The Determination of Oxidized and Reduced Diphosphopyridine Nucleotide and Triphosphopyridine Nucleotide in Animal Tissues

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Sensitive methods have been devised for the separate determination of both oxidized and reduced coenzymes in neutralized acid and alkaline tissue extracts respectively, oxidized coenzymes being relatively stable in dilute acid, whereas reduced coenzymes are relatively stable in dilute alkali (see Schlenk, 1951). Reduced diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN), continuously formed in the presence of ethanol and alcohol dehydrogenase or glucose 6-phosphate (G 6-P) and G 6-P dehydrogenase respectively, are coupled with their respective cytochrome *c* reductases and the rate of reduction of cytochrome *c* is followed spectrophotometrically.

Triphosphopyridine nucleotide (TPN). This was made from horse liver by the method of Kornberg & Horecker (1953). It contained 75% TPN (analysed with G 6-P dehydrogenase according to Kornberg, 1950) and no DPN (by the alcohol-dehydrogenase method of Racker, 1950).

Diphosphopyridine nucleotide (DPN). This was a commercial preparation (Nutritional Biochemicals Ltd.), subsequently purified by ion-exchange chromatography on Dowex-1-formate according to Kornberg & Pricer (1953). It contained 80% DPN (by the alcohol-dehydrogenase method of Racker, 1950) and no TPN.

Reduced coenzymes. DPNH and TPNH, used in the recovery experiments, were prepared by the enzymic reduction of DPN and TPN with the alcohol-dehydrogenase and glucose 6-phosphate-dehydrogenase systems respectively.

The assay of oxidized and reduced DPN by the present alcohol dehydrogenase-DPN-cytochrome *c* reductase procedure is considerably more sensitive than the estimation of DPN and DPNH with alcohol dehydrogenase and ethanol or acetaldehyde respectively (Racker, 1950). Moreover, our G 6-P dehydrogenase-TPN-cytochrome *c* reductase procedure appears to be the only method available for the estimation of both oxidized and reduced TPN, since no completely reversible TPN-specific dehydrogenase system is at present known.

EXPERIMENTAL

Materials

D-Glucose 6-phosphate (G 6-P). This was a preparation of the barium salt used previously (Glock & McLean, 1953).

Cytochrome c. This was prepared from horse heart by the method of Keilin & Hartree (1952) and purified by reprecipitation and by ion-exchange chromatography on Amberlite IRC-50 according to Margoliash (1954). The ratio $[D_{550 \text{ m}\mu} (\text{reduced})]/[D_{280 \text{ m}\mu} (\text{oxidized})]$ was 1.165, which corresponds to an Fe content of 0.40%.

Alcohol dehydrogenase. This was prepared from baker's yeast according to Racker (1950).

Glucose 6-phosphate dehydrogenase. The method of Kornberg (1950), using either bottom lager yeast (Anheuser-Busch) or top ale yeast kindly provided by Kongens Bryghus, Copenhagen, was employed.

DPN-cytochrome c reductase. This was prepared from pig hearts by the method of Mahler, Sarkar, Vernon & Alberty (1952), except that the homogenate was centrifuged at 750 *g* for 15 min. (instead of at 2000 *g* for 30 min.) and the top gelatinous mitochondrial layer scraped off and combined with the supernatant. The precipitate from stage R₃ was dissolved in 1% crystalline serum albumin (Armour

and Co. Ltd.) containing 0.01 M glycylglycine, pH 7.6, so as to contain approximately 60 units enzyme (Mahler *et al.* 1952) per ml. We are indebted to T. Walls and Co. Ltd. for a very generous supply of pig hearts.

TPN-cytochrome *c* reductase. This was prepared from brewer's yeast according to Haas, Horecker & Hogness (1940) up to and including step 4. It was then freeze-dried. Most of this work was carried out with enzymes prepared from Anheuser-Busch bottom ale yeast, but when this was no longer available Löwenbräu (Munich) bottom lager yeast was used. Employing the same definition of units as described by Mahler *et al.* (1952) for DPN-cytochrome *c* reductase, these preparations had an average activity of 10 and 12 units/mg. For the assay of TPN, the enzyme solution used contained approx. 25 units reductase/ml.

Storage of enzymes. All enzyme preparations were stored at -10° . For the assay procedure the enzymes, if not already in solution, were dissolved in 1% albumin containing 0.01 M glycylglycine, pH 7.6, and kept at 0° .

Methods

Determination of oxidized and reduced DPN and TPN. Two Potter-Elvehjem glass homogenizers containing respectively 5 ml. 0.1 N-HCl and 5 ml. 0.1 N-NaOH were placed in boiling-water baths. After decapitation of the rat, the tissue under investigation was removed rapidly, blotted free of excess of blood, and two approximately equal portions (50–250 mg.) were weighed on a torsion balance. These were transferred immediately to the two homogenizers, and after 0.5 min. in the hot extracting fluid each was homogenized for 1.5 min. and then rapidly transferred to an ice bath. 0.25 M Glycylglycine, pH 8.7 (1 ml.), was added to each and the volumes were adjusted to 15 ml. by the addition of distilled water. They were then neutralized cautiously with stirring by the dropwise addition of 5 ml. 0.1 N-HCl or 0.1 N-NaOH and finally spun for 30 min. at 0° at 20 000 g. The supernatants were poured off, filtered if necessary to remove fat globules, and stored at 0° for the assay of the coenzymes.

Both coenzymes were estimated by following the rate of reduction of cytochrome *c* at 550 m μ . in 1 cm. cells in a Hilger Uvispek spectrophotometer. Oxidized and reduced coenzymes were determined, respectively, in the neutralized acid and alkaline tissue extracts. In the case of the TPN assay, the reaction mixture consisted of 0.5 ml. 0.25 M glycylglycine, pH 8.7, 0.1 ml. 0.05 M G 6-P (Na salt), 0.1 ml. (1 mg.) G 6-P dehydrogenase, 0.1–0.5 ml. neutralized tissue extract and 0.1 ml. TPN-cytochrome *c* reductase in a total volume of 2.4 ml. The reaction was started by the addition of 0.1 ml. 0.001 M cytochrome *c* and readings were taken every minute for 6 min. against a blank cell containing only buffer, water and cytochrome *c*. DPN was determined by a comparable procedure in which G 6-P, G 6-P dehydrogenase and TPN-cytochrome *c* reductase were replaced by 0.1 ml. ethanol, 0.1 ml. (100 μ g.) alcohol dehydrogenase and 0.1 ml. DPN-cytochrome *c* reductase.

All results were corrected for the small separately determined non-enzymic reduction of cytochrome *c* by the tissue extracts and by combined reductase, dehydrogenase and substrate. The assay procedures were standardized daily by using four levels of each coenzyme (0.1–0.8 μ g. DPN and 0.05–0.4 μ g. TPN). The DPN, DPNH, TPN and TPNH contents of the tissue extracts were calculated from the

increase in density at 550 m μ . between 1 and 6 min., after correcting for the enzyme and extract blanks.

Test for coenzyme specificity. This was tested for in the DPN-cytochrome *c* reductase system, described above, by replacing DPN with 10 μ g. TPNH and in the TPN-cytochrome *c* reductase system by replacing TPN with 10 μ g. DPNH.

Tests for interfering enzymes. (a) *Enzymic breakdown of DPN and TPN.* Breakdown of DPN by the DPN-cytochrome *c* reductase preparation was determined by incubating the reductase in glycylglycine buffer at room temperature with 0.5 μ g. DPN. A control (without DPN) was set up to correct for any loss in reductase activity. After 30 min., 0.5 μ g. DPN was added to the control and ethanol, alcohol dehydrogenase and cytochrome *c* to both cells. The rate of reduction of cytochrome *c* was followed for 6 min. as in the assay procedure. A similar experiment was carried out with the TPN-cytochrome *c* reductase preparation and 0.2 μ g. TPN.

(b) *Enzymic conversion of TPN into DPN.* The usual reaction mixture for the DPN assay system was used, except that DPN was replaced by 1 μ g. TPN. Any reduction of cytochrome *c* was followed at 550 m μ .

(c) *Pyridine nucleotide transhydrogenase.* This enzyme catalyses the reaction: $\text{TPNH} + \text{DPN}^+ \rightarrow \text{TPN}^+ + \text{DPNH}$ (Kaplan, Colowick & Neufeld, 1953), and if present in the TPN-cytochrome *c* reductase preparation would tend to produce low results in the assay of oxidized and reduced TPN in tissue extracts, by converting TPNH into TPN^+ . In the test for transhydrogenase in the TPN-cytochrome *c* reductase preparation, the reaction mixture contained only glycylglycine buffer, cytochrome *c*, 2.0 μ g. TPNH and 2.0 μ g. DPN. The control contained no DPN. The reaction was started by the addition of TPN-cytochrome *c* reductase and the reduction of cytochrome *c* was followed. The transhydrogenase activity of the DPN-cytochrome *c* reductase preparation was tested for in a comparable experiment, although the reversibility of the above transhydrogenase reaction has not so far been demonstrated in animal tissues. The reaction mixture in this case contained only glycylglycine buffer, cytochrome *c*, 5.0 μ g. DPNH and 5.0 μ g. TPN. The control contained no TPN.

(d) *Oxidation of DPNH and TPNH.* This was tested for by following any reoxidation of DPNH (0.1 mg.) or TPNH (0.1 mg.) at 340 m μ . on addition of DPN-cytochrome *c* reductase or TPN-cytochrome *c* reductase respectively.

(e) *Cytochrome oxidase.* The cytochrome oxidase activities of the two combined dehydrogenase-cytochrome *c* reductase systems were tested for separately by adding them, in amounts used in the assay procedures, to partially reduced cytochrome *c* and following the reoxidation of reduced cytochrome *c* at 550 m μ .

RESULTS

Extraction procedure

The separate determination of oxidized and reduced coenzymes is possible on account of differences in stability, oxidized coenzymes being relatively stable in dilute acid and reduced coenzymes in dilute alkali (see Schlenk, 1951). This is the basis of methods of estimation of DPN and

DPNH described by Adler & Euler (1936), Euler, Schlenk, Heiwinkel & Högborg (1938), Fisher & Schlenk (1948) and very recently by Holzer, Goldschmidt, Lamprecht & Helmreich (1954). It has also been adopted in the present procedure.

Our results on the stability of oxidized and reduced DPN and TPN in 0.1M nicotinamide, 0.1N-HCl and 0.1N-NaOH at 100 and 85° are shown in Table 1. These results indicate that DPNH and TPNH are unstable both at 85 and

version of TPN into DPN on heating for 2 min. in 0.1N-HCl at 100°.

On applying this 2 min. hot-extraction procedure to the extraction of coenzymes from rat liver (see Table 2), it was found to be immaterial whether the tissue was homogenized at 85° or 100°. The time of heating, however, must not exceed 2 min. In contrast to Holzer *et al.* (1954) it was found immaterial whether the liver homogenates were neutralized before or after centrifuging.

Table 1. *The stability of oxidized and reduced DPN and TPN*

160 µg. DPN⁺, 80 µg. DPNH, 25 µg. TPN⁺ or 40 µg. TPNH were added, respectively, to 5 ml. portions of hot extracting medium. After heating for the times stated; tubes were cooled rapidly in ice, and neutralization and estimation of coenzymes carried out as described under Methods.

Extracting medium	Coenzyme	Percentage recovery of coenzymes							
		100°				85°			
		1 min.	2 min.	3 min.	4 min.	1 min.	2 min.	3 min.	4 min.
0.1M Nicotinamide	DPN ⁺	103	100	95	93	100	100	100	96
	TPN ⁺	91	93	92	80	97	96	—	—
	DPNH	70	72	64	51	69	66	65	60
	TPNH	48	29	10	5	64	45	37	27
0.1N-HCl	DPN ⁺	100	92	86	82	106	100	102	100
	TPN ⁺	104	95	77	72	104	104	100	100
	DPNH	—	0	—	—	—	—	—	—
	TPNH	—	4	—	—	—	—	—	—
0.1N-NaOH	DPN ⁺	—	0	—	—	—	—	—	—
	TPN ⁺	—	0	—	—	—	—	—	—
	DPNH	95	92	76	78	92	92	88	79
	TPNH	96	101	86	85	95	96	92	88

Table 2. *Extractions of coenzymes from liver*

Values given for three rat livers expressed as means $\pm$ S.E.M. Extraction and assay procedures as described in the Methods section.

Extracting medium	Coenzyme	Coenzyme content (µg./g. liver)	
		100°	85°
0.1N-HCl	DPN ⁺	370 $\pm$ 10	369 $\pm$ 13
	TPN ⁺	11 $\pm$ 1	22 $\pm$ 3
0.1N-NaOH	DPNH	203 $\pm$ 11	207 $\pm$ 13
	TPNH	215 $\pm$ 7	210 $\pm$ 6

100° in 0.1M nicotinamide and confirm the extreme lability of oxidized coenzymes in hot alkali and of reduced coenzymes in hot acid. There was somewhat better recovery of DPN and TPN added to hot 0.1N-HCl at 85° than at 100°, and recoveries of both oxidized and reduced DPN and TPN were good after heating for only 2 min. in 0.1N-HCl and 0.1N-NaOH respectively. For these reasons, in the final extraction procedure (see under Methods) the tissue was heated for 0.5 min. at 100° in the extracting medium, followed by homogenization for exactly 1.5 min. The average final temperature of the homogenate was 85–90°. There was no con-

The loss of DPN by adsorption reported by these workers when neutralization preceded spinning can probably be attributed to the relatively large amounts of liver used and to the small final volume of neutralized homogenate.

It has generally been assumed that if tissue extraction is carried out in neutral buffered solutions, it is possible to determine only total DPN and TPN contents. Recently, however, Spirtes & Eichel (1954) have extracted liver with hot phosphate-carbonate buffer, pH 7.4, and have found it possible to determine both DPN and DPNH in the same extract. The values they have reported for rat liver, using alcohol dehydrogenase for the assay of both DPN and DPNH, are considerably higher than those we have found employing DPN-cytochrome *c* reductase. In an attempt to elucidate these discrepancies, we have compared both methods of extraction and methods of estimation. Table 3, which incorporates these results, indicates that the total DPN (DPN⁺ + DPNH) contents of phosphate-carbonate extracts of rat liver are consistently higher when determined with alcohol dehydrogenase than with the combined alcohol dehydrogenase-DPN cytochrome *c* reductase procedure described in this paper. This

Table 3. Comparison of methods of extraction and estimation of coenzymes

Extracting medium	Method of estimation	Coenzyme content* ($\mu\text{g./g. liver}$)				$\frac{\text{DPN}^+}{\text{DPNH}}$
		DPN ⁺	DPNH	DPN ⁺ + DPNH	TPN ⁺ + TPNH	
Phosphate-carbonate†	A	386 $\pm$ 14	339 $\pm$ 18	725 $\pm$ 27	—	1.1 $\pm$ 0.05
	B	—	—	522 $\pm$ 37	203 $\pm$ 11	—
0.1N-HCl or 0.1N-NaOH	B	364 $\pm$ 13	200 $\pm$ 11	564 $\pm$ 18	207 $\pm$ 9	1.8 $\pm$ 0.1

* Values for four rat livers expressed as means $\pm$ S.E.M.

† Method of Spirtes & Eichel (1954).

A, Spectrophotometric estimation with alcohol dehydrogenase; B, spectrophotometric estimation at 550 m μ . as described in the Methods section.

is due chiefly to the much higher DPNH values obtained with the former method. On the other hand, our figures for both total DPN and DPN⁺/DPNH quotients of rat livers are in fairly good agreement with those of Holzer *et al.* (1954), who employed similar acid and alkaline extraction procedures but estimated DPN and DPNH with alcohol dehydrogenase. It is difficult to account for the high DPNH levels obtained by the procedure of Spirtes & Eichel (1954). They might be attributable to the lack of absolute coenzyme specificity of alcohol dehydrogenase (Singer & Kearney, 1954*a*), which would imply the presence of more biologically active pyridine derivatives in phosphate extracts of liver than in acid or alkaline extracts.

Coenzyme specificity

The coenzyme specificity of both DPN- and TPN-cytochrome *c* reductase systems employed in the assay procedure was found to be absolute (see under Methods).

pH/activity curves

The pH/activity curves for the DPN and TPN assay systems in 0.05M glycylglycine are given in Fig. 1. The TPN system has a broad optimum between approximately pH 8.3 and 9.9 and the DPN system from pH 7.6 to 9.0. The final assay procedure for both coenzymes was carried out in 0.05M glycylglycine at pH 8.7. Although this is not the optimum pH for either G 6-P dehydrogenase or alcohol dehydrogenase, a large excess of these enzymes was employed so that the rate of reduction of neither TPN nor DPN was limiting.

The effect of various buffers and salts on the activity of the two reductase systems is shown in Table 4. Vernon, Mahler & Sarkar (1952) have shown that purified DPN-cytochrome *c* reductase is inhibited considerably by various ions, including phosphate, chloride, sulphate, Mg⁺⁺ and Na⁺.

These results indicate the advisability of employing glycylglycine as buffer in the present assay procedure and of omitting MgCl₂ from the TPN method. Lack of inhibition of the DPN system by NaCl and sulphate can probably be attributed to the relative impurity of the reductase preparations.

Fig. 1. pH/activity curves of the DPN and TPN assay systems in glycylglycine buffer. Activities determined as described in the Methods section. ○, 6 units of DPN-cytochrome *c* reductase, 0.3 $\mu\text{g. DPN}$; ●, 2 units of TPN-cytochrome *c* reductase, 0.3 $\mu\text{g. TPN}$.

Table 4. Effect of certain buffers and salts on the activity of the DPN and TPN assay systems

Results expressed as percentage of activity of assay system measured in 0.05M glycylglycine, pH 8.7, as described in the Methods section. Activities in the presence of added salts determined in 0.05M glycylglycine, pH 8.7.

Final concn.	Percentage activity	
	DPN assay system	TPN assay system
0.05M Glycylglycine buffer, pH 8.7	100	100
0.02M Diol buffer, pH 8.7*	95	94
0.01M Phosphate buffer, pH 8.3†	—	30
0.02M-NaCl‡	99	102
0.02M-MgCl ₂ §	—	37
0.10M-(NH ₄) ₂ SO ₄	100	—

* 2-Amino-2-methyl-1,3-propanediol buffer ('Diol' buffer) as used by Vernon *et al.* (1952).

† Assay conditions of Haas *et al.* (1942).

‡ The final concn. if 1 ml. neutralized tissue extract used in assay.

§ The optimum concn. for G 6-P dehydrogenase activity.

|| The final concn. in DPN assay due to addition of alcohol dehydrogenase.

Variations in cytochrome *c* concentration

The effect of varying the concentration of cytochrome *c* on the assay of DPN and TPN by the two dehydrogenase-reductase systems is shown in Fig. 2. It is obvious that the concentration of cytochrome *c* employed in the assay procedure (0.1 ml. 0.001M cytochrome *c* in a total volume of 2.5 ml.) is optimum for both systems with the particular coenzyme concentrations used.

Fig. 2. Effect of variations in cytochrome *c* concentration on the activities of the DPN and TPN assay systems. Activities determined as described in the Methods section. ○, 6 units of DPN-cytochrome *c* reductase, 0.3 μg. DPN; ●, 2.4 units of TPN-cytochrome *c* reductase, 0.2 μg. TPN.

Fig. 3. Effect of variations in the concentration of DPN-cytochrome *c* reductase and TPN-cytochrome *c* reductase. Activities determined as described in the Methods section. ○, DPN assay system with 0.35 μg. DPN; ●, TPN assay system with 0.14 μg. TPN.

Variations in cytochrome *c* reductase concentrations

The effect of variations in concentration of DPN-cytochrome *c* reductase and TPN-cytochrome *c* reductase on the assay of DPN and TPN respectively is shown in Fig. 3. It is apparent that

Fig. 4. DPN concentration curves. ○, 6 units of DPN-cytochrome *c* reductase; ●, 18 units of DPN-cytochrome *c* reductase.

Fig. 5. TPN concentration curves. ●, 2.4 units of TPN-cytochrome *c* reductase; ○, 7.2 units of TPN-cytochrome *c* reductase.

the sensitivity of both methods of assay can be increased by employing more reductase.

Variations in coenzyme concentrations

The effects of varying the concentrations of DPN and TPN on the rate of reduction of cytochrome *c* by the two assay systems are shown in Figs. 4 and 5 respectively. Two levels of reductase were used, the lower levels (6 units of DPN-cytochrome *c* reductase and 2.4 units of TPN-cytochrome *c* reductase) corresponding to the approximate concentrations of reductases employed in the assay procedures. Although these lower levels of reductase are not optimum, the rate of reduction of cytochrome *c* is, nevertheless, directly proportional to coenzyme concentration up to a ΔD at 550 $m\mu$. of about 0.3, corresponding to approximately 30% reduction of the cytochrome *c*. The advantage of using these suboptimum concentrations of reductases in the assay procedures are, first, that the range of concentration of coenzyme that can be estimated is extended and, secondly, that the non-enzymic reduction of cytochrome *c* by the reductase preparation itself is minimized. The range of the methods can also be extended by increasing the concentration of cytochrome *c*.

Variations in volume of tissue extract

The rate of reduction of cytochrome *c* was found to be proportional to the volume of neutralized tissue extract used in both DPN and TPN assay procedures provided that not more than approximately 30% of the cytochrome *c* is reduced. Typical results for neutralized acid and alkaline extracts are shown in Fig. 6.

Recovery of added coenzymes

The results incorporated in Table 5 indicate that there is good recovery of DPN, DPNH, TPN and TPNH when added both during the extraction procedure and to the final assay system. The quantitative recovery of coenzymes added to the

assay systems indicates that neutralized liver extracts contain no substances that alter the rate of reduction of cytochrome *c* by the reductase systems. This also applies to the other tissue extracts investigated.

Lack of interference by other enzymes

The present assay procedures would be completely invalidated if the systems were contaminated with any of a variety of interfering enzymes. These include enzymes capable of breaking down DPN or TPN, of converting TPN into DPN, of oxidizing DPNH and TPNH, transhydrogenase

Fig. 6. Direct proportionality between the volume of liver extract and the rate of reduction of cytochrome *c*. Extraction and assay as described in the Methods section, using approximately 200 mg. rat liver. —, Neutralized acid extract; ---, neutralized alkaline extract; O, DPN or DPNH; ●, TPN or TPNH.

Table 5. *Recovery of coenzymes added to liver during extraction and assay procedures*

Extraction and assay procedures as described in the Methods section.

		Recovery of coenzymes*			
Extracting medium	Coenzyme	Added during extraction†		Added to assay system	
		$\mu\text{g.}$ added	Percentage recovery	$\mu\text{g.}$ added	Percentage recovery
0.1N-HCl	DPN+	60	101 $\pm$ 6	0.4	100 $\pm$ 1
	TPN+	40	98 $\pm$ 7	0.1	100 $\pm$ 2
0.1N-NaOH	DPNH	60	95 $\pm$ 8	0.4	100 $\pm$ 2
	TPNH	40	93 $\pm$ 5	0.1	99 $\pm$ 3

* Values for three rat livers expressed as means $\pm$ s.e.m.

† Coenzymes added to hot extracting medium immediately after introduction of approximately 200 mg. liver.

and cytochrome oxidase. For this reason the dehydrogenase-cytochrome *c* reductase systems used in the assays were carefully tested for the presence of these enzymes, as described under Methods. They were all found to be absent from both systems. Since diaphorase, which reacts sluggishly with oxygen (see Singer & Kearney, 1954*b*), would be included in the group of enzymes capable of re-oxidizing DPNH and TPNH, and these were found to be absent or inactive under our conditions of assay, it is obvious that the diaphorase activity of the DPN-cytochrome *c* reductase does not interfere with the assay procedure. In addition, Singer & Kearney (1950) have reported that various non-specific flavins can catalyse the non-enzymic reduction of cytochrome *c* by DPNH. These cannot be present in our tissue extracts, since the rate of reduction of cytochrome *c* by the extracts alone was not increased on the addition of DPNH.

DISCUSSION

Quantitative methods have been described for the determination of both oxidized and reduced DPN and TPN in animal tissues. These involve coupling reduced coenzymes with their respective cytochrome *c* reductases and following spectrophotometrically the rate of reduction of cytochrome *c*. These methods are so sensitive that it has been possible to determine both oxidized and reduced DPN and TPN even in tissues with very low coenzyme contents, without preliminary concentration of the tissue extracts. As far as we are aware tissue-coenzyme levels have not previously been estimated in this way, although TPN-cytochrome *c* reductase has been used for estimating TPN in pure solution (Haas, Harrer & Hogness, 1942). Apart from the recently published figures of Holzer *et al.* (1954), Frunder (1954) and Spirtes & Eichel (1954) for the DPN and DPNH contents of liver (determined spectrophotometrically with alcohol dehydrogenase), very few reliable figures are available for both DPN and DPNH contents of animal tissues (see Schlenk, 1951), and even fewer for TPN. There are none for TPNH. This scarcity of results can be attributed chiefly to errors in the extraction procedure, to the use of unreliable coenzyme standards and to the relatively low TPN content of most tissues. Moreover, the manometric methods available are not completely coenzyme specific. Thus Adler, Elliott & Elliott (1940) showed that conversion of TPN into DPN occurs in the apozymase fermentation system, and Negelein & Wulff (1937) demonstrated that 'old yellow enzyme', used as oxygen carrier in the *Zwischenferment* system of Warburg, Christian & Griese (1935), reacts with both DPNH and TPNH.

Singer & Kearney (1954*a*) have shown that Co III can replace DPN in the alcohol dehydrogenase and lactic dehydrogenase systems and reduced Co III can replace DPNH in the DPN-cytochrome *c* reductase system. This presumably means that published values for DPN contents of animal tissues involving the use of any of these enzyme systems are too high, owing to inclusion of Co III. This coenzyme is widely distributed in animal tissues, the concentration being highest in kidney and liver (Singer & Kearney, 1953).

Our most interesting finding is that whereas in liver DPN is present mainly in the oxidized form the reverse is true of TPN, which is present almost exclusively in the reduced form (see Table 2 and Fig. 6). Thus the average DPN^+/DPNH quotient in liver is 1.8, whereas the TPN^+/TPNH quotient is as low as 0.03. This is difficult to interpret and cannot be explained by the activity of the pyridine nucleotide transhydrogenase system, which favours the formation of DPNH (Kaplan *et al.* 1953).

SUMMARY

1. Sensitive methods have been described for the separate determination of DPN^+ , DPNH, TPN^+ and TPNH in animal tissues. These involve coupling reduced coenzymes with their respective cytochrome *c* reductases and following the rate of reduction of cytochrome *c* spectrophotometrically.

2. By these methods it is possible to estimate accurately as little as 0.1 μg . DPN or TPN.

3. DPN is present in rat liver mainly in the oxidized form, whereas TPN is present mainly in the reduced form. Average DPN^+ , DPNH, TPN^+ and TPNH values are, respectively, 370, 204, 6 and 205 μg . coenzyme/g. liver.

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Levels of Oxidized and Reduced Diphosphopyridine Nucleotide and Triphosphopyridine Nucleotide in Animal Tissues

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The preceding paper (Glock & McLean, 1955) describes sensitive methods for the separate determination of oxidized and reduced diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) in animal tissues. Reduced coenzymes are coupled with their respective cytochrome *c* reductases and the rate of reduction of cytochrome *c* is followed spectrophotometrically. These methods have now been applied to the estimation of DPN⁺, DPNH, TPN⁺ and TPNH in a variety of animal tissues.

EXPERIMENTAL

The preparation of substrates and enzymes and the estimation of coenzymes were carried out as described previously (Glock & McLean, 1955). Oxidized and reduced coenzymes were determined, respectively, in neutralized acid and alkaline tissue extracts.

RESULTS

Our results for the levels of oxidized and reduced DPN and TPN in a variety of animal tissues are incorporated in Table 1. The rate of reduction of cytochrome *c* was in all cases directly proportional to the volume of tissue extract. The presence of relatively high concentrations of ascorbic acid in the neutralized acid extracts of adrenals does not invalidate the assay procedures, since, although ascorbic acid reduces cytochrome *c* directly, the rate of reduction is so rapid that it is virtually

complete before the first spectrophotometric reading is taken.

In all the tissues investigated, the total DPN concentrations (DPN⁺ + DPNH) are considerably higher than the total TPN concentrations (TPN⁺ + TPNH). Moreover, whereas DPN is present chiefly in the oxidized form, TPN on the contrary is present mainly in the reduced form. Thus the DPN⁺/DPNH quotients are in all cases greater than 1 and the TPN⁺/TPNH quotients always considerably less than 1. In many tissues the levels of oxidized TPN are negligible. The highest total concentration of TPN is found in liver but fairly high concentrations also occur in adrenals, lactating mammary glands, ovary and kidney. Most of the other tissues examined contain very little TPN either in the oxidized or reduced form. The low values in brain and voluntary muscle (diaphragm), amounting to less than 5% of the total DPN concentration, are particularly interesting. The most variable results were obtained with the adrenals both for the total concentrations of DPN and TPN and for the proportions of oxidized to reduced coenzymes. This may prove to be related to the physiological state of activity of the gland, particularly since both DPN and TPN have been shown to be necessary for various steroid interconversions in adrenal homogenates (Hayano, 1954; Hayano & Dorfman, 1953; Plager & Samuels, 1953). The high TPN levels in ovary might also be associated with steroid metabolism.