6. Serine is synthesized from a non-nitrogenous C_3 precursor which is more closely related to an intermediate of the glycolytic pathway of glucose catabolism, possibly a triose or glyceric acid, than to pyruvate.

7. The conversion of glucose, labelled in $C_{(1)}$ or in all carbon atoms, into alanine has been used to calculate the relative contribution of the glycolytic and oxidative pathways of glucose catabolism. In the intact rat none of the pyruvate appears to arise by the oxidative catabolism of glucose.

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The Effects of Some Inhibitors of Oxidative Phosphorylation on the Morphology and Enzymic Activities of Mitochondria

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It has been shown in previous experiments that uncoupling of oxidative phosphorylation occurs in mitochondria isolated from fatty livers. The same type of enzymic damage has been found to occur in mitochondria from normal livers after treatment with distilled water (Harman & Feigelson, 1952; Dianzani, 1954). Since both water-treated mitochondria and mitochondria from fatty livers are swollen and have a spherical form, it seems probable that this uncoupling of oxidative phosphorylation is intimately connected with the morphological changes. Since adenosine triphosphate (ATP) is necessary for the activation of fatty acids (Lipmann, Jones, Black & Flynn, 1952), the hypothesis that the accumulation of fat within the

cell is the result of the uncoupling of the oxidative phosphorylation has been advanced (Dianzani, 1954). The mechanism of the uncoupling of oxidative phosphorylation occurring in both water-treated and 'fatty' mitochondria is not completely clear, but it seems very likely that the synthetic reaction $ADP + \sim P \rightleftharpoons ATP$ is affected (Dianzani, 1954).

Many substances have been reported to uncouple oxidative phosphorylation *in vitro*. 2:4-Dinitrophenol (DNP) and some compounds commonly used as histological stains are particularly active (Judah & Williams-Ashman, 1951; Lardy & Wellman, 1952; Potter, Siekevitz & Simonson, 1953; Chappell & Perry, 1953). The mechanism of

the action of DNP has been investigated by many authors. DNP has been found to increase the adenosine triphosphatase (ATPase) activity of mitochondria and also the activity of soluble ATPase (Lardy & Wellman, 1952).

The effect *in vivo* of the substances which uncouple oxidative phosphorylation *in vitro* has not yet been investigated. The effects of DNP and of five histological stains [methylene blue (MB), brilliant cresyl blue (BCB), thionine (Th), Janus Green B (JG) and phenosafranin (PS)] on the morphology and oxidative phosphorylation of mitochondria isolated from different organs have been studied, and the results are described in this paper. Particular attention was devoted to the study of the morphology of liver mitochondria and to the lipid content of whole liver.

MATERIAL AND METHODS

Mitochondrial preparations. Albino rats from an inbred strain, weighing 120–150 g., and albino mice from Strong A strain, weighing 20–22 g., were used. The animals were fed on a standard diet. They were killed by decapitation and the organs (liver, kidney, brain, heart and skeletal muscle) were immediately dissected, weighed and transferred to the cold room at 2°. Homogenization was made in a Potter-Elvehjem apparatus with a Lucite (polymethyl methacrylate, du Pont de Nemours) pestle, 10% (w/v) homogenates being prepared in 0.25 M sucrose. 0.88 M sucrose was used in a few instances. With brain, only the cortex was used. Differential centrifuging was done in a Servall SS-1 Type Angle Centrifuge in the cold room. Tissue debris, undamaged cells, nuclei and some mitochondria (and also myofibrils in the case of heart and skeletal muscle) were discarded by centrifuging at 1000 g for 10 min. Mitochondria were then sedimented at 12 000 g for 30 min. when 0.25 M sucrose was used as suspension medium and at 22 000 g for 30 min. when 0.88 M sucrose was employed. Mitochondria were then washed twice with sucrose. The purity and morphology of the mitochondrial preparations were studied in a Zeiss phase-contrast microscope. Brain preparations were never pure, a certain amount of myelin material being always present. As the use of more elaborate purification procedures often produced damage to mitochondria, the use of non-purified sediments was preferred.

Oxidative phosphorylation. This was determined as described elsewhere (Dianzani, 1954), with 30 μ moles of α -oxoglutarate as substrate. The reaction mixture contained: 0.02 M Na_2HPO_4 – KH_2PO_4 , pH 7.4; 0.001 M adenosine monophosphate (AMP); 0.0067 M MgSO_4 ; 0.025 M KCl; 10^{-5} M cytochrome c; 0.026 M fructose; 0.013 M KF, 0.3 ml. hexokinase solution and 0.5 ml. of the mitochondrial suspension (corresponding to 100 mg. of fresh tissue). The final volume was 3 ml. KOH (30%, w/v, 0.2 ml.) was placed in the centre well of the Warburg flasks; temp., 25°. The O_2 uptake was measured for 10 min.; after this time, 0.5 ml. of 40% (w/v) trichloroacetic acid (TCA) was added from the side arm to stop the reaction.

Inorganic orthophosphate. This was determined in the protein-free filtrate according to Fiske & Subbarow (1925), with 40% (w/v) SnCl_2 dissolved in conc. HCl as a reducing

agent. Readings of the optical density were made at 730 m μ . in a Beckman Model DU spectrophotometer.

Phosphorylated intermediates. In some experiments, the concentrations of certain phosphorylated intermediates in the protein-free filtrate were determined. The fractionation was made according to LePage (1951), by treatment with barium acetate at pH 8.2. The fraction giving insoluble barium salts was analysed for acid-labile P (increase of inorganic P after hydrolysis with N-HCl at 100° for 8 min.) and for ribose. In the experimental conditions used in this work, acid-labile P represents the inorganic P released from ATP + ADP. As ATP releases two phosphate groups/unit of ribose, and ADP only one phosphate group/unit of ribose, the relative concentrations of ATP and ADP may be deduced from the value of the molar quotient acid-labile P/ribose. The ethanol-insoluble fraction of the soluble barium salts was analysed for AMP and for fructose 6-phosphate. The concentration of AMP was determined by measuring the optical density at 260 m μ . after dissolving in acid, neutralization and dilution. Fructose 6-phosphate concentration was calculated from fructose determinations, made according to Roe (1934).

Hexokinase. This was prepared from wheat germ, and its activity was determined as described by Saltman (1953). A large excess of the enzyme was used in the phosphorylation experiments.

Measurement of enzyme activities. ATPase activity was determined in most experiments according to the method of Dubois & Potter (1943), with 0.067 M borate buffer, pH 6.9, 0.01 M ATP (Na salt) and 0.0067 M MgSO_4 . In other experiments, in which the tonicity of the reaction medium was important, the incubation medium had a different composition; this will be shown in each particular case.

Alkaline phosphatase activity was determined at pH 9.2, according to the method of Nguyen Van Thoi, Roche & Roger (1947), with 0.2 M sodium β -glycerophosphate as substrate and 0.0067 M MgSO_4 as activator. Acid phosphatase was determined at pH 5.2, with 0.067 M veronal-HCl buffer, and 0.2 M sodium β -glycerophosphate. Inorganic pyrophosphatase was determined with 0.067 M borate buffer, pH 7.4, 0.0067 M MgSO_4 and 0.01 M sodium pyrophosphate.

5'-Nucleotidase was determined by measuring the release of inorganic orthophosphate from 0.01 M AMP, with 0.067 M borate buffer, pH 7.4, and 0.0067 M MgSO_4 . In all phosphatase determinations, incubation was at 38° for 20 min., with 0.1 ml. of a 10% suspension of mitochondria in 0.25 M sucrose. In every case the enzymic reaction was stopped by addition of an equal volume of 40% (w/v) TCA.

Myokinase activity was determined according to Kielley & Kielley (1951), with a reaction mixture containing 0.05 M histidine, 0.005 M MgCl_2 , 0.04 M KCl, 0.025 M glucose, 0.0007 M ATP, 0.1 ml. hexokinase, 0.1 ml. of adenylic acid deaminase, 0.1 ml. of the mitochondrial suspension and water to 3 ml. The mixture was pre-incubated at 27° for 10 min. with hexokinase and adenylic acid deaminase; the mitochondrial suspension was then added and the incubation at 27° was continued for 5 min. The reaction was stopped by addition of an equal volume of 10% (w/v) TCA. The decrease of the optical density at 260 m μ . occurring in these conditions was taken as a measure of myokinase activity. Adenylic acid deaminase was the preparation A of Kalekar (1947) from rabbit muscle;

0.1 ml. of this preparation was enough to destroy in 5 min. at 27° 40% of added AMP, when the concentration of this was 600 $\mu\text{g./ml.}$ The enzyme was stored at -22° for 2 months without loss of activity.

Nitrogen. This was determined by a micro-Kjeldahl technique.

Total lipid content of the liver. This was determined by weighing the dry powder of the organ before and after extraction with ether for 3-4 hr. in a Kumagawa apparatus. Drying of the liver to constant weight was done in an oven at 85°.

Specimens for histological examination were prepared from each liver. Staining of fat was made with Sudan III; glycogen was stained by the method of Best (1906).

Reagents. The following substances were used as uncouplers of oxidative phosphorylation: DNP, methylene blue (3:7-bisdimethylaminophenothiazine chloride), thionine (3:7-diaminophenothiazine chloride), brilliant cresyl blue (3-amino-7-diethylamino-8-methyl-phenoxazine), Janus Green B (5-chloro-3-diethylamino-7-*p*-dimethylaminophenylazo-5-phenylphenazine) and phenosafranine, (2:8-diamino-10-hydroxy-10-phenylphenazine). DNP, JG and MB were products of the firm E. Merck, Darmstadt; BCB was furnished by Ciba Aktiengesellschaft, Basel; PS was a product of Eastman Kodak Co., Rochester 3, New York, and Th was a product of the firm G. Grüber Co., Leipzig.

ATP (sodium salt) was prepared from the dibarium salt (Schwarz Lab. Inc., New York 17, N.Y.), after solution in 0.1N-HCl, and precipitation of barium with the calculated amount of H_2SO_4 . AMP (sodium salt) was prepared by neutralization of the acid (Schwarz Lab. Inc.).

Cytochrome *c* was prepared from horse heart by the method of Keilin & Hartree (1945). Its Fe content varied from 0.35 to 0.40% in different preparations. Fe determinations were made according to the method of Elvehjem (1930). All other substances used were commercial preparations.

Statistical evaluation of results. The values obtained were submitted to statistical analysis, the standard deviation and Student's *t* test being calculated for each average. Only differences with a *t* value corresponding to a probability $P < 0.05$ were accepted as significant.

RESULTS

Inhibition of oxidative phosphorylation in vitro

The extent of the inhibition of oxidative phosphorylation caused by DNP and by the histological stains used in this work has been investigated by many authors, and particularly by Judah & Williams-Ashman (1951). It has been shown that these substances act mainly by inhibiting the uptake of inorganic orthophosphate from the reaction medium, whereas the oxygen uptake is unmodified or increased. The extent of uncoupling of the oxidative phosphorylation caused by the substances used has been the object of a preliminary study during the present investigation.

Three different concentrations of each substance were used, and their effect was studied on mito-

chondria isolated from liver, kidney, brain, heart and skeletal muscle. Table 1 shows that a 10^{-4}M concentration of the substances used inhibits the oxidative phosphorylation by about 90% in mitochondria from most organs. The percentage inhibitions produced by concentrations of 10^{-5}M were, however, not identical in all enzyme preparations. The difference was particularly striking in the case of DNP, which produced 94% inhibition in mitochondria from skeletal muscle; the inhibition did not exceed 50% in mitochondria from other organs. All the substances used affected mainly the uptake of inorganic orthophosphate, while the oxygen uptake was practically unmodified.

The effects of these substances on the morphology of mitochondria have been studied. Mitochondria isolated from approximately 100 mg. of tissue with 0.88M sucrose were suspended in a small volume of 0.88M sucrose. Small amounts of the inhibitors of oxidative phosphorylation dissolved in 0.88M sucrose were then added to the mitochondrial suspension, the final concentration of the inhibitors being 10^{-4}M . The mixture was set aside at room temperature; every 5 min. a sample of the suspension was removed and the morphology of the mitochondria was examined under the phase-contrast microscope. Mitochondria isolated from hypertonic sucrose homogenates were mainly rod-like. Their form did not show any modification after being kept for 30 min. at room temperature. In the presence of DNP or of the other inhibitors of oxidative phosphorylation, a rapid change of form from rod-like to spherical form occurred. Nearly all mitochondria were swollen 15 min. after the addition of the uncoupling agent. The change was particularly rapid with DNP. This fact agrees with the observation made by Loomis & Lipmann (1948).

Inhibition of oxidative phosphorylation in vivo

In other experiments, the influence of a single injection of the inhibitors of oxidative phosphorylation on mitochondria from different organs was studied. Albino rats were injected intraperitoneally with 3 mg. of the inhibitor. Their body temperature was measured at regular intervals before and after the injection of the substances. All animals showed an increase of body temperature of about 1-2° within 12 hr. They were killed 20 hr. after the injection, and mitochondria were isolated from their organs for the measurement of oxidative phosphorylation. Table 2 shows that all injected substances produced uncoupling of oxidative phosphorylation in mitochondria of liver, heart, kidney and skeletal muscle. P/O quotients very close to normal were, however, obtained with mitochondria isolated from brain.

Mitochondria isolated either with 0.88M or with 0.25M sucrose from organs in which uncoupling of oxidative phosphorylation had occurred exhibited a spherical form and appeared to be swollen in comparison with mitochondria from normal organs. It is clear from these experiments that the substances used are active also *in vivo*. As they produced fever, the hypothesis may be advanced that some relationship exists between the uncoupling of oxidative phosphorylation and the fever.

Production of fatty infiltration in the liver on treatment with inhibitors of oxidative phosphorylation

As uncoupling of oxidative phosphorylation occurs in fatty livers (Dianzani, 1954), the effect of prolonged treatment with the inhibitors of this enzymic reaction on the total lipid content of the liver was studied. All substances were injected into albino rats or mice intraperitoneally, using 0.2 mg. daily for mice and 2 mg. for rats, six injections

Table 1. *Inhibition of oxidative phosphorylation by three different concentrations of the inhibitors*

30 μ moles of α -oxoglutarate were used as substrate. The results represent the mean values obtained in five experiments $\pm$ S.D.

Inhibitor	Source of mitochondria (rat tissue)	P:O ratios without inhibitor added	Decrease of P:O ratios in the presence of the inhibitors (%)		
			Concn. of inhibitor (M)		
			0.1 $\times 10^{-4}$	0.5 $\times 10^{-4}$	10 $^{-4}$
DNP	Liver	3.9 $\pm$ 0.2	50.5 $\pm$ 4.6	81.6 $\pm$ 4.0	96.3 $\pm$ 2.4
	Kidney	3.6 $\pm$ 0.1	43.7 $\pm$ 4.6	73.9 $\pm$ 9.3	98.2 $\pm$ 1.0
	Heart	3.5 $\pm$ 0.2	36.4 $\pm$ 7.2	86.3 $\pm$ 3.0	96.7 $\pm$ 1.4
	Muscle	3.2 $\pm$ 0.1	94.0 $\pm$ 3.6	100	100
	Brain	3.3 $\pm$ 0.1	43.5 $\pm$ 6.5	63.1 $\pm$ 0.9	91.9 $\pm$ 5.3
PS	Liver	3.9 $\pm$ 0.1	73.6 $\pm$ 7.1	83.6 $\pm$ 2.8	98.1 $\pm$ 0.6
	Kidney	3.6 $\pm$ 0.1	64.0 $\pm$ 8.0	81.5 $\pm$ 2.4	94.6 $\pm$ 2.4
	Heart	3.4 $\pm$ 0.2	45.4 $\pm$ 5.8	63.8 $\pm$ 11.1	100
	Muscle	3.2 $\pm$ 0.3	30.5 $\pm$ 5.6	68.2 $\pm$ 11.5	93.0 $\pm$ 4.0
	Brain	3.5 $\pm$ 0.4	36.4 $\pm$ 4.6	72.5 $\pm$ 8.2	96.4 $\pm$ 0.6
BCB	Liver	3.9 $\pm$ 0.2	48.8 $\pm$ 3.6	66.6 $\pm$ 5.4	82.8 $\pm$ 6.8
	Kidney	3.7 $\pm$ 0.1	56.2 $\pm$ 4.5	70.7 $\pm$ 4.6	85.0 $\pm$ 6.4
	Heart	3.3 $\pm$ 0.1	38.6 $\pm$ 0.9	68.1 $\pm$ 4.4	83.3 $\pm$ 5.0
	Muscle	3.4 $\pm$ 0.2	29.6 $\pm$ 8.4	52.0 $\pm$ 4.0	66.3 $\pm$ 5.9
	Brain	3.5 $\pm$ 0.4	56.8 $\pm$ 4.7	72.8 $\pm$ 5.0	92.0 $\pm$ 6.1
Th	Liver	3.7 $\pm$ 0.1	88.9 $\pm$ 6.5	92.9 $\pm$ 6.4	98.5 $\pm$ 3.7
	Kidney	3.5 $\pm$ 0.1	78.8 $\pm$ 7.5	86.9 $\pm$ 7.5	91.5 $\pm$ 6.0
	Heart	3.6 $\pm$ 0.2	64.6 $\pm$ 5.9	72.0 $\pm$ 5.8	100
	Muscle	3.4 $\pm$ 0.2	24.6 $\pm$ 9.4	54.0 $\pm$ 6.2	77.1 $\pm$ 11.4
	Brain	3.6 $\pm$ 0.2	56.4 $\pm$ 0.2	58.6 $\pm$ 4.5	80.4 $\pm$ 5.0
JG	Liver	3.7 $\pm$ 0.2	74.5 $\pm$ 8.9	80.1 $\pm$ 3.5	98.9 $\pm$ 1.0
	Kidney	3.8 $\pm$ 0.3	73.0 $\pm$ 2.0	79.9 $\pm$ 3.0	96.3 $\pm$ 4.5
	Heart	3.5 $\pm$ 0.2	63.0 $\pm$ 4.0	75.7 $\pm$ 5.0	91.1 $\pm$ 4.6
	Muscle	3.5 $\pm$ 0.4	43.9 $\pm$ 6.4	69.3 $\pm$ 5.0	97.1 $\pm$ 2.0
	Brain	3.6 $\pm$ 0.2	47.5 $\pm$ 5.0	60.9 $\pm$ 4.2	84.7 $\pm$ 5.0
MB	Liver	3.9 $\pm$ 0.1	50.3 $\pm$ 2.9	60.7 $\pm$ 5.2	88.8 $\pm$ 3.4
	Kidney	3.7 $\pm$ 0.3	31.7 $\pm$ 1.6	46.6 $\pm$ 2.0	66.1 $\pm$ 4.6
	Heart	3.7 $\pm$ 0.1	10.4 $\pm$ 2.0	35.6 $\pm$ 1.9	47.3 $\pm$ 3.0
	Muscle	3.7 $\pm$ 0.2	5.8 $\pm$ 2.0	32.8 $\pm$ 2.0	47.9 $\pm$ 2.0
	Brain	3.6 $\pm$ 0.2	34.6 $\pm$ 2.6	51.9 $\pm$ 2.2	68.9 $\pm$ 2.4

Table 2. *P:O ratios in mitochondria isolated from different organs of rats injected with inhibitors of oxidative phosphorylation*

The amount of the inhibitor injected intraperitoneally was 3 mg. The results represent the mean P:O values $\pm$ S.D.

Inhibitor	No. of expts.	P:O ratios in mitochondria isolated from				
		Liver	Kidney	Heart	Skeletal muscle	Brain
DNP	6	0.65 $\pm$ 0.16	1.95 $\pm$ 0.10	1.78 $\pm$ 0.48	0.28 $\pm$ 0.20	3.1 $\pm$ 0.45
PS	3	1.20 $\pm$ 0.34	1.10 $\pm$ 0.26	1.23 $\pm$ 0.30	1.40 $\pm$ 0.52	2.70 $\pm$ 0.15
BCB	3	0.96 $\pm$ 0.15	1.80 $\pm$ 0.34	1.56 $\pm$ 0.27	0.73 $\pm$ 0.11	2.93 $\pm$ 0.08
Th	3	0.90 $\pm$ 0.1	1.33 $\pm$ 0.22	1.00 $\pm$ 0.44	1.26 $\pm$ 0.30	3.00 $\pm$ 0.34
JG	3	1.46 $\pm$ 0.32	2.00 $\pm$ 0.10	1.86 $\pm$ 0.41	1.56 $\pm$ 0.06	3.00 $\pm$ 0.26
MB	3	0.77 $\pm$ 0.05	1.73 $\pm$ 0.37	1.20 $\pm$ 0.29	0.73 $\pm$ 0.20	2.70 $\pm$ 0.34

being made in all. The animals were killed 24 hr. after the last injection. Table 3 shows that the total lipid content of the livers of treated animals is markedly increased. Those treated with DNP form the only exception, the total lipid content of their livers being very close to the normal value. Histological specimens stained with Sudan III showed a high degree of fatty infiltration of the

Table 3. Total lipid content of the liver of mice treated intraperitoneally with inhibitors of oxidative phosphorylation

Inhibitor	No. of expts.	Total lipids (mg./g. of fresh liver)
None	6	29.0±2.2
DNP	4	36.0±2.2
PS	5	68.0±3.6
BCB	4	58.0±3.4
Th	3	52.4±4.6
JG	3	48.6±2.6
MB	4	74.0±5.6

evident. In rats killed after 36 hr., glycogen was not present, but small droplets of fat became evident within the liver cells. The number of fat droplets was very significant in the liver cells of rats killed after 48–96 hr. Infiltration with glycogen became evident after 120 hr. and was substantial after 144 hr. At these stages the number of droplets of neutral fat decreased progressively. It is clear from this experiment that damage of lipid metabolism is present also in the cells of the liver of rats treated with DNP.

Mechanism of inhibition of oxidative phosphorylation produced by DNP and by other substances used

Two possible types of mechanism may have been responsible for the decreased uptake of inorganic orthophosphate from the reaction medium in oxidative phosphorylation: (i) a real decrease of the synthesis of ATP; (ii) an increased destruction

Table 4. Influence of DNP and other substances on the phosphatase activities of liver mitochondria of normal rats

The results represent mg. of inorganic orthophosphate liberated in 1 hr. by mitochondria isolated from 1 g. of liver, ±s.d. Concn. of the inhibitor, 10⁻⁴M.

Enzyme	No. of expts.	Inhibitor added						
		None	DNP	PS	BCB	Th	JG	MB
Acid phosphatase	5	2.71±0.02	3.93±0.72	2.66±0.60	3.23±0.20	2.59±0.80	1.84±0.20	2.57±0.40
Alkaline phosphatase	5	2.80±0.43	3.90±0.21	2.55±0.10	4.42±0.80	2.38±0.60	2.94±1.30	2.75±0.30
Inorganic pyrophosphatase	4	7.00±0.40	6.40±0.50	6.90±0.20	6.20±0.30	7.10±0.50	6.40±0.60	6.30±0.21
5'-Nucleotidase	4	2.68±0.31	3.65±0.60	2.34±0.27	3.32±0.17	2.72±0.60	2.50±0.18	2.70±0.81
ATPase	5	8.50±0.31	24.0±0.80	9.90±0.22	16.40±0.60	8.80±0.63	7.81±0.82	7.60±0.50

centrolobular type in all treated animals, with the exception of those treated with DNP. Fatty infiltration was already evident 36–48 hr. after the first injection. Rats treated with DNP showed a high degree of infiltration of glycogen rather than of fat. A very similar observation has been made by Barnes (1953) on the livers of rats treated with 2:5-dinitro-*o*-cresol and by Fonnesu & Severi (1955) on those of rats treated with DNP. As the reason for the different behaviour of DNP from that of other uncouplers was not clear, some experiments were carried out in order to discover the first stages of modification occurring in the liver cells after the injection of DNP. For this purpose, 2 mg. of DNP were injected into rats daily and the animals were killed 12, 24, 36, 48, 96 and 144 hr. after the first injection. Histological specimens were prepared from the livers and these were stained both with Sudan III and by the same method as that used by Best (1906) for glycogen. In the first 12–24 hr. only a slight degree of swelling was observed in liver cells, neither fat nor glycogen being morphologically

of normally formed ATP or of other phosphorylating intermediates. For this reason, the influences of all the inhibitors on ATPase, 5'-nucleotidase, inorganic pyrophosphatase and on both acid- and alkaline-phosphatase activities of mitochondria were studied. Particular attention was devoted to the study of liver mitochondria. The influence of the inhibitors was studied *in vitro* as well as *in vivo*. Table 4 shows that only DNP and BCB, in 10⁻⁴M concentration, produced any degree of stimulation of ATPase, 5'-nucleotidase and of both acid and alkaline phosphatases *in vitro*. Table 5 shows that all the inhibitors, when injected intraperitoneally in amounts of 2 mg. daily for 3 days, produced an increase of ATPase as well as of both acid and alkaline phosphatases. Since the liver of the animals contained an increased amount of fat in most cases, the possibility exists that the increase of enzymic activity results from the morphological modifications which occur in fatty livers (Dianzani, 1954; Berthet, Berthet, Appelmans & Duve, 1951).

The mechanism of ATPase stimulation produced by DNP has been extensively studied by Potter *et al.* (1953) and by Lardy & Wellman (1952). These authors reported that DNP is active on both fresh and pre-aged mitochondria. Pre-ageing consisted in incubating at 37° for 20 min. Lardy & Wellman (1952) observed that soluble ATPase was also stimulated by DNP.

Two types of mitochondrial preparation were used: the first was a suspension of liver mitochondria in 0.25 M sucrose; with the second the medium was the same, but the suspension was incubated at 28° for 30 min. It is known that ageing of mitochondria produces both damage to the membrane and activation of ATPase (Potter & Recknagel, 1951; Kielley & Kielley, 1951). In these experiments, the

Table 5. *Influence of intraperitoneal injection of DNP and of other substances on some phosphatase activities of rat-liver mitochondria*

The results are expressed similarly to those in Table 4. Into the rats was injected 2 mg. of DNP or other substance intraperitoneally for 3 days and the rats were killed 24 hr. after the last injection.

Enzyme	No. of expts.	Inhibitor injected						
		None	DNP	PS	BCB	Th	JG	MB
Acid phosphatase	5	2.71±0.02	3.28±0.09	3.36±0.12	3.29±0.11	3.00±0.11	3.20±0.10	3.94±0.04
Alkaline phosphatase	5	2.80±0.43	3.84±0.08	4.43±0.28	4.10±0.49	3.50±0.10	3.77±0.12	4.47±0.22
Inorganic pyrophosphatase	4	7.00±0.40	3.60±0.40	3.50±0.10	3.70±0.10	3.60±0.10	3.30±0.40	3.70±0.90
5'-Nucleotidase	4	2.68±0.30	2.90±0.01	2.80±0.02	3.20±0.03	3.00±0.02	3.10±0.03	2.89±0.30
ATPase	5	8.50±0.31	17.80±0.70	18.2±0.40	12.8±0.40	10.4±0.80	9.60±0.40	12.8±0.30

Table 6. *Effect of Mg²⁺, Ca²⁺, DNP and BCB on ATPase of rat-liver mitochondria*

Concn. of Mg²⁺ was 0.002 M, that of Ca²⁺ 0.001 M, those of DNP and of BCB were 3 × 10⁻⁵ M. The mitochondria used contained 0.06 mg. of N. The values are given as μmoles of inorganic orthophosphate liberated in 15 min. at 38°, ± S.D.

Substance added	Mitochondria	
	Fresh mitochondria	aged at 28° for 30 min.
None	0.25±0.02	0.65±0.03
Mg ²⁺	0.40±0.02	1.94±0.04
Ca ²⁺	0.51±0.04	0.80±0.02
Ca ²⁺ and Mg ²⁺	1.11±0.06	1.68±0.04
DNP	1.00±0.04	0.90±0.01
DNP and Mg ²⁺	0.90±0.03	1.84±0.02
DNP and Ca ²⁺	0.46±0.01	0.80±0.03
DNP, Ca ²⁺ and Mg ²⁺	0.80±0.02	1.60±0.05
BCB	0.80±0.02	0.90±0.02
BCB and Mg ²⁺	0.41±0.02	1.35±0.04
BCB and Ca ²⁺	0.98±0.03	0.82±0.02
BCB, Ca ²⁺ and Mg ²⁺	0.60±0.02	0.65±0.02
BCB, DNP, Ca ²⁺ and Mg ²⁺	0.66±0.01	0.90±0.03

In order to study the mechanism of the stimulation of ATPase which is produced by BCB, experiments were made in which the percentage activation was studied with regard to (i) the concentration of inhibitor, (ii) the concentration of enzyme, (iii) the concentration of stimulating cations (Mg²⁺ and Ca²⁺), (iv) the state of the mitochondrial membrane and (v) the behaviour of DNP under the same conditions. In a first group of experiments, the effect of BCB, DNP, Ca²⁺ and Mg²⁺ or of different mixtures of these substances on the ATPase activity of liver mitochondria was studied.

medium of Dubois & Potter (1943) was not used, in order to avoid as far as possible any osmotic damage to the mitochondria. 0.25 M Sucrose was used as a suspension medium in every case; all other added substances were also dissolved in 0.25 M sucrose. The reaction mixture contained 6 μmoles of ATP, 0.04 M-KCl, mitochondria from 10 mg. of liver, and, when present, 0.001 M-CaCl₂, 0.002 M-MgCl₂, 3 × 10⁻⁵ M DNP and 3 × 10⁻⁵ M BCB. Table 6 shows that the addition of Ca²⁺ or Mg²⁺ to fresh mitochondria produced marked activation of ATPase. When both cations were added at the same time, the degree of activation was markedly higher. This fact agrees with the results reported by other workers (Potter *et al.* 1953; Lardy & Wellman, 1952). DNP produced a marked activation when used alone, but the extent of the stimulation produced was decreased in the presence of Mg²⁺ or Ca²⁺. The same phenomenon occurred with BCB. Simultaneous addition of DNP and BCB did not increase the stimulation produced by DNP alone. The influence of the inhibitors on ATPase was very different when mitochondria were previously incubated at 28° for 30 min. ATPase activity of these mitochondria, when determined without any activator, was higher than the activity of fresh mitochondria. The effect of the addition of Mg²⁺ was very much higher, while that of Ca²⁺ was slight. The use of Ca²⁺ and Mg²⁺ together did not increase the stimulation produced by Mg²⁺ alone. Stimulations by DNP and by BCB were lower than those which occurred with fresh mitochondria, but were consistent in every case. Addition of DNP or BCB did

not increase the effect of Mg^{2+} or of Ca^{2+} . These results agree with those obtained for DNP, Ca^{2+} and Mg^{2+} by Lardy & Wellman (1952). It seems likely from these experiments that the stimulation of ATPase produced by these substances is not entirely a consequence of the modification of the form of mitochondria. Mg^{2+} , whose stimulating activity was maximal on aged mitochondria,

tions of fresh mitochondria from rat liver. The results are shown in Fig. 1. The activation produced under these conditions by DNP was proportional to the amount of enzyme used, when this did not exceed that corresponding to 0.18 mg. of N. With larger amounts of the enzyme, the stimulation produced by DNP was proportionally lower. In the case of BCB, the increase of ATPase activity

Fig. 1. Influence of the concentration of enzyme on the stimulation of ATPase activity by DNP, BCB, Mg^{2+} and Ca^{2+} . A, DNP; B, BCB. Mitochondria were isolated from rat liver. The concentration of DNP was $3 \times 10^{-5} M$, that of BCB $10^{-4} M$, and those of Ca^{2+} and Mg^{2+} 0.0075 M. The final volume was 3 ml.

Fig. 2. Influence of concentration of Mg^{2+} and Ca^{2+} on the stimulation of ATPase by DNP and BCB. A, aged mitochondria; B, non-aged mitochondria. The concentration of DNP was $3 \times 10^{-5} M$, that of BCB $10^{-4} M$. The N content of the mitochondria was 0.06 mg.

probably exerts its effect directly on the enzyme, while Ca^{2+} , which had only a slight influence on aged mitochondria, probably exerts its influence through a morphological modification of the particles. This was also the conclusion of Slater & Cleland (1953). The fact that Ca^{2+} and Mg^{2+} have a synergistic action on fresh mitochondria agrees with this interpretation.

In a second group of experiments, the influence of $3 \times 10^{-5} M$ DNP, $10^{-4} M$ BCB, 0.0075 M- Ca^{2+} or 0.0075 M- Mg^{2+} was studied on different concentra-

tions of fresh mitochondria from rat liver. The results are shown in Fig. 1. The stimulation produced under these conditions by DNP was proportional to the amount of enzyme used, when this did not exceed that corresponding to 0.18 mg. of N. With larger amounts of the enzyme, the stimulation produced by DNP was proportionally lower. In the case of BCB, the increase of ATPase activity

was smaller than that produced by DNP; when BCB was used together with Ca^{2+} , the stimulation of ATPase was proportionally higher for low concentrations of the enzyme than for high concentrations. The stimulation produced by Mg^{2+} alone was slight, but that found with the mixture of Ca^{2+} and Mg^{2+} increased progressively with the increase of the amount of the enzyme used.

In a third group of experiments, the influence of different concentrations of Ca^{2+} or Mg^{2+} , alone or in the presence of DNP or BCB, on ATPase activity

of both fresh and aged mitochondria was investigated. It is clear from Fig. 2 that the greatest stimulation was exerted, with fresh mitochondria, by $3 \times 10^{-3} \text{ M-Mg}^{2+}$. With aged mitochondria, the degree of stimulation increased up to a concentration of $7.5 \times 10^{-3} \text{ M}$. This fact agrees with the idea that Mg^{2+} acts directly on the enzyme. For Ca^{2+} , increase of concentration up to $3 \times 10^{-3} \text{ M}$ produced an increase of the activation of ATPase on fresh as

procedure of Lardy & Wellman (1952), was investigated (Table 7). It is clear from this table that Mg^{2+} produced activation of soluble ATPase, while Ca^{2+} was inactive. Both DNP and BCB exerted a strong stimulating activity. On the basis of these results, the action of Ca^{2+} seems to be mainly concerned with the damage of the mitochondrial membrane, whereas Mg^{2+} , DNP and BCB seem to act directly on the enzyme.

Fig. 3. Influence of concentration of BCB and DNP on the stimulation of ATPase activity of rat-liver mitochondria. A, aged mitochondria; B, non-aged mitochondria. The concentration of Mg^{2+} and of Ca^{2+} , when present, was 0.0075 M . All the other experimental conditions were as in Fig. 2.

Table 7. Influence of Ca^{2+} , Mg^{2+} , DNP and BCB on the soluble ATPase of rat-liver mitochondria

The results represent μmoles of inorganic orthophosphate liberated in 10 min. at 38° by an amount of enzyme containing 0.378 mg. of N. Concentration of ATP, 0.01 M .

Concn. of the substance tested (10^{-3} M)	Substance added			
	Ca^{2+}	Mg^{2+}	DNP	BCB
0	0.55	0.55	0.55	0.55
0.75	0.60	0.77	0.67	0.62
1.50	0.64	0.77	0.74	0.60
2.70	0.56	0.79	1.00	0.64
4.50	0.60	0.79	1.04	0.69

well as on aged mitochondria. The addition of BCB or of DNP decreased the effect of Ca^{2+} as well as that of Mg^{2+} . These results agree with similar observations made by Lardy & Wellman (1952) on DNP. Fig. 3 shows the influence of increasing concentrations of DNP and BCB in either the presence or the absence of 0.0075 M-Mg^{2+} , on ATPase activity of both fresh and aged mitochondria. $0.1 \times 10^{-4} \text{ M}$ DNP, which produced 60–70% inhibition of oxidative phosphorylation, strongly increased the ATPase activity of fresh mitochondria. In another group of experiments, the effect of DNP and of BCB on soluble ATPase, prepared from fresh mitochondria, of rat liver according to the

Myokinase

As the uncoupling of oxidative phosphorylation produced by the substances studied may be a consequence of a decrease in the formation of ADP through the myokinase reaction, the influence of these substances on myokinase was studied both *in vitro* and *in vivo*. Table 8 shows that, of all the substances used, only phenosafranine inhibits significantly myokinase activity of rat-liver mitochondria *in vitro* as well as *in vivo*. Since in the systems for

Table 8. Influence of DNP and of other substances on myokinase activity of rat-liver mitochondria

The results represent μmoles of ADP which disappeared from the reaction medium in 5 min. in the presence of the mitochondria isolated from 1 g. of liver. In the experiments *in vitro*, the concentration of the substance tested was 10^{-4} M . The results represent the mean of five experiments $\pm \text{s.d.}$

Substance added	Expts. <i>in vitro</i>	Expts. <i>in vivo</i>
None	22.2 ± 1.8	22.2 ± 1.8
DNP	24.3 ± 0.9	24.1 ± 1.3
PS	12.9 ± 1.7	16.4 ± 0.4
BCB	24.0 ± 0.9	22.5 ± 1.3
Th	19.5 ± 1.2	20.3 ± 0.7
JG	18.9 ± 0.4	21.6 ± 1.2
MB	23.4 ± 0.8	22.2 ± 1.9

the determination of myokinase activity an inhibition of ADP splitting may be due to damage of hexokinase or of adenylic deaminase as well as to a real inhibition of myokinase, the influence of all the substances on hexokinase and on adenylic deaminase was studied. The results obtained were, however, completely negative.

Analysis of phosphorylated intermediates

The results of analysis of the phosphorylated intermediates that accumulate in the reaction medium under the conditions of oxidative phosphorylation are reported in Table 9. Sodium L-glutamate (30 μ moles) was used as a substrate instead of α -oxoglutarate in these experiments. It was found that in the presence of DNP the amount of AMP which disappeared from the reaction medium was decreased by only 34 %, while P uptake decreased by about 87 %. The sum of ATP and ADP (acid-labile P) decreased by about 52 %, and the molar quotient acid-labile P/ribose decreased from 1.9 to 1.22. It is clear from these results that relative accumulation of ADP occurs in the reaction system. The mechanism of the uncoupling seems then to be related to inhibition of the reaction $\text{ADP} + \sim\text{P} \rightleftharpoons \text{ATP}$. Nearly the same type of modification in the distribution of phosphorylated intermediates occurred with MB, Th, JG and BCB. With PS the mechanism of inhibition of oxidative phosphorylation seems to be different from that occurring with other substances. In fact, the amount of AMP which disappeared from the reaction medium with PS was low, as also was the concentration of ADP. The molar quotient acid-labile P/ribose was 1.51. It appears probable then that with PS the inhibition of myokinase exerts a role in the inhibition of oxidative phosphorylation. It is also possible, however, that the synthesis of ATP from $\text{ADP} + \text{P}$ is also directly affected. In fact, in some experiments, in which ADP was used instead of AMP as phosphate acceptor for the measurement of oxidative phosphorylation, very low P:O ratios were obtained in the presence of PS.

DISCUSSION

The results obtained in the present investigation may be summarized as follows: (i) DNP, BCB, MB, Th, JG and PS, which uncouple oxidative phosphorylation *in vitro*, produce the same type of damage also *in vivo* of mitochondria isolated from liver, heart, kidney and skeletal muscle. The uncoupling of oxidative phosphorylation is in every case accompanied by the change of the form of mitochondria from rod-like to sphere-shaped and by consequent swelling. (ii) Prolonged treatment with many substances which uncouple oxidative phosphorylation produces a high degree of fat

Table 9. Analysis of phosphorylated intermediates contained in the reaction medium after 10 min. respiration in the system used for the determination of oxidative phosphorylation

30 μ moles of glutamate were added as a substrate. The concentration of the inhibitors, when added, was 3×10^{-4} M. The results refer to mitochondria isolated from 100 mg. of liver.											
Substance added	P disappearing (μ atoms)	O taken up (μ atoms)	P:O	P disappearing (μ g.)	Acid-labile P (μ g.)	Ribose (μ g.)	Acid-labile P/ribose	ATP formed (μ g.)	ADP formed (μ g.)	AMP disappearing (μ g.)	Fructose-6-phosphate formed (μ g.)
None	19.0	6.5	2.9	584	118	300	1.90	581	85	460	2500
DNP	2.4	7.0	0.34	75	57	225	1.22	69	321	300	102
PS	4.7	5.0	0.90	144	31	100	1.51	28	35	50	600
MB	9.4	6.0	1.56	288	65	280	1.13	20	176	160	1080
None	16.1	5.4	2.6	432	108	280	1.88	518	89	450	1980
Th	0.9	1.2	0.7	28	20	70	1.39	42	84	105	60
JG	1.2	1.4	0.8	36	26	100	1.21	30	141	160	80
BCB	1.4	1.0	1.4	43	36	120	1.45	88	136	165	60
None	18.6	7.0	2.65	576	125	335	1.80	545	172	510	1800
DNP	1.4	7.5	0.20	45	36	145	1.20	39	198	160	80
PS	1.3	5.6	0.23	40	31	100	1.51	93	105	145	70
MB	6.4	6.0	1.06	200	62	250	1.20	67	341	331	1020
None	15.8	6.0	2.63	490	102	265	1.87	489	132	430	2700
Th	1.2	3.0	0.40	37	24	80	1.45	59	91	120	114
JG	1.2	3.0	0.40	37	28	112	1.20	30	154	158	82
BCB	1.5	4.2	0.36	47	42	140	1.45	107	158	220	45

infiltration of centrolobular type in the liver. Of all the substances used, only DNP produces accumulation of glycogen in the liver. In the first few hours after the beginning of the treatment with DNP, a deposition of fat was, however, present in the liver cells, before the beginning of glycogen infiltration. (iii) DNP and BCB produce activation of latent ATPase activity in mitochondria previously aged at 28° for 30 min. The same substances also produce stimulation *in vitro* of 5'-nucleotidase and of both alkaline and acid phosphatases. All the substances produce stimulation of ATPase, 5'-nucleotidase and acid and alkaline phosphatases *in vivo*. (iv) The mechanism of the action of BCB seems to be substantially similar to that of DNP with regard to ATPase activation. The activity of DNP is, however, larger than that of BCB. Both substances produce stimulation of soluble ATPase as well as of ATPase bound to the structure of the mitochondria. This fact agrees with the hypothesis that the activation of ATPase produced by DNP and BCB is not primarily concerned with a modification of the form of the mitochondria. It seems probable, on the contrary, that the morphological damage of these cell particles is a result of the activation of ATPase and of loss of ATP. (v) Myokinase activity of liver mitochondria is not modified by any of the substances, with the exception of PS, which inhibits this enzyme *in vitro* as well as *in vivo*.

It seems likely from these experiments that the substances exert their action on oxidative phosphorylation through different mechanisms. DNP and BCB, which stimulate ATPase activity of aged mitochondria and also stimulate soluble ATPase, act probably by such a mechanism. In fact, it seems evident that at least for DNP and BCB the activation of ATPase and the loss of ATP precede the morphological damage to the mitochondria. It is doubtful, however, if the activation of ATPase is entirely responsible for the uncoupling of oxidative phosphorylation. In fact, in the reaction mixture used for the phosphorylation measurements, the presence of 0.013M-KF decreases the activity of ATPase. The hypothesis that the same enzyme produces both synthesis and destruction of ATP cannot be excluded; in this case the direction of the reaction would be reversed by DNP (Lardy & Wellman, 1952). The fact that MB, Th and JG, which, like DNP and BCB, uncouple oxidative phosphorylation, do not, however, strongly activate ATPase *in vitro*, indicates that the mechanism of action of these substances is probably different.

The mechanism of action of PS seems to be mainly concerned with the inhibition of myokinase, with consequent decrease of the amount of ADP available for the synthesis of ATP. The existence of

a direct damage also to the enzyme which synthesizes ATP from ADP + P cannot, however, be definitely excluded.

The fact that all the substances produced uncoupling of oxidative phosphorylation *in vivo* seems noteworthy. In fact, all the histological dyes used in this work, as well as DNP, are well-known fever-producing agents. The inhibition of oxidative phosphorylation was particularly clear in mitochondria from skeletal muscles. DNP was more active than the other substances in this respect. Mitochondria isolated from the organs of animals treated with DNP and with other uncouplers were spherical, and this form corresponds to a higher oxidative activity (Harman, 1950; Dianzani, 1953). In addition, Green & Stoner (1950) have established that the oxygen uptake of the tissues is markedly decreased in animals treated with ATP. The presence of metabolic and morphological damage in skeletal muscle in fever has been described by many authors (Vernoni, 1954). In view of these findings, the hypothesis that the morphological modifications of mitochondria, and especially of those contained in skeletal muscle, may have an important role in the pathogenesis of fever should be given full consideration.

All the substances used produced accumulation of fat within the liver cells. This fact favours the hypothesis that uncoupling of oxidative phosphorylation is an important step in the pathogenesis of fatty liver degeneration. It is, however, not yet clear whether the uncoupling of oxidative phosphorylation is itself directly responsible for the fatty infiltration through a decrease of the activation of fatty acids or whether its action is mediated through other mechanisms concerned with the modification of the form of the mitochondria. A redistribution of cytochrome c (Dianzani & Viti, 1955) and of pyridine nucleotides (Dianzani, 1955) occurs in homogenates prepared from fatty livers, and this is probably due to the increased permeability of morphologically modified mitochondria. Accumulation of glycogen in the liver resulting from treatment with 2,5-dinitro-*o*-cresol was reported by Barnes (1953). Fonnesu & Severi (1955) have reported accumulation of glycogen in the liver of rats treated with DNP. The mechanism of glycogen accumulation is difficult to understand. It is interesting, however, to remember that treatment of rats with ATP introduced intraperitoneally produces glycogen depletion in the liver (Green & Stoner, 1950). It is therefore probable that the accumulation of glycogen is due to a deficiency of ATP. The reason why DNP produces mainly glycolytic infiltration, while other uncouplers produce fatty infiltration in the liver, is not clear. The difference in behaviour may be perhaps related to the extent of damage produced in skeletal

muscle, which is particularly great with DNP. Further experiments on these problems are in progress.

SUMMARY

1. The influence of 2:4-dinitrophenol, methylene blue, brilliant cresyl blue, thionine, phenosafranine and Janus Green B on the oxidative phosphorylation of mitochondria isolated from liver, kidney, brain, heart and skeletal muscle of rats has been studied *in vitro* as well as *in vivo*.

2. All the substances used produced a high degree of inhibition of oxidative phosphorylation both *in vitro* and *in vivo*. In every case, uncoupling of oxidative phosphorylation was accompanied by a change of form of the mitochondria from rod-like to spherical and by swelling.

3. 2:4-Dinitrophenol and brilliant cresyl blue produce activation of adenosinetriphosphatase of liver mitochondria *in vitro*. 5'-Nucleotidase and both acid and alkaline phosphatases are also stimulated. All the substances used stimulated adenosinetriphosphatase, 5'-nucleotidase and both acid and alkaline phosphatases *in vivo*.

4. 2:4-Dinitrophenol and brilliant cresyl blue stimulate adenosinetriphosphatase activity of fresh as well as of aged mitochondria. Also soluble adenosinetriphosphatase is activated by these substances.

5. Phenosafranine strongly inhibits myokinase of liver mitochondria, both *in vitro* and *in vivo*.

6. Prolonged treatment of rats with the substances produces fatty infiltration of the liver. 2:4-Dinitrophenol produces glycogen accumulation instead of fatty infiltration.

7. The significance of the phenomena is discussed.

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Studies on the Metabolism of the Protozoa

7. COMPARATIVE CARBOHYDRATE METABOLISM OF ELEVEN SPECIES OF TRYPANOSOME*

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From the numerous papers on the carbohydrate metabolism of trypanosomes which have appeared during the past twenty years, it has become obvious that, with the possible exception of *Trypanosoma cruzi* (Brand, Tobie, Kissling &

Adams, 1949) and the plant trypanosome *Strigomonas oncopelti* (Ryley, 1955), cellular motility depends on a supply of extracellular monosaccharide, which is incompletely broken down to a mixture of organic acids; much of this work is admirably summarized by Brand (1951). Trypanosomes of the *brucei-evansi* group [for classification

* Part 6: Manners & Ryley (1955).