DISTRIBUTION OF ENZYMES CLEAVING PYRIDINE NUCLEOTIDES IN ANIMAL TISSUES*

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A number of enzymes capable of splitting the pyridine nucleotides have been found in animal tissues. A portion of the DPNase[1] that hydrolyzes the nicotinamide ribose bond of DPN and TPN was reported in the microsomes of rat liver (1). The DPN pyrophosphorylase, first described by Kornberg (2), has been found by Hogeboom and Schneider to be largely localized in the nucleus (3). In previous investigations (4) an enzyme from pigeon liver which splits DPNH and not DPN at the pyrophosphate linkage was described. The present communication deals with the intracellular distribution of enzymes from various species that attack the pyridine coenzymes at the pyrophosphate linkage. The distribution and properties of DPNases from different species and tissues will also be presented.

Materials and Methods

Monoesterase was prepared from human hypertrophied prostate glands according to the method of Markham and Smith (5). Yeast alcohol dehydrogenase was obtained from the Worthington Biochemical Sales Co.

The 3-acetyl pyridine analog of DPN (3AP-DPN) was prepared as described by Kaplan and Ciotti (6). Reduced pyridine nucleotides were prepared enzymatically by yeast alcohol dehydrogenase (7). The millimolar extinction coefficients used were as follows: 7.8 for reduced 3AP-DPN and 6.3 for DPNH (6, 8, 9). DPN was obtained from the Pabst Laboratories.

Protein concentration was measured by a modified method of Lowry *et al.* (10). Phosphate concentration was determined by the method of Fiske and SubbaRow (11).

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[1] The following abbreviations will be used throughout this paper: DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; 3AP-DPNH, reduced 3-acetylpyridine analog of DPN; NMN, nicotinamide mononucleotide; ADPR, adenosine ribose phosphate; DPNase, enzyme that splits DPN at the nicotinamide ribose bond; ADH, yeast alcohol dehydrogenase; tris, tris (hydroxymethyl) aminomethane.

Rats of the Wistar strain, hamsters, rabbits, and albino pigeons were obtained commercially. The mice were an F_1 hybrid strain (BALB/CAN × DBA/2J).

Tissue Fractionation.—The centrifugal fractionation procedure was modified from that of Dounce *et al.* (12). The tissue was homogenized in 0.44 M sucrose in a well worn, loose fitting TenBroeck homogenizer (clearance of 0.5 to 1.0 mm.), made up to a final volume 10 times its original weight by addition of 0.44 M sucrose, and strained through two layers of cheesecloth. Twenty ml. of the homogenate was centrifuged in 4 x 10 cm. celluloid tubes at 610 *g* for 10 minutes on head No. 269 of the International refrigerated centrifuge. The pellet was resuspended in the plastic centrifuge tube using an ice-filled test tube as a pestle, and washed twice in a volume of sucrose solution comparable to 0.1 to 0.2 of the originating homogenate's volume. The washes and first supernatant fluid were combined and centrifuged at 13,000 *g* for 10 minutes in the multispeed attachment. Resuspension and washing were done as before. Combined washings and supernatant fluid were centrifuged in the Spinco model L at 105,000 *g* for 60 minutes. The supernatant fluid was termed the soluble fraction and the pellet, the microsomal fraction. The nuclear and mitochondrial fractions were the particles sedimenting at 600 *g* and 13,000 *g*, respectively.

All the fractions were routinely observed by light microscope. In no case were erythrocytes or nuclei seen in the mitochondrial fraction. The nuclear fraction always contained many small particles, in addition to erythrocytes, occasional whole cells, and the spherical nuclei. A fractionation of pigeon liver using the sucrose–citric acid solution recommended by Dounce *et al.* (12) gave almost identical distribution of nucleotide pyrophosphatase activity. Although the subcellular fractions in all cases were not definitely established cytologically, the changes in enzyme activity with different centrifuged fractions suggest that distinct separation had been achieved. For example, in cases in which mitochondria were completely inert, the nuclei and microsomes contained activity. However, we are aware of the possibility that what are called mitochondria in liver may not be identical with the kidney fraction which sediments at the same gravitation.

Assay for DPNH Pyrophosphatase.—Reaction mixture consisted of 0.01 M $MgCl_2$, 0.1 M tris pH 7.5, 1.7 to 2 mM DPNH, an aliquot of the tissue fraction, and 0.01 M KCN or 0.1 M nicotinamide. After the disappearance of DPNH aliquots were assayed at zero and subsequent times by yeast alcohol dehydrogenase and acetaldehyde as described elsewhere (4). The pyrophosphatase assay was performed at 37°C. The maximal amount of particulate fraction or homogenate was governed by the contribution it made to the 340 mμ absorption; the amount of active fraction was chosen so that a linear rate of DPNH splitting occurred. The DPNH concentration was always in considerable excess of saturating concentrations. The inhibition of the DPNH oxidase by nicotinamide will be described.

Assay for DPN Pyrophosphatase.—Reaction mixture was the same as that for the DPNH pyrophosphatase assay except that DPN replaced DPNH; cyanide was omitted, and nicotinamide added whenever a tissue preparation containing DPNase was to be assayed. The amount of tissue fraction or homogenate used was the same as for DPNH pyrophosphatase. For measuring the disappearance of DPN, aliquots were assayed at zero and subsequent times with yeast alcohol dehydrogenase and ethanol.

Assay for DPNase.—Reaction mixture consisted of 0.1 M tris or 0.1 M tris-maleate buffer, 1.5 to 2 mM DPN, and 0.01 M $MgCl_2$. The pH of the reaction mixture was varied with different tissues. The destruction of DPN at the nicotinamide ribose bond was determined by measuring the decrease in cyanide reaction as described by Colowick *et al.* (13).

Units of Activity.—All three assays are expressed as μmoles pyridine nucleotide split per hour per gram of fresh weight of the tissues from which the fraction was prepared.

RESULTS

DPNH Pyrophosphatase Distribution in Fractions of Various Tissues.— Table I compares the activity of the fractions of livers from various animals; it shows that the pigeon liver soluble fraction is unique in that it contains over 50 per cent of the DPNH splitting activity. The activity in the pigeon nuclear and mitochondrial fractions together comprised a minor part of the total activity (less than 10 per cent). Occasionally the mitochondria had no

TABLE I

Distribution of DPNH and DPN Pyrophosphatases

Organ	DPNH pyrophosphatase activity						DPN phyrophosphatase activity					
	Hom	Nuc	Mt	Mi	Sol	Rec	Hom	Nuc	Mt	Mi	Sol	Rec
						per cent						*per cent*
Pigeon liver 1	16.9	1.0	0	9.9	10.8	128	—	—	—	—	—	—
" " 2	14.5	0.9	1.3	5.1	9.3	114	1.7	0.4	1.0	1.2	0	150
" " 3	8.1	1.0	0.3	2.7	4.8	109	3.5	0.5	0.3	2.4	0	92
Rat liver 1	41.7	9.0	1.8	21.9	1.9	83	17.7	3.0	0	11.1	1.3	87
" " 2*	—	—	—	—	—	—	—	—	—	—	—	—
" " 3*	—	8.0	1.4	16.2	0.3	—	—	5.4	0.4	9.1	0	—
Mouse liver	25.0	4.2	4.2	15.0	1.8	100	9.7	2.0	1.4	7.3	0	110
Hamster liver	21.5	0	1.4	13.6	4.0	88	15.3	2.9	0	10.4	0	87
Rabbit liver	7.8	3.3	0.3	1.8	4.3	124	2.8	1.3	0.2	0.5	0	71
Rabbit kidney	54.6	5.4	9.3	41.4	3.0	107	20.7	3.3	3.9	18.0	0	122
Rabbit brain	0	0	0	0	0	—	0	0	0	0	0	—

Key to column headings: hom, homogenate; nuc, nuclear fraction; mt, mitochondrial fraction; mi, microsomal fraction; sol, soluble fraction; rec, recovery of activity in fraction as compared to activity in homogenate.

Values in table are μmoles DPNH or DPN split per 60 minutes per gram of fresh weight of the organ.

* Rat livers 2 and 3 were fractionated in 0.25 M sucrose.

demonstrable activity; thus it appears that the activity in other cases may have been due to microsomal contamination. As with the purified enzyme (4), only DPNH, but not DPN, was split by the soluble fraction of pigeon liver. In these data values less than 1 μmole/60 minutes/gram are not very accurate or significant.

A survey of other animals demonstrated that the soluble fraction of the livers of rat, mouse, and hamster either contained very little or no pyrophosphatase activity. The soluble fraction of the rabbit liver resembled that of the pigeon liver in that it too contained an active pyrophosphatase that distinguished DPNH from DPN. It may also be noted that the mitochondria of all species contained a uniformly low percentage of the liver homogenate's total pyridine nucleotide pyrophosphatase activity.

TABLE II

Distribution of DPNase and Protein

Organ	DPNase activity						Protein distribution					
	Hom	Nuc	Mt	Mi	Sol	Rec	Hom	Nuc	Mt	Mi	Sol	Rec
						per cent						*per cent*
Pigeon liver 1	0	0	0	0	0		118.0	29.5	19.7	65.7	53.7	142
" " 2	0	0	0	0	0		84.0	5.1	20.7	19.7	38.2	99
" " 3	5.4	—	—	3.9	—	72	89.0	12.4	21.0	20.6	28.9	93
Rat liver 1	—	—	—	—	—		105.0	9.1	19.8	23.9	34.8	85
" " 2	25.7	4.1	0	23.4	0	107	62.0	4.1	10.3	23.6	22.9	98
" " 3	15.8	1.2	0	15.3	0	105	116.0	12.5	24.9	36.2	47.2	104
Mouse liver	75.0	12.6	11.0	57.6	1.5	110	119.0	8.9	27.3	39.7	43.9	100
Hamster liver	19.5	1.9	0	12.6	0	75	141.0	15.1	27.7	27.2	53.1	87
Rabbit liver	50.4	10.7	9.9	22.3	0	86	63.0	20.7	5.1	6.3	42.3	117
Rabbit kidney	14.4	2.4	0	6.6	0	62	116.4	21.3	15.6	27.2	39.8	89
Rabbit brain	117.0	14.7	15.3	111.0	0	120	64.0	6.9	14.4	24.3	15.2	95

The pH of the different assays was as follows:

pH 7.5—pigeon 1 and 2, rat, mouse, and rabbit brain.

pH 6.5—pigeon 3 and rabbit liver.

pH 5.5—hamster and rabbit kidney.

DPNase values in this table are μmoles DPN split per 60 minutes per gram of fresh weight. DPNase and protein assays were performed on the same preparations as those reported in Table I.

Protein values expressed as milligrams per gram of fresh weight of organ.

TABLE III

Specific Activities of Fractions

Organ	DPNH pyrophosphatase					DPN pyrophosphatase					DPNase				
	Hom	Nuc	Mt	Mi	Sol	Hom	Nuc	Mt	Mi	Sol	Hom	Nuc	Mt	Mi	Sol
Pigeon liver 1	0.143	0.034	0	0.151	0.205	—	—	—	—	—	0	0	0	0	0
" " 2	0.173	0.176	0.063	0.259	0.243	0.022	0.079	0.048	0.061	0	0	0	0	0	0
" " 3	0.091	0.081	0.014	0.131	0.160	0.039	0.040	0.014	0.116	0	0.061	—	—	0.189	—
Rat liver 1	0.397	0.990	0.091	0.916	0.054	0.169	0.330	0	0.465	0.037	—	—	—	—	—
" " 2	—	—	—	—	—	—	—	—	—	—	0.415	1.0	0	0.990	0
" " 3	—	0.640	0.056	0.448	0.006	—	0.432	0.056	0.251	0	0.136	0.096	0	0.423	0
Mouse liver	0.210	0.472	0.154	0.378	0.041	0.082	0.225	0.051	0.184	0	0.630		0.403	1.45	0.034
Hamster liver	0.152	0	0.051	0.500	0.076	0.108	0.192	0	0.383	0	0.138	0.126	0	0.463	0
Rabbit liver	0.124	0.160	0.059	0.286	0.101	0.045	0.063	0.039	0.080	0	0.800	0.518	1.94	3.54	0
Rabbit kidney	0.470	0.254	0.596	1.52	0.075	0.178	0.155	0.250	0.662	0	0.124	0.112	0	0.242	0
Rabbit brain	0	0	0	0	0	0	0	0	0	0	1.83	2.13	1.06	4.57	0

μmoles split per 60 minutes per milligrams of protein calculated from values in Tables I and II.

A comparison of the pyrophosphatase activity of different rabbit organs showed a marked variation in activity. The rabbit kidney was many times more active than the liver while the brain was completely devoid of dinucleotide pyrophosphatase.

The specific activities in terms of μmoles per milligram of protein per 60 minutes, may be calculated from the protein values for each fraction; these protein determinations are presented in Table II and the specific activities are given in Table III. The values may be of interest in determining a suitable source to be used in purifying any of these enzymes.

DPN Pyrophosphatase Distribution in Fractions of Various Tissues.—The main purpose of testing DPN as a substrate for pyrophosphatase was to establish the specificity of the enzyme toward DPNH. In this respect, Table I shows that the soluble fraction of pigeon liver is specific for DPNH, as is that of rabbit liver. The soluble fractions of other species had such low activity on DPNH that the lack of activity on DPN is without significance. The microsomes were uniformly high in terms of the per cent of the homogenate's activity, as well as specific activity. In the particles of all species studied the pyrophosphatases attacked DPNH faster than DPN.

DPNase Distribution in Fractions of Various Tissues.—As reported by Sung and Williams for rat liver (1), some of the DPNase activity was found in the microsomes. As shown in Table II the microsomes of nearly all the types of organs examined contained a large per cent of the DPNase activity of the homogenate although the total recovery values did not approach 140 per cent, as was previously reported (1). It is of interest that rabbit brain, which contains the highest level of DPNase, has no DPN or DPNH pyrophosphatase activity.

Although considerable DPNase activity was found in the rat, rabbit, and mouse livers, the fractions from hamster and pigeon liver were found to be inactive at pH 7.5. This was particularly surprising in the case of the hamster. It was thought that the hamster homogenate might contain an inhibitor; however, the homogenate did not influence the activity of the DPNase of rat liver microsomes. Exposure to sonic vibration, freezing and thawing, and dialysis did not activate the hamster preparation. Finally it was found that by carrying out the DPNase assay between pH 5 and 6.5 activity could be detected in the hamster liver. The pH optima for the DPNases from various tissues are given in Fig. 1.

The pH optimum for the mouse and rat liver microsomal DPNase was shown to be between 6 and 6.5. At pH 7.5, the activity was about 75 per cent of the optimum.

The liver microsomal DPNase of the hamster resembled that of the mouse in having a sharply defined optimum near pH 6, but differed from that of both rat and mouse in that the activity at pH 7.5 was negligible.

Comparison of different organs of the rabbit revealed further differences in the pH characteristics of DPNases. The kidney and liver microsomes were optimally active at pH 6–6.5 but the brain microsomes and the blood DPNase did not exhibit a definite pH optimum (Fig. 1).

In contrast to the brain of the rabbit, the pigeon brain did exhibit a sharp pH optimum, as did a homogenate of breast muscle. The spleen and kidney homogenates were completely inactive at pH 5.5, 7.0, and 8.0. In the fractionation studies of pigeon liver the DPNase had been routinely assayed at pH 7.5 and activity could not be demonstrated. Following the discovery of the more acidic DPNase of hamster the pigeon liver was reexamined at lower

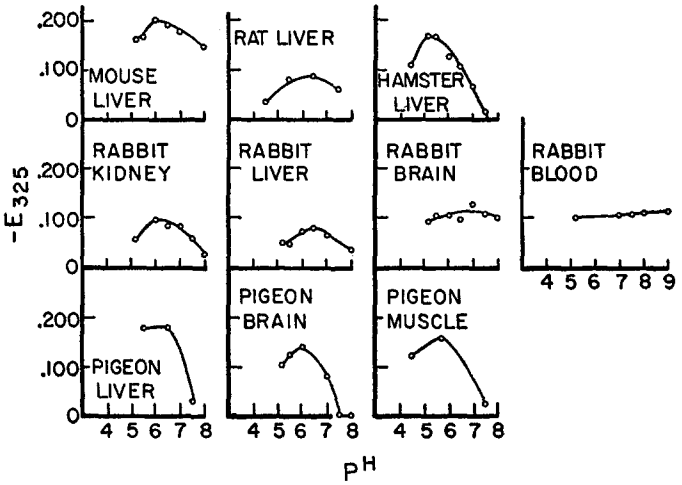

FIG. 1. Effect of pH on DPNases from various animals. Reaction consisted of 0.1 M trismaleate for the pH range 5.2–9.0 or of acetate for pH range 4.0–5.5. Activity in the two buffers was usually comparable. DPN concentration was 1.2–1.6 $\times$ 10^{-3} M which saturated the enzyme. Course of reaction followed by decrease in E_{325} of aliquots placed in 1 M KCN. All rates are in arbitrary time units since no attempt was made to use equivalent amounts of tissue. For the hamster, rat, mouse, rabbit liver, rabbit kidney, rabbit brain, and pigeon liver assays the microsome fractions were used; for the pigeon muscle and brain assays, the whole homogenate. Rabbit blood was a sucrose homogenate of the clot, 0.125 M sucrose final concentration.

pH values. As shown in Table II and Fig. 1, the homogenate and microsomes of pigeon liver contained a DPNase active at pH 6.5; these preparations were active after storage for 2 days at 0°. Assays on preceding days had been questionable. An attempt to repeat these observations was unsuccessful since no activity could be found at pH 5.5 or 6, and none appeared during storage for a week at 0°. The erratic appearance and disappearance of the DPNase in pigeon liver is suggestive of some further requirement for enzymatic activity which is not clearly understood at present and would appear to warrant further study.

Specific activities of the homogenates and fractions were calculated from the data in Tables I and II and appear in Table III.

Competition of DPNase and Pyrophosphatase for DPN.—In Experiment 1 of Table IV, rat liver microsomes were incubated with DPN, and the destruction of DPN was followed simultaneously by yeast alcohol dehydrogenase and by cyanide. The amounts of DPN lost according to the enzymatic and cyanide assays were virtually the same, indicating that there was essentially no pyrophosphatase activity but that the DPNase type of splitting was the predominant mechanism of cleavage in the microsomes. This was surprising since

TABLE IV

Competition between Rat Liver Microsomal DPNase and Pyrophosphatase for DPN

Substrate and enzyme	Time	DPN destroyed		Phosphate released/DPN split
		Dehydrogenase	KCN	
	min.	*μmoles/ml.*	*μ moles/ml.*	
Exp. 1				
1.2 μmoles DPN/ml.	0	0	0	0
0.2 ml. microsomes/ml.	15	0.61	0.72	—
	30	0.93	0.92	—
	60	1.19	1.08	2.0
Exp. 2				
1.9 μmoles DPN/ml.	0	0	0	
0.1 ml. microsomes/ml.	15	—	0.24	
	30	—	0.39	
	60	0.9	0.52	

Reaction consisted of 0.01 M $MgCl_2$, 0.2 M tris (pH 7.5), prostatic monoesterase, and substrate and enzyme as shown. Assay by alcohol dehydrogenase and 1 M KCN on identical aliquots taken at the same time. Phosphate determined in trichloroacetic acid supernatant (5 per cent final concentration). Incubation at 37°.

the microsomes have an active nucleotide pyrophosphatase when assayed in the presence of sufficient nicotinamide to inhibit the DPNase activity.

In Experiment 2 of Table IV, the concentration of DPN was increased and the amount of microsomes decreased; the pyrophosphatase is then apparent since more DPN is lost by the enzymatic assay than by the cyanide assay. A possible explanation for the results of Experiment 1 is that the DPNase, being a somewhat more active enzyme, started splitting DPN rapidly, releasing ADPR. The pyrophosphatase, if it is similar to that of pigeon liver, splits ADPR more readily than DPN and therefore, by acting on ADPR, might not attack DPN. The phosphate released by the added monoesterase at the completion of Experiment 1 was exactly twice the amount of DPN split, showing that the pyrophosphatase was functioning. Thus, in Experiment 1, almost all the DPN was split at the nicotinamide ribose bond, and the ADPR was subse-

quently split by the pyrophosphatase,[2] while, in Experiment 2, intact DPN was apparently available for both enzymes.

Evidence for Pyrophosphatase Activity in Pigeon and Rat Liver Fractions.—Enzymatic attack of DPNH could conceivably occur in several ways so as to yield a product inactive in the ADH assay. The presence of pyrophosphatase was established by including a monoesterase in the incubation mixture. When a dinucleotide is split, the two mononucleotides produced make available two monoesterphosphate groups for hydrolysis by the monoesterase. Thus, the appearance of 2 moles of inorganic phosphate for each mole of dinucleotide destroyed is indicative of a pyrophosphatase type of splitting of the dinucleotide. The ratio of phosphate produced to DPNH destroyed is presented in Table V. In this experiment no inhibitor of the DPNH oxidase was added since both cyanide and nicotinamide inhibit the monoesterase. The evidence in

TABLE V

Ratio of Phosphate Released per Mole of DPNH Split by Pigeon Liver Fractions

Fraction	DPNH split	Inorganic P liberated	P released/DPNH split
Homogenate	0.24	0.37	1.5
Nuclei	0.15	0.28	1.9
Microsomes	0.43	0.58	1.4
Soluble	0.17	0.56	2.1

DPNH and phosphate values expressed as μmoles per milliliters per 30 minutes. Reaction mixture contained 0.01 M $MgCl_2$, 0.1 M tris (pH 7.5), monoesterase, 1.9 mM DPNH, arbitrary amount of pigeon liver fraction. Incubation at 37°.

Table V shows that between 1.5 and 2 moles of phosphate were liberated from each mole of DPNH inactivated by the pigeon liver fractions. The amount of DPNH oxidized was not great enough to affect the balance; although the lower ratios in the homogenates and microsomes may be the result of the higher DPNH oxidase activity of these fractions. The monoesterase did not release phosphate from ADP or ATP under similar conditions nor does it split DPNH. Some experiments, yielding comparable results, were done in which the pyrophosphatase incubation was performed prior to monoesterase addition; the pyrophosphatase was stopped by heating, and the phosphate liberated by subsequent incubation with monoesterase.

In a similar manner fractions of a rat liver were assayed, and the ratio of phosphate liberated to DPNH split was shown to be close to 2. Only the pigeon and rat livers were studied in this way; the evidence for pyrophosphatase in other organs is taken from the ADH assay alone, as compared to the cyanide assay.

[2] The microsomal DPNase was unable to split NMN in this assay.

Differences between Particulate and Soluble Pyrophosphatases.—Additional demonstration of the difference between the pyrophosphatases of the pigeon liver fractions was sought to determine whether or not the soluble fraction's activity was the result of elution of the active protein from one of the particulate fractions. This point was approached by comparing the rate at which each fraction split 3AP-DPNH and DPNH. Results of such an experiment are shown in Fig. 2. In this particular fractionation the mitochondria, as well as the microsomes and nuclei, contained some DPNH pyrophosphatase activity, and the specificity of this fraction was also investigated. The three particulate fractions split DPNH at a somewhat faster rate than 3AP-DPNH. The soluble

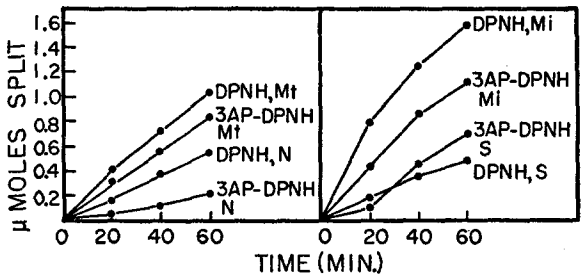

FIG. 2. Comparison of pigeon liver fraction by their rates of splitting DPNH and 3AP-DPNH. Reaction consisted of 0.01 M $MgCl_2$, 0.2 M tris (pH 7.5), 0.04 M KCN, and either 2.3×10^{-3} M DPNH or 2.7×10^{-3} M 3AP-DPNH; incubated at 37° and aliquots assayed with alcohol dehydrogenase and acetaldehyde. The enzyme added in each case was one of the pigeon liver fractions: N, nuclear; Mt, mitochondrial; Mi, microsomal; S, soluble.

fraction differed considerably in that DPNH was split more slowly than 3AP-DPNH over an hour period and in that the rate of splitting of the analog increased during incubation, whereas the rate of DPNH destruction remained linear. The rate of splitting of each substrate with the particulate fractions was either maintained or the rate decreased slowly. The relative activities among the fractions do not represent distribution of activity since an arbitrary amount of each fraction was chosen to produce an appreciable splitting.

Reexamination of the soluble fraction at closer time intervals is shown in Fig. 3; the rate of splitting of the 3AP-DPNH again is seen to increase with time of incubation. This phenomenon has been observed with several pigeon liver preparations. During these studies it was found that the E_{360} of the 3AP-DPNH increased about 5 per cent when it was split by either the soluble fraction of pigeon liver or by snake venom diesterase. The values presented in Figs. 2 and 3 have been corrected for this change. Therefore, the acceleration of splitting of 3AP-DPNH appears to be characteristic of the pyrophosphatase of the soluble fraction.

A potent inhibitor of the specific DPNH pyrophosphatase of pigeon liver

was shown previously (4) to be 5′-adenylic acid. The particulate and the soluble pyrophosphatase of pigeon liver were assayed in the presence of 5′-adenylic acid; the soluble enzyme was more strongly inhibited. This may indicate differing sensitivity to the inhibitor, but the presence of a monoesterase in the particulate fractions could also explain such results.

Another type of evidence, pertaining to the intracellular location of the DPNH pyrophosphatase, was obtained by varying the fractionation procedure. Soluble fractions were prepared in three ways: first, as described in Methods,

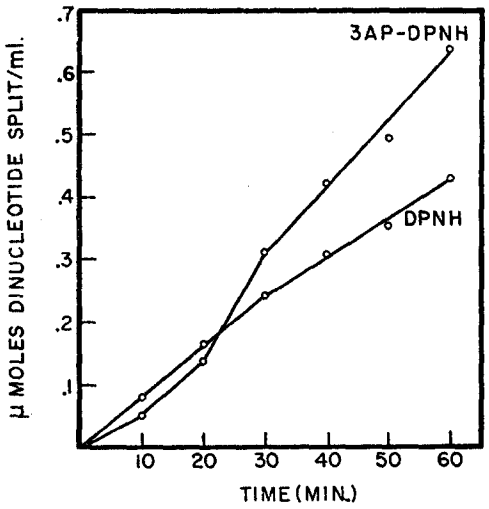

FIG. 3. Rates of splitting of DPNH and 3AP-DPNH by soluble fraction from pigeon liver. Reaction mixture consisted of 0.01 M $MgCl_2$, 0.1 M tris (pH 7.5), 0.04 M KCN, and either 2.4 × 10^{-3} M DPNH or 2.7 × 10^{-3} M 3AP-DPNH. Incubation was at 37°, aliquots were assayed for reduced dinucleotide with alcohol dehydrogenase and acetaldehyde.

where each fraction, including the microsomal, was washed; second, the same way except that none of the fractions were washed; and third, a portion of the homogenate was centrifuged at 105,000 *g* to remove the particulate material in one step. The DPNH splitting activities of the soluble fractions obtained by these three methods were virtually identical whether expressed in relation to the weight of the liver or to the protein content of the fraction. The activity of the microsomal fraction was not affected by washing.

From the differences between the particulate fractions and the soluble fraction of the pigeon liver in their rate of splitting DPNH and 3AP-DPNH as well as the lack of evidence for washing the active enzyme from particulate fractions, it is concluded that the DPNH pyrophosphatase found in the pigeon liver soluble fraction, as defined centrifugally, probably does occur in the soluble cytoplasm of the cell.

Cyanide Activation of DPN Pyrophosphatase.—Previously it was mentioned that cyanide was not present during the assay for DPN pyrophosphatase. In Table VI it may be seen that, in the presence of cyanide, the soluble fraction of pigeon liver split oxidized DPN and that the microsomal fraction was stimulated. The activity of these fractions on DPNH was unaffected by cyanide.

TABLE VI

Effect of Cyanide on Splitting of DPNH and DPN by Pigeon Liver Fraction

Fraction	DPN splitting activity		DPNH splitting activity	
	−CN	+CN	−CN	+CN
Soluble	0	0.059	0.177	0.177
Microsomes	0.067	0.090	0.205	0.193

DPN splitting activity expressed at the $-\Delta E_{340}/60$ minutes/aliquot obtained on assay with alcohol dehydrogenase and ethanol. DPNH splitting activity expressed as the $+\Delta E_{340}/60$ minutes/aliquot obtained on assay with alcohol dehydrogenase and acetaldehyde. Reaction mixture contained 0.01 M $MgCl_2$, 0.2 M tris pH 7.5, 1.6 mM DPN, 0.01 M KCN where indicated, and microsomal or soluble fraction where indicated.

TABLE VII

Nicotinamide Inhibition of DPNH Oxidation by Pigeon Liver Particles

Addition	Rate of oxidation			
	Mitochondria		Microsomes	
	Rate	Inhibition	Rate	Inhibition
		per cent		*per cent*
None	0.328	0	0.268	0
Nicotinamide 0.2 M	0.072	78	0.054	80
Nicotinamide 0.02 M	0.252	23	0.162	40

Rate: the $-\Delta E_{340}$ in a period of 15 seconds to 3 or 5 minutes, for mitochondria and microsomes respectively, is the rate recorded above. Particles were preincubated for 5 minutes at room temperature (*ca.* 25°) before adding DPNH.

Reaction mixture contained 0.01 M $MgCl_2$, 0.1 M tris pH 7.5, 0.14 mM DPNH, 3×10^{-6} M cytochrome *c*, and either mitochondria or microsomes that were isolated from 0.036 gm. of pigeon liver.

The addition reaction of DPN with cyanide results in a ring structure similar to that of DPNH (15). The cyanide addition product of DPN probably resembles DPNH sufficiently for it to be attacked by the soluble enzyme and to be split more rapidly than DPN by the microsomal pyrophosphatase. The quantities of the two liver fractions were chosen to give comparable rates of DPNH splitting and do not represent equivalent amounts of liver.

Inhibition of DPNH Oxidase by Nicotinamide.—As was previously noted,

nicotinamide was included in some DPNH pyrophosphatase assays to inhibit DPNH oxidation. In Table VII is shown the inhibitory effect of nicotinamide on the rate of DPNH oxidation by mitochondria and microsomes of pigeon liver. Inhibition of the DPNH oxidase of several other species was also observed. Larner *et al.* (14) have observed that 1 per cent nicotinamide prevented destruction of DPN by a brain homogenate and that 4 per cent nicotinamide (0.33 M) inhibited oxygen uptake. The addition of DPN did not reverse the inhibition of oxygen uptake in these studies.

DISCUSSION

Marked variation of the distribution and characteristics of the three enzymes studied was observed when organs of different species were fractionated. The soluble fractions of pigeon and of rabbit livers contain pyrophosphatases that split DPNH but not DPN. Three rodent livers had insignificant pyridine nucleotide pyrophosphatase activity in their soluble fractions under the conditions of the assay.

The behavior of the DPNases examined provides another example of the variation among the species and even among the organs of a single species. The pH optimum for hamster liver DPNase was about pH 5.5, but the enzyme was virtually inactive above pH 7. Two other rodent livers, rat and mouse, had acidic pH optima but retained a large part of their activity at pH 7.5–8. The variation among the rabbit organs and between rabbit brain and pigeon brain leads to the conclusion that a single type of enzyme may have distinctly different properties in different animals or even in different organs of the same animal. It would appear that caution is necessary in declaring that an enzyme is absent because it was inactive according to an assay designed for another organ or animal.

DPNase distribution was reported by Sung and Williams (1) for rat livers. In addition to the microsomes, the soluble fraction also had high levels of the enzyme, according to their procedure, and the total recovery of activity among the fractions was about 140 per cent. They did not describe their homogenization technique, but it is conceivable that it was severe enough to disrupt a number of the microsomes, releasing DPNase activity into the soluble fraction. In our studies, none of the soluble fractions of any of our preparations was observed to have significant DPNase activity.

SUMMARY

1. The distribution of DPN and DPNH pyrophosphatases and DPNase in centrifugally prepared fractions of organs of several species of animals is reported.

2. A DPNH pyrophosphatase was found in the soluble fraction of pigeon and of rabbit liver. This enzyme did not split DPN but accounted for over 50 per cent of the DPNH pyrophosphatase activity of the whole homogenates.

3. All the organs tested, including the pigeon liver and rabbit liver, contained a microsomal pyrophosphatase that attacked both DPNH and DPN. This microsomal enzyme split DPNH faster than DPN in all cases.

4. DPN pyrophosphatase and DPNase activity were generally concentrated in the microsomal fraction of liver, of kidney, and of brain.

5. The DPNase of hamster liver was virtually inactive at pH 7.5 but was optimally active at pH 5.5. Considerable difference was found with respect to pH on the activity of DPNase from organs of different animals.

6. The inhibition of mitochondrial and microsomal DPNH oxidation by nicotinamide was noted during the course of these experiments.

7. The significance of some of the distribution patterns is discussed.

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