Determination of the Reduced and Oxidized Pyridine Nucleotides in Animal Tissues

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The determination of the concentration of the reduced and oxidized pyridine nucleotide in biological material is a matter of interest, because of the key role of these substances in intermediary metabolism. When the work reported in this paper was begun a sensitive method suitable for the determination of $0{\cdot}3 \times 10^{-9}$ mole in 1 ml., elaborated by Glock & McLean (1955), was available but difficulties were encountered in preparing the diphosphopyridine nucleotide-linked cytochrome *c* reductase required for the determination. After the publication of the fluorimetric procedure of Lowry, Roberts & Kapphahn (1957) it was decided to employ this method but it was found necessary to introduce modifications.

The concentrations of reduced coenzymes in liver reported by Lowry *et al.* were considerably lower than the corresponding data presented by Glock & McLean, and a comparison of the extraction procedures used by these two groups of workers showed the method of Glock & McLean to be superior in that their alkaline extract (pH about 13) contained more reduced coenzyme than the extract of Lowry *et al.* made at pH 8. Accordingly, an extraction procedure similar to that of Glock & McLean was adopted. A further modification of the method of Lowry *et al.* was necessary because of the oxidation of the reduced coenzymes during subsequent manipulation of the alkaline extract. The rate of oxidation is very rapid after adjustment of the alkalinity to pH 8·0. This invalidates the assumption that all the coenzymes in this extract are in the reduced form. The error introduced by this oxidation was more serious when the extracts were stored (even at $-15°$) before estimation. However, by combining the selective destruction of the oxidized coenzymes by alkali and of the reduced coenzymes by acid (see Schlenk, 1951) with dehydrogenase reactions specific for diphosphopyridine nucleotide and triphosphopyridine nucleotide respectively, a satisfactory procedure for the estimation of both oxidized and reduced di- and triphosphopyridine nucleotides in concentrations as low as $1{\cdot}5\ \mu$M was obtained.

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Whilst this work was in progress, another method was published by Jacobson & Astrachan (1957). Their procedure requires the preparation of five enzymes and the sensitivity is less than that of the other methods.

This paper presents the results obtained in an investigation of a number of factors influencing the precision and accuracy of the method; and a series of values for the level of coenzymes in several tissues from different animals and for the levels of oxidized and reduced coenzymes during the incubation of tissue preparations *in vitro*.

EXPERIMENTAL

Diphosphopyridine nucleotide (DPN) was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. It contained 91% of DPN (by the alcohol dehydrogenase method of Racker, 1950) and no triphosphopyridine nucleotide (TPN). TPN was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. It contained 92% of TPN when analysed by fluorimetry against a DPN standard solution prepared from the DPN described above.

Reduced diphosphopyridine nucleotide (DPNH) was obtained from Sigma Chemical Co. and contained 62% of DPNH when analysed by fluorimetry. Reduced triphosphopyridine nucleotide (TPNH) was obtained from Sigma Chemical Co. and contained 27% of TPNH when analysed by fluorimetry.

Glucose 6-phosphate dehydrogenase (G 6-P dehydrogenase) was prepared by the method of Kornberg (1950) and stored in the lyophylized form at $-20°$. Alcohol dehydrogenase was prepared from baker's yeast by the method of Racker (1950). The crystalline enzyme was stored in half-saturated $(NH_4)_2SO_4$ at $-20°$.

Nicotinamide and ethylenediaminetetra-acetic acid (disodium salt; EDTA) were obtained from British Drug Houses Ltd. 2-Amino-2-hydroxymethylpropane-1:3-diol (tris) was obtained from Sigma Chemical Co. Marsalid phosphate and isoniazid were kindly supplied by Roche Products Ltd., Welwyn Garden City, Herts. Acetaldehyde was redistilled before use and 10 mM-solutions were prepared as required.

Glucose 6-phosphate (G 6-P) was obtained as the barium salt heptahydrate from British Drug Houses Ltd. The barium was removed with Dowex-50 H^+ resin and the glucose 6-phosphoric acid neutralized by the addition of $NaHCO_3$ solution. The final strength of the solution was 0·1 M. Hydrogen peroxide (3%, w/v) was prepared by

dilution of 30% (w/v) reagent obtained from British Drug Houses Ltd., and was stored at 2°.

Experimental animals. Rats were of the white Wistar strain. Mice were CBA strain and the New Zealand strain of obese mice (N.Z.O.) was used.

Sheep heart was obtained from the abattoir, the tissue being transferred as speedily as possible to a Dewar flask containing crushed ice.

Preparation of homogenate. Tissues were minced in the Fischer mincer (Établissement Jouan, Paris) and portions of the mince were weighed into cooled beakers and then transferred with cold homogenizing medium into a cold stainless-steel homogenizer of the Potter–Elvehjem type. The pestle was rotated at 2000 rev./min.

Preparation of minces. Tissues were minced in the Fischer mincer, portions were weighed into cooled beakers and suspended in cold medium.

Preparation of chopped tissue. The mechanical tissue chopper (McIlwain & Buddle, 1953) was used. Prisms of dimensions 0·35 mm. × 0·35 mm. × 3 mm. were prepared and suspended in cold medium. The entire preparation was carried out in the cold room at 2°. The above-mentioned preparations were suspended in the medium (without calcium) with added phosphate buffer, pH 7·4, of Krebs & Eggleston (1940).

Measurement of fluorescence. The fluorescence developed from samples containing pyridine nucleotides was measured in the Farrand fluorimeter model A, Farrand Optical Co. Inc., New York 70, N.Y. The primary filter was Farrand no. 5860 and the secondary filter comprised Farrand no. 3389 and 4308 together with Wratten no. 2B. The instrument was regularly standardized against a solution of quinine sulphate at 0·1 mg./l. of 0·1 N-H_2SO_4.

Extraction procedures

Whole tissue. Two small glass homogenizers containing the following extracting fluids were heated in boiling water. Acid medium: 5 ml. of 0·1 M-tris–HCl buffer, pH 8·2; 2 ml. of water; 1 ml. of N-HCl. Alkaline medium: 5 ml. of tris–HCl buffer; 2 ml. of water; 1 ml. of N-NaOH.

The test animal was killed, the liver removed as quickly as possible and two portions of 300–500 mg. were weighed and placed in the hot extracting fluids. The time from killing the animal to placing the samples in the medium was less than 2 min.

During the first heating for 10 sec., the liver was broken into small pieces with a glass rod and then homogenized for about 30 sec., after which it was briefly heated again. After a total heating time of 2 min., rapid cooling was achieved by immersion of the homogenizers in liquid air and by stirring the contents until they froze. The mixtures were thawed, and stirred during adjustment to pH 8·0 (checked with indicator papers) with 1 ml. of either N-HCl or N-NaOH. The solutions were transferred to chilled graduated tubes and the volumes made up to 10 ml. The extracts were then centrifuged at 20 000 *g* for 15 min. in a refrigerated centrifuge. It was useful to employ an upper layer of *n*-hexane during centrifuging to prevent retention of particles by fat at the upper surface of the aqueous layer. The supernatant aqueous layers were removed with Pasteur pipettes, immediately frozen and retained for analysis. Subsequently the extract prepared in the acid medium is designated 'acid extract' and that prepared in the alkaline medium 'alkaline extract'.

Homogenate, mince and chopped tissue. For each analysis of a homogenate, mince or chopped tissue, two small conical flasks (about 15 ml. volume) were used. One contained 1 ml. of tris–HCl (0·1 M, pH 8·2) and 0·25 ml. of N-NaOH, the other contained 1 ml. of tris–HCl (0·1 M, pH 8·2) and 0·25 ml. of N-HCl. The flasks were heated in boiling water.

Samples (1 ml.) of tissue preparation were added to the appropriate flasks, which were periodically shaken in the water bath during heating for 2 min. The flasks were removed and cooled rapidly in liquid air until the contents were frozen. The subsequent treatment was similar to that described for whole-tissue extracts.

Solutions required for estimations

Glucose 6-phosphate dehydrogenase. The activity of aqueous solutions of the freeze-dried enzyme was adjusted so that 10 µl. reduced 10^{-8} mole of DPN at pH 8·2 (tris–HCl buffer) in a total volume of 0·5 ml. in 10 min.

Alcohol dehydrogenase. The activity of aqueous solutions of the stock suspension was adjusted so that 10 µl. reduced 10^{-8} mole of DPN at pH 8·2 (tris–HCl buffer) in a total volume of 0·5 ml. in 10 min.

Approximately N-hydrochloric acid and -sodium hydroxide. Solutions of identical normality were prepared. These will be referred to as 'standard' HCl and 'standard' NaOH.

Sodium hydroxide–hydrogen peroxide solution. This was prepared immediately before use by adding 0·1 ml. of the stock 3% H_2O_2 to each 3 ml. of 9 N-NaOH. The appearance of a fine precipitate in this solution did not affect the reproducibility of the results.

Standard DPN solution. A solution (20 µM) of DPN was prepared in tris–HCl buffer, pH 8·2, at a molarity close to that of the test solutions (about 0·03 M).

Analytical procedure

The acid and alkaline extracts from tissue preparations were analysed according to the scheme shown in Fig. 1.

Acid extract. To a sample (0·5 ml.) of the acid extract (solution A, Fig. 1) in a 3 ml. tube was added 10 µl. of G 6-P dehydrogenase solution and 10 µl. of G 6-P. The solutions were mixed and allowed to stand at room temperature (about 20°) for 20 min. before further manipulation.

Alkaline extract. The alkaline extract (solution A′, Fig. 1) contains the coenzymes which in the original tissue preparation were in the reduced form. It was found that a proportion of the reduced coenzymes in this extract became reoxidized during the lengthy manipulations involved in both the extraction and estimation; when storage of this extract was necessary reoxidation continued even at −15°. It was therefore necessary to separate oxidized from reduced coenzymes by treating one sample (0·5 ml.) of the alkaline extract with 30 µl. of standard HCl at room temperature for 5 min. and another sample of 0·5 ml. with 30 µl. of standard NaOH at 60° for 15 min. Then each solution was adjusted to pH 8·2 by the addition of 30 µl. of standard alkali or acid to give solution B (containing oxidized coenzymes) and solution C (containing reduced coenzymes). The solutions were stirred continually during adjustment to pH 8·2.

Solution B. This volume (0·5 ml.) was given the same treatment as the 0·5 ml. sample of 'acid extract' above.

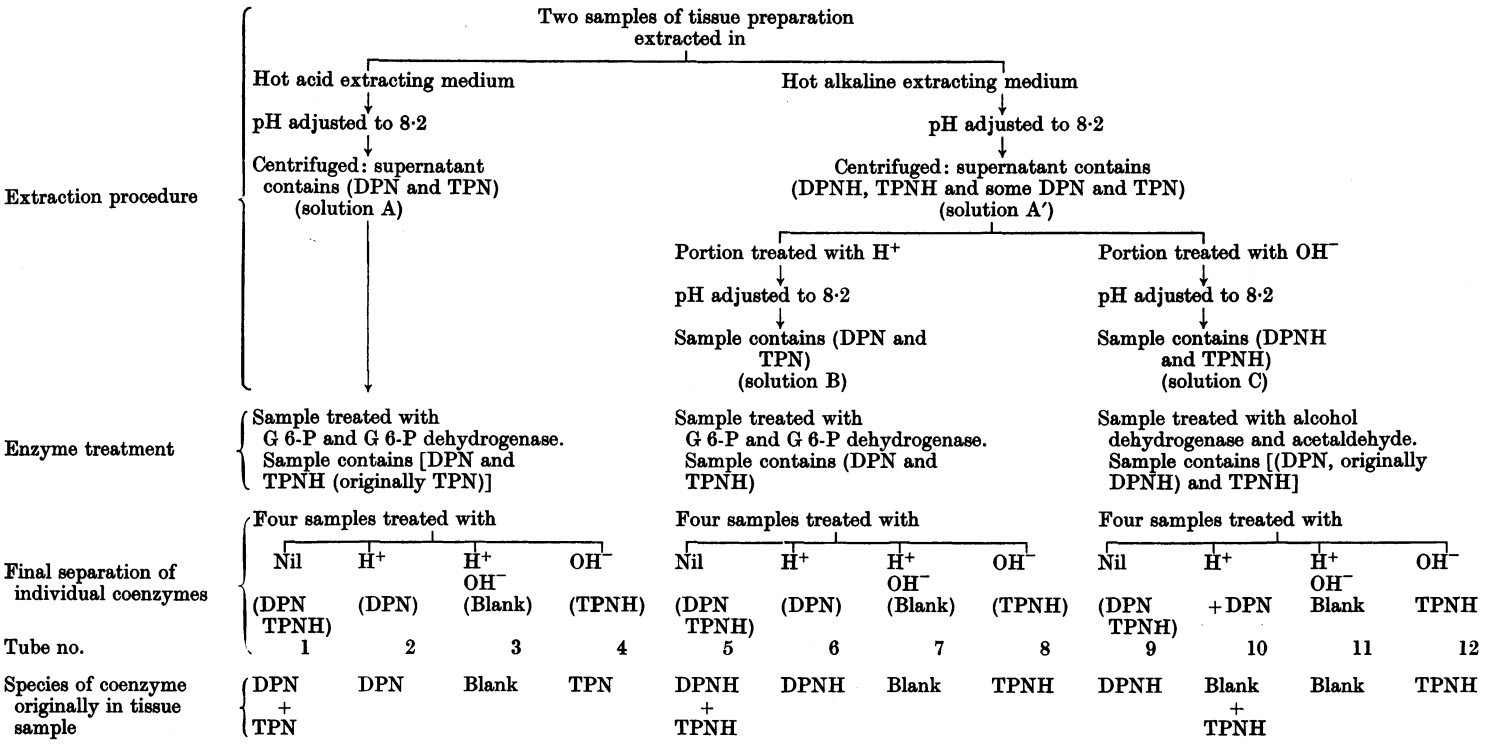

Fig. 1. Scheme of analytical procedure. Fluorescence was developed in each of the tubes 1 to 12 by the addition of the appropriate amounts of conc. $NaOH–H_2O_2$ mixture. Values obtained in the pairs of tubes 5 and 9, 6 and 10, 8 and 12, are combined. H^+ indicates 'standard' acid, OH^- indicates 'standard' alkali.

Solution C. To this 10 μl. of 0·035 M-EDTA, 10 μl. of alcohol dehydrogenase solution and 5 μl. of 0·01 M-acetaldehyde were added and the mixture was allowed to stand at room temperature for 20 min. before further manipulation.

From this point in the analysis each of the solutions A, B and C received the following identical treatment.

Four samples, each of 25 μl., were placed in fluorimeter tubes. The first of these tubes received no standard acid or alkali. To the second tube 2 μl. of standard HCl was added; to the third tube 2 μl. of standard HCl was added and, at the end of 5 min., 7 μl. of standard alkali; to the fourth tube 5 μl. of standard alkali was added. Both tubes receiving alkali were heated at 60° for 15 min. and then cooled.

To every fluorimeter tube 0·1 ml. of the NaOH–H_2O_2 mixture was added and, after mixing, the tubes were heated in a water bath at 38° for 60 min. It was necessary to use distilled water in the bath to prevent the possible contamination of the outside of the tubes by fluorescent material.

The fluorescence from a known amount of coenzyme was determined by including with each batch of estimations three tubes containing 25 μl. of standard 20 μM-DPN solution. The DPN in one of these samples was destroyed by adding 5 μl. of standard NaOH and heating at 60° for 15 min. The fluorescence developed in this sample was subtracted from the mean value obtained from the other two standard DPN samples.

When values for total oxidized and total reduced coenzyme only were required, the procedure was shortened by treating samples of solutions A and A′ (see Fig. 1) with the concentrated NaOH–H_2O_2 mixture, and fluorescence developed in the usual way. Each determination involved the production of a blank with the standard acid and standard alkali treatment.

Owing to the relatively low concentrations of TPN in the tissues studied, it was sometimes desirable to increase the volume of sample finally analysed from 25 to 75 μl. Certain modifications of the method were then necessary. The quantity of standard acid and alkali added to destroy the coenzymes was increased threefold, and since the final concentration of alkali in the mixtures which were heated at 38° for 60 min. must exceed 6 N, the concentrated NaOH–H_2O_2 mixture was prepared by adding 0·16 ml. of 3% (w/v) H_2O_2 to each 3 ml. of 15 N-NaOH. Analyses of the same solution by the standard method and with this modification gave comparable results.

Blanks. The blanks obtained with a particular tissue were reasonably constant. For example, with the 'acid' extract from rat-liver mince, mean value from twenty-four experiments for the galvanometer readings was 13, with S.D. of 3; and for the alkaline extract was 20, S.D. of 6. Blank values at the upper limit of these ranges were not correlated with a low observed amount of coenzyme, either in simultaneous analyses of the livers of several different animals or in analyses of different extracts of the same liver. Some of the highest total coenzyme concentrations observed were associated with relatively high blank values.

Infrequently, after incubation of tissues, the fluorescence in the blank samples treated with standard acid followed by standard alkali (see Fig. 1) increased greatly (up to four times the original values) and exceeded that found in the samples treated with either standard acid or standard alkali alone. When this occurred, an alternative method for producing the blank was employed, in which the oxidized coenzymes were first reduced enzymically and then all the coenzymes in the sample were destroyed with standard acid.

RESULTS

Recovery of added coenzymes. The recoveries (see Table 1) were determined by adding known amounts of coenzymes to tissue preparations during extraction. The coenzyme was added after heating a suspension of rat-liver mince for 30 sec., and heating was then continued for a further 2 min. before freezing. The endogenous coenzyme was determined in a separate extract.

Factors influencing the sensitivity of the method. The presence of tris–HCl buffer in solutions of the coenzymes reduced the fluorescence developed. Accordingly, all standard coenzyme solutions were made in this buffer.

Lowry *et al.* (1957) have shown that certain compounds decrease the fluorescence developed from coenzyme samples, and note that H_2O_2 reduces this effect. With the NaOH–H_2O_2 mixtures described in this paper, pyruvate, α-oxoglutarate and

Table 1. *Recovery of coenzymes added to rat-liver mince during extraction*

Values given are means ± S.E.M. The amount of coenzyme added was one to three times the endogenous amount.

Coenzyme added	No. of expts.	Recovery (%)
DPN	4	93 ± 6
TPN	5	93 ± 3
DPNH	4	104 ± 5
TPNH	8	88 ± 7

Table 2. *Coenzymes extracted in 'neutral', acid and alkaline media*

The tissue preparation was a 1 in 15 suspension of rat-liver mince (Fischer mincer). The acid and alkaline extractions were performed as described in the Experimental section. The 'neutral' extraction was made in tris–HCl buffer (pH 8·2, 0·1 M), and the supernatant after centrifuging was analysed for total oxidized and reduced coenzymes in the manner described for the neutralized supernatant from the alkaline extract (see Fig. 1 down to solutions B and C).

	Coenzyme content (μm-moles/g. fresh wt.)				
	DPN	TPN	DPNH	TPNH	Total
Acid extract	485	103	—	—	1174
Alkaline extract	—	—	302	284	
'Neutral' extract	445	115	215	114	889

fructose at the concentrations greater than about 5 mM reduced the fluorescence, whereas succinate and citrate did not, i.e. compounds containing keto groups are responsible for the interference. At concentrations less than 5 mM in the final sample, pyruvate and α-oxoglutarate had no effect.

Extraction procedure. A comparison of the extraction made with the acid and alkaline media used by Glock & McLean (1955) and the single extraction in tris–HCl buffer, pH 8·2, used by Lowry *et al.* (1957) showed that the former procedure was to be preferred (see Table 2).

No significant variation was found in the amount of coenzyme extracted during times of heating ranging from 1·5 to 4·5 min. Outside these limits values were lower. As a routine, heating times of 2·0–2·5 min. were used.

The efficiency of the extraction was not affected by homogenizing the tissue preparation during the heating.

Oxidation of reduced coenzymes in neutralized alkaline extracts. It was found that on storage of the neutralized alkaline extracts, considerable oxidation of the reduced coenzymes took place. Consequently it was necessary to treat the alkaline extract as a mixture of both oxidized and reduced coenzymes (Table 3). When this was done, satisfactory recoveries of all the coenzymes were obtained after storage. Storage of the alkaline extract without neutralization reduces the degree of oxidation; but even with the shortest possible delay in estimation, no alkaline extract was found to contain only reduced coenzymes. In eight analyses of freshly prepared alkaline extracts, the mean values for the diphosphopyridine nucleotide and the triphosphopyridine nucleotide in the oxidized form were 31 and 18% respectively. Consequently, all alkaline extracts were analysed for both oxidized and reduced coenzymes and the sum of the values was taken as the original level of reduced coenzymes.

Initial values. The contents of the individual coenzymes in the livers of various animals are shown in Table 4.

The effect of starvation on the concentration and total content of coenzymes in rat livers was investigated. The weight of individual livers invariably fell with starvation. The concentration of coenzyme showed an initial increase to a higher level, which was either maintained or subsequently diminished, whereas the total quantity of coenzyme did not usually decrease.

Method of tissue preparation for use in metabolic experiments. The effect on the initial level of coenzyme and the suitability for metabolic studies are factors which must influence the choice of tissue preparation. No great differences were found between the levels of coenzymes in different preparations of a particular tissue (whole tissue, homogenate, mince and chopped tissue), though the values obtained with the last-mentioned were usually the lowest. In view of the lability of the coenzymes (see Tables 6 and 7) these low values are probably due to the length of time taken over this preparation. Further it was clear that portions of whole tissue could not be used in metabolic experiments. Accordingly, most of the subsequent work was done with either minces or homogenates.

Appreciable losses of coenzymes occurred in whole and minced liver kept at 0° (10–20% in 20 min.), though under similar conditions no

Table 3. *Behaviour of coenzymes during storage*

Rat-liver mince (1 g.; Fischer mince) was suspended in 14 ml. of medium with 25 mM-nicotinamide. The samples of extract not used for immediate analysis were frozen at −15°.

Coenzyme		Coenzyme content (μm-moles/g. fresh wt.) Analysed at once		Analysed after 24 hr.	
DPN		485		515	
TPN		103		115	
DPNH	as DPN	22	302	86	288
	as DPNH	280		202	
TPNH	as TPN	54	284	163	287
	as TPNH	230		124	

Table 4. *Coenzyme content of various tissues*

Figures in parentheses indicate the number of animals used. Values given are means ± S.E.M.; those marked with an asterisk were obtained from estimation of total oxidized and reduced coenzyme only.

Tissue	Preparation	Coenzyme content (μm-moles/g. fresh wt.) DPN	TPN	DPNH	TPNH	Total
Rat liver	Whole tissue (9)	—	—	—	—	899 ± 60*
Rat liver	Fischer mince (14)	—	—	—	—	895 ± 80*
Rat liver	Whole tissue (8)	485 ± 33	25 ± 6	153 ± 24	251 ± 24	912 ± 62
Rat liver	Fischer mince (14)	308 ± 41	64 ± 11	221 ± 38	261 ± 44	854 ± 81
Pigeon liver	Whole tissue (6)	596 ± 26	36 ± 17	93 ± 9	189 ± 36	924 ± 76
Pigeon liver	Fischer mince (5)	733 ± 53	82 ± 18	143 ± 60	288 ± 69	1246 ± 87
Mouse liver	Fischer mince (6)	513 ± 66	28 ± 7	137 ± 22	126 ± 12	801 ± 68
'Obese mouse' liver	Fischer mince (5)	434 ± 144	25 ± 3	162 ± 16	129 ± 12	750 ± 146

significant loss was seen in mince suspended in medium containing 0·05 M-nicotinamide. It follows that to obtain the most reliable initial values, any preparation should be carried to the stage of suspension in medium containing nicotinamide as speedily as possible. The relatively greater stability of the suspended-tissue preparation was clearly shown with pigeon liver, where the values obtained for whole tissue were significantly lower than for minced tissue (Table 4).

Measurement of coenzymes during incubation of various tissues. During the incubation of tissue preparations considerable destruction of coenzymes occurred (see Table 6). Three inhibitors of coenzyme-splitting enzymes were tested, namely nicotinamide, isoniazid and marsilid (see Zatman, Kaplan, Colowick & Ciotti, 1954). The last-named two substances markedly reduced the fluorescence obtained. When corrections for the interference were applied it was seen that the protective action of these compounds was not as effective as that of nicotinamide. When rat-liver mince was incubated at 38° for 20 min. 76 % of the total coenzyme was destroyed. In the presence of nicotinamide (50 mM), isoniazid (5 mM) and marsilid (15 mM), the losses were 38, 56 and 96 % respectively. The most satisfactory concentrations of nicotinamide were 25–50 mM, but even in the presence of this inhibitor and at 0° there was a considerable loss of coenzyme. The rate of destruction does not increase greatly as the temperature is raised from 25° to 37°.

Incubations of pigeon liver. Pigeon-liver homogenates were incubated in 0·01 M-lactate under aerobic and anaerobic conditions and with and without nicotinamide (Table 5). The effect of anaerobiosis was to increase the amount of TPNH found after incubation, though there was no marked difference in the levels of DPNH in oxygen or in nitrogen. The presence of nicotinamide increased the stability of the oxidized coenzymes, particularly DPN. The highest value for total coenzyme was found in oxygen and with nicotinamide, the lowest in oxygen and without nicotinamide. Nicotinamide also reduced the rate of endogenous respiration.

It was expected that in nitrogen the coenzymes would be predominantly in the reduced form, and this was so for the triphosphopyridine nucleotides; the constancy of the levels of DPNH in both oxygen and nitrogen may be due to the activity of DPNH pyrophosphatase of liver (see Jacobson & Kaplan, 1957). In oxygen, a marked increase in the proportion of oxidized coenzyme (notably DPN) was demonstrated only in the presence of nicotinamide, presumably because of the inhibition of diphosphopyridine nucleotidase activity. The values for total pyridine nucleotide are consistently high in the presence of nicotinamide because of a high proportion of the relatively stable TPNH.

Nicotinamide in pigeon-liver homogenates to which no substrate has been added has the effect of inhibiting the reduction of both TPN and DPN. In the presence of added lactate this effect is not found. These results are consistent with the existence of two distinct pathways for the reduction of pyridine nucleotides, one dependent on endogenous substrate (inhibited by nicotinamide) and the other on added lactate (not inhibited by nicotinamide).

Rat-liver incubations. When suspensions of rat-liver mince, final concentration 1 g. in 15 ml. of medium, with and without nicotinamide, were incubated at 37°, the results confirmed the marked protective effect of nicotinamide (Table 6). Measurements of the oxygen consumed by tissue in the two solutions showed that nicotinamide inhibited the respiration initially but maintained it at a higher level on prolonged incubation. It has been shown with pigeon-liver homogenates that nicotinamide in the absence of substrate inhibits

Table 5. *Coenzyme content of pigeon-liver homogenate found with and without addition of nicotinamide in the presence of lactate*

Pigeon-liver mince (1 g.) was suspended in 14 ml. of medium (Expt. 1) or of medium with 50 mM-nicotinamide (Expt. 2). All cups contained lactate (0·01 M). Incubations were carried out for 30 min. in Warburg vessels at 40°; the values given for Expts. 1 and 2 were obtained at the end of the incubation and are compared with initial levels of coenzyme in pigeon liver.

	Coenzyme content (μm-moles/g. fresh wt.)				
	Expt. 1		Expt. 2		
Coenzyme	O_2	N_2	O_2	N_2	Normal whole pigeon liver
DPN	238	202	346	223	584
TPN	51	44	65	21	14
DPN + TPN	289	246	411	244	598
DPNH	157	137	140	182	98
TPNH	36	225	120	213	149
DPNH + TPNH	193	362	260	395	247
Total	482	608	671	639	845

the reduction of coenzyme and also the oxygen consumption. The decrease in the level of reduced coenzymes in both tissues may account for the lower rate of endogenous respiration.

When suspensions of rat-liver mince were incubated in the presence and absence of pyruvate or lactate the only significant difference in the distribution of the coenzymes was a tendency for the level of DPNH to be maintained by the substrate (see Table 7). The effect produced by lactate on the distribution of coenzymes was not altered significantly by using a starved rat.

It was thought that as the proportion of undamaged cells in chopped tissue would be greater than in mince or homogenate, and that consequently the liberation of pyridine nucleotide-splitting enzymes into the tissue suspension would be reduced, a greater stability of coenzymes might be obtained with chopped tissue. To remove the enzymes released during chopping the preparation was washed twice with medium. However, no such increase in stability was achieved. Unlike the homogenate or mince the washed, chopped tissue comprises a suspension of mainly intact cells in a medium very low in substrate released from damaged cells. Consequently, a consistent difference observed was the extent to which the coenzymes became oxidized during incubation of chopped tissue, in contrast with homogenate and mince.

It was considered that chopped tissue offers a better possibility of observing a clear-cut effect of added substrate on the levels of oxidized and reduced coenzymes.

Sheep-heart incubations. The levels of oxidized and reduced coenzymes found after incubation of sheep-heart homogenates were virtually unaffected by the addition of pyruvate. The ability of sheep-heart homogenate to maintain the levels of reduced coenzymes was inferior to that of rat-liver mince whether or not pyruvate was added.

A similar effect to that which was found with washed chopped rat liver was also seen with the same preparation of sheep heart.

Table 6. *Effect of nicotinamide on the coenzyme level and respiration of rat-liver mince*

Rat-liver mince (1 g.) was suspended in 11 ml. of medium with or without 25 mM-nicotinamide. Incubations were carried out in Warburg vessels at 38° with air as the gas phase. Each vessel contained 3 ml. of suspension and 1 ml. of medium.

	Without nicotinamide				With nicotinamide			
	Coenzyme content (μm-moles/g. fresh wt.)			Increments of O_2 uptake	Coenzyme content (μm-moles/g. fresh wt.)			Increments of O_2 uptake
Time (min.)	Oxidized	Reduced	Total	(μl./g. fresh wt.)	Oxidized	Reduced	Total	(μl./g. fresh wt.)
0	310	320	630	—	635	455	1090	—
20	87	130	217	707	374	260	634	468
40	37	0	37	498	308	116	424	280
64	23	0	23	264	161	101	262	192
90	21	0	21	170	150	74	224	190
120	18	0	18	112	62	32	94	185
140	16	0	16	75	45	27	72	150

Table 7. *Coenzyme content of rat-liver mince incubated with and without pyruvate or lactate*

Rat-liver mince (1 g.) was suspended in 14 ml. of medium with 0·1 M-nicotinamide. The final concentration of pyruvate (Expt. 1) or of lactate (Expt. 2) was 10 mM. Incubations were carried out in Warburg vessels at 38° with air as the gas phase.

		Coenzyme content (μm-moles/g. fresh wt.)									
		DPN		TPN		DPNH		TPNH		Total	
Expt. no.	Time (min.)	No substrate	Pyruvate	No substrate	Pyruvate	No substrate	Pyruvate	No substrate	Pyruvate	No substrate	Pyruvate
1	0	346	302	112	150	148	181	158	196	764	829
	10	273	200	112	68	86	239	103	88	574	595
	17	145	180	100	61	92	149	43	112	480	502
2	0	293	329	60	57	89	90	128	106	570	582
	2	272	290	54	29	60	106	117	149	503	574
	8	228	265	35	56	81	96	115	137	459	554
	15	197	228	24	50	56	102	120	106	396	486
	30	132	134	0	18	7	91	101	103	240	346

DISCUSSION

Aspects of the method of estimation of coenzymes. The method for the analysis of pyridine nucleotides described above permits satisfactory estimation of DPN, TPN, DPNH and TPNH in concentrations as low as 1·5 μM. The sensitivity of this method is similar to that of Glock & McLean (1955) and Lowry *et al.* (1957), and ten times greater than that of Jacobson & Astrachan (1957). The method has two advantages over that of Lowry *et al.* (1957). First, the extraction procedure is improved. The method of Lowry *et al.* gives only 70 % of the total nucleotide extractable in acid and alkaline medium, most of the deficit being in the reduced coenzymes. Secondly, the present method permits complete estimation of the extracted reduced nucleotides, even when these have been oxidized. The procedure of Lowry *et al.* (1957) results in underestimation of TPNH and overestimation of DPNH because it does not allow for this oxidation. An advantage over the method of Glock & McLean (1955) is the greater ease of preparation of the necessary enzymes. Numerous preparations of DPN–cytochrome *c* reductase made in this Laboratory for the procedure of Glock & McLean (1955) had only a small fraction of the required activity. Variation of the volume of the sample in which the fluorescence is developed may be made without major modification of the method, the upper limit of this volume being determined by the need for producing a concentration (6 N or greater) of sodium hydroxide during the development of the fluorescence. Sample volumes of 75 μl. have been used either when the concentration of total nucleotides or the relative concentration of one nucleotide was low. This variation was most frequently used to improve the reliability of endogenous TPN estimation.

It is essential to make frequent checks on the activity of the enzymes used in the method. Inactivity of either enzyme will not be detected by a discrepancy between the values for total nucleotides obtained (*a*) directly and (*b*) by addition of the values found for the individual species (e.g. Fig. 1: compare tube 1 with tube 2 plus tube 4). Other manipulative errors (e.g. failure to acidify a sample) will be detected by such a comparison.

Keto compounds present in the tissue extract were found to reduce the fluorescence, a finding compatible with the known ability of these compounds to quench fluorescence (Guinn, 1957; Lowry *et al.* 1957). During the extraction procedure substances which do not themselves quench fluorescence may be converted into compounds which do so interfere. For example, sucrose does not affect the estimation, but during the heating at low pH, fructose, which does interfere, is formed. Not only is the total amount of coenzyme underestimated but, because the alkaline treatment does not result in the same hydrolysis of sucrose, the estimation of the relative amounts of oxidized and reduced coenzymes is grossly in error.

It is clear that the rate of oxidation of the reduced nucleotides in the alkaline extracts adjusted to pH 8·0 is such that adequate allowance for this must be made in the analytical procedure. Lowry *et al.* have also noted an oxidation of DPNH in storage of pure solutions (i.e. other than tissue extracts), though the amount of DPN found was small. Singer & Kearney (1950) have shown that the rapid oxidation of reduced coenzymes in air is mediated by a number of free flavins, including riboflavin, which will be present in most tissue extracts. The main disadvantage of the modification described in this paper is the extent to which it extends the manipulations involved. Unsuccessful attempts were made either to prevent the oxidation by the addition of a number of anti-oxidants and reducing compounds, or to complete it by adding riboflavin or by shaking the sample in an atmosphere of oxygen.

Coenzyme content of animal tissues. The values presented in this paper for the levels of coenzymes in rat liver agree fairly closely with those of Glock & McLean (who used a method in which oxidation during storage cannot result in the underestimation of reduced nucleotides), but differ, especially in the amount of TPNH, from those of Lowry *et al.* (1957). This difference is probably related to the modifications of the methods of extraction and estimation. The magnitudes of the standard errors of the mean given in Table 5 are greater than those presented by Glock & McLean (1955). Values for the total coenzyme level of rat-liver mince as high as 1400 μm-moles/g. fresh wt. and as low as 600 μm-moles/g. fresh wt. have been observed. With rat liver, it has been shown that damage to the tissue lowers the level of coenzymes and the initial values obtained in experiments necessitating longer handling of tissue preparations before extraction were also somewhat lower. The levels of coenzymes found in extracts of solid pieces of pigeon liver were significantly lower than those found in minces of the same tissue. It seems possible that partial inactivation of coenzyme-splitting enzymes may take place when the tissue is minced and suspended in buffer at pH 7·4, since Jacobson & Kaplan (1957) have shown that these enzymes are inactive at this pH; removal of connective tissue and blood vessels during mincing may also increase the apparent concentration of the coenzymes in minces. The initial amount of reduced coenzyme in minced tissue was greater than that of comparable whole-tissue preparations, and this difference may reflect the ease with which

endogenous substrate and the coenzymes interact when cells are disrupted.

Measurement of coenzymes during the incubation of tissue preparations. The rapid loss of coenzyme during the incubation of the various tissue preparations makes it difficult to examine the factors controlling the coenzyme level. The addition of nicotinamide reduced the loss but did not prevent it. However, nothing more effective has been found. Nicotinamide does not only protect the coenzymes from destruction but also reduces the initial rate of endogenous respiration and prevents the reduction of coenzyme by endogenous substrate, an effect also noted in unpublished experiments with mitochondria. It is advisable therefore to keep the concentration of nicotinamide at a minimum. A comparison of initial values for the oxidized and reduced coenzymes of rat-liver minces prepared with and without nicotinamide suggests that the oxidized coenzymes are more rapidly destroyed, and this preferential destruction means that the observed ratios of oxidized and reduced coenzyme are altered in a way which may not be paralleled in the intact animal. The addition of substrates producing a greater proportion of reduced coenzyme may also help to maintain the total coenzyme level. A precise interpretation of all such experiments is difficult and only clear-cut shifts in ratios can be taken as having a significant relationship to the effect of the metabolites added in the experiments. Results are more meaningful when the time of incubation is short.

Changes in the levels of oxidized and reduced coenzyme during short periods of incubation of homogenates and minces of rat liver, pigeon liver and sheep-heart muscle were not influenced greatly by the addition of substrate. Sometimes, with longer periods of incubation, most of the reduced coenzymes were removed in the absence of added substrate, whereas with lactate and pyruvate the disappearance of TPNH and DPNH was greatly diminished. However, there was always a considerable loss of total coenzyme. It was possible to demonstrate more rapid and clear-cut effects of added substrate with washed, chopped tissue, but the total coenzyme level in these preparations was always low. Thus as short times of incubation with added substrate did not affect the ratios of oxidized and reduced coenzymes, and as longer incubations or the use of washed tissue resulted in considerable loss of total coenzymes, it was impossible to correlate the ratios of oxidized and reduced coenzymes with the influence of added substrate. None of the preparations used seems to be suited for investigating the influence of levels of pyridine nucleotides on metabolic reactions. Accordingly, the investigations are being pursued with mitochondrial preparations.

SUMMARY

1. A fluorimetric method for the estimation of di- and tri-phosphopyridine nucleotide and reduced di- and tri-phosphopyridine nucleotide in animal tissues is described. Acid and alkaline extractions of the tissues are used for the oxidized and reduced coenzymes respectively. After extraction, the four species of coenzymes are separated by treatment with specific enzymes and selective destruction of oxidized and reduced forms with acid and alkali. The fluorescence of the separated coenzymes is developed by heating with 6N-sodium hydroxide.

2. The method can be used for the estimation of pyridine nucleotides in concentrations as low as 1·5 μM, and quantities as small as $0{\cdot}1 \times 10^{-9}$ mole have been determined.

3. Conditions affecting the accuracy and specificity of the method have been investigated.

4. Values for the concentrations of the coenzymes in various tissues immediately after death are presented (see Table 5).

5. The suitability of a number of tissue preparations for use in metabolic experiments involving incubation and the subsequent estimation of the coenzymes has been investigated.

6. There is a rapid destruction of coenzymes on incubation of homogenates and minced or chopped tissues. Pyruvate and lactate usually decreased the rate of destruction and in some experiments maintained a high level of reduced coenzymes.

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