tissue extract and 0.8 ml. of a solution containing 25 mg. of glycine, 14 mg. of NaOH and 0.04 ml. of ethanol; the final pH was about 9.5. The coenzyme was reduced on addition of 0.025 ml. of alcohol-dehydrogenase solution.

For NADP, each cuvette (2cm. light-path) contained 1 ml. of tissue extract, 1.5 ml. of 0.2 M-tris buffer, pH 7.4, 0.1 ml. of 0.1 M-DL-isocitrate and 0.1 ml. of 0.1 M-$MgCl_2$; the final pH was about 7.5. The coenzyme was reduced on addition of 0.02 ml. of isocitrate-dehydrogenase solution.

For $NADH_2$, each cuvette (2 cm. light-path) contained 2 ml. of tissue extract, 0.5 ml. of 0.2 M-tris buffer, pH 7.4 and 0.1 ml. of 50 mM-dihydroxyacetone phosphate; the final pH was about 7.4. The coenzyme was oxidized on addition of 0.025 ml. of glycerol-phosphate-dehydrogenase solution.

For $NADPH_2$, each cuvette (2 cm. light-path) contained 2 ml. of tissue extract, 0.5 ml. of 0.2 M-tris buffer, pH 7.4 and 0.1 ml. of 0.1 M-oxidized glutathione. The coenzyme was oxidized on addition of 0.025 ml. of glutathione-reductase solution.

(ii) Extraction with cold 0.5 M-trichloroacetic acid. Approx. 1 g. of tissue was weighed and homogenized at 2 000 rev./min. for 2 min. in 15 ml. of ice-cold 0.5 M-trichloroacetic acid with a Potter-Elvehjem glass homogenizer and teflon pestle (A. H. Thomas & Co., Baltimore, U.S.A.). The homogenizer was rinsed with 5 ml. of 0.5 M-trichloroacetic acid and the volume of the combined suspension was measured. The clear extract obtained by centrifuging at 2 500 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 on 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 0.3 M-tris, 0.1 ml. of 100 mM-DL-isocitrate and 0.1 ml. of 0.1 M-$MgCl_2$; the final pH was 7.5. The coenzyme was reduced on addition of 0.02 ml. of isocitrate-dehydrogenase solution.

(iii) Extraction with hot NaOH solution. The procedure was the same as in (i) above, except that 0.1N-NaOH was used and the pH was adjusted to 7.4 with 1 M-maleic acid before centrifuging.

(iv) Extraction with hot trichloroacetic acid. The extraction and assays were carried out as in (ii) above, with the heating procedure as in (i).

(v) Extraction with hot Na_2CO_3 solution. Approx. 1g. of tissue was weighed and homogenized at 2 000 rev./min. for 2 min. in 15 ml. of 0.1 M-Na_2CO_3 in a Potter-Elvehjem homogenizer in a bath of boiling water. The homogenizer containing the carbonate solution was pre-heated 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 the oxidized coenzymes (see (ii) above).

The extract was immediately poured into a polythene bottle in liquid nitrogen, the homogenizer was rinsed with 5 ml. of 0.1 M-Na_2CO_3, and the rinsings were combined with the homogenate. The extract was thawed, adjusted to pH 7.5 with 1.0 M-maleic acid (approx. 0.6 - 1.0 ml.) and the volume was 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 60 000 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 0.2 M-tris-HCl buffer (pH 7.5) and 0.025 ml. of 0.5 M-acetaldehyde. The coenzyme was oxidized on 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 0.2 M-tris-HCl buffer (pH 7.5) and 0.2 ml. of 0.1 M-oxidized glutathione. The coenzyme was oxidized on addition of 0.02 ml. of glutathione-reductase solution.

All assays were carried out at 25° in duplicate or triplicate. Extinctions were read against air, and when the first extinction change resulting from oxidation or reduction of the coenzyme was complete, a second portion of enzyme was added and the corresponding change in extinction (due to the extinction of the solution of enzyme) was subtracted from the first change.

Results and Discussion

As shown in the Experimental section, accurate and specific assays of the four nicotinamide nucleotide coenzymes were possible by direct spectrophotometry with ethanol-alcohol dehydrogenase for NAD, acetaldehyde-alcohol dehydrogenase for $NADH_2$, isocitrate-isocitrate dehydrogenase for NADP and oxidized glutathione-glutathione reductase for $NADPH_2$.

Although fluorimetric methods and coupled assays based on rates are reproducible when used in conjunction with suitable controls, the spectrophotometric assays used in the present investigations are more direct and rapid. With a photometric accuracy of 0.002 the smallest amount of coenzyme that could be measured with 10% error in 5 ml. of solution in a 4 cm. cuvette is 4 µm-mole. In the procedure described here this is derived from 0.1 g., so that a coenzyme concentration of 40 µm-moles/g. fresh wt. of tissue can be measured with this photometric accuracy.

While satisfactory assays were available for the coenzymes when they had been obtained in solution, the preparation of extracts that provide a true measure of the coenzyme content of tissues is much more difficult. Although many studies of coenzyme concentrations in tissue have been described, few authors have compared the results obtained when different methods of extraction are applied to the same tissue. Table 6 lists some studies of this kind. One of

the main difficulties is the absence of uniform material for analysis. There is considerable variation of coenzyme concentrations in the same organ (e.g. rat liver) in a group of animals handled as far as possible in identical conditions (cf. Table 5, reference nos. (15)-(18), (25), (29)).

A major difficulty is to ensure reproducible treatment of the tissue samples during homogenization at 100°. In the earlier experiments (Table 7 (a) to (j); Table 8), the homogenizer vessel containing the extracting medium and the pestle was heated in a boiling-water bath for 10 min., the tissue sample introduced, the contents dispersed using the power-driven pestle and the homogenizer and contents reheated in the water bath. In experiments (k) of Table 7, and in the studies of coenzymes during growth (Section II), an electrically-heated water bath, fitted with a perspex and rubber collar was used (see Plate 1). This enabled the homogenization to be carried out while the tissue sample was being heated in boiling water.

Also, in these later studies, instead of using an ice-water bath to cool the tissue sample and extracting medium after homogenizing, the contents of the homogenizer were frozen by pouring into a polythene bottle which was immersed in liquid nitrogen. These procedures enabled a more exact control of the conditions of heating, extracting and cooling the sample.

It was considered that there could be advantages in obtaining a single extract which would contain both the oxidized and reduced

Plate 1

Power-driven homogenizer with teflon pestle in an electrically-heated water bath fitted with a rubber and perspex collar.

coenzymes. Since these coenzymes have been shown to be more stable when heated in tris buffer than in phosphate (Colowick, Kaplan & Ciotti, 1951), 0.2 M-tris-maleate buffer, pH 8.4, was used. The variability in the values obtained when using this extraction method on fresh and frozen rat-liver tissue and compared with cold and hot trichloroacetic acid and hot NaOH (see Table 7, (a) to (d)) was largely due to difficulties in controlling extraction conditions at 100°; rapid inactivation of dehydrogenases is essential and this is more easily effected in acid or alkali.

Due to difficulties in obtaining disease-free rats at this stage, chickens were used as a readily available source of liver tissue. As is seen in Table 7 (f) and (j), consistent results could be obtained with the hot tris-maleate extraction but in a number of instances no $NADPH_2$ was found in the extract. In each case the enzyme system was active and the assay of $NADPH_2$ added to the extract was satisfactory; again, oxidation by dehydrogenases in the initial stages of extraction is a probable source of error.

Since it was intended to use these extraction methods in a study of coenzymes in sheep liver during growth, these comparisons were repeated using adult-sheep liver (Table 7, (h) to (j).
In order to study changes in coenzyme content of the tissue resulting from storage of the samples at room temperature the tris-maleate extraction was repeated at various times from the death of the animal. By adhering to a strict schedule for handling the tissue and extractions, a more consistent series of results was

Table 6. <u>Comparison of methods of extraction of nicotinamide nucleotide coenzymes from adult-rat liver</u>

Reference & extraction medium	Coenzyme content (μm-moles/g.fresh wt.; mean values ± S.E.M.)					
	[NAD]	[$NADH_2$]	[NAD] + [$NADH_2$]	[NADP]	[$NADPH_2$]	[NADP] + [$NADPH_2$]
(a) Glock & McLean (1955a)						
Acid extract	546±19	-	846	-	-	-
Alkaline extract	-	300±16		-	-	-
Phosphate-carbonate buffer, pH 7.4	579±21	508±27	1087	-	-	-
(b) Basham *et al.*, (1959)						
Acid extract	485	-	787	103	-	387
Alkaline extract	-	302		-	284	
0.1M-tris-HCl buffer, pH 8.2	445	215	660	215	114	329
			([NAD] + [NADP])		([$NADH_2$] + [$NADPH_2$])	
(c) Lindall & Lazarow (1964)						
Acid extract			588		-	
Alkaline extract			-		497	
0.05M-tris buffer, pH 8.2			537		789	

Table 7. Comparison of methods for extracting nicotinamide nucleotide coenzymes from liver tissue

For details of extraction and assay methods, see pp. 55-60.

Coenzyme content, mean value (μm-moles/g. fresh wt.).

(a) Nicotinamide-adenine dinucleotide in rat liver after freezing: Extraction with cold 0.5M-trichloroacetic acid:

	[NAD]	
	Mean	Range of values
Male rats	665	(609, 670, 608, 565, 773)
Female rats	538	(521, 520, 489, 535, 545, 603)

(b) Fresh and frozen rat liver tissue: extraction with hot tris-maleate.

	[NAD]	[$NADH_2$]	[NADP]	[$NADPH_2$]
Fresh	480	73	109	120
Frozen	294	25	198	35

(c) Fresh rat liver tissue: hot trichloroacetic acid extraction.

Time from death of animal	[NAD]		[NADP]	
4 min.	258	-	32	-
10 min.	310	-	60	-
15 min	292	-	18	-

Table 7 (continued)

(d) Fresh rat-liver tissue: hot NaOH extraction

Time from death of animal	[NAD]	[$NADH_2$]	[NADP]	[$NADPH_2$]
5½ min.	-	88	-	232
9½ min.	-	95	-	268
15 min.	-	.60	-	275

(e) Hot trichloroacetic acid extraction : chicken and chicken-embryo liver:

	[NAD] Mean	[NAD] Range of values
Mean of two groups of livers from 8 embryos (3 days before hatching)	930	(860, 1000)
Mean of 4 birds; (6 weeks old)	420	(350, 430, 450, 450)

(f) Comparison of hot tris-maleate and cold trichloroacetic acid extraction: 12 week-old cockerel

Extraction	[NAD]	[$NADH_2$]	[NAD] + [$NADH_2$]	[NADP]	[$NADPH_2$]	[NADP] + [$NADPH_2$]	Total
tris-maleate	395	128	523	26	156	182	705
TCA	406	-	-	102	-	-	
tris maleate	321	194	515	45	140	185	700
TCA	436	-	-	139	-	-	-

Table 7 (continued)

(g) Hot tris-maleate extraction: 11 week-old cockerel

	[NAD]	[$NADH_2$]	[NAD] + [$NADH_2$]	[NADP]	[$NADPH_2$]	[NADP] + [$NADPH_2$]	Total
(1)	331	181	512	34	118	152	664
(2)	356	153	509	42	104	146	655

(h) Tris-maleate and hot trichloroacetic acid extraction:

Ewe: A8-560, 2 years old.

Extraction	Time from death	[NAD]	[$NADH_2$]	[NAD]+ [$NADH_2$]	[NADP]	[$NADPH_2$]	[NADP]+ [$NADPH_2$]	Total
tris-maleate	5 min.	660	125	785	257	215	472	1257
hot TCA	9 min.	640	-	-	164	-	-	-
tris-maleate	15 min.	576	116	692	288	146	434	1126

(i) Tris maleate, hot trichloroacetic acid and hot NaOH extraction: ewe: A7-234, 3 years old: fresh tissue

Extraction	Time from death	[NAD]	[$NADH_2$]	[NAD]+ [$NADH_2$]	[NADP]	[$NADPH_2$]	[NADP]+ [$NADPH_2$]	Total
tris-maleate	5½ min.	656	180	836	240	198	438	1274
hot NaOH	9 min.	-	40	-	-	308	-	
tris-maleate	11 3/4 min.	664	125	789	312	183	495	1289
hot TCA	14½ min.	610	-	-	150	-	-	

(alkaline + acid extractions ,1108)

Table 7 (continued)

(j) A7-234, frozen tissue: 12 hr. after death of animal

Extraction	[NAD]	[$NADH_2$]	[NAD] + [$NADH_2$]	[NADP]	[$NADPH_2$]	[NADP] + [$NADPH_2$]	Total
tris-maleate	400	144	544	342	92	437	981
hot NaCH	-	70	-	-	171	-	1095
hot TCA	622	-	-	232	-	-	
cold TCA	790	-	-	345	-	-	(1135)

Table 7 (continued)

(k) Fresh and frozen adult-rat liver; extraction using cold trichloroacetic acid and hot Na_2CO_3

Coenzyme content (μm-mole/g. fresh wt.)

	[NAD]	[$NADH_2$]	[NAD] + [$NADH_2$]	[NADP]	[$NADPH_2$]	[NADP] + [$NADPH_2$]	Total
A, fresh	555	150	705	83	258	334	1039
A, frozen	-	-	-	-	-	-	-
B, fresh	673	124	797	105	193	298	1095
B, frozen	740	135	875	131	288	429	1304
C, fresh	655	150	805	204	234	438	1243
C, frozen	645	116	761	115	166	281	1042
D, fresh	566	160	726	120	238	358	1084
D, frozen	600	134	734	160	210	370	1004
E, fresh	583	192	775	120	255	375	1150
E, frozen	575	153	728	135	205	340	1068
Mean and range (B to E)							
fresh	620	156	776	137	230	367	1143
	(566 to 673)	(124 to 192)	(726 to 805)	(105 to 204)	(193 to 255)	(298 to 438)	(1084 to 1243)
frozen	640	135	775	135	217	352	1127
	(575 to 740)	(116 to 153)	(728 to 875)	(115 to 160)	(205 to 288)	(281 to 429)	(1004 to 1304)

obtained. From Table 7 (h) to (j), it is seen that the extract with hot tris-maleate buffer contains more NADP than that with hot trichloroacetic acid but that cold trichloroacetic acid is as effective in extracting NADP as is the tris-maleate buffer. The $NADH_2$ content of the hot NaOH extract was less than that in the tris-maleate buffer.

The extraction with hot tris-maleate buffer was used for a series of animals during September, 1960. These results are given in Table 8. In several experiments, the extracts appeared to be unstable and increase in extinction at 340 mμ in the control extract made accurate assay difficult.

Lowry, Roberts & Kapphahn (1957) used 0.05 M-tris buffer, pH 8.2, for extraction of these coenzymes from rat-liver tissue (see Table 5) and reported very low values for $NADPH_2$. Bassham et al., (1959) and Lindall & Lazarow (1964) have compared this extraction medium with separate acid and alkaline extractions (see Table 6 (b) & (c)) and also found a low $NADPH_2$ content. This was due to oxidation of the $NADPH_2$ in the extract, giving rise to high values for NADP.

From these results and from the data in Table 7 it is clear that spurious values could be obtained with the single-extraction procedure. It therefore was decided to use a double-extraction procedure, similar to that of Jacobson & Kaplan (1957); cold 0.5 M-trichloroacetic acid was used for NAD and NADP and hot 0.1 M-Na_2CO_3 for $NADH_2$ and $NADPH_2$. The two extractions were carried out simultaneously on separate samples of the tissue. The use of

sodium carbonate solution gave a less-coloured extract, more suitable for spectrophotometry, than one obtained with sodium hydroxide. Values obtained for adult-rat liver, using this two-extraction method, are of the same order as those obtained in recent studies (see Table 5).

When studies of coenzymes and growth had been completed, reports of the work of Lowry and co-workers (1961, 1963) suggested that it was necessary to freeze rat-liver tissue rapidly after death of the animal in order to obtain values for coenzyme content which will reflect the *in vivo* levels. A further group of adult rats was taken and the two-extraction procedure carried out on fresh and frozen samples from the same tissue. The results in Table 7 (k) show that under the extraction conditions used here, there was no significant difference found between fresh and rapidly frozen rat-liver tissue.

Klingenberg & Bucher (1960), in discussing the nicotinamide nucleotide coenzyme content of livers and mitochondria, commented that "the extraction and enzymatic analytical determination of pyridine nucleotides from tissues apparently still has some pitfalls." Although this is so, many of the discrepancies would appear to be due to insufficient attention being given to factors which are easily controlled and also to inadequate parameters being used as the basis for comparison. It is clear that in comparison of results obtained by different workers for any one tissue, such factors as age and sex of animal, diet, freedom from disease, differences between strains or varieties, physiological state of the tissue

Table 8 Nicotinamide nucleotide coenzymes in the livers of lambs during growth (Series I): hot tris-maleate buffer extraction

Coenzyme content. (μm-moles/g. fresh wt.)

Age.	[NAD]	[$NADH_2$]	[NAD] + [$NADH_2$]	[NADP]	[$NADPH_2$]	[NADP] + [$NADPH_2$]	Total
Prenatal	906	33	939	45	167	212	1151
	625	16	641	83	94	177	818
	405	45	450	21	96	117	567
	666	35	701	73	133	206	907
Newborn	686	25	711	41	132	173	884
	696	54	750	41	146	187	937
Postnatal (days)	461	35	496	106	91	197	693
1	424	21	445	113	108	221	666
2	402	50	452	38	131	169	621
4	728	27	755	138	194	332	1087
	401	20	421	81	149	230	651
8	441	28	469	149	169	318	787
9	391	84	475	197	45	242	717
	567	35	602	162	140	302	904
20	445	40	485	104	182	286	771
22	510	30	540	186	171	357	897
(years) 2	660	125	785	239	235	474	1257
3	656	180	836	257	215	472	1274

immediately before sampling, method of killing animals, sampling procedure, extraction procedure, stability of the extract and method of coenzyme assay, will all affect the final result. Normal animal variations must be taken into consideration and sufficient data ought to be obtained so as to allow statistical evaluation of the significance of the variation. It is not always clear how an author has calculated a "standard error" and data would be of considerably more use if all the values found for concentrations were given, or at least the range of values stated. If experimental details of this sort were available, then many of the apparent conflicts in the literature on coenzyme levels would probably be resolved.

SECTION II

Nicotinamide Nucleotide Coenzyme Concentrations and Growth of the Liver in Sheep and Rats

Introduction

In recent studies of the interaction of the cell nucleus with the cytoplasm the aspect of genetical control of growth and metabolism that has received most attention is the transfer of information from deoxyribonucleic acid to sites of protein synthesis as ribonucleic acid with a specific sequence of nucleotides. However, the rates of reactions in the cell depend not only on the enzymes present, but also on the concentrations of coenzymes and other essential cofactors. The possibility that enzymes in the nucleus might control the concentrations of coenzymes in the cytoplasm is therefore of great interest in the interpretation of the control of growth and differentiation.

The presence in the nucleus of one of the essential enzymes of the metabolic pathway leading to nicotinamide nucleotide coenzymes is an unusual example of a nuclear enzyme catalysing one reaction in a sequence that is otherwise apparently localised in the cytoplasm. This led Morton to suggest (1958, 1961) that the nuclear adenylyl-transferase that catalyses formation of nicotinic acid-adenine dinucleotide from nicotinic acid nucleotide and adenosine triphosphate might limit the supply of NAD and its derivatives and thus control the rate of growth and cell division.

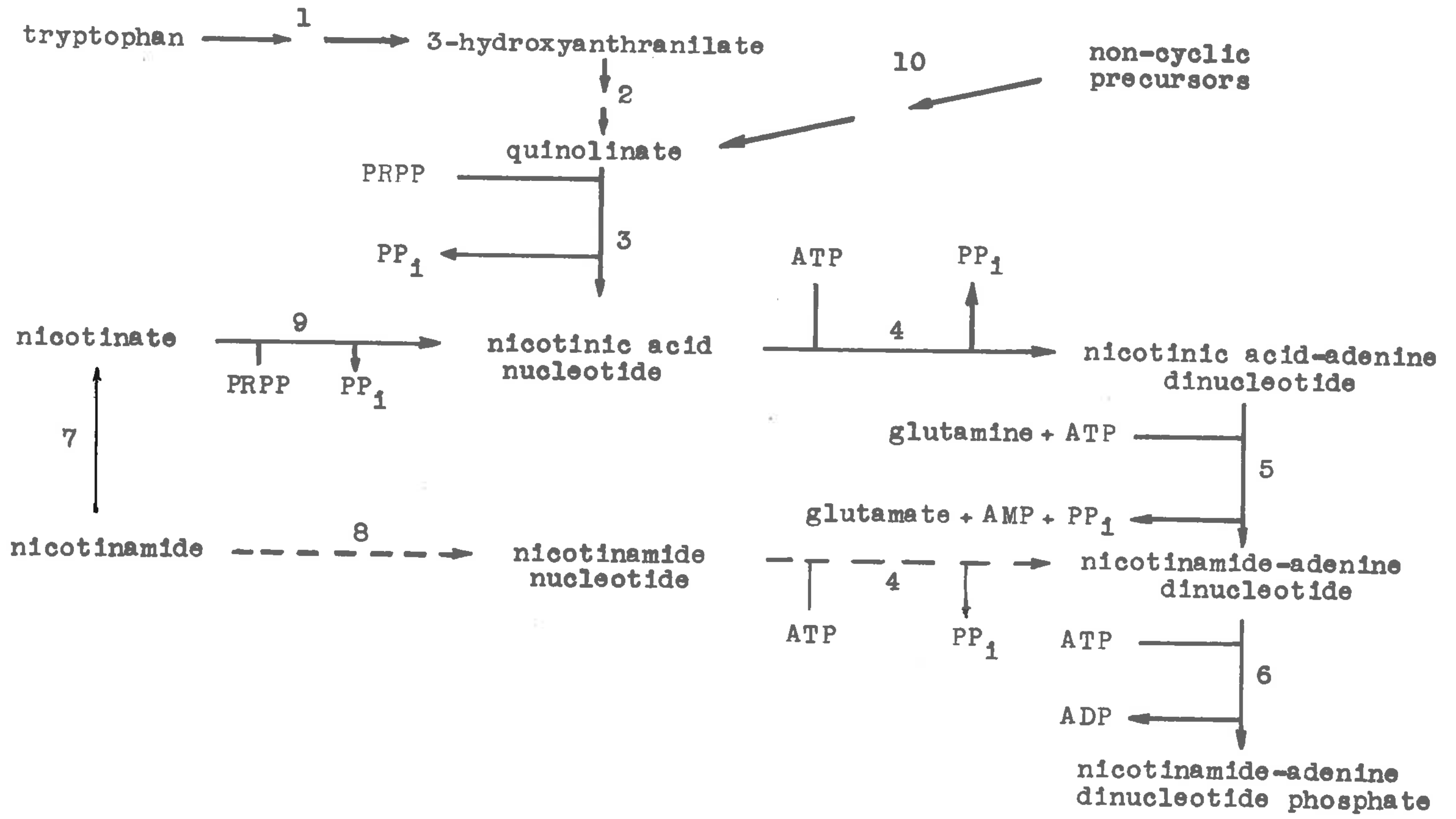
As discussed in the general introduction (p.1) and as shown in more detail in Figure 2, animal tissues are known to contain pathways leading to NAD and NADP from tryptophan, nicotinic acid and nicotinamide. In some respects the first of these pathways, through hydroxyanthranilate and quinolinate, is analogous to the '*de novo*'pathways of biosynthesis of purine and pyrimidine nucleotides; this analogy is even more marked in the case of plants and micro-organisms that make quinolinate from precursors other than tryptophan (Gholson *et al*. 1964). Conversely, the pathways from nicotinamide and nicotinate resemble the 'salvage' pathways of purine and pyrimidine nucleotide biosynthesis. Kornberg (1957) has pointed out the significance of these direct syntheses of purine and pyrimidine nucleotides by phosphoribosyl transfer from 5-phosphoribosyl-pyrophosphate to free purines and pyrimidines that have been liberated by the breakdown of nucleic acids and nucleotides.

It is not possible at present to state which are the rate-limiting steps of biosynthesis of NAD and NADP in animal tissues under physiological conditions; this is particularly true of tumour tissues and tissues in stages of rapid development. In normal adult liver the demonstration that there is little accumulation of [^{14}C]-nicotinic acid nucleotide during conversion of [^{14}C]-nicotinate into NAD is no evidence that the conversion of nicotinic acid nucleotide into its adenylyl derivative is not rate limiting in the overall conversion of tryptophan, nicotinate and nicotinamide into NAD (see Preiss & Handler, 1958*a*).

Figure 2

Pathways for synthesis of nicotinamide nucleotide coenzymes in animal tissues

1. See Mitchell & Nyc (1948)
2. 3-Hydroxanthranilic oxidase (Decker *et al.* 1961)
3. See Nishizuka & Hayaisha (1963),
4. ATP : NMN-adenylyltransferase
5. NAD synthetase
6. NAD kinase
7. Nicotinamide deamidase
8. NMN pyrophosphorylase
9. Nicotinic acid mononucleotide pyrophosphorylase
10. See Ortega & Brown (1960)



tryptophan
1
3-hydroxyanthranilate
2
10
non-cyclic
precursors
quinolinate
PRPP
3
PPi
ATP
PPi
nicotinate
9
PRPP
PPi
nicotinic acid
nucleotide
4
nicotinic acid-adenine
dinucleotide
7
glutamine + ATP
5
glutamate + AMP + PPi
nicotinamide
8
nicotinamide
nucleotide
4
ATP
PPi
nicotinamide-adenine
dinucleotide
ATP
6
ADP
nicotinamide-adenine
dinucleotide phosphate

The pathways of biosynthesis of NAD and NADP afford many potential sites of metabolic control. Conversion of nicotinamide into nicotinate by nicotinamidase is inhibited in liver, and this inhibition is under hormonal control (Greengard, Petrack, Craston & Kalinsky, 1963). Conversion of quinolinate into nicotinic acid nucleotide in crude extracts of liver is inhibited by unidentified material that may be adsorbed on charcoal and is also inhibited by NAD or its breakdown products in this system (Gholson et al., 1964). This raises the possibility of 'feed back' control of biosynthesis of NAD.

A further possibility of control of the formation of NAD arises from the separation of the nuclear transferase, which requires a supply of ATP for formation of nicotinic acid-adenine dinucleotide, from the main sources of ATP in the cytoplasm. Atkinson, Jackson & Morton (1964) were only able to extract this enzyme from disrupted nuclei in conditions that extracted nucleic acid, and the enzyme may be bound at a specific site in the nucleus. Baltus (1954) found the enzyme in the nucleolar fraction of starfish oocytes. Formation of nucleic acids in the nucleus also requires a supply of ATP and the inverse relationship of NAD biosynthesis to rate of cell division and synthesis of nucleic acid might result from competition for ATP. Atkinson, Jackson & Morton (1961) found that the nuclear transferase also catalysed nucleotidyl transfer from guanosine triphosphate and deoxyadenosine triphosphate; competition for these precursors of nucleic acids by the nuclear

transferase might also contribute to the interaction of pyridine nucleotide metabolism and nucleic acid metabolism (cf. Morton, 1961; Revel & Mandel, 1962; Chambon, Weill & Mandel, 1963).

The activity of ATP; NMN adenylyltransferase in tumours and embryonic tissues is lower than that in normal adult tissue. The contrast is particularly marked when the activity is expressed on a 'per nucleus' basis. Some results reported by Branster & Morton (1956) and by Branster (1958) are listed in Table 9. Similar results by Dawkins (1959) were discussed in the general introduction. Stirpe & Aldridge (1961) found lower activities of the adenylyltransferase in nuclei from regenerating rat liver than in normal adult liver. de Burgh (1957) had shown that regenerating mouse liver had a decreased capacity to cause a net increase in the level of NAD on injection of nicotinamide.

In Table 10, the levels of nicotinamide nucleotide coenzymes in several normal adult and tumour tissues are compared.

It has been known since the work of von Euler (1938) and Bernheim & Felsovanyi (1940) that low levels of nicotinamide nucleotide coenzymes are present in tumour and embryonic tissues. A comparison of Sections A & B of Table 10, shows that when expressed on a fresh wt. basis, the coenzyme content of tumours is much lower than that of the corresponding normal adult tissue, and this is especially marked with levels of NADP and $NADPH_2$. This decrease in coenzyme level is paralleled by a decrease in the activity of NMN-adenylyltransferase (compare Table 9).

Table 9. Comparative values of ATP: NMN-adenylyltransferase activities of cell nuclei in normal and tumour tissues. (Branster & Morton, 1956; Branster, 1958).

Tissue		Enzymic Activity (μ-moles of NAD formed/10^9 nuclei/hr.)	
		Mean	Range
A. Normal Tissues			
Fowl	liver	24	-
Mouse	liver,		
	foetal	2	(2 to 3)
	7-17 days old	12	(11 to 13)
	2 months old	30	-
	adult	44	(42 to 45)
	mammary gland,		
	lactating	13.2	(10 to 17)
	non-lactating	7.3	(6 to 8)
Human	liver	10	-
	lymph gland	6	-
B. Tumours			
Mouse	mammary carcinoma (C_3H)	2.8	(2 to 3)
	hepatoma	7	(5 to 9)
	Ehrlich ascites	2	(1 to 3)
Rat	Walker sarcoma	4	-
Human	Sarcoma	4	-
	squamous carcinoma	3	-

Usually, enzymic activities or amounts of tissue constituents are expressed on the basis of units of activity/unit of fresh wt., dry wt. or nitrogen. This will only be valid if there are no marked differences in cell sizes, dry wts. or nitrogen contents of the tissues being compared. For example, when expressed on a fresh wt. basis, dye-induced hepatomas contain 48% of the total NAD and 14% of the total NADP of normal rat liver (see Glock & McLean, 1957; and Table 10). The DNA phosphorus content per nucleus of hepatoma and normal rat liver is the same (Davidson & Leslie, 1950) but the amounts of DNA phosphorus/g. fresh wt. of the tissues are 546 & 249 μg., respectively. Hence, there are about 2.2 times as many nuclei per g. of tissue in hepatoma as compared with normal adult liver. When expressed on the basis of amount per nucleus, therefore, the results of Glock & McLean (1957) show that hepatoma contains about 20% of the NAD of normal adult rat liver. This agrees well with the relative activities of NMN-adenylyltransferase in nuclei from hepatoma and normal adult liver (see Table 9).

On the basis of this data, Morton (1958) proposed that in cells that were growing rapidly, the synthesis of new cellular material occurred against a limiting rate of supply of NAD from the nucleus, and that in normal embryonic tissues, this would lead to a decline with time in the rate of cell division, allowing differentiation of the adult tissue. If a genetic lesion prevented the increase in NMN-adenylyltransferase and hence in amount of NAD which would normally follow cell division, then new

Table 10. Levels of nicotinamide nucleotide coenzymes in normal and tumour tissues

Tissue	Coenzyme content (μm-moles/g. fresh wt.)					Reference
	[NAD]	[$NADH_2$]	[NADP]	[$NADPH_2$]	Total	
A. NORMAL						
1. Liver						
(a) Rat	555	306	8	266	1135	Glock & McLean (1955b)
	446	166	44	425	1081	Jacobson & Kaplan (1957)
	485	152	25	251	914	Bassham et al. (1959)
	628	252	115	502	1497	Lowry et al. (1961)
	(other values, see Table 5)					
(b) Mouse	657	183	57	328	1195	Jacobson & Kaplan (1957)
2. Mammary gland-rat	340	125	< 3	68	536	Glock & McLean (1955b)
3. Skeletal Muscle-rat	417	40	10	12	479	Glock & McLean (1955b)

Tissue	Coenzyme content (μm-moles/g. fresh wt.) [NAD]	[$NADH_2$]	[NADP]	[$NADPH_2$]	Total	Reference
B. TUMOUR						
1. Hepatoma-rat	376	76	-	-	-	Jedeikin & Weinhouse (1955)
	382	38	<4	37	431	Glock & McLean (1957)
2. Mammary gland carcinoma-mouse	149	48	-	-	-	Fisher & Schlenk (1948)
	156	57	-	-	-	Jedeikin & Weinhouse (1955)
3. Sarcoma-rat	141	81	-	-	-	Fisher & Schlenk (1948)
37	157	63	-	-	-	Jedeikin & Weinhouse (1955)
	166	33	<4	<4	207	Glock & McLean (1957)
Jensen	177	62	<4	6	249	Glock & McLean (1957)
-mouse						
Crocker	168	92	7	12	279	Glock & McLean (1957)
4. Ascites- Ehrlich	446	0	-	-	-	Jedeikin & Weinhouse (1955)
Ehrlich	198	54	<4	<4	260	Glock & McLean (1957)

cell division would be induced. Morton (1958) considered that tumour cells differed from normal embryonic cells in that they lacked an inherited potential for increasing the amount of transferase following cell division and that tumour cells may arise from normal adult cells by depletion of enzyme systems associated with the synthesis of NMN-adenylyltransferase.

Further information was therefore needed on amounts of nicotinamide nucleotide coenzymes in tissue that was undergoing normal growth and differentiation, together with data that would allow growth rate of the tissue to be measured. Rats and lambs were chosen as the experimental animals and sufficient parameters were measured so that growth of the whole animal and of the liver could be assessed. The variety of reference bases which were measured (dry wt., nitrogen, nucleic acid phosphorus and number of nuclei per g. fresh wt.), also allowed results to be expressed on the basis of per nucleus ('per cell') during growth.

Experimental

Animals

All sheep and lambs used in this study were obtained from the "Glenthorne" Experimental Station, C.S.I.R.O., Division of Biochemistry and General Nutrition, Adelaide. The sheep had been grazing on an improved mixed pasture and the lambs running with their dams in a separate area on the same type of pasture.

Male albino rats were obtained from the same colony as those used in Section I.

The animals used in Series I and III were lambs and sheep and in Series II and IV, lambs, sheep and rats.

Series I . Two Merino ewes and sixteen wether lambs, ranging in age from about one week prenatal to 22 days old were obtained during September, 1960.

Series II. 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, 1961.

The rats used ranged in age from 4 to 28 days and included a group of four 7 month-old rats.

Series III. Merino lambs (48 animals) ranging in age from about 2 weeks before birth to 50 days after birth, five wethers (6 months to 4 years old) and two adult ewes (5-6 years old) were used; the gestation period of Merino sheep under local conditions

is about 150 days.

The lambs listed in Table 10 were born during May, 1962, from ewes which had been grazing on an improved mixed pasture, dry until the end of April, and becoming increasingly green after autumn rains. These ewes were also offered a supplementary feed of chaffed wheaten hay and during the last few weeks of pregnancy an additional ration of grain (oats and wheat).

Series IV. A further group of lambs (Table 11) born during August-September, 1962, were also used. These animals grazed on an improved mixed pasture (spring growth) and the lambs ran with their dams in a separate area on the same type of pasture.

The pre-natal lambs were removed from the uterus within 30 sec. of slaughtering the ewe, and placed on a weighed stainless steel tray so that the body wt. of the animal could be determined after the liver samples had been removed. The curved crown-rump length (Cloette, 1939) was measured after all samples had been obtained. The newborn lambs (1-5 hr. old) were unsuckled; the other lambs and sheep were removed from their source of food and water about 2 hr. and the rats about 30 min. before slaughter.

The adult rats used were six months old.

Assay procedures

Sampling procedure. This was the same as that used in Section I for fresh tissue. Livers from the young rats were pooled from groups of animals at the various ages.

Extraction and assay of coenzymes. The oxidized and reduced coenzymes were extracted simultaneously from separate samples of the

tissue. Cold trichloroacetic acid and hot Na_2CO_3 solutions were used as described in Section I. A standardized time schedule was used and extraction of both oxidized and reduced nicotinamide nucleotides was commenced within 3 min. of the death of the animal, except with the pooled livers, when the time was extended by 2 to 3 min. All assays were completed within 5 hr. of the death of the animal.

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 0.2 M-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 three thousand nuclei were counted in each sample. All counts were made by the one operator with the one haemocytometer throughout the work. As a test of this procedure, deoxyribonucleic acid was estimated by the method described below. With pooled livers from the two groups of 28-day-old rats the values were 219 and 304 µg. DNA phosphorus/g. fresh wt. These samples contained 313 and 359 x 10^6 nuclei/g., corresponding to values of 0.70 and 0.85 x 10^{-12}g. DNA phosphorus/nucleus respectively, which is in good agreement with published values (see Davidson, 1960). This indicates that reliable counts of nuclei were obtained.

Nucleic acids. These were extracted as described by Schneider (1945) except that perchloric acid (5% w/v) was used to remove acid-soluble material and DNA was estimated colorimetrically with

p-nitrophenyl hydrazine (Webb and Levy, 1955); the N-NaOH used to develop the colour was saturated with trisodium phosphate at 20°. The DNA of about a third of the samples was also estimated by Burton's (1956) procedure. The values obtained by Burton's method were up to 5% lower than those obtained by Webb and Levy's technique. Phosphorus was determined by Allen's (1940) procedure. Calf-thymus DNA (Zamenhof, 1958) was used as a standard; as determined by Wyatt's (1955) method, the molar ratio of uracil/thymine was no greater than 0.02/1 and the recovery of bases was 96% of the total phosphorus.

The tissue samples were treated with perchloric acid and lipid solvents and the dried powder was stored at -15° for less than 5 days before extraction with trichloroacetic acid and estimation of deoxyribose. It was found that storage of the dried powder for more than one week or storage of the trichloroacetic acid extract for more than 12 hr. gave lower values of DNA.

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

Reference bases. So that changes in amounts of coenzymes and in enzyme activities could be adequately assessed in a tissue that is rapidly developing, a number of parameters other than calendar age were determined. Tables 11, 12 and 13 show the values for calendar age, body wt., liver fresh wt., liver dry wt., percentage dry wt., and the number of nuclei and the amount of nitrogen/g. fresh wt. of liver. The derived values for the number of nuclei, and the amounts of nitrogen, per liver, are also given. The crown-rump length was measured in prenatal lambs (Table 12) but it varied too much in relation to other parameters to be a reliable guide to the stage of development.

Values of DNA phosphorus and RNA phosphorus/g. fresh wt. of liver were determined in lambs and sheep from 4 days to 4 years old and in adult rats. The results are given in Table 13, together with the derived values for the amounts of nucleic acid phosphorus/liver and DNA phosphorus/nucleus. Some samples deteriorated on storage (see p.84) and, for these, values of total nucleic acid phosphorus only are shown. RNA phosphorus was determined by difference between total nucleic acid phosphorus and DNA phosphorus. From these, the DNA phosphorus/nucleus was calculated. The mean value for the RNA phosphorus: DNA phosphorus ratio was 1.40 (range 0.84-2.18) for 19 animals (sheep and lambs). The ratio showed no trend during development. This value was about half of that

Table 11. <u>Reference bases and nicotinamide nucleotide coenzymes in livers of sheep and rats.</u>
(<u>Series II</u>)

Species and Age (days)	Body wt. (kg.)	Liver (fresh wt. g.)	Liver (dry wt. g.)	Nuclei x 10^{-6}/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	267	365	68	433	20	234	254	687
	4.6	141	35.7	242	442	48	490	10	85	95	585
	4.8	114	29.1	294	330	33	363	20	143	163	526
	5.0	124	31.5	293	322	101	423	27	154	181	604
14	6.2	134	36.4	299	381	17	398	10	235	245	643
	7.8	190	50.1	231	220	15	235	27	230	257	492
	8.2	193	52.1	257	247	54	301	10	176	186	487
	8.8	215	59.4	250	279	18	297	10	109	119	416
29	7.3	164	45.9	287	353	46	399	52	277	329	728
	10.5	245	68.6	292	149	58	207	40	201	241	448
	10.7	222	60.8	299	109	51	160	42	257	299	459
56	11.7	264	73.0	282	508	56	564	34	160	194	758
	16.0	346	99.0	258	349	36	385	33	160	193	578
	18.8	361	104.0	260	507	102	609	38	182	220	829
	19.8	357	103.0	254	400	48	448	47	219	266	714
	19.9	324	91.2	309	417	62	479	50	263	313	792

(years)											
3	48.0	780	235	203	736	111	847	15	195	210	1057
3	50.2	836	251	191	670	116	786	28	258	286	1072
3	51.9	930	277	232	679	107	786	26	297	323	1109
3	53.2	815	244	240	608	36	644	33	212	245	889
Rats (days)	g.										
4(17)*	8.3	0.28	0.07	271	321	71	392	5	151	156	548
7(13)*	13.4	0.44	0.12	424	413	120	533	21	234	255	788
28(5)*	39.8	1.62	0.47	323	470	113	583	20	296	316	899
(3)*	56.5	2.69	0.78	313	473	83	556	5	274	279	835
(3)*	68.0	3.40	1.00	359	455	96	551	15	305	320	871
(months)											
7	520	14.8	4.61	188	435	162	597	30	364	394	991
7	560	17.3	5.33	185	433	98	531	25	394	419	950
7	595	17.9	5.45	181	328	80	408	25	276	301	709
7	623	17.3	5.58	181	500	93	593	37	487	524	1117

*Samples were taken from the pooled livers of the number of animals shown.

Table 11 (continued)

Species and Age (days)	Nitrogen (g./liver)	10^{-8} x Nuclei /liver	Coenzyme content (μmoles/liver) [NAD]	[NADH$_2$]	[NAD] + [NADH$_2$]	[NADP]	[NADPH$_2$]	[NADP] + [NADPH$_2$]	Total	Coenzyme ratio
Sheep										
4	3.94	339	46.4	8.6	55.0	2.5	29.8	31.3	86.1	1.70
	4.23	342	62.3	6.8	69.1	1.4	13.3	14.7	83.8	5.16
	3.65	335	37.6	3.8	41.4	2.3	16.3	18.6	60.0	2.23
	4.09	364	40.0	12.4	52.4	3.3	19.1	22.4	74.8	2.34
14	4.29	401	51.1	2.3	53.4	1.3	31.5	32.8	86.2	1.62
	5.89	471	41.8	2.9	44.7	5.1	43.7	48.8	93.5	0.91
	5.60	439	47.7	10.4	58.1	1.9	34.0	35.9	94.0	1.62
	6.67	496	60.0	3.9	63.9	2.2	23.4	25.6	89.5	2.50
29	5.08	538	57.8	7.5	65.3	8.5	45.4	53.9	120	1.21
	7.35	716	36.5	15.2	51.7	9.8	49.2	59.0	111	0.86
	7.10	664	24.2	11.3	35.5	9.3	57.1	66.4	102	0.54
56	8.71	744	134	14.5	148	9.0	42.2	51.2	199	2.91
	10.7	894	121	12.5	134	11.4	55.4	66.8	201	1.99
	11.9	938	183	37.0	220	13.8	65.9	79.7	300	2.77
	12.1	908	143	17.1	160	16.8	78.2	95.0	255	1.69
	10.4	1000	135	20.1	155	16.2	84.2	101	256	1.53

(years)										
3	27.3	1585	574	86.5	661	11.7	152	164	825	4.03
	30.1	1596	560	97.0	657	23.4	216	239	896	2.75
	32.6	2160	632	100	932	24.2	276	300	1232	2.43
	285	1955	496	29.3	525	26.9	175	202	727	2.63
Rats										
(days)	(mg./liver)									
4(17)*	8	0.8	0.10	0.01	0.11	0.00	0.04	0.04	0.15	2.51
7(13)*	13	1.9	0.18	0.06	0.24	0.01	0.10	0.11	0.35	2.09
28(5)*	55	5.2	0.76	0.19	0.95	0.03	0.48	0.51	1.46	1.84
(3)*	91	8.4	1.27	0.23	1.50	0.00	0.74	0.75	2.25	1.99
(3)*	102	12.2	1.58	0.29	1.87	0.05	1.04	1.09	2.96	1.72
(months)										
7	503	27.8	6.43	2.41	8.84	0.45	5.38	5.83	14.67	1.52
	606	32.1	7.50	1.68	9.18	0.43	6.82	7.25	16.43	1.27
	627	32.4	5.37	0.94	7.31	0.44	4.94	5.38	12.69	1.36
	588	31.3	8.65	1.62	10.27	0.49	8.42	8.91	19.18	1.13

* Samples were taken from the pooled livers of the number of animals shown.

Table 12. Reference bases and concentrations of nicotinamide nucleotide coenzymes in livers of lambs and sheep. (Series III)

			Liver			Nuclei		Coenzyme content (μm-moles/g.fresh wt.)						
Age	Body wt. (kg.)	C/R length (cm.)	fresh wt. (g.)	dry wt. (g.)	%	$\times 10^{-6}$ /g.	$\times 10^{-8}$ /liver	[NAD]	[$NADH_2$]	[NAD] + [$NADH_2$]	[NADP]	[$NADPH_2$]	[NADP] + [$NADPH_2$]	Total
Pre-natal	3.26	48.3	68.1	17.3	25.3	304	207	-	-	-	-	-	-	-
	3.43	49.5	67.0	17.4	26.0	335	225	562	41	603	10	63	73	676
	3.34	52.0	69.8	17.4	24.9	374	261	460	31	491	5	79	84	575
	3.06	46.6	71.2	17.9	25.1	365	260	241	24	265	5	124	129	394
	2.78	49.5	86.0	19.0	22.1	496	426	204	39	243	8	68	76	319
	3.54	50.8	82.7	20.7	25.0	392	324	578	77	655	5	77	82	737
	4.93	55.9	93.3	25.3	27.1	288	269	543	44	587	5	55	60	647
	3.68	50.8	106	27.5	25.9	350	371	338	38	376	5	115	120	496
Post-natal (hr.)														
5	3.9	51.4	60.6	15.3	25.2	369	224	407	65	472	5	117	122	594
3	3.5	51.3	64.5	17.8	27.6	376	242	487	83	570	5	91	96	666
3	4.0	55.2	70.2	18.3	26.0	347	243	480	-	-	5	-	-	-
2	4.5	56.6	71.1	18.8	26.5	412	293	584	60	644	5	120	125	769
1	3.5	52.7	71.4	19.5	27.3	365	262	575	79	654	5	61	66	720
2	5.1	55.9	95.6	26.5	27.7	329	315	763	44	807	5	184	189	996

(days)														
1	4.1	55.9	79.5	21.6	27.1	314	250	408	90	498	15	139	154	652
1	3.8	53.4	93.0	22.3	23.9	291	270	566	66	632	10	92	102	734
1	4.9	59.4	97.1	24.3	25.0	316	307	578	57	635	5	81	86	721
1	5.5	59.7	129.5	32.3	24.9	296	384	321	75	396	5	98	103	499
2	4.1	54.6	97.5	25.2	25.9	297	289	314	44	358	5	65	70	428
3	5.2	60.7	136.3	35.5	26.0	309	421	260	58	318	5	63	68	386
3	5.5	60.4	154	40.6	26.4	-	-	377	59	436	29	235	264	700
5	5.8	62.2	146	36.2	24.8	298	436	329	42	371	5	92	97	468
14	8.0	73.7	174.5	48.3	27.7	272	475	414	62	476	10	114	124	600
18	8.8	71.8	180.8	51.3	28.4	254	459	448	56	504	5	135	140	644
24	10.0	76.2	211.6	60.5	28.6	256	542	472	73	545	5	184	189	734
22	11.3	77.5	228.7	64.6	28.2	264	604	352	69	421	20	166	186	607
27	9.3	71.7	182.5	53.5	29.3	305	556	460	54	514	10	91	101	615
50	15.3	85.4	286.4	80.0	28.0	284	813	474	59	533	15	178	193	726
(years)														
5	33.5	-	493	145	29.3	275	1355	585	58	643	15	197	212	855
6*	36.1	-	720	220	30.5	258	1858	612	79	691	15	256	271	962

*Lactating 24 days

Table 12 (continued)

Age	Liver nitrogen (mg./g. fresh wt.)	Liver nitrogen (g./liver)	Coenzyme content (µmoles/liver) [NAD]	[NADH$_2$]	[NAD] + [NADH$_2$]	[NADP]	[NADPH$_2$]	[NADP] + [NADPH$_2$]	Total	Coenzyme Ratio
Prenatal	20.8	1.27	-	-	-	-	-	-	-	-
	20.6	1.38	37.6	2.8	40.4	0.7	4.2	4.9	45.3	8.26
	23.6	1.65	32.1	2.2	34.3	0.3	5.5	5.8	40.1	5.85
	23.3	1.66	17.2	1.7	18.9	0.4	8.8	9.2	28.1	2.05
	21.5	1.85	17.6	3.3	20.9	0.7	5.8	6.5	27.4	3.20
	22.9	1.89	47.8	6.4	54.2	0.4	6.4	6.8	61.0	8.0
	21.5	2.02	50.7	4.1	54.8	0.5	5.1	5.6	60.4	9.78
	22.2	2.34	35.8	4.1	39.9	0.5	12.2	12.7	52.6	3.13
Postnatal (hr.)										
5	28.0	1.70	24.7	3.9	28.6	0.3	7.1	7.4	36.0	3.87
3	24.8	1.56	31.4	5.4	36.8	0.3	5.9	6.2	43.0	5.94
3	27.1	1.95	33.7	-	-	0.4	-	-	-	-
2	26.7	1.90	41.5	4.3	45.8	0.4	8.5	8.9	54.7	5.15
1	24.2	1.73	41.1	5.6	46.7	0.4	4.3	4.7	61.4	9.91
2	27.2	2.60	73.0	4.2	77.2	0.4	17.6	18.0	95.2	4.27
(days)										
1	30.5	2.43	32.4	7.2	39.6	1.2	11.0	12.2	51.8	3.23
1	30.5	2.84	52.6	6.2	58.8	0.9	8.6	9.5	68.3	6.20

1	27.5	2.67	56.1	5.5	61.6	0.5	7.9	8.4	70.0	7.38
1	31.6	4.09	41.6	9.7	51.3	0.6	12.7	13.3	64.6	3.84
2	29.0	2.83	30.6	4.3	34.9	0.5	6.3	6.8	41.7	5.12
3	30.9	4.22	35.5	7.9	43.4	0.7	8.5	9.2	52.6	4.68
3	30.3	4.67	58.1	9.1	67.2	4.4	36.2	40.6	107.8	1.65
5	29.6	4.32	48.0	6.2	54.2	0.7	13.5	14.2	68.4	3.78
14	29.5	5.14	72.3	10.8	83.1	1.7	19.9	21.6	104.7	3.84
18	32.5	5.87	81.1	10.1	91.2	0.9	24.4	25.3	116.8	3.60
24	30.4	6.42	99.9	15.4	115.3	1.1	38.9	40.0	155	2.88
22	29.5	6.76	80.6	15.8	96.4	4.6	38.0	42.6	139.0	2.26
27	30.7	5.60	84.0	9.8	93.8	1.8	16.6	18.4	112.2	5.08
50	32.6	9.32	136	17.0	152	4.3	51.0	55.3	207	2.76
(years)										
5	31.6	15.9	288	28.6	317	7.4	97.1	105	422	3.03
6*	30.7	22.1	441	56	497	11.0	184	195	692	2.55

*Lactating ewe, 24 days

Table 13. Reference bases and the concentration of nucleic acid phosphorus and of nicotinamide adenine dinucleotide in the livers of sheep and rats. (Series IV)

Age	Body wt. kg.	Liver wt. Fresh g.	Liver wt. Dry g.	Liver wt. %	Liver nitrogen mg./g. fresh wt.	Liver nitrogen g./liver	Nuclei $\times 10^{-6}$/g. fresh wt.	Nuclei $\times 10^{-8}$/ liver
Sheep (days)								
4	4.0	88.6	22.5	25.5	29.9	2.65	264	234
	4.0	84.5	23.0	27.2	29.4	2.47	269	228
	4.8	100	26.8	26.8	32.5	3.25	365	365
	5.3	108	29.3	27.1	29.6	3.16	380	411
	6.0	144	38.2	26.5	31.4	4.6	338	477
	6.7	161	42.4	26.3	29.5	4.75	307	498
7	6.5	154	40.6	26.4	31.5	4.82	262	403
	7.2	177	46.3	26.2	31.6	5.44	250	442
	6.1	181	51.0	28.2	28.2	5.14	199	360
	8.0	218	58.2	26.7	30.4	6.6	233	507
14	10.0	284	81.1	28.2	29.4	8.32	271	498
15	8.0	199	54.5	27.4	29.8	5.92	249	495
14	8.5	218	57.9	26.6	29.6	6.44	250	543
14	8.4	220	61.4	28.0	29.7	6.52	243	534

(days)								
30	12.5	232	62.8	27.1	33.1	7.68	312	725
29	11.9	260	71.8	27.6	33.3	8.66	269	698
28	13.3	280	77.7	27.7	33.9	9.49	317	890
26	15.6	340	94.9	27.8	29.6	10.6	289	985
49	15.8	303	88.4	29.1	33.1	10.0	265	802
	19.1	336	96.4	28.7	33.9	11.4	261	875
(months)								
6	28.3	691	202	29.3	34.2	23.6	213	1474
	23.1	681	205	30.3	35.4	24.1	217	1480
(years)								
3	56.6	921	275	29.9	33.8	31.1	188	1735
4	57.6	857	250	29.3	34.9	29.9	234	2000
	57.8	884	272	30.7	36.4	32.2	209	1845
Rats (months)	g.							
6	477	17.2	5.32	30.9	31.6	0.544	127	21.9
	464	16.7	5.34	32.0	32.9	0.549	152	25.4
	494	17.2	5.50	32.0	32.4	0.557	143	24.5
	500	19.4	6.11	31.5	28.7	0.557	155	30.1
	510	19.8	6.22	31.4	27.4	0.543	150	29.7

Table 13 (continued)

Animals	Age (days)	Nucleic acid phosphorus DNA µg./g.	RNA µg./g.	DNA mg/liver	RNA mg/liver	RNA/DNA	DNA µµg./nucleus	[NAD] µm-moles / g.fresh wt.	µmoles /liver
Lambs	4	264	384	23.4	34.0	1.45	1.00	-	-
		347	517	29.3	43.6	1.49	1.29	432	36.5
		428	360	42.8	36.0	0.84	1.17	-	-
		354	416	38.2	44.9	1.18	0.93	-	-
		390	452	56.2	65.0	1.16	1.15	-	-
		316	476	50.8	76.6	1.51	1.03	370	59.6
	7	-	-	-	-	-	-	310	47.6
		279	361	49.4	63.4	1.29	1.12	374	66.2
		208	454	37.6	82.2	2.18	1.04	396	71.6
		272	541	59.2	117.8	1.99	1.17	374	81.3
	14	271	391	76.9	111.0	1.44	1.00	260	73.8
	15	302	398	60.1	79.2	1.32	1.21	370	73.6
	14	325	381	70.4	82.9	1.17	1.30	277	60.3
	14	317	443	69.6	97.3	1.40	1.30	310	68.0

	30	378	346	87.6	80.2	0.92	1.21	475	110
	29	288	412	75.0	107.4	1.43	1.07	460	120
	28	304	403	85.2	113.0	1.33	0.96	435	122
	26	317	473	108.0	161.0	1.49	1.10	420	143
	49	-	714*	-	-	-	0.89	504	153
	49	-	625*	-	-	-	0.81	584	196
Sheep	(months)								
	6	242	348	167.2	240.5	1.44	1.13	595	412
		-	526*	-	-	-	0.82	636	433
	(years)								
	3	192	318	176.8	293.0	1.66	1.02	717	660
	4	-	510*	-	-	-	0.95	824	706
		-	440*	-	-	-	0.69	910	805
Rats	(months)								
	6	159	506	2.74	8.70	3.18	1.25	405	6.97
		187	526	3.12	8.78	2.81	1.22	465	7.76
		162	508	2.79	8.74	3.14	1.13	415	7.14
		-	-	-	-	-	-	385	7.48
		-	480*	-	-	-	-	460	9.12

* Total nucleic acid phosphorus

obtained for three adult rats (mean 3.04, range 2.81-3.18).

The DNA phosphorus/nucleus was found to be 1.12 μμg (range 0.93-1.30μμg.) in these lambs and sheep, and 1.20 μμg (range 1.13-1.25 μμg.) in the adult rats. In a further group of pen-fed ewes aged 3 years, a mean value of 1.03 μμg. (range 0.91-1.19 μμg.) for seven animals was obtained.

The percentage dry wt. of the livers in the lambs in Series II (4 to 56 days old) varied between 25.3 and 28.9%, with a mean of 27.3% (16 animals), and in the four adult sheep increased to a mean of 30.0% (range 29.8-30.2%). In the sheep and lambs of Series III and IV, (cf. Tables 12 and 13) the percentage dry wt. of the livers of animals up to 5 days old varied between 22.1% and 27.7%, with a mean of 25.9% (28 animals), and in the five adult sheep increased to a mean of 29.9% (range 29.3-30.7%). In the rats (Series II, Table 11) the percentage dry wt. of the liver showed an increase from 25.2% (mean for 17 animals, 4 days old) to 31.1% (range 30.4 to 32.2% for four animals) in the adult.

The total nitrogen/g. fresh wt. of liver in the lambs in Series II, varied between 29 and 34 mg./g., with a mean of 31.6 mg./g. (16 animals) and in the four adult sheep increased to a mean of 35.3 mg./g. (range 35 to 36 mg./g.). In the animals of Series III and IV (Tables 12 and 13) the total nitrogen/g. fresh wt. of liver was lower in the eight prenatal lambs (mean 22.1, range 20.6-23.6 mg./g.) than in the six newborn lambs (mean 26.3, range 24.2-28.0 mg./g.) and than in the older lambs and adults (41 animals; mean 31.3, range 27.5-36.4 mg./g.). During growth

of the rat after birth (cf. Table 11) the total nitrogen content of the liver increased from a mean of 29.6 mg./g. fresh wt. (30 animals, range 29-30 mg./g.) in rats up to 7 days old, to a mean of 32.9 mg./g. (11 animals, range 30-34 mg./g.) in rats 28 days old and reached a mean value of 34.5 mg./g. (4 animals, range 34-35 mg./g.) in the adult.

The number of nuclei/g. fresh wt. of liver differed little in any of the groups of lambs but the number was greater than that found in the livers of adult sheep. In the young rats there were almost twice as many nuclei/g. fresh wt. of liver as in the adult.

<u>Concentrations of nicotinamide nucleotide coenzymes in livers of sheep and rats during growth.</u> Table 11 shows the concentrations of nicotinamide nucleotide coenzymes/g. fresh wt. of tissue, in thelivers of sheep and rats of various ages ranging from four days to maturity (animals of Series II). An analysis of variance between and within age classes was carried out on these results and showed little 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 1030 μm-moles/g. fresh wt.). A similar rise in concentration of coenzymes in the liver was also shown during growth of the rats.

Table 12 shows the concentrations of nicotinamide nucleotide coenzymes in livers of prenatal and postnatal lambs of various ages.

A wide variation was observed in the values obtained for the livers of foetal and newborn animals, but the general trend was for the concentration of total [NAD]/g. fresh wt. to be higher in the prenatal and immediately postnatal lambs than in lambs 2-5 days old. The concentration of total [NADP]/g. fresh wt. in the livers of foetal and newborn animals was lower than in the young lambs and adult sheep. Values obtained for lambs during their subsequent development agreed well with those obtained in the previous series of lambs and sheep (see Table 11).

Table 13 shows the concentration of NAD in the livers of postnatal lambs and of adult rats.

Table 8 shows the concentrations of nicotinamide nucleotide coenzymes/g. fresh wt. in the livers of lambs and sheep ranging from about one week prenatal to the adult animal (Series I). These values were obtained by using a single-extraction method which although less precise, gave results which showed trends in concentrations which are similar to the results obtained in the later studies (cf. Tables 11, 12 & 13).

Total amounts of nicotinamide nucleotide coenzymes in the livers of sheep and rats during development.

From the data in Table 12 for sheep and rats (Series III), regression analysis was carried out using calendar age, body wt., liver dry wt., and the total number of nuclei/liver as parameters of relative maturity; the total amounts of [NAD], [NADP] and [NAD] +

[NADP] per liver were the dependent variates. It was necessary to take logarithms of both the dependent and determining variates to produce homoscedasticity and linearity. Details of the statistical treatment are given in the Appendix. The notation used in the regression equations is as follows, all being on a total liver basis.

$$x_2 = \log \text{(liver dry wt. g.)}$$

$$x_3 = \log (\text{no. of nuclei} \times 10^{-8})$$

$$x_4 = \log ([\text{NAD}] + [\text{NADH}_2])$$

$$x_5 = \log ([\text{NADP}] + [\text{NADPH}_2])$$

$$x_6 = \log \text{(total coenzymes)}$$

Sheep

$x_4 = -0.3795 + 1.3001\ x_2$ (curve B, Fig.3)

$x_4 = -2.3317 + 1.5421\ x_3$ (curve E, Fig.4)

$x_5 = -0.2941 + 1.1037\ x_2$ (curve C, Fig.3)

$x_5 = -2.0816 + 1.3548\ x_3$ (curve F, Fig.4)

$x_6 = -0.0462 + 1.2265\ x_2$ (curve A, Fig.3)

$x_6 = -1.9335 + 1.4708\ x_3$ (curve D, Fig.4)

Rats

$x_4 = 0.2541 + 0.9802\ x_2$ (curve B, Fig.5)

$x_4 = -0.9037 + 1.2163\ x_3$ (curve E, Fig.6)

$x_5 = 0.0255 + 1.1235\ x_2$ (curve C, Fig. 5)

$x_5 = -1.3002 + 1.3929\ x_3$ (curve F, Fig.6)

$x_6 = 0.4587 + 1.0330\ x_2$ (curve A, Fig 5)

$x_6 = -0.7604 + 1.2809\ x_3$ (curve D, Fig 6)

These twelve regression curves, which are all highly significant ($P < 0.001$), are presented in Figs. 3, 4, 5 and 6.

It can be seen that for the sheep (Fig. 3) the regression curve of ([NADP] + [$NADPH_2$]) on liver dry wt. was almost linear indicating that with increasing size of liver the concentration of this coenzyme remained unchanged. However, with ([NAD] + [$NADH_2$]), and thus also with total coenzymes, there was a greater increase in the amount of coenzymes per unit increase in wt. of liver in the larger livers, i.e. in the more mature animals. A similar trend was shown in Fig. 4 when expressed on a nuclear basis.

In the liver of the rat (Fig. 5) the regression of ([NAD] + [$NADH_2$]) on liver dry wt. was linear indicating a constant rate of increase of this coenzyme as the liver increased in total dry wt. This also applied to ([NADP] + [$NADPH_2$]) and consequently to total coenzymes. However on a nuclear basis (Fig. 6) the gradient of the regression curve of both coenzymes on nuclei increased during development, i.e. animals with larger numbers of liver nuclei contained proportionally a greater amount of coenzymes in the liver.

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.

<u>Sheep</u>

log (body wt., kg.) = 0.4039 + 0.4340 log (age, days)

log (liver dry wt., g.) = 1.2749 + 0.3725 log (age, days)

<u>Rats</u>

$$\log(\text{body wt., g.}) = 0.1889 + 1.0986 \log(\text{age, days})$$

$$\log(\text{liver dry wt., g.}) = -1.7733 + 1.0817 \log(\text{age, days})$$

A similar regression analysis was carried out on the data from the sheep and lambs in Series III (Table 12), using values for the dry wt. of the liver and the number of nuclei and amounts of coenzymes in the whole liver of each animal, and gave the following equations:

A, $\log(\text{total coenzymes}) = 0.2772 + 1.0606 \log W$;

B, $\log([NAD] + [NADH_2]) = 0.2613 + 1.0062 \log W$;

C, $\log([NADP] + [NADPH_2]) = -0.8834 + 1.3395 \log W$;

D, $\log(\text{total coenzymes}) = -1.4834 + 1.2978 \log N$;

E, $\log([NAD] + [NADH_2]) = -1.3873 + 1.2228 \log N$;

F, $\log([NADP] + [NADPH_2]) = -3.2169 + 1.6814 \log N$;

where W = liver dry wt. (g.) and N = number of nuclei x 10^{-8}.
In each case the regression is highly significant ($P < 0.001$).
A comparison was made of these six regressions and those obtained previously for the growing lambs in Series II (see Table 11 and p.95; equations A-F, Figs 3 & 4). Since only in one case, namely regression B, do the coefficients differ significantly and these only at the 0.05 level the two sets of data may be considered compatible. The difference in regression B was due to higher levels of ($[NAD] + [NADH_2]$) in the livers of the young lambs in the present series. The means of each of the regression of the present series differ significantly from those of the previous series as would be expected from the differences in the stages of development of the animals in

each group. Further details of this statistical treatment are given in the Appendix.

The means, variances and intercorrelations of the logarithms of the 1961 and 1962 data on the growing lamb (Series II & III) are given in Table 14. These statistics were obtained using a stock programme on the I.B.M. 1620 Computer of the University of Adelaide.

In order to assess the rate of change of number of nuclei per unit change of coenzymes, the regression of x_3 on x_6 was calculated and the resulting regression equation was differentiated after conversion to the original units, using the data from the lambs and sheep of Series II (see Table 11; Figs 3 & 6).

<u>Sheep</u>

$$N = 27.04\ C^{0.6278}$$

$$\text{whence}\quad \frac{dN}{dC} = 16.98\ C^{-0.3722}$$

<u>Rats</u>

$$N = 3.98\ C^{0.7687}$$

$$\frac{dN}{dC} = 3.06\ C^{-0.2313}$$

where N is the number of nuclei/liver and C is the total amount of coenzymes/liver. Fig. 7 (<u>a</u> and <u>b</u>) shows the curves obtained for the sheep and rat respectively and indicates the change in number of nuclei per unit change in total coenzymes relative to the amount of coenzymes in normal liver tissue during growth and development.

Table 14

Correlation matrix of data from lambs and sheep of Series II and III.

Variates:

x_2 = log (liver dry wt., g.);

x_3 = log (no. of nuclei x 10^{-8}/liver);

x_4 = log (total NAD/liver, μ-moles);

x_5 = log (total NADP/liver, μ-moles);

x_6 = log (total coenzymes/liver, μ-moles).

Series II (61/5/20).

Correlation matrix:

	x_2	x_3	x_4	x_5	x_6
x_2,	1.0000	.9880	.9365	.9417	.9745
x_3,		1.0000	.9134	.9506	.9609
x_4,			1.0000	.8508	.9816
x_5,				1.0000	.9323
x_6,					1.0000

Sample means and variances:

x_2,	1.876088	± 0.101309
x_3,	2.847710	± 0.068503
x_4,	2.059591	± 0.195272
x_5,	1.776421	± 0.139144
x_6,	2.225489	± 0.160512

Table 14 (continued)

Series III (62/5/27)

Correlation matrix :

	x_2	x_3	x_4	x_5	x_6
x_2,	1.0000	.9660	.9302	.9532	.9492
x_3,		1.0000	.8760	.9272	.9001
x_4,			1.0000	.8974	.9950
x_5,				1.0000	.9303
x_6,					1.0000

Sample means and variances

x_2,	1.511217	±	0.086365
x_3,	2.591684	±	0.051855
x_4,	1.781856	±	0.101055
x_5,	1.140808	±	0.170537
x_6,	1.880002	±	0.107816

Figure 3.

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) $= -0.0462 + 1.2265 \log W$;

B, log ([NAD] + [$NADH_2$]) $= -0.3795 + 1.3001 \log W$;

C, log ([NADP] + [$NADPH_2$]) $= -0.2941 + 1.1037 \log W$;

where W is the liver dry wt. (g.) In each case $P < 0.001$. 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. selected from the range of values shown in Table 11; for clarity, not all observations are illustrated. ●, (total coenzymes); ▲, ([NAD] + [$NADH_2$]); ■, ([NADP] + [$NADPH_2$]).

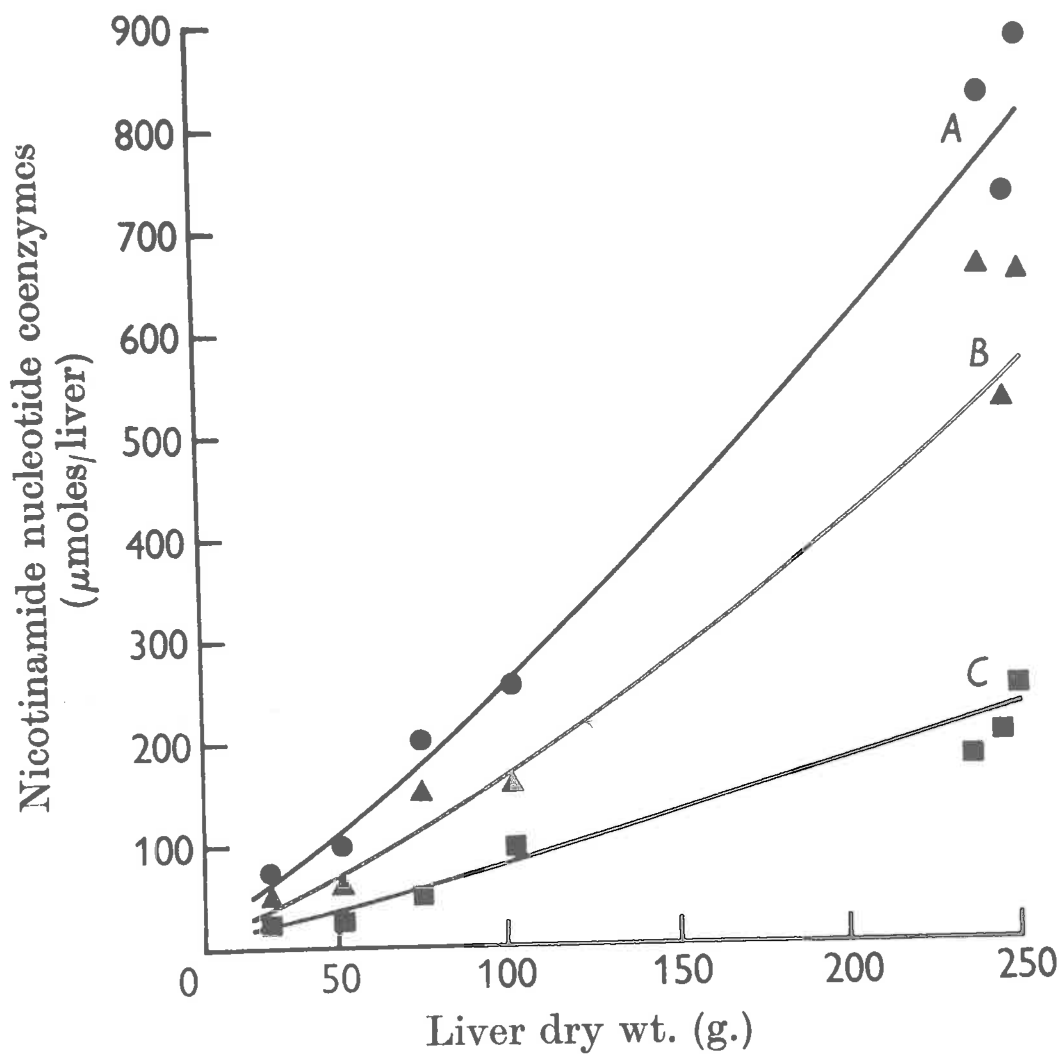

Nicotinamide nucleotide coenzymes
(μmoles/liver)
900
800
700
600
500
400
300
200
100
0
50
100
150
200
250
Liver dry wt. (g.)
A
B
C

Figure 4.

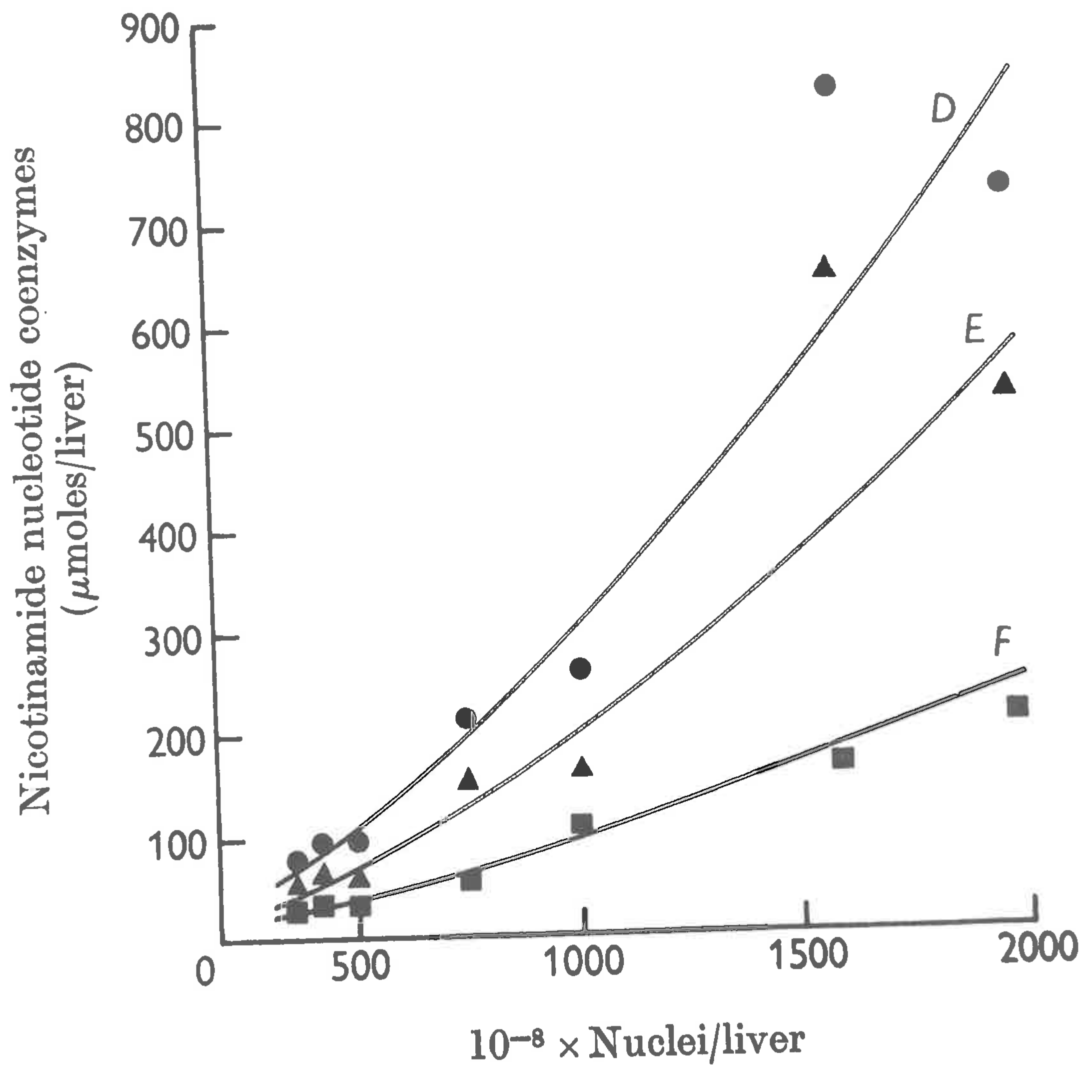
Changes in amounts of nicotinamide nucleotide coenzymes of liver during development of the sheep. Curves were drawn from the regression equations:

D, log (total coenzymes) $= -1.9335 + 1.4708 \log N$;

E, log ([NAD] + [$NADH_2$]) $= -2.3317 + 1.5421 \log N$;

F, log ([NADP] + [$NADPH_2$]) $= -2.0816 + 1.3548 \log N$;

where N is the number of nuclei x 10^{-8}. In each case $P < 0.001$. To illustrate the fit of observations to the regression curves, some estimates of nicotinamide nucleotide coenzymes are shown for various values of number of nuclei selected from the range of values shown in Table 11; for clarity, not all observations are illustrated. ●, (total coenzymes); ▲, ([NAD] + [$NADH_2$]); ■, ([NADP] + [$NADPH_2$]).



Nicotinamide nucleotide coenzymes
(μmoles/liver)
900
800
700
600
500
400
300
200
100
0
500
1000
1500
2000
D
E
F
10⁻⁸ × Nuclei/liver

Figure 5.

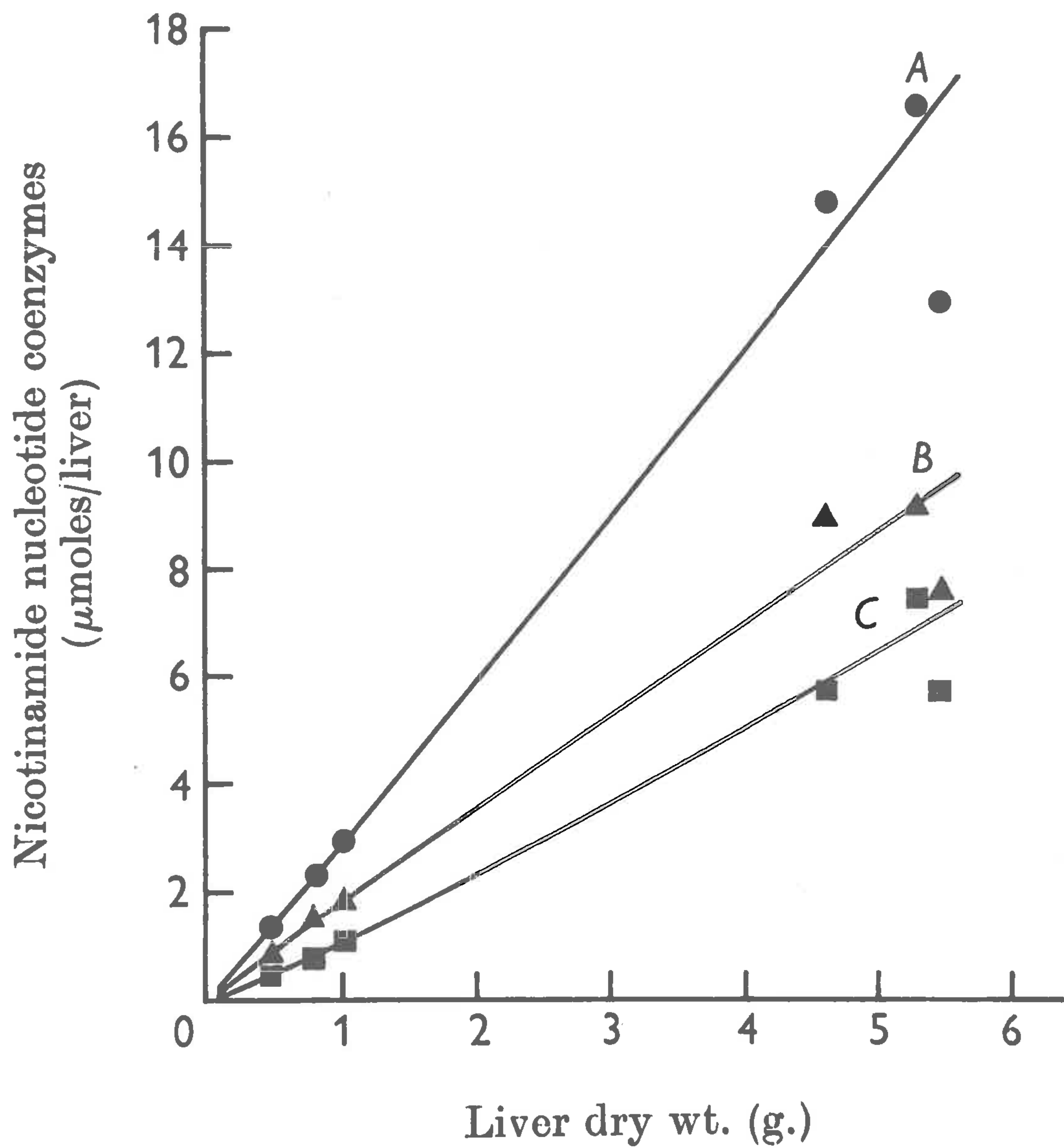
Changes in amounts of nicotinamide nucleotide coenzymes of liver during development of the rat. The lettering and symbols are as for Fig. 3. Curves were drawn from the regression equations:

A, log(total coenzymes) = 0.4587 + 1.0330 log W;

B, log([NAD] + [$NADH_2$]) = 0.2541 + 0.9802 log W;

C, log([NADP] + [$NADPH_2$]) = 0.0255 + 1.1235 log W;

In each case $P < 0.001$.



Nicotinamide nucleotide coenzymes (μmoles/liver)
Liver dry wt. (g.)
A
B
C
0
1
2
3
4
5
6
2
4
6
8
10
12
14
16
18

Figure 6.

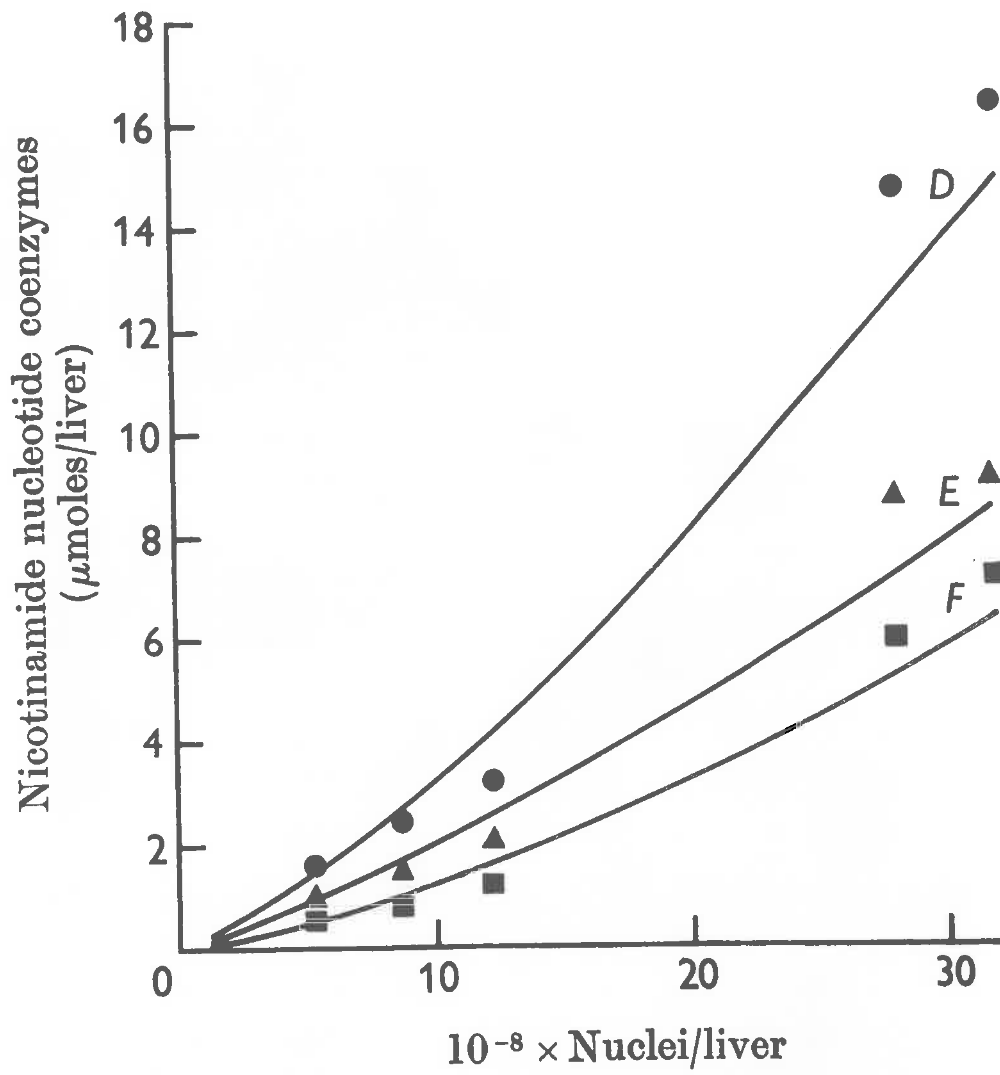
Changes in amounts of nicotinamide nucleotide coenzymes of liver during development of the rat. Lettering and symbols as for Fig. 4. Curves were drawn from the regression equations:

D, log (total coenzymes) = -0.7604 + 1.2809 log N;

E, log ($[NAD] + [NADH_2]$) = -0.9037 + 1.2163 log N;

F, log ($[NAD] + [NADPH_2]$) = -1.3002 + 1.3929 log N;

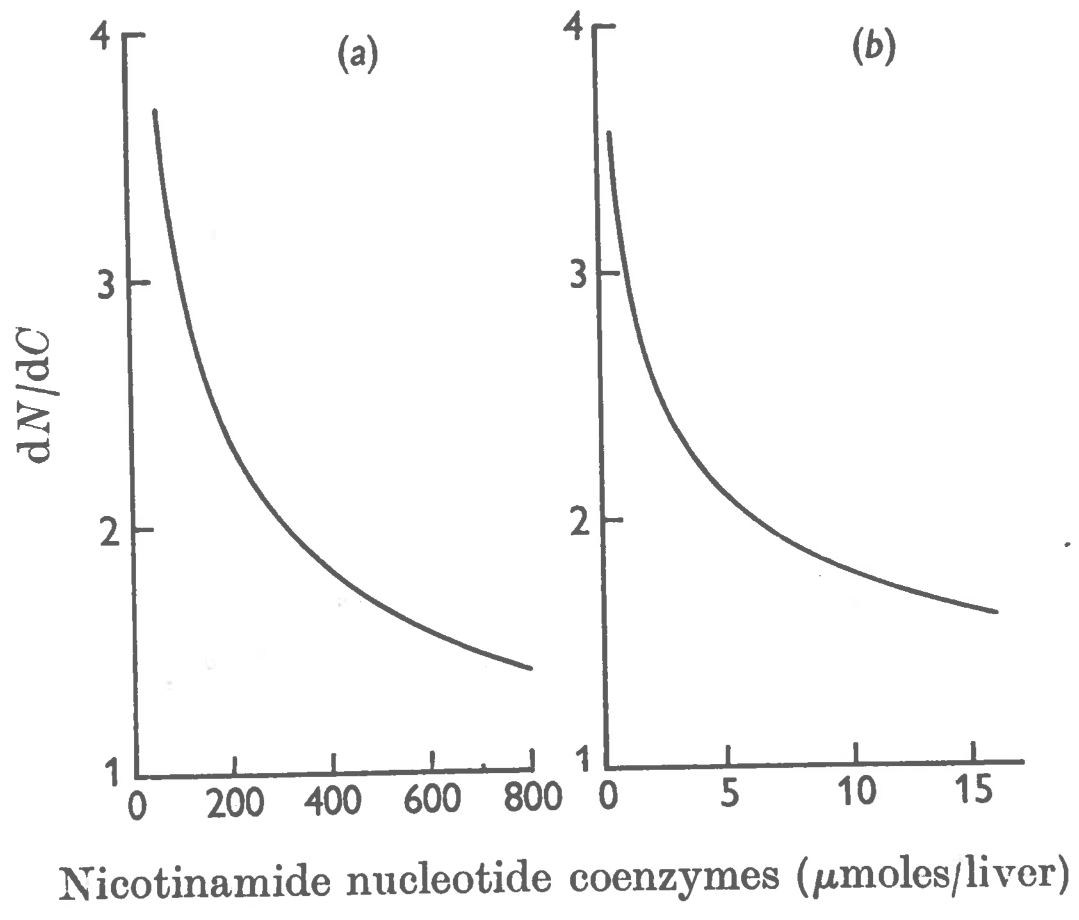
In each case $P < 0.001$.



18
16
14
12
10
8
6
4
2
0
10
20
30
D
E
F
Nicotinamide nucleotide coenzymes
(µmoles/liver)
10⁻⁸ × Nuclei/liver

Figure 7.

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. 4 and 6, curves D). For sheep, $dN/dC = 16.98C^{-0.3722}$; for the rat, $dN/dC = 3.06C^{-0.2313}$.


(a)
(b)
dN/dC
4
3
2
1
0
200
400
600
800
0
5
10
15
Nicotinamide nucleotide coenzymes (μmoles/liver)

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Discussion

Reference bases in liver tissue

A number of studies (see Kosterlitz, 1958) have clearly indicated that in any evaluation of analyses done on liver tissue, the choice of a reference base depends on the type of experiment. In the first instance the data in Table 11, (Series II) were grouped under ages and an analysis of variance between and within age classes 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 calendar age, which makes no allowance for varying growth rates within an animal group. The information gained from the regression analysis of the data in Table 11 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. Regression analysis on the basis of age, although not quite as precise, still gave a highly significant relationship resulting in the same general conclusions.

Since the concentration of coenzyme at the active centre of the relevant enzymes is the most direct factor controlling the rate of enzymic processes involving the coenzyme, concentration/ unit fresh wt. of tissue is probably the best approximation to concentration. However, the coenzymes are not uniformly distributed

in the tissue sample; there is heterogeneity of cell types and inter-cellular spaces exist; within the cell there is also great heterogeneity, and concentrations reported here represent the mean concentration over the whole volume occupied by nuclei, mitochondria, membranes, intercellular spaces etc. This is one of the main problems with work of the type reported here, but reliable methods are not yet available to measure coenzyme concentrations at active centres of enzymes in intact tissue.

In comparison of coenzyme concentrations in tissues, amount/g. fresh wt. has been the most commonly used reference base (see Table 5). 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. 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 amount 'per cell', using the nuclear count as the reference base the value for NAD at 7 days was less than half that obtained for the adult rat (23×10^{-7} μm-moles/

cell) and even after one month had only reached two thirds of the adult level. It is obvious that multiple reference bases are needed for comparative studies.

When a further study of nicotinamide nucleotide coenzymes in the livers of prenatal and newborn lambs was carried out (Series III; Table 12), the same parameters were measured and the dry wt. of the liver and the total number of nuclei per liver were again used as measures of the relative stage of development. In addition, nucleic acid phosphorus was determined for most of the animals used in Series IV (see Table 13).

Little information is available on the DNA content of lamb and sheep liver. Values of 5.4 and 6.1 x 10^{-12}g. DNA/nucleus for livers of two sheep were reported by Vendrely and Vendrely (1949); these results were based on estimations with Dische's (1930) diphenylamine reaction in the nucleic acid extract obtained by modification of Schneider's (1945) and Schmidt and Thannhauser's (1945) procedures. The mean DNA content of the nuclei in the livers of sheep and lambs used in this study (19 animals) was found to be 1.12 x 10^{-12}g. DNA phosphorus/nucleus (equivalent to 11.2 x 10^{-12}g. DNA/nucleus) and is thus much higher than the values obtained by Vendrely and Vendrely (1949). However, values reported by Masters (1963) for DNA phosphorus (236-252 μg./g. fresh wt.) and for the ratio of RNA/DNA (1.64 - 1.92) in the livers of young and adult Merino sheep, agree well with those reported here (see Table 13, and Table 15).

The mean value of DNA phosphorus/nucleus in the livers of six-month old rats (1.12 x 10^{-12}g. see Table 13) was in good agreement with results of other workers (0.91 - 1.14 x 10^{-12}g. DNA phosphorus/nucleus; see Leslie, 1955).

Whereas there was no trend in the amount of DNA phosphorus/nucleus during development in the sheep, the mean value of 1.2 x 10^{-12}g. for livers of rats at six months was higher than the value (0.77 x 10^{-12}g.; see p.83) at one month due to the increased proportion of tetraploid nuclei in the older rats. The expression of coenzyme or enzyme levels as functions of either DNA phosphorus in the liver or of number of nuclei in the liver of sheep is equally valid, but in corresponding studies in rats allowance would have to be made for the increase in the average amount of DNA of the nuclei in the liver during development.

<u>Nicotinamide nucleotide coenzymes and growth</u>

Low nicotinamide nucleotide coenzyme concentrations 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 lower nicotinamide nucleotide coenzyme concentrations than the comparable tissue 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).

If the data of Oide (1958) are taken and the mitotic rate derived and plotted against nicotinamide nucleotide coenzyme concentration for regenerating rat liver, a significant negative correlation is shown (see Morton, 1961; and Fig. 8).

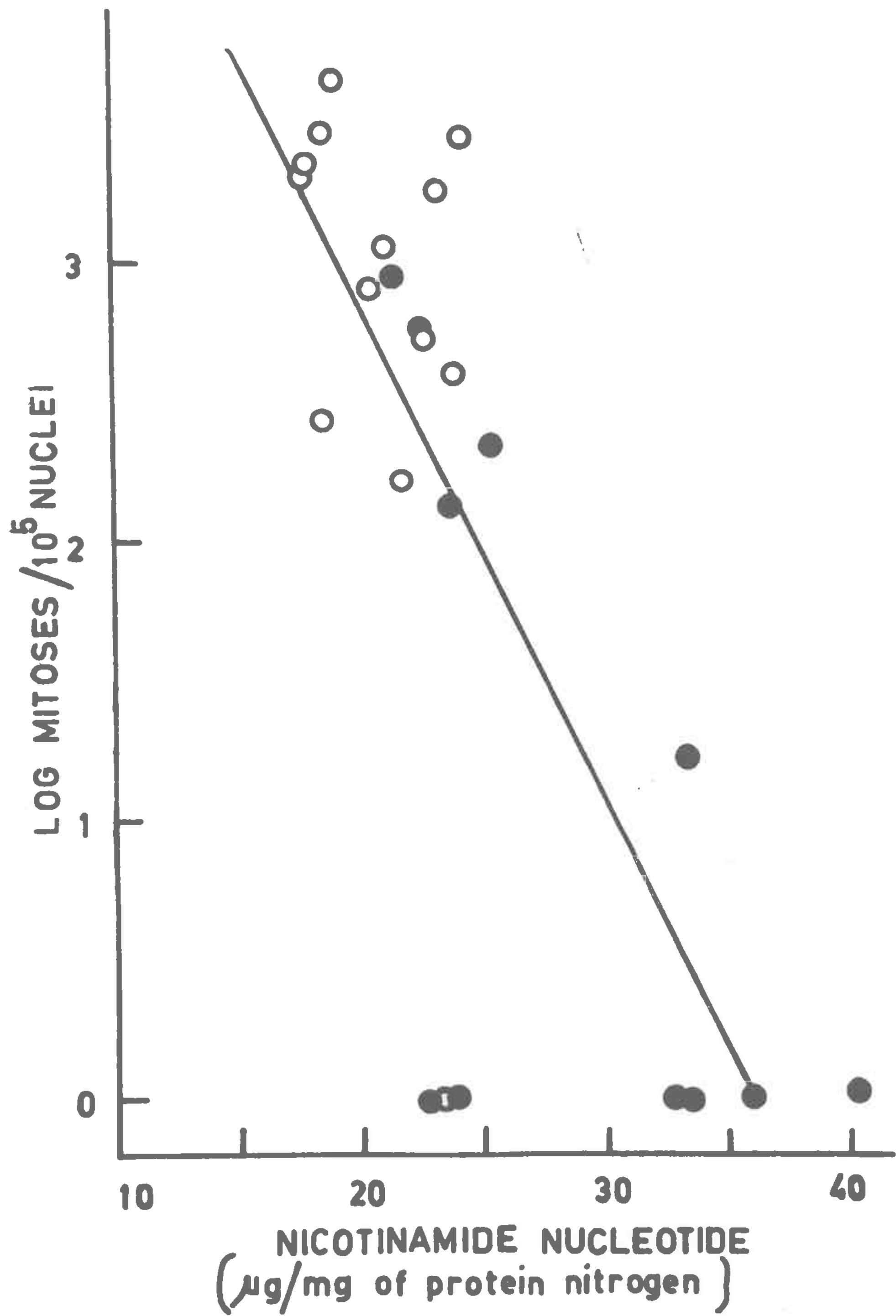
Fujii & Mizuno (1958) also showed that nicotinamide (25 mM) will prevent cleavage of fertilized sea-urchin eggs. On removal of the eggs to fresh nutrient solution, in the absence of nicotinamide, normal cell division is resumed. This suggests that the inhibition of cell division may operate through a specific physiological mechanism rather than resulting from general toxicity through denaturation of proteins.

From Fig. 7 it can be seen that during normal growth of the liver in both sheep and rats total nicotinamide nucleotide coenzyme concentration has reached a maximum value when growth has ceased. The total coenzyme concentration per average cell was found to be of the same order (490 µm-moles/10^8 nuclei) in the adult liver tissue of both rat and sheep. In young animals of both species the concentration was about half this value.

The third group of lambs and sheep (Series III, Table 12) included animals from about two weeks before birth to maturity. The regression equations A - F (see p. 97) that relate coenzyme content to stage of liver development in the sheep, confirm the conclusions from the data of Series II (Table 12; Figs. 3 & 4) which were derived with animals from four days old to maturity.

Figure 8.

Relationship between mitotic rate and concentration of nicotinamide nucleotide coenzymes in regenerating liver. Plotted from the results of Oide (1958) following Morton, (1961). $b = -1.654$; $P < 0.001$. ○, Untreated animals; ●, Animals after implantation of nicotinamide.


LOG MITOSES/10^5 NUCLEI
3
2
1
0
10
20
30
40
NICOTINAMIDE NUCLEOTIDE
(µg/mg of protein nitrogen)

The small difference ($P < 0.05$) in the regression equation B, and the corresponding equation derived in the previous study (Fig. 3, B) is due to a higher concentration of NAD in the livers of animals during the first two weeks after birth. In this study the growth rate of the young lambs was less than in the previous work, probably reflecting a poorer nutritional status of the lambs, which were running with older dams on autumn pasture. It was shown from the data from Series II (see Fig. 7) that the nicotinamide nucleotide coenzyme concentration is lower in a more rapidly growing liver. This later study, (Series III, Table 12) with a larger number of young animals also confirms the relationship between the rate of change of number of nuclei as a function of the change in concentration of total coenzymes in the liver.

The results in this section confirm the earlier observations with other tissues that the total concentration of nicotinamide nucleotide coenzymes increases as the liver grows and the rate of cell division decreases.

It can not be concluded that the rate of division and the coenzyme concentrations are causally related or that either is controlled by any one enzyme involved in the biosynthesis of pyridine nucleotide coenzymes. However, it is likely that experiments to discover the reason for the rise in coenzyme levels as the rate of cell division decreases will provide valuable information on the relationship of nucleic acid and nucleotide metabolism and on the interaction of nucleus and cytoplasm.

One of the main purposes of the programme of study of formation of NAD was the design of inhibitors of the reactions leading to this compound (Morton, 1958). Most emphasis was given to inhibition of the adenylyltransferase, and powerful competitive inhibition of this enzyme by 6-thioinosine 5'-triphosphate was observed (Atkinson, Jackson, Morton & Murray, 1962). There is no known way to generate this compound inside cells and at present it is difficult to envisage specific competitive inhibition of enzymes in cells when both substrates are nucleotides. The low permeability of cells to nucleotides and their rapid hydrolysis by enzymes raises problems in chemotherapy with these compounds.

Recent advances in knowledge of pyridine nucleotide biosynthesis indicate new possibilities of inhibition of this process. Analogues of nicotinic acid are ineffective antimetabolites in animal systems (Woolley, 1963). It is now clear that pathways through quinolinic acid would prevent total inhibition of nicotinic acid nucleotide biosynthesis by inhibitors of the 'salvage' pathway from nicotinamide and nicotinic acid.

SECTION III

Carbohydrate Metabolism and Nicotinamide Nucleotide Coenzymes in the Liver of the Developing Lamb

Introduction

Although observations have been made on the concentrations of nicotinamide nucleotide coenzymes in the tissues of laboratory animals, there appears to be little information on concentrations of the oxidized and reduced forms of these substances in ruminant tissues.

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, 1958a; Gallagher & Buttery, 1959). Moreover, the metabolism of the young lamb more closely resembles that of the non-ruminating animal than that of the 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 inter-relationships between glucose and lower fatty acids has still to be elucidated (Lindsay, 1959; Jarrett & Filsell, 1961; Jarrett, Jones & Potter, 1964). Since nicotinamide nucleotide coenzymes are required for both oxidative metabolism and reductive syntheses, further information is needed on the supply and demand for these coenzymes in animals that predominantly use acetate for both

oxidative metabolism and for lipogenesis (see Annison & Lewis, 1959).

In the first instance, the post-natal lambs and rats of Series II (from 4 days old to maturity) were studied and marked changes in amounts of coenzymes were shown (Table 11). In view of the comparatively high proportion of NAD found in the livers of some peri-natal lambs (Series I, Table 8), it was decided to extend the study of coenzymes to the prenatal and newly born animal.

A number of physiological adjustments take place in the newly born animal. Some of these are associated with cardio-vascular changes (Dawes, 1961; Mott, 1961), and others can be more closely related to metabolism. During the later part of foetal development, the glycogen content of the liver rises to high levels and falls rapidly immediately after birth in many animals (Shelley, 1960, 1961; Ballard & Oliver, 1963; Dawkins, 1963). Similar changes occur in the lipid content of the liver of the guinea pig in the peri-natal period (Raiha, 1961). Dawkins (1959) has shown that respiratory enzymes in the liver of the newborn rat increased in activity in the first few days after birth. In the liver of the prenatal guinea pig, activities of glucuronyl transferase, tryptophan pyrrolase and glucose 6-phosphatase are low, and rise toward adult values soon after birth, whereas that of glucose 6-phosphate dehydrogenase shows a gradual decrease and that of 6-phosphogluconate dehydrogenase shows little change during development (Nemeth & Dickerman, 1960). These changes in relative enzymic activities

and in carbohydrate and lipid contents reflect the changes in functional activities of the liver after birth (see Villee, 1961).

The oxidative pentose phosphate pathway is a potential source of $NADPH_2$ for reductive syntheses (see Dickens, 1961; Lowenstein, 1961), and the formation of this compound by enzymic reduction is important in the control of metabolism (see McLean, 1964). The relative activities of glucose 6-phosphate dehydrogenase and of 6-phosphogluconate dehydrogenase were therefore determined in liver extracts of the lambs, sheep and rats from Series III and IV.

Although the exogenous supply of glucose to the adult sheep is limited, the phosphorylation of glucose is necessary for its subsequent metabolism. Apparent glucokinase activity in extracts of lamb and sheep liver was tested by following reduction of NADP in the presence of added glucose and ATP.

Since considerable differences in nicotinamide nucleotide coenzyme concentrations and in the activities of glucokinase and of glucose 6-phosphate and 6-phosphogluconate dehydrogenases were found in the livers of lambs during development, the effects of intravenous infusion of glucose, acetate and propionate on the levels of these coenzymes and enzymes in the livers of adult sheep were than investigated.

In this section changes in amounts of coenzymes and in enzymic activities are discussed with reference to the intermediary metabolism of the liver in the peri-natal animal and to metabolic

differences between ruminants and monogastric animals.

Experimental

Animals

The lambs, sheep and rats used for the study of change in enzymic activities associated with carbohydrate metabolism during development were those of Series III and IV.

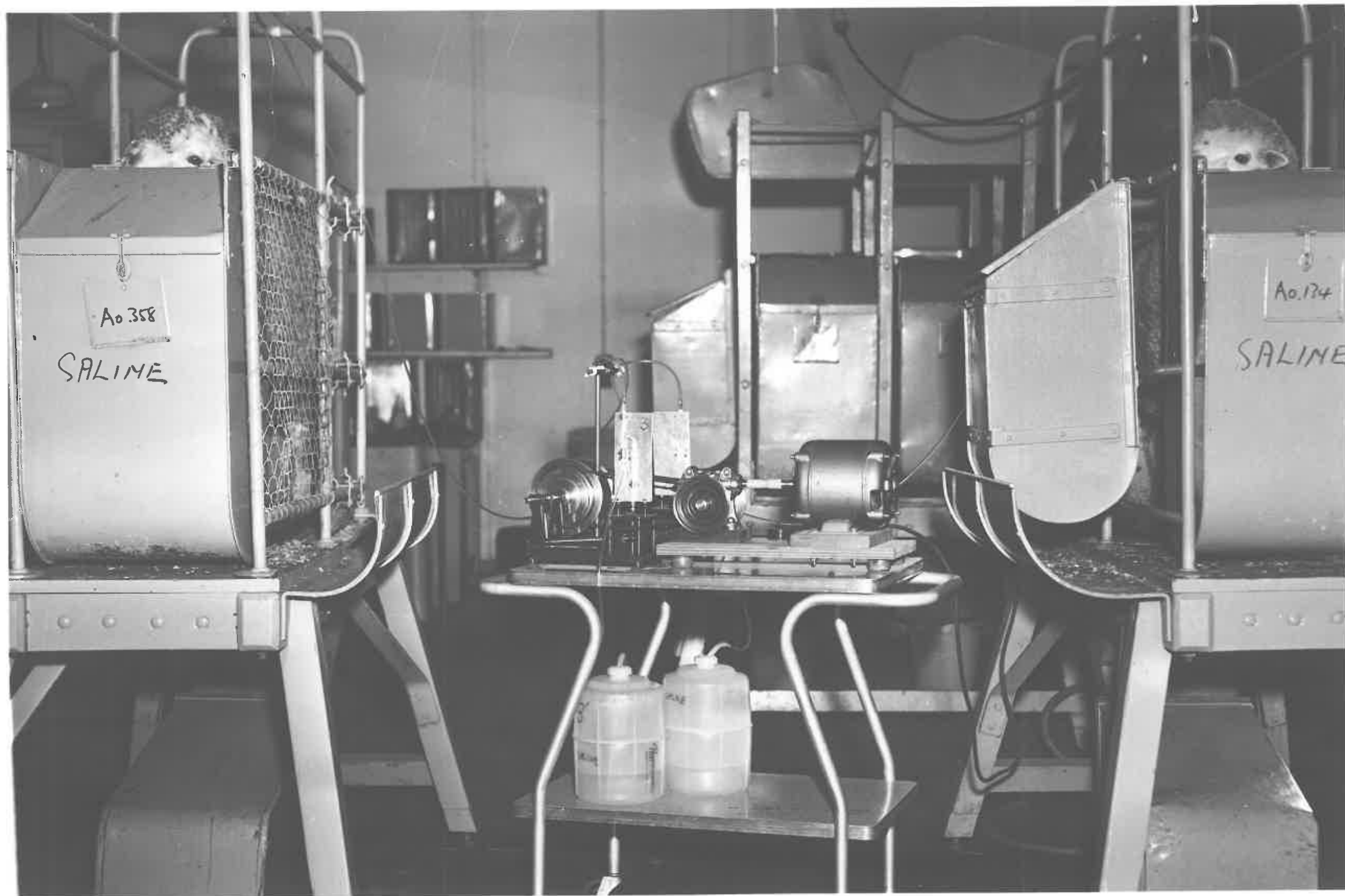
Series V. Eleven Merino ewes, 2 to 4 years old, that had been accustomed to being handled and had been trained to remain placid in metabolism cages were used. They were given 1000 g. of a mixture of two parts of chaffed wheaten hay and one part of chaffed lucerne hay each morning and allowed free access to water.

Technique of infusion. A polythene tube filled with heparin was secured into an external jugular vein and the appropriate solution was infused. A constant rate of infusion (1000 ml. per 24 hr.) was maintained by using a modified Dale-Schuster pump mechanism attached to a glass syringe, fitted with a glass two-way valve (see Plates 2 & 3); the infusion was continued for 10 days.

The solutions used for infusion were 0.155M-sodium chloride (control group), 0.556M-glucose, 1.90M-acetic acid and 1.08M-propionic acid. The sodium chloride solution was isotonic with sheep blood and the acetic and propionic acid solutions were neutralised to pH 7 with sodium hydroxide solution. The amounts of glucose, acetate and propionate were chosen so as to be isocaloric and represent 400 Calories per litre. When infused at the rate of 1 lr./24 hr., these solutions supply approx. 40% of the maintenance requirement of the

Plate 2

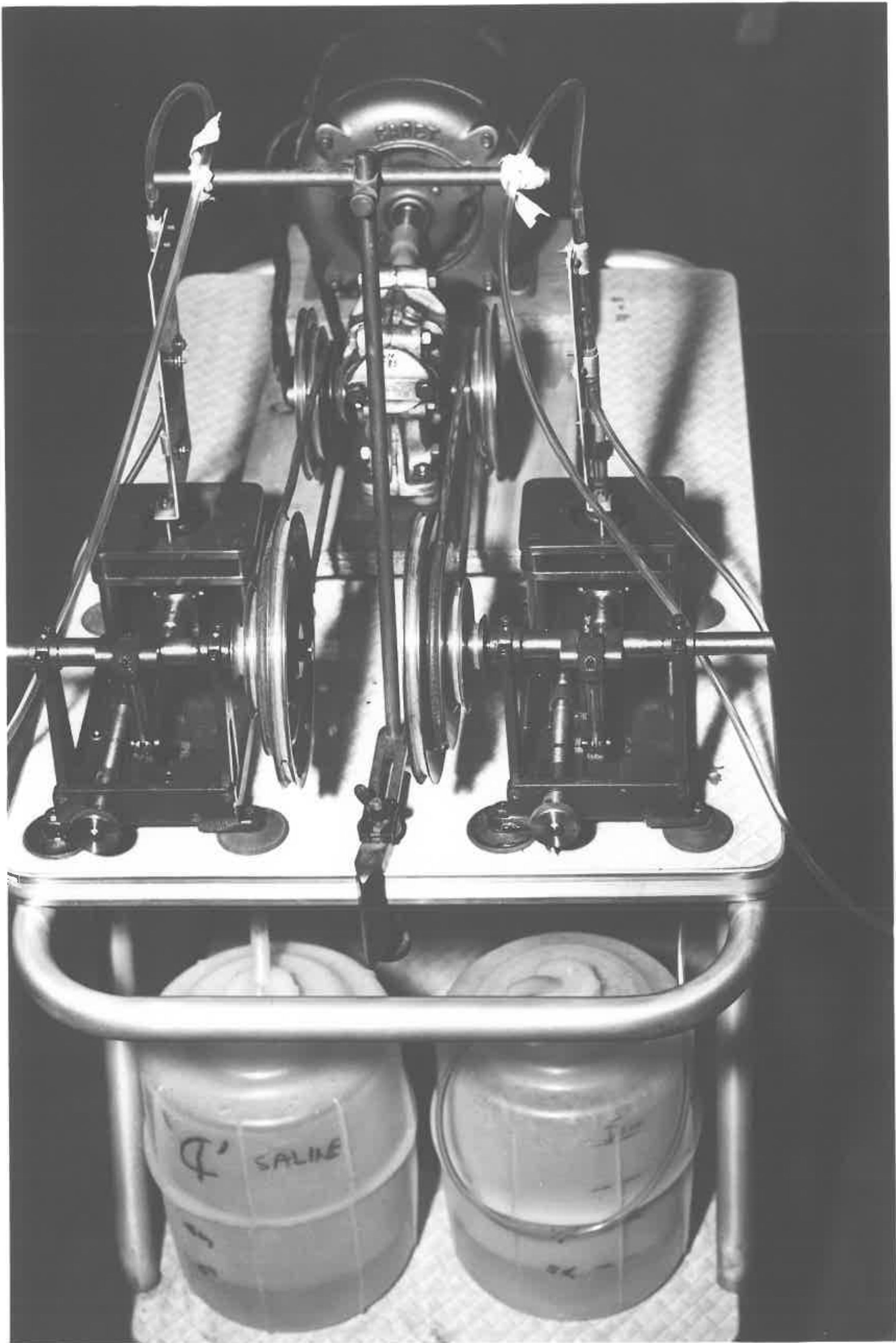
Sheep in metabolism cages during intravenous infusion, showing polythene reservoir and tubing and modified Dale-Schuster pump mechanism.



Ao.358
SALINE
Ao.134
SALINE

Plate 3

Modified Dale-Schuster pump mechanism attached to motor. Glass pump with two-way valves attached by polythene tubing to reservoir and to intravenous catheter.


SALINE

animal under the experimental conditions used (see Marston, 1948).

Assay Procedures

Sampling procedure was the same as that used in Section I.

Extraction and assay of coenzymes and determinations of dry wt., nitrogen, nucleic acids and nuclear count were carried out as in Section II.

Blood glucose was determined on the protein-free filtrate (Somogyi, 1952) by the colorimetric procedure of Nelson (1944).

Total volatile fatty acids in blood were determined by a micro-modification of the method of Friedmann (1938) involving an electrometric titration of the distillate.

Relative enzymic activities. Rates of reduction of NADP with glucose 6-phosphate, 6-phosphogluconate and with glucose and ATP were measured in the presence of tissue extracts; the methods used were based on those of Glock and McLean (1953) and McLean (1958).

Approx. 2 g. of tissue was weighed and transferred to 18 ml. of ice-cold 0.15M-KCl- 0.16mM-$KHCO_3$ in a Potter Elvehjem glass mortar with a teflon pestle (A. H. Thomas & Co., Baltimore, U.S.A.). The tissue was dispersed by hand for 30 sec. and then homogenized at 1000 rev./min. for 1 min. The homogenate was centrifuged at 60,000 g for 30 min. at 2° and the supernatant was dialysed at 2° against the KCl-$KHCO_3$ solution for about 18 hr. The extract was again centrifuged at 28 000 g for 15 min. at 2°. Reduction of NADP was measured at 340 mμ in cuvettes of 1 cm. light path at 25° with a Beckman DK-2A recording spectrophotometer. Each cuvette contained

0.5 ml. of 25mM-glycylglycine-KOH buffer at pH 7.6, 0.5 ml. of 0.1 M-$MgCl_2$, 0.2 ml. of 1.5 mM-NADP and liver extract (0.1 ml. for dehydrogenase activity and 0.2 ml. for glucokinase activity) in a total volume of 2.4 ml. The glucose 6-phosphate, 6-phosphogluconate and glucose respectively were added to the test cuvettes in each of the appropriate assays (see below).

Rate of reduction of NADP with glucose 6-phosphate. The reaction was started by the addition of 0.1 ml. of 50 mM-glucose 6-phosphate. The rate of increase of extinction at 340 mμ was linear for at least 8 min. The results are expressed as μmoles of $NADPH_2$ formed/min./g. fresh wt.

Rate of reduction of NADP with 6-phosphogluconate. The reaction was started by the addition of 0.1 ml. of 0.1 M-6-phosphogluconate. The rate of change of extinction at 340 mμ declined somewhat with time, particularly after the first two minutes. Results were expressed as μmoles of $NADPH_2$ formed/min./g. fresh wt., calculated from the rate of change of extinction in the first 2 min. The same rate was observed on addition of 0.1 ml. of 50 mM-6-phosphogluconate to the test cuvette.

Rate of reduction of NADP with glucose and ATP. ATP (0.4 ml. of 20 mM-solution) was added to both test and blank cuvettes. The reaction was commenced by the addition of 0.1 ml. of 2.5 M-glucose. After a lag period of up to 2 min. the rate of increase of extinction was constant for at least 16 min. The results were expressed as μmoles of $NADPH_2$ formed/min./g. fresh wt. as estimated after the lag period. With lower concentrations of glucose, lower rates were

observed; from the results an apparent Michaelis constant (K_m) for glucose was estimated at 14 mM using a liver homogenate from a 28-day-old lamb. All results shown in Fig. 11 are given for a substrate concentration of 0.1 M-glucose.

Chemicals. Glucose 6-phosphate (sodium salt) and 6-phosphogluconate (sodium salt) were obtained from C.F.Boehringer and Soehne (Germany) and Sigma Chemical Co., (U.S.A.) respectively. Glucose (A.R. grade) was obtained from British Drug Houses Ltd., ATP (sodium salt) was from either C. F. Boehringer and Soehne (Germany) or from California Corpn. for Biochemical Research (U.S.A.).

Results

Coenzyme ratio. From the values of ([NAD] + [$NADH_2$]) and of ([NADP] + [$NADPH_2$]) obtained from the regression equations B and C (Section II, p. 95) for rat liver, the coenzyme ratio, ([NAD]+[$NADH_2$]): ([NADP] + [$NADPH_2$]), was calculated for selected liver dry wts. The plot of these values against liver dry wt. gave the curve shown in Fig 9. Since the regression equations from the studies on lambs and sheep of Series II and III have been shown to be compatible (see p. 97), the results of both series were pooled and regression analysis gave the following equation:

$$y = 3.728 - 3.473x + 0.879x^2,$$

where $y = \log$ ([NAD] + [$NADH_2$]): ([NADP] + [$NADPH_2$]),

and $x = \log$ (liver dry wt., g.).

The curve in Fig. 10 shows these relationships. When the coenzyme ratio was derived from regression equations E and F (see p. 95 and Fig. 6), and the values plotted against number of nuclei, essentially the same curves were obtained.

Enzymic activities. The rates of reduction of NADP in the presence of added glucose and ATP, glucose 6-phosphate and 6-phosphogluconate under the conditions used here reflect the relative activities of glucokinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Fig. 12 and 13 show these activities for lambs and sheep (μmoles $NADPH_2$ produced/min./g. fresh wt.) plotted against liver dry wt. Logarithms were taken of both the dependent and determining variates to produce homoscedasticity

Figure 9

Change in the coenzyme ratio, ([NAD] + [$NADH_2$]) : ([NADP] + [$NADPH_2$]), in the liver during development of the rat. The curve was derived from regression equations B and C (see p. 95).

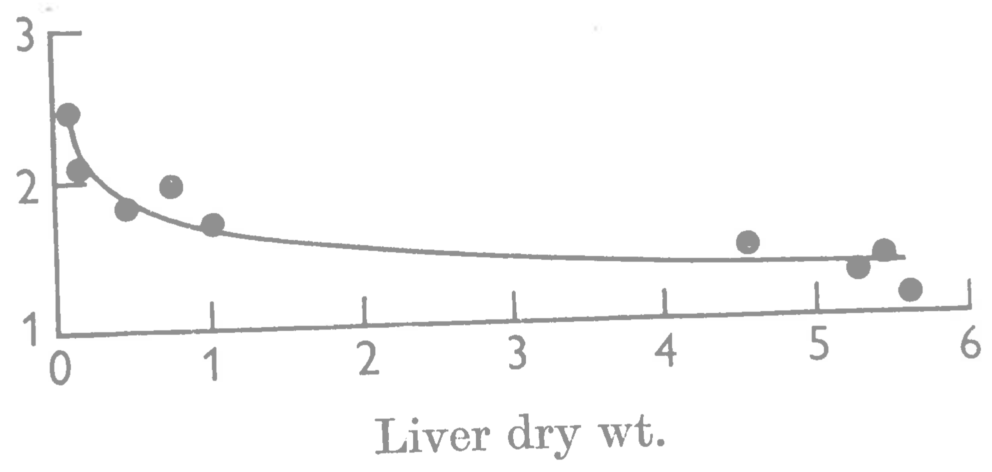

Coenzyme ratio ([NAD]+[NADH₂]) : ([NADP]+[NADPH₂])
3
2
1
0
1
2
3
4
5
6
Liver dry wt.

Figure 10

Change in coenzyme ratio,

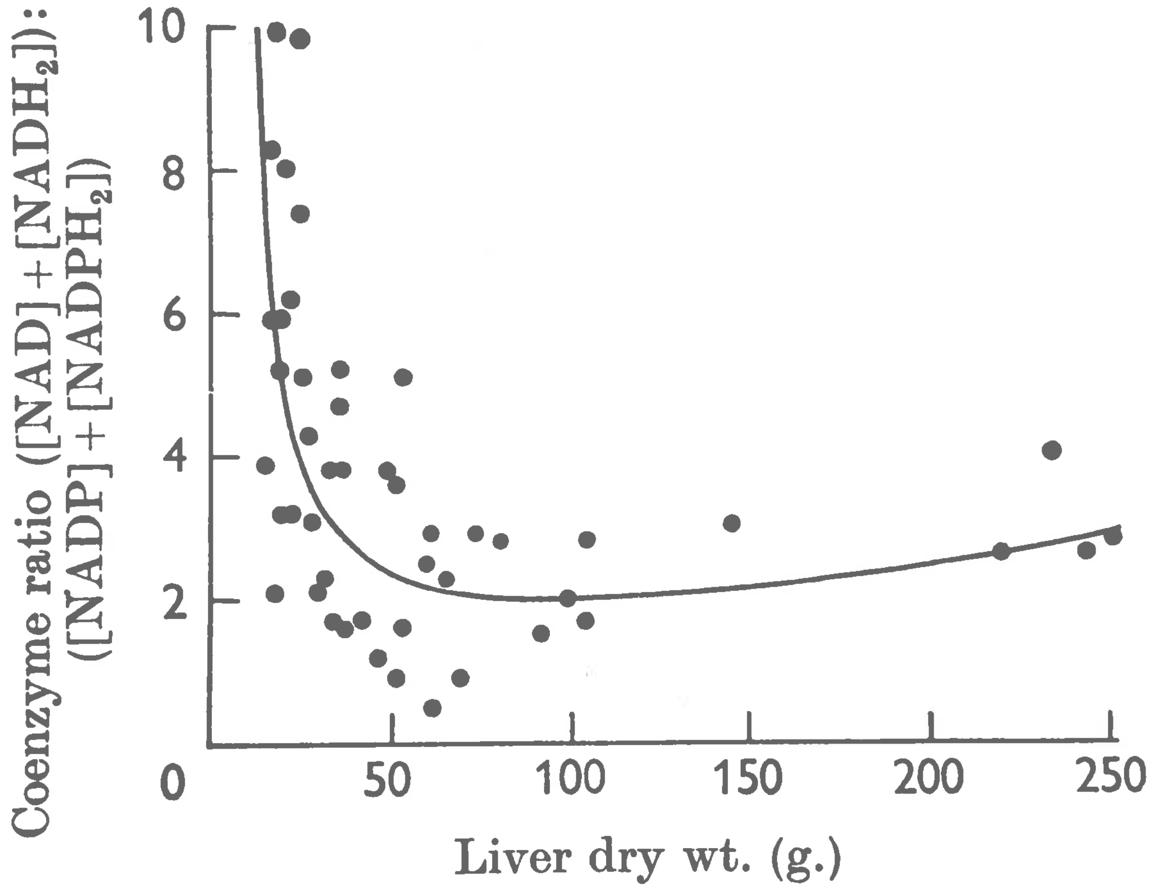
$([NAD] + [NADH_2]) : ([NADP] + [NADPH_2])$, in the liver during development of the sheep. The curve was derived from the regression equation;

$$y = 3.728 - 3.473x + 0.879x^2 ;$$

where $y = \log ([NAD] + [NADH_2]) : ([NADP] + [NADPH_2])$,

$x = \log$ (liver dry wt. (g.)).

$P < 0.001$.



Coenzyme ratio ([NAD]+[NADH$_2$]):
([NADP]+[NADPH$_2$])
10
8
6
4
2
0
50
100
150
200
250
Liver dry wt. (g.)

and linearity and regression analysis gave the curves shown.

The rate of reduction of NADP with 6-phosphogluconate was greater than that with glucose 6-phosphate. The relative activity with 6-phosphogluconate for prenatal and newborn lambs was 3.5-7.0 μmoles $NADPH_2$/min./g. fresh wt. and declined during further development to values ranging from 0.4 - 2.5. The relative activity with glucose 6-phosphate also showed a wide scatter, falling from about 1.1 to about 0.4 μmoles $NADPH_2$/min./g. fresh wt. in the liver of the mature animal. With glucose and ATP the values ranged from 0.3 - 1.0 μmoles $NADPH_2$/min./g. fresh wt. in the prenatal and newly born lambs and fell to 0.01 - 0.05 in mature wethers.

Some of the wide variation in relative enzymic activities in the very young lambs could well have been due to differences in the previous nutritional history of lambs taken at random from the flock.

With extracts of rat liver (see Table 13, for rats listed in order of increasing body wt.) the following values were obtained: with glucose 6-phosphate, 6.25, 5.78, 5.58, 1.55, and 2.55; with 6-phosphogluconate, 1.33, 1.39, 1.32, 1.21 and 1.26; with glucose and ATP, 0.36, 0.33, 0.34, 0.14, and 0.32 μmoles $NADPH_2$/min./g. fresh wt. respectively.

Details of the statistical treatment of these results are given in the Appendix.

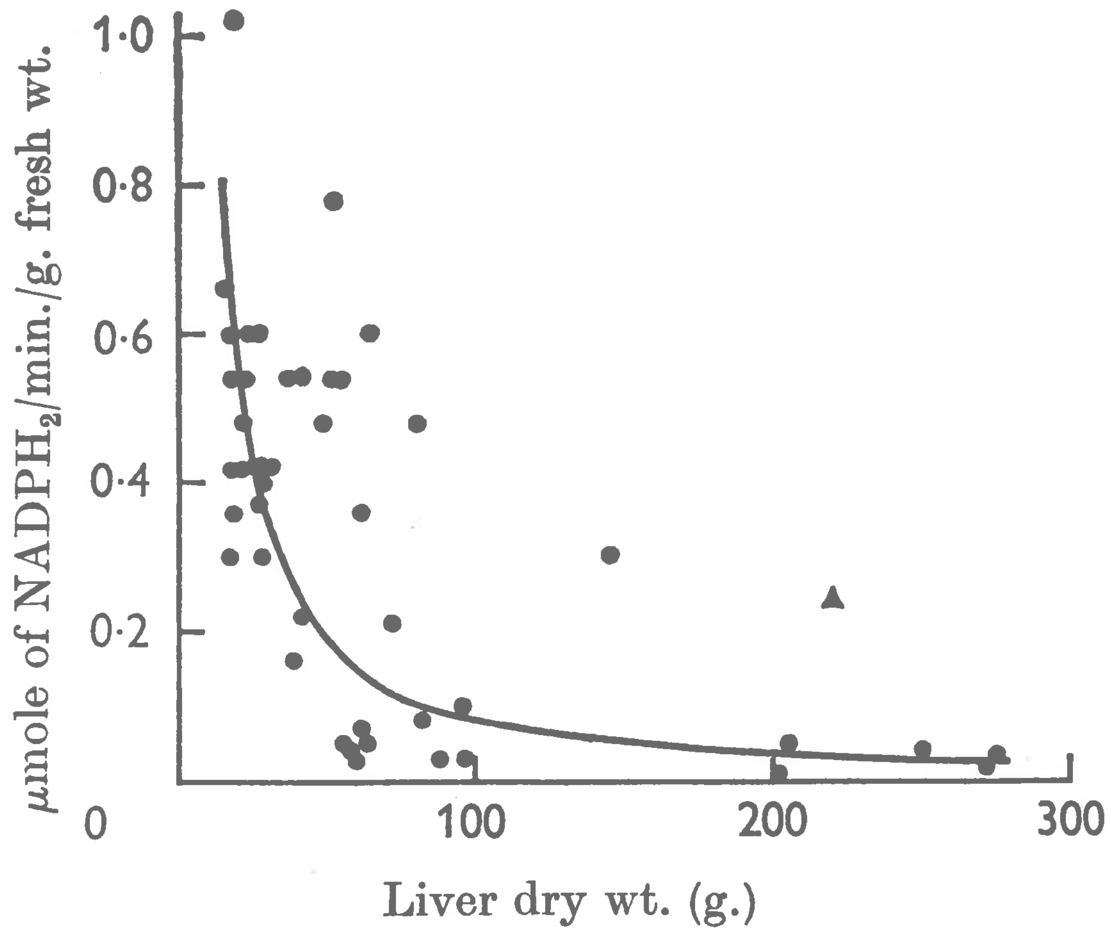
Figure 11

Change in the rate of reduction of NADP, with glucose and ATP by homogenates of sheep and lamb liver during development. The curve was drawn from the regression equation:

$$\log y = 1.1382 - 1.2020 \log x;$$

where y is µmole of $NADPH_2$/min./g. fresh wt., and x is the liver dry wt. (g.); $P < 0.001$.

▲, Value for lactating ewe.



μmole of NADPH₂/min./g. fresh wt.
1·0
0·8
0·6
0·4
0·2
0
100
200
300
Liver dry wt. (g.)

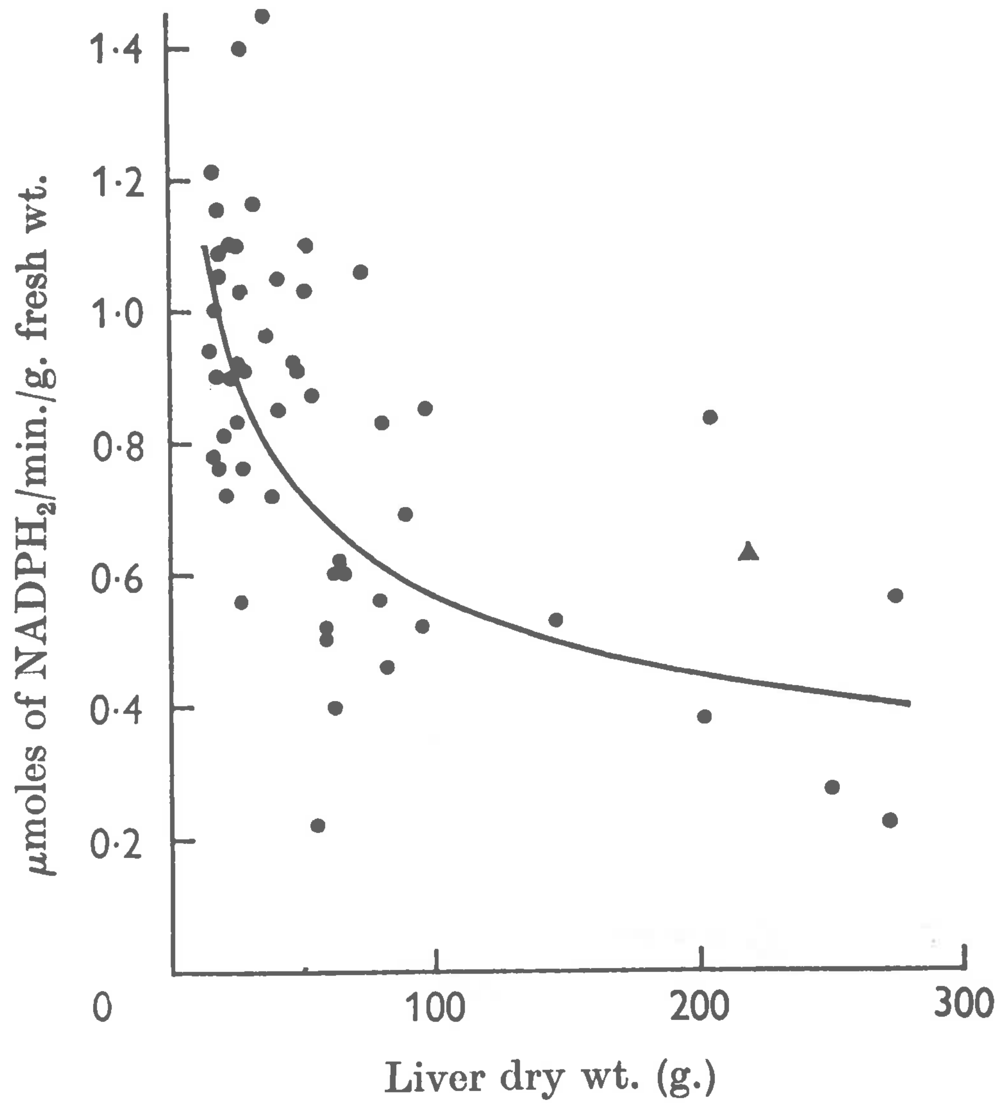
Figure 12

Change in the rate of reduction of NADP with glucose 6-phosphate, by homogenates of sheep and lamb liver during development. The curve was drawn from the regression equation:

$$\log y = 0.4455 - 0.3459 \log x ;$$

where y is µmoles of $NADPH_2$/min./g. fresh wt., and x is the liver dry wt. (g.) $P<0.001$.

▲, Value for lactating ewe.



μmoles of NADPH₂/min./g. fresh wt.
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
100
200
300
Liver dry wt. (g.)

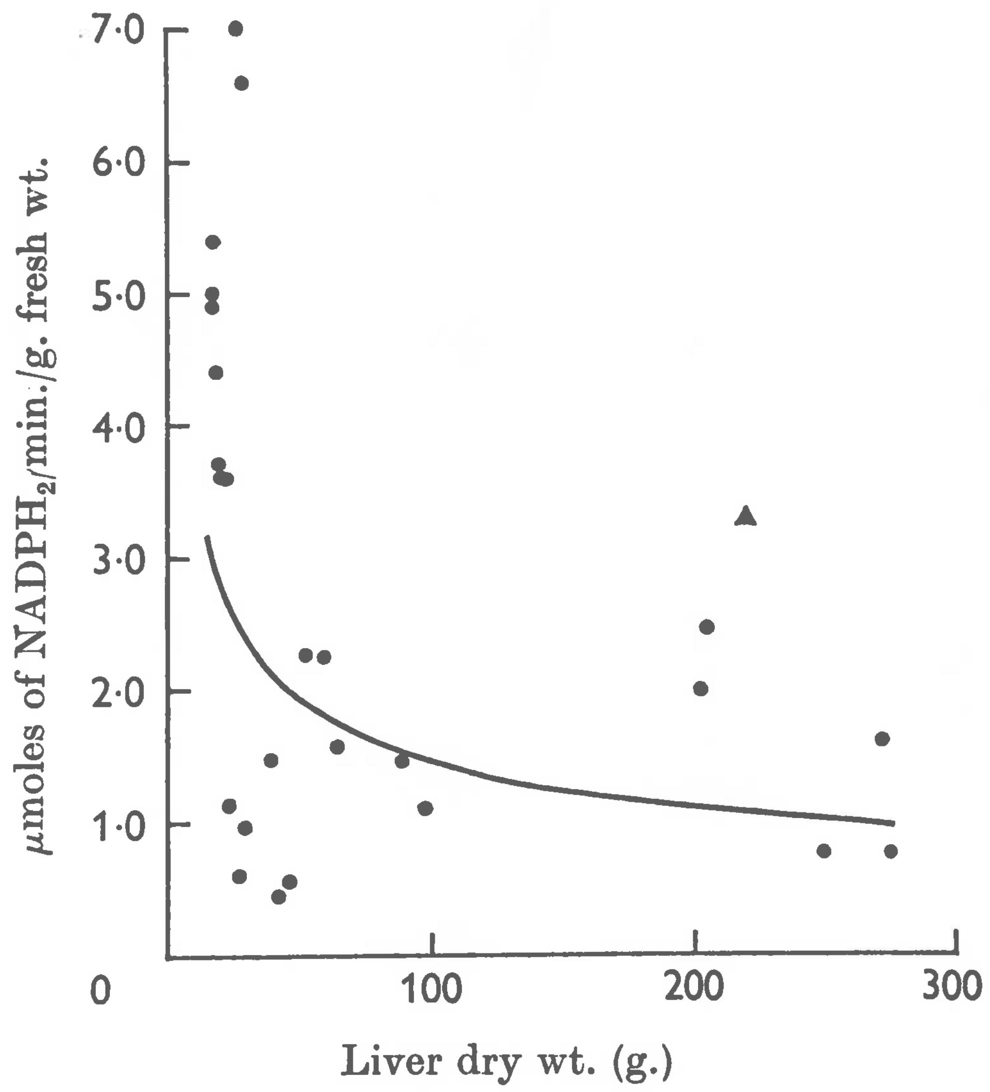
Figure 13

Change in the rate of reduction of NADP with 6-phosphogluconate, by homogenates of sheep and lamb liver during development. The curve was drawn from the regression equation,

$$\log y = 0.9705 - 0.4025 \log x,$$

where y is µmoles of $NADPH_2$/min./g. fresh wt., and x is the liver dry wt. (g.). $P < 0.05$.

▲, Value for lactating ewe.


7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
100
200
300
μmoles of NADPH2/min./g. fresh wt.
Liver dry wt. (g.)

Infusion experiments. Table 15 lists the values found for body wt. liver fresh wt. and dry wt., nitrogen, nucleic acid phosphorus and number of nuclei/g. fresh wt., together with nicotinamide nucleotide coenzyme content and the activities of glucokinase and of glucose 6-phosphate dehydrogenase in the livers of these ewes. For comparison, some data for animals that were not infused, but fed wheaten-hay chaff under the same conditions, are also given.

Samples of blood were taken towards the end of the period of infusion and glucose and total volatile fatty acids were determined. The blood-glucose level in the animals infused with saline varied between 36 and 67 mg./100 ml. and between 34 and 54 mg./100 ml. in the animals that had been infused with the glucose solution. The total volatile fatty acid concentration in the blood of the animals that had been infused with acetate and propionate varied between 0.3 and 1.2 μmoles/lr. These values for glucose and total volatile fatty acids are within the normal range for sheep under these conditions (see Jarrett & Potter, 1957).

	Coenzyme content (μm-moles/g. fresh wt.)							Coenzyme ratio	$NADPH_2$ formed (μmoles/min./g. fresh wt.)	
	[NAD]	$[NADH_2]$	[NAD] + $[NADH_2]$	[NADP]	$[NADPH_2]$	[NADP] + $[NADPH_2]$	Total		with glucose 6-phosphate	with glucose and ATP
A	475	57	532	23	192	215	737	2.48	0.81	0.13
B	770	66	836	75	160	235	1071	3.56	0.84	0.11
C	250	50	300	5	126	131	431	2.29	0.44	0.12
D	342	57	399	16	169	179	578	2.23	0.76	0.18
E	575	28	603	5	215	220	823	2.74	0.62	0.02
F	655	58	713	14	300	314	1027	2.27	0.43	0.01
G	740	44	784	28	215	243	1027	3.22	0.63	0.05
H	820	62	882	5	244	249	1131	3.54	0.81	0.07
I	718	29	747	18	245	263	1010	2.84	-	-
J	735	83	818	19	280	299	1117	2.74	-	-
K	761	81	842	42	327	369	1211	2.28	-	-

Discussion

Intermediary metabolism in the liver of the developing lamb

A number of studies on the development of enzyme systems in the maturing mammalian liver have indicated a changing pattern to enable the liver to perform its important role of metabolic homoeostasis after birth. In the later stage of foetal development liver metabolism is characterised by an increasing rate of synthesis and deposition of glycogen, the concentration of which falls rapidly in the first few hours after birth and after remaining low for several days, gradually rises towards the adult level (Villee, 1954; Shelley, 1960, 1961; Ballard & Oliver, 1963; Dawkins, 1963). The ability of the liver to regulate blood-glucose concentration is linked with the development of glucose 6-phosphatase activity, which appears during the latter part of gestation and increases rapidly immediately after birth in all species that have been examined e.g., rat, rabbit, guinea pig, sheep, man (see Dawkins, 1961, 1963).

The phosphorylation of glucose is necessary for its subsequent metabolism by tissues and the occurrence of different forms of glucokinase (E C 2.7.1.2.) in mammalian liver has been shown. These forms may be distinguished by chromatographic behaviour (Moore & Angeletti, 1961), apparent Michaelis constant for glucose (Vinuela, Salas & Sols, 1963; Walker, 1963; Walker & Rao, 1964; Ballard & Oliver, 1964), and pH optimum (Ballard &

Oliver, 1964).

In the guinea pig and the rat, one form of this enzyme occurs in foetal liver and a second form appears soon after birth (Walker, 1962, 1963; Ballard & Oliver, 1964). Both of these 'foetal' and 'adult' isoenzymes of glucokinase are present in the adult liver. The kinetic properties of these forms in different species are summarised in Table 16.

The 'foetal' form of the enzyme is a relatively non-specific hexokinase (of the type ATP:D-hexose 6-phosphotransferase, E C 2.7.1.1), and the 'adult' isoenzyme is more specific (of the type ATP:D-glucose 6-phosphotransferase, E C 2.7.1.2) (see Vinuela *et al.*, 1963; Walker & Rao, 1964). The relative activities of these two isoenzymes in the liver of the rat change following starvation (Vinuela *et al.* 1963), and in alloxan diabetes (Cahill, Ashmore, Earl & Zottu, 1958). This change is due to decrease in the activity of the 'adult' isoenzyme (Oliver & Cooke, 1964).

The liver cell is freely permeable to glucose (see Cahill *et al.* 1958) and the initial phosphorylation of glucose appears to be a rate-limiting step in glucose utilization by the liver (Di Pietro *et al.* 1962; Walker & Rao, 1964; Ballard & Oliver, 1964). The Michaelis constant of the 'adult' glucokinase (10-30m*M*) is such that large variations in the rate of glucose phosphorylation would occur with change in blood glucose concentration over the normal range of about 3-10 m*M*.

Although the supply of exogenous glucose to the liver of

the mature ruminant is limited, it has been possible to show glucokinase activity in homogenates from the livers of adult sheep as well as in prenatal, newly born and postnatal lambs (see Fig. 11). Gallagher & Buttery (1959) were unable to demonstrate hexokinase activity in liver homogenates from newly born lambs and from adult sheep. This failure would appear to be for two reasons. Firstly, there is a high glucose 6-phosphatase activity present in homogenates of ruminant liver, causing rapid hydrolysis of glucose 6-phosphate formed via the glucokinase reaction (Raggi, Kronfeld & Kleiber, 1960). This glucose 6-phosphatase activity is associated with the microsomal fraction and may be removed by high-speed centrifugation; Gallagher & Buttery (1959) used centrifugation at 9 000g whereas centrifugation at 60 000g was used in the experiments reported here. (see Di Pietro et al. 1962; Walker, 1962; Raggi, Kronfeld, Bartley & luick, 1963).

Secondly, Gallagher & Buttery (1959) used a glucose concentration of 10 mM, whereas the apparent K_m for this enzyme, from the liver of a 28-day-old lamb, was 14mM (see p. 115).

The values obtained for glucokinase activity in homogenates from the livers of foetal and newly born lambs (Fig. 11) were of the same order as those found in adult rats (0.14-0.36 µmoles of $NADPH_2$/min./g. fresh wt.) and much higher than those in adult wethers. Raggi et al. (1963), using similar method, have reported soluble glucokinase activity in the liver of the cow (0.17 µmole of $NADPH_2$/min./g. fresh wt.) which is similar to the activity found here for a lactating ewe (see Fig. 11).

Ballard & Oliver (1964) have shown that glucokinase activity in the liver of the rat rises during development from the foetus to the adult, the change being due to the increase in activity of the 'adult' isoenzyme, which has a high K_m for glucose (cf. Table 16). During development of the lamb from foetus to adult, a marked fall occurs in apparent glucokinase activity of the liver (see Fig. 11); it is suggested that this fall is due to decrease in activity of a glucokinase which has a high K_m for glucose. Jarrett & Filsell (1958) have shown that the apparent hexokinase activity, as measured by the ability of intestinal mucosa to utilize glucose, is greater in 2-day-old lambs and adult rats than in older lambs and adult sheep.

Glucose 6-phosphate may be oxidized via the glycolytic and pentose phosphate pathways, both of which operate from an early stage of development in foetal liver (Villee & Loring, 1961). Figs. 12 & 13 show that the relative activities of the first two enzymes involved in the oxidative pentose phosphate pathway, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, decrease during development of the liver in sheep. Nemeth and Dickerman (1960) have reported a similar fall in activity of glucose 6-phosphate dehydrogenase during development of guinea-pig liver with little change in activity of 6-phosphogluconate dehydrogenase.

With adult rats the rates of reduction of NADP with 6-phosphogluconate were less than with glucose 6-phosphate in contrast to the lambs and sheep. The values obtained with the

<u>Table 16</u>. <u>Kinetic properties of glucokinase isoenzymes of mammalian liver</u>

Reference	Species	Apparent $K_{\underline{m}}$ for glucose	
		'foetal'	'adult'
Lange & Kohn (1961)	rat	40 μ*M*	-
Di Pietro *et al.* (1962)	rat	-	10 - 40 m*M*
Walker (1962)	guinea pig	30 μ*M*	30 m*M*
Walker (1963)	rat	37 μ*M*	10 m*M*
Vinuela *et al.* (1963)	rat	10 μ*M*	10 m*M*
Ballard & Oliver (1964)	rat	50 - 80 μ*M* (pH optimum, 9.0)	10 - 30 m*M* (pH optimum, 7.0)

liver from a lactating ewe (24 days) are shown in Figs. 12 & 13 and are in each case greater than those for the adult wethers; they were of the same order as those reported by McLean (1958).

These higher apparent enzymic activities in the young lamb are consistent with the hypothesis that many aspects of the carbohydrate metabolism of these animals differ from those of the adult sheep and more closely resemble those of non-ruminants (Jarrett & Potter, 1952; Jarrett & Filsell, 1960, 1964; Jarrett, Jones & Potter, 1964). The decrease in relative activities of glucokinase, of glucose 6-phosphate dehydrogenase and of 6-phosphogluconate dehydrogenase during growth of the lamb is consistent with the changing pattern of metabolism during development in ruminants.

Of the total nicotinamide nucleotide coenzymes found in the livers of sheep and rats in this study, the concentration of NAD was always greater than that of $NADH_2$ and the concentration of $NADPH_2$ 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 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. It is apparent that a higher NAD concentration was present in the livers of prenatal and newborn lambs than in lambs more than one day postnatal (see Tables 11 & 12, p. 86a & 88a) and regression equations B, p. 97. Since hypoxia may be

one of the main dangers to the animal at this time (Dawes, 1961) a high ratio of [NAD] : [$NADH_2$] would facilitate anaerobic glycolysis under conditions where anoxia limits the reoxidation of $NADH_2$. The relative resistance of newborn lambs to hypoxia has been clearly demonstrated (Mott, 1961).

When solutions of saline, glucose, acetate and propionate were infused intravenously for 10 days into ewes, there was a decrease in total nicotinamide nucleotide coenzyme concentration of the livers of the animals infused with glucose (see Table 15). This change was largely due to a decrease in concentration of NAD but the groups of animals used were not sufficiently large to allow estimation of the statistical significance of the difference. In these animals (C & D, Table 15) there was some rise in glucokinase activity of the liver. The much lower levels of glucokinase activity in the livers of the ewes that were infused with acetate (E & F, Table 15) may be significant but this requires further investigation.

Information on the levels of nicotinamide nucleotide coenzymes in the tissues of ruminants may be of value in understanding the aetiology of bovine ketosis and pregnancy toxaemia in the sheep. Kronfeld & Kleiber (1959) have suggested that the metabolic defects in acetonaemia are most simply explained by a shortage of both oxidized and reduced forms of NAD and of NADP. Kronfeld & Raggi (1964) have shown that the concentrations of NAD, $NADH_2$, NADP and $NADPH_2$ in mammary tissues of ketotic cows are reduced to about half

of the concentrations found in the mammary tissues of normal cows (values expressed as µm-moles/g. protein).

Patterson (1963) has determined the concentrations of nicotinamide nucleotide coenzymes in the livers of healthy pregnant and lactating sheep and found values similar to those reported here (see Table 12, p.88a). Moreover, no significant difference could be shown in activities of isocitrate dehydrogenase, of glucose 6-phosphate dehydrogenase and of 6-phosphogluconate dehydrogenase of liver tissue and from other groups of well-nourished and under-nourished pregnant ewes. A moderate degree of ketonaemia (more than 10 mg. 'acetone'/100 ml. of plasma) was established in the under-nourished group. These results showed that levels of $NADPH_2$ in liver tissue are not markedly low late in pregnancy and also that the capacity of the liver tissue for production of $NADPH_2$ is not decreased in hyperketonaemic pregnant ewes (which did not show clinical symptoms of pregnancy toxaemia). Further data are obviously required before the role of the pyridine nucleotide coenzymes in these diseases can be clarified.

Although the total coenzyme concentration/average cell was of the same order in the liver tissue of both young rats and lambs (250 µm-moles/10^8 nuclei) and rose to about twice this value in the adults of both species, the coenzyme ratio, $([NAD] + [NADH_2]) : ([NADP] + [NADPH_2])$, showed a marked fall during development (see Figs. 9 & 10). The coenzyme ratio is particularly high in the livers of the prenatal and newborn lambs, falling from 6-10 to 2-4

in the older lambs and adult sheep (Fig. 10). A similar decline occurs in the coenzyme ratio of liver tissue during development of the rat, falling from about 2.5 in the 4-day-old animal to about 1.3 in the adult.

Calculations from the data of Nemeth & Dickerman (1960) indicate a similar fall in coenzyme ratio in the liver of the guinea pig during development. Villee (1962) has reported values for nicotinamide nucleotide coenzymes in human foetal liver and these data indicated a coenzyme ratio of about 8. Roux, Gordon, Dinnerstein and Romney (1962) have determined these coenzymes in the liver of the rabbit during development but the change in coenzyme ratio cannot be calculated from the information available.

It would appear that a high coenzyme ratio is characteristic of mammalian liver in the prenatal and newborn animal, falling rapidly around the time of birth and then attaining a characteristic value for adult liver.

The difference in coenzyme ratio between adult sheep and rats is largely due to a greater amount of NAD in the liver of the sheep and of $NADPH_2$ in the liver of the rat (cf. Table 11). It is suggested that differences in relative amounts of pyridine nucleotide coenzymes in a tissue may be related to emphasis on particluar metabolic pathways. The coenzyme content of various tissues of the rat and guinea pig is given in Table 7.

It is clear from this table that the coenzyme ratio is relatively high in tissues where there is an emphasis on oxidative

Table 17. Nicotinamide nucleotide coenzyme content of rat and guinea-pig tissues (Glock & McLean, 1955b)

Tissue	Coenzyme content (μm-moles/g.fresh wt.)					Coenzyme ratio*
	[NAD]	[$NADH_2$]	[NADP]	[$NADPH_2$]	Total	
Rat						
diaphragm	435	207	< 3	17	661	33
brain	200	132	< 3	10	344	27
pancreas	173	117	< 3	16	298	16
cardiac muscle	447	273	5	43	773	15
kidney	334	318	4	70	726	8.8
mammary gland (18 days lactation)	340	125	< 3	66	533	6.8
adrenal	473	231	22	151	877	4.1
liver	555	306	8	267	1136	3.1
Guinea pig						
ovary	321	57	8	150	536	2.4

* Coenzyme ratio = ([NAD] + [$NADH_2$]) : ([NADP] + [$NADPH_2$])

metabolism e.g. muscle and brain, and much lower in those tissues where lipid synthesis is more important e.g. adrenal and ovary. In lactating mammary gland, where oxidative metabolism and lipid synthesis are both occurring extensively, the coenzyme ratio has an intermediate value.

McLean (1958b) has shown that during the lactation cycle in the rat, the changes in nicotinamide nucleotide coenzyme content of the mammary gland (largely in NAD and $NADPH_2$) are paralleled by changes in enzymic activities, notably of glucose 6-phosphate dehydrogenase and of 6-phosphogluconate dehydrogenase. Further studies (McLean, 1964; Greenbaum & Darby, 1964) have shown a close relationship between the oxidative pentose phosphate pathway and fatty acid synthesis in this tissue.

For the rat, the lower coenzyme ratio of liver tissue, due to a higher concentration of $NADPH_2$, may be associated with the relatively greater activity of the oxidative pentose phosphate pathway as compared to that in the sheep (see McLean 1958a; Raggi et al. 1961).

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; Holdsworth, Neville, Nader, Jarrett & Filsell, 1964). 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; Holdsworth et al. 1964). It may be that the relatively higher amount of NAD in sheep liver is related to this activity. From a study of developmental changes in lactic dehydrogenases, Fine, Kaplan & Kuftinec (1963) have suggested that oxidative metabolism may be greater in the livers of ruminants than in those of other mammals.

It is suggested that the difference in proportions of NAD and $NADPH_2$ shown here reflect a comparative emphasis on oxidative metabolism in the liver of the sheep and on reductive syntheses in the liver of the rat.

SECTION IV

Synthesis and Properties of Nicotinic Acid Nucleotides

Introduction

Atkinson & Morton's (1960) method for the preparation ofnicotinic acid nucleotide and of nicotinic acid-adenine dinucleotide gave a more direct approach to these compounds than was previously available (see General Introduction pp.10-12). Since these compounds are intermediates in the biosynthesis of NAD, it was necessary to show that the products obtained by this new method were identical with the naturally occurring compounds.

In this section, methods for the preparation of nicotinic acid nucleotide and nicotinic acid-adenine dinucleotide are described. Nicotinic acid nucleotide and ATP were converted quantitatively into nicotinic acid-adenine dinucleotide and pyrophosphate using ATP:NMN-adenylyltransferase. This dinucleotide was also prepared by a modification of the method of Lamborg et al. (1958) and the dinucleotides prepared by these two methods had the same R_F in two solvent systems and were converted into NAD by NAD synthetase.

The preparation of pure nicotinic acid-hypoxanthine dinucleotide is described and the quantitative cleavage of the pyrophosphate bond of nicotinic acid-adenine dinucleotide by acetic anhydride in pyridine is shown.

The possibility that nicotinic acid-adenine dinucleotide is a substrate of NAD kinase, or inhibits this enzyme was also investigated. The work described in this section was carried out in collaboration with Dr. M. R. Atkinson.

Enzyme preparations

Nucleotide pyrophosphatase (dinucleotide nucleotidehydrolase; EC 3.6.1.9) from potato was prepared by the method of Atkinson, Jackson & Morton (1961). The product had an activity of 1 μmole of NAD hydrolysed/min./ml.; the protein concentration was 8 mg./ml.

ATP:NMN-adenylyltransferase (EC 2.7.7.1) was prepared from pig-liver nuclei as described by Atkinson, Jackson & Morton (1961). The preparation had an activity of 15 μmoles of NAD synthesized/min./ml. and contained 18 mg. of protein/ml.

NAD kinase (ATP:NAD 2'-phosphotransferase; EC 2.7.1.23) prepared from pigeon liver by the method of Wang, Kaplan & Stoltzenbach (1954) was obtained from Sigma Chemical Co.. The product had an activity of 93 μm-moles of NADP formed/hr./mg. protein at pH 7.5 and 37°; the protein concentration was 6 mg./ml.

NAD(P) glycohydrolase (NAD(P) nucleosidase; EC 3.2.2.6) was prepared from pig brain by the method of Zatman, Kaplan & Colowick (1954). The preparation had an activity of 7 μmoles of NAD hydrolysed/hr./ml. at pH 7.6 and 37°.

NAD glycohydrolase (NAD nucleosidase; EC 3.2.2.5) was prepared from Neurospora crassa (wild type) by Kaplan's (1955) method by Dr. J. F. Jackson. At pH 7.2 and 37° the product hydrolysed 12 μmoles of NAD/min./mg. of protein.

NAD synthetase (deamido-NAD: L-glutamine amidoligase

(AMP); EC 6.3.5.1) was prepared from pig liver by Preiss & Handler's (1958b) method.

Other enzymes and chemicals were from the sources given in Sections I and III (pp. 35 and 115).

Results and Discussion

(i) Preparation of nicotinamide nucleotide from nicotinamide-adenine dinucleotide with nucleotide pyrophosphatase from potato

Nicotinamide-adenine dinucleotide tetrahydrate (1.0 g., about 1.35 m-moles) was dissolved in water (10 ml.) and titrated to pH 7.2 at 20° with 3.5N potassium hydroxide in the Radiometer Automatic Recording Titrater. The solution was kept at pH 7 with potassium hydroxide after addition of 12 ml. of potato nucleotide pyrophosphatase. Hydrolysis of the pyrophosphate bond resulted in a corresponding consumption of alkali. The reaction was complete in 90 hr., 0.85 ml. of alkali having been used.

Assay with alkaline ethanol and yeast alcohol dehydrogenase showed that not more than 0.25 μmole of NAD remained after hydrolysis. The product had 7.78×10^3 A.U. 325/CN^-*, corresponding to 1232 μmoles of nicotinamide nucleotide (c. 90%) calculated from a millimolar extinction coefficient of the cyanide complex of 6.3 at 325 mμ (Jackson, 1960).

The solution was cooled to 0°, mixed with perchloric acid (72%; 3ml.) and centrifuged. The residue was washed with cold water (5 ml.). The combined solutions contained 5.42×10^3 A.U. 325/CN^-. After being shaken for 1 hour at 2° with Nuchar C (3 g.) the solution contained 758 A.U. 325/CN^- and after treatment for 30 minutes with a further 1 g. of Nuchar C the solution contained 255 A.U. 325/CN^-

* A.U./CN^- = absorbance in M-KCN x dilution x volume, with a cuvette of 1 cm. light path.

(5% of the material in the original supernatant). The charcoal was washed with water (5 x 100 ml.) at 2° and washed four times with portions of ethanol-amyl alcohol-water (5:5:2).

Extract no.	Volume	A.U.325/CN⁻
1	65 ml.	4230
2	19	243
3	53	352
		4825 (93% of possible units)

The extracts were evaporated at 25° and poured onto a column of Dowex-1 acetate (10 cm. x 6 cm.^{2}; x4; 200-400 mesh) and displaced with water at 2°. Fractions 2-8 (15 ml.) contained material (R_F0.75 - 0.80 in Preiss and Handler's (1958a) solvent B) which absorbed light at 254 mμ, and showed blue fluorescence in light of 360 mμ wavelength after exposure to ammonia and ethyl-methyl ketone. No other material absorbing light at 254 mμ was present in these fractions. Material which absorbed at 254 mμ but gave no fluorescent compound was present in fractions 12 - 18.

Fractions 2-9 were evaporated at 20-25° and the residue was made up to 10 ml. in water and contained 3.42 x 10^3 A.U.325/CN⁻ (543 μmoles). Recovery of nicotinamide nucleotide from the perchloric acid extract was about 63% and from the dinucleotide about 40%.

(ii) <u>Conversion of nicotinamide nucleotide into nicotinic acid nucleotide with nitrous anhydride</u>

Nicotinamide nucleotide (900 μmoles) was dried at 20°/0.1 mm.

for 16 hours, suspended in acetic acid (20 ml.) and cooled in an ice bath. Dry nitrous anhydride from arsenious oxide (50 g.) and nitric acid (50 ml.) was passed into the cooled solution. For 5 min. the solution was green, and gas was evolved at the surface of the insoluble solid. Gas evolution then ceased, and the solution became brown. After 15 min. volatile material was removed in a stream of dry air at 20°, and the white powder remaining in the flask was stored at -10°.

Chromatography of this material on a Dowex-1 formate column (13 cm. x 6 cm.^{2}; x 8; 200-400 mesh) without neutralisation of the nucleotide did not give complete retention of nicotinic acid nucleotide on the resin.

The product was washed in with water (50 ml.) and then eluted with a linear gradient of 200 ml. of M-formic acid added to 200 ml. of 0.2 M-formic acid. Material with absorption at 266 mμ was displaced from the column between 0 and 100 ml. and between 135 and 360 ml. These fractions were freeze-dried separately and dissolved in 12 ml. and 15 ml. of water respectively. Material from the first fraction had maximum extinction at 322 mμ in M-potassium cyanide (3.36 x 10^3 A.U.); that from the second fraction had maximum extinction at 315-320 mμ (544 A.U.).

The first fraction was freeze-dried again and finally dried over potassium hydroxide and sulphuric acid at 2°/0.01 mm. The residue dissolved completely in acetic acid (20 ml.); in molar aqueous potassium cyanide the solution had maximum extinction at 320 mμ (3.48 x 10^3 A.U.) and an absorption minimum at 286 mμ.

In water the material had maximum extinction at 267 mμ (2.75 x 10^3 A.U.). The solution was treated with nitrous anhydride as before. The product had maximum extinction at 315 mμ in M-potassium cyanide. It was dissolved in 10 ml. water, the aqueous phase was extracted with ether (3 x 10 ml.) and freed of ether in an air stream. After adjustment to pH 7.5 with potassium hydroxide the solution was poured on a column of Dowex-2 formate (8 cm. x 4 cm.^{2}; x4; 200-400 mesh) and washed in with water at 2°. Material with extinction at 266 mμ emerged in the first 50 ml.

This material had maximum extinction at 315 mμ (3.35 x 10^3 A.U.) and accounted for 97% of the A.U. 315/CN^- applied to the column. Failure to retain nicotinic acid nucleotide in both these experiments was probably due to the high concentration of acetic acid or potassium acetate in the sample applied to the column. The eluate was mixed with perchloric acid (70%; 3 ml.) and kept for 2 hours at 0° (final pH 2.1). Potassium perchlorate was removed by filtration and washed with cold water (2 x 10 ml.). The combined filtrate had maximum extinction at 315 mμ in M-potassium cyanide (2.89 x 10^3A.U). Maximum extinction was developed in 15 minutes at 20°. Decolorising charcoal (B.D.H.) was washed with hot N-hydrochloric acid, hot trichloroacetic acid (5%) and then with water until the washings were neutral. The charcoal was then dried at 100°. The acid filtrate after potassium perchlorate precipitation was shaken with 2 ml. of packed dry charcoal for 15 minutes at 2°. After filtration 773 A.U. 315/CN^- (23%) remained in solution. After

treatment with a further 2 ml. portion of charcoal at 2° for 15 minutes 455 A.U. 315/CN^-(14%) remained in solution, and after treatment with a further 1 ml. for 2 hours at 2°, 231 A.U. 315/CN^- (7%) remained. This was discarded. The combined charcoal was washed with water (200 ml.) at 2°. The water removed 300 A.U. 315/CN^-(10% of adsorbed units). The charcoal was extracted at 2° with portions of ethanol-amyl alcohol-water (4:1:4 by volume) and portions of each extract were examined in M-potassium cyanide.

Extract number	Vol.	Time of extraction (hr.)	A.U. 315/CN^-
1	10 ml.	0.25	204
2	10	0.25	685
3	10	0.25	625
4	15	16	418
5	15	0.5	281
6	15	0.5	132
7	15	0.5	98
			2643 (111% of calc.; possibly due to extraneous colour).

Charcoal was removed from the combined eluates by filtration (Whatman No. 544 paper) and the filtrate concentrated in a stream of air at 15° to 67 ml. The solution contained 2.15 x 10^3 A.U. 315/CN^- and showed a single component absorbing light at 254 mμ after electrophoresis for 4 hr. at 9 volts/cm. and 2° on Whatman No.3 MM paper in 0.05M-disodium phosphate-1mM EDTA (anionic migration: 8.5 cm.; migration of nicotinamide nucleotide in this system:

6 cm.). The eluate contained no material which gave a fluorescent product after exposure to ammonia and ethyl-methyl ketone and examination with light from a filtered high pressure mercury lamp.

This eluate was poured on a Dowex-2 formate column (dimension as before; washed with ethanol before use). Only 90 A.U. 315/CN$^-$ passed through the column with a water wash. The column was then washed with a linear gradient of 150 ml. of M-formic acid added to 150 ml. of 0.25M-formic acid. Material with extinction at 266 mμ started to emerge after 275 ml. of the formic acid gradient. Elution was continued with a gradient of 120 ml. of 2M-formic acid into 120 ml. of M-formic acid and displacement of material with absorption at 266 mμ was complete in a further 150 ml. This material was concentrated by freeze-drying to 10 ml. and added slowly to acetone (90 ml.) at 2°. The mixture was centrifuged and the precipitate was kept at 2°. This material crystallised as colourless branched prisms and had maximum extinction in water or in 0.04M-phosphate, pH 7 at 265 mμ (1238 A.U.) and in molar aqueous potassium cyanide at 315 mμ (1338 A.U.); $E_{315/CN^-}/E_{265} = 1.08$.

ε (Based on phosphorus analysis carried out by Mr. K. R. Gayler) was 4.6×10^3 at 265 mμ (pH 7) and 5.0×10^3 at 315 mμ (15 min. after dissolving the sample in M-KCN). Naylor (1963) found that the corresponding extinction coefficients of analytically-pure nicotinic acid riboside were 4.6×10^3 at 265 mμ and 4.7×10^3 at 316 mμ.

This material contained a single component (R_F 0.30) which absorbed light at 254 mμ after chromatography (Whatman No.1; ascending) in Preiss and Handler's (1958) solvent C; R_F0.76 in Preiss and Handler's solvent B). On electrophoresis on Whatman No.3 MM paper for 4 hr. at 12.5 volts/cm. at 2° in 0.04M-potassium citrate pH 4.5, there was also a single component (11 cm. migration towards anode). No material which fluoresced at 360 mμ, after exposure to ammonia and ethyl-methyl ketone, could be detected in either chromatographic or electrophoretic separation. After this treatment the papers were warmed at 100° for 20 min. and were exposed to cyanogen bromide vapour. When sprayed with an acidic ethanolic solution of p-aminobenzoic acid an orange colour developed in the region of ultra-violet absorption, probably because of hydrolysis of the nicotinic acid-glycosidic linkage in these conditions.

(iii) Conversion of nicotinic acid nucleotide to nicotinic acid-adenine dinucleotide with adenosine triphosphate and ATP:NMN-adenylyl-transferase from pig-liver nuclei

Adenosine triphosphate (207 μmoles), nicotinic acid nucleotide (chromatographically and electrophoretically homogeneous, 314 A.U. 266; 68 μmoles), and magnesium chloride (500 μmoles) were diluted to 55 ml. and adjusted to pH 7.8 with 4M-potassium hydroxide (0.13 ml.). Nicotinamide nucleotide adenylyltransferase (0.2 ml.) was added. After 1 hr. the pH had fallen to 7.75 and after 3.5 hr. to 7.6. After 11 hr. at 20° the solution was frozen, but chromatography in Preiss and Handler's solvent B and electrophoresis at pH 4.5

in 0.04M-potassium citrate indicated that adenylyl transfer was almost complete after 1 hr.

The reaction mixture was fractionated on Dowex-2 formate essentially as described by Preiss and Handler (1958a) for a system of nicotinic acid metabolites. (Dowex-2 formate; x 8; 200-400 mesh; 13 cm. x 4.5 cm^2.).

Fraction No.	A.U. 260	µmoles(approx.)
I; nicotinic acid nucleotide	83	19
II; nicotinic acid-adenine dinucleotide	827	54
III; adenosine diphosphate	184	12
IV; adenosine triphosphate	2120	138

Within the limits of the analytical method, nicotinic acid riboside 5'-phosphate was converted into its phospho-adenylyl derivative without any destruction by the enzyme from pig liver nuclei.

(iv) Preparation of nicotinic acid-adenine dinucleotide by exchange reaction with ethyl nicotinate and NAD(P) nucleosidase from pig brain

Nicotinamide-adenine dinucleotide (820 µmoles) was dissolved in water (10 ml.) and after the addition of 2 ml. of M-KH_2PO_4 was adjusted to pH 7.5 with potassium hydroxide (3.48N; 0.38 ml.). This solution was then incubated at 38° with 90 ml. of the NAD(P) nucleosidase preparation from pig brain and 1.0 ml. of ethyl nicotinate, following the method of Lamborg et al. (1958). NAD was assayed by

using ethanol and glycinate at pH 9.5 and yeast alcohol dehydrogenase; quaternary pyridinium compounds were assayed by extinction at 320 mμ in M-KCN. The amount of ethylnicotinate-adenine dinucleotide was obtained by difference. The incubation was continued for 7.5 hr. and residual NAD was hydrolysed by treatment with NAD nucleosidase from *Neurospora*. At this stage the ethylnicotinate-adenine dinucleotide showed an extinction maximum at 321 mμ in M-KCN.

Summary of recovery of pyridinium quaternary compounds in exchange reaction

	Incubation time (hr.)			Treatment with	Hydro-lysis	Charcoal adsorption & elution	Dowex-1 formate
	0	4.25	7.5	NADase			
NAD (μmoles)	820	77	20	0	-	-	-
nicotinate nucleotide (μmoles)	-	350	370	290	233	170	160
% yield	-	43	45	35	29	21	20

After the hydrolysis, the extinction maximum of nicotinic acid-adenine dinucleotide in M-KCN was at 318 mμ. The nucleotides were separated by elution from Dowex-1 formate using a formic acid gradient, following the method of Preiss & Handler (1958a). Nicotinic acid-adenine dinucleotide was obtained in a yield of 8% (65 μmoles). This method also gave 48 μmoles of nicotinic acid nucleotide.

The nicotinic acid-adenine dinucleotide prepared by adenylyl transfer (see (iii) above) and that prepared by the exchange reaction were compared by paper chromatography in two different solvents and gave the following results:

	Compound	R_F (in Preiss & Handler's (1958a) solvents)	
		B	C
a)	Dinucleotide (adenylyl transfer)	0.27	0.13
b)	Dinucleotide (nicotinate-exchange)	0.26	0.12
c)	Mixture (a) + (b)	0.27	0.13
	(Trace contaminant: adenosine diphosphate ribose from (b))		0.22

Material which fluoresced at 360 mμ after exposure to ethyl-methyl ketone/ammonia, or gave a colour with acidic p-aminobenzoic acid after exposure to cyanogen bromide was absent.

(v) Conversion of nicotinic acid-adenine dinucleotide into nicotinamide-adenine dinucleotide with NAD synthetase

0.35 Ml. of the equilibrium reaction mixture from (iii) above was incubated with NAD synthetase substrate (see Preiss & Handler, 1958b), (0.25 ml.) and 0.4 ml. of NAD synthetase prepared from pig liver. One sample was mixed with 1.5 ml. of 0.5 N-trichloroacetic acid at zero time, the other after 30 min. at 38°. 2.0 Ml. of supernatant and 3.0 ml. of ethanol-glycinate (pH 9.5) were mixed and examined in a 4 cm. cuvette at 340 mμ before and after addition of alcohol dehydrogenase. The absorbance change

in the zero-time blank was 0.021, and that in the test was 0.527. Net synthesis of NAD in 0.5 hr. was 0.126 µmole. The sample was expected to contain 0.40 µmole of nicotinic acid-adenine dinucleotide.

The enzyme was subsequently shown to destroy NAD rapidly, and to give incomplete net conversion of nicotinic acid-adenine dinucleotide prepared by the exchange reaction (see (iv) above) to NAD (approximate yield, 11%).

The spectrum of the test solution was read against that of the blank after reduction with ethanol and alcohol dehydrogenase, and showed maximum extinction at 341 mµ and minimum extinction at 310 mµ.

After removal of volatile material by freeze-drying the nicotinic acid-adenine dinucleotide described above was titrated potentiometrically to pH 7.5. No orthophosphate ester dissociation could be detected in the range pH 6.5- pH 7.5. In water at pH 6 the material had maximum extinction at 260 mµ. In *M*-potassium cyanide after 15 min. at 20° the maximum extinction was at 316.5 mµ. $E_{316.5/CN^-}/E_{260aq.} = 0.34$.

(vi) <u>Conversion of nicotinamide-adenine dinucleotide into nicotinic acid-hypoxanthine dinucleotide with nitrous anhydride</u>

Nicotinamide-adenine dinucleotide tetrahydrate (500 µmoles) was suspended in dry acetic acid (12 ml.) and cooled to the freezing point of the suspension while dry nitrous anhydride

from 25 g. of arsenious oxide and 50 ml. of concentrated nitric acid was condensed into the reaction vessel. The final volume of deep green solution was about 20 ml. During 25 min. at about 10-15° (cooling bath) gas was evolved. Dry air was then passed through the solution for 10 min., when the suspension (about 12 ml.) was almost colourless. A second portion of nitrous anhydride was condensed into the reaction mixture as before, but no gas evolution was observed, and after 40 min. at 10° volatile material was removed in a stream of dry air, at first at 10°, and after removal of nitrogen oxides, at 25°. The residue was dissolved in water (10 ml.), extracted with ether (3 x 10 ml.) and the ether was removed in an air stream. The solution was adjusted to pH 6 with 5M-ammonium hydroxide and diluted to 16.5 ml. In water at pH 6 the product had maximum absorption at 249 mμ (6.47×10^3 A.U.). In M-potassium cyanide the product had an extinction maximum at 316 mμ (2.32×10^3 A.U., corresponding to 480 μmoles of nicotinic acid nucleotide derivative for a millimolar extinction coefficient of 4.8).

The product was poured on a column of Dowex-1 formate (14 cm. x 4 cm.^{2}, x 8; 200-400 mesh) at 2° and washed in with 30 ml. of water. The column was washed with 250 ml. of 0.2 M-formic acid and then with 350 ml. of 2M-formic acid. Little material absorbing light at 254 mμ was removed by these solutions. On elution with M-ammonium formate in 4M-formic acid material with extinction at 254 mμ emerged between 40 ml. and 120 ml., but mainly between 50 and 90 ml. This material had maximum extinction at 249 mμ in water and at 317 mμ in M-potassium cyanide. After freeze-drying, the

material was freed of ammonia by passage through two columns of Dowex-50 (H^+) (8 cm. x 6.5 cm.^{2} ; 200-400 mesh) at 2°. All material absorbing light at 249 mμ was washed through with water, and the eluate was filtered (Whatman No.544 paper) and diluted to 56 ml. This material had maximum extinction in water at 249 mμ (4.82×10^3 A.U.) and in M-potassium cyanide at 316 mμ (4.52×10^3 A.U.; equilibrium was attained after 13 min. at 20°; $E_{316CN^-}/E_{249H_2O} = 0.32$).

This material was dried to constant weight (0.250 g.) during 18 hrs. at 0.01 mm.

	C	H	N	P
$C_{21}H_{25}N_5O_{16}P_2.4H_2O$ requires:	34.2	4.5	9.5	8.4
Found:	34.45	4.5	9.75	8.7

Phosphorus analysis was by Mr. A. C. Jennings and other analyses by the Australian Micro-analytical Service.

The yield of dinucleotide was 339 μmoles (based on tetrahydrate). Extinction coefficients were 14.2×10^3 at 249 mμ in water and 4.5×10^3 at 316 mμ in M-potassium cyanide.

Material which had been stored at pH 1 for 5 days was examined by electrophoresis (Whatman No. 3 MM) in 0.05 M-disodium phosphate- 1 mM-EDTA for 4 hr. at 12.5 volts/cm. in a room at 2°; subsequent experiments showed a temperature rise in these conditions. The main anionic component (about 98% of the extinction at 254 mμ) migrated 10.0 cm. A trace of a more rapidly-moving component (? inosine diphosphate ribose; about 2% of the extinction) moved

11.1 cm. towards the anode. The main band (nicotinic acid-hypoxanthine dinucleotide) showed blue fluorescence under a filtered high pressure mercury lamp after treatment with methanolic 0.5M-potassium cyanide.

(vii) Hydrolysis of nicotinic acid-hypoxanthine dinucleotide with nucleotide pyrophosphatase from potato

Nicotinic acid-hypoxanthine dinucleotide (2.54 mM, 5 ml.) was titrated potentiometrically at 20° with 43.5 mM-potassium hydroxide from pH 2.6 to pH 7.5 (0.85 ml. alkali was used). Dissociations with pK about 2.6 and greater than 8 were evident, but no orthophosphate dissociation near pH 6-7 could be detected.

When solutions of nucleotide pyrophosphatase (2 ml.) and the dinucleotide were mixed in the 'pH-state' at pH 7.5, rapid hydrolysis took place. The reaction rate was constant until about half the dinucleotide had been hydrolysed, and declined to half the initial rate when 95% of the dinucleotide had been hydrolysed (i.e. at a concentration of about 8×10^{-5}M). The initial rate of hydrolysis was about 0.34 µmole/min.

The hydrolysis of 12.7 µmoles of dinucleotide consumed 0.51 ml. of 43.5 mM-potassium hydroxide. If dissociation of orthophosphates was complete at this pH the expected consumption of alkali would be 0.58 ml. The observed consumption of 86% of the alkali needed for titration of the orthophosphates formed is in good agreement with the expected extent of dissociation at this pH i.e. about 90%.

Chromatography in Preiss & Handler's (1958a) solvent B showed the presence of nicotinic acid nucleotide (R_F 0.80) and inosine 5'-phosphate (R_F0.47). After hydrolysis was complete the solution was cooled to 0° and mixed with perchloric acid (72%; 0.6 ml.). After 10 min. at 0° the suspension was centrifuged for 10 min. at 12 000 g and the supernatant was brought to pH 7 with 4N-potassium hydroxide. After 16 hr. at -15° potassium perchlorate was removed by centrifuging at 15 000 g for 10 min. and the supernatant was stored at -15° and used as a source of nicotinic acid nucleotide for synthesis of nicotinic acid-adenine dinucleotide.

(viii) Attempted conversion of nicotinamide-adenine dinucleotide into nicotinic acid-adenine dinucleotide by non-enzymic method

Nicotinamide-adenine dinucleotide tetrahydrate (0.50 g., containing 630 μmoles of NAD on enzymic assay) was suspended in 3 ml. of dry pyridine and cooled in ice while 3 ml. of acetic anhydride was added. The sample was then shaken at 20° for 46 hr. During this time the solid dissolved slowly, and finally only a small insoluble residue was present. On chromatography on paper in pyridine-water (2:1;v/v) material that absorbed at 254 mμ and gave an ethyl-methyl ketone reaction was present at R_F 0.60 and R_F 0.72. Yellow material was present at R_F 0.85. After removal of solvent at 30°/0.05 mm. for 1 hr. the residue was dissolved in 5 ml. of dry acetic acid and 1 ml. of acetic anhydride. The solution was frozen in dry ice-cellosolve and an equal volume of

dry nitrous anhydride was condensed in during 30 min. The temperature of the mixture was raised to 13° during 10 min. in a water bath. There was considerable gas evolution, and the green solution became brown. After a further 15 min. at 13° volatile material was removed in a stream of nitrogen, and then in a freeze-drying apparatus. The residue was finally dried for an hour at 50°/0.1 mm. and evaporated to dryness again with 30 ml. of dry methanol. After 34 hr. at 2° to decompose mixed anhydrides, the solution was saturated with dry ammonia at 0°. Precipitation of colourless material started immediately. After 22 hr. at 0° the ammonia was removed at 5° in a stream of nitrogen, and the pale powder was washed with dry acetone at -15° (3 x 25 ml.). The residue was dissolved in 25 ml. water and placed on a column of Dowex-2 formate (13 cm. x 3.5 cm.^{2}; x 8; 200-400 mesh) and washed with water. After 350 ml. of effluent had been collected $E^{0.2}_{260\ m\mu}$ had declined to 0.020. Elution with 4N-formic acid was started. Material with extinction at 260 mμ emerged after 50 ml., and the effluent was collected. At 350 ml. $E^{0.2}_{260}$ had declined to 0.15. The column was then washed with 0.5 M-ammonium formate - 1.5 N-formic acid and all material with extinction at 260 mμ eluted with this solution was freeze-dried.

Fractions were examined for extinction at 260 and 315 mμ in water and in M-potassium cyanide.

Fraction No.	Vol. of formic acid (ml.)	A.U. 260	A.U. 315/CN$^-$ *
I	30-70	16	
II	70-95	1688	90
III	95-118	3582	1160
IV	118-148	605	480
V	148-173	125	
VI	173-223	65	
			1730

* Measured in M-KCN-ethanol (2:1; v/v).

Nucleotides were identified by paper chromatography in Preiss & Handler's (1958a) solvent B. Fraction II contained much more AMP than nicotinic acid nucleotide; fraction III contained about equal amounts of these nucleotides and fraction IV contained more nicotinic acid nucleotide than AMP. Fractions (III and IV) and (V and VI) were freeze dried separately and the residues were dissolved in 12 ml. of water and frozen.

The only nicotinic acid nucleotide detected was the mononucleotide (about 360 µmoles). Approximately an equal molar proportion of adenosine 5'-phosphate was present. This method of cleavage of the pyrophosphate linkage with acetic anhydride in pyridine provides a convenient non-enzymic synthesis of nicotinamide nucleotide and of nicotinic acid nucleotide.

(ix) <u>Effect of nicotinic acid-adenine dinucleotide on the formation of nicotinamide-adenine dinucleotide phosphate from NAD and ATP with NAD kinase</u>

In order to test the possibility that nicotinic acid-adenine dinucleotide is a substrate of NAD kinase or inhibits this enzyme, the formation of NADP from NAD and ATP was measured at two levels of NAD and four levels of nicotinic acid-adenine dinucleotide. The reaction mixtures contained ATP (5 μmoles), $MgCl_2$ (10 μmoles), tris-HCl (100 μmoles, pH 7.4) NAD (0.5 and 1.0 μmole), nicotinic acid-adenine dinucleotide (0.5, 1.0, 2.5 and 5 μmoles), and NAD kinase (0.2 ml.); the total volume was 1.0 ml. Blank determinations, without the addition of nicotinic acid-adenine dinucleotide were also carried out. The reaction was stopped at suitable times by the addition of 1.5 ml. of 0.5<u>M</u>-trichloroacetic acid. After centrifugation the NADP formed was determined with isocitrate dehydrogenase (see Section I, p.41).

<u>Nucleotide</u>	<u>Concentration</u> (m<u>M</u>)									
NAD	0.5	0.5	0.5	0.5	0.5	1.0	1.0	1.0	1.0	1.0
nicotinic acid-adenine dinucleotide	0	0.5	1.0	2.5	5.0	0	0.5	1.0	2.5	5.0
NADP formed	.019	.018	.016	.018	.018	.024	-	.026	.026	.024

The solutions containing 0.5 and 1.0 μmole of NAD were incubated at 38° for 20 and 25 min., respectively. From these results the K_m of the kinase for NAD was estimated at 5.5×10^{-4}; Wang <u>et al.</u>

(1954) give a value of 6×10^{-4} M. It is clear that there was no inhibition of NADP formation by nicotinic acid-adenine dinucleotide under these conditions.

In order to check the possibility that nicotinic acid-adenine dinucleotide phosphate (the nicotinic acid analogue of NADP) could be formed under these conditions, the experiment was repeated with larger amounts of the nucleotides. Tube A contained ATP (10 μmoles), $MgCl_2$ (20 μmoles), tris HCl (200 μmoles, pH 7.4) and NAD (12.5 μmoles); in tube B, the NAD was replaced by nicotinic acid-adenine dinucleotide (12.5 μmoles); the total volume was 2.5 ml. NAD kinase (0.6 ml.) was added to each and the tubes were incubated at 38° for 9 hr. The contents of the tubes were frozen and stored overnight at -15°. After thawing, hydrogen peroxide (30%; 0.25 ml.) and perchloric acid (72%; 0.1 ml.) were added. The nucleotides were adsorbed on Norit and eluted as described by Threlfall (1957). The nucleotides in 25 μl. samples from each tube were separated by electrophoresis on Whatman 3 MM paper in 0.04 M-sodium citrate, pH 4.5, for 7 hr. at 12 volts/cm.

In sample A, AMP, ADP, nicotinic acid-adenine dinucleotide and ATP were the only nucleotides detected. The paper with sample B was dried and spots absorbing light at 254 mμ corresponding to NAD, AMP, ADP, NADP and ATP were detected. Ascending paper chromatography in pyridine: water (2:1; v/v) was carried out, at right angles to the direction in which the voltage had been applied. The paper was dried and then exposed to ethyl-methyl ketone/ammonia vapour.

When examined under ultraviolet light, spots corresponding to NAD and NADP showed a bright blue fluorescence.

Samples A and B were also examined by ascending paper chromatography in acetone: water (2:1 v/v) and descending paper chromatography in n-butyric acid: water : ammonia (14 M) (66: 33: 1 ; v/v). No nucleotides with the expected properties of nicotinic acid-adenine dinucleotide phosphate could be detected.

APPENDIX

Statistical Analysis of Data from Lambs, Sheep and Rats of Series II (1961), Series III (1962) and Series IV (1962)

A standard regression analysis of variance is presented together with the regression coefficient and its standard deviation. The relevant regression equations are also given.

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Key to Symbols

b	=	regression coefficient
C	=	total amount of coenzymes/liver, μmoles
Corr.	=	corrected
df	=	degrees of freedom
MS	=	mean sum of squares
N	=	total number of nuclei/liver
n	=	number of values
NS	=	not significant
*	=	significant at 0.05 level
* *	=	significant at 0.01 level
* * *	=	significant at 0.001 level
Sx	=	sum of values for x, etc.
SD	=	standard deviation
SE(b)	=	standard error of b
SP	=	sum of products
SS	=	sum of squares
t	=	Student's t
W	=	liver dry wt., g.
x_0	=	log (age, days)
x_1	=	log (body wt., Kg.)
x_2	=	log (liver dry wt., g.)
x_3	=	log (no. of nuclei x 10^{-8}/liver)
x_4	=	log ([NAD + $NADH_2$]/liver, μmoles)

Key to Symbols (continued)

x_5 = log([NADP + $NADPH_2$]/liver, µmoles)

x_6 = log(total coenzymes/liver, µmoles)

y_1 = coenzyme ratio =
([NAD] + [$NADH_2$]) : ([NADP] + [$NADPH_2$])

y_2 = enzymic activity (µmoles of $NADPH_2$ formed/
min./g. fresh wt. of liver tissue)

(a) Lambs and sheep, Series II : $\log_{10}$ data, n = 20

No.	Variate x_0	x_1	x_2	x_3
1	0.60206	0.66276	1.55267	2.53403
2	0.60206	0.69897	1.49831	2.56110
3	0.60206	0.62325	1.52375	2.53020
4	0.60206	0.68124	1.46389	2.52504
5	1.14613	0.79239	1.56110	2.60314
6	1.14613	0.89209	1.69984	2.64246
7	1.14613	0.94448	1.77379	2.73078
8	1.14613	0.91381	1.71684	2.69548
9	1.46240	1.02119	1.83632	2.85491
10	1.46240	0.86332	1.66181	2.67302
11	1.46240	1.02938	1.78390	2.82217
12	1.74819	1.06819	1.86332	2.87157
13	1.74819	1.20412	1.99564	2.95134
14	1.74819	1.29667	2.01284	2.95809
15	1.74819	1.29885	1.95999	3.00000
16	1.74819	1.27416	2.01703	2.97220
17	3.03941	1.70070	2.39967	3.20305
18	3.03941	1.71517	2.44248	3.33445
19	3.03941	1.68124	2.37107	3.20004
20	3.03941	1.72591	2.38739	3.29113

(a) Lambs and sheep, Series II : $\log_{10}$ data, (continued)

No.	Variate		
	x_4	x_5	x_6
1	1.83948	1.12710	1.91645
2	1.71933	1.35025	1.87390
3	1.74036	1.49136	1.93450
4	1.61700	1.26951	1.77815
5	1.72591	1.51587	1.93450
6	1.65031	1.68931	1.97128
7	1.80482	1.40824	1.95134
8	1.76418	1.55509	1.97313
9	1.70501	1.77232	2.04107
10	1.81558	1.73239	2.07703
11	1.55145	1.82282	2.00902
12	2.17319	1.70927	2.30146
13	2.12451	1.82478	2.30103
14	2.20412	1.97772	2.40654
15	2.19088	2.01114	2.40960
16	2.34242	1.90037	2.47641
17	2.81757	2.37840	2.95231
18	2.86451	2.47712	3.01369
19	2.82020	2.21433	2.91635
20	2.72099	2.30103	2.86094

(a) Crude and corrected sums of squares and products

(Log_{10} data : sheep : n = 20)

	x_1	x_2	x_3	x_4	x_5	x_6
Totals	22.08789	37.52165	56.95420	41.19182	35.52842	45.09870
means	1.104395	1.876083	2.847710	2.059591	1.776421	2.254935
x_1	27.001214 2.607470	-	-	48.379679 2.887660	41.735148 2.497757	52.545428 2.738672
x_2		72.318612 1.924901	108.414649 1.563872	79.781853 2.502601	68.778663 2.124417	86.969855 2.360974
x_3			163.490645 1.301601	119.309503 2.007146	102.938040 1.763403	130.342414 1.914396
x_4				88.548467 3.710166	-	-
x_5					65.757179 2.643748	-
x_6						104.743983 3.049346

(a) Regression analysis : $\log_{10}$ data : sheep ; Series II

1) Regression of x_4 on x_1

Variation due to	df	SS	MS	VR
Regression	1	3.197958	3.197958	112.38***
Residual	18	0.512208	0.028456	
Total	19	3.710166		

b = 1.107457 (SE (b) = 0.1045 $x_4 = 0.8365 + 1.1075x_1$

2) Regression of x_4 on x_2

Regression	1	3.253680	3.253680	128.30***
Residual	18	0.456486	0.025360	
Total	19	3.710166		

b = 1.300119 SE(b) = 0.1148 $x_4 = -0.3795 + 1.3001x_2$

3) Regression of x_4 on x_3

Regression	1	3.095138	3.095138	90.59***
Residual	18	0.615028	0.034168	
Total	19	3.710166		

b = 1.542059 SE(b) = 0.1620 $x_4 = -2.3317 + 1.5421x_3$

4) Regression of x_3 on x_2

Regression	1	1.270557	1.270557	736.55***
Residual	18	0.031044	0.001725	
Total	19	1.301601		

b = 0.812443 SE (b) = 0.0299 $x_3 = 1.3235 + 0.8124x_2$

5) Regression of x_5 on x_1

Regression	1	2.392660	2.392660	171.53***
Residual	18	0.251088	0.013949	
Total	19	2.643748		

b = 0.957924 SE (b) = 0.0731 $x_5 = 0.7185 + 0.9579x_1$

(a) Regression analysis : log_{10} data : sheep; Series II (continued)

6) Regression of x_5 on x_2

Variation due to	df	SS	MS	VR
Regression	1	2.344613	2.344613	141.08***
Residual	18	0.299135	0.016619	
Total	19	2.643748		

b = 1.103650 SE (b) = 0.0929 $x_5 = -0.2941 + 1.1037x_2$

7) Regression of x_5 on x_3

Variation due to	df	SS	MS	VR
Regression	1	2.389050	2.389050	168.84***
Residual	18	0.254698	0.014150	
Total	19	2.643748		

b = 1.354795 SE (b) = 0.1043 $x_5 = -2.0816 + 1.3548x_3$

8) Regression of x_6 on x_1

Variation due to	df	SS	MS	VR
Regression	1	2.876476	2.876476	299.51***
Residual	18	0.172870	0.009604	
Total	19	3.049346		

b = 1.050318 SE (b) = 0.0607 $x_6 = 1.0950 + 1.0503x_1$

9) Regression of x_6 on x_2

Variation due to	df	SS	MS	VR
Regression	1	2.895836	2.895836	339.57***
Residual	18	0.153510	0.008528	
Total	19	3.049346		

b = 1.226543 SE (b) = 0.0666 $x_6 = -0.0462 + 1.2265x_2$

10) Regression of x_6 on x_3

Variation due to	df	SS	MS	VR
Regression	1	2.815695	2.815695	216.91***
Residual	18	0.233651	0.012981	
Total	19	3.049346		

b = 1.470801 SE (b) = 0.0999 $x_6 = -1.9335 + 1.4708x_3$

(a) Regression analysis : $\log_{10}$ data : Series II

Sheep

11) Regression of x_1 on x_0

Variation due to	df	SS	MS	VR
Regression	1	2.497309	2.497309	408.06***
Residual	18	0.110161	0.006120	
Total	19	2.607470		

b = 0.434009 $x_1 = 0.4039 + 0.4340x_0$

12) Regression of x_2 on x_0

Regression	1	1.839799	1.839799	389.13***
Residual	18	0.085102	0.004728	
Total	19	1.924901		

b = 0.372519 $x_2 = 1.2749 + 0.3725x_0$

Rats

11) Regression of x_1 on x_0

Regression	1	4.430566	4.430566	685.95***
Residual	7	0.045211	0.006459	
Total	8	4.475777		

b = 1.098558 $x_1 = 0.1889 + 1.0986x_0$

12) Regression of x_2 on x_0

Regression	1	4.295521	4.295521	371.87***
Residual	7	0.080855	0.011551	
Total	8	4.376376		

b = 1.081687 $x_2 = -1.7733 + 1.0817x_0$

(a) Points for plotting regression lines of Series II

Sheep

log x_2	x_2	x_4	x_5	x_6
1.4	25.1	27.6	17.8	46.9
1.5	31.6	37.2	23.0	62.2
1.6	39.8	50.2	29.6	82.5
1.7	50.1	67.6	38.2	109.4
1.8	63.1	91.3	49.3	145.1
1.9	79.4	123.2	63.5	192.4
2.0	100.0	166.2	81.9	255.2
2.1	125.9	224.2	105.6	338.5
2.2	158.5	302.5	136.1	449.0
2.3	199.5	408.0	175.5	595.5
2.4	251.2	550.4	226.3	789.8
2.5	316.2	742	291.7	1047.6

logx_3	x_3	x_4	x_5	x_6
2.50515	320	34.0	20.5	56.4
2.60206	400	48.0	27.8	78.3
2.69897	500	67.6	37.6	108.7
2.81291	650	101.4	53.6	159.9
2.90309	800	139.6	71.0	217.0
3.00000	1000	197.0	96.1	301.2
3.07918	1200	260.9	123.0	393.9
3.17609	1500	368.1	166.5	546.9
3.25527	1800	487.6	213.1	715.1
3.30103	2000	573.6	245.8	835.0
3.34242	2200	664.5	279.7	960.6

(b) Rats, Series II : $\log_{10}$ data

No.	Variate			
	x_0	x_1	x_2	x_3
1	0.60206	0.91908	-1.14874	-0.11351
2	0.84510	1.12710	-0.93930	0.27184
3	1.44716	1.59988	-0.32790	0.71933
4	1.44716	1.75205	-0.11014	0.92634
5	1.44716	1.83251	0.00170	1.08707
6	2.32222	2.74819	0.72673	1.50650
7	2.32222	2.71600	0.66370	1.44404
8	2.32222	2.79449	0.74663	1.49554
9	2.32222	2.77452	0.73640	1.51054

(b) Rats, Series II : $\log_{10}$ data (continued)

No.	Variate		
	x_4	x_5	x_6
1	-0.95078	-1.35655	-0.80688
2	-0.62893	-0.95079	-0.45967
3	-0.02457	-0.29073	0.16343
4	0.17555	-0.12378	0.35218
5	0.27277	0.03706	0.47173
6	0.96284	0.86034	1.21564
7	0.94645	0.76567	1.16641
8	1.01157	0.94988	1.28279
9	0.86392	0.73078	1.10344

(b) Crude and corrected sums of squares and products

($\log_{10}$ data : rats : $n = 9$)

	x_1	x_2	x_3	x_4	x_5	x_6
Totals	18.26382	0.34908	8.84769	2.62882	0.62189	4.48907
means	2.029313	0.038787	0.983077	0.292091	0.069099	0.498786
x_1	41.538790 4.475777	-	-	9.625818 4.291119	6.193440 4.931431	13.638313 4.528584
x_2		4.389915 4.376376	3.821211 3,478039	4.391480 4.289518	4.941182 4.917062	4.695018 4.520903
x_3			11.517208 2.819251	6.013479 3.429147	4.538231 3.926866	8.024192 3.611093
x_4				4.997820 4.229966	-	-
x_5					5.608176 5.565204	-
x_6						6.937044 4.697961

(b) **Regression analysis : $\log_{10}$ data : rats, Series II**

1) Regression of x_4 on x_1

Variation due to	df	SS	MS	VR
Regression	1	4.114079	4.114079	248.51***
Residual	7	0.115887	0.016555	
Total	8	4.229966		

b = 0.958743 SE (b) = 0.0608 $x_4 = -1.6535 + 0.9587x_1$

2) Regression of x_4 on x_2

Variation due to	df	SS	MS	VR
Regression	1	4.204384	4.204384	1150.31***
Residual	7	0.025582	0.003655	
Total	8	4.229966		

b = 0.980153 SE (b) = 0.0289 $x_4 = 0.2541 + 0.9802x_2$

3) Regression of x_4 on x_3

Variation due to	df	SS	MS	VR
Regression	1	4.170983	4.170983	495.01***
Residual	7	0.058983	0.008426	
Total	8	4.229966		

b = 1.216333 SE (b) = 0.0547 $x_4 = -0.9037 + 1.2163x_3$

4) Regression of x_3 on x_2

Variation due to	df	SS	MS	VR
Regression	1	2.764103	2.764103	350.86***
Residual	7	0.055148	0.007878	
Total	8	2.819251		

b = 0.794730 SE(b) = 0.0424 $x_3 = 0.9523 + 0.7947x_2$

5) Regression of x_5 on x_1

Variation due to	df	SS	MS	VR
Regression	1	5.433473	5.433473	288.72***
Residual	7	0.131731	0.018819	
Total	8	5.565204		

b = 1.101804 SE (b) = 0.0648 $x_5 = -2.1668 + 1.1018x_1$

(b) Regression analysis : $\log_{10}$ data : rats , Series II (continued)

6) Regression of x_5 on x_2

Variation due to	df	SS	MS	VR
Regression	1	5.524548	5.524548	951.20***
Residual	7	0.040656	0.005808	
Total	8	5.565204		

b = 1.123547   SE (b) = 0.0364 $x_5 = 0.0255 + 1.1235x_2$

7) Regression of x_5 on x_3

Variation due to	df	SS	MS	VR
Regression	1	5.469636	5.469636	400.62***
Residual	7	0.095568	0.013653	
Total	8	5.565204		

b = 1.392876   SE (b) = 0.0696 $x_5 = -1.3002 + 1.3929x_3$

8) Regression of x_6 on x_1

Variation due to	df	SS	MS	VR
Regression	1	4.582014	4.582014	276.62***
Residual	7	0.115947	0.016564	
Total	8	4.697961		

b = 1.011798   SE (b) = 0.0608 $x_6 = -1.5545 + 1.0118x_1$

9) Regression of x_6 on x_2

Variation due to	df	SS	MS	VR
Regression	1	4.670203	4.670203	1177.86***
Residual	7	0.027758	0.003965	
Total	8	4.697961		

b = 1.033024   SE(b) = 0.0301 $x_6 = 0.4572 + 1.0330x_2$

10) Regression of x_6 on x_3

Variation due to	df	SS	MS	VR
Regression	1	4.625339	4.625339	445.82***
Residual	7	0.072622	0.010375	
Total	8	4.697961		

b = 1.280870   SE (b) = 0.0607 $x_6 = -0.7604 + 1.2809x_3$

(b) Points for plotting regression lines of Series II

Rats

log x_2	x_2	x_4	x_5	x_6
-1.15	0.071	0.134	0.054	0.187
-0.95	0.112	0.210	0.091	0.300
-0.75	0.178	0.330	0.152	0.483
-0.55	0.282	0.519	0.256	0.777
-0.35	0.447	0.815	0.429	1.252
-0.15	0.708	1.280	0.719	2.013
0.05	1.122	2.010	1.207	3.239
0.25	1.778	3.156	2.025	5.212
0.45	2.818	4.956	3.397	8.386
0.65	4.467	7.783	5.699	13.495
0.75	5.623	0.754	7.382	17.119

log x_3	x_3	x_4	x_5	x_6
-0.1	0.794	0.094	0.036	0.129
0.1	1.259	0.165	0.069	0.233
0.3	1.995	0.289	0.131	0.421
0.5	3.162	0.506	0.249	0.759
0.7	5.012	0.887	0.473	1.368
0.9	7.943	1.553	0.898	2.468
1.1	12.59	2.718	1.706	4.452
1.3	19.95	4.750	3.240	8.030
1.5	31.62	8,334	6,153	14.484
1.6	39.81	11.027	8.480	19.453

(c) Derivation of rate of change of number of nuclei on coenzymes for sheep of Series II

$$x_3 = 1.432051 + 0.627805x_6$$

i.e. $N = 27.0428\ C^{0.627805}$

$$\frac{dN}{dC} = 16.9776\ C^{-0.372195}$$

x_6	C	N	$\frac{dN}{dC}$
1.77815	60	353.5	3.70
2.00000	100	487.1	3.06
2.30103	200	752.7	2.36
2.47712	300	971.0	2.03
2.60206	400	1163.	1.83
2.69897	500	1338	1.68
2.77815	600	1500	1.57
2.84510	700	1653	1.48
2.90309	800	1797	1.41
2.95424	900	1935	1.35
3.00000	1000	2068	1.30
3.02119	1050	2132	1.27

(c) <u>Derivation of rate of change of number of nuclei on coenzymes for rats of Series II</u>

$x_3 = 0.599685 + 0.768651x_6$

$N = 3.97818\ C^{0.768651}$

$\frac{dN}{dC} = 3.057832\ C^{-0.231349}$

x_6	C	N	$\frac{dN}{dC}$
-0.82391	0.15	0.926	4.74
-0.30103	0.50	2.34	3.59
0.00000	1.0	3.98	3.06
0.30103	2.0	6.78	2.60
0.47712	3.0	9.26	2.37
0.69897	5.0	13.7	2.11
0.90309	8.0	19.7	1.89
1.07918	12.0	26.9	1.72
1.20412	16.0	33.5	1.61
1.30103	20.0	39.8	1.53

(d) Lambs and sheep (Series III) : $\log_{10}$ data

No.	Variate				
	x_2	x_3	x_4	x_5	x_6
1	1.24055	2.35218	1.60638	0.69020	1.65610
2	1.24055	2.41664	1.53529	0.76343	1.60314
3	1.25285	2.41497	1.27646	0.96379	1.44871
4	1.27875	2.62941	1.32015	0.81291	1.43775
5	1.31597	2.51054	1.73400	0.83251	1.78533
6	1.40312	2.42975	1.73878	0.74819	1.78104
7	1.43933	2.56937	1.60097	1.10380	1.72099
8	1.18469	2.35025	1.45637	0.86923	1.55630
9	1.25042	2.38382	1.56585	0.79239	1.63347
10	1.27416	2.46687	1.66087	0.94939	1.73799
11	1.29003	2.41830	1.66932	0.67210	1.78817
12	1.42325	2.49831	1.88762	1.25527	1.97864
13	1.33445	2.39794	1.59770	1.08636	1.71433
14	1.34830	2.43136	1.76938	0.97772	1.83442
15	1.38561	2.48714	1.78958	0.92428	1.84510
16	1.50920	2.58433	1.71012	1.12385	1.81023
17	1.40140	2.46090	1.54283	0.83251	1.62014
18	1.55023	2.62428	1.63749	0.96379	1.72099
19	1.55871	2.63949	1.73400	1.15229	1.83506

(d) Lambs and sheep (Series III) : $\log_{10}$ data (continued)

No.	Variate				
	x_2	x_3	x_4	x_5	x_6
20	1.68395	2.67669	1.91960	1.33445	2.0200
21	1.71012	2.66181	1.95999	1.40312	2.06742
22	1.78176	2.73400	2.06181	1.60206	2.19033
23	1.81023	2.78104	1.98408	1.62941	2.14301
24	1.72835	2.74507	1.97220	1.26482	2.04999
25	1.90309	2.91009	2.18184	1.74273	2.31597
26	2.34242	3.26902	2.69636	2.29003	2.84011
27	2.16137	3.13191	2.50106	2.02119	2.62531

(d) Crude and corrected sums of squares and products

(Log_{10} data : Series III : n=27)

	x_2	x_3	x_4	x_5	x_6
Sum	40.80286	69.97548	48.11010	30.80182	50.76004
mean	1.51122	2.59168	1.78186	1.14081	1.88000
SS	63.907476	182.702646	88.352690	39.572939	98.23168
Corr. SS	2.245500	1.348283	2.627441	4.433972	2.803217
x_2			74.964170	49.555980	79.090982
			2.259367	3.007745	2.381545
x_3			126.334907	82.095639	133.303787
			1.648709	2.267041	1.749781

(d) Regression analysis : log_{10} data : Sheep Series III

1) Regression of x_4 on x_2

Variation due to	df	SS	MS	VR
Regression	1	2.273320	2.273320	160.49***
Residual	25	0.354121	0.014165	
Total	26	2.627441		

b = 1.006175 SE (b) = 0.07942 $x_4 = 0.2613 + 1.0062x_2$

2) Regression of x_5 on x_2

Variation due to	df	SS	MS	VR
Regression	1	4.028737	4.028737	248.55***
Residual	25	0.405235	0.016209	
Total	26	4.433972		

b = 1.339454 SE (b) = 0.08496 $x_5 = -0.8834 + 1.3395x_2$

3) Regression of x_6 on x_2

Variation due to	df	SS	MS	VR
Regression	1	2.525832	2.525832	227.65***
Residual	25	0.277385	0.011095	
Total	26	2.803217		

b = 1.060586 SE (b) = 0.07029 $x_6 = 0.2772 + 1.060x_2$

(d) Regression analysis : $\log_{10}$ data : sheep ; Series III
(continued)

4) Regression of x_4 on x_3

Variation due to	df	SS	MS	VR
Regression	1	2.016076	2.016076	82.44***
Residual	25	0.611365	0.024455	
Total	26	2.627441		

b = 1.222821 SE (b) = 0.1347 $x_4 = -1.3873 + 1.2228x_3$

5) Regression of x_5 on x_3

Variation due to	df	SS	MS	VR
Regression	1	3.811867	3.811867	153.19***
Residual	25	0.622105	0.024884	
Total	26	4.433972		

b = 1.681428 SE (b) = 0.1359 $x_5 = -3.2169 + 1,6814x_3$

6) Regression of x_6 on x_3

Variation due to	df	SS	MS	VR
Regression	1	2.270839	2.270839	106.64***
Residual	25	0.532378	0.021295	
Total	26	2.803217		

b = 1.297785 SE (b) = 0.1257 $x_6 = -1.4834 + 1.2978x_3$

(e) Crude and corrected sums of squares and products

(Log$_{10}$ data : Series II and Series III)

n = 47	x_2	x_3	x_4	x_5	x_6
Sum	78.32451	126.92968	89.30192	66.33024	95.85874
SS	136.226089	346.193292	176.901158	105.330119	202.976151
Corr. SS	5.699943	3.403001	7.223662	11.719465	7.467683
x_2			154.746023	118.334644	166.060838
			5.926255	7.796696	6.314267
x_3			245.644410	185.033679	263.646202
			4.472833	5.900145	4.767070

Summation of the corrected sums of squares and products for Series II and Series III

	x_2	x_3	x_4	x_5	x_6
SS	4.170401	2.649884	6.337607	7.077720	5.852563
x_2			4.761968	5.132162	4.742519
x_3			3.655855	4.030444	3.664177

(e) Regression analysis : $\log_{10}$ data : sheep : Series (II + III)

1) Regression of x_4 on x_2

Variation due to	df	SS	MS	VR
Regression	1	6.161553	6.161553	261.005***
Residual	45	1.062309	0.023607	
Total	46	7.223862		

b = 1.039704 SE (b) = 0.06436 $x_4 = 0.1674 + 1.0397x_2$

2) Regression of x_5 on x_2

Variation due to	df	SS	MS	VR
Regression	1	10.664750	10.664750	455.020***
Residual	45	1.054715	0.023438	
Total	46	11.719465		

b = 1.367855 SE (b) = 0.06412 $x_5 = -0.8682 + 1.3679x_2$

3) Regression of x_6 on x_2

Variation due to	df	SS	MS	VR
Regression	1	6.994801	6.994801	665.664***
Residual	45	0.472882	0.010508	
Total	46	7.467683		

b = 1.107777 SE(b) = 0.04294 $x_6 = 0.1935 + 1.1078x_2$

(e) Regression analysis : $\log_{10}$ data : sheep : Series (II + III) (continued)

4) Regression of x_4 on x_3

Variation due to	df	SS	MS	VR
Regression	1	5.878998	5.878998	196.714***
Residual	45	1.344864	0.029886	
Total	46	7.223862		

b = 1.314379   SE (b) = 0.09371 $x_4 = -1.6496 + 1.3144x_3$

5) Regression of x_5 on x_3

Variation due to	df	SS	MS	VR
Regression	1	10.229709	10.229709	308.999***
Residual	45	1.489756	0.33106	
Total	46	11.719465		

b = 1.733806   SE (b) = 0.09863 $x_5 = -3.2711 + 1.7338x_3$

6) Regression of x_6 on x_3

Variation due to	df	SS	MS	VR
Regression	1	6.677916	6.677916	380.508***
Residual	45	0.789767	0.017550	
Total	46	7.467683		

b = 1.400843   SE (b) = 0.07181 $x_6 = -1.7436 + 1.4008x_3$

(e) **Analysis of variance of regressions : log_{10} data : sheep: Series (II + III)**

Variation due to	df	SS	MS	VR
x_4 on x_2				
Means	1	0.886255		47.01***
Between Regressions	1	0.089552		4.75*
Common Regression	1	5.437448		288.44
Deviations	43	0.810607	0.018851	
Total	46	7.223862		
x_5 on x_2				
Means	1	4.641745		283.36***
Between Regressions	1	0.057630		3.52
Common Regression	1	6.315720		385.55
Deviations	43	0.704370	0.016381	
Total	46	11.719465		
x_6 on x_2				
Means	1	1.615119		161.17***
Between Regression	1	0.028545		2.85
Common Regression	1	5.393123		538.18
Deviations	43	0.430895	0.010021	
Total	46	7.467683		

(e) Analysis of variance of regressions : $\log_{10}$ data : sheep : Series (II + III) (continued)

Variation due to	df	SS	MS	VR
x_4 on x_3				
Means	1	0.886255		31.07
Between Regressions	1	0.067493		2.37
Common Regression	1	5.043721		176.84
Deviations	43	1.226393	0.028521	
Total	46	7.223862		
x_5 on x_3				
Means	1	4.641745		227.64
Between Regressions	1	0.070657		3.47
Common Regression	1	6.130260		300.64
Deviations	43	0.876803	0.020391	
Total	46	11.719465		
x_6 on x_3				
Means	1	1.615119		90.66
Between Regression	1	0.019824		1.11
Common Regression	1	5.066710		284.41
Deviations	43	0.766029	0.017815	
Total	46	7.467683		

(e) Points for plotting regression lines of Series III

Regression	x_4 on x_2		x_5 on x_2		x_6 on x_2	
x_2	Log_{10}	Original	Log_{10}	Original	Log_{10}	Original
1.1	1.3681	23.3	0.5901	3.89	1.4439	27.8
1.2	1.4687	29.4	0.7240	5.30	1.5499	35.5
1.3	1.5694	37.1	0.8580	7.21	1.6560	45.3
1.4	1.6700	46.8	0.9919	9.82	1.7620	57.8
1.5	1.7706	59.0	1.1259	13.36	1.8681	73.8
1.6	1.8712	74.3	1.2598	18.19	1.9742	94.2
1.7	1.9718	93.7	1.3938	24.76	2.0802	120.2
1.8	2.0725	118.2	1.5277	33.71	2.1863	153.6
1.9	2.1731	149.0	1.6617	45.89	2.2923	196.0
2.0	2.2737	187.8	1.7956	62.46	2.3984	250.3
2.1	2.3743	236.8	1.9296	85.04	2.5045	319.5
2.2	2.4749	298.5	2.06350	115.74	2.6105	407.8
2.3	2.5756	376.4	2.19745	157.56	2.7166	520.7
2.4	2.6762	474.5	2.33140	214.49	2.8226	664.7

(e) Points for plotting regression lines of Series (II + III)

Regression	x_4 on x_2		x_5 on x_2		x_6 on x_2	
x_2	Log_{10}	Original	Log_{10}	Original	Log_{10}	Original
1.1	1.3111	20.5	0.6365	4.33	1.4121	25.8
1.2	1.4150	26.0	0.7733	5.93	1.5229	33.3
1.3	1.5190	33.0	0.9101	8.13	1.6336	43.0
1.4	1.6230	42.0	1.0469	11.14	1.7444	55.5
1.5	1.7270	53.3	1.1837	15.27	1.8552	71.6
1.6	1.8309	67.8	1.3204	20.91	1.9660	92.5
1.7	1.9349	86.1	1.4572	28.66	2.0768	119.3
1.8	2.0389	109.4	1.5940	39.26	2.1875	154.0
1.9	2.1428	138.9	1.7308	53.80	2.2983	198.8
2.0	2.2468	176.5	1.8676	73.72	2.4091	256.5
2.1	2.3508	224.3	2.00439	101.02	2.5199	331.1
2.2	2.4547	284.9	2.14118	138.41	2.6307	427.3
2.3	2.5587	362.0	2.27797	189.66	2.7414	551.3
2.4	2.6627	459.9	2.41476	259.87	2.8522	711.6
2.5	2.7667	584.4	2.55155	356.08	2.9630	918.3

(f) Coenzyme ratio : $\log_{10}$ data : Sheep : n = 47

Series II		Series III	
$(\log W)^2$	$\log y_1$	$(\log W)^2$	$\log y_1$
2.41078	0.71238	1.53896	0.91618
2.24493	0.36908	1.53896	0.77186
2.32181	0.24900	1.56963	0.31267
2.14297	0.34749	1.63520	0.50724
2.43703	0.21004	1.73178	0.90149
2.88946	-0.03900	1.96875	0.99059
3.14633	0.39658	2.07167	0.49717
2.94754	0.20909	1.40349	0.58714
3.37207	-0.06731	1.56355	0.77346
2.76161	0.08319	1.62348	0.71148
3.18230	-0.27137	1.66418	0.99722
3.47196	0.46392	2.02564	0.63235
3.98258	0.29973	1.78076	0.51134
4.05152	0.22640	1.81791	0.79166
3.84156	0.17974	1.91992	0.86530
4.06841	0.44205	2.27768	0.58627
5.75842	0.43917	1.96392	0.71032
5.96571	0.38739	2.40321	0.67370
5.62197	0.60587	2.42958	0.58171
5.69963	0.41996	2.83569	0.58515
		2.92451	0.55687
		3.17467	0.45975
		3.27693	0.35467
		2.98719	0.70738
		3.62175	0.43911
		5.48693	0.40633
		4.67152	0.47987

(f) Various regressions were calculated in an attempt to relate the coenzyme ratio, ([NAD] + [$NADH_2$]): ([NADP] + [$NADPH_2$]), to liver dry wt., W., e.g.

$\log_{10}$ (coenzyme ratio) on $\log_{10}W$,

$$\log_{10}W + (\log_{10}W)^2,$$

$$\frac{1}{\log_{10}W} + \log_{10}W,$$

$$\frac{1}{\log_{10}W} + (\log_{10}W)^2,$$

$$\frac{1}{\log_{10}W} + \log_{10}W + (\log_{10}W)^2.$$

There was little to choose between any of these but the regression of $\log_{10}$ (coenzyme ratio) on $\log_{10}W + (\log_{10}W)^2$ was slightly better than the others. This analysis is presented on the following page.

(f) Regression of $\log_{10}(y_1)$ on $\log_{10}W$ and $(\log_{10}W)^2$

	SD	t(on 44 df)
b_1 = -3.473008	± 0.9210	3.771***
b_2 = 0.879165	± 0.2561	3.433**

Analysis of Variance

Variation due to	df	SS	MS	VR
Regression	2	1.208972	0.604486	11.97***
Deviations	44	2.222551	0.050513	
Total	46	3.431523		

$$\log(y_1) = 3.7282 - 3.4730 \log W + 0.8792 (\log W)^2$$

(g) Enzymic activities : $\log_{10}$ data : sheep; Series III

log W	log (100 y_2) glucose ATP	glucose 6-phosphate	6-phospho- gluconate
1.23805	1.77815	2.00000	2.69020
1.24055	1.77815	1.89209	2.73239
1.24055	1.47712	2.08279	2.69897
1.25285	1.73239	2.06070	2.56820
1.27875	-	1.88081	1.56820
1.31597	1.73239	1.85733	2.55630
1.40312	1.62325	1.91908	-
1.43933	1.47712	1.88081	2.81954
1.18469	1.81954	1.97313	-
1.25042	1.62325	2.03743	-
1.26245	1.55630	1.95424	-
1.27416	2.00860	2.02119	2.64345
1.29003	1.73239	1.90848	2.55630
1.42325	1.62325	2.01284	2.84510
1.33445	1.68124	2.14613	-
1.34830	1.62325	2.04139	-
1.38561	1.77815	2.04139	-
1.50920	1.62325	2.06446	-
1.40140	1.77815	1.96379	-
1.55023	1.73239	1.98227	-
1.60853	1.73239	2.02119	-
1.55871	1.73239	2.16137	-
1.68395	1.68124	1.95904	-
1.71012	1.73239	2.04139	-
1.78176	1.55630	1.77815	-
1.81023	1.77815	1.77815	-
1.72835	1.73239	1.93952	-
1.90309	1.68124	1.91908	-

(g) Enzymic activities : $\log_{10}$ data : sheep Series IV

log W	glucose ATP	log (100 y_2) glucose 6-phosphate	6-phospho-gluconate
1.35218	-	1.95424	2.04922
1.42813	1.56820	1.74819	1.77815
1.46687	1.60206	1.95904	1.98677
1.58206	1.20412	1.85733	2.17026
1.62737	-	-	-
1.60853	1.34242	1.92942	1.65321
1.66558	-	1.96379	1.74819
1.70757	1.89209	2.01284	2.35603
1.76492	0.47712	1.71600	2.35411
1.90902	0.90309	1.66276	-
1.73640	0.69897	1.34242	-
1.76268	0.60206	1.69897	-
1.78817	0.84510	1.60206	-
1.79796	0.69897	1.79239	2.19590
1.85612	1.32222	2.02531	-
1.89042	-	1.74819	-
1.97727	1.00000	1.71600	-
1.94645	0.47712	1.83885	2.16732
1.98408	0.47712	1.92942	2.04139
2.30535	0.00000	1.57978	2.30103
2.31175	0.69897	1.91908	2.39270
2.43933	0.47712	1.74819	1.88081
2.39794	0.60206	1.43136	1.89209
2.43457	0.30103	1.34242	2.20412

(g) Enzymic activities : Regression analysis : Series (III + IV)

(i) Regression of log ($100y_2$) on log W

y_2 with glucose + ATP

$n = 47$

$S W = 77.33452$

$S W^2 = 132.703142$

$S(W-\bar{W})^2 = 5.455738$

$b = -1.202036$

$S y_2 = 62.99466$

$S(y_2)^2 = 97.353703$

$S(y_2-\bar{y}_2)^2 = 12.921209$

$S W y^2 = 97.094383$

$S(W-\bar{W})(y_2-\bar{y}_2) = -6.557994$

Analysis of Variance

Variation due to	df	SS	MS	VR
Regression	1	7.882946	7.882946	70.41***
Residual	45	5.038264	0.111961	
Total	46	12.921210		

$y_2 = 3.3182 - 1.2020W$

(g) Enzymic activities : Regression analysis : Series (III + IV) (continued)

(i) Regression of log ($100y_2$) on log W

y_2 with glucose 6-phosphate

$n = 51$

$S\,W = 83.52145$

$S\,W^2 = 142.514579$

$S(W-\bar{W})^2 = 5.733547$

$b = -0.345855$

$S\,y_2 = 95.83629$

$S(y_2)^2 = 181.795774$

$S(y_2-\bar{y}_2)^2 = 1.705686$

$S\,W\,y_2 = 154.965763$

$S(W-\bar{W})(y_2-\bar{y}_2) = -1.982979$

Analysis of Variance

Variation due to	df	SS	MS	VR
Regression	1	0.685825	0.685825	32.95***
Residual	49	1.019862	0.020814	
Total	50	1.705687		

$y_2 = 2.4455 - 0.3459W$

(g) Enzymic activities : Regression analysis : Series (III + IV) (continued)

(i) Regression of log $(100y_2)$ on log W

y_2 with 6-phosphogluconate

$n = 26$

$S\,W = 43.18676$

$S\,W^2 = 76.091417$

$S(W-\bar{W})^2 = 4.356946$

$b = -0.402524$

$S\,y_2 = 59.84995$

$S(y_2)^2 = 140.862700$

$S(y_2-\bar{y}_2)^2 = 3.092834$

$S\,W\,y_2 = 97.658736$

$S(W-\bar{W})(y_2-\bar{y}_2) = -1.753779$

Analysis of Variance

Variation due to	df	SS	MS	VR
Regression	1	0.705940	0.705940	7.10*
Residual	24	2.386894	0.099454	
Total	25	3.092834		

$$y_2 = 2.9705 - 0.4025W$$

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