TABLE 1

R_F VALUES

(40% butanol—10% ethanol—50% water [neutral] Whatman No. 1 filter paper downward migration)

	R_F	Silver Nitrate	Aniline Hydrogen Phthalate	Ninhydrin
3:4-dimethyl-D-mannose	0.53	+	+	−
3:4:6-trimethyl-D-mannose	.70	+	+	−
2:3:4:6-tetramethyl-D-mannose	.68	+	+	−
2:3-dimethyl-D-glucose	.51	+	+	−
2:3:4-trimethyl-D-glucose	.70	+	+	−
2:3:6-trimethyl-D-glucose	.66	+	+	−
2:3:4:6-tetramethyl-D-glucose	.85	+	+	−
1:3:4-trimethyl-D-fructose	.67	+	−	−
1:3:4:6-tetramethyl-D-fructose	.75	+	−	−
D-glucosamine hydrochloride	.1	+	−	+
N-acetyl-D-glucosamine	.24	+	−	+
N-carbobenzoxy α methyl glucosaminide	.82	−	−	−
3-methyl N-acetyl glucosamine	.34	+	−	−
4:6-dimethyl N-acetyl glucosamine	.50	+	+	−
3:4:6 trimethyl N-acetyl glucosamine	.72	+	+	−
glucosaminic acid	.02	−	−	+
α methyl 2(acetamido-) glucopyranoside	.31	−	−	+
β methyl 2-(acetamido-) glucofuranoside	.42	±	±	−
N-(2: 4 dinitrophenyl) D-glucosamine	.75	+	+	−
1:3:4:6 tetracetyl DNP-D-glucosamine	.93	+	+	−
DNP glucosamine diethyl mercaptal	.89	−	−	−
tetracetyl DNP glucosamine diethyl mercaptal	.96	−	−	−
DNP glucosaminic acid	.60	−	−	−
DNP chondrosamine	.61	+	+	−
1:3:4:6-tetracetyl DNP chondrosamine	.92	+	+	−
DNP glycine	.42	−	−	−
DNP tyrosine	.77	−	−	−
DNP histidine	(i) .64 (ii) .85	−	−	−
DNP arginine	.65	−	−	−
DNP valine	.72	−	−	−
DNP aspartic acid	.28	−	−	−
DNP alanine	.58	−	−	−
DNP tryptophane	0.69	−	−	−

readily separated using Whatman No. 1 filter paper and neutral N-butanol (40%)—ethanol (10%)—water (50%) as the eluting agent. The yellow band could be observed directly, and on spraying the chromatogram with aniline hydrogen phthalate the other sugars were revealed and the identity of the colored band confirmed as an amino sugar derivative by its specific color change from yellow to brown.

A typical mixture separated has the following composition: glucose (0.31 mg), rhamnose (1.44 mg), arabinose (0.26 mg), 2:3:4:6 tetramethyl glucose (0.23 mg), DNP tetracetylglucosamine (0.35 mg).

The reaction with 2:4-dinitrofluorobenzene could be carried in the presence of neutral sugars. Thus a mixture containing, for example, glucosamine (2 mg), arabinose (4 mg), glucose (10 mg), and glucuronic acid (5 mg) in 1.0 ml 1% sodium bicarbonate and 1.0 ml ethanol reacted at room temperature when shaken with 2:4-dinitrofluorobenzene (0.05 ml, i.e., excess). Two drops of the resulting reaction mixture were applied to the chromatogram. The DNP amino sugar was clearly separated, the unchanged fluoro reagent moved with the solvent front, and the remaining sugars were observed by treatment with aniline hydrogen phthalate.

It was also found possible to separate DNP glucosamine from DNP chondrosamine and free amino acids, the latter being observed by their coloration with ninhydrin.

The problem of the separation of DNP amino sugars and DNP amino acids (*6*) has been investigated. This involved the determination of the R_F values of numerous DNP amino acids as summarized in Table 1. It was found that a distinct separation could be achieved between many DNP amino acids, DNP amino sugars, and free amino acids. DNP amino acids were without effect on silver nitrate solution, aniline hydrogen phthalate, or ninhydrin.

The results are being applied to the investigation of the hydrolyzates from various mucopolysaccharides.

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Proteins of Liver and Hepatoma Mitochondria

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The cytoplasmic inclusions known under the term mitochondria or chondriosomes are among the most prominent and widely distributed structures of living cells (*1*). As a result of cytological studies carried out in the past 50 years, a number of theories have arisen concerning the physiological significance of mitochondria, including the still-tenable and intriguing view that they are self-duplicating and autonomous cell organelles (*2*). Until recently, however, most ideas pertaining to their function have been based largely on conjecture.

With the advent of improvements in the technique of cell fractionation by means of differential centrifugation, permitting the isolation of intracellular components in cytologically defined states (*3*), the importance of the role played by the mitochondrion in cellular metabolism has become increasingly apparent.

TABLE 1

SEDIMENTATION CONSTANTS OF MITOCHONDRIAL PROTEINS

Preparation	Method of disintegration of mitochondria	No. experiments	Sedimentation constant*				Range of total protein concentration ($N \times 6.25$) (percentage)
			Component 1	Component 2	Component 3	Component 4	
Normal liver mitochondria	Apparatus of Milner *et al.* (*cf.* Fig. 1*A*)	4‡	3.8 (3.7–3.9)	5.1 (5.0–5.3)	7.5† (6.9–8.1)	*Ca.* 10–11†	1.4–2.3
	Sonic vibrations (*cf.* Fig. 1*B*)	3§	3.7 (3.6–3.8)	6.28 (6.24–6.33)	Absent	*Ca.* 11–13†	1.7–2.2
Hepatoma 98/15 mitochondria	Apparatus of Milner *et al.* (*cf.* Fig. 2*A*)	4‖	3.7 (3.6–3.8)	Absent	7.6† (7.2–8.1)	*Ca.* 12–15†	0.6–2.6
	Sonic vibrations (*cf.* Fig. 2*B*)	3¶	3.8 (3.8–3.9)	"	8.0† (7.4–8.4)	*Ca.* 12–13†	0.5–1.6

* Values are corrected to water and 20° and are expressed as Svedberg units.

† Polydisperse.

‡ Solvents included 0.1 *M* KCl – 0.002 *M* KH_2PO_4 – 0.016 *M* K_2HPO_4, pH 7.7; 0.1 *M* KCl – 0.05 *M* sodium veronal – 0.01 *M* veronal, pH 8.6; 0.15 *M* KCl; 0.004 *M* KH_2PO_4 – 0.032 *M* K_2HPO_4, pH 7.7; 0.1 *M* sodium veronal – 0.02 *M* veronal, pH 8.6.

§ Solvents included 0.002 *M* KH_2PO_4 – 0.016 *M* K_2HPO_4, pH 7.7; 0.1 *M* KCl – 0.002 *M* KH_2PO_4 – 0.016 *M* K_2HPO_4, pH 7.7.

‖ Solvents included 0.1 *M* KCl – 0.002 *M* KH_2PO_4 – 0.016 *M* K_2HPO_4, pH 7.7; 0.1 *M* KCl – 0.05 *M* sodium veronal – 0.01 *M* veronal, pH 8.6; 0.008 *M* KH_2PO_4 – 0.064 *M* K_2HPO_4, pH 7.7.

¶ Solvents included 0.004 *M* KH_2PO_4 – 0.032 *M* K_2HPO_4, pH 7.7; 0.1 *M* KCl – 0.002 *M* KH_2PO_4 – 0.016 *M* K_2HPO_4, pH 7.7.

Among the enzyme systems and related compounds associated with mitochondria isolated from rat or mouse liver are cytochrome oxidase (*4–6*), cytochrome c (*7, 8*), succinoxidase (*3–6*), octanoic acid oxidase (*9, 10*), oxalacetic acid oxidase (*11*), glutamic dehydrogenase (*12*), adenosinetriphosphatase (*5, 13*), triphosphopyridine nucleotide-cytochrome c reductase (*14*), diphosphopyridine nucleotide-cytochrome c reductase (*15, 16*), riboflavin (*17*), and a relatively small amount of pentose nucleic acid (*3, 5, 13*). It is thus evident that the mitochondrion is responsible for at least several of the phases of the Krebs cycle and, through its content of cytochrome oxidase and related systems, accounts for most if not all of the respiratory activity of the cell. Furthermore, certain aerobic reactions carried out by preparations of isolated mitochondria, including the synthesis of *p*-aminohippuric acid and the maintenance of high-energy phosphate bonds (*18*) lead to the suggestion that additional important synthetic reactions may take place in this cellular component.

A problem of considerable importance concerns the possible changes in biochemical properties undergone by the mitochondrion during the process of neoplasia. Along this line, a series of experiments has been initiated in which a comparison is being made of the biochemical propertities of cell fractions derived from normal C3H mouse liver and from C3H mouse hepatoma 98/15. This tumor was considered particularly suitable for such an investigation because of its high degree of differentiation and its histologic resemblance to normal liver (*19*); thus any marked differences between these two closely related tissues are probably more significant than differences of the same magnitude between a normal tissue and an anaplastic tumor. To date the results have indicated that several of the quantitative biochemical differences between the two tissues are a reflection of differences in the mitochondria (*6, 13, 16*). Similar data have been reported for the mitochondria of rat liver and rat liver tumors induced with carcinogenic azo dyes (*20*). In the latter case it was also found that alterations characteristic of tumor mitochondria were present, although to a lesser degree, in the liver mitochondria of animals fed carcinogenic azo dyes for periods insufficient to produce tumors (*21*). Furthermore, the extent of these mitochondrial alterations was roughly proportional to the carcinogenicity of the dyes fed (*21*). Thus the implication is made that mitochondria are intimately concerned in the carcinogenic process and not merely altered as a result of the carcinogenic process. These findings lend added significance to studies of the mitochondria of normal liver and hepatoma.

A consideration of certain aspects of the structure of mitochondria has recently led to a new method of study of these cellular elements. Previous cytological observations of the behavior of the isolated particles when subjected to changes in the osmotic pressure of the suspending media (*3, 22*) have provided evidence for the existence of a well-defined mitochondrial membrane. This evidence has been substantiated by electron microscopy (*23, 24*) which, in addition, has revealed the presence of internal structures of a particulate nature. In the latter respect, Claude (*22*) found that after prolonged contact with a relatively large volume of distilled water, mitochondria isolated from guinea pig liver underwent lysis, yielding particulate material of small size and a soluble fraction that in-

cluded proteins and dialyzable compounds. This finding suggested that the membranes of isolated mitochondria are capable of retaining soluble substances, including compounds of relatively low molecular weight. In fact, as noted in a recent publication (*25*), the disruption by means of intense sonic vibrations of the membranes of rat liver mitochondria resulted in the release into solution of approximately 60% of the total nitrogen. Furthermore, most of the nitrogen represented proteins that could be separated and characterized on the basis of sedimentation constants in the analytical centrifuge. The latter finding offered a unique method for the comparison of mitochondria of tumors and normal tissues and has been applied with the following results to C3H mouse liver and hepatoma 98/15.

Homogenates of perfused C3H mouse liver (10–15 livers) and of hepatoma 98/15 (30–50 small, nonnecrotic hepatomas) were prepared in 0.25 *M* sucrose and were fractionated according to a procedure described previously (*6*). The mitochondria were washed twice with 0.25 *M* sucrose and were finally suspended either in distilled water or in dilute potassium phosphate buffer (0.002 *M* KH_2PO_4 – 0.016 *M* K_2HPO_4, pH 7.7). The suspensions were then either forced through a small orifice under 20,000 psi pressure at 3° in a stainless steel apparatus similar to that described by Milner *et al.* (*26*) or were subjected to sonic vibrations (9 kc) for 15 min at 3° in a Raytheon magnetostriction oscillator, Type R-22-3. After either of these procedures, the originally opaque suspensions of mitochondria were only slightly opalescent and contained few particles large enough to be seen in the phase contrast microscope. The preparations were then centrifuged at 3° for 30 min at 115,000–150,000 × *g* in the Type A or C preparative rotor of the Spinco ultracentrifuge, yielding gelatinous, transparent, reddish pellets and clear yellow supernatants, the latter containing 50–65% of the original total nitrogen. The supernatants were then dialyzed against appropriate solutions (Table 1). Ten to 20% of the nitrogen diffused through cellophane during dialysis, but most if not all of the colored substances were retained. At the same nitrogen concentration the supernatant obtained from normal liver mitochondria was much more deeply colored than that from hepatoma mitochondria.

The dialyzed supernatants were then studied in the Spinco analytical ultracentrifuge at a speed of 59,780 rpm. Figs. 1 and 2 are typical photographs showing the sedimentation of the soluble proteins obtained from the mitochondria of normal liver and of hepatoma 98/15. Table 1 summarizes the results of 14 separate experiments with the two tissues. As shown in Fig. 1*A*, most of the protein obtained from normal liver mitochondria by disintegration in the apparatus of Milner *et al.* (*26*) was represented by a slowly sedimenting, broad peak, which eventually was resolved into two distinct components (Nos. 1 and 2, Fig. 1*A*). These components were present in approximately equal concentration and were characterized by average sedimentation constants of 3.8 and 5.1 *S* (Svedberg units) (Table 1). Two other, smaller peaks (Nos. 3 and 4, Fig. 1*A*), both of which appeared to be polydisperse, sedimented at more rapid rates. The average sedimentation constant of component No. 3 was 7.5 *S*, the rather wide range shown in the table probably being due to difficulties in precisely locating the position of this peak. The sedimentation constant of component No. 4 (Fig. 1*A*) could not be determined accurately but was estimated to be approximately 10–11 *S* (Table 1). A variety of solvents was used without appreciable effect on the sedimentation constants (Table 1). Disintegration of the mitochondria in phosphate buffer rather than in water also had no effect on the results. In additional experiments it was found that the soluble proteins of mitochondria isolated from regenerating C3H mouse liver gave a sedimentation pattern identical with that obtained with normal liver mitochondria (Fig. 1*A*).

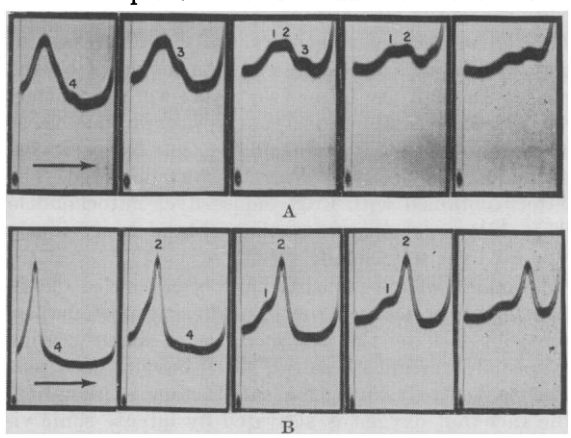

FIG. 1. Sedimentation of proteins of normal liver mitochondria. Photographs taken at 16-min intervals; speed, 59,780 rpm; solvent, 0.1 *M* KCl – 0.002 *M* KH_2PO_4 – 0.016 *M* K_2HPO_4. *A*, mitochondria disintegrated in water by apparatus of Milner *et al.*; total protein concentration, 1.5%; av temp during run, 20.6°. *B*, mitochondria disintegrated in water by sonic vibrations; total protein concentration, 1.7%; av temp during run, 21.2°.

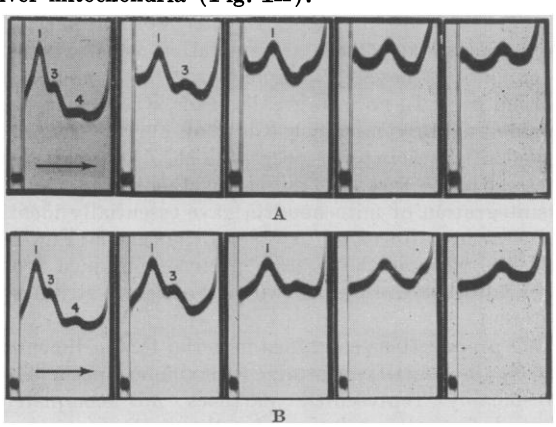

FIG. 2. Sedimentation of proteins of hepatoma mitochrondria. Photographs taken at 16-min intervals; speed 59,780 rpm; solvent, 0.1 *M* KCl – 0.002 *M* KH_2PO_4 – 0.016 *M* K_2HPO_4. *A*, mitochondria disintegrated in water by apparatus of Milner *et al.*, total protein concentration, 1.5%; av temp during run, 20.0°. *B*, mitochondria disintegrated in water by sonic vibrations; total protein concentration, 1.6%; av temp during run, 22.2°.

A distinctly different pattern was obtained when normal liver mitochondria were distintegrated by means of sonic vibrations (Fig. 1*B*). In this case most of the protein was represented by a sharp, symmetrical peak (No. 2, Fig. 1*B*), having a sedimentation constant significantly higher (Table 1) than that of component No. 2 of Fig. 1*A*. Also present were two other components, the first sedimenting slowly (No. 1, Fig. 1*B*), the second sedimenting rapidly and present in small amounts (No. 4, Fig. 1*B*). As indicated by the data of Table 1, the latter two peaks were characterized by sedimentation constants approximating those of components Nos. 1 and 4 of Fig. 1*A*. No peak corresponding to No. 3 of Fig. 1*A* was observed. The pattern obtained with C3H mouse liver mitochondria (Fig. 1*B*) was similar to that obtained previously with rat liver mitochondria (*25*).

A number of experiments have been carried out in an attempt to account for the different protein patterns obtained by the two methods of disintegration of normal liver mitochondria, but it has not been possible as yet to arrive at a satisfactory explanation. The fact that oxygen is activated by intense sonic vibrations (*27*) brings up the possibility that this or a similar chemical reaction might account for the present results. In view of previous findings (*25*) and of the fact that mitochondrial preparations show high catalase activity (*28*), it seems unlikely, however, that oxygen activation could play an important role. It should also be pointed out that, of the two methods of disintegration of liver mitochondria, only sonic vibrations yielded a component whose behavior in the analytical centrifuge suggested a homogeneous protein (component No. 2, Fig. 1*B*).

As shown in Fig. 2, three components were observed in the preparations obtained from hepatoma mitochondria, the most prominent being represented by a reasonably sharp, slowly sedimenting peak (No. 1) corresponding in sedimentation constant to component No. 1 of normal liver (Table 1). The two remaining peaks (Nos. 3 and 4, Fig. 2) were apparently polydisperse and gave sedimentation constants approximating those of components Nos. 3 and 4 of normal liver. In no instance, however, was a peak corresponding to component No. 2 of normal liver detected in the hepatoma preparations. Of additional interest is the fact that the two methods used for the disintegration of mitochondria gave essentially identical protein patterns in the case of the tumor (Fig. 2*A* and *B*), whereas the protein patterns obtained from liver mitochondria by the two methods were strikingly different.

All preparations contained material that sedimented before the centrifuge attained maximum speed. This presumably represented particles not completely cleared from the solutions by the preliminary preparative centrifugation. In addition, there was present a variable amount of material of relatively low molecular weight that did not completely clear the meniscus (*cf.* Fig. 2*A*). The rapidly sedimenting particles and the low molecular weight material accounted for a maximum of 10–30% of the total nitrogen of the individual dialyzed preparations.

It is evident from the present results that, within the limits of sensitivity of the analytical centrifuge, a qualitative difference exists between the mitochondria of hepatoma 98/15 and the mitochondria of normal C3H mouse liver. This conclusion is indicated by the fact that a prominent component of the proteins of normal liver mitochondria could not be detected in corresponding preparations obtained from hepatoma mitochondria and is further supported by the fact that liver mitochondria responded differently to the two methods of disintegration, whereas hepatoma mitochondria did not. Whether the apparent absence of component No. 2 (Fig. 1) from the tumor represents a difference of fundamental significance between this tissue and normal or regenerating liver, or represents simply the loss of some specialized function incidental to the process of neoplasia, is a problem for the future. In an attempt to answer the questions arising from the present investigation, studies of the biochemical properties of the mitochondrial proteins are being conducted, and other methods designed to provide independent verification of the results, including electrophoretic analysis and immunological tests, are being explored.

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