count for its very high affinity, *i.e.*, it may be acting as a transition-state analog.

Tetrahydropteridines with only hydrogen substituents in the pyrazine ring appear to be bound loosely at the cofactor site. The two inhibitory compounds, 4-keto-H_4Pt (II) and 4-amino-H_4Pt (III), have poor affinity for the enzyme (K_i = 0.6 and 0.8 mM, respectively). In each case inhibition is mixed with respect to cofactor and substrate, suggesting that the two inhibitors may be only loosely bound at the active site. 2-Amino-4-keto-H_4Pt (VIII) has cofactor activity, but catalyzes a nonstoichiometric reaction, since about two tetrahydropteridines are oxidized for each tyrosine formed. A similar nonstoichiometric reaction has been observed when 2-amino-4-keto-7-methyl-H_4Pt is used as cofactor (Storm and Kaufman, 1968). In contrast, with the 6-methyl and 6,7-dimethyl analogs, one tyrosine is formed for each tetrahydropteridine oxidized. It thus appears that at least a methyl substituent is required at the 6 position to hold the tetrahydropteridine specifically at the cofactor site in order to produce a stoichiometric reaction or, in the case of inhibitory compounds, to cause competitive inhibition. The competitive inhibition with respect to cofactor, observed with 2-amino-4-keto-6,7-diphenyl-H_4Pt, indicates that a 6-phenyl substituent also allows specific binding at the cofactor site. This leaves the 7-phenyl group as the cause of the inhibition by the diphenyl compound, possibly by blocking the phenylalanine site. It will be of interest to determine whether 7-phenyltetrahydrobiopterin inhibits the enzyme in the same manner. The corollary, that 2-amino-4-keto-6-phenyl-H_4Pt would be a cofactor, rather than an inhibitor, is at present under investigation.

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Biosynthesis of Glutamate Dehydrogenase in Rat Liver. Demonstration of Its Microsomal Localization and Hypothetical Mechanism of Transfer to Mitochondria†

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ABSTRACT: The time-dependent incorporation of [^{14}C]isoleucine into rat liver glutamate dehydrogenase subunits showed that the fraction of this enzyme associated with the microsomes was labeled before the glutamate dehydrogenase in the mitochondria. The microsomal glutamate dehydrogenase extracted in phosphate buffer was present in the same active form as the mitochondrial enzyme. Purified radioactive glutamate dehydrogenase added to rat liver homogenates was bound weakly to mitochondrial membranes and preferentially to microsomal membranes during fractionation of the tissue. This indicates that a redistribution of the soluble enzyme could occur during the separation of the different cellular constituents. A possible mechanism by which glutamate dehydrogenase could be temporarily bound to the microsomes by specific lipid–protein interaction and then translocated into the mitochondria is discussed.

Although mitochondrial biogenesis has been studied for nearly a century, the mode of replication of these particles is far from being clearly understood. As early as 1890, Altmann described mitochondria as being semiautonomous organelles capable of self-replication within the cell. It is generally accepted now that mitochondria have a limited capacity for protein synthesis and that most mitochondrial proteins are made at other sites within the cell (Beattie, 1971). This applies not only for mammalian cells but also for those of lower organisms such as *Neurospora crassa* (Sebald *et al.*, 1969). In general, studies have shown that the more soluble mitochondrial

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proteins are synthesized outside the mitochondria and that most likely only a few of the less soluble mitochondrial proteins are synthesized by mitochondria.

The choice of glutamate dehydrogenase (EC 1.4.1.3) as a model for studying the synthesis of soluble proteins was dictated by various considerations: rat liver glutamate dehydrogenase is located in the mitochondrial matrix (Norum *et al.*, 1966; Brdiczka *et al.*, 1968) and in part may be bound to the inner membrane (Schnaitman and Greenwalt, 1968). It is easily solubilized and present in fairly large amounts in rat liver mitochondria (Pette, 1966). Moreover, Stratman *et al.* (1972) have recently demonstrated that mitochondrial ATPase after cold dissociation into inactive subunits could be transferred *in vitro* specifically to the inner mitochondrial membrane. Glutamate dehydrogenase is a large protein whose smallest active unit has a molecular weight of 312,000 (Cohen and Mire, 1971). It can polymerize up to a molecular weight of several millions (Eisenberg and Tomkins, 1968) but the protomer is composed of six identical inactive subunits of mol wt 52,000 (Appella and Tomkins, 1966). These characteristics first demonstrated with the beef liver enzyme and verified with the rat liver enzyme (Sedgwick-King and Frieden, 1970; Ifflaender and Sund, 1972) made glutamate dehydrogenase a choice enzyme to test the possibility of its biosynthesis on the microsomes and its subsequent transfer into the mitochondria.

Experimental Section

Materials. Female rabbits (White New Zealand) weighing 3 kg and male rats (Sprague-Dawley) of about 200 g were used. L-[^{14}C]Isoleucine, uniformly labeled (200 mCi/mmol), was purchased from Nuclear Dynamics Inc. and [^{35}S]methionine was purchased from Amersham/Searle. Bovine liver glutamate dehydrogenase was obtained from Sigma Chemical Co. in suspension in 2 M $(NH_4)_2SO_4$, pH 7.0. Complete Freund adjuvant was from Difco and nucleotides from Boehringer.

DEAE-Sephadex A-25, Sephadex G-25, and G-200 were from Pharmacia, Upsala, Sweden. All other chemicals were from Mallinckrodt and Schwarz-Mann in the highest available purity.

Fractionation Procedure. All operations were done at 0–4°. Rat livers were homogenized in medium A (0.25 M sucrose–5 mM Tris-Cl–1 mM EGTA,[1] pH 7.4, at 1:5 (w/v)). After sedimentation of the nuclear fraction at 600 g for 10 min, mitochondria were separated from the supernatant fraction by centrifugation at 6500*g* for 15 min (Sottocasa *et al.*, 1967). Mitochondria were washed twice with one-half of the initial volume of medium A. Microsomes were obtained by centrifuging the 6500*g* supernatant fraction first at 19,000*g* for 15 min in order to remove residual mitochondria and then at 105,000*g* for 1 hr.

Both microsomes and mitochondria were suspended in 0.01 M Tris-acetate buffer–0.1 mM EDTA, pH 7.4, at about 20 mg of protein/ml and sonicated twice for 20 sec at 2 A (Branson sonifier) in ice to prevent the temperature from rising above 8°. The sonicated suspensions were sedimented at 105,000*g* for 1 hr. The supernatant fractions obtained will be referred to as the "Tris-acetate soluble fractions." The pellets were homogenized in 0.1 M phosphate (K^+) buffer, pH 7.4, incubated at 30° for 15 min or alternatively sonicated in ice, 2 × 20 sec at 4 A. Between each sonication, the temperature was lowered below 5°. The supernatant fractions (105,000*g*, 60 min) prepared from these fractions will be called the "phosphate soluble fractions."

The contamination of mitochondria by microsomes was monitored by using glucose 6-phosphatase as a marker enzyme and the contamination of microsomes by mitochondria was monitored by assays for glutamate dehydrogenase, as described by Schnaitman and Greenawalt (1968). In later experiments, the presence of mitochondria in microsomes was detected by the latent glutamate dehydrogenase activity unmasked by treatment with 0.5 mg of Lubrol WX/mg of protein.

When indicated, rats were fasted 24 or 48 hr before the experiments. "Injected rats" were first fasted 24 hr, and then injected intraperitoneally with a complete mixture of amino acids corresponding to the amounts in 100 ml of blood plasma as described by Exton and Park (1967) in which unlabeled isoleucine was replaced by 100 μCi of L-[^{14}C]isoleucine. L-[^{14}C]Isoleucine was chosen because it is an indispensable amino acid for the rat, slowly metabolized, and present in high content in rat liver glutamate dehydrogenase (Sedgwick-King and Frieden, 1970).

Isolation, Purification, and Counting of Labeled Proteins. To study the incorporation of amino acids into rat proteins, the procedure described by Hochberg *et al.* (1972b) was used. In addition, glutamate dehydrogenase was isolated with its specific antibody as described below.

To follow the redistribution of radioactive glutamate dehydrogenase during subcellular fractionation, ^{35}S-labeled glutamate dehydrogenase was added to medium A prior to homogenizing the liver tissue. An aliquot of each subcellular fraction was precipitated with 8% trichloroacetic acid. The precipitate was collected by filtration on fiber glass disks (Gelman) and washed twice with 5 ml of 10% trichloroacetic acid. The disks were dried and counted as described by Hochberg *et al.* (1972b).

Analytical Procedures. Proteins were determined with the biuret method (Layne, 1957) or with the Lowry method (Lowry *et al.*, 1951). The protein concentration in solutions of glutamate dehydrogenase was measured from the optical density at 280 nm, assuming an absorbance of 0.97 per milligram of enzyme (Olson and Anfinsen, 1953). Glutamate dehydrogenase activity was measured according to Arnold and Maier (1971).

Purification of Rat Liver Glutamate Dehydrogenase. All operations were conducted at 0–4°. Mitochondria prepared from rat liver according to Johnson and Lardy (1967) were homogenized in 0.02 M Tris-acetate–2 mM EDTA, pH 7.4, for 3 min in a Waring Blendor at full speed. After stirring for 1 hr and centrifuging (23,500*g*, 25 min) the supernatant was concentrated by ultrafiltration in an Amicon cell equipped with an XM-50 membrane. After centrifugation at high speed (200,000*g* for 60 min), a fractionation by 0.55–1.37 M $(NH_4)_2SO_4$ was carried out at pH 6.5 according to Arnold and Maier (1971). The pellet was dissolved in 0.025 M phosphate (K^+), pH 7.1, and applied onto a column (30 × 2.5 cm) of calcium phosphate containing 10 g of cellulose (Whatmann CF 11) per gram of calcium phosphate gel (dry weight) prepared according to Swingle and Tiselius (1951). The column was equilibrated with 0.025 M phosphate (K^+). Glutamate dehydrogenase was eluted with a linear gradient of phosphate (K^+) (250 ml (0.025 M)–250 ml (0.2 M)) at a flow rate of 20 ml/hr. The enzyme was precipitated from the column effluent by addition of crystalline $(NH_4)_2SO_4$ up to a concentration of 1.78 M. After centrifugation (17,000*g* for 20 min), the sediment was dissolved in 0.025 M phosphate (K^+), pH 7.1.

[1] Abbreviations used are: EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetate.

TABLE I: Purification of Glutamate Dehydrogenase from Rat Liver.[a]

Fraction	Vol (ml)	Proteins (Total mg)	Enzyme Act. Total Units	Enzyme Act. Units/mg of Protein	Purification (Fold)	Recovery (%)
Homogenized mitochondria	145	3451	3710	1.1	1.0	100
Mitochondrial supernatant	140	1099	3000	2.7	2.5	80.5
High-speed supernatant	38	471	2386	5.1	4.7	75.0
$(NH_4)_2SO_4$ fractionation	38	300	2354	7.7	7.3	63.4
Calcium phosphate eluate	180	90	1600	17.7	16.5	43.0
1.78 M $(NH_4)_2SO_4$	3	63	1410	22.4	21	37.0
1st crystals	1	24	1171	49	35	31.6
2nd crystals	1	15	1200	80	75	32.0
3rd crystals	1	6.5	975	160	140	26.3
4th crystals	1	4.05	730	180	172	19.7

[a] Enzyme activity was measured at 25° in 50 mM triethanolamine buffer (pH 7.6) containing 5 mM EDTA, 0.2 mM NADH, 3 mM α-ketoglutarate, 40 mM $(NH_4)_2SO_4$, and 2 mM ADP. Initial rate of oxidation of NADH was followed at 340 nm after enzyme addition. No significant oxidation could be detected when α-ketoglutarate was omitted. Glutamate dehydrogenase activity is expressed in micromoles of NADH oxidized per minute.

$(NH_4)_2SO_4$ was added up to a concentration of 0.6 M. The pellet (17,000*g* for 20 min) was discarded. To crystallize the enzyme the solution was brought to a faint turbidity with a saturated solution of $(NH_4)_2SO_4$, pH 7.0, and allowed to stand for several days at 4° with occasional stirring. Crystallizations were repeated as long as the specific activity was not the same in the crystals and in the mother solution (three or four times). Crystals were suspended in 2.0 M $(NH_4)_2SO_4$ at pH 7.0. The specific activity was constant for at least 4 months. Table I shows a typical example of this purification. This preparation was made from eight rats weighing 200 g. When the specific activity before the crystallization steps was lower than 15, an additional purification on a DEAE-Sephadex column was performed according to Sedgwick-King and Frieden (1970).

Homogeneity was checked according to Arnold and Maier (1971). The purified enzyme passed through a Sephadex G-200 column was eluted with 0.025 M phosphate (K^+) as a symmetrical homogeneous peak. Analytical ultracentrifugation revealed a single peak. In gel electrophoresis, only a single protein band was detected. It moved toward the anode.

To prepare radioactive glutamate dehydrogenase, six rats weighing about 150 g were fasted for 24 hr, injected with 1 ml of [^{35}S]methionine (30 Ci/mmol) in 0.9% NaCl (0.58 mCi/rat), and killed after 2 hr. The enzyme was purified by the same procedure.

Immunological Procedures. Bovine liver glutamate dehydrogenase prepared by Sigma Chemical Co. was used for the preparation of antibodies. Its purity was checked as described for rat liver glutamate dehydrogenase. Samples showing a possible contamination were recrystallized twice. Antibodies were prepared according to Talal *et al.* (1964) or according to Lehmann (1971). Both techniques gave similar yield of antibodies, the variations observed depending only on individual rabbits.

Antisera were tested qualitatively by Ouchterlony double diffusion analysis (1958) in 1% agarose–0.9% NaCl–50 mM phosphate (K^+), pH 7.6. Their titer was determined by a quantitative precipitin technique (rat or bovine liver enzyme was incubated with the antiserum at 25° for 1 hr in the presence of 0.9% NaCl–0.1 M borate (Na^+) buffer, pH 8.4 (final volume, 0.8 ml). After 24 hr at 0–4°, the immunoprecipitates were collected by centrifugation (2500*g*, 30 min) and washed four times with 5 ml of the borate–saline buffer. Their protein content was measured by the method of Lowry *et al.* (1951).

Immunochemical analyses in mitochondrial or microsomal soluble fractions were carried out under similar conditions. However, before the preparation of the immunoprecipitates, these soluble fractions were incubated at 25° for 1 hr and centrifuged (20,000*g*, 20 min). When radioactive glutamate dehydrogenase preparations were assayed they were previously treated with Sephadex G-25 to remove the free labeled isoleucine as described by Volfin *et al.* (1969) and the immunoprecipitates were washed with borate–saline buffer containing 10 mM unlabeled isoleucine. Nonimmune serum was used as a control for nonspecifically precipitating material and was always close to blank values in protein determinations and close to background levels in radioactivity measurements.

Gel analyses were performed according to the technique of Cashmann and Pitot (1971), modified as follows: 7.5% sodium dodecyl sulfate–polyacrylamide gels (15 × 0.6 cm) were prepared as described by Schnaitman (1969). Washed immunoprecipitates were air-dried at 37°, dissolved in 100 μl of 8 M urea–0.1% sodium dodecyl sulfate–10 mM dithiothreitol–0.1 M phosphate (Na^+), pH 7.1, and incubated at 45° for 1 hr. Samples were layered onto the gels with 5 μl of 0.05% Bromophenol Blue as a tracking dye and electrophoresis was carried out for 12 hr at 6 mA/gel. Under these conditions, the dye moved about 13 cm. Gels were fixed by soaking 18 hr in 20% sulfosalicylic acid, then immersed in 0.02% Coomassie Blue dissolved in 12% trichloroacetic acid–3.5% sulfosalicylic acid–25% methanol for 1 hr at 60°, and destained with 7.5% acetic acid–25% ethanol. Gels containing the assays and gels prepared with known amounts of rat liver glutamate dehydrogenase precipitated with the same antiserum and treated by the same way were photographed together. The photographs were scanned with a Joyce-Loebl microdensitometer. The

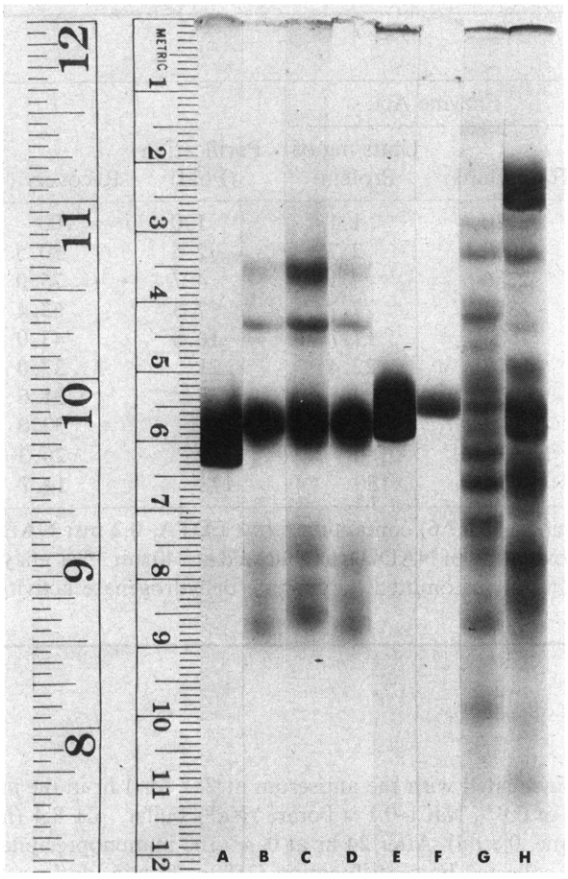

FIGURE 1: Sodium dodecyl sulfate–gel electrophoresis of glutamate dehydrogenase: purified enzymes, crude soluble fractions, and immunoprecipitates: A, beef liver glutamate dehydrogenase, 100 μg; B, immunoprecipitate prepared with the Tris-soluble mitochondrial fraction containing 4.95 glutamate dehydrogenase units; C, immunoprecipitate prepared with 30 μg of purified rat liver enzyme, 190 units/mg; D, immunoprecipitate prepared with the phosphate-soluble microsomal fraction containing 3.2 glutamate dehydrogenase units; E, purified rat liver enzyme, 100 μg; F, purified rat liver enzyme, 20 μg; G, phosphate-soluble microsomal fractions, 200 μg of protein; H, Tris-soluble mitochondrial fraction, 200 μg of protein. All samples were treated as described in the Experimental Section.

areas of the peaks corresponding to glutamate dehydrogenase subunits were estimated by multiplying the height of the peak by its width at half-height. Within experimental error, the quantity of enzyme determined by this procedure was identical with that obtained from the quantity of protein in the immunoprecipitates. Because significant counts could be detected in the area of the gel where the tracking dye migrates, this gel electrophoresis technique was always employed to assess the radioactivity incorporated into the subunits of glutamate dehydrogenase.

To measure the radioactivity on the gel, the bands corresponding to glutamate dehydrogenase subunits were cut out, dissolved in 0.2 ml of H_2O_2 at 70° for 4 hr in capped vials, and counted either as described by Stratman *et al.* (1972) or after adding 15 ml of scintillation solution containing 100 ml of Biosolv BSIII (Beckman), 4 g of 2,5-diphenyloxazole, and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene per liter of toluene. The two techniques gave equivalent counting efficiencies of 60%.

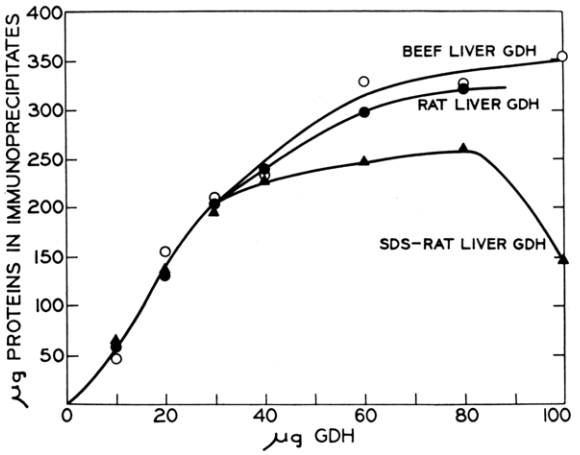

FIGURE 2: Immunoprecipitin curves obtained with beef liver and rat liver glutamate dehydrogenase (active or inactive): beef liver, 190 units/mg of protein; rat liver, 210 units/mg of protein; sodium dodecyl sulfate–rat liver enzyme, no activity. One milligram of active rat liver glutamate dehydrogenase was incubated (30 min) in 0.2 ml of 5 mM sodium dodecyl sulfate–10 mM dithiothreitol–10 mM Tris-acetate, pH 7.2, before the preparation of the immunoprecipitates. The preparation of immunoprecipitates, three washings, and the determination of their protein contents are described under Immunological Procedures. For each assay, the value of the control prepared with a normal serum was subtracted; 0.2 ml of serum was used.

Results

Properties of the Antiglutamate Dehydrogenase Serum. As Arnold and Maier (1971) have shown that an antiserum prepared with beef liver glutamate dehydrogenase reacts as well with rat liver glutamate dehydrogenase, antiserum was prepared with commercial beef liver glutamate dehydrogenase. The specificity of this antiserum was checked qualitatively by immunodiffusion and immunoelectrophoresis according to Arnold and Maier (1971). No difference could be demonstrated between the precipitin lines obtained with purified rat or beef liver glutamate dehydrogenase. Several precipitin lines could be detected which confirms the data of Talal *et al.* (1964). No difference was apparent between the purified rat liver glutamate dehydrogenase and the Tris soluble fraction of mitochondria.

When a crude Tris-acetate soluble fraction of mitochondria or a Tris-phosphate soluble fraction of microsomes was submitted to sodium dodecyl sulfate gel electrophoresis after treatment with sodium dodecyl sulfate and urea, the electrophoretogram obtained presented a complex pattern of bands (Figure 1, H and G) and it was hazardous to decide which band corresponded to the band of purified rat liver glutamate dehydrogenase (Figure 1, F and E). Therefore, to determine the specific radioactivity of the glutamate dehydrogenase directly on these gels, glutamate dehydrogenase was first separated from other soluble proteins by immunoprecipitation.

Immunoprecipitates were dissociated in sodium dodecyl sulfate and urea and analyzed on gel electrophoresis. Similar patterns were obtained if the immunoprecipitates were prepared with purified rat liver glutamate dehydrogenase (Figure 1, C), beef liver glutamate dehydrogenase (not shown), the Tris-soluble fraction of mitochondria (Figure 1, B), and the phosphate-soluble fraction of microsomes (Figure 1, D). The band corresponding to enzyme subunits (Figure 1, F and E) was well separated from the other bands. These studies demonstrated that the antiserum was specific toward glu-

TABLE II: Solubilization of Glutamate Dehydrogenase Associated with Mitochondria and Microsomes.[a]

Treatment of Rats	No. of Expt	% Enzyme Solubilized by Sonication in Tris-Acetate–EDTA		% Enzyme Extracted by 0.1 M Phosphate
		Mitochondria	Microsomes	Microsomes
Normally fed	5	79.2 ± 4.1	2.3 ± 1.1	71.9 ± 8.7
Fasted 1 day	3	67.3 ± 1.3	0.7 ± 0.45	91.0 ± 9.0
Fasted 2 days	3	78.3 ± 3.8	3.0 ± 1.6	79.2 ± 11.4
Injected[b]	9	79.8 ± 8.2	5.9 ± 2.1	75.0 ± 4.0

[a] Mitochondria and microsomes were prepared as described under Fractionation Procedure, suspended in 0.01 M Tris-acetate buffer–0.1 mM EDTA, pH 7.4, sonicated, and centrifuged (105,000g × 60 min). The per cent enzyme solubilized by sonication represents the per cent of glutamate dehydrogenase found in this supernatant as compared to the whole sonicate. Microsomal pellets obtained after this treatment were suspended in 0.1 M phosphate buffer, pH 7.4, incubated at 30° for 15 min, and centrifuged (105,000g × 60 min). The per cent enzyme extracted by 0.1 M phosphate represents the per cent of activity in this supernatant as compared to the initial activity found in the microsomes. Glutamate dehydrogenase activity was measured as in Table I. Each value is followed by the standard error of the mean: $(\Sigma(x - \bar{x})^2/N(N-1))^{1/2}$. [b] Injected rats are fasted 1 day, injected with amino acids, and killed 2 hr later.

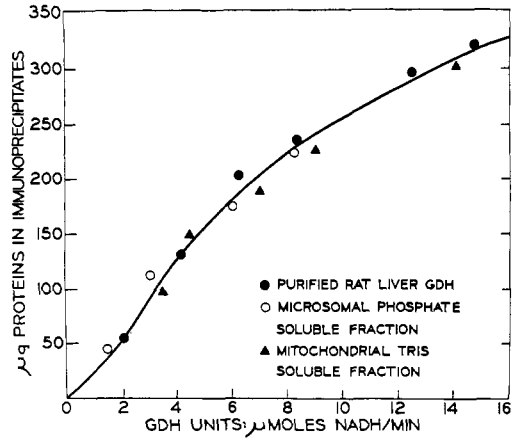

FIGURE 3: Immunoprecipitin curves obtained with purified rat liver enzyme, mitochondrial extracts, and microsomal extracts: purified rat liver enzyme, 210 units/mg of protein. Mitochondrial soluble fractions (5.3 glutamate dehydrogenase units/mg of protein, 18.4 mg of protein/ml) and microsomal phosphate soluble fractions (1.4 glutamate dehydrogenase unit/mg of protein, 21.8 mg of protein/ml) were isolated as described under Fractionation Techniques. For immunoprecipitations, see Figure 1, but 0.1 ml of serum was used. No enzyme activity could be detected in the supernatants.

tamate dehydrogenase and confirmed that rat and beef liver enzymes were not immunologically different qualitatively.

Figure 2 shows that the immunoprecipitation of rat and beef liver glutamate dehydrogenase in active form was also quantitatively very similar. The immunoprecipitin curve obtained with rat liver enzyme dissociated with sodium dodecyl sulfate was similar for a low concentration of glutamate dehydrogenase but exhibited a typical dissolution of the antigen–antibody complex by excess of antigen. This was not observed with native glutamate dehydrogenase. The maximal sodium dodecyl sulfate concentration present (10 μM) was not sufficient to dissociate the complex.

Quantitative measurements of immunoprecipitates with purified rat liver enzyme, rat liver enzyme present in the Tris-soluble fraction of mitochondria, and phosphate-soluble fraction of microsomes yielded coincidental plots (Figure 3), indicating that the intrinsic specific activity of glutamate dehydrogenase was identical for the purified enzyme and for the enzyme associated with the mitochondria or with the microsomes.

Association of Glutamate Dehydrogenase with Microsomal and Mitochondrial Membranes. A mild sonication of mitochondria in Tris-acetate (low ionic strength medium) solubilized 67–90% of the enzyme activity (Table II). The same treatment solubilized only 0.7–5.8% of the enzyme activity linked to the microsomes. A medium of higher ionic strength, such as 0.1 M phosphate (K^+), and either an incubation at 30° for 15 min or a sonication (2 × 20 sec, 4 A), were necessary to solubilize 72–91% of the enzyme bound to the microsomes. Fasting the rats or injecting amino acids to fasted rats did not change significantly the amount of enzyme or the way it could be released from either mitochondria or microsomes.

Table III shows that glutamate dehydrogenase could be partially extracted from the microsomes when substrates and effectors of the enzyme were added to the low ionic strength buffer. Glutamate (20 mM) alone had no effect but glutamate with 2 mM NAD^+ or 2 mM NAD^+ + 0.5 mM GTP, respectively, could solubilize 34.3 and 53% of the enzyme activity.

The glutamate dehydrogenase activity bound to the microsomes could not be attributed to small mitochondria contaminating these microsomes. Treatment with Lubrol WX or sonication increased the glutamate dehydrogenase activity of the mitochondria from 18 to 20 times, but neither of these treatments had any effect on enzyme activity in the microsomal

TABLE III: Solubilization of Glutamate Dehydrogenase from Microsomes in the Presence of Effector.[a]

Incubation Medium	Effectors (mM)			% Enzyme Solubilized
	Glu	NAD^+	GTP	
Tris-acetate–EDTA				2.9
Tris-acetate–EDTA	20			0.5
Tris-acetate–EDTA	20	2		34.3
Tris-acetate–EDTA	20	2	0.5	53.0
0.1 M phosphate (K)				88.0

[a] Microsomes were obtained from rats fasted one day and suspended in Tris-acetate–EDTA buffer as described in Table II or in 0.1 M potassium phosphate buffer, pH 7.4. Effectors were added when indicated. Final volume was 2 ml. Incubation was at 30° for 15 min and centrifugation at 105,000g for 1 hr. Enzyme activity was measured in 5-μl aliquots of the complete incubation mixture and of the supernatant to determine the percentage of total activity found in the supernatants.

TABLE IV: Glutamate Dehydrogenase and Glucose 6-Phosphatase Activity in Hepatic Mitochondrial and Microsomal Fractions.[a]

	Rats Normally Fed	24-Hr Fasted Rats	Injected Rats
	Glucose 6-Phosphatase		
No. of expts	4	4	
Sp act. in homogenate	0.046 ± 0.030	0.076 ± 0.007	
Sp act. in microsomes	0.143 ± 0.026	0.218 ± 0.008	
Sp act. in mitochondria	0.012 ± 0.030	0.014 ± 0.001	
% microsomes in mitochondria	7.6 ± 1.2	6.6 ± 0.6	
% microsomes in homogenate	31.0 ± 2.3	34.7 ± 1.3	
	Glutamate Dehydrogenase		
No. of expts	9	9	13
Total act. in homogenate	1876 ± 22	1594 ± 121	1767 ± 72
Sp act. in microsomes	0.19 ± 0.03	0.39 ± 0.05	0.47 ± 0.03
Sp act. in mitochondria	3.24 ± 0.66	4.07 ± 0.45	3.98 ± 0.33
Sp act. in postmicrosomal supernatant	0.004 ± 0.001	0.014 ± 0.001	0.015 ± 0.001
% mitochondria in microsomes	5.6 ± 0.1	9.8 ± 0.7	10.6 ± 0.6
% mitochondria in homogenate	33.4 ± 1.5	29.8 ± 4.8	28.0 ± 1.8

[a] Mitochondria and microsomes were prepared as described in the Experimental Section. Glucose 6-phosphatase activity was measured in initial homogenate, in mitochondria, and microsomes according to Swanson (1950). Specific activity is expressed in micromoles of P_i liberated per minute and per milligram of protein. Glutamate dehydrogenase was measured in homogenate, mitochondria, and microsomes in the presence of Lubrol WX (0.5 mg/mg of protein). Specific activity is expressed in micromoles of NADH oxidized per minute per milligram of protein. Total activity corresponds to the whole liver homogenate of one rat (weighing 200 ± 5 g before fasting). The per cent of one organelle contained in another fraction was calculated according to Schnaitman and Greenawalt (1968).

fraction. Therefore, in this fraction, glutamate dehydrogenase was not present within functional mitochondrial membrane.

Table IV shows the quantitative distribution of enzymes characteristic of mitochondria and microsomes, respectively. Assuming according to Schnaitman and Greenawalt (1968) that all glutamate dehydrogenase is located in mitochondria and all glucose 6-phosphatase is in microsomes, an apparent contamination of 5.6% of mitochondria in microsomes and 7.6% of microsomes in mitochondria from normal rats can be calculated. These figures are close to those described by Sottocasa *et al.* (1967). Since sonication or Lubrol did not enhance the enzyme in the microsomal fraction, this activity must be of microsomal origin or could represent enzyme released from mitochondria, by damage during homogenization, and adsorbed to microsomes. Starving the rats, with or without injection of amino acids, slightly diminished the total glutamate dehydrogenase activity in the whole homogenate. Enzyme specific activity was not significantly modified in the mitochondria but doubled in the microsomes. Rather than an augmentation of the contamination of microsomes by mitochondria, this increase in the microsomal glutamate dehydrogenase specific activity must reflect a diminution of the content of other proteins by fasting, a fact that is well documented (*cf.* Freedland and Szepezi, 1971).

TABLE V: Specific Enzymatic Activity of Glutamate Dehydrogenase in the Tris-Soluble Fraction of Mitochondria and the P_i-Soluble Fraction of Microsomes.[a]

Fraction	Enzyme Act. ± SEM[b] (μmol of NADH Oxidized per min per mg of Enzyme)
Tris-acetate soluble fraction of mitochondria	180 ± 11
P_i-soluble fraction of microsomes	178 ± 15

[a] Enzyme activity was determined as described in Table I. Twice as much antiserum as is required to precipitate all of the glutamate dehydrogenase activity was added and the immunoprecipitates were analyzed with the sodium dodecyl sulfate gel procedure (see Experimental Section). The milligrams of glutamate dehydrogenase present in each solution were estimated by comparison to a standard curve made with purified rat liver enzyme treated with the antiserum at the same time as the soluble fractions. The specific activity was measured by dividing the enzymic activity introduced by the milligrams of glutamate dehydrogenase found in the immunoprecipitates. The values are the average of 14 experiments. [b] SEM = standard error of the mean.

Incorporation of [^{14}C]Isoleucine into Rat Liver Glutamate Dehydrogenase. The time-dependent incorporation of [^{14}C]-isoleucine into rat liver proteins associated with different cell fractions is shown in Figure 4. The specific radioactivity of the microsomal proteins attained its maximum after 1 hr, declined rapidly between 1 and 2 hr, and declined slowly thereafter. In the postmicrosomal supernatant, the incorporation arrived at a plateau after 2 hr. The incorporation into whole mitochondria was at a maximum after 1 hr and then decreased slowly. In the soluble fraction, the plateau was reached at about 90 min. The highest specific radioactivity was found in the microsomes followed by the postmicrosomal supernatant while the lowest one corresponded to the soluble mitochondrial proteins.

The kinetics of labeling of purified microsomal and mitochondrial glutamate dehydrogenase indicates that the enzyme associated with the microsomes was labeled first and to a

TABLE VI: Redistribution of Glutamate Dehydrogenase during Subcellular Fractionation.[a]

Fraction	Radioactivity (cpm/mg of Protein)	Radioactive + Endogenous Enzyme	
		(Enzyme Unit/mg of Protein)	(cpm/Enzyme Unit)
Purified enzyme introduced	10,166	180	56.5
Homogenate	6.3 ± 0.25	0.7 ± 0.04	9.0
Postmicrosomal supernatant (PMS)	1.3 ± 0.1	0.057 ± 0.001	22.8
Microsomes	42.6 ± 1.1	1.35 ± 0.08	31.5
Mitochondria	17.8 ± 2.8	3.73 ± 0.14	4.77
Washing of mitochondria	36.8 ± 2.2	0.25 ± 0.04	147.0
Tris-soluble mitochondria	24.8 ± 2.6	4.94	5.02
Tris-soluble microsomes	77.7 ± 3.2	1.05 ± 0.04	74.0
P_i mitochondria	9.5 ± 1.9	0.85	10.0
P_i microsomes	12.3 ± 1.0	1.49 ± 0.01	8.25

[a] Each value (average of three experiments) is followed by the standard error of the mean. ^{35}S-Labeled glutamate dehydrogenase (0.6 mg) was added to 10 ml of 0.25 M sucrose–5 mM Tris-Cl–1 mM EGTA, pH 7.4, used to homogenize 2 g of rat liver (24-hr fasted). Fractionation procedure and radioactivity determinations are described in the Experimental Section. Enzyme activity was measured in the presence of Lubrol (0.5 mg/mg of protein); PMS = 105,000g × 1 hr supernatant fraction; microsomes = 105,000g × 1 hr pellet; mitochondria = 6500g × 15 min pellet of mitochondria washed twice; washing of mitochondria = supernatant of the first washing of the mitochondria; Tris-soluble mitochondria and Tris-soluble microsomes = Tris-acetate soluble fractions of mitochondria and microsomes, respectively. For the P_i mitochondria and P_i microsomes (after extraction of the Tris-acetate soluble fraction), the pellet of mitochondria and microsomes was homogenized in 0.1 M phosphate (Na^+), pH 7.4.

higher extent than the enzyme in the mitochondria. The maximal incorporation was obtained after 90 min in the microsomes. The specific activity of the enzyme associated with the microsomes was then three times higher than the specific activity of enzyme extracted from mitochondria. At the same time, the specific activity of the microsomal proteins was about four times higher than the specific activity of the mitochondrial proteins.

Table V shows that the enzymatic activity of glutamate dehydrogenase extracted from mitochondria or microsomes was identical. For these experiments, an aliquot of the mitochondrial or microsomal soluble fractions containing a known glutamate dehydrogenase activity was added to a twofold excess of antiserum. The immunoprecipitates were collected and analyzed on gel electrophoresis. The actual amount of enzyme was determined by comparison to a standard curve made with purified rat liver glutamate dehydrogenase. The supernatant fraction obtained after centrifuging the immunoprecipitates contained no more enzyme activity and the addition of a new amount of antiserum to this supernatant fraction did not precipitate any more material.

Redistribution of Glutamate Dehydrogenase during Subcellular Fractionation. To interpret these results, it was important to know how any glutamate dehydrogenase released in a soluble form from mitochondria during cell disruption and fractionation would be subsequently redistributed. This was studied by adding radioactive glutamate dehydrogenase to the homogenization medium and following the location of the radioactivity among the cell fractions. The results presented in Tables VI and VII show that the added radioactive glutamate dehydrogenase did not remain in the soluble fraction but bound to the membranes. The specific radioactivity present in the postmicrosomal supernatant fraction was lower than the specific radioactivity in the homogenate (Table VI) and represented only 3.5% of the total radioactivity introduced (Table VII). The radioactive glutamate dehydrogenase bound to the mitochondria was partially removed by the washings. The highest specific radioactivity was linked to the microsomes and could be extracted at least partially by sonication in 0.01 M Tris-acetate–1 mM EDTA. The specific radioactivity was higher in the Tris-soluble fraction of the microsomes than in the total microsomes (Table VI). Moreover, 67% of the radioactivity passed into the soluble fraction of microsomes by sonication while only 31.3% of the enzymatic activity bound to the microsomes was extracted by this sonication (Table VII). On the contrary, only 57% of the radioactivity associated with the mitochondria was extracted by sonication while 88.1% of the total enzymatic activity was solubilized (Table VII). Since the radioactive glutamate dehydrogenase and the endogenous glutamate dehydrogenase do not extract in the same way, there must have been a pool

TABLE VII: Per Cent of Glutamate Dehydrogenase Rendered Soluble from Different Fractions.[a]

	% of Radioactive Enzyme Solution	% of Enzymatic Found in Solution
PMS/homogenate[b]	3.5 ± 0.4	1.4 ± 0.04
Tris-soluble fraction of mitochondria/mitochondria	56.8 ± 5.2	88.0 ± 6.0
Tris-soluble fraction of microsomes/microsomes	67.3 ± 2.2	31.3 ± 2.1

[a] Conditions as in Table VI. The percentages are calculated by comparing the total radioactivity or the total enzymatic activity present in each fraction. [b] PMS, 105,000g × 1 hr supernatant fraction.

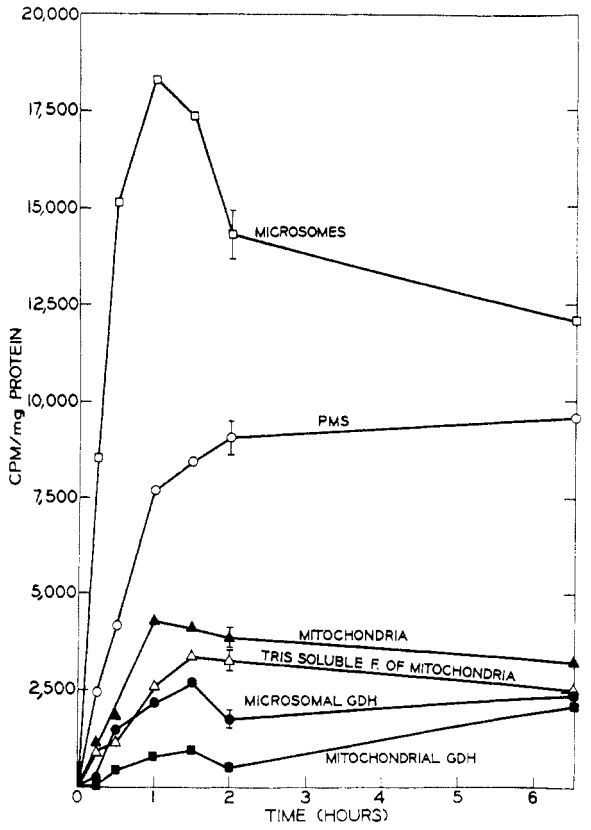

FIGURE 4: Time-dependent incorporation of [^{14}C]isoleucine into proteins of different cell fractions and into immunoprecipitated glutamate dehydrogenase. Rats were injected with 100 μCi of [^{14}C]isoleucine (200 mCi/mmol) and labeled mitochondrial, microsomal, and postmicrosomal proteins were isolated as described in the Experimental Section. Specific radioactivity of glutamate dehydrogenase was determined after immunoprecipitation, dissociation of the immunoprecipitates, and separation of the dissolved proteins on sodium dodecyl sulfate–gel electrophoresis. The amount of enzyme present was determined by scanning the gels' photographs and the radioactivity was measured after cutting the corresponding bands as described in the Experimental Section; PMS, 105,000g $\times$ 1 hr supernatant fraction.

of glutamate dehydrogenase in the microsomes and in the mitochondria that was not in equilibrium with the radioactive enzyme.

The ratio counts per minute per glutamate dehydrogenase unit in the Tris-soluble fraction of the mitochondria is only half that in the mitochondrial pellet: P_i mitochondria. This could mean either that the radioactive glutamate dehydrogenase linked to the pellet was inactive or that the radioactive enzyme was more tightly bound to the membrane than endogenous one. The ratio counts per minute per glutamate dehydrogenase unit cannot be used to calculate the endogenous glutamate dehydrogenase associated with the different fractions, for the added enzyme bearing the ^{35}S does not equilibrate with the endogenous enzyme and there is evidence that the radioactive enzyme was denatured during the fractionation process. Without denaturation, the ratio counts per minute per enzyme unit could never be higher in any fraction than 56, the ratio in the initially added glutamate dehydrogenase. In the washings of the mitochondria and in the Tris-soluble fraction of the microsomes, this ratio greatly exceeded the theoretical maximum indicating the presence of denatured radioactive glutamate dehydrogenase.

This conclusion was confirmed by the results presented in Table VIII. The glutamate dehydrogenase present in the Tris-acetate soluble fraction of the microsomes was treated with the antiglutamate dehydrogenase serum and the immunoprecipitable proteins were determined. The specific enzymatic activity of the glutamate dehydrogenase calculated as in Table V was diminished by about 35% which demonstrated a partial denaturation during the preparation of the microsomal sample. Moreover, the specific radioactivity of the glutamate dehydrogenase diminished slightly indicating that a small amount of endogenous enzyme was present in the microsomes.

TABLE VIII: Properties of Glutamate Dehydrogenase in the Tris-Soluble Microsomal Fraction.[a]

	Purified Enzyme Introduced in Homogenate	Tris-Soluble Microsomal Fraction	
		Total Extract	Immunoprecipitate
Enzyme unit/mg of protein	180	1.3	116[b]
Cpm/mg of protein	9400	94.0	8500[c]
Cpm/unit	52	73.5	73.5

[a] Conditions as in Table VI, except that 1.65 mg of [^{35}S]-glutamate dehydrogenase (9400 cpm/mg) was homogenized with 3.5 g of rat liver. The Tris-acetate soluble fraction obtained from the microsomes was treated with the enzyme antiserum to determine the content of precipitable material. [b] Specific enzymatic activity of the enzyme as in Table V. [c] Specific radioactivity of the enzyme component of the precipitate.

Discussion

The immunodiffusion test and the sodium dodecyl sulfate gel electrophoresis obtained with purified enzymes, mitochondrial or microsomal crude fractions demonstrate the specificity of the antiserum. The atypical precipitin curve obtained with the purified enzyme may be explained by the fact that glutamate dehydrogenase exists in different immunologically distinct molecular forms (Talal *et al.*, 1964) since this curve becomes typical with glutamate dehydrogenase dissociated with sodium dodecyl sulfate where only subunits are present (Talal *et al.*, 1964).

The time-dependent incorporation of [^{14}C]isoleucine into glutamate dehydrogenase showed that the enzyme solubilized from the microsomes was labeled before and to a higher extent than the one extracted from the mitochondria. These results indicate that glutamate dehydrogenase must be synthesized on the microsomes and then be incorporated into the mitochondria. The fact that the same enzymic activity per milligram of glutamate dehydrogenase was found in the phosphate-soluble fraction of microsomes, in the Tris-acetate soluble fraction of mitochondria (Table V), and in the purified rat liver glutamate dehydrogenase (Table I) (Figure 3) indicates that these fractions contain the enzyme in its active form. As the antiserum was able to precipitate the subunits of glutamate dehydrogenase as well as the active enzyme (Figure 2) and as the antiserum was able to precipitate inactivated radioactive glutamate dehydrogenase in the Tris-acetate soluble fraction (Table VIII), the mitochondrial and micro-

somal soluble fractions studied must not contain enzyme subunits, at least not subunits similar to those obtained with sodium dodecyl sulfate treatment. However, these results do not exclude the possibility that inactive glutamate dehydrogenase subunits are present in the microsomes but not extracted in this phosphate-soluble fraction. The conclusion that biogenesis of the enzyme occurs on the microsomes is in agreement with results obtained by Solomon (1959), who observed that glutamate dehydrogenase activity of embryonic chick liver started to increase in the mitochondrial fraction after the twelfth day of incubation and diminished drastically in the supernatant fraction at 15 days of incubation. He concluded that this might be due to the transfer of this enzyme from extramitochondrial sources to the mitochondria. This also agrees with the results of Balinsky *et al.* (1970) which showed that the synthesis *de novo* of glutamate dehydrogenase in amphibian premetamorphic tadpoles was inhibited by inhibitors specific of cytoribosomal protein synthesis. However, the transfer must not be simple since the specific radioactivity of glutamate dehydrogenase at 2 hr in the mitochondrial soluble fraction is lower than at 90 min.

The glutamate dehydrogenase activity found in the microsomal fraction cannot be attributed to small functional mitochondria which would sediment with the microsomes because the activity in this fraction is not increased by sonication or detergent treatment. But this activity could partly come from damaged mitochondria and subsequent redistribution. Indeed, when radioactive glutamate dehydrogenase was added to the homogenate, it was preferentially bound to the microsomes (Table VI). This radioactive enzyme was extracted preferentially to endogenous enzyme by sonication in Tris-acetate (Table VII) and it was partially denatured (Table VIII). Without added enzyme, 0.7–5.9% of glutamate dehydrogenase activity in the microsomal fraction was extracted by sonication in Tris-acetate (Table II), and this percentage was increased when enzyme had been added to the homogenate. These results indicate that the enzyme redistributed during subcellular fractionation ought to be partly removed from the microsomes by this procedure. However, about 33% of the radioactive glutamate dehydrogenase linked to the microsomes remained there after this sonication. Therefore, the truly microsomal enzyme may be diluted by any enzyme released from mitochondria damaged by homogenization. Walter and Anabitarte (1971) found that when soluble glutamate dehydrogenase was added to rat liver homogenates it could be recovered in the cytosolic fraction only in the presence of 50 mM phosphate. The differential release of enzyme activity from the microsomes in the presence of Tris-acetate or phosphate reported here (Table III) explains their results.

By analogy with the work published by González-Cadavid *et al.* (1968) on cytochrome *c*, it is unlikely that glutamate dehydrogenase is synthesized in mitochondria that are preferentially damaged during cell disruption.

If glutamate dehydrogenase is synthesized on the microsomes, it has to be transferred to the mitochondria by some specific mechanism. It has been recently demonstrated that isolated glutamate dehydrogenase interacts with phospholipids (Julliard and Gautheron, 1972; Dodd, 1972). The effects observed depend on experimental conditions and particularly on ionic strength; the lipid–enzyme interaction decreases with increasing ionic strength. But both reports reveal that the charge type of the lipid head determines the extent of lipid–protein complexing. Phosphatidylcholine and phosphatidylethanolamine showed little or no binding. Cardiolipin strongly and reversibly inhibited the enzyme (apparent K_i ranging from 24 nM to 20 μM). Another anionic phospholipid, phosphatidylserine, also inhibited the enzyme but less effectively (apparent K_i ranging from 2 to 8 μM). Moreover, phosphatidylserine inhibition was released by increasing the concentration of glutamate in the presence of 0.5 mM NAD^+, suggesting a competition between the binding of glutamate and phosphatidylserine to the enzyme.

Phosphatidylserine is present in microsomal membranes but can barely be detected in inner mitochondrial membranes of rat liver (Colbeau *et al.*, 1971). On the contrary, cardiolipin is characteristic of inner mitochondrial membranes (Getz *et al.*, 1962). The suggestion that the binding of glutamate dehydrogenase with microsomes could be of the same type as the interaction observed *in vitro* between the purified enzyme and phosphatidylserine appears in Table III. Enzyme activity (88%) is released by incubation in 0.1 M phosphate (Na), whereas almost no release occurred in a low ionic strength buffer such as 0.01 M Tris-acetate. Addition of effectors that increase the affinity of glutamate dehydrogenase for glutamate, *e.g.*, NAD^+ (Frieden, 1959, 1962) or GDP + NAD^+ (Dalziel and Egan, 1972), also increases the release of glutamate dehydrogenase (Table III). The binding to phosphatidylserine would explain the liaison of soluble enzyme with the microsomes, and the fact that the affinity of glutamate dehydrogenase for cardiolipin is higher than for phosphatidylserine could explain the normal location of the enzyme in the mitochondria. As cardiolipin is synthesized by the mitochondria (Dennis and Kennedy, 1972), the transfer of glutamate dehydrogenase from microsomes to mitochondria may be correlated with the synthesis of new cardiolipin. Cardiolipin molecules not already associated with glutamate dehydrogenase or other proteins may aid in the transport of enzyme subunits from the cytosol to the matrix space of mitochondria.

If this sort of mechanism were a general one and if soluble mitochondrial enzymes must first bind to the mitochondrial membrane before being transferred to the matrix, the incorporation of radioactive amino acids into the inner mitochondrial membrane should precede the labeling of proteins in the soluble fraction of mitochondria. This could explain why radioactive amino acids are incorporated more rapidly *in vivo* into proteins associated with the mitochondrial membranes than into fractions containing the more soluble proteins (Truman, 1963; Beattie, 1968; Beattie *et al.*, 1966; Kadenbach, 1969; this paper, Figure 4).

Acknowledgments

The authors are very grateful to Drs. F. W. Stratman, R. N. Zahlten, and A. A. Hochberg for their very helpful cooperation and very stimulating discussions.

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