# UNCOUPLING OF OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA FROM FATTY LIVERS*

by

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Mitochondria have been described by several authors as the site of oxidative phosphorylation within the cell (CROSS *et al.*, HUNTER AND HIXON, LEHNINGER AND SMITH, GREEN, LINDBERG AND ERNSTER, SLATER AND HOLTON, JUDAH AND WILLIAMS-ASHMAN and others[1-7]). Since oxidative phosphorylation is an aspect of the problem of the utilization of energy liberated by oxidative processes, it seemed very interesting to study the efficiency of this system in those cases in which the cell metabolism is pathologically modified. Fatty liver degeneration (steatosis), in which pathological accumulation of neutral fats occurs within the cells, is one of these cases.

Increase of $O_2$ uptake by liver slices during the earlier stages of liver steatosis has been described by many authors (MEIER AND THOENES[8], CALIFANO[9], ENNOR[10], BIAGINI[11]). This increase has been recently found to be related to the change in the form of mitochondria from rod- to sphere-shaped, which occurs in steatotic livers[12]. In fact, sphere-shaped mitochondria obtained by treatment with distilled water show in many cases higher oxidative activities than the normal ones (HARMAN[13], DIANZANI[14]). Uncoupling of oxidative phosphorylation has been reported by GÖRANSON AND ERULKAR[15], JUDAH AND WILLIAMS-ASHMAN[6] and HARMAN AND FEIGELSON[16] to occur in mitochondria as a consequence of osmotical damage. Owing to the parallelism which exists in many cases between normal mitochondria treated with distilled water and those isolated from steatotic livers, it seemed interesting to check this parallelism also with regard to oxidative phosphorylation. The results of such an investigation are described in this paper.

## MATERIAL AND METHODS

*Enzyme preparations.* Albino rats from a homogeneous strain, each weighing 150–170 g, and fed with a standard diet containing all vitamins and dietary factors, were used for this investigation.

Liver steatosis was obtained by subcutaneous injection of either carbon tetrachloride (0.2 ml of 20% olive oil solution daily) or of 0.5% phosphorated oil (0.1 ml per day). The animals were killed by dislocation of the head and the livers and the kidneys immediately removed, weighed and homogenized with a POTTER-ELVEHJEM[17] glass homogenizer in the cold room at +2° C. 10% homogenates were prepared either with distilled water, or with 0.25 *M* sucrose (isotonic for red cells), or with 0.88 *M* sucrose (hypertonic for red cells). Isolation of mitochondria was made by differential

* Following abbreviations were used in this paper: ATP = adenosine triphosphate; ADP = adenosine diphosphate; AMP = adenosine-5-monophosphate; PP = inorganic pyrophosphate; P = inorganic orthophosphate; CoA = Coenzyme A.

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centrifugation in a Serval Type SS-1 Angle Centrifuge (Ivan Sorvall Inc., New York) placed in the cold room. Undamaged cells, nuclei, tissue débris, red cells and some mitochondria were discarded by a first centrifugation at 1500 $\times g$ for 15 minutes. Mitochondria were then sedimented at 12000 $\times g$ for 30 minutes (with 0.25 *M* sucrose or water homogenates) or at 22,000 $\times$ *g* for 30 minutes (with 0.88 *M* sucrose homogenates). Washings were made by resuspension of the sediment and resedimentation. The microsome fraction was prepared only in some cases, by centrifugation at 22,000 $\times g$ for 2 hours of the 0.25 *M* sucrose homogenates. Final suspension of the mitochondria was made with the same fluid media used for the preparation of the homogenates, and the volume was that of the original homogenate.

Hexokinase was prepared from wheat germ according to the method of SALTMAN[18]. These preparations contained only a small adenosine triphosphatase and no myokinase activity. 0.3 ml of the enzyme suspension were used per flask. This amount was calculated to be largely in excess with respect to the amount of hexose added in the phosphorylation experiments.

Adenylic acid deaminase was the preparation A of KALCKAR[12] from rabbit muscle. 0.1 ml of this preparation were found able to destroy 38% of added adenylic acid in 5 minutes at 28° C, when the concentration of this substance was 540 $\mu$g/ml. This preparation was stored in the frozen state at —22° C for more than 1 month without apparent loss in activity.

*Estimations.* Inorganic orthophosphate phosphorus was determined by the method of FISKE AND SUBBAROW[20]; the colour was developed with 40% $SnCl_2$ in concentrated HCl. Absorption was measured at 730 m$\mu$ in a Beckman Mod. DU spectrophotometer. In the experiments in which inorganic pyrophosphate was the substrate, its concentration at the time of colour development was less than $10^{-4}$ *M*. This concentration is far below that which diminishes the amount of the colour developed with the Fiske-Subbarow method (NAGANNA *et al.*[21]).

$O_2$ uptake was measured manometrically, using Warburg flasks equipped with one side arm and with air as a gas phase.

P:O ratios ($\mu$atoms of inorganic orthophosphate phosphorus disappearing: $\mu$atoms $O_2$ taken up) were calculated by determining both $O_2$ and P uptake in a medium including the following substances: 0.02 *M* $Na_2HPO_4$–$KH_2PO_4$ buffer, pH 7.4, 0.001 *M* adenosine-5-phosphate, 0.0067 *M* Mg sulphate, 0.025 *M* KCl, 0.00001 *M* cytochrome *c*, 0.01 *M* substrate, 0.026 *M* fructose and 0.3 ml hexokinase. 0.2 ml of 30% KOH were placed in the central well for absorbing $CO_2$. 0.5 ml of the enzyme preparation were added immediately afterwards. 0.013 *M* KF was added in most experiments. 0.026 *M* glucose was used instead of fructose in some instances. Final volume in the principal compartment of the flasks was 3 ml. Substrates used were in most cases L(+)-glutamate, pyruvate, L(+)-ascorbate, $\alpha$-ketoglutarate, all added as sodium salts. With pyruvate as a substrate, 0.001 *M* succinate was used to prime the reaction. In the case of the oxidation of ascorbate, the concentration of cytochrome *c* used was 0.0001 *M*. A blank for autoxidation of ascorbate was included and the values subtracted. The solution of ascorbate was prepared immediately before use, the other solutions were conserved in the frozen state at —5° C. The temperature chosen for the experiments was 15° C in most cases, 25° C or 38° C in the others. After the 5 minutes required for temperature equilibration, the first reading was made. Oxygen uptake was measured during the next 10 minutes; after that, the reaction was stopped by addition of trichloroacetic acid from the side arm (0.5 ml of 90% solution). The contents of the flasks were made up to 5 ml with water. They were then filtered and samples of the filtrate taken for chemical estimations. A blank at zero time was made in every case. Another blank for endogenous $O_2$ uptake of the enzyme preparations was made with homogenates as enzyme material, but was found unnecessary with mitochondria. No inorganic orthophosphate phosphorus uptake was found to be associated with endogenous respiration.

In most cases the filtrate after deproteinization was analysed only for the disappearance of inorganic orthophosphate P. In a group of experiments, however, the other phosphorus fractions were also determined. The fractionation of the P compounds was made according to the procedure of LEPAGE[22], with barium acetate at pH 8.2 as a precipitating agent. The Ba-insoluble fraction was analysed for 7′ P, by determining the increase of inorganic orthophosphate P concentration after hydrolysis for 8 minutes at 100° C with 1 *N* HCl. Ribose was also determined on this fraction according to MEJBAUM[23]. The percentages of both adenosine triphosphate and adenosine diphosphate present in this fraction were then calculated from the molar ratio of 7′ P to ribose. The amount of ATP plus ADP in this fraction was also checked by measurement of the absorption at 260 m$\mu$ in the Beckman spectrophotometer. The Ba-soluble fraction was treated with 4 volumes of cold 95% ethanol. The precipitate was redissolved with 1 *N* HCl and barium removed with a slight excess of $H_2SO_4$; the $BaSO_4$ precipitate was washed with water and the washing fluid added to the first supernatant. The combined fluids were then analysed for the adenosine-5-phosphate and fructose-6-phosphate content.

Adenosine-5-phosphate was determined by measurement of the absorption at 260 m$\mu$ and by plotting the obtained values against a titration curve. Fructose-6-phosphate was determined by estimation of the fructose content of the fraction according to ROE[24]. Since it gives only 60.5% of the theoretical value in the fructose test (LEPAGE[22]), the actual data obtained were multiplied by the factor 2.39.

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Hexokinase activity was determined according to the procedure of SALTMAN[18]. Adenosine triphosphatase activity was determined according to the DUBOIS AND POTTER's method[23], at pH 6.9, with 0.067 *M* borate buffer.

Alkaline phosphatase activity was estimated as by NGUYEN-VAN THOAI *et al.*[26], at pH 9.2, with 0.2 *M* β-glycerophosphate as a substrate. 0.0067 *M* $MgSO_4$ was added as an activator of the enzyme.

Acid phosphatase was determined at pH 5.2 with 0.067 *M* veronal-HCl buffer and 0.2 *M* sodium β-glycerophosphate as a substrate.

Inorganic pyrophosphatase activity was determined at pH 7.4, with 0.067 *M* borate buffer and sodium pyrophosphate (0.01 *M*) as a substrate.

The hydrolysis of fructose-6-phosphate was measured with 0.01 *M* substrate in 0.067 *M* veronal-acetate buffer, pH 7.4. The temperature was 38° C for all the phosphatase determinations, and the amount of enzyme material used was 0.1 ml of a 10% homogenate or a 10% cytoplasmic fraction suspension.

Myokinase activity was determined according to KIELLEY AND KIELLEY[27], with incubation mixtures of the following composition: 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 adenylic acid deaminase, 0.1 ml of the liver fraction and water to 3 ml. The medium was preincubated at 27° C with hexokinase and deaminase for 10 minutes. The liver fraction was then added and the incubation at 27° C continued for 5 minutes. The reaction was stopped by the addition of 2 ml 40% trichloroacetic acid. Absorption at 260 mμ was then measured on neutralized samples after convenient dilution.

Adenylic acid deaminase was determined according to KALCKAR[19], by measuring the decrease of the optical density at 260 mμ, with 0.1 *M* sodium succinate buffer, pH 5.9, 0.001 *M* adenosine-5-phosphate as a substrate and 0.1 ml of the enzyme preparation. The incubation temperature was 27° C and the time of the experiment 5 minutes. After this time, the reaction was interrupted with trichloroacetic acid and the measurement of the optical density made as for the myokinase tests.

Glucose was determined according to the method of HAGEDORN AND JENSEN[28]. Nitrogen estimations were made by the usual micro Kjeldhal technique.

The total lipid contents of the liver were determined by weighing the dry material obtained from fresh liver by heating it in a stove at 100° C until constant weight, before and after extraction with ether for 4 hours in a Kumagawa-Suto glass apparatus.

*Reagents.* Sodium adenosine triphosphate was prepared from the dibarium salt received from Schwarz Laboratories. Sodium adenosine-5-phosphate was prepared in solution by neutralization of the acid (Schwarz Lab. Inc.) with 1 *N* NaOH. Both substances were chromatographically pure.

Sodium pyrophosphate and sodium β-glycerophosphate were Merck products. Sodium α-ketoglutarate was obtained from Hoffman-La Roche. Fructose-6-phosphate was prepared by acid hydrolysis of hexosediphosphate (barium salt, Schwarz Lab. Inc.) according to the method of NEUBERG, LUSTIG AND ROTHENBERG[29].

Cytochrome *c* was prepared from horse heart by the procedure of KEILIN AND HARTREE[30]. It contained from 0.37 to 0.41% Fe in various preparations, as found with the method of ELVEHJEM[31]. The other substances used were commercial preparations.

*Morphological examinations* were made with the Zeiss-Winkel phase contrast microscope. The presence of steatosis in the liver was checked also on histological specimens stained with Sudan III.

## EXPERIMENTAL AND RESULTS

### *Oxidative phosphorylation in normal tissues*

The oxidative phosphorylation has been studied in normal tissues from different species by many authors and their optimal conditions have been described. A detailed description of the preliminary work made for the present investigation is thus unnecessary. Some points need, however, to be discussed.

The amount of material used has been found to exert a marked influence on the values of P:O ratios. In fact, with small amounts of enzyme, lower ratios were obtained. The best results were obtained with enzyme material corresponding to 250 mg of fresh tissue (about 7 mg N for homogenates, 1.5–2 mg N for mitochondria), but 100 mg is also a suitable amount. In these conditions, P:O ratios very close to the theoretical values were obtained: 3 for the oxidation of glutamate and pyruvate, 4 for ketoglutarate and 1 for ascorbate. It has been shown that the temperature of the reaction plays an

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important role (HUNTER AND HIXON[2], JUDAH AND WILLIAMS-ASHMAN[7]). The determinations in the present work were made at 15°, at 25° and at 38° C.

It is evident from Table I that no practical difference exists between the values obtained at 15° C and those at 25° C. The values were, however, markedly lower at 38° C.

The importance of the addition of a trapping agent to the reaction mixture has been demonstrated by many authors (KIELLEY AND KIELLEY[27], JUDAH AND WILLIAMS-ASHMAN[7], LINDBERG AND ERNSTER[5], KREBS *et al.*[32]). All these workers have used hexokinase preparations from yeast. Since a hexokinase preparation from wheat germ was used in this work, its influence on P:O ratios has been checked, either with glucose or with fructose as phosphate acceptors. It was confirmed that the P:O ratios were lower when hexokinase plus hexose was omitted. No practical difference between glucose and fructose was found.

The influence of the addition of 0.013 *M* KF was also investigated. The P:O ratios were lower when fluoride was omitted.

In some experiments, the distribution of oxidative phosphorylation within the cell was studied. Only mitochondria were found capable of these processes; both microsomes and nuclei were devoid of oxidative phosphorylations capacities. Their addition to mitochondria did not influence the values of $O_2$ and P uptake.

*Uncoupling of oxidative phosphorylation by treatment with distilled water*

It has been shown that mitochondria undergo morphological changes and swelling when suspended in distilled water or in hypotonic solutions and that these changes are characterized by an increase in respiratory activity[13,14] and by an inhibition of phosphorylative processes[16]; conversely, substances active as inhibitors of oxidative phosphorylation (2,4-dinitrophenol, usnic acid, Janus Green B) produce change of the form of mitochondria from rod-like to sphere-shaped[16].

Some of the aspects of the relation between the form and the function of mitochondria have been the object of the present study. Homogenates were prepared from the same rat liver with both distilled water and 0.25 *M* sucrose. Washings and resuspensions necessary for the isolation of mitochondria were made with the fluid previously used for the preparation of the homogenates. Amounts of enzyme material corresponding to the same nitrogen content were used for the determinations of P:O ratios. Table II shows that almost complete inhibition of oxidative phosphorylation occurs in both homogenates and mitochondria as a consequence of water treatment. The P:O ratios obtained with water homogenates were, however, very close to the normal ones when an amount of enzyme corresponding to 250 mg of fresh tissue was used. The possible cause for this discrepancy is that these homogenates were prepared from 1.5 g of tissue, which were added to only 3 ml with water, 0.5 ml of this suspension being used for the experiment. The concentration of the material was then, probably, too high to permit the water to exert its influence. In fact, the phenomenon did not occur with mitochondria.

In some experiments, 0.88 *M* sucrose was used for the preparation of the homogenates. The main part of the mitochondria isolated from these homogenates was rod-shaped, approximatively 30% being spherical. The oxidative activities and P uptake of these mitochondria were about the same as for mitochondria isolated from 0.25 *M* homogenates, when studied with the same reaction medium.

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TABLE I

INFLUENCE OF THE TEMPERATURE ON P:O RATIOS OF NORMAL RAT LIVERS OR KIDNEYS

(The amount of enzyme material used was 250 mg in the case of liver, 100 mg in that of kidney. The homogenates were prepared with 0.25 *M* sucrose. Reaction mixtures as described in the text). ($\sigma$ means standard deviation).

| *Temperature* | *Enzyme from* | *Substrate* | *Number of experiments* | *μ Atoms P taken up* $\pm \sigma$ | *μ Atoms O taken up* $\pm \sigma$ | *P:O* |
|---|---|---|---|---|---|---|
| 15° C | liver | glutamate | 5 | 7.8 ± 1.1 | 2.6 ± 0.4 | 3.0 |
| | homogenate | ascorbate | 5 | 2.5 ± 0.47 | 3.2 ± 0.9 | 0.78 |
| | | pyruvate | 5 | 8.1 ± 2.3 | 2.8 ± 0.6 | 2.8 |
| | | α-ketoglutarate | 3 | 12.0 ± 2.2 | 3.0 ± 0.4 | 4.0 |
| 15° | liver | glutamate | 6 | 5.6 ± 1.4 | 1.85 ± 0.47 | 3.0 |
| | mitochondria | ascorbate | 4 | 2.4 ± 0.7 | 2.5 ± 0.8 | 0.9 |
| | | pyruvate | 5 | 6.0 ± 1.0 | 2.0 ± 0.6 | 3.0 |
| | | α-ketoglutarate | 3 | 9.4 ± 1.5 | 2.5 ± 0.5 | 3.7 |
| 15° | kidney | glutamate | 2 | 3.7 ± 0.1 | 1.3 ± 0.1 | 2.8 |
| | homogenate | ascorbate | 2 | 3.0 ± 0.1 | 3.2 ± 0.1 | 0.9 |
| | | pyruvate | 2 | 3.5 ± 0.2 | 1.2 ± 0.1 | 2.9 |
| | | α-ketoglutarate | 2 | 13.6 ± 0.2 | 3.2 ± 0.1 | 4.2 |
| 15° | kidney | glutamate | 2 | 2.5 ± 0.3 | 1.0 ± 0.2 | 2.5 |
| | mitochondria | ascorbate | 2 | 2.4 ± 0.1 | 2.4 ± 0.1 | 1.0 |
| | | pyruvate | 2 | 2.5 ± 0.5 | 0.9 ± 0.3 | 2.8 |
| | | α-ketoglutarate | 2 | 10.4 ± 0.8 | 2.5 ± 0.4 | 4.1 |
| 25° | liver | glutamate | 3 | 9.0 ± 0.4 | 3.0 ± 0.2 | 3.0 |
| | homogenate | ascorbate | 3 | 3.1 ± 0.3 | 3.1 ± 0.1 | 1.0 |
| | | pyruvate | 3 | 11.0 ± 1.8 | 3.6 ± 0.2 | 3.0 |
| | | α-ketoglutarate | 3 | 12.5 ± 0.4 | 3.4 ± 0.4 | 3.7 |
| 25° | liver | glutamate | 3 | 6.2 ± 1.2 | 2.4 ± 0.5 | 2.6 |
| | mitochondria | ascorbate | 3 | 1.7 ± 0.4 | 1.9 ± 0.2 | 0.9 |
| | | pyruvate | 3 | 6.4 ± 2.2 | 2.2 ± 0.4 | 2.9 |
| 25° | kidney | glutamate | 2 | 4.0 ± 0.2 | 1.5 ± 0.2 | 2.6 |
| | homogenate | ascorbate | 2 | 2.5 ± 0.2 | 2.4 ± 0.1 | 1.0 |
| | | pyruvate | 2 | 4.1 ± 0.2 | 1.5 ± 0.2 | 2.7 |
| | | α-ketoglutarate | 2 | 8.6 ± 1.0 | 2.2 ± 0.3 | 3.9 |
| 25° | kidney | glutamate | 2 | 2.6 ± 0.1 | 1.0 ± 0.1 | 2.6 |
| | mitochondria | ascorbate | 2 | 1.9 ± 0.1 | 2.2 ± 0.1 | 0.8 |
| | | pyruvate | 2 | 2.8 ± 0.2 | 1.0 ± 0.1 | 2.8 |
| 38° | liver | glutamate | 3 | 13.3 ± 1.7 | 7.4 ± 1.0 | 1.7 |
| | homogenate | ascorbate | 2 | 4.0 ± 0.4 | 10.0 ± 1.0 | 0.4 |
| | | pyruvate | 3 | 15.6 ± 2.0 | 7.2 ± 0.6 | 2.1 |
| | | α-ketoglutarate | 2 | 17.2 ± 2.8 | 8.1 ± 1.0 | 2.1 |
| 38° | liver | glutamate | 3 | 7.0 ± 0.9 | 5.4 ± 0.4 | 1.3 |
| | mitochondria | ascorbate | 2 | 3.8 ± 0.3 | 9.6 ± 0.2 | 0.4 |
| | | pyruvate | 2 | 5.1 ± 0.4 | 2.1 ± 0.2 | 2.4 |
| | | α-ketoglutarate | 2 | 14.0 ± 1.0 | 6.2 ± 0.6 | 2.2 |
| 38° | kidney | glutamate | 1 | 10.2 | 5.1 | 2.0 |
| | homogenate | ascorbate | 1 | 2.8 | 8.2 | 0.3 |
| | | pyruvate | 1 | 10.8 | 5.1 | 2.1 |
| | | α-ketoglutarate | 1 | 12.9 | 5.9 | 2.1 |
| 38° | kidney | glutamate | 1 | 3.9 | 2.0 | 1.9 |
| | mitochondria | ascorbate | 1 | 2.5 | 6.4 | 0.4 |
| | | pyruvate | 1 | 4.0 | 1.8 | 2.2 |
| | | α-ketoglutarate | 1 | 10.2 | 5.0 | 2.0 |

TABLE II

UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY DISTILLED WATER (TEMPERATURE 25° C)

(Reaction mixtures as described in the text)

| *Amount of material used as mg of wet liver* | *Number of experiments* | *Suspension medium* | *Substrate* | *Homogenates* | | | *Mitochondria* | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *μatoms P taken up* ± σ | *μatoms O taken up* ± σ | *P:O* | *μatoms P taken up* ± σ | *μatoms O taken up* ± σ | *P:O* |
| 100 | 3 | 0.25 *M* sucrose | glutamate | 6.3 ± 0.9 | 2.6 ± 0.4 | 2.4 | 3.1 ± 0.3 | 1.0 ± 0.1 | 3.1 |
| | | | ascorbate | 3.1 ± 0.2 | 3.5 ± 0.2 | 0.8 | 2.5 ± 0.2 | 3.3 ± 0.3 | 0.7 |
| | | | pyruvate | 6.6 ± 0.9 | 2.8 ± 0.4 | 2.3 | 3.3 ± 0.3 | 1.4 ± 0.07 | 2.3 |
| 100 | 3 | water | glutamate | 0.2 ± 0.1 | 2.8 ± 0.4 | 0.09 | 0.2 ± 0.4 | 1.1 ± 0.2 | 0.18 |
| | | | ascorbate | 0.1 ± 0.2 | 3.7 ± 0.1 | 0.03 | 0 | 3.4 ± 0.7 | 0 |
| | | | pyruvate | 0.2 ± 0.1 | 3.0 ± 0.4 | 0.07 | 0 | 1.7 ± 0.4 | 0 |
| 250 | 7 | 0.25 *M* sucrose | glutamate | 7.9 ± 0.9 | 2.7 ± 0.2 | 2.9 | 5.5 ± 0.7 | 1.9 ± 0.1 | 2.9 |
| | | | pyruvate | 9.2 ± 1.3 | 3.1 ± 0.4 | 2.9 | 5.3 ± 0.8 | 1.9 ± 0.2 | 2.7 |
| | | | ascorbate | 3.3 ± 0.4 | 3.6 ± 0.3 | 0.9 | 2.8 ± 0.2 | 3.1 ± 0.4 | 0.9 |
| 250 | 7 | water | glutamate | 6.6 ± 0.9 | 2.9 ± 0.1 | 2.2 | 0.5 ± 0.3 | 2.0 ± 0.3 | 0.25 |
| | | | ascorbate | 2.1 ± 0.2 | 3.9 ± 0.6 | 0.28 | 0.3 ± 0.3 | 3.2 ± 0.4 | 0.1 |
| | | | pyruvate | 7.3 ± 1.1 | 3.2 ± 0.1 | 2.2 | 0.2 ± 0.1 | 1.9 ± 0.2 | 0.1 |

It has been suggested by HARMAN AND FEIGELSON[16] that the degenerative changes produced by hypotonic solutions are provoked by uncoupling of oxidative phosphorylation in isolated mitochondria. The results obtained with 0.25 *M* sucrose solution, which is a hypotonic one, are, however, very satisfactory with regard to the intensity of P as well as of $O_2$ uptake, notwithstanding the form of mitochondria, which is almost exclusively spherical. Practically no difference was found between the results obtained with 0.25 *M* and those with 0.88 *M* sucrose, when the determination of P:O ratios was made as soon as the enzyme preparation was ready. It seemed, however, interesting to note the effect which was exerted by ageing on the phosphorylative ability of mitochondria suspended either in 0.25 *M* or in 0.88 *M* sucrose. Fig. 1 shows that the P:O ratios, which are practically the same when determined without ageing, fall down more

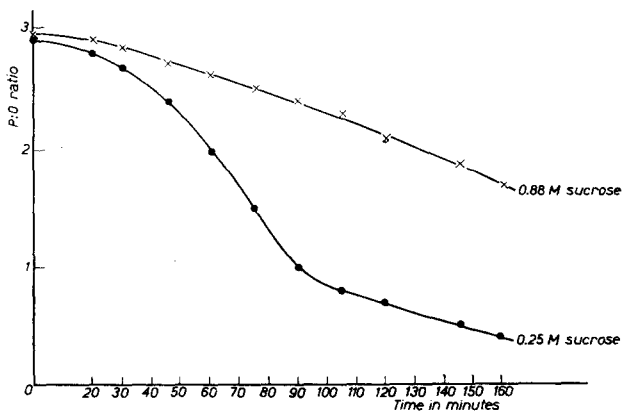


Fig. 1. Effect of ageing at 27° C on the P:O ratios of mitochondria from normal liver suspended either in 0.88 *M* or in 0.25 *M* sucrose. 30 *μM* sodium glutamate added as a substrate: Reaction mixture as indicated in the text. P:O ratios were determined at 25° C.

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rapidly for mitochondria suspended in 0.25 *M* than for those in 0.88 *M* sucrose. This fact agrees with the hypothesis that the extent of uncoupling of oxidations and phosphorylation is proportional to the extent of degenerative changes of mitochondria. The hypothesis of HARMAN AND FEIGELSON, who claimed that the morphological changes of mitochondria are a consequence of the loss of oxidative phosphorylation, is however contradicted by the fact that mitochondria isolated from 0.25 *M* sucrose, which are sphere-shaped, possess as high phosphorylative abilities as those isolated with 0.88 *M* sucrose, which are rod-like. Loss of phosphorylative abilities as a consequence of morphological degenerative processes seems then to be the more probable hypothesis.

*Uncoupling of oxidative phosphorylation in mitochondria from fatty livers*

Mitochondria from fatty livers undergo swelling *in vivo* (ZOLLINGER[33], DIANZANI[12]). They have a spherical form also when isolated from 0.88 *M* sucrose homogenates (DIANZANI[12], DIANZANI AND BAHR[34]). The succinoxidase activity is higher than that of normal mitochondria when studied in hypertonic media[12]. A strong parallelism exists between mitochondria from fatty livers and those with artificial osmotic changes. Table III shows that this parallelism exists also with regard to the oxidative phosphorylation. The extent of uncoupling is not as large as for mitochondria treated with distilled water, but it must be remembered that, especially in the livers from animals treated with $CCl_4$, regenerative processes were always present. This is evident particularly in animals treated for many days (20–30) with $CCl_4$. The histological picture of these livers was that of cyrrhosis with the presence of many regenerative areas. P:O ratios of these liver preparations were higher than those treated for a shorter time, in which regeneration was not important. Uncoupling was particularly striking in livers from animals treated with phosphorated oil. In all animals, a close parallelism existed between the decrease of P:O ratios and the increase of the lipid content of the liver. The effect of the suspension in distilled water of mitochondria isolated from fatty livers was investigated in some experiments. Complete inhibition of the residual P uptake was observed. Mitochondria from fatty livers were isolated also from homogenates prepared with 0.88 *M* sucrose, but no real difference from the values obtained with 0.25 *M* sucrose was observed. In some experiments, the behaviour of oxidative phosphorylation was studied also in the kidneys of treated rats. A decrease of P:O ratios was found also in these experiments. The histological picture of these kidneys was that of cloudy swelling, and fat was not morphologically present.

*Phosphatase activities of normal and steatotic livers*

The decrease of inorganic orthophosphate P uptake, which occurs in normal mitochondria treated with distilled water and in mitochondria from steatotic livers, may be due either to a real inhibition of the fixation of inorganic orthophosphate on the organic acceptor or to a strong increase of the hydrolysis of formed organic phosphates. The last possibility was supported by the observation of KIELLEY AND KIELLEY[27] and of POTTER *et al.*[35], who found an increase of adenosine triphosphatase activity as a consequence of ageing of mitochondria. DE DUVE, BERTHET *et al.*[36] obtained activation of mitochondria acid phosphatase on treating it with distilled water and ageing. The study of the behaviour of some phosphatase activities in water homogenates and mitochondria and in fatty livers was then taken up, in order to estimate the eventual influence of these enzymes on the decrease of P:O ratios. The steps for the fixation of inorganic

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TABLE III

UNCOUPLING OF OXIDATIVE PHOSPHORYLATION IN FATTY LIVERS

(The values were obtained with amounts of enzyme material corresponding to 250 mg of wet liver. Temperature 25° C)

| Exp. No. | Treatment with | Number of injections received | Total lipids in 1 g of wet liver in mg | Substrate | Homogenates | | P:O | Mitochondria | | P:O |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | μatoms P | μatoms O | | μatoms P | μatoms O | |
| 1 | $CCl_4$ | 1 | 59.8 | α-ketoglutarate | 5.2 | 3.5 | 1.5 | 2.4 | 2.0 | 1.2 |
| 2 | $CCl_4$ | 3 | 63.6 | glutamate | 5.4 | 3.3 | 1.6 | 2.2 | 2.8 | 0.7 |
| | | | | pyruvate | 2.9 | 2.6 | 1.1 | 0.8 | 1.0 | 0.8 |
| | | | | ascorbate | 1.1 | 3.1 | 0.4 | 0.6 | 2.4 | 0.2 |
| 3 | $CCl_4$ | 5 | 70.2 | glutamate | 0.6 | 3.0 | 0.2 | 0.4 | 2.1 | 0.2 |
| | | | | pyruvate | 0.6 | 2.1 | 0.3 | 0.4 | 1.2 | 0.3 |
| | | | | ascorbate | 0.3 | 3.1 | 0.1 | 0.3 | 3.0 | 0.1 |
| 4 | $CCl_4$ | 6 | 73.5 | glutamate | 2.4 | 4.7 | 0.5 | 1.5 | 2.2 | 0.7 |
| 5 | $CCl_4$ | 8 | 78.6 | glutamate | 1.9 | 2.7 | 0.7 | 0.4 | 2.1 | 0.2 |
| | | | | pyruvate | 2.0 | 2.9 | 0.7 | 0.6 | 1.9 | 0.3 |
| | | | | ascorbate | 0.7 | 3.7 | 0.2 | 0.2 | 1.9 | 0.1 |
| 6 | $CCl_4$ | 20 | 69.6 | glutamate | 15.7 | 7.6 | 2.1 | 4.4 | 2.2 | 2.0 |
| 7 | $CCl_4$ | 30 | 62.7 | glutamate | 4.6 | 4.4 | 1.1 | 2.0 | 2.8 | 0.7 |
| 8 | phosphorated oil | 1 | 49.0 | glutamate | 2.0 | 2.9 | 0.7 | 2.0 | 2.5 | 0.8 |
| | | | | pyruvate | 2.2 | 1.4 | 1.7 | 1.1 | 1.1 | 1.0 |
| | | | | ascorbate | 0.8 | 3.1 | 0.2 | 0.3 | 2.4 | 0.1 |
| 10 | phosphorated oil | 2 | 53.0 | glutamate | 3.8 | 2.9 | 1.3 | 1.6 | 2.0 | 0.8 |
| | | | | pyruvate | 2.2 | 1.4 | 1.7 | 1.1 | 1.1 | 1.0 |
| | | | | ascorbate | 1.2 | 2.7 | 0.4 | 1.2 | 2.5 | 0.5 |
| 9 | phosphorated oil | 1 | 56.8 | α-ketoglutarate | 6.8 | 3.8 | 1.7 | 2.4 | 3.7 | 0.6 |
| 11 | phosphorated oil | 2 | 52.0 | glutamate | 6.0 | 3.0 | 2.0 | 2.8 | 2.2 | 1.3 |
| 12 | phosphorated oil | 3 | 75.0 | glutamate | 1.1 | 2.7 | 0.4 | 1.1 | 2.0 | 0.5 |
| | | | | pyruvate | 1.1 | 1.4 | 0.7 | 0.9 | 1.2 | 0.7 |
| | | | | ascorbate | 0.3 | 2.9 | 0.1 | 0.24 | 2.2 | 0.1 |
| 13 | phosphorated oil | 3 | 62.4 | α-ketoglutarate | 5.8 | 3.4 | 1.7 | 1.9 | 2.7 | 0.7 |
| 14 | phosphorated oil | 3 | 68.0 | α-ketoglutarate | 2.1 | 2.4 | 0.8 | 2.1 | 2.1 | 1.0 |
| | | | | citrate | 2.0 | 2.0 | 1.0 | 2.0 | 1.8 | 1.1 |
| 15 | phosphorated oil | 4 | 70.0 | glutamate | 4.0 | 4.2 | 0.9 | 0.9 | 2.6 | 0.3 |
| 16 | phosphorated oil | 4 | 76.0 | α-ketoglutarate | 3.7 | 4.2 | 0.9 | 2.1 | 2.3 | 0.9 |

The amount of total lipid found in 1 g of wet liver from 21 normal rats was mg $45.7 \pm 6.5$, where $\pm 6.5$ means the standard deviation.

orthophosphate P are not completely known, but the main authors suggest that the first acceptor is ADP, which is transformed to ATP. In the presence of hexose plus hexokinase the phosphate is transferred from ATP onto the sugar. Inorganic pyrophosphate was shown to accumulate in the systems for oxidative phosphorylation where no trapping agent was present (CROSS *et al.*[1], KORNBERG AND LINDBERG[3,7], GREEN *et al.*[38]). Little is known about its mode of formation, but an eventual connection with the metabolism of adenylnucleotides has been suggested (GREEN[39], LINDBERG AND ERNSTER[5]).

*References p. 531/532.*

TABLE IV

OXIDATIVE PHOSPHORYLATION IN THE KIDNEY OF RATS SUBMITTED TO STEATOGEN TREATMENT

The values were obtained with 100 mg of wet tissue. The reaction mixture was that indicated in the text. Temperature 25° C

| *Exp. No.* | *Treatment with* | *Number of injections received* | *Total lipids in the liver mg/g* | *Substrate* | *Homogenates* | | *P:O* | *Mitochondria* | | *P:O* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *μ atoms P* | *μ atoms O* | | *μ atoms P* | *μ atoms O* | |
| 1 | phosphorated oil | 2 | 52.0 | glutamate | 4.9 | 1.8 | 2.1 | 3.2 | 1.6 | 2.0 |
| | | | | pyruvate | 3.8 | 2.0 | 1.9 | 3.2 | 1.8 | 1.7 |
| 2 | phosphorated oil | 3 | 68.0 | glutamate | 6.1 | 2.4 | 2.6 | 4.0 | 2.0 | 2.0 |
| | | | | pyruvate | 5.8 | 2.2 | 2.6 | 4.0 | 2.0 | 2.0 |
| | | | | α-ketoglutarate | 8.4 | 2.6 | 3.2 | 6.9 | 2.1 | 3.3 |
| 3 | $CCl_4$ | 6 | 73.4 | glutamate | 5.0 | 2.6 | 1.9 | 4.6 | 2.4 | 1.9 |
| | | | | pyruvate | 4.6 | 2.4 | 1.9 | 3.0 | 2.0 | 1.5 |
| 4 | $CCl_4$ | 8 | 78.6 | glutamate | 3.4 | 2.4 | 1.4 | 2.4 | 1.5 | 1.6 |
| | | | | pyruvate | 3.6 | 2.3 | 2.0 | 3.6 | 2.0 | 1.8 |

All the steps in this reaction chain which might be considered responsible for an eventual splitting of fixed phosphate have been investigated, *i.e.* adenosine triphosphatase for ATP, inorganic pyrophosphatase for PP, acid and alkaline phosphatases as generic phosphate-splitting enzymes, and hydrolysis of fructose-6-phosphate. Adenosine triphosphatase has been the object of particular attention. In fact, the observation by KIELLEY AND KIELLEY[27] mentioned above, that undamaged mitochondria, which show high P:O ratios, are practically devoid of ATPase activity, while an increase of ATPase with accompanying decrease of P:O ratios occurs as a result of ageing, show that a modification of ATPase can exert some influence on the P:O ratios. The question now arises if the increase of ATPase is directly responsible for the inefficiency of phosphorylative processes.

These observations by KIELLEY AND KIELLEY have been confirmed by LARDY AND WELLMAN[40] and by COPENHAVER AND LARDY[41]. Negative results have however been reported by CHRISTIE AND JUDAH[42] and by CHAPPELL AND PERRY[43] for heart and muscle mitochondria. The activity and the distribution of ATPase among the homogenate fractions of normal and fatty livers are given in Tables V and VI. One may see from Table V that ATPase activity of normal livers is about 40% higher with water homogenates than with those prepared with 0.25 *M* sucrose. In fatty livers, whose ATPase activity is only 10% higher than normal, acceleration by distilled water does not occur either in homogenates or in mitochondria. Mitochondria isolated with 0.25 *M* sucrose had nevertheless a good ATPase activity, which was about 45% of that of the unfractionated homogenate. Good distribution figures, similar to those reported by SCHNEIDER *et al.*[44] and by CHRISTIE AND JUDAH[42], were obtained. Distribution was not affected by homogenization in distilled water and was not modified in fatty livers.

Influence of ageing at 27° C on the homogenates and mitochondria ATPase activity was also investigated. Fig. 2 shows that ageing produces increase of mitochondrial ATPase activity only with 0.25 *M* sucrose homogenates. The maximal values were reached roughly after 105 minutes: the values at this time were about the same as

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TABLE V

HYDROLYSIS OF SOME PHOSPHATES BY LIVER HOMOGENATES AND MITOCHONDRIA FROM NORMAL RATS AND FROM RATS WITH LIVER STEATOSIS

(The values are given as mg P liberated in 1 h by enzyme preparations corresponding to 1 g of wet liver. Time of each experiment was 15 minutes and temperature 38° C. Liver steatosis was obtained either by 3 injections of phosphorated oil or by 6 injections of $CCl_4$. Homogenates were prepared with 0.25 *M* sucrose or with water.)

| *Substrate* | *Normal rats* | | | | | *Rats with liver steatosis* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Number of experiments* | *Homogenates* | | *Mitochondria* | | *Number of experiments* | *Homogenates* | | *Mitochondria* | |
| | | *in 0.25 M sucrose* | *in water* | *in 0.25 M sucrose* | *in water* | | *in 0.25 M sucrose* | *in water* | *in 0.25 M sucrose* | *in water* |
| Adenosine-triphosphate | 6 | 28.1 ± 2.0 | 39.2 ± 3.8 | 11.1 ± 0.8 | 15.9 ± 1.1 | 6 | 31.8 ± 4.5 | 32.9 ± 4.5 | 12.3 ± 3.2 | 14.3 ± 5.7 |
| Inorganic pyrophosphate | 5 | 33.1 ± 2.4 | 34.4 ± 3.2 | 7.79 ± 0.34 | 3.95 ± 0.32 | 5 | 31.1 ± 4.9 | 30.7 ± 4.65 | 3.83 ± 0.46 | 3.26 ± 0.7 |
| Fructose-6-monophosphate | 2 | 1.32 ± 0.1 | 1.6 ± 0.1 | 0 | 0 | 2 | 1.11 ± 0.26 | 1.15 ± 0.07 | 0 | 0 |
| β-glycero-phosphate, pH 5.2 | 3 | 2.18 ± 0.22 | 9.38 ± 0.41 | 1.36 ± 0.14 | 1.8 ± 0.17 | 3 | 8.45 ± 0.54 | 8.65 ± 0.6 | 3.66 ± 0.22 | 3.46 ± 01 |
| β-glycero-phosphate, pH 9.2 | 3 | 2.63 ± 0.09 | 5.55 ± 0.15 | 1.33 ± 0.09 | 1.01 ± 0.11 | 3 | 5.74 ± 0.08 | 5.6 ± 0.2 | 0.81 ± 0.05 | 8.1 ± 0.02 |

Total lipid content of 1 g liver was mg 42.9 ± 2.3 for normal rats; mg 68.6 ± 8.5 for rats with liver steatosis.

those obtained with non-aged mitochondria isolated from water homogenates. Increase of ATPase did not occur with "water" mitochondria, which, on the contrary, showed progressive inactivation. Ageing did not produce activation of ATPase either with homogenates prepared with 0.25 *M* sucrose or with distilled water. The enzyme activity, on the contrary, decreased as a consequence of incubation. It is not improbable that

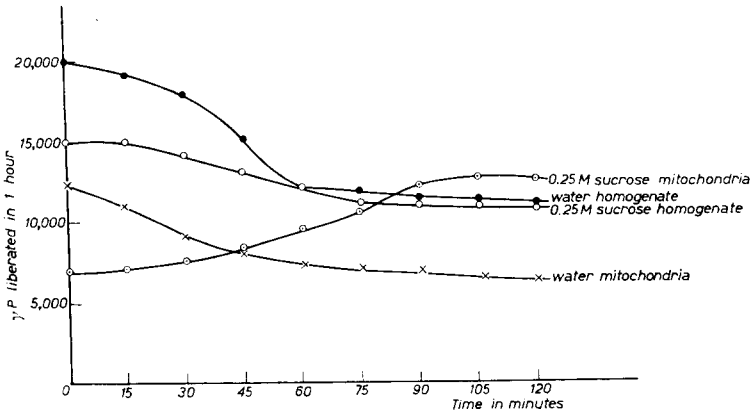


Fig. 2. Effect of ageing at 27° C on the adenosine triphosphatase activities of both homogenates and mitochondria from normal livers, either suspended in 0.25 *M* sucrose or in water. Reaction mixture as indicated in the text. Temperature of the determinations 38° C. The values are given as μg P liberated in 1 h by enzyme preparations corresponding to 0.5 g of fresh tissue.

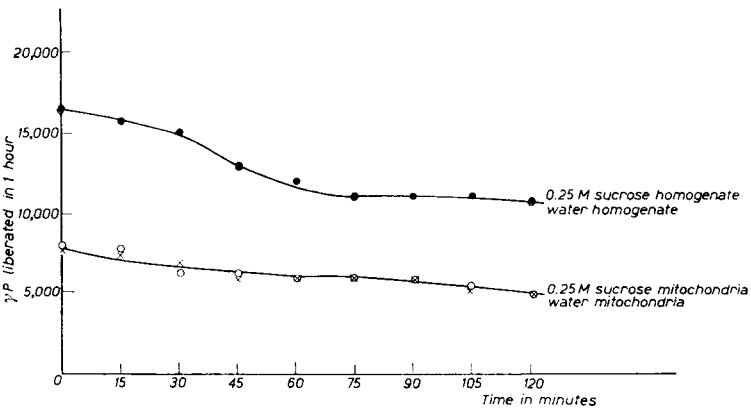


Fig. 3. Effect of ageing at 27° C on the adenosine triphosphatase activities of both homogenates and mitochondria from fatty liver, either suspended in 0.25 *M* sucrose or in distilled water. Reaction mixture as indicated in the text. The same conditions as described for Fig. 2. Liver steatosis was obtained by 3 injections of phosphorated oil. Total lipid content in the case shown in the figure was 63.6 mg per gram of wet liver.

this phenomenon is concerned with the activity of proteases which are present in the fluid part of the homogenates. Fig. 3 shows that ageing does not produce increase of ATPase activity of both water and 0.25 *M* sucrose mitochondria from fatty livers. Also in this case inactivation was observed with homogenates.

The more logical conclusion from these experiments is that the increase of ATPase activity produced by distilled water and that produced by ageing has a similar nature.

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Mitochondria from fatty livers, whose ATPase activity is not increased by ageing, behave as normal aged mitochondria. It therefore seems probable that the increase of ATPase in these conditions is due to the structural changes occurring within the mitochondria. It is, however, improbable that the increase of ATPase is entirely responsible for the decrease in P:O ratios; in fact, the extent of activation occurring with distilled water on ATPase is far below that of inactivation of oxidative phosphorylation.

TABLE VI

DISTRIBUTION OF SOME PHOSPHATASE ACTIVITIES IN CELL FRACTIONS OF NORMAL AND OF FATTY LIVERS OF RAT

(Mean values of 4–5 experiments are given. They are expressed as the percent of enzymic activity recovered in each fraction.)

| *Enzyme* | *Cell fractions* | *Normal livers* | | *Fatty livers* | |
|---|---|---|---|---|---|
| | | *0.25 M sucrose homogenates* | *water homogenates* | *0.25 M sucrose homogenates* | *water homogenates* |
| Adenosine-triphosphatase | Nuclear fraction | 20.0 | 19.7 | 25.3 | 25.3 |
| | Mitochondria | 48.7 | 52.4 | 43.8 | 43.8 |
| | Microsomes | 16.1 | 13.1 | 18.5 | 18.5 |
| | Supernatant | 15.2 | 14.8 | 12.4 | 12.4 |
| Inorganic pyrophosphatase | Nuclear fraction | 28.1 | 12.9 | 10.2 | 6.5 |
| | Mitochondria | 22.4 | 9.9 | 12.6 | 11.1 |
| | Microsomes | 20.6 | 5.9 | 8.2 | 7.2 |
| | Supernatant | 28.9 | 71.3 | 69.0 | 75.2 |
| Acid phosphatase | Nuclear fraction | 12.4 | 6.4 | 9.4 | 8.6 |
| | Mitochondria | 58.5 | 16.5 | 22.6 | 20.4 |
| | Microsomes | 3.5 | 4.0 | 4.2 | 4.5 |
| | Supernatant | 25.6 | 73.1 | 63.8 | 66.5 |
| Alcaline phosphatase | Nuclear fraction | 10.6 | 8.4 | 8.6 | 9.0 |
| | Mitochondria | 49.5 | 15.6 | 16.9 | 15.0 |
| | Microsomes | 6.4 | 4.2 | 4.8 | 4.1 |
| | Supernatant | 33.5 | 71.8 | 69.7 | 71.9 |

The results concerning the hydrolysis of inorganic pyrophosphate are shown in Tables V and VI. It is clear that pyrophosphatase activity of homogenates from fatty livers is not higher than that of normal ones. Water homogenates do not show higher activity than 0.25 *M* sucrose homogenates do. Some difference exists, however, with regard to pyrophosphatase activity of mitochondria. These possess only a weak enzyme activity, but the values obtained for mitochondria from fatty livers are lower than those obtained for normal ones. Also the values given by mitochondria isolated from water homogenates of normal livers are lower than those obtained for the same particles isolated from 0.25 *M* sucrose homogenates. With fatty livers, the results were practically the same either with mitochondria from water homogenates or with those from 0.25 *M* sucrose homogenates. The study of the distribution of pyrophosphatase within the cell gives an explanation of these results. In fact, in sucrose homogenates of normal livers 22% of the pyrophosphatase activity is associated with the mitochondrial fraction and about 30% is present in the supernatant, while only 10% of the enzyme is present in mitochondria in water homogenates, and the content of the supernatant is increased

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to about 70%. Since pyrophosphatase is a soluble enzyme, the difference of distribution is probably due to its extraction from particulate material which occurs after water treatment. A very similar phenomenon was described by WALKER[45] for liver mitochondria β-glucuronidase and by DE DUVE *et al.*[46,36] for acid phosphatase. SWANSON[47], who studied the pyrophosphatase distribution in liver homogenates prepared with 0.15 *M* KCl, found the main part of the enzyme in the soluble part of the cytoplasm.

Distribution of the enzyme in fatty livers resembles closely that of water homogenates from normal livers. It is clear that the difference of distribution is the reason for the decrease of pyrophosphatase activity found in mitochondria from both fatty and water-treated livers in the experiments mentioned above.

Effect of ageing on pyrophosphatase activity was also investigated, but no increase was found in both homogenates and mitochondria: on the contrary, a progressive inactivation occurred also in these experiments. No difference was noticed between normal and fatty livers in this respect.

Hydrolysis of fructose-6-phosphate was investigated for both homogenates and mitochondria. Homogenates had a very weak activity and mitochondria none. All the activity was present in the supernatant fluid. The hydrolysis activity was found to be slightly higher in water than in sucrose homogenates from normal liver, but no difference was detected between normal and steatotic livers. The slight increase produced by distilled water was not observed with homogenates from fatty livers.

Acid and alkaline phosphatase were studied as examples of aspecific phosphatases. Results very similar to those described by BERTHET *et al.*[36-46] were found for acid phosphatase. These workers have reported that the enzyme is strongly activated by ageing or by treatment with distilled water. This treatment produces the transfer of the enzyme, which is originally mitochondria-linked, to the supernatant fluid. A very similar phenomenon was found to occur also for alkaline phosphatase. The activities of both acid and alkaline phosphatases of fatty livers were originally high, and no further activation was produced by treatment with distilled water. Distribution figures of enzyme activities showed that only a very small part of the activity is mitochondria-linked in water homogenates, about 70% being present in the supernatant fluid. 0.25 *M* sucrose homogenates of normal livers show, on the contrary, that a large part of the activities is associated with mitochondria. 0.25 *M* sucrose homogenates of fatty livers show the same distribution as normal water homogenates.

The effect of ageing at 27° C was also studied. It was seen that alkaline phosphatase activity of mitochondria isolated from normal 0.25 *M* homogenates increases about 20% after 20 minutes standing at 27° C, whilst the activity of homogenates increases about 50%. Acid phosphatase increases about 30% for mitochondria and 60% for homogenates. No effect was determined by ageing on phosphatase activities of both homogenates and mitochondria from fatty livers.

From all these experiments on phosphatase activities concerned with the splitting of the substrates formed during the phosphorylative reaction, it may be concluded that, although some modifications are produced by distilled water and by steatosis, their effectivity as the sole agent of the decrease in P:O ratios is improbable. In fact, the reaction mixture for the determination of P:O ratios contained also 0.013 *M* KF, which is a powerful inhibitor of all tested phosphatase activities. It is clear from Table VII that the extent of the phosphatase activation mentioned before must be strongly diminished in the presence of KF.

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TABLE VII

INHIBITION OF SOME PHOSPHATASE ACTIVITIES BY 0.013 *M* KF

| *Enzyme* | *Inhibition %* |
|---|---|
| ATPase | 40 |
| Inorganic pyrophosphatase | 88 |
| Acid phosphatase | 70 |
| Alkaline phosphatase | 55 |
| Hydrolysis of fructose-6-phosphate | 65 |

*Analysis of the phosphorylative intermediates*

In some experiments phosphorylative intermediates were analysed in the deproteinized filtrate after the measurement of oxygen uptake, with α-ketoglutarate as a substrate. The disappearance of inorganic orthophosphate P, the formation of easily hydrolyzable P (7′ P, HCl 1 *N*, 100°), the ribose content of this fraction and the disappearance of adenylic acid and fructose-6-monophosphate formation were all investigated. The relative amounts of ADP and ATP present in the 7′ P fraction were calculated from the molar ratio 7′ P/ribose. Some of most representative experiments of this type are recorded in Table VIII. It appears from this table that in homogenates and mitochondria prepared with distilled water the decrease of 7′ P is not proportional to the decrease of inorganic orthophosphate uptake. It may be calculated from the 7′ P/ribose ratios that the amount of ATP present in this fraction is strongly decreased and that accumulation of ADP occurs. The decrease of the disappearance of AMP from the reaction mixture is also not proportional to the decrease of inorganic orthophosphate P uptake. Fructose-6-monophosphate, which accumulates in normal experiments as the end product of the reaction, is formed only in small amounts.

The results obtained with fatty livers preparations were very similar to those obtained with normal water treated preparations. The reason for the accumulation of ADP in water treated and "fatty" preparations has been investigated. ADP may be formed in the above system: 1. from ATP plus fructose in the presence of hexokinase; 2. from ATP plus AMP by the myokinase reaction. The first reaction cannot be held responsible for the accumulation of ADP, because of the strong decrease in the formation of fructose-6-monophosphate. Moreover, no influence of either "water" or "fatty" homogenates and mitochondria was found on the added hexokinase in controlled experiments. The myokinase activity of both normal and treated preparations was then investigated, in order to study the importance of case No. 2. Table IX shows that there is practically no difference in myokinase activity between normal and treated livers. This means that ADP could not have been accumulated as a consequence of an increase in the myokinase reaction. It is generally accepted (KIELLEY AND KIELLEY[27], LINDBERG AND ERNSTER[5], SLATER[48], KREBS[49] and others) that the primary phosphate acceptor during the oxidative phosphorylation is ADP. Reaction ADP plus P brings about the synthesis of ATP, which functions as a phosphate reservoir. A possible damage to this reaction, but with normal myokinase activity, may be accounted for by 1. decrease of ATP, 2. accumulation of ADP, 3. normal or about normal rate of disappearance of added AMP from the reaction mixture. Since all these phenomena have been found to occur in both water and "fatty" liver preparations, it is suggested that uncoupling of oxidative phosphorylation is due to the inefficiency of the reaction ADP + P → ATP.

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## TABLE VIII

ANALYSIS OF PHOSPHORYLATIVE INTERMEDIATES

(The data represent the values obtained in single experiments, with an enzyme material corresponding to 250 mg of wet liver and with the reaction mixture as indicated in the text. 30 $\mu M$ α-ketoglutarate added as a substrate. The values are given as $\mu g$ of substance formed or disappeared.)

| *Exp. No.* | *Treatment* | *Enzyme material from* | *Inorganic orthophosphate P disappeared* | *7' P* | *Ribose in 7' P fraction* | *Molar ratio 7' P/ribose* | *ATP formed* | *ADP formed* | *AMP disappeared* | *fructose-6-phosphate formed* |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | None | 0.25 *M* sucrose homogenate | 328.6 | 69.7 | 182.0 | 1.85 | 317 | 79 | 280 | 929 |
| | | water homogenate | 161.2 | 36.3 | 108.4 | 1.62 | 125 | 92 | 160 | 160 |
| | | 0.25 *M* sucrose mitochondria | 421.6 | 79.6 | 205.7 | 1.87 | 377 | 72 | 320 | 1075 |
| | | water mitochondria | 117.8 | 47.2 | 197.0 | 1.16 | 41 | 238 | 230 | 125 |
| 2 | None | 0.25 *M* sucrose homogenate | 345 | 70.3 | 188.8 | 1.80 | 306 | 97 | 280 | 1020 |
| | | water homogenate | 150 | 39.0 | 121.0 | 1.56 | 119 | 118 | 165 | 158 |
| | | 0.25 *M* sucrose mitochondria | 370 | 73.0 | 194.1 | 1.82 | 336 | 80.1 | 310 | 1058 |
| | | water mitochondria | 102 | 45.0 | 181.5 | 1.20 | 49 | 248 | 250 | 130 |
| 3 | phosphorus 1 injection | 0.25 *M* sucrose homogenate | 211 | 53.6 | 165.0 | 1.57 | 166 | 159 | 240 | 480 |
| | | water homogenate | 124 | 34.0 | 117.1 | 1.40 | 74 | 140 | 170 | 110 |
| | | 0.25 *M* sucrose mitochondria | 74.4 | 38.0 | 128.2 | 1.43 | 89 | 149 | 195 | 70 |
| | | water mitochondria | 40.3 | 25.0 | 102.6 | 1.18 | 24.5 | 141 | 140 | 20 |
| 4 | phosphorus 3 injections | 0.25 *M* sucrose homogenates | 180 | 45.0 | 139.4 | 1.56 | 137 | 136 | 210 | 440 |
| | | water homogenates | 108 | 32.0 | 116.2 | 1.33 | 57.8 | 177 | 215 | 195 |
| | | 0.25 *M* sucrose mitochondria | 59 | 36.0 | 124.4 | 1.40 | 56 | 106 | 125 | 72 |
| | | water mitochondria | 12 | 13.0 | 57.1 | 1.10 | 7.1 | 80.5 | 85 | 420 |
| 5 | $CCl_4$ 6 injections | 0.25 *M* sucrose homogenate | 161 | 47.0 | 147.6 | 1.54 | 138 | 149 | 220 | 500 |
| | | water homogenate | 127 | 38.0 | 131.2 | 1.40 | 83 | 157 | 180 | 162 |
| | | 0.25 *M* sucrose mitochondria | 75 | 35.0 | 122.4 | 1.38 | 72.5 | 149 | 175 | 102 |
| | | water mitochondria | 16 | 15.0 | 64.9 | 1.12 | 9.8 | 90.8 | 190 | 29 |

TABLE IX

MYOKINASE ACTIVITY OF NORMAL AND FATTY LIVERS
INFLUENCE OF TREATMENT WITH DISTILLED WATER

(The values are given as $\mu M$ ADP disappearing per mg of the enzyme preparation in 5 minutes.)
(Mean values of 3 experiments are represented.)

| | *Normal livers* | *Livers from animals treated with $CCl_4$ (4 injections)* | *Livers from animals treated with phosphorated oil (3 injections)* |
|---|---|---|---|
| 0.25 $M$ sucrose homogenates | $6.47 \pm 0.39$ | $6.26 \pm 0.82$ | $7.34 \pm 0.6$ |
| Water homogenates | $6.19 \pm 0.6$ | $5.69 \pm 0.98$ | $7.04 \pm 0.67$ |
| 0.25 $M$ sucrose mitochondria | $22.3 \pm 1.7$ | $20.5 \pm 3.4$ | $22.4 \pm 1.6$ |
| Water mitochondria | $21.4 \pm 2.2$ | $19.4 \pm 3.1$ | $21.3 \pm 1.5$ |

## DISCUSSION

It has been found in the experiments described in this paper that uncoupling of oxidative phosphorylation occurs in both homogenates and mitochondria from normal livers when distilled water is used for the preparation of the homogenates. A very similar phenomenon occurs also with 0.25 $M$ sucrose homogenates and mitochondria from fatty livers. The changes determined by distilled water were similarly found in 0.25 $M$ sucrose homogenates of fatty livers.

Since a common feature of "water-treated" and "fatty" mitochondria is the change of the shape and the size, one may think that these morphological changes are related to the enzymic modifications. The fact that the modified form of mitochondria is characterized by loss of the ability of utilizing the energy produced by oxidations for synthetic reactions is very suggestive, especially with regard to the pathogenesis of some regressive processes of the cell. It is not yet completely clear whether the morphological change is itself responsible for the uncoupling of oxidative phosphorylation, or conversely. HARMAN AND FEIGELSON[16] have shown that some substances known as inhibitors of oxidative phosphorylation produce also swelling and degenerative changes of mitochondria. Fluoride, which is an inhibitor of ATPase, protects mitochondria from both morphological and biochemical changes. These arguments, however, do not seem completely sufficient for affirming that uncoupling of oxidative phosphorylation is responsible for the change in the form. The fact that mitochondria suspended in 0.25 $M$ sucrose are spherical and possess a high phosphorylative power similar to that of the rod-like mitochondria suspended in 0.88 $M$ sucrose, is in itself a more favourable argument for the first hypothesis, even though it is not conclusive.

The fact that many of the morphological and biochemical modifications present in liver steatosis are identical with those determined by distilled water on normal mitochondria opens new problems, particularly with regard to the pathogenesis of liver steatosis and to the accumulation of fat within the cell. The presence of osmotic changes within the living liver cell during steatosis may be advanced as a working hypothesis. In fact, both chemical poisons used in this work, *i.e.* $CCl_4$ and phosphorated oil, do not possess the ability to uncouple oxidative phosphorylation *in vitro*. In some experiments in which the poisons were added to normal homogenates and to 0.25 $M$ sucrose suspensions of normal mitochondria in much larger amount than that capable of producing

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steatosis *in vivo*, no influence on the P:O ratios was observed, not even after 20′ incubation at 38° C. It seems then improbable that the mode of action of the steatogen poisons used is a direct one. The accumulation of fat within the cell may perhaps be concerned with the inefficiency of oxidative phosphorylation. In fact, the uncoupling of oxidative phosphorylation results in a loss of utilization of energy and in a deficient synthesis of ATP. It has been recently found (LIPMANN *et al.*[50], HILZ AND LYNEN[51], GREEN[52], HELL[53], LEHNINGER AND GREVILLE[54]) that oxidation of fatty acids takes place by way of a reaction including CoA and ATP: R–COOH + SH–CoA + ATP $\rightleftharpoons$ R–CO–S–CoA + AMP + PP. It is clear that a decrease of the concentration of ATP within the cell may produce a deficient oxidation of fatty acids. A decrease of easily hydrolyzable P (including ATP plus ADP) in the skeletal muscle and the kidneys of guinea pig has been reported by FONNESU AND SEVERI[55] to occur during cloudy swelling, a pathological modification which is also characterized by morphological changes of mitochondria (ANITSCHKOW[56], ZOLLINGER[57]).

Uncoupling of oxidative phosphorylation in the kidneys of treated animals, showing the histological picture of cloudy swelling, was found during the present research. It is not improbable that the fatty degeneration is a further stage, within certain types of cells, of the same process which produces cloudy swelling. Further work on these problems is in progress.

## SUMMARY

The author confirms that uncoupling of oxidative phosphorylation occurs in both homogenates and mitochondria from livers and kidneys of rats after treatment with distilled water. Uncoupling of oxidative phosphorylation was found also to occur in both homogenates and mitochondria from fatty livers, without previous treatment with water. ATPase activity, and also pyrophosphatase, acid and alkaline phosphatases, and the hydrolysis of fructose-6-monophosphate were investigated in order to see if the observed uncoupling could be referred to an increase of these phosphatase activities. Increase of ATPase and of acid and alkaline phosphatases activities were noticed. A modification of the distribution of inorganic pyrophosphatase and of both acid and alkaline phosphatases within the cell was found. The extent of increase of phosphatase activities does not seem, however, sufficient for explaining the uncoupling of phosphorylations from oxidations. The analysis of phosphorylated intermediates formed during the oxidative phosphorylation reaction showed in both "water-treated" and "fatty" preparations a decrease of the formation of ATP, decrease of the formation of fructose-6-monophosphate, accumulation of ADP and normal rate of disappearance of added AMP. On the base of these observations, the author thinks that uncoupling of oxidative phosphorylation is due to the inefficiency of the formation of ATP from ADP + P.

## RÉSUMÉ

L'auteur confirme que la phosphorylation oxydative est inhibée dans les homogénats et les mitochondries isolés de foies et de reins de rat à la suite d'un traitement par de l'eau distillée. Le même phénomène a été observé dans les homogénats et les mitochondries isolés de foies gras, sans traitement préalable à l'eau. Les activités ATPasique, pyrophosphatasique, celle des phosphatases acide et alcaline et l'hydrolyse du fructose-6-monophosphate ont été étudiées pour voir si l'inhibition de la phosphorylation oxydative pouvait être due à une augmentation éventuelle de ces activités enzymatiques. On a observé une augmentation de l'ATPase et des phosphatases acide et alcaline et une irrégularité de la distribution dans la cellule de la pyrophosphatase et des phosphatases acide et alcaline. L'augmentation des activités phosphatasiques n'était pas suffisante pour expliquer à elle seule l'inhibition de la phosphorylation oxydative. L'analyse des intermédiaires phosphorylés qui sont formés pendant la réaction phosphorylante a montré une diminution de la formation de l'ATP et du fructose-6-phosphate, une accumulation de l'ADP et une vitesse normale de consommation de AMP par les préparations traitées avec l'eau distillée ou provenant de foies gras. L'auteur conclut que l'inhibition de la phosphorylation oxydative qui se produit dans ces cas est due à une déficience de la réaction ADP + P → ATP.

*References p. 531/532.*

ZUSAMMENFASSUNG

Der Verfasser bestätigt dass die oxydative Phosphorylation in aus Rattenlebern und -nieren isolierten Homogenaten und Mitochondrien durch Behandlung mit distilliertem Wasser gehemmt wird. Dasselbe Phänomen wurde auch in Homogenaten und Mitochondrien aus verfetteten Lebern, welche nicht mit Wasser vorbehandelt worden waren, nachgewiesen. Die Aktivität einiger Phosphatasen, und zwar von ATPase, anorganischer Pyrophosphatase, saurer und alkalischer Phosphatase, und die Hydrolyse von Fructose-6-monophosphat, wurden studiert. Eine geringe Steigerung der ATPase und der sauren und alkalischen Phosphatasen, und eine Änderung der Verteilung der anorganischen Pyrophosphatase und der sauren und alkalischen Phosphatasen wurden nachgewiesen, aber die beobachteten Steigerungen waren nicht genügend gross, um die Hemmung der oxydativen Phosphorylation zu erklären. Die Analyse der organischen Phosphate, die durch die Reaktion der oxydativen Phosphorylation gebildet werden, zeigte in mit Wasser behandelten und in verfetteten Homogenaten und Mitochondrien eine Verminderung der ATP- und Fructose-6-phosphat-Bildung, eine Anhäufung von ADP und eine normale Verbrauchsgeschwindigkeit von AMP an. Der Verfasser denkt, dass die Unwirksamkeit der Reaktion ADP + P→ATP für die Hemmung der oxydativen Phosphorylation, die in mit Wasser behandelten und verfetteten Homogenaten und Mitochondrien aus Rattenleber nachgewiesen wurde, verantwortlich ist.

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