The Content of Adenosine Polyphosphates in Fatty Livers

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It has been reported in a previous paper (Dianzani, 1954) that uncoupling of oxidative phosphorylation occurs in fatty livers. Adenosine triphosphatase (ATPase) activity of mitochondria isolated from fatty livers is increased. Conversely, many substances which inhibit oxidative phosphorylation produce fatty infiltration of the liver when administered intraperitoneally to rats or mice (Dianzani & Scuro, 1956). A decrease of adenosine triphosphate (ATP) and of phosphocreatine has been shown to occur in many organs of rats treated with 3:5-dinitro-*o*-cresol, a substance which inhibits oxidative phosphorylation (Parker, 1954). From these facts one may expect that ATP concentration in fatty liver is decreased.

Only scanty indirect data are available in the literature on organophosphorus compounds in fatty liver. Flock, Bollman & Mann (1936*a*, *b*) found a decrease of inorganic orthophosphate in the liver of dogs fed on a hyperlipidic diet, but reported a normal distribution of organophosphorus compounds in the liver of dogs treated with carbon tetrachloride. Kaplan & Greenberg (1944) observed a decrease of high-energy phosphates in fatty livers of rats fed on a hyperlipidic diet. Wagtendonk (1944) and Wagtendonk & Lamfrom (1945) described a marked decrease of ATP and phosphocreatine in liver, kidneys and muscles of rats fed on a diet deficient in vitamin E. It is well known that this deficiency produces fatty infiltration and necrosis in the liver, as well as muscular dystrophy (Himsworth, 1952). Ennor & Stocken (1948), on the contrary, found in the liver of guinea pigs treated with carbon tetrachloride a marked increase of those acid-soluble organophosphorus compounds having insoluble barium salts. This fraction contains mainly ATP, adenosine diphosphate (ADP) and hexose diphosphate. The results of Ennor & Stocken were confirmed by Rowles (1952), who used the enzymic method of Rowles & Stocken (1950) for the determination of ATP. The discrepancy in these results may be perhaps attributed to the different methods that have been used. Since new specific methods are now available for estimation of ATP, the problem of the content of ATP in fatty livers has been again investigated, and the results are described here.

MATERIAL AND METHODS

Production of fatty livers. Albino rats from an inbred strain, weighing 120–150 g., were used in most experiments. Guinea pigs weighing 250–300 g. were used in some instances. The animals were fed on a standard diet. Fatty infiltration of the liver was obtained by three different procedures: (1) by daily subcutaneous injection of CCl_4 [a 20% (v/v) solution in olive oil, 0·2 ml. for rats, 0·4 ml. for guinea pigs]; (2) by daily subcutaneous injection of a 0·5% solution of white phosphorus in olive oil (0·1 ml. for rats, 0·3 ml. for guinea pigs); (3) by feeding rats on a diet deficient in choline. The composition of this diet has been described in another paper (Dianzani, 1955). A control group of rats fed on the same diet received a daily dose of 5 mg. of choline given subcutaneously.

Preparation of homogenates and extracts. The animals were killed by decapitation in the cold room, and their organs (liver, kidney, heart and leg muscle) were immediately dissected out and weighed as quickly as possible. Homogenates (10%, w/v) were prepared with 0·25 M sucrose in a Potter–Elvehjem apparatus provided with a Lucite (polymethylmethacrylate, du Pont de Nemours) pestle. Cold trichloroacetic acid [0·1 ml. of 40% (w/v)] was immediately added. The whole procedure lasted approx. 30 sec. In some experiments, the organs were frozen with compressed gaseous CO_2 and then ground in a mortar chilled at −20°. Homogenization was then carried out in the usual way. When it was important to suspend the tissues in very small amounts of fluid the homogenization was done in a small mortar.

When mitochondria were to be isolated, 10% (w/v) homogenates in 0·25 M sucrose were first centrifuged at 1000 *g* for 10 min. in a Serval SS 1-Type angle centrifuge (Ivan Sorvall Inc., New York) placed in the cold room. The sediment was discarded and the mitochondria were then sedimented by centrifuging at 12 000 *g* for 30 min. They were used without any other washing.

ATP estimation. Four different methods (*A*, *B*, *C* and *D*) were employed for ATP estimation. Methods *A* and *B* were used with liver, kidney, heart and skeletal muscle; methods *C* and *D* were used only with liver.

Method *A* was the chemical fractionation procedure for phosphorus compounds in protein-free extracts, as described by LePage (1951). Deproteinization of homogenates was made by adding trichloroacetic acid. The mixture was filtered after standing for 10 min. in the cold room at 2°. The filtrate was neutralized with N-NaOH and then analysed for total phosphorus, 'apparent' inorganic phosphorus and 'true' inorganic phosphorus. This last was determined by adding to the neutralized filtrate 0·2 vol. of 10% (w/v) $CaCl_2$ saturated with $Ca(OH)_2$ at pH 8·8 (LePage, 1951). The

difference between the values for 'apparent' inorganic phosphorus and for 'true' inorganic phosphorus was taken to be the phosphorus in phosphocreatine. Fractionation of organic phosphates was made with barium acetate at pH 8·2. The fraction giving insoluble barium salts was analysed for acid-labile P (increase of inorganic phosphate after hydrolysis with N-HCl at 100° for 8 min.) and for ribose and fructose. This fraction contains mainly ATP and ADP, as well as hexose diphosphate. As hexose diphosphate was found to be hydrolysed to the extent of about 20% in N-HCl at 100° for 8 min., the amount of phosphate released from hexose diphosphate in each experiment was calculated from fructose estimations. These were made according to Roe (1934). The values obtained were then subtracted from those for acid-labile P in order to obtain the data for P released from ATP plus ADP. The relative concentrations of ATP and ADP were deduced from the value of the molar quotient acid-labile P/ribose. Ribose was determined by the method of Meijbaum (1939). All phosphorus determinations were made by the method of Berenblum & Chain (1938).

Method *B* was the enzymic procedure developed by Holloway (1954), which was adapted for tissue extracts. The enzymic reaction involved in this method is the conversion of 3-phosphoglycerate into 1:3-diphosphoglycerate in the presence of ATP and of a phosphoglycerate kinase from green peas. When 3-phosphoglycerate and the enzyme are in excess, the amount of 1:3-diphosphoglycerate which is formed is a measure of the amount of ATP present in the reaction medium. 1:3-Diphosphoglycerate is estimated by the hydroxamic acid assay described by Lipmann & Tuttle (1945) with some modifications introduced by Holloway (1954). Phosphoglycerate kinase was prepared according to Holloway (1954). The reaction medium contained ATP (potassium salt) or tissue extract, 0·056 M potassium 3-phosphoglycerate, 0·4 ml. of hydroxylamine reagent, 0·3 ml. of phosphoglycerate kinase and water to 2 ml. Incubation time was 2 hr. at 38°. Hydroxylamine reagent is 2·5 M hydroxylamine hydrochloride solution, pH 7·3, containing 0·015 M-$MgCl_2$. The reaction was stopped by the addition of 3 ml. of $FeCl_3$ reagent and 2·5 ml. of ethanol. The $FeCl_3$ reagent contained 8·3 g. of $FeCl_3, 6H_2O$, 0·20 g. of trichloroacetic acid and 42 ml. of conc. HCl made up to 500 ml. with water. The reaction mixture was then centrifuged at 3000 *g* before the optical density at 490 mμ. was read. The accuracy of the method was previously tested with different known amounts of ATP. Plotting the optical density against ATP concentration gave a straight line. The accuracy of the estimation was found to be ±7%, within the range 0·2–2 μmoles of ATP. The reaction is specific for ATP. ADP, phosphocreatine and adenosine monophosphate, and different mixtures of these substances, gave negative results. As the sensitivity of the method is not high, large amounts of tissues must be used in order to prepare extracts for estimation of ATP. For this purpose, 1–2 g. of tissue was homogenized in a mortar with a small volume of water (3–4 ml.) containing 0·2 ml. of 40% (w/v) trichloroacetic acid. Precipitated proteins were discarded after centrifuging and the supernatant was neutralized with 30% (w/v) KOH. A portion (1 ml.) of the neutral fluid was used in each determination. Small amounts of substances giving positive colour reaction in the absence of the enzyme are present in tissue extracts, and a blank at zero time is therefore necessary. In some experiments, known amounts of ATP were added to the homogenate immediately before the addition of trichloroacetic acid, in order to study the extent of loss of ATP during the procedure. The recovery of added ATP was 95±6%.

Method *C* was the enzymic procedure recently described by Slater (1953). This method permits the separate estimation of hexose diphosphate, hexose monophosphate and high-energy phosphate (~P). It depends on the enzymic conversion of phosphorylated sugars into dihydroxyacetone phosphate, which then reacts with reduced diphosphopyridine nucleotide (DPN) in the presence of glycerol phosphate dehydrogenase. The amount of reduced DPN reacting is determined spectrophotometrically at 340 mμ. Slater's (1953) procedure *B* (*b*) measures the total ~P content of an extract, i.e. ATP, ADP, phosphocreatine and phosphopyruvate, as well as phosphoglycerate. Slater's procedure *C* measures only ATP, so that from the two measurements it is possible to calculate the amount of ADP plus phosphocreatine plus phosphopyruvate plus phosphoglycerate by difference. Since phosphopyruvate and phosphoglycerate concentrations in liver tissue are usually low (LePage, 1951; Sacks, 1950; Albaum, Tepperman & Bodansky, 1946), one may assume that these values are due mainly to ADP plus phosphocreatine. Rabbit-muscle fractions *A* and *B*, necessary for the method, were prepared as described by Slater (1953). Hexokinase was a Sigma type II preparation from yeast (Sigma Chemical Co., 4648 Easton Avenue, St Louis, Missouri). Reduced DPN was obtained according to Ohlmeyer (1938). Tissue extracts were prepared from 10% (w/v) homogenates by addition of 0·5 ml. of 10% (w/v) trichloroacetic acid. The high sensitivity of this method permits the estimation of 0·01–0·02 μmole of ATP with considerable accuracy.

Method *D* was the chromatographic procedure developed by Eggleston & Hems (1952).

Adenosine triphosphatase was determined according to the method of Dubois & Potter (1943). Their reaction medium, however, was made isotonic by preparing all the solutions in 0·25 M sucrose.

Spectrophotometric determinations were made in a Beckman DU spectrophotometer.

Nitrogen was determined by the usual micro-Kjeldahl technique.

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

Specimens for histological examination were prepared from each liver.

Reagents. ATP (sodium salt) of chromatographic-purity grade was obtained from Schwarz Lab. Inc., New York 17, N.Y. It gave only one spot in chromatograms. ATP (potassium salt) was prepared from the dibarium salt by solution of the latter in N-HCl, precipitation of barium with the calculated amount of H_2SO_4 and neutralization with 0·1 N-KOH. ADP (sodium salt) was prepared from the dibarium salt (Schwarz) by a similar procedure. Adenosine monophosphate (sodium salt) was prepared by neutralization of the acid (Schwarz). DPN of 90–95% purity was obtained from the Sigma Chemical Co. 3-Phosphoglycerate (potassium salt) was obtained from the dibarium salt (Sigma) by the same procedure as for the potassium salt of ATP. All other substances used were Merck (Darmstadt) products.

Table 1. *Content of some phosphorus fractions in liver, kidney, heart and skeletal muscle of normal rats and of rats with fatty infiltration of the liver*

Results are given as mg./g. wet wt. ± standard deviation.

Treatment	Organ	Total P	'True' inorganic P	Phospho-creatine P	Acid-labile P	Ribose in previous fraction	Molar quotient acid-labile P/ribose	Nitrogen	Total lipids
None (7 rats)	Liver	1·030±0·030	0·311±0·089	0·052±0·013	0·241±0·042	0·639±0·117	1·82±0·05	28·3±2·5	36·4±3·8
	Kidney	0·980±0·090	0·387±0·177	0·046±0·019	0·114±0·025	0·292±0·101	1·88±0·07	27·0±2·2	—
	Heart	1·050±0·071	0·578±0·173	0·052±0·011	0·115±0·018	0·297±0·039	1·87±0·09	27·6±1·9	—
	Muscle	1·290±0·268	0·751±0·168	0·049±0·019	0·206±0·026	0·554±0·073	1·80±0·03	27·1±0·8	—
One injection of CCl_4 (6 rats)	Liver	0·960±0·070	0·240±0·060	0·036±0·013	0·160±0·020	0·500±0·060	1·54±0·02	28·0±1·4	42·6±2·5
	Kidney	0·990±0·100	0·360±0·120	0·039±0·013	0·100±0·020	0·285±0·070	1·69±0·04	27·0±0·5	—
	Heart	1·060±0·020	0·575±0·090	0·048±0·010	0·120±0·020	0·300±0·040	1·93±0·15	26·7±1·8	—
	Muscle	1·296±0·220	0·750±0·120	0·040±0·020	0·210±0·030	0·556±0·070	1·82±0·10	26·1±0·7	—
Six injections of CCl_4 (6 rats)	Liver	0·954±0·077	0·245±0·053	0·024±0·008	0·121±0·051	0·470±0·216	1·25±0·11	25·0±1·2	76·8±6·9
	Kidney	0·976±0·085	0·326±0·060	0·026±0·018	0·078±0·016	0·242±0·036	1·55±0·12	26·8±0·5	—
	Heart	1·073±0·019	0·519±0·096	0·040±0·012	0·101±0·019	0·279±0·060	1 74±0·11	27·2±0·9	—
	Muscle	1·427±0·127	0·806±0·061	0·045±0·012	0·188±0·039	0·536±0·135	1·69±0·08	26·9±1·9	—
One injection of white phosphorus (6 rats)	Liver	0·960±0·070	0·240±0·060	0·036±0·013	0·160±0·020	0·500±0·060	1·54±0·02	28·0±1·4	—
	Kidney	0·990±0·100	0·360±0·120	0·039±0·013	0·100±0·020	0·285±0·070	1·69±0·04	27·0±0·5	—
	Heart	1·060±0·020	0·575±0·090	0·048±0·010	0·120±0·020	0·300±0·040	1·93±0·15	27·5±0·8	—
	Muscle	1·296±0·220	0·750±0·120	0·040±0·020	0·210±0·030	0·556±0·070	1·82±0·10	26·1±0·7	—
Three injections of white phosphorus (6 rats)	Liver	0·901±0·102	0·210±0·057	0·029±0·015	0·095±0·041	0·372±0·129	1·23±0·10	25·8±0·7	74·9±5·7
	Kidney	0·899±0·100	0·260±0·032	0·039±0·013	0·085±0·018	0·284±0·011	1·45±0·19	26·9±0·2	—
	Heart	1·079±0·078	0·473±0·083	0·045±0·009	0·093±0·014	0·261±0·043	1·73±0·05	26·9±1·9	—
	Muscle	1·499±0·074	0·734±0·066	0·052±0·014	0·225±0·059	0·642±0·183	1·70±0·19	26·8±0·1	—
Feeding on deficient diet for 1 week	Liver	0·935±0·080	0·210±0·057	0·022±0·020	0·126±0·020	0·390±0·050	1·54±0·10	29·0±0·8	46·9±1·2
	Kidney	0·930±0·104	0·260±0·032	0·038±0·015	0·116±0·025	0·315±0·027	1·78±0·15	28·0±1·2	—
	Heart	1·219±0·114	0·473±0·083	0·041±0·001	0·121±0·010	0·340±0·028	1·80±0·03	27·6±0·9	—
	Muscle	1·260±0·029	0·734±0·066	0·036±0·004	0·195±0·037	0·520±0·076	1·82±0·10	26·6±0·9	—
Feeding on deficient diet for 6 weeks	Liver	0·891±0·029	0·315±0·074	0·013±0·001	0·072±0·013	0·316±0·056	1·09±0·04	25·2±0·8	69·6±6·8
	Kidney	1·095±0·102	0·360±0·062	0·026±0·007	0·094±0·030	0·294±0·121	1·59±0·24	27·4±0·6	—
	Heart	1·246±0·088	0·678±0·030	0·042±0·007	0·141±0·012	0·386±0·037	1·78±0·15	28·0±1·7	—
	Muscle	1·483±0·145	0·864±0·163	0·043±0·015	0·185±0·031	0·515±0·070	1·75±0·15	26·7±0·5	—
Feeding on deficient diet + choline for 6 weeks (6 rats)	Liver	1·080±0·180	0·320±0·071	0·054±0·012	0·236±0·025	0·620±0·102	1·84±0·12	29·0±0·9	35·8±0·7
	Kidney	0·970±0·087	0·328±0·087	0·043±0·010	0·110±0·043	0·320±0·056	1·68±0·15	27·0±1·4	—
	Heart	1·250±0·123	0·570±0·076	0·045±0·012	0·100±0·030	0·270±0·018	1·73±0·14	28·0±0·8	—
	Muscle	1·470±0·200	0·700±0·098	0·046±0·010	0·230±0·060	0·600±0·120	1·85±0·13	26·8±1·2	—

Statistical evaluation of results. The values obtained were submitted to statistical analysis, the standard deviation and the 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

Adenosine triphosphate content of rat organs

The results of the investigation on some rat organs for ATP content are represented in Tables 1–3. Table 1 reports the results which were obtained by the method of chemical fractionation of phosphate compounds in tissue extracts (LePage, 1951). Table 2 reports results obtained by the enzymic procedure of Holloway (1954). Table 3 gives a comparative picture of the values obtained by four different methods for ATP and ADP in the liver of normal and of treated rats. It is interesting to note that the values for ATP content of normal organs found by these methods do not differ much, and that they lie within the range of the standard deviation. This proves the reliability of all the methods used. The values found for ATP agree with those reported by other workers (Wildman, Campbell & Bonner, 1949; Rapoport, 1945; Venkataraman, Venkataraman, Schulman & Greenberg, 1950; Kaplan & Greenberg, 1944; Albaum, Tepperman & Bodansky, 1946; Albaum, Pankin & Harvill, 1953; Proger, Decaneas & Schmidt, 1945). The values obtained for ADP were always lower than those found for ATP. The molar ratio ATP:ADP was nearly 3:1 in all tissues studied. This value agrees substantially with those found for rat liver by Albaum, Tepperman & Bodansky (1946), by Albaum, Pankin & Harvill (1953) and by Oliver (1953), but differs remarkably from those reported by LePage (1946) (1:17), by Ennor & Stocken (1948) (from 1:1 to 1:7), by Rapoport (1945) (1:2) and by Rowles (1952) (9:1).

It is clear from Tables 1–3 that there is a marked decrease of ATP in all the types of fatty livers studied, the decrease being shown by all four methods of estimation. Table 1 shows that phosphocreatine also is decreased in fatty livers. It was found by the chemical fractionation method that the decrease of acid-labile P was not proportional to that of ribose contained in the same fraction. Calculation of relative amounts of ATP and ADP present in the fraction with insoluble barium salts, on the basis of the molar quotient acid-labile P/ribose, showed that the decrease of acid-labile P in fatty livers is due mainly to loss of ATP, whereas the ADP concentration increases significantly. This was confirmed by the data obtained by method *C*, which is an enzymic one. The molar ratio ATP:ADP, which was about 3:1 in normal livers, attained the value 1:15 in the livers of rats fed for six weeks on a diet deficient in choline (method *A*).

Table 2. *ATP content of some organs from normal and from treated rats, as determined by method* B *(see text)*

Values are given as mg./g. wet wt. ±standard deviation.

Treatment	No. of expts.	Liver		Kidney		Heart		Skeletal muscle	
		Nitrogen	ATP	Nitrogen	ATP	Nitrogen	ATP	Nitrogen	ATP
None	10	28·3±0·7	0·922±0·122	26·3±0·5	0·456±0·091	27·4±4·8	0·454±0·092	24·7±0·8	0·743±0·114
One injection of CCl_4	6	26·4±0·6	0·600±0·065	26·5±0·3	0·470±0·035	27·0±2·3	0·500±0·060	26·0±0·5	0·820±0·120
Six injections of CCl_4	8	26·2±0·1	0·466±0·051	25·1±0·2	0·348±0·113	26·9±1·8	0·467±0·136	25·4±1·4	0·607±0·105
One injection of white phosphorus	6	26·4±0·6	0·620±0·070	26·0±0·1	0·420±0·060	27·0±1·6	0·490±0·030	27·8±0·3	0·800±0·125
Three injections of white phosphorus	11	25·8±1·8	0·393±0·218	27·1±2·0	0·602±0·322	26·5±2·0	0·608±0·099	25·9±1·6	0·600±0·264
Feeding on deficient diet for 1 week	6	26·9±2·1	0·670±0·124	28·0±1·5	0·396±0·082	28·0±1·2	0·550±0·094	27·0±1·1	0·910±0·052
Feeding on deficient diet for 5 weeks	7	24·3±3·6	0·240±0·114	27·4±1·4	0·296±0·123	26·8±1·4	0·608±0·058	27·7±1·9	0·914±0·228
Feeding on deficient diet for 5 weeks and treatment with choline	6	27·5±2·5	0·900±0·070	27·0±1·5	0·478±0·085	27·2±2·4	0·520±0·060	27·5±2·4	0·900±0·120

Table 3. *ATP and ADP content of the liver of normal rats and of rats with fatty infiltration of the liver. Comparison of the results obtained by four different methods*

Method *A* is the chemical procedure of LePage (1951). Data for ATP and ADP were calculated from those of acid-labile P and of the molar quotient acid-labile P/ribose. Method *B* is the enzymic method of Holloway (1954). Method *C* is the enzymic procedure of Slater (1953) and method *D* the chromatographic method of Eggleston & Hems (1952). The values are given as mg./g. of tissue (wet wt.) ± standard deviation. The values reported under ADP for method *C* are to be referred to ADP + phosphocreatine + phosphopyruvate + phosphoglycerate.

Treatment	Method	Nitrogen	Lipids	ATP	ADP	No. of expts.
None	*A*	28·3±2·5	36·4±3·8	1·077±0·192	0·298±0·107	7
	B	28·3±0·7	34·2±1·5	0·922±0·122	—	10
	C	28·6±1·4	36·1±4·8	0·917±0·196	0·289±0·045	7
	D	28·4±1·0	35·4±2·6	0·940±0·110	0·240±0·070	6
One injection of CCl_4	*A*	28·0±1·6	42·6±2·5	0·534±0·075	0·530±0·065	6
	B	26·4±0·6	40·4±5·6	0·600±0·065	—	6
Six injections of CCl_4	*A*	25·0±1·2	76·8±6·9	0·165±0·079	0·624±0·077	6
	B	26·2±0·1	70·4±4·9	0·466±0·051	—	8
	C	25·6±2·6	63·1±6·5	0·384±0·171	0·445±0·057	6
	D	25·0±3·6	75·6±8·9	0·430±0·160	0·450±0·120	6
One injection of white phosphorus	*A*	28·4±1·4	45·7±2·9	0·471±0·080	0·506±0·070	6
	B	26·4±0·6	40·4±5·6	0·620±0·070	—	6
Three injections of white phosphorus	*A*	25·8±0·7	74·9±5·7	0·119±0·113	0·503±0·149	6
	B	25·8±1·8	82·8±9·6	0·393±0·218	—	11
	D	24·0±1·5	79·8±9·2	0·380±0·150	0·500±0·090	6
Feeding on diet deficient in choline for 1 week	*A*	29·0±0·8	46·9±1·2	0·371±0·040	0·399±0·040	6
	B	26·9±2·1	40·9±3·6	0·670±0·124	—	6
	C	27·5±0·4	44·7±2·4	0·490±0·070	0·360±0·025	6
	D	27·0±2·5	46·5±4·8	0·560±0·128	0·320±0·040	6
Feeding on diet deficient in choline for 5–6 weeks	*A*	25·2±0·8	69·6±6·8	0·035±0·020	0·451±0·079	6
	B	24·3±3·6	75·6±4·8	0·240±0·114	—	6
	C	25·0±1·2	76·0±8·6	0·383±0·114	0·417±0·054	6
	D	24·2±2·6	63·6±4·5	0·159±0·068	0·560±0·052	6
Feeding on diet deficient in choline for 5–6 weeks and parenteral administration of choline	*A*	29·0±0·9	35·8±0·7	1·080±0·040	0·260±0·039	6
	B	27·5±2·5	34·2±1·9	0·900±0·070	—	6
	C	27·6±1·5	38·1±2·4	0·851±0·058	0·256±0·026	6
	D	29·6±2·4	39·5±4·6	0·920±0·060	0·265±0·040	6

The extent of the decrease of ATP was particularly great in the livers of rats receiving six injections of carbon tetrachloride, or three injections of white phosphorus, and of those fed for six weeks on the deficient diet. It was, however, noticeable also in the livers of rats fed on this diet for only one week, as well as in the livers of rats which received only one injection of carbon tetrachloride or phosphorus. Since the total-lipid content of the livers of these rats was not significantly increased, one may assume that depletion of ATP occurs before fatty infiltration. Nitrogen content of fatty livers was decreased, but to a considerably lesser extent than ATP, so that the decrease of ATP was significant also on a nitrogen-content basis.

ATP content of other tissues (kidney, heart and skeletal muscle) remained unaffected during the first stages of the treatments. After longer treatments, however, ATP decreased in the kidney, but it remained unaffected in both heart and skeletal muscle. Histological specimens of the kidneys in which ATP was decreased showed regressive processes such as cloudy swelling, tubular atrophy, haemorrhagic changes. No histological damage was found during the early stages of treatment, when ATP content was normal. This means that decrease of ATP is not a feature peculiar to fatty livers only, but probably occurs also in other regressive processes. In fact, decrease of P:O ratios was found also in cloudy swelling (Dianzani, 1954; Fonnesu & Severi, private communication). Also ~P was found to be decreased in the kidneys of guinea pigs treated with diphtheria toxin; here again histological examination showed cloudy swelling (Fonnesu & Severi, 1953).

ATP content of guinea-pig liver

The experiments of Ennor & Stocken (1948) and those of Rowles (1952), who found an increase of ~P in the liver after carbon tetrachloride poisoning, were made on guinea pigs. It seemed possible therefore that the discrepancy in the data found for rats during the present investigation, and those reported by these workers, could be referred to a species difference. The experiments were therefore repeated with guinea pigs. The results, which are given in Table 4, show that a decrease of ATP was found also in the liver of guinea pigs treated with

carbon tetrachloride or with white phosphorus. The extent of this decrease was, however, higher for rats than for guinea pigs.

Adenosine phosphates of isolated mitochondria

In some experiments, the adenosine phosphates of isolated mitochondria were determined in both normal and fatty livers, by the chromatographic method of estimation. The results, which are reported in Table 5, show that the percentage distribution of adenosine phosphates in liver mitochondria from normal rats resembles strongly that found by Bartley & Davies (1954) for sheep kidney-cortex mitochondria. These authors noted that the relative proportions of these compounds are much nearer the equilibrium of myokinase than those found in whole homogenate, and suggested that this was due to the presence of myokinase within mitochondria. It is clear from Table 5 that the amount of adenosine nucleotides is decreased in mitochondria from fatty livers. All three phosphates are decreased, but ATP is affected more than ADP.

Loss of adenosine phosphates from mitochondria after incubation in hypotonic medium

The decrease of adenosine phosphates in mitochondria from fatty livers could perhaps be due to their release into the surrounding medium. This was found to be the case for cytochrome *c* (Dianzani & Viti, 1955) and for pyridine nucleotides (Dianzani, 1955), and also for ATP (Blaschko, Born, D'Iorio & Eade, 1956) with ox adrenal-medullary granules submitted to hypotonic swelling. To test this possibility, mitochondria isolated from both normal and fatty livers were suspended either in 0·25 M sucrose or in water. Potassium fluoride (0·01 M) was added to the suspension medium in order to inhibit ATPase, preliminary experiments having shown that this concentration inhibits the enzyme to an extent of about 60%. The suspensions were allowed to stand at 28° for 10 min. and were then centrifuged at 12 000 *g* for 30 min. at 0°. The sediment was suspended in the same volume of water, and 0·1 ml. of 10% (w/v) trichloroacetic acid was added to both resuspended sediment and supernatant. The adenosine phosphates of the protein-free neutralized fluids were then determined by paper chromatography. It was found (Table 5) that a portion of the adenosine nucleotides is displaced from mitochondria into the surrounding medium as a consequence of incubation. With normal mitochondria, this portion was relatively small when the incubation medium was isotonic sucrose; it was, however, much higher when the incubation medium was water. With mitochondria isolated from fatty

Table 4. *ATP and ADP contents of the liver of normal guinea pigs and of guinea pigs with fatty infiltration of the liver*

Values are given as mg./g. wet wt. ± standard deviation. Method *A* is the chemical-fractionation procedure developed by LePage (1951). Method *D* is the chromatographic procedure.

Treatment	No. of expts.	Method	Nitrogen	Lipids	ATP	ADP
None	6	*A*	32·6 ± 2·5	30·6 ± 2·5	0·610 ± 0·045	0·193 ± 0·050
		D	32·4 ± 1·4	31·4 ± 2·7	0·635 ± 0·037	0·200 ± 0·050
Six injections of CCl_4	4	*A*	27·0 ± 2·4	62·5 ± 3·4	0·410 ± 0·050	0·315 ± 0·060
		D	26·6 ± 1·9	66·4 ± 7·8	0·395 ± 0·065	0·325 ± 0·135
Three injections of white phosphorus	4	*A*	27·0 ± 1·9	70·4 ± 5·6	0·360 ± 0·110	0·347 ± 0·100
		D	28·6 ± 2·5	79·4 ± 6·4	0·420 ± 0·080	0·315 ± 0·059

Table 5. *Release of adenosine nucleotides from mitochondria of normal and of fatty livers of rats after suspension in either isotonic or hypotonic medium*

Values are given as mg./10 g. of wet liver ± standard deviation. The chromatographic method was used and the number of the experiments was six in each case. Fatty infiltration of the liver was obtained by feeding rats on the diet deficient in choline for 4 weeks. AMP, adenosine monophosphate.

Source of mitochondria	Type of adenosine nucleotide	Nucleotide content before incubation	Nucleotide content after incubation in 0·25 M sucrose			Nucleotide content after incubation in water		
			Sediment	Supernatant	Recovery (%)	Sediment	Supernatant	Recovery (%)
Normal livers	ATP	0·800 ± 0·090	0·600 ± 0·070	0·100 ± 0·020	87·5	0·350 ± 0·090	0·200 ± 0·080	68·7
	ADP	0·980 ± 0·120	0·900 ± 0·060	0·160 ± 0·050	108·2	0·570 ± 0·065	0·560 ± 0·070	115·3
	AMP	1·300 ± 0·220	1·120 ± 0·140	0·200 ± 0·060	101·5	0·640 ± 0·110	0·690 ± 0·115	102·3
Fatty livers	ATP	0·180 ± 0·100	0·060 ± 0·050	0·060 ± 0·040	66·6	0·055 ± 0·010	0·060 ± 0·018	63·8
	ADP	0·520 ± 0·120	0·315 ± 0·068	0·290 ± 0·072	116·3	0·300 ± 0·075	0·310 ± 0·072	117·3
	AMP	0·650 ± 0·180	0·290 ± 0·075	0·340 ± 0·069	96·9	0·300 ± 0·090	0·345 ± 0·080	99·2

livers, the amount of adenosine nucleotides released into the surrounding medium was high, regardless of tonicity. Percentage recovery of ATP, calculated from the sum of ATP found in the sediment plus that found in the supernatant, was higher with normal mitochondria incubated with isotonic sucrose, than with the same mitochondria incubated with water. The reverse occurred for ADP. This means that a portion of ATP is destroyed through ATPase action during the incubation time. Here again, the change of incubation medium did not alter the behaviour of mitochondria from fatty liver.

DISCUSSION

This investigation shows that a marked decrease of ATP occurs in fatty livers of both rats and guinea pigs. Since the fatty livers have been produced by three different types of treatment, it seems evident that the decrease of ATP is a feature of this type of regressive process. Since the ATPase activity of fatty livers is increased, the question arises whether the loss of ATP occurs *in vivo*, or whether it is produced after the death of the animal during the preparation of homogenates. As this process lasted only about 30 sec., this second hypothesis seemed improbable. Some experiments were made, however, in which 0·25 M sucrose containing 0·01 M potassium fluoride was used for the preparation of the homogenates, in order to inhibit ATPase. Other samples from the liver of the same rats were homogenized in 0·25 M sucrose as usual. No difference was found between the values obtained for ATP and ADP either in the presence or in the absence of 0·01 M potassium fluoride. The hypothesis that loss of ATP occurs during homogenization as a consequence of ATPase activation is then highly improbable.

The results described in this paper with regard to ~P content in fatty livers agree with those of Kaplan & Greenberg (1944), who found decrease of these substances in the liver of rats fed on a hyperlipidic diet, and with those of Wagtendonk (1944), who found a marked decrease of ATP and phosphocreatine in fatty livers of rats fed on a diet deficient in vitamin E. They do not confirm, however, the results obtained by Ennor & Stocken (1948) and by Rowles (1952) on guinea pigs treated with carbon tetrachloride. In fact, Ennor & Stocken found an increase of acid-labile P, a fraction which contains mainly ATP, ADP and hexose diphosphate. The values for normal content of acid-labile P in guinea-pig extracts obtained by these workers, are, however, very low in comparison with those reported by other authors. Ennor & Stocken obtained for normal guinea pigs 5·3 mg./100 g. of wet liver. Fonnesu & Severi (1953) found 14·0 ± 1·5 mg./100 g. of wet liver; Wagtendonk (1944) obtained a figure of 14·2 ± 0·9. The values reported by this last author refer to 15 min. hydrolysis with N sulphuric acid at 100°, whereas those reported by Ennor & Stocken and by Fonnesu & Severi refer to 7 min. with N hydrochloric acid. It seems, however, improbable that this increase in the hydrolysis time is entirely responsible for the difference between the values of Wagtendonk and those of Ennor & Stocken. In fact, these authors stated that the release of inorganic orthophosphate P from the fraction with insoluble barium salts increases by only 2·0 mg./100 g. during the interval 7–15 min. Rowles (1952), who made enzymic determinations by the apyrase method of Rowles & Stocken (1950), reported a value of 3·3 mg./g. of liver nitrogen for the acid-labile P of adenosine polyphosphates of normal guinea pigs. The values for nitrogen content in the liver of these animals are not given. If one assumes, however, that the value found in the present investigation (32·6 mg./g.) is the normal nitrogen content, the values reported by Rowles correspond to 10·8 mg./100 g. of wet tissue, a value which is much higher than that obtained by Ennor & Stocken. A possible reason for the low values found by these authors may be found in their method of preparing extracts, which included freezing of the liver with liquid air before homogenization. Freezing is known to produce damage to mitochondria (Williams, 1952; Porter, Deming, Wright & Scott, 1953; Witter, Cottone & Stotz, 1954) as well as loss of soluble coenzymes from them. ATP requirements for choline and citrate oxidations are increased in these mitochondria and ATPase activity is also increased (Witter, Cottone & Stotz, 1954). Some ATPase estimations which were made during the present investigation confirmed this. The ATPase activity of mitochondria isolated from non-frozen liver was found to be able to release from ATP 7·55 ± 0·31 mg. of inorganic P/hr./g. of wet liver, and the value for mitochondria isolated from frozen liver was 13·6 ± 0·8. (The number of experiments was six for each group.) The fact that Ennor & Stocken obtained by the chemical method of

Table 6. *Influence of previous freezing on ATP and ADP content of normal guinea-pig liver*

Values are given as mg./g. of wet liver.

Expt. no.	Method	Non-frozen liver ATP	Non-frozen liver ADP	Frozen liver ATP	Frozen liver ADP
1	*A*	0·635	0·200	0·375	0·280
	D	0·660	0·210	0·390	0·290
2	*A*	0·595	0·215	0·360	0·275
	D	0·610	0·210	0·360	0·260
3	*A*	0·620	0·198	0·355	0·242
	D	0·640	0·205	0·345	0·250

estimation ATP:ADP ratios ranging from 1:1 to 1:7, and that the ratio was 3:1 in the present experiments, strongly supports this interpretation. In other experiments by Ennor & Stocken, in which the relative amount of ATP and ADP were calculated by the myosin ATPase method, ATP:ADP ratios ranged from 90:1 to 2:1.

In some experiments which were made during the present investigation, extracts were prepared from frozen livers, freezing being obtained very quickly with compressed gaseous carbon dioxide. The ATP content of normal guinea-pig liver was found to be about 40% lower by this method than by the procedure consisting of direct homogenization without previous freezing. ADP was higher under the same conditions (Table 6). Lower values for adenosine polyphosphates in frozen than in non-frozen livers were observed also by Rowles (1952). If this explanation for the low values reported by Ennor & Stocken is valid, one may perhaps explain also why these authors found higher acid-labile P levels in fatty than in normal livers. In fact, fatty livers contain much more ADP than the normal ones; they must then be affected by ATPase activation to a much lesser degree. The increase of ADP in fatty livers may be due either to increased rate of synthesis or to decreased disappearance. Since ATPase is increased and oxidative phosphorylation is inhibited, whereas the myokinase reaction is quite normal, it seems probable that all these phenomena contribute to the increase of ADP.

The decrease of ATP is particularly marked in mitochondria. Also ADP and adenosine monophosphate are decreased in these particles. One of the reasons for this decrease seems to be their displacement from the mitochondria into the surrounding medium. This was found to happen also with cytochrome *c* and the pyridine nucleotides. Loss of adenosine nucleotides was observed also with normal mitochondria suspended in water; it seems probable that it is concerned with swelling of mitochondria and with their increased permeability. Loss of soluble coenzymes and cofactors seems then to be a rather general feature of swollen mitochondria. In fatty livers, the decrease of these factors (ATP and DPN) seems to occur before the beginning of fatty infiltration; it is then probable that this phenomenon is responsible for accumulation of fat. In fact, ATP is necessary for the activation of fatty acid oxidation, and DPN is an obligatory hydrogen acceptor in the fatty acid cycle (Lipmann, Jones, Black & Flynn, 1952; Lynen & Ochoa (1953).

SUMMARY

1. The content of adenosine phosphates in liver, kidney, heart and skeletal muscle has been studied by four different methods in normal rats and in rats with fatty livers. The fatty infiltration of the liver was obtained by three different procedures (treatment with carbon tetrachloride; treatment with white phosphorus; feeding on a diet deficient in choline).

2. A marked decrease of adenosine triphosphate (ATP) was found in fatty livers by all four methods of estimation. Adenosine diphosphate (ADP) was increased.

3. Decrease of ATP and increase of ADP occurred in the livers of treated animals before the beginning of fatty infiltration.

4. ATP decreased slightly also in the kidneys of treated animals, but only after long treatment. Histological examination of these kidneys showed cloudy swelling, tubular atrophy, haemorrhagic changes. No change was found in the ATP content in the heart or skeletal muscle.

5. The concentration of adenosine phosphates was much decreased in mitochondria isolated from fatty livers. Mitochondria isolated from normal livers released more adenosine phosphates into the surrounding medium when they were suspended in water than when in isotonic sucrose. Mitochondria from fatty livers released about the same amount of adenosine phosphates both in hypotonic and in isotonic media.

6. Possible explanations of the experimental results are discussed.

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The Transformation of Purines into Pteridines

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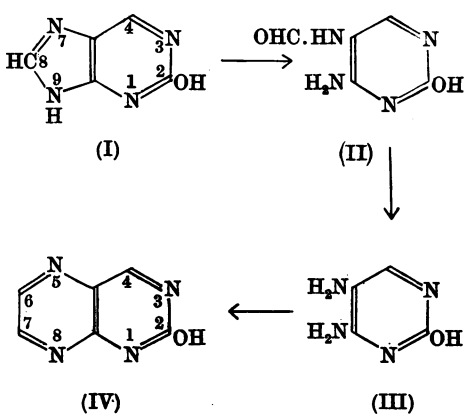
It has been demonstrated (Albert, 1954) that 2-hydroxypurine (I), incubated in water with glyoxal at 37°, gives 2-hydroxypteridine (IV), and that other purines can be converted into pteridines. It was suggested that this reaction has biological importance, and recent work has confirmed this (e.g. Ziegler-Günder, Simon & Wacker, 1956). Quantitative aspects of the chemical transformation are explored in the present communication.

MATERIALS

2-Hydroxypurine was obtained by the action of nitrous acid on 2-aminopurine (Albert & Brown, 1954), 2-hydroxy-9-methylpurine by the action of formic acid on 5-amino-2-hydroxy-4-methylaminopyrimidine (Brown & Mason, 1957) and 9-methylpurine by the action of formic acid on 5-amino-4-methylaminopyrimidine (Albert & Brown, 1954).



Hypoxanthine, xanthine and guanine were commercial preparations recrystallized to chromatographic purity and maximal absorption at $\lambda_{max.}$ (see Mason, 1954).

2-Hydroxy- (Albert, Brown & Cheeseman, 1951), 2:6-dihydroxy- (Albert, Lister & Pedersen, 1956), 4:6-dihydroxy- and 4:6:7-trihydroxy-pteridine (Albert & Brown, 1953) and xanthopterin (Albert & Wood, 1952) were made by standard methods. 6:7:8-Trimethyl-2-pteridone was kindly prepared by Dr D. J. Brown, from diacetyl and 5-amino-2-hydroxy-4-methylaminopyrimidine (Brown & Mason, 1957). Ethyl glyoxylate hemiacetal was prepared from ethyl tartrate (Rigby, 1950). Glyoxylic acid (crystalline) and glyoxal (50% syrup) were purchased. 4:5-Diamino-2-hydroxypyrimidine was prepared as by Johns (1911).

A Hilger Uvispek spectrophotometer with 4 cm. cells was used.

EXPERIMENTAL

2-Hydroxypurine to 2-hydroxypteridine. 2-Hydroxypurine monohydrate (0·46 g.; 0·003 mole), glyoxal (1·38 g. of 50% syrup; 0·012 mole) and water (6·5 ml.) were set aside at 37° for a week, then at 0° for 3 days (final pH was 2). The solid, recrystallized from 50 parts of boiling water (with charcoal), gave a yield of 16% of 2-hydroxypteridine monohydrate. This was chromatographically [on paper, in *n*-butanol–5N acetic acid (7:3), also in 3% (w/v) aqueous NH_4Cl] and spectrometrically identical with authentic 2-hydroxypteridine. (Found for material dried at 120°/0·1 mm.: C, 43·6; H, 3·8; N, 33·3. Calc. for $C_6H_4ON_4,H_2O$: C, 43·4; H, 3·6; N, 33·7%.)

From the same quantities of materials plus 5N-H_2SO_4 (1·8 ml.) set aside at 37° for 24 hr. (final pH, 0·5), 0·26 g. (52%) of pure 2-hydroxypteridine monohydrate was isolated; but 84% was demonstrated to have been formed, by spectrometric examination (pH 7) of the neutral molecule at 230 and 307 mμ. When the incubation was prolonged for a week, or when a higher temperature was used, the