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Some Observations on the Instability of Adenine Nucleotides in Bird-liver Preparations

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In the course of a study of purine metabolism in suspensions of bird liver it was observed that incubation in the presence of 2:4-dinitrophenol (DNP) results in a considerable formation of free purines. It seemed unlikely that these would be of synthetic origin, and an investigation of possible catabolic sources showed that the liberated purines could be accounted for almost entirely by an extensive breakdown of the free adenine nucleotides in the tissue. The nucleic acid content of such preparations remained relatively unaffected. Other inhibitors of oxidative phosphorylation and, furthermore, the addition of magnesium ions gave similar results. Investigations with the soluble and insoluble fractions of the suspensions and with chick-liver slices are also described.

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MATERIALS AND METHODS

Chicks. Day-old chicks were obtained from hatcheries and fed on chick mash, which was gradually supplemented with, and finally replaced by, crushed oats and greenstuffs at 8 weeks. Chicks were used from 2–12 weeks old. They were usually allowed to feed until shortly before being killed, but in two experiments (88 and 90) they were starved for 20 hr. before death.

Pigeons. Adult pigeons were obtained from dealers and used at once.

Uric acid. Uric acid was determined by the colorimetric method of Brown (1945), modified as described by Dresel & Moyle (1950).

Pentose. Pentose was determined by the method of Mejbaum (1939) with modifications suggested by Ogur & Rosen (1950).

Inorganic, 10 min. acid-labile and total phosphates. These were determined by the method of Fiske & Subbarow (1925), using the micromodifications of Mitchell & Moyle (1951).

Nucleic acids. Nucleic acids were determined essentially by the method of Schmidt & Thannhauser (1945), modified as follows: the preliminary extraction of the trichloroacetic acid precipitate with lipid solvents was omitted, and the ribonucleic acid (RNA) estimated by ultraviolet absorption at 260 $m\mu$, using the molar extinction coefficient of 10 000 for an average tetranucleotide (Wyatt, 1950; Mitchell & Moyle, 1951).

Hypoxanthine. Hypoxanthine was determined enzymically by the method of Kalckar (1947).

Inosine and inosinic acid. These were determined collectively after conversion to hypoxanthine with 0.5N- H_2SO_4 (Kalckar, 1947).

Adenosine monophosphate (5-AMP), Adenosine diphosphate (ADP), Adenosine triphosphate (ATP), and yeast adenylic acid. These were determined spectrophotometrically, using an adenylic acid deaminase (preparation A, Kalckar, 1947), a pyrophosphatase from potato and an adenosine deaminase and phosphatase preparation from intestinal mucosa (Kalckar, 1947). The last was found to contain some xanthine oxidase activity which had to be allowed for in the present work. Furthermore, it appears that this preparation will also estimate certain dinucleotides containing adenylic acid, such as, for example, diphosphopyridine nucleotide.

Derivatives of guanine. These were determined collectively as hypoxanthine after acid hydrolysis and treatment with a preparation containing nucleoside phosphorylase and guanase activity (Kalckar, 1947).

Diphosphopyridine nucleotide. This was determined spectrophotometrically with a lactic dehydrogenase preparation. Spectrophotometric determinations were carried out with a Beckman model DU spectrophotometer.

Preparation of suspensions. The bird was decapitated and bled, the liver excised, and, after removal of the gall bladder, cooled rapidly to 0° in saline. A suitable portion was weighed, quickly chopped on a cold tile and then ground up, usually with 1.5 vol. ice-cold 0.13M-KCl-0.03M-potassium phosphate buffer, pH 7.4, in a Potter-Elvehjem glass homogenizer for 1–2 min. Such a preparation was essentially free from cells other than erythrocytes. The suspension was strained through muslin, and samples pipetted into flasks which already contained all other constituents for incubation.

Preparation of soluble (S) and insoluble (I) fractions: the suspension was centrifuged at 2000 *g* for 15 min. The supernatant was decanted and made up to the volume of the original suspension with 0.13M-KCl-0.03M-potassium phos-

phate buffer, pH 7.4 (S). The residue was washed twice with 0.9% KCl in the cold and then made up to the same volume with the above KCl-phosphate mixture (I); this preparation is similar to the cyclophorase preparation of Green, Loomis & Auerbach (1948).

Incubations were carried out in air except where otherwise stated.

2:4-Dinitrophenol (DNP). This was recrystallized from water and used at a concentration of 1.8×10^{-4} M throughout.

RESULTS

The additional uric acid formed on incubation of chick-liver suspensions in the presence of DNP often amounted to more than 3 times that formed in the control flasks and reached values up to 0.7 mg./g. wet weight liver. DNP caused no rise in uric acid content if the proteins of the suspension were precipitated prior to incubation, nor if the system was kept at 0° instead of being incubated at higher temperatures.

Effect of DNP on tissue nucleic acids. With the experimental conditions used, the breakdown of nucleic acids during incubation of the tissue alone amounted to 2–18%. The additional breakdown of ribonucleic acids (RNA) due to DNP was 6–9% in one experiment and in all others not more than 2%. The deoxyribonucleic acids (DNA) were probably unchanged, the apparent increases being due to artifact (see below). For comparative purposes it is assumed that nucleic acids are composed of tetranucleotides, each of which would give rise to two molecules of free purine. The results summarized in Table 1 show that the losses of RNA due to DNP were very much less than would be required to account for the gain in free purine.

The apparent small increase in RNA in some experiments led to the suspicion that DNP itself might contribute to the ultraviolet absorption by being carried down with the original protein precipitate and then being redissolved in *N*-NaOH during the Schmidt & Thannhauser procedure. A determination of the absorption at 360 $m\mu$. of the neutralized RNA extract (Table 1, Exp. 96) showed that some 20% of the added DNP was present in

Table 1. *Effect of DNP on nucleic acid content of the liver suspension*

(Samples of suspension incubated with 0.13M-KCl containing 0.03M-potassium phosphate buffer, pH 7.4, except in Exp. 96 where phosphate buffer was 0.012M and flasks also contained 0.016M- $KHCO_3$; gas phase 95% O_2 + 5% CO_2 in Exp. 96 and air in all others. Time of incubation 15 min. in Exps. 85, 86 and 88, and 30 min. in Exps. 59, 95 and 96. Temp. 38°.)

Exp. no.	Wet wt. liver in flask (mg./ml.)	Total RNA + DNA after incubation without DNP (μ moles purine/g. wet wt.)	Changes due to incubation with DNP		
			RNA	DNA	Free purine
59 (chick)	50	15.4	-0.20	+0.19	+2.98
85 (chick)	148	14.9	-0.26	-0.08	+0.42
86 (chick)	169	14.2	+0.08	+0.03	+0.62
88 (chick)	85	15.3	-0.09	+0.10	+1.72
95 (chick)	56	17.2	-1.07	+1.24	+4.29
95 (chick)	167	17.0	-0.77	+0.17	+3.29
96 (pigeon)	116	18.1	+0.29	+0.39	+2.85

Table 2. *Effect of DNP on adenine nucleotides of the liver suspension*

(For conditions of incubation see Table 1. Substrates were added to Exp. 90 (0.02M- α -ketoglutarate) and Exp. 92 (0.01M-glutamate). Time of incubation 15 min. in Exps. 88, and 92, and 30 min. in Exps. 90, 95 and 96.)

Exp. no.	Wet wt. liver in flask (mg./ml.)	AMP, ADP and ATP after incubation			Adenine lost due to DNP (%)	Purine gained due to DNP		Purine gained accounted for as adenine lost (%)
		(μmoles adenine/g. wet wt.)				Uric acid (μmoles/g. wet wt.)	Hypo- xanthine (μmoles/g. wet wt.)	
		0°	38°	38° + DNP				
88 (chick)	85	2.00	1.82	0.38	79	1.66	—	87
90 (chick)	150	1.29	0.86	0.28	68	0.48	0.62	53
95 (chick)	55	—	4.58	0.67	85	4.29	0.00	91
92 (pigeon)	116	2.79	2.82	2.23	21	—	0.33*	179
96 (pigeon)	116	—	3.27	0.74	77	—	2.30*	110

* These figures include both free hypoxanthine and hypoxanthine in the form of inosine and inosinic acid.

this fraction, and it was calculated that this accounted exactly for the apparent 2% gain in RNA. The losses in RNA shown in Table 1 are therefore a little low, but the error incurred is too small to affect the conclusions materially.

The apparent gain in DNA, which, in some cases, corresponded rather closely to the loss in RNA, is most probably an artifact. It seems conceivable that the DNP carried down with the precipitate of nucleic acids inhibits the hydrolysis of RNA, some of which is therefore reprecipitated with the DNA. Alternatively, the DNP carried down with the protein may be in the form of a phosphate derivative, thus estimating as DNA.

Effect of DNP on nucleotide content of the suspension. Although, in the intact tissue, adenine mononucleotides may be present largely in the form of adenosine polyphosphates (Davidson & Weymouth, 1944; Rapoport & Nelson, 1945; see also Table 5), it seems that dispersal causes a very considerable loss of pyrophosphate bonds, 80–100% of the adenine mononucleotides being recovered as 5-AMP, even in samples not incubated at 38°. In most cases incubation at 38° brought about very little change in the ratio of AMP to ADP and ATP, with the exception of Exp. 92 (Table 2), where the adenosine polyphosphate content rose from 20 to 49% during incubation, the total adenine mononucleotide content remaining essentially unchanged.

Table 2 shows that incubation in the presence of DNP results in a very considerable loss of adenine mononucleotides, amounting, in the case of the chick, to 68–85% of the nucleotides normally recovered after 15–30 min. incubation. An attempt to correlate these losses with the corresponding gain in free purines was not entirely satisfactory, but the figures appear to be of a similar order and suggest that the adenine mononucleotides should be considered as the chief source of the additional purine liberated in the presence of DNP.

Purine derivatives other than those discussed above would also be present in suspensions, for

instance nucleotides resulting from nucleic acid breakdown and coenzymes. It was found that endogenous yeast adenylic acid (2- or 3-AMP), guanylic acid and diphosphopyridine nucleotide were not broken down to a significantly greater extent in the presence of DNP. In the case of diphosphopyridine nucleotide, most of that present initially was lost even on incubation without DNP. Other coenzymes, if affected, would probably be present in insufficient amounts to add appreciably to the total purine liberated.

Effect of incubation of the suspension on composition of insoluble fraction. Samples of a potassium chloride suspension were incubated 30 min. at 0° or 38° both with and without DNP. They were then centrifuged and washed according to Green's procedure for the cyclophorase preparation (Green *et al.* 1948). The residue (R_2L) was taken up in 0.9% potassium chloride and the protein precipitated with perchloric acid, the final concentration of acid being 3% (w/v). The supernatant should contain nucleotides and cofactors which had remained attached to the insoluble fraction of the suspension during incubation and washing. The ultraviolet absorption at 260 m μ . pentose, inorganic, 10 min. acid-labile, and total phosphates were estimated. The results of several experiments are summarized in Fig. 1. Incubation at 38° in the presence of DNP led to a considerable loss of phosphorus-containing components from the insoluble fraction. Molar ratios indicated that a large part of these were probably of nucleotide structure. At 0°, DNP removed material estimating as inorganic P (Teply, 1949) and some 10 min. acid-labile P, but no whole nucleotides.

Nucleotide content of the soluble and insoluble fractions of the suspension after separate incubation. Table 3 illustrates the results of an experiment where the effect of DNP on the complete suspension (*H*), the insoluble fraction (*I*) and the soluble fraction (*S*) were studied side by side. Samples of each preparation were incubated 30 min. at (a) 0°, (b) 38° and (c) 38° in the presence of DNP.

α -Ketoglutarate was added throughout in order to allow for the removal of endogenous substrates from (I). After incubation, samples (a), (b) and (c) from (H) and (I) were centrifuged and washed with 0.9 % potassium chloride, and the nucleotide content of the insoluble and supernatant fractions analysed separately after precipitation of the proteins with perchloric acid. It was found that the effect of DNP in suspensions is not reproducible in the separated soluble and insoluble fractions. De-

nucleotides lost from the insoluble material. Part of the surplus nucleotide probably resulted from nucleic acid breakdown.

Thus, although the enzymes responsible for degradative changes of free nucleotides are absent from the insoluble fraction, the nucleotides are more easily dislodged when the latter is incubated alone than when incubated as the suspension. It would be of interest to study the effect of added adenine nucleotides in such preparations.

Fig. 1. Fate of perchloric-acid-soluble components attached to insoluble fraction after incubation of suspension at 38° and treatment with DNP. For experimental technique see text. Recoveries expressed as percentage of amounts found after incubation at 0° without DNP. A=ultraviolet absorption at 260 m μ .; R=pentose; P₀=organic P-10 min. acid-labile P; P₁₀=10 min. acid-labile P; P₁=inorganic P. ■, ▨, ▩, ▪, ▫, correspond to results from five experiments.

gradative changes similar to those caused by DNP in the suspension were observed in the soluble fraction on incubation alone, and DNP caused very little, if any, additional breakdown. Incubation of the insoluble fraction alone resulted in a 75 % loss of attached nucleotides, but these were recovered, apparently unchanged, in the supernatant obtained after recentrifuging. In fact, the recovery of adenine nucleotides in the supernatant was 160 % of the

Effect of other inhibitors of oxidative phosphorylation. Changes in the purine-containing components of chick-liver suspensions, similar to those reported above, were caused by other inhibitors of oxidative phosphorylation. Thus, a liberation of uric acid occurred after incubation with azide (0.017 M), calcium chloride (0.005 M) and cyanide (0.001 M). Arsenite (0.009 M) gave rise to an accumulation of hypoxanthine rather than uric acid, owing

Table 3. *Effect of incubation with and without DNP on adenine nucleotide content of the soluble and insoluble fractions of a chick-liver suspension*

(For conditions of incubation see Table 2, Exp. 90. Time of incubation 15 min. For preparation of fractions see methods.)

	Suspension			Insoluble fraction			Soluble fraction		
	0°	38°	38° + DNP	0°	38°	38° + DNP	0°	38°	38° + DNP
Perchloric acid extracts from supernatants									
5-AMP, ADP and ATP (μ moles)	0.82	0.55	0.16	0.06	0.21	0.07	0.70	0.07	0.11
Adenine estimated with adenosine deaminase (μ moles)	0.11	0.02	0.13	0.10	0.24	0.28	0.14	0.24	0.07
Total adenine nucleotides	0.93	0.57	0.29	0.16	0.45	0.35	0.84	0.31	0.18
Perchloric acid extracts from insoluble material									
5-AMP, ADP and ATP (μ moles)	0.16	0.10	0.05	0.14	0.04	0.03	—	—	—
Adenine estimated with adenosine deaminase (μ moles)	0.21	0.11	0.01	0.10	0.02	0.00	—	—	—
Total adenine nucleotides	0.37	0.21	0.06	0.24	0.06	0.03	—	—	—
Change in adenine nucleotides (μ moles)		-0.52	-0.95	—	+0.11	-0.02	—	-0.53	-0.66
Change in free purines (μ moles uric acid and hypoxanthine)		+0.47	+1.28	—	+0.03	+0.10	—	+0.81	+0.82

to the inhibition of xanthine oxidase by this reagent (Barry, Bunbury & Kennaway, 1928). However, 0.02M-sodium sulphate, which, according to Lehninger (1949), is a potent uncoupling agent of oxidative phosphorylation, did not increase uric acid formation or inhibit oxidative phosphorylation in the present system. Finally, anaerobiosis alone was found to be as effective as DNP in stimulating the breakdown of adenine nucleotides in the suspension. It seems, then, that impairment of oxidative phosphorylation, however this may be effected, will lead to an increased breakdown of free nucleotides in the suspension.

Effect of magnesium. The experiment illustrated in Table 4 shows that incubation of the suspension with magnesium chloride (0.005M) results in an extensive breakdown of adenine nucleotides, similar to that obtained with DNP.

Table 4. *Comparison of effects of magnesium ions and DNP on adenine nucleotides of the suspension*

(For conditions of incubation see Table 1, Exp. 88; concentration of magnesium ions was 0.005M.)

Additions	None	DNP	MgCl ₂
Adenine as AMP, ADP and ATP (μmoles)	1.55	0.32	0.44
Uric acid (μmoles)	0.83	2.25	1.78
Additional uric acid accounted for as adenine lost (%)	—	87	117

Inhibition of oxidative phosphorylation in chick-liver slices. The effect of anaerobiosis and of treatment with DNP on the nucleotide content of chick-liver slices is illustrated in Table 5. Although, as was usually the case with suspensions, incubation of the tissue alone resulted in the loss of some adenine nucleotides, this was much increased during anaerobiosis as well as by the addition of DNP. The colorimetric method of determining uric acid was insufficiently sensitive to decide conclusively whether the adenine nucleotides were broken down completely to free purines.

Table 5. *Effect of DNP and of anaerobiosis on the nucleotide content of chick-liver slices*

(300–350 mg. wet weight slices were incubated 30 min. at 37° in Krebs-Ringer phosphate containing 0.2% glucose. After incubation they were dropped into 10% (w/v) perchloric acid and ground up rapidly in a small, loosely fitting, glass homogenizer. After centrifuging the supernatant was decanted and the residue washed once with 1% perchloric acid. The combined supernatants were neutralized and the adenine nucleotide content determined.)

Exp. no.		Slices precipitated at start	Incubation		
			Aerobic	Aerobic plus DNP	Anaerobic
			(μmoles/g. wet wt.)		
89	5-AMP	0.43	0.25	0.59	—
	ADP and ATP	1.20	1.03	0.32	—
	5-AMP, ADP and ATP	1.63	1.28	0.91	—
91	5-AMP	0.50	0.27	0.50	0.45
	ADP and ATP	1.03	1.03	0.27	0.30
	5-AMP, ADP and ATP	1.53	1.30	0.77	0.75

DISCUSSION

It is hoped that the present investigation has served to emphasize the instability of bird-liver adenine nucleotides. A very extensive loss of easily hydrolysable phosphate occurs on grinding of the tissue alone, but a more complete degradation of adenine nucleotides is observed after incubation of suspensions or slices in the absence of active phosphorylating processes. The nucleic acids appear to be relatively more stable, for, under conditions where the addition of DNP resulted in the complete breakdown of 60–80% of the adenine nucleotides, the breakdown of nucleic acids was scarcely accelerated. LePage (1949) also noted only a slow loss of nucleic acids from rat-liver suspensions when no system for maintaining ATP was present.

The enzymes responsible for the degradation of 5-AMP appear to be confined to the soluble fraction of the suspension, as Potter, Lyle & Schneider (1951) also concluded. Since oxidative phosphorylation is carried out chiefly in the mitochondria, it is not surprising that the factors tending to restrain adenine nucleotide breakdown were found to be associated with the insoluble fraction. However, the regulative mechanism appears to be complex. In most experiments the endogenous nucleotides, although present largely in the form of 5-AMP, were relatively resistant to breakdown during incubation unless inhibitors of oxidative phosphorylation were added. *In vitro*, there appears to be little uptake of radioactive phosphate into 5-AMP as opposed to ATP and flavin adenine dinucleotide (Hummel & Lindberg, 1949; Good & Leaf, 1950), and it seems that the loss of 5-AMP cannot therefore be attributed, simply, to a removal of synthetic activity.

The present work may have little bearing on studies pertaining to the action of DNP on insoluble and mitochondrial preparations, since no significant changes in the nucleotide content of such preparations were observed.

The acceleration of adenine nucleotide breakdown by magnesium ions is of particular interest. Although alkaline phosphatases are activated by magnesium ions (Roche & Nguyen-van Thoai, 1950), the rate of breakdown of 5-AMP in rabbit-liver extracts is not stimulated by magnesium ions (Conway & Cooke, 1939). Swanson (1951) has demonstrated a stimulation of adenosine triphosphatase in rat-liver suspensions by 0.005M-magnesium, and the action of magnesium in unmasking adenosine triphosphatase activity in mitochondria (Potter & Simonson, 1952) may also be significant. Thus magnesium ions, like inhibitors of oxidative phosphorylation, may stimulate nucleotide breakdown by reducing the level of easily hydrolysable phosphate. This does not, however, explain why Swanson did not also observe an increased rate of breakdown of 5-AMP, as would be expected in the light of the present work. This discrepancy may be due to species difference.

The stability of adenine nucleotides in tissue preparations has not been studied extensively, but observations similar to the above have been made by other workers. A ready deamination of 5-AMP in muscle breis in the absence of phosphorylating processes was demonstrated by Ostern & Mann (1935). Eiler & Allen (1938) showed that liberation of adenosine from purine nucleotides in rabbit blood occurs in the absence of glycolysis. Jeener (1943) observed an extensive loss of adenine nucleotides from liver slices of the rabbit, rat and pigeon during anaerobiosis. Finally, Rapoport (1945) has pointed out that fasting of rats alone might lead to appreciable losses in the adenine nucleotide of the liver. A loss in adenine nucleotides in the absence of phos-

phorylating processes thus seems to be fairly universal, but the extent of the breakdown may vary from tissue to tissue and also with the type of preparation used.

SUMMARY

1. Incubation of potassium chloride suspensions of bird livers with 2:4-dinitrophenol results in the formation of free purines such as hypoxanthine and uric acid.

2. Most of the liberated purine is derived from the adenine nucleotides of the suspension, which are, in part, attached to the particles. No significant increase in nucleic acid breakdown was noted.

3. Other inhibitors of oxidative phosphorylation, such as azide, cyanide, arsenite, calcium ions and anaerobiosis, exert a similar effect, and so do magnesium ions.

4. Incubation of the soluble fraction of the suspension alone results in a rapid breakdown of adenine nucleotides, whereas incubation of the particles alone results in a dislodgement of attached nucleotides, but no breakdown, even in the presence of 2:4-dinitrophenol.

5. Similar effects are obtained with chick-liver slices, incubated anaerobically or in the presence of 2:4-dinitrophenol.

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