CONTENT AND DISTRIBUTION OF PYRIDINE NUCLEOTIDES IN FATTY LIVERS

by

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The relationship between the form of mitochondria and their enzymic activities has been outlined in previous work from several laboratories[1-6]. Since mitochondria from fatty livers show large morphologic modifications, resembling strongly those which are produced in normal particles by the hypotonicity of the suspension medium, the hypothesis has been advanced that osmotic damage occurs in mitochondria from fatty livers and that the accumulation of the fat within the cells may be in some manner related to the structural changes.

Uncoupling of oxidative phosphorylation has been found in mitochondria from fatty livers as well as in normal mitochondria treated with distilled water; in addition, an increase of the activity of ATPase and of both acid and alkaline phosphatase has been reported to occur in water-treated mitochondria (DE DUVE *et al.*[7]) as well as in those isolated from fatty livers[6]. The fact that a large part of acid phosphatase is displaced from mitochondria into the surrounding medium on treatment with distilled water (DE DUVE *et al.*[7]) and that the same type of modification has been found with regard to the intracellular distribution of this enzyme in fatty livers[6], leads one to consider the possibility that an increase of permeability of mitochondria to soluble substances may be the cause of some metabolic features of fatty infiltration.

An investigation on the content and the distribution of cytochrome *c* in the liver cell[8] has shown that a displacement of cytochrome *c* from mitochondria into the surrounding fluid occurs in fatty livers. The possibility then arises that a similar type of displacement occurs for other soluble coenzymes.

The content and the distribution of pyridine nucleotides in both normal and fatty liver cells have been investigated and the results are described in this paper.

Abbreviations

The following abbreviations were used: DPN = diphosphopyridine nucleotide, PN = pyridine nucleotides, PNH = pyridine nucleotides in their reduced form, DPNH = reduced diphosphopyridine nucleotide, PN/PNH = ratio between the oxidized and the reduced forms of pyridine nucleotides, DPNase = diphosphopyridine nucleotide nucleosidase, NMN = nicotinamide mononucleotide, ATP = adenosine triphosphate, ATPase = adenosine triphosphatase, PP = inorganic pyrophosphate, CoA = coenzyme A, TPP = thiamine pyrophosphate.

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References p. 405.

MATERIAL AND METHODS

Albino rats of the Wistar strain fed on a standard diet and weighing 120–150 g were used for this research.

Fatty infiltration of the liver was obtained in 3 different ways: (1) by treatment with CCl_4; this substance was administered daily by subcutaneous injection of 0.2 ml of a 20% solution in olive oil, (2) by treatment with a white phosphorus solution (0.2 ml of a 0.5% solution in olive oil being injected daily by the subcutaneous method), (3) by feeding with a diet deficient in choline. The type of diet used for this purpose was very similar to that used by RIDOUT *et al.*[9] and contained the following substances: casein (vitamin-free test casein for biological test procedures, General Biochem. Inc., Chagrin Falls, Ohio) 8%, gelatin 12%, salts mixture No. 185 (McCollum and Simmonds) 5%, amylum oryzae 2%, sucrose 60%, beef fat 10%, peanut oil (containing 20 mg 2-methyl, 1,4-naphtoquinone, 3000 I.U. Vitamin A and 700 I.U. Vitamin D) 2%, vitamin powder 1%. The vitamin powder contained 50 mg thiamine-HCl, 25 mg riboflavine, 20 mg pyridoxine, 100 mg Ca pantothenate, 100 mg nicotinamide per 100 g sucrose. Some control animals fed on this deficient diet received 10 mg choline subcutaneously every second day.

The animals were killed by dislocation of the spinal cord; the liver was immediately taken out and transferred to the cold room at + 2° C. 10% homogenates were prepared with distilled water or with 0.25 *M* sucrose, or also with 0.88 *M* sucrose in a glass homogenizer of the Potter-Elvehjem type with lucite pestle. Differential centrifugation of the homogenates was made either in a refrigerated Zernike centrifuge, or in the Spinco preparative ultracentrifuge. 4 fractions were collected: (1) nuclear fraction, (2) mitochondria, (3) microsomes, (4) supernatant fluid. Nuclei were sedimented by centrifugation at 700 × *g* for 10 minutes with water or with 0.25 *M* sucrose as suspension fluids, at 1,500 × *g* for 10 minutes with 0.88 *M* sucrose. They were washed twice and washing fluids were added to the supernatant. Mitochondria were separated from this at 10,000 × *g* for 30 minutes in the case of water or 0.25 *M* sucrose, at 26,000 × *g* for 30 minutes in that of 0.88 *M* sucrose. Sedimentation of microsomes was obtained after centrifugation at 26,000 × *g* for 1 hour with water or 0.25 *M* sucrose homogenates, at 92,000 × *g* for 1 hour with 0.88 *M* sucrose homogenates.

Dehydrogenase activities were studied with Warburg manometers at 37°.5 C, in a medium of the following composition: 0.025 *M* KCl, 0.0067 *M* $MgSO_4$, 0.00002 *M* cytochrome *c*, 0.067 *M* Na_2HPO_4–KH_2PO_4 buffer, pH 7.4, 0.01 *M* substrate (added from the side arm after 5 minutes necessary for temperature equilibration), 0.5 ml enzyme (corresponding to 50 mg tissue). 0.1 ml 30% KOH were placed in the central well. 1 μM DPN was added, when used, from the side arm. The gaseous environment was air. O_2 uptakes were measured during 20 minutes. Substrates used were: L(+)-glutamate, L(+)-malate, α-ketoglutarate, pyruvate, all being employed as sodium salts. When oxidation of pyruvate was studied, 0.0001 *M* L(+)-malate was used as a primer.

Pyridine nucleotides were determined by 2 independent methods, both determinations being made on each tissue sample. The first method was that described by ROBINSON *et al.*[10] as a development of the original procedure of HUFF AND PERLZWEIG[11]: the principle of this method consists in the formation of a fluorescent condensation compound[12] from acetone and N'-substituted nicotinamide derivatives in the presence of strong alkali, followed by heating in acid solution. According to the original method of ROBINSON *et al.*, a small piece of tissue is transferred into a weighed beaker containing 20 mg of $Ce(SO_4)_2$ and a 2% solution of nicotinamide, in order to inhibit the DPN splitting through DPN-ase activity of the homogenates. The beaker is weighed again and the tissue is then homogenized in a Waring blendor; the proteins are discarded by treatment with 10% trichloroacetic acid. In the present investigation some modifications were introduced in this procedure. Homogenization was made in a Potter-Elvehjem apparatus. Since DPNH is very rapidly and completely destroyed in acid media at room temperature (WARBURG AND CHRISTIAN[13], VON EULER, SCHLENK *et al.*[14]) probably by substitution of acid radicals across one of double bonds of the nicotinamide ring (KARRER *et al.*[15]), 0.5 ml of 30% H_2O_2 were added to the homogenates immediately after trichloroacetic acid, as it was suggested by FEIGELSON *et al.*[16]. This resulted in obtaining values approximately 30% higher than those found without the addition of H_2O_2. The addition of $Ce(SO_4)_2$ was omitted, as no difference was found when this substance was either present or absent. In addition, $Ce(SO_4)_2$ produced irreversible clumping and agglutination of the cytoplasmic particles, and this precluded its use in fractionation experiments. Homogenization in sucrose or water containing 2% nicotinamide was found to inhibit completely the DPN-nucleosidase without producing morphological changes in mitochondria. This fact permitted us to obtain reproducible results in the fractionation experiments. Fluorimetric estimations were made with a Cohen type fluorimeter provided with a 3289 Corning Filter.

The second method of determination of pyridine nucleotides consisted in reading the extinction of the reduced form of pyridine nucleotides at 340 mμ in a Beckman spectrophotometer, after reduction with an excess of sodium dithionite in alkaline medium (FEIGELSON *et al.*) and oxidation of dithionite, which had not reacted by bubbling compressed air through the solution for 5 minutes. An extinction coefficient of $6.22 \cdot 10^6 \cdot cm^2 \cdot mole^{-1}$ was used, as suggested by HORECKER AND KORN-

References p. 405.

BERG[17]. Preparation of the homogenates and deproteinization were made as in the case of the first procedure.

When the PN/PNH ratios were to be calculated, both reduced and oxidized forms of pyridine nucleotides were determined on the same liver, according to a principle described by FEIGELSON *et al.*[16]. 2 small pieces (400–600 mg) of tissue were submitted to homogenization either in 2.5% trichloroacetic acid containing 0.5 ml of 30% H_2O_2, or in 2.5% trichloroacetic alone. As DPNH is instantly destroyed in acid medium, the values obtained with the homogenate prepared without hydrogen peroxide were about 30% lower than those obtained with that prepared in the presence of H_2O_2. The latter account for total pyridine nucleotides content of the liver sample, while the first ones represent only the oxidized form. The amount of PNH can be calculated by difference. In the original method of FEIGELSON *et al.*[16], pyridine nucleotides are determined by measurement of the extinction at 340 mμ after reduction with sodium dithionite. In the present investigation, the more sensible fluorimetric estimation was used.

Synthesis of DPN *in vitro* from NMN + ATP was studied according to KORNBERG[18]. The reaction mixture contained 0.1 ml 0.02 *M* ATP, 0.05 ml 0.05 *M* NMN, 0.2 ml 0.25 *M* glycylglycine buffer, pH 7.4, 0.1 ml 0.15 *M* $MgCl_2$, 0.1 ml 2 *M* nicotinamide, 0.1 ml 10% liver homogenate (corresponding to 10 mg tissue) in 0.25 *M* sucrose, water to 1 ml. The mixture was incubated for 20 minutes at 38° C, after which time the reaction was stopped by addition of 1 ml 10% trichloroacetic acid. A blank at 0 time was made. Estimation of formed DPN was made by reading the extinction at 340 mμ after reduction with sodium dithionite.

Destruction of DPN was studied according to the procedure described by SUNG AND WILLIAMS[19], with an incubation medium of the following composition: 1.9 ml 0.25 *M* sucrose, 1 ml DPN solution in phosphate buffer, pH 7.4 (containing 260–280 μg DPN), 0.1 ml 10% homogenate or corresponding fraction. The mixture was incubated for 20 minutes at 38° C, after which time the reaction was stopped by addition of 5 ml 2% trichloroacetic acid, followed immediately by 1 ml of 30% hydrogen peroxide. Blanks at 0 time were made. Both fluorimetric and spectrophotometric estimations of DPN were made for each experiment. As the fluorimetric method is specific for N′-substituted nicotinamide derivatives, the measurement in the decrease of the intensity of this reaction in the presence of the enzyme is a measure of the splitting of the glucosidic bond between nicotinamide and the rest of the molecule, *i.e.* of DPN-nucleosidase. The extent of decrease of optical density at 340 mμ after reduction with an excess of sodium dithionite is a measure of the disappearance of DPN produced through any method of destruction. The difference between the values obtained with the spectrophotometric method and those obtained with the fluorimetric one has been taken as a measure of the DPN destruction in any other way than with DPN-nucleosidase.

Nitrogen was determined on each homogenate and cytoplasmic fraction by the usual micro-Kjeldahl technique.

Total lipids content of liver was determined in each animal by weighing the dry powder of the organ before and after extraction for 3–4 hours with ether in a Soxhlet apparatus. The liver was dried by exposing it in a stove at 85° C until it reached a constant weight, and the drying was accomplished above anhydrous $CaCl_2$.

Morphologic examination of mitochondria was made with a phase contrast microscope.

The obtained values were studied statistically, the standard deviation $\left(\sigma = \pm\sqrt{\frac{\Sigma d^2}{n-1}}\right)$, the standard error $\left(\varepsilon = \sigma(\sigma) = \pm\frac{\sigma}{\sqrt{2n}}\right)$ and the "t" test of Student-Fisher being calculated for each average datum. Only the data with a "t" value corresponding to a probability $P < 0.05$ were accepted as significant. The value of the standard error is given in the tables after each average datum.

Reagents

Sodium L(+)-glutamate and sodium L(+)-malate were prepared in solution by neutralization of the acids. Sodium pyruvate was a Hoffmann-La Roche preparation. Diphosphopyridine nucleotide was a commercial product (Sigma) of announced 90% purity. This degree of purity was confirmed by direct estimation.

ATP sodium salt was prepared in solution from the dibarium salt (Schwarz Lab.) by solution in HCl, precipitation of barium with H_2SO_4 and neutralization with 1 *N* NaOH.

Nicotinamide mononucleotide was prepared from DPN with a potato pyrophosphatase according to KORNBERG *et al.*[20].

All other substances used were commercial preparations.

References p. 405.

EXPERIMENTAL

1. *Oxidation of L(+)-glutamate*

Oxidation of L(+)-glutamate in the rat liver is produced by a specific dehydrogenase, which transfers H^+ from substrate to DPN with production of NH_3 and α-ketoglutarate. As other coenzymes do not seem to be involved in this reaction, the study of the oxidation of L(+)-glutamate in the presence or in the absence of DNP appeared to be a rather simple indirect method to test the behaviour of DNP in fatty liver.

In previous experiments it was observed that the oxidation of L(+)-glutamate in the absence of added DPN is strongly decreased in fatty livers, and particularly in mitochondria isolated from them. The addition to these in the Warburg flasks of small amounts (0.5 ml) of the supernatant fluids after the sedimentation of mitochondria produced an activation of the oxidation of glutamate. This activation occurred with supernatant fluids from both normal and fatty liver homogenates. Mitochondria from normal livers were not activated by the addition of supernatant fluids. Since the supernatant fluid contains itself a very slight amount of glutamic dehydrogenase activity, it seemed very likely that some coenzymes or activators of the enzymic system were contained in this cytoplasmic fraction. A series of experiments was then made in which the oxidation of L(+)glutamate either in the presence or in the absence of DPN added was tested in both homogenates and mitochondria of normal and of fatty livers.

The results, which are represented in Table I, showed that the addition of DPN produces an almost complete restoration of glutamic dehydrogenase activity of both homogenates and mitochondria of fatty livers. The enzymic activity of normal homogenates is stimulated by the addition of DPN more than that of mitochondria. This fact shows that the amount of DPN contained in normal mitochondria is approximately sufficient for the maximal functioning of the enzyme. This fact agrees with the previous statement by HUENNEKENS AND GREEN[21]. The fact that the stimulation of the oxidation of glutamate by DPN is more pronounced in the homogenates than in mitochondria is probably related to the cytoplasmic distribution of the DPN-splitting enzymes. In fact,

TABLE

OXIDATION OF L(+)-GLUTAMATE BY

The data for glutamic acid dehydrogenase activity are given as μl O_2 consumed in 1 h by 1 mg N of the standard error is given after each average value.

Type of treatment	*Number of expts.*	*Lipids content of liver*	*Homogenates*	
			Nitrogen	*Glutamic*
				without DPN added
None	7	35.4 ± 0.7	30.5 ± 0.5	55.1 ± 1.1
CCl_4 (1 injection)	3	39.6 ± 1.5	29.3 ± 0.4	42.0 ± 0.7
CCl_4 (3 injections)	8	72.1 ± 2.6	29.2 ± 0.2	35.7 ± 0.9
Phosphorus (1 injection)	3	38.2 ± 0.7	29.0 ± 0.16	35.0 ± 0.4
Phosphorus (3 injections)	5	59.8 ± 1.0	30.9 ± 0.2	33.3 ± 0.9
Feeding with deficient diet for 1 week	8	38.8 ± 0.6	29.2 ± 0.2	30.4 ± 1.0
Feeding with deficient diet for 4 weeks	7	87.5 ± 2.8	27.8 ± 0.4	29.6 ± 1.9
Feeding with deficient diet and parentheral administration of choline	3	36.4 ± 1.0	30.2 ± 0.3	54.8 ± 2.3
None, homogenates prepared with distilled water	4	36.0 ± 0.3	30.6 ± 0.4	47.7 ± 1.1

References p. 405.

these are localized principally in microsome and supernatant fractions, while they are practically missing in mitochondria (SUNG AND WILLIAMS[19]).

The decrease of the oxidation of glutamate occurring in fatty livers was stronger for mitochondria than for homogenates. The extent of activation by addition of DPN was very much higher for mitochondria than for homogenates, about the same values as those found in normal animals being attained in both cases. The most probable interpretation of this fact is that the amount of DPN present in fatty livers is not sufficient for the maximal activity of glutamic dehydrogenase, and that this insufficiency is particularly remarkable in mitochondria.

It is particularly interesting to remark that the decrease of oxidation of glutamate occurs in the liver of treated animals before the increase of lipids. In fact, this decrease, and also the restoration of full activity on addition of DPN, are present also in rats treated with only 1 injection of CCl_4 or of phosphorus solution, and in those fed for 1 week with a diet deficient in choline, whose lipids content of the liver was quite normal.

The hypothesis then arose that a decrease of DPN took place in the liver of treated animals before the beginning of the infiltration of fat in the cells. Since the type of alteration occurring in fatty liver mitochondria resembles in many cases that produced on normal mitochondria by treatment with distilled water, the effect of treatment with distilled water on the oxidation of glutamate by both homogenates and mitochondria was studied. As Table I shows, the oxidation of glutamate in the absence of DPN is decreased in water homogenates of normal livers and particularly in mitochondria isolated from them. Also in this case, restoration of the activity occurs on addition of DPN.

The distribution of glutamic dehydrogenase among the cell fractions, as studied with a complete reaction system, is the following in the case of normal livers: Nuclear fraction 12.2%; Mitochondria 63.4%; Microsomes 6.4%; Supernatant fluid 18%. No difference in distribution was found in fatty livers.

A very recent report by JUDAH AND CHRISTIE[22], which appeared when this investigation was in progess, has confirmed the statement of a low oxidation of glutamate in the liver of rats treated with CCl_4 and its partial reversal on addition of DPN.

I

LIVER HOMOGENATES AND MITOCHONDRIA

enzymic preparation. Those for N or lipids content of the liver are given as mg per g of wet tissue. The The homogenates were prepared with 0.25 *M* sucrose.

Homogenates			*Mitochondria*		
dehydrogenase		*Nitrogen*	*Glutamic dehydrogenase*		
with DPN added	*Increase % by DPN*		*without DPN added*	*with DPN added*	*Increase % by DPN*
88.5 ± 2.5	66.0 ± 2.4	7.1 ± 0.1	150.9 ± 2.8	197.9 ± 5.1	31.1 ± 2.1
66.9 ± 1.6	59.0 ± 1.2	6.8 ± 0.3	101.8 ± 9.1	187.2 ± 10.0	84.0 ± 7.9
65.8 ± 1.6	85.3 ± 6.1	6.9 ± 0.2	66.2 ± 2.4	155.9 ± 4.7	137.9 ± 7.2
66.0 ± 1.2	88.6 ± 3.5	7.1 ± 0.2	69.1 ± 2.3	164.1 ± 4.6	137.0 ± 10
59.7 ± 2.3	85.6 ± 2.9	7.3 ± 0.1	51.4 ± 9.3	145.6 ± 7.4	214.0 ± 26
66.3 ± 4.2	118.0 ± 8.5	6.9 ± 0.1	63.1 ± 2.1	155.9 ± 4.5	147.1 ± 11
55.5 ± 2.2	92.7 ± 6.8	6.3 ± 0.1	56.8 ± 2.6	171.1 ± 6.7	208.7 ± 18
89.8 ± 3.5	63.8 ± 3.3	7.0 ± 0.1	156.8 ± 3.1	198.4 ± 5.9	26.5 ± 2
88.8 ± 0.6	88.0 ± 3.8	7.6 ± 0.1	69.0 ± 6.0	195.2 ± 8.7	195.8 ± 23

References p. 405.

2. Content and distribution of pyridine nucleotides in normal and in fatty livers

In order to verify the hypothesis reported above, the content and distribution of the pyridine nucleotides in the liver cell of both normal and treated rats was investigated. As was affirmed by HUENNEKENS AND GREEN[20], the enzymes of the cyclophorase complex do not require extra-addition of DPN, and this shows the presence of a strong linkage between DPN and the protein part of the enzyme.

TABLE

CONTENT AND DISTRIBUTION OF PYRIDINE NUCLEOTIDES

The values are given as μg PN/g of wet liver. The homogenates were prepared either with distilled water, the spectrophotometric method. The standard

Cytoplasm fractions	*Homogenates prepared with distilled water*			
	Method A		*Method B*	
	µg/g	*%*	*µg/g*	*%*
Homogenate	888 ± 5.8		893 ± 9	
Nuclear fraction	93 ± 6	10.6	100 ± 8	11.3
Mitochondria	133 ± 8	15.1	126 ± 6	14.2
Microsomes	40 ± 2	4.5	46 ± 3	5.2
Supernatant	614 ± 13	69.8	613 ± 10	69.3
Total	880	100	885	100
Number of experiments	5			

The average value for the PN content in the homogenate of 18 normal rats was 881 μg ± 62 as determined was 92.5% with the first method, 92.9% with the second one. The difference between the values obtained content of 1 g liver was mg 34.6 ± 0.9 in 18 experiments.

TABLE

CONTENT AND DISTRIBUTION OF PYRIDINE NUCLEOTIDES

The homogenates were prepared with 0.88 *M* sucrose.

Type of treatment	*No. of expts.*	*Method of estimation*	*Lipids in 1 g wet liver*	*Homogenate*
1 injection CCl_4	3	A	39.6 ± 1.5	536 ± 15
		B		547 ± 19
3 injections CCl_4	5	A	69.6 ± 4.0	565 ± 27
		B		604 ± 28
1 injection phosphorus solution	3	A	36.4 ± 1.2	602 ± 11
		B		621 ± 10
3 injections phosphorus solution	5	A	62.5 ± 2.8	625 ± 18
		B		669 ± 27
Feeding on a deficient diet for 6–8 days	8	A	37.4 ± 2.5	529 ± 15
		B		540 ± 10
Feeding on a deficient diet for 26–30 days	7	A	86.8 ± 6.4	466 ± 17
		B		491 ± 16
Feeding on a diet deficient in choline and injected with choline	4	A	35.4 ± 2.3	881 ± 9
		B		908 ± 3

References p. 405.

The results of the investigation on the content and the distribution of pyridine nucleotides in normal livers are given in Table II. It is evident from this table that the fluid used for the preparation of the homogenates influences the distribution of pyridine nucleotides among the different cell fractions. When the homogenate is prepared either with 0.88 *M* or with 0.25 *M* sucrose, about 35% of pyridine nucleotides are recovered in mitochondria, and about 40% in supernatant fluid. With water homogenates the dis-

II

IN LIVER FRACTIONS OF NORMAL RATS

or with 0.25 *M* sucrose, or also with 0.88 *M* sucrose. Method A was the fluorimetric method, method B was error is given after each average value.

Homogenates prepared with 0.25 M sucrose				*Homogenates prepared with 0.88 M sucrose*			
Method A		*Method B*		*Method A*		*Method B*	
μg/g	%	*μg/g*	%	*μg/g*	%	*μg/g*	%
926 ± 31		977 ± 52		829 ± 5		863 ± 17	
110 ± 6	13.8	95 ± 2	11.1	107 ± 7	13.4	126 ± 4	15.5
223 ± 6	28.0	300 ± 19	35.1	265 ± 4	33.3	296 ± 17	36.3
88 ± 6	11.2	105 ± 5	12.3	56 ± 4	7.0	60 ± 6	7.3
374 ± 5	47.0	355 ± 22	41.5	369 ± 5	46.3	334 ± 12	40.9
795	100	855	100	797	100	816	100
5				5			

with the fluorimetric method, 941 μg ± 95 as determined with the spectrophotometric one. Average recovery with the first method and those obtained with the second one was 6.4% in the case of homogenates. Lipids

III

IN LIVER FRACTIONS OF RATS WITH LIVER STEATOSIS

The same type of representation as in Table II.

Nuclear fraction		*Mitochondria*		*Microsomes*		*Supernatant*	
μg/g	%	*μg/g*	%	*μg/g*	%	*μg/g*	%
108 ± 4	21.3	64 ± 4.5	12.7	22 ± 0.8	4.3	312 ± 7	61.7
106 ± 8	20.1	76 ± 6.0	14.4	27 ± 0.8	5.1	318 ± 12	60.4
66 ± 7	11.6	91 ± 19	15.9	33 ± 3	5.8	381 ± 22	66.7
78 ± 7	12.6	107 ± 13	17.2	40 ± 4	6.5	395 ± 15	63.7
105 ± 6	18.2	101 ± 1.2	17.5	33 ± 1.2	5.8	337 ± 4	58.5
108 ± 7	18.2	107 ± 8	18.2	29 ± 0.4	4.9	348 ± 4	58.7
82 ± 4	13.2	100 ± 8	15.9	39 ± 2.9	6.1	406 ± 13	64.8
83 ± 2	12.9	105 ± 8	16.3	39 ± 1.6	6.1	415 ± 16	64.7
83 ± 6	17.3	59 ± 8	12.2	24 ± 1.0	4.9	315 ± 10	65.6
90 ± 6	17.9	63 ± 4.5	12.6	28 ± 1.0	5.6	320 ± 6	63.9
69 ± 4	14.6	67 ± 7	14.2	24 ± 1	5.1	312 ± 12	66.1
70 ± 4	14.3	69 ± 5	14.1	28 ± 1	5.7	323 ± 12	65.9
136 ± 2	17.4	265 ± 2	34.1	39 ± 0.3	5.0	337 ± 8	43.5
141 ± 5	17.2	281 ± 3	34.3	43 ± 1.0	5.2	356 ± 7	45.3

References p. 405.

tribution of pyridine nucleotides is quite different, the amount of those which are situated in mitochondria being only 15% and those present in the supernatant fluid about 70%. This fact means that a displacement of DPN from the mitochondria to the supernatant fluid occurs as a consequence of the treatment with distilled water. Some experiments were made in order to test the nature of the binding between mitochondria and pyridine nucleotides. When mitochondria isolated from 0.88 *M* sucrose homogenates are washed thrice with 0.88 *M* sucrose, only a small part (5–10%) of their pyridine nucleotides are transferred into the washing fluids. The amount of pyridine nucleotides lost by water-treated mitochondria after 3 washings with distilled water is about 70%. This fact eliminates the possibility that the amount of pyridine nucleotides recovered in the mitochondrial fraction is the result of a contamination with other fractions, and one may thus assume that a portion of the DPN is firmly bound to the structure.

Table III shows the results of the investigation on the content and distribution of pyridine nucleotides in fatty livers. It is evident from this table that the content of pyridine nucleotides is strongly decreased in fatty livers. The extent of decrease per g tissue is about 36% in the case of rats treated with 3 injections of CCl_4, 29% in that of rats treated with 3 injections of phosphorus solution, and 47% in the case of rats fed on a diet deficient of choline. As the extent of the decrease of nitrogen per g liver was respectively 4.3, 4.9 and 8.8%, the real decrease of pyridine nucleotides in fatty livers is highly significant. The decrease of pyridine nucleotides occurs before the accumulation of fat within the liver cell; in fact, about the same percentage of decrease found in fatty livers was observed in rats treated only with 1 injection of CCl_4 (extent of decrease 39%), with 1 injection of phosphorus (decrease 31%), and in those fed on a deficient diet for 6–8 days (decrease 41%). The lipid content of the liver was not increased in these animals. These facts agree with the result of a very recent investigation by FRUNDER[23], which appeared when the present work was well advanced. The extent of the decrease found by this author (10%) with CCl_4 poisoning of mice is however remarkably lower than that observed in the present experiments.

The distribution of pyridine nucleotides among the cell fractions shows that with all types of treatments used, the percentage of coenzyme linked to mitochondria is

TABLE IV

DIFFERENCE IN RELEASE OF PN FROM MITOCHONDRIA ISOLATED EITHER FROM NORMAL OR FROM STEATOTIC LIVER INTO THE INCUBATION FLUID

Mitochondria isolated from 2–3 g liver were suspended in 20 ml 0.25 *M* sucrose and incubated at room temperature for 1 h. PN content of the suspension was determined at 0 time on an aliquot of the suspension. After 1 h incubation, the remaining suspension was submitted to centrifugation at 16,000 × *g* for 30 minutes and PN were determined on both sediment and supernatant by the fluorimetric method.

Type of treatment	*Number of expts.*	*PN content per g wet liver µg*	*Lipid content per g wet liver mg*	*% distribution after incubation*		
				Sediment	*Supernatant*	*Recovery %*
None	6	232 ± 6	36 ± 0.3	69.9 ± 2.2	30.1 ± 2.2	93.0 ± 0.9
Feeding with a deficient diet for 4 weeks	6	118 ± 2	84.8 ± 1.1	22.5 ± 1.5	77.5 ± 2.4	89.6 ± 2.3
Injected with 3 injections CCl_4	3	108 ± 1	65.1 ± 0.3	32.9 ± 1.6	67.1 ± 1.6	95.6 ± 1.2

References p. 405.

strongly decreased, while that recovered in the supernatant fluid is increased. The type of distribution occurring in fatty liver is very similar to that found in normal water homogenates. The extent of decrease of pyridine nucleotides recovered in mitochondria from fatty livers is then higher than that occurring in the homogenate. The values of this decrease were respectively 75% for liver mitochondria of rats treated with 1 injection of CCl_4, 65% in those of rats treated with 3 injections of the same substance, 63% in mitochondria of rats treated with 1 injection of phosphorus, 63% in those of rats treated with 3 injections of phosphorus. The extent of decrease was 78 and 75% in mitochondria of rats fed on diet deficient of choline for 1 and 4 weeks respectively.

An attempt was made to discover the cause for this preferential decrease of pyridine nucleotides in mitochondria. Mitochondria isolated from 2–3 g liver with 0.25 *M* sucrose and washed once were suspended in 10 ml 0.25 *M* sucrose and incubated at room temperature (± 18° C) for 1 h. The pyridine nucleotide content of the suspension at 0 time was determined. After 1 h incubation, the suspension was submitted to centrifugation at 16000 × *g* for 30 minutes and pyridine nucleotides were determined in both sediment and supernatant fluid.

As shown in Table V, mitochondria from normal livers release into the supernatant fluid about 30% of their pyridine nucleocide content, while those from fatty livers release about 70%. This means that the binding between mitochondria and DPN is particularly weak in fatty livers, and that the relative increase of pyridine nucleotides contained in the supernatant fraction of the homogenates is probably a consequence of this condition.

Phase contrast examination of mitochondria isolated from all types of fatty livers obtained revealed an increase of size with respect to the normal particles. Mitochondria isolated from these livers with 0.88 *M* sucrose are sphere-shaped, while those isolated from normal livers in the same conditions are mainly rod-like. The same type of morphologic modification was evident also in mitochondria of rats treated with only

TABLE V

PN/PNH RATIOS IN THE LIVER OF NORMAL AND STEATOTIC RATS

The values are referred to 1 g of fresh liver.

Type of treatment	*Number of experiments*	*Pyridine nucleotides content* μg	*Oxidized pyridine nucleotides* μg	*Reduced pyridine nucleotides* μg	*PN/PNH*
None	7	879 ± 18	670 ± 18	209 ± 5	3.2 ± 0.12
Feeding on deficient diet for 1 week	6	609 ± 8	404 ± 11	205 ± 6	2.0 ± 0.09
Feeding on deficient diet for 4 weeks	7	566 ± 21	352 ± 21	214 ± 6	1.6 ± 0.08
Feeding on deficient diet and injected with choline	3	860 ± 11	660 ± 6	200 ± 5	3.3 ± 0.10
3 injections of CCl_4	3	675 ± 12	416 ± 2.5	259 ± 13	1.6 ± 0.08
3 injections of phosphorus solution	3	606 ± 7	396 ± 2.5	210 ± 5.4	1.9 ± 0.04

References p. 405.

1 injection of steatogenic poisons, or fed on a diet deficient of choline for 1 week. It was never observed, however, in the animals fed on a diet deficient of choline for 4 weeks, which received choline parentherally.

3. *PN/PNH ratios in normal and in fatty livers*

Table V shows the results of an investigation on the ratios between the oxidized and the reduced form of pyridine nucleotides in both normal and fatty liver.

The value of this ratio for normal liver is about 3. This value agrees with that obtained by FEIGELSON *et al.*[16], who used the same estimation method. The value of the ratio for the fatty livers is strongly decreased, being 1.6 for rats fed on a deficient diet for 4 weeks and for those treated with CCl_4. It is interesting to remark that practically the same degree of decrease has been found also in the livers of rats fed on the deficient diet only for 1 week. This means that the modification of the ratio occurs before the deposition of fat within the cell. Treatment with choline prevents both accumulation of fat and decrease of the ratio PN/PNH.

4. *Synthesis and destruction of DPN in vitro in both normal and fatty livers*

An attempt to investigate the cause of the absolute decrease of pyridine nucleotides in fatty livers was made by studying the activity of enzymic systems which produce the synthesis and the decomposition of pyridine nucleotides. The problem of the synthesis of these substances is not yet completely clear. The most possible way of synthesis is that described by KORNBERG[18]: NMN + ATP $\rightleftarrows$ DPN + PP. The importance of this reaction in fatty livers was investigated. The results, which are represented in Table VI, show that the intensity of the synthesis of DPN through this reaction is not decreased. Since uncoupling of oxidative phosphorylation occurs in mitochondria from fatty livers, the possibility exists, however, that the intensity of the reaction is decreased *in vivo* through a decrease of ATP.

TABLE VI

SYNTHESIS OF DPN *in vitro* BY LIVER HOMOGENATES OF NORMAL AND STEATOTIC RATS

Type of treatment	*Number of experiments*	*μM DPN/h/100 mg liver tissue*	*μM DPN/h/mg N*
None	5	1.07 ± 0.4	0.35 ± 0.03
Feeding on a diet deficient in choline for 4 weeks	3	0.93 ± 0.07	0.34 ± 0.04
3 injections CCl_4	3	1.03 ± 0.001	0.35 ± 0.04
3 injections phosphorus solution	3	0.96 ± 0.02	0.34 ± 0.04

The enzymic decomposition of DPN occurs through the following possible mechanisms: nucleotide pyrophosphatase action, pyrophosphorolysis, nucleosidase action, deamination (SINGER AND KEARNEY[14]). The first type of splitting occurs principally in vegetable material (potato), but it has been described as being present also in kidney particles (KORNBERG AND LINDBERG[20], KORNBERG AND PRICE[25]). Deamination has been described in molds (KAPLAN *et al.*[26]), but its importance in animal tissues has not yet been investigated. Pyrophosphorolytic cleavage through the reversal of the KORNBERG reaction can occur in rat liver. The most important agent of decomposition of DPN in

References p. 405.

this tissue seems to be however the hydrolysis of the glycosydic bond between nicotinamide and the rest of the molecule, which is performed by DPN-nucleosidase (DPNase) action. SUNG AND WILLIAMS[19] have studied the cytoplasmic distribution of this enzyme. They reported that the enzyme is missing in mitochondria and that it is situated principally in the microsomes and in the supernatant fraction. The sum of the activities exerted by the 4 different cytoplasm fractions is about 2–3 times higher than the activity of the unfractionated homogenate. Artificial reconstitution of the homogenate by mixing the isolated fractions results in obtaining about the same value found for the unfractionated homogenate. The cytoplasm fractions appear then to be mutually inhibitory.

The destruction of DPN through DPNase action and in any other possible way are reported in Table VII. It is clear from this table that the activity of enzymic systems destroying DPN is not increased in fatty livers *in vitro*. Liver mitochondria are completely devoid of DPNase activity and contain only a small amount of enzymes destroying DPN by other mechanisms. This fact is of particular importance in order to understand the functioning of intramitochondrial enzymes requiring DPN.

The possibility was investigated that DPNase or other types of DPN decomposition appear or increase in mitochondria as a consequence of ageing at 28° C or of treatment with distilled water, as ATPase does, but negative results were obtained.

5. *Oxidation of other substrates requiring DPN*

Oxidation of some other substrates requiring DPN (L(+)-malate, pyruvate and α-ketoglutarate) was also investigated. The enzyme systems producing oxidation of the last two substrates are more complex than those which oxidize respectively glutamate and malate. In fact, recent investigation have found that CoA and DPN are necessary for the oxidation of α-ketoglutarate to succinate through the following reactions: (1) α-ketoglutarate + HS-CoA + DPN $\rightleftarrows$ succinil-S-CoA + CO_2 + DPNH (2) succinil-S-CoA + H_2O $\rightleftarrows$ succinate + CoA-SH (KAUFFMANN *et al.*[28], SANADI AND LITTLEFIELD[27]), Probably also TPP and α-lipoic acid are required (OCHOA[29], REED AND DE BUSK[30], GOLDBERG AND SANADI[31]). Requirements of DPN, CoA, TPP and α-lipoic acid for oxidation of pyruvate are, however, well established (GUNSALUS *et al.*[32], OCHOA[33]).

Table VIII shows that also the oxidation of malate, ketoglutarate and pyruvate is decreased in fatty livers and that the extent of this decrease is higher in mitochondria than in homogenates. The increase of activity occurring on addition of DPN is higher for mitochondria than for homogenates, with the exception of the oxidation of ketoglutarate. In fact, in the case of this substrate, no significant difference between homogenates and mitochondria has been found in the stimulation of oxidative activity by DPN.

DISCUSSION

It seems clear from the experiments described above that a marked deficiency of pyridine nucleotides exists in fatty livers. Since this deficiency is present when the deposition of fat within the cells is not yet evident, the hypothesis of a close relationship between the decrease of pyridine nucleotides and the pathogenesis of fatty infiltration seems to be supported.

DPN is necessary for many reactions connected with the metabolism of fatty acids: (1) the oxidation of β-hydroxybutyrate to acetoacetate (2) 3 steps of the Krebs cycle; (a) oxidation of *d*-isocitrate to α-ketoglutarate (b) oxidation of α-ketoglutarate to succi-

References p. 405.

TABLE

DESTRUCTION OF DPN *in vitro* BY HOMOGENATES

The data are given as μg DPN destroyed in 20 minutes by 10 mg tissue or

Type of treatment	*Number of experiments*	*Type of enzymic destruction*	*Homogenate values per 10 mg tissue*
None	9	nucleosidase	138.0 ± 3.3
		any other	41.4 ± 2.1
Feeding on deficient diet for 4 weeks	8	nucleosidase	101.0 ± 7.7
		any other	42.0 ± 2.2
3 injections CCl_4	3	nucleosidase	137.0 ± 3.7
		any other	35.0 ± 2.6
3 injections phosphorus solution	3	nucleosidase	137.0 ± 4.5
		any other	35.0 ± 1.8

TABLE

OXIDATION OF L(+)-MALATE, α-KETOGLUTARATE AND

The values are given as μl O_2 consumed in 1 h per mg N of the enzymic preparation. Those for lipid and

Type of treatment	*No. of expts.*	*Lipids in the liver*	*Enzyme preparation*	*N content*
None	7	37.0 ± 0.5	homogenate	30.6 ± 0.2
			mitochondria	7.2 ± 0.1
Feeding for 4 weeks with deficient diet	5	85.5 + 3.8	homogenate	27.2 ± 0.6
			mitochondria	6.2 ± 0.2
3 injections CCl_4	3	64.6 ± 1.7	homogenate	29.2 ± 1
			mitochondria	6.7 ± 0.01
3 injections phosphorus solution	3	65.2 + 2.0	homogenate	29.9 ± 0.04
			mitochondria	6.7 ± 0.04

nate (c) oxidation of malate to oxaloacetate. It is clear that an eventual damage of the Krebs cycle represents an obstacle also for the normal oxidation of fatty acids.

The cause of the decrease of pyridine nucleotides in fatty livers is not completely clear. Possible causes may be grouped in 2 principal mechanisms: (a) decrease of the rate of synthesis, (b) increase of the rate of decomposition. Since the synthesis of DPN *in vitro* through the KORNBERG reaction is not diminished, a possible damage to the responsible enzyme, which has been found to be situated in the nucleus (HOGEBOOM AND SCHNEIDER[34]), can be excluded. The possibility exists, however, that the rate of the Kornberg reaction is decreased *in vivo* as a consequence of the decrease of ATP concentration. As oxidative phosphorylation is uncoupled in mitochondria of fatty livers *in vitro*, it is highly probable that the same lesion is operating *in vivo*. Further experiments to verify this hypothesis are in progress.

The rate of decomposition of DPN is also not modified *in vitro*, but also in this case it cannot be excluded that the imbalance of the Kornberg reaction is modified *in vivo*

References p. 405.

VII

OF NORMAL AND FATTY LIVERS OF RAT

corresponding fraction. The homogenates were prepared with 0.25 *M* sucrose.

Homogenate values per mg nitrogen	*Nuclear fraction*	*Mitochondria*	*Microsomes*	*Supernatant*
460.0 ± 3	67 ± 4	0	67.0 ± 3	51.0 ± 3.0
124.3 ± 2.0	18.8 ± 3.4	19.0 ± 0.7	15.0 ± 2.4	32.4 ± 3.7
388.0 ± 6.2	35.0 ± 7.2	0	52.0 ± 2.5	47.0 ± 2.5
109.3 ± 2.0	18.0 ± 1.3	21.0 ± 1.6	22.0 ± 1.8	31.0 ± 3.1
456.0 ± 8.3	48.0 ± 3.3	0	59.0 ± 3.1	46.0 ± 2.0
105.4 ± 2.2	16.0 ± 3.0	18.6 ± 0.9	13.0 ± 2.2	25.0 ± 1.4
442.0 ± 10.0	50.0 ± 1.6	0	54.0 ± 3.5	45.0 ± 3.1
108.7 ± 1.5	15.0 ± 1.8	21.0 ± 2.5	14.0 ± 1.6	31.0 ± 1.5

VIII

PYRUVATE BY NORMAL AND FATTY LIVERS OF RAT

N contents are given as mg per g of wet liver. The homogenates were prepared with 0.25 *M* sucrose.

L(+)malate			*v-ketoglutarate*			*pyruvate*		
without DPN added	*with DPN added*	*Increase %*	*without DPN added*	*with DPN added*	*Increase %*	*without DPN added*	*with DPN added*	*Increase %*
30.6 ± 1.0	58.5 ± 2.1	88.8 ± 4.4	24.8 ± 0.7	42.2 ± 1.3	70.2 ± 2.1	30.9 ± 1.3	35.8 ± 1.5	23.9 ± 2.1
89.3 ± 3.9	134.6 ± 3.7	50.7 ± 5.0	104.2 ± 3.5	138.2 ± 4.5	32.6 ± 1.1	104.6 ± 8	121.0 ± 7.9	15.6 ± 1.5
19.5 ± 1.5	50.2 ± 4.8	157.4 ± 13.6	18.1 ± 1.3	38.5 ± 2.3	112.7 ± 8	16.3 ± 1.3	25.4 ± 1.3	55.8 ± 5.6
40.6 ± 0.6	134.8 ± 1.7	232.0 ± 5.2	89.4 ± 4.9	169.2 ± 8.1	89.2 ± 15	72.4 ± 3.6	128.4 ± 11	77.3 ± 7
22.5 ± 1.4	62.4 ± 4.8	173.3 ± 2.7	19.1 ± 0.1	51.9 ± 4.6	171.7 ± 26	20.8 ± 0.2	31.3 ± 0.7	50.0 ± 1.9
43.1 ± 3.9	134.3 ± 7.0	211.6 ± 5.8	49.8 ± 10	122.7 ± 5	146.4 ± 8	37.1 ± 1.3	91.4 ± 6.2	173.3 ± 8.8
23.8 ± 0.6	67.6 ± 3.1	184.9 ± 26.6	20.9 ± 0.3	55.6 ± 1.3	166.0 ± 5.1	21.9 ± 0.3	45.6 ± 3.0	108.3 ± 12
37.4 ± 2.5	173.2 ± 1.2	370.3 ± 28.2	55.1 ± 0.5	149.7 ± 4.5	171.7 ± 4.8	22.2 ± 1.3	81.9 ± 2.2	268.9 ± 1.0

towards the pyrophosphorolytic cleavage of DPN. Another possible cause which may be considered as responsible for the decrease of pyridine nucleotides is the partial displacement of these compounds from mitochondria into the supernatant fluid, where the DPNase is very active.

The modification of the PN/PNH ratio may be particularly important in order to understand the mechanism of the fat accumulation within the cells. In fact, proportional increase of the reduced form of DPN may provoke predomination of synthesis as compared to breakdown of fatty acids (LYNEN[35]).

The cause of the decrease of the PN/PNH ratio is also difficult to explain, but it is probable that the loss of both DPN and cytochrome *c* from mitochondria, while the protein part of the enzymes is almost normal, may be responsible for the reduction of remaining DPN. Another possible cause may be the selective decomposition of the oxidized form of pyridine nucleotides which have escaped from mitochondria by DPNase contained in microsomes and in supernatant fractions. In fact, DPNase is active only

References p. 405.

on the oxidized form of pyridine nucleotides, and cannot attack the reduced form (McILWAIN AND RODNIGHT[36], ZATMAN, KAPLAN AND COLOWICK[37]).

Further experiments to study the problem of the functioning *in vivo* of the Krebs cycle in animals with fatty livers are in progress.

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SUMMARY

Oxidation of some substrates requiring DPN (glutamate, malate, pyruvate, ketoglutarate) is strongly decreased in fatty livers of rats, obtained either by injection of steatogenic poisons (CCl_4, P) or by feeding with a diet deficient of choline. The decrease of oxidative power is particularly strong in mitochondria. Addition to these mitochondria of the supernatant fluid produces stimulation of the oxidative activity. Almost complete restoration of activity is produced both in homogenates and in mitochondria by addition of DPN. The concentration of pyridine nucleotides in fatty livers is strongly decreased. The extent of this decrease is particularly remarkable in mitochondria. A redistribution of pyridine nucleotides, with displacement of these substances from mitochondria into the supernatant fluid, occurs in fatty liver homogenates. The PN/PNH ratio is decreased in fatty livers. Synthesis of DPN *in vitro* through the KORNBERG reaction and destruction of DPN are not modified. Both decrease of oxidation of glutamate and decrease and displacement of DPN occur in the livers of treated animals before the beginning of the accumulation of fat within the cells. The possible causes for the described phenomena and their importance for the pathogenesis of liver steatosis are discussed.

RÉSUMÉ

L'oxydation de quelques substrats qui demandent le DPN (glutamate, malate, pyruvate, cétoglutarate) est diminuée remarquablement dans le foie gras, obtenu soit à la suite d'un traitement au CCl_4 ou avec phosphore, soit par alimentation avec une nourriture dépourvue de choline. La diminution est particulièrement forte dans les mitochondries isolées. Si on ajoute aux mitochondries de la liqueur surnageante, on obtient une stimulation de l'oxydation. Une restauration presque complète de l'activité est produite par l'addition de DPN.

La concentration de pyridine-nucléotides dans le foie gras est fortement diminuée, et le pourcentage de diminution est plus haut dans les mitochondries que dans l'homogénat entier. On observe dans le foie gras une redistribution des pyridine-nucléotides, charactérisée par le passage d'une partie de ces substances des mitochondries à la liqueur surnageante. Le rapport PN/PNH est diminué dans le foie gras. La synthèse de DPN *in vitro* n'est pas diminuée et sa destruction n'est pas augmentée. La modification de la distribution des pyridine-nucleotides et la diminution de ces substances précèdent l'accumulation de graisse dans les cellules. L'auteur discute les causes qui peuvent avoir déclenché les phénomènes observés.

ZUSAMMENFASSUNG

Die Oxydation einiger Substrate die DPN reduzieren (Glutaminsäure, Äpfelsäure, Benztraubensäure, α-Ketoglutarsäure) ist in Fettlebern von Ratten stark vermindert, unabhängig von der Art der Behandlung die die Leberverfettung verursacht hat (CCl_4- oder Phosphor-Vergiftung, Nährung mit einem Cholin-freien Futter). Die Verminderung der Oxydation ist in Mitochondrien besonders stark. Der Zusatz der überstehenden Flüssigkeit zu diesen Mitochondrien stimuliert die oxydative Aktivität. Der Zusatz von DPN verursacht eine fast vollständige Wiederherstellung der normalen Aktivität. Der Gehalt der Leber an Pyridinnucleotiden ist bei Leberverfettung stark herabgesetzt; auch diese Verminderung ist in den Mitochondrien besonders stark. Die Verteilung der Pyridinnucleotide zwischen den verschiedenen Cytoplasma-Fraktionen ist bei Leberverfettung verändert: der Gehalt der Mitochondrien an Pyridinnucleotiden ist vermindert und derjenige der überstehenden Flüssigkeit ist gesteigert. Das Verhältnis PN/PNH ist bei Fettlebern vermindert. Die Synthese und die Spaltung des DPN *in vitro* sind in Fettlebern nicht verändert. Die Verminderungen der Oxydation der Glutaminsäure und des DPN-Gehaltes finden schon in der Leber der behandelten Tiere statt, wenn die Anhäufung der Lipoide in den Leberzellen noch nicht bemerkbar ist.

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