A STUDY OF THE NUCLEOSIDE TRI- AND DIPHOSPHATE ACTIVITIES OF RAT LIVER MICROSOMES

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ABSTRACT

Rat liver microsomes catalyze the hydrolysis of the triphosphates of adenosine, guanosine, uridine, cytidine, and inosine into the corresponding diphosphates and inorganic orthophosphate. The activities are stimulated by $Na_2S_2O_4$, and inhibited by atebrin, chlorpromazine, sodium azide, and deaminothyroxine. Sodium deoxycholate inhibits the ATPase activity in a progressive manner; the release of orthophosphate from GTP and UTP is stimulated by low, and inhibited by high, concentrations of deoxycholate, and that from CTP and ITP is unaffected by low, and inhibited by high, concentrations of deoxycholate. Subfractionation of microsomes with deoxycholate into ribosomal, membrane, and soluble fractions reveals a concentration of the triphosphatase activity in the membrane fraction. Rat liver microsomes also catalyze the hydrolysis of the diphosphates of the above nucleosides into the corresponding monophosphates and inorganic orthophosphate. Deoxycholate strongly enhances the GDPase, UDPase, and IDPase activities while causing no activation or even inhibition of the ADPase and CDPase activities. The diphosphatase is unaffected by $Na_2S_2O_4$ and is inhibited by azide and deaminothyroxine but not by atebrin or chlorpromazine. Upon fractionation of the microsomes with deoxycholate, a large part of the GDPase, UDPase, and IDPase activities is recovered in the soluble fraction. Mechanical disruption of the microsomes with an Ultra Turrax Blender both activates and releases the GDPase, UDPase, and IDPase activities, and the former effect occurs more readily than the latter. The GDPase, UDPase, and IDPase activities of the rat liver cell reside almost exclusively in the microsomal fraction, as revealed by comparative assays of the mitochondrial, microsomal, and final supernatant fractions of the homogenate. The microsomes exhibit relatively low nucleoside monophosphatase and inorganic pyrophosphatase activities, and these are unaffected by deoxycholate or mechanical treatment. Different approaches toward the function of the liver microsomal nucleoside tri- and diphosphatases are reported, and the possible physiological role of the two enzymes is discussed.

Occurrence of a Mg^{++}-activated ATPase[1] in rat liver microsomes was first demonstrated by Novikoff *et al.* (1) in 1952. Later, Abood and Romanchek (2) examined this enzyme in conjunction with studies of cytoplasmic fibrillar activities, and Reid *et al.* (3) in relation to hormonal status. Ernster *et al.* (4) recently investigated the distri-

[1] Abbreviations: A, adenosine; C, cytidine; G, guanosine; I, inosine; U, uridine; MP, monophosphate; DP, diphosphate; TP, triphosphate; P_i inorganic orthophosphate DOC, deoxycholate; NADH, diphosphopyridine nucleotide, reduced form; NADPH, triphosphopyridine nucleotide, reduced form; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; tris buffer, tris(hydroxymethyl)aminomethane.

bution of this ATPase in subfractions of deoxycholate-treated microsomes and found it to be concentrated in a membrane fraction which also contained the bulk of the NADH–cytochrome *c* reductase and glucose-6-phosphatase.

Continued interest in studies of the liver microsomal ATPase arose from the recognition of striking similarities between this enzyme and the Mg^{++}-activated ATPase of liver mitochondria (5) in regard to several properties such as nucleoside non-specificity (6), stimulation by sodium dithionite (7, 8), and inhibition by atebrin (8), chlorpromazine (9), azide (1, 10), and deaminothyroxine (11). It was also felt to be of interest to compare this ATPase with microsomal ATPases from other tissues, primarily skeletal muscle (12–14) and nervous tissue (15–22), which have lately been intensely studied in relation to muscle fiber relaxation and active ion transport, respectively.

In the course of our investigations, we also detected a nucleoside diphosphatase in the microsomes. This enzyme reacts predominantly with GDP, UDP, and IDP, and is present in the freshly prepared microsomes in a latent state, its activity being greatly enhanced by various treatments of the microsomes. A diphosphatase with this substrate specificity has been known for some time (23–25) but its microsomal localization has not been recognized. Yet very recently Novikoff and Goldfischer (26) presented histochemical evidence for a concentration of this enzyme in the cytoplasmic membranes of the Golgi region.

It is the purpose of this paper to describe our studies along the above lines and to discuss some functional aspects of the nucleoside tri- and diphosphatase activities of liver microsomes. A preliminary account of this work has appeared (27).

EXPERIMENTAL

Preparation Procedures

Wistar rats (150 to 200 gm) of both sexes were used. Preparation of liver microsomes, and, when desired, fractionation of the microsomes with deoxycholate, were performed as previously described (4). Mechanical disruption of microsomes was carried out with an Ultra Turrax Blender (24,000 RPM) in the cold for 1 minute, using a suspension of microsomes in 0.035 M tris buffer, pH 7.5 (about 4 mg microsomal protein/ml medium). Centrifugation after treatment was made in a Spinco model L centrifuge, rotor 40, at 105,000 *g* for 1 hour.

When mitochondrial and microsomal fractions were both desired, the procedure described by Ernster *et al.* (28) was employed. The protein content of the microsomes and mitochondria (as estimated by the biuret method (29)) was 15 to 25 mg protein/gm liver and 20 to 30 mg/gm liver, respectively.

Assays and Analyses

The standard assay system for the nucleoside di- and triphosphatase activities contained the following: 5 mM nucleoside di- or triphosphate (purchased from Sigma Chemical Co.), 4 mM $MgCl_2$, 50 mM tris buffer, pH 7.5, and microsomes or microsomal subfraction corresponding to 40 mg wet weight liver, in a final volume of 0.5 ml. Incubation was made at 30°C for 20 minutes, after which time the samples were fixed with 0.5 ml cold 1 M perchloric acid, and transferred to an ice bath. P_i was determined according to the modified (30) Martin and Doty (31) method.

Glucose-6-phosphatase activity was assayed as described previously (4).

RESULTS

Effects of Cations

Fig. 1 *a* illustrates the effect of varying concentrations of Mg^{++} on the ATPase activity of rat liver microsomes. Maximal activity ensued at a Mg^{++}:ATP ratio of 1. This finding is in accordance with those earlier reported with the microsomal ATPases from skeletal muscle (12) and nervous tissue (16), although with rat brain microsomes, as recently found by Järnefelt (21), only that part of the ATPase requires Mg^{++} for maximal activity which is not stimulated by Na^{+}. Comparison of the present data with the Mg^{++} requirement of the ATPase of structurally damaged (DOC-treated) rat liver mitochondria (10) is difficult, for, there, two optimal Mg^{++} concentrations are found, one at about the level of that of ATP, and the other considerably lower. The maximal liver microsomal ATPase activity was linear with the enzyme concentration, and ranged from 20 to 40 μmoles ATP split in 20 minutes per gram liver, or 1 to 2 μmoles ATP split in 20 minutes per milligram microsomal protein. On a protein basis, this activity is about 2.5 to 5 times lower than the maximal Mg^{++}-stimulated ATPase activity of rat liver mitochondria (elicited by treating the mitochondria with 0.1 per cent DOC). This situation is opposite to that found with skeletal muscle and brain, where the microsomal

ATPase activity exceeds several times the maximal ATPase activity of the mitochondria.

As shown in Fig. 1 *b*, the ATPase activity of liver microsomes was also stimulated to some extent by Ca^{++}, whereas the same ion slightly inhibited the enzyme when this was maximally activated by Mg^{++}. Strong inhibition by Ca^{++} has been reported in the case of the muscle microsomal ATPase (12), while in the case of brain microsomes Ca^{++} has been shown to inhibit only the Na^{+}-stimulated fraction of the ATPase activity (20). With mitochondria, Ca^{++} has been found to stimulate the ATPase activity (32), but this effect, again, is not readily comparable with that found with the microsomes, since in mitochondria Ca^{++} induces structural damage and thereby promotes the manifestation of the latent ATPase.

ATPases which are stimulated by Na^{+} or Na^{+} + K^{+} have been described as occurring in membraneous elements of nervous tissue (15–22), kidney (33, 34) and red blood cells (35). When rat liver microsomes were incubated in a Na^{+}- and K^{+}-free medium, added Na^{+} (20 to 100 mM), alone or in combination with K^{+} (5 mM), had no effect on the ATPase activity. However, a Na^{+}-stimulated ATPase has recently been demonstrated in a "cell membrane" fraction from liver, obtained by differential centrifugation under special conditions (36).

Effects of Various Compounds

Myers and Slater (7) and Löw (8) have demonstrated a stimulation, by about 50 per cent, of the Mg^{++}-activated ATPase of rat liver mitochondria by $Na_2S_2O_4$. A similar effect of $Na_2S_2O_4$ is obtained with the microsomal ATPase, as shown in Fig. 2. As in the case of mitochondria, the $Na_2S_2O_4$ effect was not duplicated by enzymically acting reducing agents such as NADH or NADPH, nor was the ATPase activity an the absence of $Na_2S_2O_4$

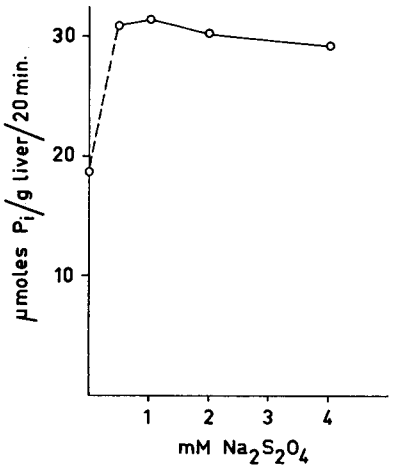

FIGURE 2

Stimulation of microsomal ATPase by $Na_2S_2O_4$.

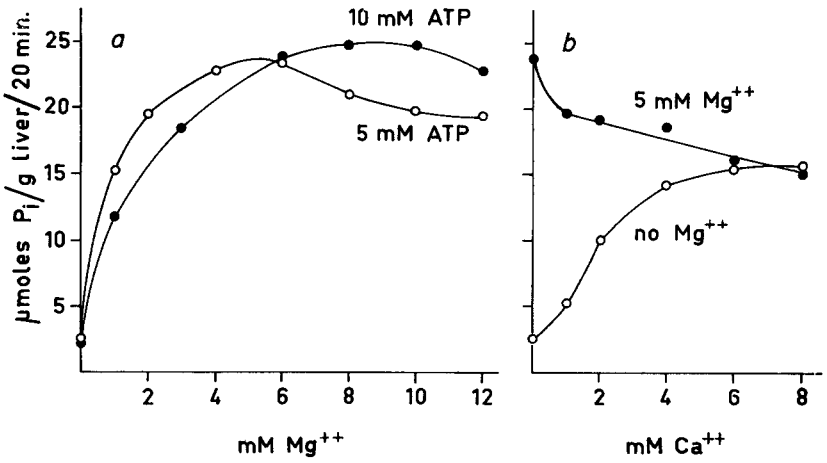

FIGURE 1

a. Activation of rat liver microsomal ATPase by Mg^{++}.

b. Effect of Ca^{++} on microsomal ATPase in presence and absence of Mg^{++}. Concentration of ATP, 5 mM.

lowered by oxidizing agents such as 2,6-dichlorphenolindolphenol, cytochrome *c*, FAD, FMN, or the oxidized form of the pyridine nucleotides.

Atebrin (8), chlorpromazine (9), azide (1, 10), and deaminothyroxine (11) have previously been found to inhibit the Mg^{++}-activated liver mitochondrial ATPase. The effects of the same compounds on the liver microsomal ATPas are illustrated by the data in Fig. 3. Atebrin and chlorpromazine, both of which are inhibitors of flavoenzymes, suppressed the microsomal ATPase activity to an extent comparable to the effects found with mitochondrial ATPase; atebrin gave half inhibition at a concentration of about 1 mM, and chlorpromazine at a concentration of about 0.25 mM. Because of reports in the literature that high concentrations of atebrin may inhibit enzymes unrelated to flavin, such as glucose-6-phosphate dehydrogenase (37, 38) and aliesterase (38), it was of interest to test the effect of atebrin on another microsomal enzyme, glucose-6-phosphatase. It can be seen in Fig. 3 that atebrin up to a concentration of 8 mM did not inhibit this enzyme.

Another point of interest in connection with the atebrin inhibition of the microsomal ATPase was the effect of added flavin nucleotides. FMN or FAD in concentrations up to 5 mM, which had no effect on the ATPase activity when added alone, did not counteract the inhibition caused by 4 mM atebin. This finding is similar to that previously reported with the Mg^{++}-activated mitochondrial ATPase of liver (8), but is in contrast to those reported with the dinitrophenol-induced liver

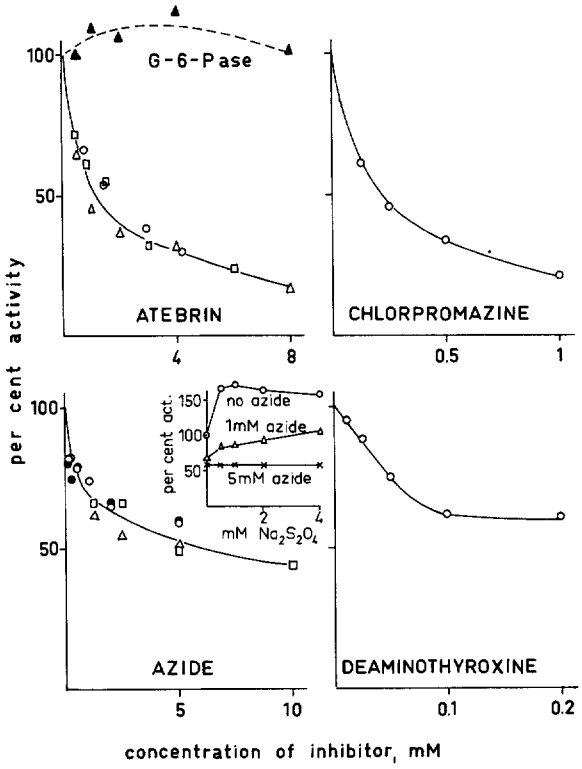

FIGURE 3

Effects of atebrin, chlorpromazine, azide, and deaminothyroxine on microsomal ATPase. Also indicated are the effect of atebrin on glucose-6-phosphatase (G-6-Pase, upper left diagram) and the combined effects of $Na_2S_2O_4$ and azide on the ATPase (insert in lower left diagram). Different symbols used within the same diagram represent different experiments. Among the experiments with azide, the solid and open circles originate from the same experiment, the assays having been performed in the absence and presence of 0.1 mM 2,4-dinitrophenol.

mitochondrial ATPase (8) and with the Mg^{++}-activated ATPase of both the mitochondria (13) and microsomes (14) from skeletal muscle. Whether or not such an effect with added flavin nucleotides ensues appears thus to be dependent upon the tissue and the type of ATPase studied, suggesting that this effect is not simply a matter of binding between atebrin and the flavin nucleotides as recently implied by Hemker and Hülsmann (38).

The effects of azide and deaminothyroxine on the microsomal ATPase (Fig. 3) differ from those described for the mitochondrial ATPase (10, 11) in that the inhibitions are only partial, amounting to about 50 per cent with azide and 40 per cent with deaminothyroxine. The concentrations required for half maximal inhibition are about 1 mM for azide and 0.05 mM for deaminothyroxine. The latter concentration is about equal to that necessary in the case of mitochondria, whereas the concentration of azide required for half maximal inhibition was 5 to 10 times higher than with mitochondrial ATPase (10, 11). A further difference in regard to the azide inhibition was that, in mitochondria, $Na_2S_2O_4$ effectively counteracted the azide effect (11); in microsomes, on the contrary, azide even removed the stimulation of the ATPase by $Na_2S_2O_4$ (see Fig. 3). The same was true of the combined effects of $Na_2S_2O_4$ and atebrin or chlorpromazine.

Pullman *et al.* (39) have reported that an ATPase extracted from beef heart electron transport particles can be stimulated slightly with 2,4-dinitrophenol, and that this stimulation becomes larger if the basic ATPase activity is partially inhibited by azide. In our system 10^{-4} M dinitrophenol did occasionally stimulate the ATPase activity by about 10 per cent, but no azide effect of the type described by Pullman *et al.* could be observed.

Amytal inhibits the mitochondrial dinitrophenol-induced ATPase (10), especially in combination with atebrin (8) or chlorpromazine (9), but is without effect upon the mitochondrial Mg^{++}-activated ATPase. This compound had essentially no effect on the microsomal Mg^{++}-activated ATPase (10 to 15 per cent inhibition by 2 to 8 mM amytal).

Another important difference between mitochondrial and microsomal ATPases is in their behavior toward oligomycin, a compound which has been shown by Lardy to act as a strong inhibitor of mitochondrial ATPases (40). Liver microsomal ATPase was unaffected by oligomycin even at concentrations exceeding several times (on the protein basis) that required for inhibition of mitochondrial ATPases.

Nucleoside Specificity and Effects of DOC

Further lines of comparison of the ATPase of liver microsomes with that of liver mitochondria and with microsomal ATPases from other tissues involved an investigation of the nucleoside specificity and the effect of DOC. It has been shown that whereas dinitrophenol-induced ATPase of liver mitochondria is specific for ATP, mitochondria which have been treated so as to exhibit a Mg^{++}-activated ATPase split other nucleoside triphosphates as well (6). DOC inhibits the mitochondrial dinitrophenol-induced ATPase and stimulates the Mg^{++}-activated ATPase (10). The microsomal Mg^{++}-activated ATPase, on the other hand, has been reported to be inhibited by increasing concentrations of DOC as disclosed by studies with skeletal muscle (12) and nervous tissue (21).

Data relating to the nucleoside specificity and response to DOC of the liver microsomal enzyme are shown in Fig. 4. As can be seen in Fig. 4 *a*, liver microsomes catalyzed a splitting of GTP, UTP, ITP, and CTP as well as ATP. The ATPase activity was inhibited by increasing concentrations of DOC, as was the case with microsomes from skeletal muscle (12) and brain (21). However, the GTPase and UTPase activities behaved in a different manner toward DOC, both revealing a diphasic response: stimulation at low, and inhibition at high, concentrations of DOC. The ITPase and CTPase activities occupied an intermediate position, describing a plateau at low, and a decreasing activity with increasing, concentrations of DOC. The findings with the GTPase and UTPase were reminiscent of the effect of DOC on glucose-6-phosphatase reported earlier by Hers and de Duve (41). Fig. 4 *b* illustrates this similarity. It appeared, therefore, that there might be two different types of enzyme: an ATPase which is inhibited in a straightforward manner by DOC, and a GTP-UTPase whose activity, like that of the glucose-6-phosphatase, increases at moderate, and then falls again with increasing, DOC concentrations; both types of enzyme would react with ITP and CTP, the activities with these nucleotides thus

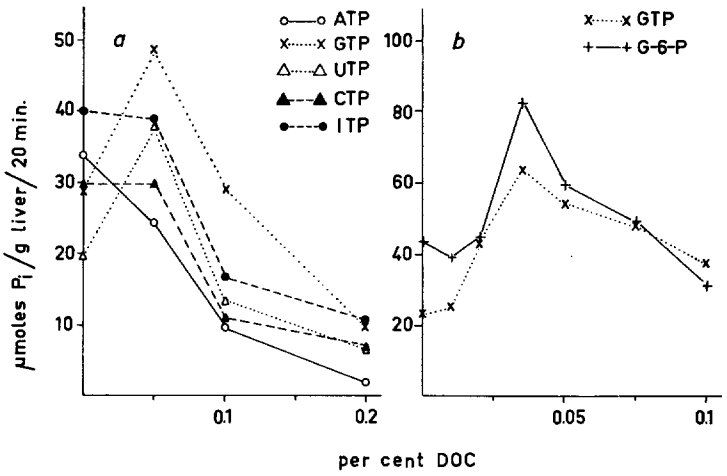

FIGURE 4

a. Effect of DOC on microsomal nucleoside triphosphatase activities.

b. Comparison of effects of DOC on GTPase and glucose-6-phosphatase (G-6-Pase) activities.

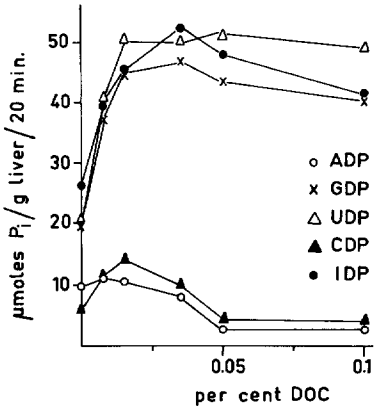

FIGURE 5

Effect of DOC on microsomal nucleoside diphosphatase activities.

revealing a plateau at moderate concentrations of DOC.

An alternative interpretation of the above findings, however, was suggested when the nucleoside diphosphatase activities of the microsomes were investigated. As shown in Fig. 5, in the absence of DOC the microsomes exhibited relatively moderate diphosphatase activities with ADP, GDP, UDP, and IDP. However, when increasing concentrations of DOC were added, three of the diphosphatase activities, those with GDP, UDP, and IDP, showed a marked increase, whereas the remaining two were essentially unaffected. It appeared possible, therefore, that the transient increase found with the GTP and UTP at medium concentrations of DOC (cf. Fig. 4 *a*) might be a reflection of the increasing diphosphatase activities. Such would be the case if the triphosphates were hydrolyzed only to the level of the diphosphates, and this hydrolysis would then proceed to the monophosphate level when GDP and UDPase reactions were enhanced by moderate concentrations of DOC. In accordance with this reasoning, ITPase and CTPase activities (both of which showed a plateau at medium DOC concentrations) should have increased and decreased, respectively, since the IDPase was increasing and the CDPase essentially constant with increasing concentrations of DOC. It is possible, however, that the situation is further complicated by the affinities of the individual diphosphates toward the diphosphatase, as well as by the slight increase, at low DOC concentrations, in those diphosphatase activities (CDPase and ADPase) in which no further increase ensued upon further increasing the concentration of DOC. These aspects might be clarified in the future by following the individual mono- and diphosphates formed during the triphosphatase reactions in addition to the net liberation of P_i. It may be added that the microsomes catalyzed the hydrolysis of the five nucleoside monophosphates and of inorganic pyrophosphate only at low rates which were not enhanced by DOC.

Effects on the tri- and diphosphatase activities

similar to those described above were obtained with another anionic detergent, Lubrol W.

Fractionation with DOC

It has been reported (4) that fractionation of liver microsomes with 0.26 per cent DOC under suitable conditions gives rise to three fractions: a tightly packed pellet, consisting mainly of RNP particles (P fraction); a loose sediment, containing smooth surfaced vesicles and representing a fraction of the endoplasmic membranes (M fraction); and a clear supernatant. Studies of the distribution of various microsomal enzymes among these fractions revealed that some of the enzymes, such as the NADH–cytochrome *c* reductase, glucose-6-phosphatase, and ATPase, were concentrated in the M fraction, whereas others, including NADPH–cytochrome *c* reductase, cytochrome b_5, and a pyridine nucleotide unspecific flavoenzyme, called DT-diaphorase (28), were either evenly distributed between the M fraction and the clear supernatant or even concentrated in the latter. None of the enzymes investigated was concentrated in the P fraction.

An extension of these studies was considered to be of interest in regard to the distribution of tri- and diphosphatase activities. Relevant data are summarized in Table I. As can be seen, all tri- and diphosphatase activities were predominantly recovered in the M fraction and/or the clear supernatant. The P fraction exhibited slight but definitely not insignificant triphosphatase activities (*see* further Table II), whereas the diphosphatase activities of this fraction were negligible or nil. The total recoveries were below 100 per cent in the case of the ATPase and slightly above 100 per cent with the remaining triphosphatases and with the ADPase and CDPase as well. Very high overrecoveries of GDPase, UDPase, and IDPase activities were obtained, a result which is in accordance with the stimulating effect of DOC on these activities (*see* Fig. 5).

TABLE I

Nucleoside Tri- and Diphosphatase Activities of Rat Liver Microsomes and of Microsomal Subfractions Obtained by Treatment with DOC According to Ernster et al. (4)

The protein contents (mg protein/g liver) were: microsomes, 21.7; P fraction, 4.41; M fraction, 3.64; clear supernatant, 14.7.

	Microsomes: μmoles P_i/20 min.		P fraction: μmoles P_i/20 min.		M fraction: μmoles P_i/20 min.		Clear supernatant: μmoles P_i/20 min.	
Substrate	Per gm liver	Per mg protein	Per gm liver	Per mg protein	Per gm liver	Per mg protein	Per gm liver	Per mg protein
ATP	38.4	1.77	2.1	0.47	28.8	7.92	2.9	0.19
CTP	31.2	1.44	2.1	0.47	33.2	9.12	7.2	0.48
GTP	48.0	2.21	0.9	0.20	38.0	10.45	22.6	1.52
UTP	30.8	1.42	1.2	0.27	26.9	7.39	13.9	0.93
ITP	52.4	2.41	1.1	0.25	40.9	11.25	16.3	1.09
ADP	18.7	0.86	0.0	0.00	15.8	4.35	5.3	0.35
CDP	11.5	0.53	0.3	0.06	7.7	2.12	10.1	0.68
GDP	34.6	1.59	0.3	0.06	30.3	8.33	101.3	6.80
UDP	33.6	1.55	0.3	0.06	36.1	9.65	107.7	7.24
IDP	43.7	2.01	0.5	0.12	44.2	12.14	89.5	6.01

On a gram liver basis, the bulk of the triphosphatase activities as well as that of ADPase was recovered in the M fraction, whereas the bulk of GDPase, UDPase, and IDPase activities was found in the clear supernatant; the CDPase activity was slightly higher in the clear supernatant than in the M fraction. On a milligram protein basis, all tri- and diphosphatase activities were 4 to 6 times higher in the M fraction than in the original microsomes. The activities of the triphosphatase and of the ADPase found in the clear supernatant were lower, on the milligram protein basis, than in the original microsomes, whereas the activities of the GDPase, UDPase, and IDPase in this fraction were higher than, and the activity of the CDPase was about equal to, that in the original microsomes.

TABLE II

Effect of $Na_2S_2O_4$ on Microsomal Nucleoside Tri- and Diphosphatase Activities under Various Conditions

DOC, when present in the assay (Experiment 2), was added in a final concentration of 0.05 per cent.

In Experiment 4, the microsomes were treated with Lubrol W, centrifuged at 105,000 *g* for 2 hours, and the pellets assayed.

Experiment no.	Substrate	Source of enzyme activity	μmoles P_i/20 min. per g liver: Without $Na_2S_2O_4$	μmoles P_i/20 min. per g liver: With 2 mM $Na_2S_2O_4$	Per cent stimulation by 2 mM $Na_2S_2O_4$
1	ATP	Microsomes	41.9	69.1	65
	ITP	"	47.9	58.6	23
	GTP	"	30.8	48.4	57
	UTP	"	24.9	28.1	13
2	ATP	Microsomes	18.5	27.0	46
	ATP	" + DOC	11.5	12.3	7
	UTP	"	8.8	10.7	22
	UTP	" + DOC	17.5	15.3	−13
	UDP	"	7.8	8.5	9
	UDP	" + DOC	51.5	50.0	−3
3	ATP	Microsomes	46.5	74.3	60
	ATP	P fraction	3.8	5.1	34
	ATP	M fraction	15.7	15.8	1
	ATP	Clear supernatant	13.0	9.5	−27
	GTP	Microsomes	35.8	51.1	43
	GTP	P fraction	3.0	5.8	93
	GTP	M fraction	15.7	15.5	−1
	GTP	Clear supernatant	30.3	29.6	−8
4	GTP	Microsomes	27.5	34.5	26
	GTP	Pellet after treatment with 0.25% Lubrol W	15.5	25.5	65
	GTP	Pellet after treatment with 0.5% Lubrol W	5.5	14.5	164

It is noteworthy that the GDPase, UDPase, and IDPase activities had a higher specific activity in the M fraction than in the clear supernatant, in spite of the fact that the major part of these activities was recovered in the latter fraction. This indicates that the stimulation and solubilization of these activities ensuing upon destruction of the microsomes are sequential rather than coincident events, a finding to be further substantiated later (*see* Table IV).

Variations in the Effects of Different Agents

In Table II, data illustrating the effect of $Na_2S_2O_4$ on the nucleoside tri- and diphosphatase activities under various conditions are summarized. In the fresh microsomes, $Na_2S_2O_4$ stimulated the GTPase and ATPase to about the same extent, by approximately 60 per cent (Experiment 1). The UTPase and ITPase, however, were only slightly stimulated (the effect on the CTPase was not tested). When DOC was added to the assay (Experiment 2), no stimulation of the triphosphatases by $Na_2S_2O_4$ ensued. The diphosphatases, as illustrated by the UDPase, were not stimulated by $Na_2S_2O_4$ in either the absence or the presence of DOC. Fractionation of microsomes with DOC into P fraction, M fraction, and clear supernatant (Experiment 3) resulted in a loss of the $Na_2S_2O_4$ stimulation of the triphosphatase activities in the two latter fractions. The slight activity found in

the P fraction, however, retained its response to $Na_2S_2O_4$ in the case of the ATPase, and the stimulation was even greater than with the original microsomes in the case of the GTPase.

A distinctive effect of $Na_2S_2O_4$ on the particle-bound GTPase is further indicated by the data reported in Table II, Experiment 4. Microsomes were treated with 0.25 and 0.5 per cent Lubrol W, then recentrifuged, and the pellets were tested for ATPase and GTPase activities in the absence and presence of $Na_2S_2O_4$. Rendi and Hultin (42) have shown that this detergent (which has an advantage over DOC in that it forms no precipitate with Mg^{++}) solubilizes membranous material from the microsomes, leaving behind the RNP particles. It may be seen that treatment of microsomes with increasing concentrations of Lubrol W and subsequent centrifugation resulted in pellets whose GTPase activities were increasingly stimulated by $Na_2S_2O_4$.

Data illustrating the effects of atebrin, chlorpromazine, azide, and deaminothyroxine on tri- and diphosphatase activities under various conditions are shown in Table III. Activities with ATP, UTP, and UDP as substrates were measured both in the absence and in the presence of DOC. The effects of the various agents were tested with ATPase only in the absence, and with UDPase only in the presence, of DOC, *i.e.* under conditions of maximal activity. In addition, they were tested with UTPase both in the absence and in the presence of DOC, in order to obtain an estimate of the influence of DOC as such on the effects of the various agents. Such an influence proved to be negligible or absent in the case of atebrin and chlorpromazine, the effects of these two agents on the UTPase being about equal with or without DOC. The effects of azide and deaminothyroxine, on the other hand, were somewhat increased as the UTPase activity was stimulated by DOC. From an examination of the data concerning the effects of atebrin and chlorpromazine, it is obvious that the UDPase is much less inhibited than the ATPase or, more generally, that the inhibitory effect of the flavin antagonists on the triphosphatases is probably not shared by the diphosphatases. The finding that the UTPase is less inhibited by these agents than the ATPase is consistent with the above conclusion that the total UTPase activity measured with the microsomes also involves a splitting of the UDP by the UDPase. The data with azide are more difficult to interpret since this compound inhibited both the ATPase and the UDPase to a rather large extent, but the UTPase less strongly. However, when the absolute amounts of P_i liberated (rather than the per cent inhibition) are calculated, it may be seen that 5 mM azide allowed the liberation of roughly the same amount of P_i in all four assays, *i.e.*, 6.8, 6.0, 8.2, and 5.7 μmoles with ATPase (no DOC), UTPase (no DOC), UTPase (DOC), and UDPase (DOC), respectively. Deaminothyroxine caused about the same extent of inhibition of the ATPase and UDPase.

Mechanical Disruption of Microsomes

Mechanical disruption of microsomes by a rapidly rotating Ultra Turrax Blendor was previ-

TABLE III

Effect of Various Inhibitors on Microsomal Nucleoside Tri- and Diphosphatase Activities with and without 0.05 per cent DOC

Substrate	DOC in test	Enzyme activity (μmoles P_i/20 min.)	Per cent inhibition by: Atebrin, 2 mM	Chlorpromazine, 0.25 mM	Azide, 5 mM	Deaminothyroxine, 0.1 mM
ATP	−	18.5	63	69	63	41
ATP	+	11.5				
UTP	−	8.8	36	36	32	21
UTP	+	17.5	29	36	53	42
UDP	−	7.8				
UDP	+	51.5	19	9	89	48

ously found to lead to a solubilization of DT-diaphorase, while leaving the NADH- and NADPH-specific diaphorases attached to the membranes (43). This treatment differs from the fractionation with DOC, which releases both DT-diaphorase and NADPH-diaphorase (4).

Since it was found that DOC solubilizes a large part of the GDP-UDP-IDP–specific diphosphatase while leaving the bulk of the nucleoside triphosphatase attached to the membranes (*see* Table I), it was of interest to investigate how the two types of enzyme behaved upon mechanical treatment of the microsomes. As can be seen in Table IV, Experiment 1, a single treatment of the microsomes for 1 minute with an Ultra Turrax Blender and subsequent centrifugation at high speed (see under "Experimental") brought almost none of the ATPase or UTPase activities into solution, but it released a considerable UDPase activity. This release, however, was not complete, as in the case of DT-diaphorase (43), the diphosphatase activity found in the pellet being about equal to that released. The sum of the pellet and supernatant UDPase activities largely exceeded that of the original microsomes, showing that the mechanical disruption, similarly to the DOC treatment, resulted in an activation of the UDPase. No similar increase, as could be anticipated, was found with the ATPase activity. The sum of the pellet and supernatant UTPase activities also was about equal to that of the original microsomes; however, when the pellet and supernatant were assayed in combination, the resulting activity exceeded significantly both that obtained in the original microsomes and the sum of the pellet and supernatant activities. This, again, fits logically with the above assumption that the increase in UTPase activity occurring upon destruction of the microsomal membranes results from a combined activity of UTPase and UDPase. In Experiment 2 of Table IV, results with all five tri- and diphosphatases are shown. The data with the supernatant fraction are well illustrative of the GDP-UDP-IDP specificity of the diphosphatase, which in this fraction was 10 to 20 times more active with these three diphosphates than with ADP or CDP. Also the triphosphatase activities in this fraction were quite low; the relatively high activities with GTP and ITP were probably due to contamination of the substrates with the corresponding diphosphates. Experiment 2 is noteworthy also from the viewpoint that in this particular case the GDPase, UDPase, and IDPase activities happened to be relatively high even in the fresh microsomes and only little further activation occurred upon disruption. Still, even under these circumstances, the mechanical treatment did not release the diphosphatase activity completely, there remaining about one-third of the total activities of GDPase, UDPase, and IDPase in the pellet

Some attempt was made to establish the sequence of events in the activation and solubilization mechanisms of the diphosphatase activity by subjecting microsomes to repeated Ultra Turrax treatments. The outcome of such an experiment is shown in Fig. 6. The activities are plotted against the number of treatments, both as total activities recovered in pellet and supernatant(s)

TABLE IV

Effect of Treatment of Microsomes with Ultra Turrax Blender on Nucleoside Tri- and Diphosphatase Activities

Single treatment for 1 minute as described under "Experimental."

Experiment no.	Fraction	Triphosphatases					Diphosphatases				
		A	C	G	U	I	A	C	G	U	I
		μmoles P_i/g liver/20 min.					*μmoles P_i/g liver/20 min.*				
1	Microsomes	19.0			15.8					14.3	
	Supernatant	1.0			3.8					18.5	
	Pellet	16.0			14.3					18.0	
	Supernatant + pellet	16.3			24.1					40.2	
2	Microsomes	38.4	23.1	52.0	40.4	61.5	14.4	16.3	65.5	61.5	78.8
	Supernatant	1.0	4.8	17.3	5.8	16.2	2.9	5.8	47.1	48.1	62.5
	Pellet	36.6	31.8	32.7	23.1	43.3	14.4	10.6	20.2	22.1	31.8

together, and as activities released into the supernatant(s). It may be seen that at no point did the GDPase activity released reach the total GDPase activity; in other words, the activation which occurs upon the mechanical disruption apparently precedes the actual release of the enzyme. The possible implication of this finding will be discussed later. Fig. 6 also shows that the total ATPase activity did not change upon repeated treatments and was released only to a very slight extent.

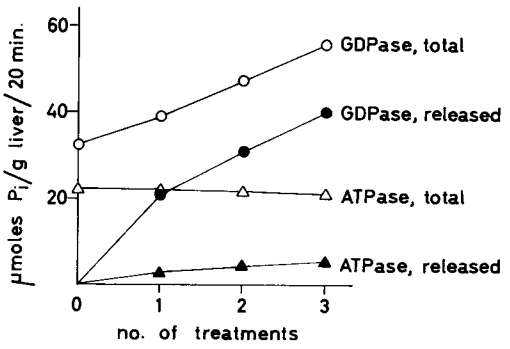

FIGURE 6

Effect of repeated treatments with Ultra Turrax Blender on activity and solubility of microsomal GDPase and ATPase. Repeated treatments and centrifugations were made as described under "Experimental." After each centrifugation, activities were determined in both pellet and supernatant. Activities "released" denote, at the three points indicated, those measured in supernatant 1, supernatants 1 plus 2, and supernatants 1 plus 2 plus 3, respectively. The four points indicating "total" activities represent those found with the original microsomes, supernatant 1 plus pellet 1, supernatant 1 plus supernatant 2 plus pellet 2, and supernatant 1 plus supernatant 2 plus supernatant 3 plus pellet 3, respectively. 16 per cent of the total microsomal protein was released in supernatant 1, and about 10 per cent in each of supernatants 2 and 3.

Diphosphatase Activity in Other Fractions

Previous reports in the literature of a nucleoside diphosphatase acting specifically on GDP, UDP, and IDP described this enzyme as occurring in the mitochondrial fraction (23–25). In order to correlate these reports with the present findings, a reinvestigation of the cytoplasmic distribution of this enzyme was warranted. Fig. 7 compares the diphosphatase activities of the mitochondrial, microsomal, and final supernatant fractions of a 0.25 M sucrose homogenate of rat liver. The activities were assayed both in the absence of DOC and in the presence of 0.05 per cent DOC, the latter in order to induce maximal GDPase, UDPase, and IDPase activities. The data clearly show, first, that the bulk of the GDPase, UDPase, and IDPase activity was in the microsomes; and, second, that only in the microsomal fraction did DOC activate the enzyme. The reason for the failure to detect this distribution of the enzyme in the earlier literature is unclear.

In the above fractionation no separate lysosome fraction was obtained. Because activation upon disruption is a known property of lysosomal enzymes (44), a closer investigation of the possible lysosomal localization of the diphosphatase appeared to be an important question. In a number of experiments, therefore, a fractionation scheme was used which has been devised by de Duve *et al.* (45) to obtain predominantly lysosomes as an intermediate fraction between mitochondria and microsomes. The fractionation was monitored by means of suitable enzyme activities, that is, cytochrome oxidase for the mitochondria, acid phosphatase for the lysosomes, and glucose-6-phosphatase for the microsomes. It was found that the GDP-UDP-IDPase followed in its distribution the glucose-6-phosphatase and thus that the enzyme is truly microsomal. This conclusion is in agreement with recent histochemical observations of Novikoff and Goldfischer (26), who found a concentration of the diphosphatase in the membrane elements of the Golgi region (which is recovered in the microsomal fraction) whereas the lysosomal particles were devoid of diphosphatase activity.

Attempts at "Integration"

Mg^{++}-stimulated ATPase activity appearing upon structural damage of liver mitochondria is generally regarded as a modified function of the transphosphorylating system involved in electron transport–coupled phosphorylation (10, 46–48). Because of the similarities in certain properties between the mitochondrial and microsomal ATPases as well as of the presence in microsomes of electron-transporting enzymes, and since microsomes actually represent severely "damaged" structures in comparison with the native endoplasmic membranes, the possibility might exist that the microsomal ATPase also represents a modified reflection of oxidative phosphorylation. In order to investigate this question, some attempts were made to prepare microsomes by

methods which could conceivably allow a better preservation of the structural or functional integrity of the endoplasmic membranes. One such method consisted of using 0.88 M (rather than 0.25 M) sucrose as the preparation medium. This has been shown to preserve the elongate, double-layered character of the membrane fragments, preventing them from becoming round vesicles (49). In another attempt, the Mg^{++}-containing buffered salt medium of Keller and Zamecnik (50) was used instead of sucrose, since this is known to yield microsomes active in amino acid incorporation. As criteria for "integration" of the ATPase activity the following indications were considered valid (on the basis of experience with mitochondria as a model): (*a*) the preparation should show a lower Mg^{++}-stimulated ATPase activity than is found in the microsomes prepared in the usual way; (*b*) this ATPase activity should be stimulated by 2,4-dinitrophenol; (*c*) the preparation should exhibit a P_i-ATP exchange reaction. However, none of these criteria was fulfilled with either of the modified techniques used. It may also be mentioned that, conversely, when the microsomes were prepared in the usual way and exposed afterward to treatments such as aging at 30°C and incubation in hypertonic medium, which are known to enhance mitochondrial ATPase activity, no significant change occurred in the activity of the microsomal ATPase.

It may, finally, be mentioned that the microsomes exhibited no polynucleotide phosphorylase activity of the type described in microorganisms by Grunberg-Manago *et al.* (51). The diphosphatase activities found in the microsomes involved a true splitting of the diphosphates into orthophosphate and the corresponding monophosphate rather than a condensation of the latter into polynucleotides. This could be ascertained both by chromatographic assay of the reaction products and by showing that the reactions involved no exchange between P^{32} and the phosphate groups of diphosphates, as in the case of polynucleotide phosphorylase reaction. These tests were performed both with the single diphosphates and with the different diphosphates in combination.

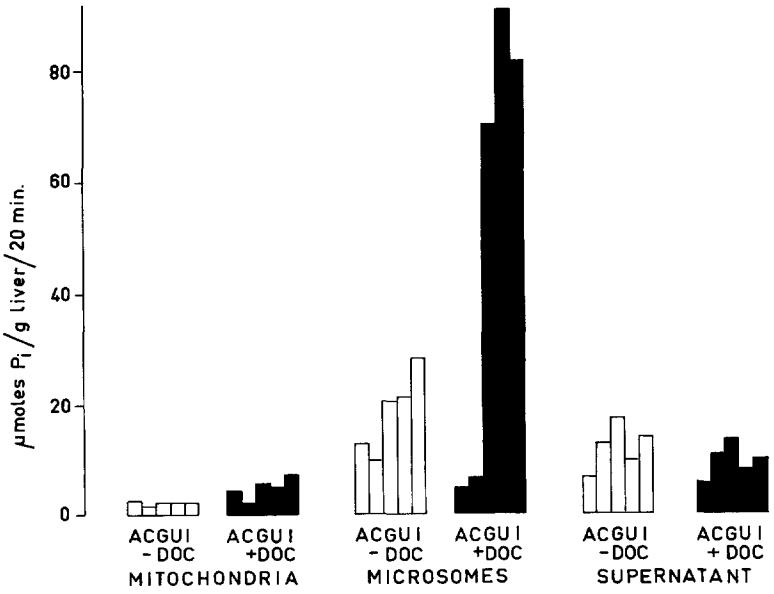

FIGURE 7

Distribution of nucleoside diphosphatase activities in the mitochondreal, microsomal, and final supernatant fractions of rat liver. Activities were assayed in the absence and presence of 0.05 per cent DOC.

DISCUSSION

The present data establish the occurrence in the microsomal fraction of rat liver of two types of

nucleoside polyphosphatase: a nucleoside triphosphatase, catalyzing the splitting of ATP, UTP, GTP, CTP, and ITP into the corresponding diphosphates and inorganic orthophosphate; and a nucleoside diphosphatase, whose activity is predominant with GDP, UDP, and IDP, and which catalyzes the splitting of these compounds into the corresponding monophosphates and inorganic orthophosphate. Both enzymes appear to be concentrated in the membranous elements of the microsomal fraction, yet the triphosphatase is tightly bound to the membranes and in a fashion which is essential for the enzymic activity, whereas the diphosphatase is associated with the membranes by a relatively loose linkage whose rupture does not inactivate the enzyme. Some triphosphatase, but not diphosphatse, is consistently found in the ribosomal fraction, and, although this phenomenon has not yet been investigated in detail, the striking stimulation of the GTPase activity of this fraction by $Na_2S_2O_4$ may be of interest in view of the known actions of GTP (50) and of certain reducing agents (42, 52) on ribosomal protein synthesis.

The nucleoside triphosphatase of the liver microsomal membranes strikingly resembles in several respects the ATPase of structurally damaged or fragmented liver mitochondria. The properties in common are: (*a*) activation by added Mg^{++}, (*b*) non-specificity toward the nucleoside, (*c*) stimulation by $Na_2S_2O_4$, and (*d*) inhibition by atebrin, chlorpromazine, azide, and deaminothyroxine. However, there are some differences in regard to the effects of the inhibitors, namely: (i) the azide inhibition of the microsomal triphosphatase is much weaker, on a concentration basis, than that of the mitochondrial enzyme; (ii) $Na_2S_2O_4$ relieves the inhibition by azide (and to some extent also by atebrin and chlopromazine) of the mitochondrial, but not the microsomal, enzyme; (iii) the azide and deaminothyroxine inhibitions can reach completeness in the case of the mitochondria, but are only partial with the microsomes; (iv) the microsomal ATPase is not inhibited by oligomycin. These differences in themselves would appear sufficient to preclude the remote possibility that the triphosphatase activity observed with the microsomes originates from contaminating mitochondrial fragments. This possibility, as discussed previously (4), is also excluded by the fact that the microsomal preparations exhibited no succinoxidase activity and, furthermore, that the triphosphatase activity was concentrated in the same membrane subfraction of the DOC-treated microsomes as was the typical microsomal enzyme glucose-6-phosphatase.

Although no organized features of the microsomal ATPase have been revealed by our studies using the mitochondrial ATPase and oxidative phosphorylation as a model, it is still conceivable, and in fact highly probable, that the microsomal enzyme as well may prove to be involved in some integrated mechanism of energy transfer. Such functions have actually been demonstrated with microsomal ATPases from other tissues, notably skeletal muscle and nervous tissue. The skeletal muscle microsomal (sarcotubular) ATPase has been implicated in muscle fiber relaxation (12–14), and the microsomal ATPase from nervous tissue in active cation transport (15–22). So far as a special function of the liver microsomal ATPase is concerned, this cannot be assessed on the basis of the available information. Among the possibilities now being considered is an ATP-dependent reduction of pyridine nucleotides by electron donors with a higher oxidation-reduction potential, similar to the mechanisms involved in the process of reversed oxidative phosphorylation, now being widely studied in mitochondria (see 53–56). Such a possibility would appear tempting in view of the known occurrence both of pyridine nucleotide–cytochrome reductases (4, 45, 57, 58) and of reduced pyridine nucleotide–dependent reductive enzymes (59–61) in liver microsomes.

It seems rather significant that both the mitochondrial (8, 9, 13) and several microsomal (13 and this work) ATPases can be influenced in their activity by flavins and/or flavin inhibitors, a finding which, as has been suggested (8, 9, 13, 62–65), may be indicative of the involvement of high energy flavin derivatives as intermediates in these reactions. This feature, together with the recently described effects of amytal on both mitochondrial (66) and microsomal (13, 14) energy transfer reactions, further amplifies the concept (4, 67, 68) of the existence of a basically common enzymic principle in biological membrane structures whose function may manifest itself as active transport of ions or electrons in one instance and as electron transport–coupled phosphorylation in another, according to the actual physiological function of the membrane.

A nucleoside diphosphatase with a substrate specificity similar to that described here has been

reported in previous literature as occurring in liver extracts and liver and kidney mitochondria (23–25). The "latent" property of the enzyme has also been noticed (23, 24), in that freezing and thawing of the mitochondria was needed to obtain maximal activity. On the basis of the present data it appears safe to conclude that the localization of this enzyme in rat liver is predominantly in the microsomal membranes. Our conclusion is in agreement with the histochemical findings of Novikoff and Goldfischer (26) cited earlier in this paper (see under "Results").

The activation of the diphosphatase upon disruption of the membranes by either DOC or mechanical treatment is an interesting property of this enzyme. Such a property may be due to a simple inclusion of the enzyme within the lumen of the membrane as in a sac, making it inaccessible to its substrates, a situation analogous to that visualized by de Duve (44) in the case of the lysosomes. Alternatively, the enzyme may be part of the membrane, which would govern its activity in both a qualitative and a quantitative manner similar to that found with the mitochondrial ATPases. The finding that mechanical treatment activated the enzyme without fully releasing it from the structure would appear to eliminate the first of these two alternatives. The kinetics of the release and activation (*see* Fig. 6) suggest that the enzyme is bound to the membranes in such a manner that the disruption has a dual effect: it fragments the membrane, thus facilitating access of the substrate to the enzyme; and it dissociates the enzymes from the fragments.

Whatever the precise mechanism of the activation of the nucleoside diphosphatase by structural disruption may be, the phenomenon itself suggests that this enzyme may have its metabolic function primarily related to the splitting of nucleoside diphosphates formed within the endoplasmic canals. As already pointed out by Gibson *et al.* (25), the nucleoside diphosphatase may serve a function in promoting reactions involving the corresponding triphosphates as reactants by shifting the equilibrium toward the formation of the diphosphate. It is conceivable that the nucleoside diphosphatase present in the endoplasmic canals may have such a function in removing GDP formed in the GTP-dependent incorporation of amino acids (50) and/or in removing UDP formed in the various uridine nucleotide-linked conjugation reactions (69) taking place in the membranes. The GMP and UMP so formed may then leave the membranes and be transformed into the corresponding triphosphatase by way of the nucleoside monophosphate kinase system (70, 71). During this transformation the diphosphates are formed as intermediates, but these would not be readily attacked by the diphosphatase because of the relative inaccessibility of this enzyme to external substrates. A counterpart of such a possible regulation mechanism is found in mitochondria, whose membrane is readily permeable to ATP and AMP but not to ADP (72). A function of the microsomal diphosphatase in connection with its third substrate, IDP, cannot yet be visualized; however, the distinctive abundance in liver microsomes of bound inosine, observed by Siekevitz (73), may be indicative of a function of inosine nucleotides in microsomal activities.

This work has been supported by a grant from the Swedish Cancer Society.

Received for publication, July 27, 1962.

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