Nitrogen Metabolism in the Isolated Perfused Rat Liver

NITROGEN BALANCE, REDOX STATE AND RATES OF PROTEOLYSIS

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Nitrogen balances were measured in isolated perfused rat livers in the presence and absence of nitrogen donors. In all instances the balance apparently was incomplete. The expression $[\text{alanine}]/[\alpha\text{-oxoglutarate}]/[\text{pyruvate}]/[\text{glutamate}]$ remained fairly constant under the metabolic conditions studied, indicating that it may be at near-equilibrium. The source of the extra nitrogen seems to be derived from increased hepatic proteolysis. The addition of a nitrogen donor to the perfusate arrested proteolysis, as did the addition of pyruvate. The free mitochondrial $[\text{NAD}^+]/[\text{NADH}]$ ratio, calculated from the glutamate dehydrogenase and β -hydroxybutyrate dehydrogenase reactants, showed similar values and exhibited parallel changes under most metabolic situations studied. These results suggest that, under the reported experimental conditions, both dehydrogenases share a common mitochondrial NAD pool. Glutamate dehydrogenase plays an important role in hepatic nitrogen metabolism *in vivo*.

When livers are isolated from the rest of the organism, and their vascular system is perfused, a net release of urea and other nitrogenous products occurs (Salaskin, 1898). There is a lack of accurate information on the source of nitrogen used for the synthesis of these products. The formation of urea and other nitrogenous products *de novo* must involve the transamination of pre-existing amino acids to form glutamate and/or an increased rate of proteolysis.

Braunstein & Asarkh (1945) and Braunstein (1957) postulated that glutamate dehydrogenase plays a central role in nitrogen metabolism, since it is the most abundant enzyme in mammalian tissues capable of oxidizing amino acids. However, it also has been suggested that glutamate dehydrogenase plays its major role in glutamate synthesis rather than oxidative deamination (Vinogradov, 1968), since isolated liver mitochondria were found to oxidize glutamate preferentially by the transamination pathway (Müller & Leuthardt, 1950; Borst, 1962). In the present paper nitrogen-balance studies, carried out to determine the relative importance of each metabolic route for glutamate metabolism in the whole organ, are reported. Also, since glutamate dehydrogenase is an NAD(P)-linked enzyme, attention has been given to changes in the mitochondrial redox state, which may control whether the enzyme is used for glutamate degradation or glutamate synthesis. A requisite for this type of

explanation is that redox couples of the mitochondrial dehydrogenases should be in equilibrium, and at present there is no general agreement whether or not equilibrium exists between the different mitochondrial dehydrogenases. Several investigators have reported that the reactants of the β -hydroxybutyrate dehydrogenase and glutamate dehydrogenase reactions are at near-equilibrium with the mitochondrial $[\text{NAD}^+]/[\text{NADH}]$ couple in rat liver (Williamson *et al.*, 1967b; Brosnan *et al.*, 1970) whereas others have found no correlation (Chamalaun & Tager, 1970; Henley & Laughrey, 1970; Haeckel & Haeckel, 1970).

It was therefore the purpose of the present study using the isolated perfused rat liver (a) to obtain information as to the source of nitrogen for the synthesis of the various nitrogenous products during perfusion with or without an added nitrogen donor; (b) to determine the fate of glutamate metabolism under these conditions; (c) to determine whether or not an equilibrium exists between the different mitochondrial dehydrogenase systems; and (d) to quantify the importance of the rate of proteolysis in the nitrogen economy in the absence of nitrogen donors and under variable cellular redox states.

Materials and Methods

Reagents

Most reagents were Sigma Grade (from Sigma Chemical Co., St. Louis, Mo., U.S.A.). Enzymes and

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nucleotides were purchased from Boehringer Mannheim Corp., New York, N.Y., U.S.A. [$U-^{14}C$]Valine and [$U-^{14}C$]alanine were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. Stripped *E. coli* tRNA was obtained from General Biochemical Inc., Chagrin Falls, Ohio, U.S.A.

Animals and perfusion technique

Male Sprague-Dawley rats (180–220 g), starved overnight, were used for all experiments. Rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (3 mg/100 g body wt.). The portal vein was cannulated *in situ* and then infused with warm oxygenated Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932). The liver was then excised from the animal, trimmed of adherent tissue and transferred to the perfusion apparatus. The perfusion apparatus and glassware were similar to that described by Miller *et al.* (1951).

The livers were perfused for 1 h with 100 ml of recirculating cell-free buffer containing 4% (w/v) bovine serum albumin (Cohn fraction V; Armour Laboratories, Kankakee, Ill., U.S.A.). Before the experiments the albumin was dissolved in a small volume of buffer and dialysed against two changes of 2 litres of buffer for 18 h at 4°C. All albumin and buffer solutions were passed through a Millipore filter (0.45 μ m) before the perfusion. The perfusion medium was kept at pH 7.4 by exposing it to a humidified mixture of $O_2 + CO_2$ (95:5).

Substrates were added (see the Results section) to obtain a concentration of 10 mM.

Sampling of medium and determinations

For the determinations of NH_3 and urea, samples of perfusate were collected under a thin layer of toluene to prevent losses of NH_3 . Urea was measured with either an autoanalyser (Skeggs, 1957) or a Boehringer Mannheim urea reagent kit. The NH_3 was determined by the procedure of Seligson & Seligson (1951). Additional perfusate samples (0.5 ml) were deproteinized in cold 6% (w/v) $HClO_4$ and neutralized to pH 6–7 with 5 M- K_2CO_3 for determinations of metabolites. All procedures were carried out at 4°C.

Liver biopsies, taken at either 0 or 60 min, were immediately frozen in aluminium clamps (Wollenberger *et al.*, 1960), which had been cooled in liquid N_2 or in a bath of solid CO_2 in methanol. The frozen sample was weighed, pulverized and then rapidly homogenized in 6 vol. of cold 6% $HClO_4$ –40% (v/v) ethanol. After centrifugation in the cold, the clear supernatant was adjusted to pH 5 with 5 M- K_2CO_3 + 0.5 M-triethanolamine.

Glutamate was measured fluorimetrically as described by Bernt & Bergmeyer (1963). Glutamine

was measured as glutamate after acid hydrolysis. Aspartate was determined fluorimetrically by the method of Pfeleiderer (1963). Valine and alanine were determined by an isotope-dilution method involving aminoacylation of tRNA as reported by Parrilla *et al.* (1973). Lactate was measured by the method of Hohorst (1963), pyruvate by the method of Bücher *et al.* (1963), and α -oxoglutarate as described by Bergmeyer & Bernt (1963). β -Hydroxybutyrate and acetoacetate were determined as described by Williamson *et al.* (1962).

All the results were expressed as either μ mol or nmol/g wet wt. The intracellular concentrations of the different metabolites were assumed to be the same as the extracellular ones.

$[NAD^+]/[NADH]$ ratios were calculated as described by Holzer *et al.* (1956). Lactate dehydrogenase was used for calculating cytosolic $[NAD^+]/[NADH]$ and β -hydroxybutyrate and glutamate dehydrogenases for the calculations of mitochondrial $[NAD^+]/[NADH]$. The following K values were used (Krebs & Veech, 1969): K (lactate dehydrogenase), 1.11×10^{-7} M; K (glutamate dehydrogenase), 3.87×10^{-6} M; K (β -hydroxybutyrate dehydrogenase), 4.93×10^{-5} M.

Statistical evaluation of the data was carried out with an Olivetti programmed desk calculator, model P-102.

Results

Nitrogen production by livers perfused in the absence of a nitrogen donor

Substrate-free perfused livers (Table 1) showed a net nitrogen production of 12.2 μ g-atoms of N/h per g liver wt. This value was calculated by taking into account the changes in the most important nitrogenous constituents in the perfusate as well as in the tissue. Tissue glutamine was the main nitrogen donor, whereas alanine, ammonia and urea were depleted to a smaller extent. Glutamate concentration changed very slightly.

These results strongly suggest that the liver must catabolize some protein in order to supply the required nitrogen atoms. When livers were perfused with pyruvate or lactate (Tables 2 and 3), nitrogen production was slightly increased to 19.2 and 18.4 μ g-atoms of N/h per g liver wt. respectively. This increase was mainly due to a higher alanine release in both instances. The higher alanine release can possibly be explained on the basis that pyruvate availability was increased and that the glutamate–pyruvate transaminase reactants were at near-equilibrium. Glutamine release was much higher in the presence of lactate, perhaps as a way of preventing an intracellular accumulation of glutamate brought about by increased NADH generation. Urea production was very similar in both cases,

Table 1. *Nitrogen disposition in isolated rat livers perfused with a substrate-free medium*For experimental details see the text. Values are means of at least six individual experiments $\pm$ S.E.M.

Metabolite	Concn. in perfusate (μ mol/g fresh wt.) (60 min)	Nitrogen content in perfusate (μ g-atoms/g fresh wt.)	Concn. in liver (μ mol/g fresh wt.)		Nitrogen content in liver (μ g-atoms/g fresh wt.)	Nitrogen formed (μ g-atoms/g fresh wt.)
			0min	60min		
Glutamate	0.8 $\pm$ 0.7	0.8	1.1 $\pm$ 0.08	0.9 $\pm$ 0.04	-0.2	0.6
Glutamine	2.0 $\pm$ 0.1	4.0	5.0 $\pm$ 0.43	3.2 $\pm$ 0.21	-3.6	0.4
Alanine	3.0 $\pm$ 0.2	3.0	1.5 $\pm$ 0.13	1.0 $\pm$ 0.10	-0.5	2.5
Ammonia	1.1 $\pm$ 0.2	1.1	1.9 $\pm$ 0.20	1.3 $\pm$ 0.10	-0.6	0.5
Urea	4.3 $\pm$ 0.3	8.6	1.4 $\pm$ 0.11	1.2 $\pm$ 0.09	-0.4	8.2
Aspartate	<1.0	—	0.6 $\pm$ 0.04	0.6 $\pm$ 0.08	0	
						12.2

Table 2. *Nitrogen disposition in isolated rat livers perfused with pyruvate (10 mM)*For experimental details see the text. Values are means of at least six individual experiments $\pm$ S.E.M.

Metabolite	Concn. in perfusate (μ mol/g fresh wt.) (60min)	Nitrogen content in perfusate (μ g-atoms/g fresh wt.)	Concn. in liver (μ mol/g fresh wt.)		Nitrogen content in liver (μ g-atoms/g fresh wt.)	Nitrogen formed (μ g-atoms/g fresh wt.)
			0min	60min		
Glutamate	1.4 $\pm$ 0.1	+1.4	1.1 $\pm$ 0.08	1.4 $\pm$ 0.26	+0.3	1.7
Glutamine	1.9 $\pm$ 0.1	+3.8	4.7 $\pm$ 0.43	3.7 $\pm$ 0.40	-2.0	1.8
Alanine	10.0 $\pm$ 0.8	+10.0	1.6 $\pm$ 0.13	0.8 $\pm$ 0.06	-0.8	9.2
Ammonia	1.3 $\pm$ 0.1	+1.3	1.9 $\pm$ 0.20	1.1 $\pm$ 0.20	-0.8	0.5
Urea	3.0 $\pm$ 0.3	+6.0	1.3 $\pm$ 0.11	1.2 $\pm$ 0.07	-0.2	5.8
Aspartate	<1.0	—	0.6 $\pm$ 0.07	0.8 $\pm$ 0.05	+0.2	0.2
						19.2

Table 3. *Nitrogen disposition in isolated rat livers perfused with lactate (10 mM)*For experimental details see the text. Values are means of at least six individual experiments $\pm$ S.E.M.

Metabolite	Concn. in perfusate (μ mol/g fresh wt.) (60min)	Nitrogen content in perfusate (μ g-atoms/g fresh wt.)	Concn. in liver (μ mol/g fresh wt.)		Nitrogen content in liver (μ g-atoms/g fresh wt.)	Nitrogen formed (μ g-atoms/g fresh wt.)
			0min	60min		
Glutamate	1.20 $\pm$ 0.20	+1.2	1.1 $\pm$ 0.08	1.8 $\pm$ 0.07	+0.7	1.9
Glutamine	3.90 $\pm$ 0.10	+7.8	4.9 $\pm$ 0.43	3.6 $\pm$ 0.29	-2.6	5.2
Alanine	6.00 $\pm$ 0.80	+6.0	1.5 $\pm$ 0.13	1.7 $\pm$ 0.17	+0.2	6.2
Ammonia	0.53 $\pm$ 0.09	+0.5	1.9 $\pm$ 0.20	1.8 $\pm$ 0.50	-0.1	0.4
Urea	2.30 $\pm$ 0.30	+4.6	1.3 $\pm$ 0.11	1.1 $\pm$ 0.13	-0.2	4.4
Aspartate	<1.0	—	0.8 $\pm$ 0.06	1.1 $\pm$ 0.10	+0.3	0.3
						18.4

although one would expect a more favourable condition for urea synthesis in the presence of lactate because the increased mitochondrial efflux of aspartate (R. Parrilla, unpublished work) would favour the synthesis of argininosuccinate in the cytosol. Glutamate dehydrogenase has to be involved for glutamate deamination in both cases to account for the nitrogen production, and the rate-limiting factor

in urea synthesis was probably the availability of amino acids.

Nitrogen balance in isolated rat livers perfused with glutamine or alanine as nitrogen donors

Tables 4 and 5 show the nitrogen balance for some of the most important nitrogenous

Table 4. *Nitrogen balance in isolated rat livers perfused with glutamine (10mm)*
For experimental details see the text. Values are means of at least six individual experiments $\pm$ S.E.M.

Metabolite	Concn. in perfusate (μ mol/g fresh wt.)		Nitrogen content in perfusate (μ - atoms/g fresh wt.)	Concn. in liver (μ mol/g fresh wt.)		Nitrogen content in liver (μ -atoms/g fresh wt.)	Nitrogen balance (μ -atoms/g fresh wt.)	
	0min	60min		0min	60min		Formed	Utilized
Metabolite	—	—	—	—	—	—	—	—
Glutamate	—	6.0 $\pm$ 0.7	+6.0	1.1 $\pm$ 0.08	11.0 $\pm$ 0.52	+10.0	16.0	—
Glutamine	109 $\pm$ 9.5	37.3 $\pm$ 5.2	-143.4	5.0 $\pm$ 0.23	6.2 $\pm$ 0.80	+2.4	2.4	143.4
Alanine	—	13.5 $\pm$ 1.2	+13.5	1.5 $\pm$ 0.13	4.2 $\pm$ 0.60	+2.7	16.2	—
Ammonia	—	85.0 $\pm$ 7.0	+85.0	1.9 $\pm$ 0.20	4.3 $\pm$ 0.70	+2.4	87.4	—
Urea	—	19.0 $\pm$ 2.6	+38.0	1.2 $\pm$ 0.09	3.0 $\pm$ 0.21	+3.6	41.6	—
Aspartate	—	<1.0	—	0.6 $\pm$ 0.08	2.5 $\pm$ 0.17	+1.9	1.9	—
							165.5	143.4

Table 5. *Nitrogen balance in isolated livers perfused with alanine (10mm)*
For experimental details see the text. Values are means of at least six individual experiments $\pm$ S.E.M.

Metabolite	Concn. in perfusate (μ mol/g fresh wt.)		Nitrogen content in perfusate (μ - atoms/g fresh wt.)	Concn. in liver (μ mol/g fresh wt.)		Nitrogen content in liver (μ -atoms/g fresh wt.)	Nitrogen balance (μ -atoms/g fresh wt.)	
	0min	60min		0min	60min		Formed	Utilized
Metabolite	—	—	—	—	—	—	—	—
Glutamate	—	4.0 $\pm$ 0.5	+4.0	1.6 $\pm$ 0.16	5.3 $\pm$ 0.23	+3.7	7.7	—
Glutamine	—	5.8 $\pm$ 0.7	+11.6	3.2 $\pm$ 0.29	5.3 $\pm$ 0.26	+4.2	15.8	—
Alanine	142 $\pm$ 12	60.3 $\pm$ 7.0	-81.7	1.5 $\pm$ 0.13	3.4 $\pm$ 0.07	+1.9	—	81.7
Ammonia	—	24.0 $\pm$ 2.0	+24.0	1.9 $\pm$ 0.20	2.5 $\pm$ 0.80	+0.6	24.6	—
Urea	—	17.0 $\pm$ 1.0	+34.0	1.3 $\pm$ 0.80	4.7 $\pm$ 0.30	+6.8	40.8	—
Aspartate	—	<1.0	—	0.4 $\pm$ 0.03	1.1 $\pm$ 0.09	+0.7	0.7	—
							89.6	81.7

constituents in the tissue and perfusate in the presence of a nitrogen donor. In both instances the balance was incomplete, suggesting that the liver persists in degrading endogenous protein in spite of a nitrogen-donor supply, albeit at a slower rate than in the presence of a nitrogen-free substrate or no substrate at all.

Since glutamine deamination (Table 4) provides enough NH_3 for urea synthesis, glutamate would be transaminated to supply the aspartate needed in the cytosol. However, one would expect alanine to be metabolized, through transaminating and deaminating pathways of glutamate catabolism, in order to deliver both NH_3 and aspartate to the urea-synthesizing system.

Glutamine (Table 4) stimulated urea synthesis about fivefold over basal production (Table 1) and NH_3 accumulated in the perfusate in large amounts. Also there was a large hepatic accumulation of glutamate. A permeability barrier for glutamate in the hepatic cell has been previously reported by Ross *et al.* (1967) and Chamalaun & Tager (1970) under similar experimental conditions. The nitrogen balance obtained indicates that part of the glutamate derived from glutamine utilization had to be deaminated in order to account for the NH_3 that accumulated in the perfusate. The rate-limiting step that prevented the utilization of NH_3 for urea synthesis has not yet been determined. Aspartate production may not be the limiting step, since it accumulated in the tissue. Alanine (Table 5) also stimulated urea production about fivefold, but the accumulation of NH_3 was much smaller than with glutamine (Table 4). To explain the nitrogen balance obtained, it has to be assumed that glutamate was deaminated to provide NH_3 and transaminated to form aspartate. This means that glutamate is metabolized through the glutamate dehydrogenase pathway.

Rates of valine release

Table 6 shows the influence of different substrates on the rate of valine release by the perfused

Table 6. Rates of valine release by the isolated perfused rat liver

Valine was measured enzymically by a procedure involving the aminoacylation of tRNA. For other details see the text. Values are means of at least six experiments $\pm$ S.E.M.

Substrate	Valine production ($\mu\text{mol/h}$ per g fresh wt.)
None	3.04 ± 0.21
Pyruvate (10mM)	1.53 ± 0.12
Alanine (8–10mM)	1.44 ± 0.24
Glutamine (8–10mM)	1.09 ± 0.16

liver. Valine is poorly oxidized by the liver (Mortimore & Mondon, 1970) and its rate of appearance in the perfusate can be accepted as an index of net proteolysis. Under our experimental conditions an inverse relationship between nitrogen utilization and valine release seems to exist in the presence of different substrates.

The highest rate of valine release was produced in the absence of any substrate, suggesting that more endogenous protein has to be utilized to meet the energy requirements in the absence of exogenous fuel.

Hepatic steady-state concentration of the reactants of the glutamate-pyruvate transaminase

Transaminating activity has been reported to increase under situations of increased fuel mobilization. Since no net change in flux can be produced through an equilibrium enzyme, attention has been given to the question of whether or not transaminase reactions are near equilibrium *in vivo* (Williamson *et al.*, 1967b). The reactants of glutamate-pyruvate transaminase were determined in livers perfused with different substrates to ascertain whether or not this reaction would influence the flux of nitrogen.

Table 7 shows that the expression:

$$\frac{[\text{alanine}][\alpha\text{-oxoglutarate}]}{[\text{pyruvate}][\text{glutamate}]}$$

remained fairly constant in spite of large variations in the concentration of some reactants. These values agree very well with the value of 1.51 reported by Krebs (1953) for the experimentally determined equilibrium constant. In addition, Williamson *et al.* (1967b) reported that this reaction was near equilibrium *in vivo*, and similar conclusions were reached by Parrilla & Toews (1974) in their work on prolonged starvation in the rat.

Redox state and the nicotinamide nucleotide system

Since most of the nicotinamide nucleotides within the cell are protein bound (Bücher, 1970) the measurement of their total content may not be a good indication of the redox state of the cell. Holzer *et al.* (1956) developed a procedure for calculating the concentration of the free $[\text{NAD}^+]/[\text{NADH}]$ ratio by using the measured concentrations of the reactants of a dehydrogenase reaction, which is presumed to be near equilibrium, and its equilibrium constant. The expression used is the following:

$$\frac{[\text{NAD}^+]}{[\text{NADH}]} = \frac{1}{K} \times \frac{[\text{reactant oxidized}]}{[\text{reactant reduced}]}$$

Table 8 shows the results obtained when this approach was utilized to elucidate whether, under

Table 7. *Hepatic concentrations of the glutamate-pyruvate transaminase reactants*Results are mean values of six experiments $\pm$ S.E.M. obtained from freeze-clamped tissues after 1 h of perfusion.

Substrate	Content (nmol/g fresh wt.)				$\frac{[\text{Alanine}][\alpha\text{-Oxoglutarate}]}{[\text{Pyruvate}][\text{Glutamate}]}$
	Alanine	α -Oxoglutarate	Pyruvate	Glutamate	
None	1030 $\pm$ 109	92 $\pm$ 10	66 $\pm$ 4	970 $\pm$ 40	1.74 $\pm$ 0.33
Glutamine (8–10mm)	4250 $\pm$ 606	310 $\pm$ 38	80 $\pm$ 7	11000 $\pm$ 520	1.76 $\pm$ 0.16
Alanine (8–10mm)	3448 $\pm$ 76	246 $\pm$ 20	188 $\pm$ 15	3450 $\pm$ 130	1.93 $\pm$ 0.22
Pyruvate (10mm)	780 $\pm$ 62	452 $\pm$ 49	190 $\pm$ 25	1420 $\pm$ 260	1.25 $\pm$ 0.14
Lactate (10mm)	1760 $\pm$ 178	507 $\pm$ 47	228 $\pm$ 24	1870 $\pm$ 70	2.06 $\pm$ 0.13

Table 8. *Influence of different substrates on the calculated $[\text{NAD}^+]/[\text{NADH}]$ ratios*Results are mean values of six experiments $\pm$ S.E.M. obtained from freeze-clamped tissues after 1 h of perfusion.

Substrate added (10mm)	Metabolite (nmol/g fresh wt.)				
	None	Glutamine	Alanine	Pyruvate	Lactate
Glutamate	970 $\pm$ 40	11000 $\pm$ 520	3450 $\pm$ 130	1420 $\pm$ 260	1870 $\pm$ 70
α -Oxoglutarate	92 $\pm$ 10	310 $\pm$ 38	246 $\pm$ 20	452 $\pm$ 49	507 $\pm$ 47
Ammonia	1340 $\pm$ 260	4300 $\pm$ 701	2540 $\pm$ 812	1120 $\pm$ 240	710 $\pm$ 63
Mitochondrial $[\text{NAD}^+]/[\text{NADH}]$	26 $\pm$ 7	30 $\pm$ 6	39 $\pm$ 7	92 $\pm$ 15	44 $\pm$ 3
β -Hydroxybutyrate	145 $\pm$ 15	104 $\pm$ 8	86 $\pm$ 3	313 $\pm$ 36	102 $\pm$ 7
Acetoacetate	216 $\pm$ 20	252 $\pm$ 24	192 $\pm$ 8	1740 $\pm$ 221	310 $\pm$ 8
Mitochondrial $[\text{NAD}^+]/[\text{NADH}]$	31 $\pm$ 4	41 $\pm$ 6	45 $\pm$ 3	122 $\pm$ 26	61 $\pm$ 3
Lactate	200 $\pm$ 12	264 $\pm$ 17	880 $\pm$ 84	244 $\pm$ 26	810 $\pm$ 106
Pyruvate	26 $\pm$ 2	80 $\pm$ 6	188 $\pm$ 15	190 $\pm$ 25	228 $\pm$ 106
Cytosolic $[\text{NAD}^+]/[\text{NADH}]$	1352 $\pm$ 240	2730 $\pm$ 700	1924 $\pm$ 250	8780 $\pm$ 470	2612 $\pm$ 167

our experimental conditions, glutamate dehydrogenase and β -hydroxybutyrate dehydrogenase reactions were in equilibrium with the same nicotinamide nucleotide pool. The calculation of the free mitochondrial $[\text{NAD}^+]/[\text{NADH}]$ ratio, by using the reactants of either dehydrogenase, resulted in extremely similar values. Changes in the cytosolic free $[\text{NAD}^+]/[\text{NADH}]$ ratio under the different conditions paralleled those of the mitochondrial free $[\text{NAD}^+]/[\text{NADH}]$ ratio. The absence of substrate produced the most reduced state of the NAD couple, whereas pyruvate produced the most oxidized. Alanine and glutamine gave intermediate values. The higher mitochondrial $[\text{NAD}^+]/[\text{NADH}]$ ratios than those encountered *in vivo* (Williamson *et al.* 1967a) are presumably due to the absence of free fatty acids.

Discussion

The data reported for nitrogen balances under various conditions suggest that glutamate dehydrogenase must operate *in vivo* in the direction of glutamate deamination in order to supply the NH_3 needed for the synthesis of urea and/or the release of NH_3 . These results are in contrast with those obtained with isolated mitochondria by Muller & Leuthardt (1950) in which 90% of the glutamate was oxidized via the transaminating pathway. It was

suspected that glutamate could be metabolized differently *in vivo*, since Hoek *et al.* (1969), using liver homogenates, found a much higher rate of glutamate deamination than in isolated mitochondria. Chamalaun & Tager (1970) also concluded that glutamate was oxidized through the glutamate dehydrogenase pathway, especially when alanine was used as a substrate in an isolated perfused liver preparation. The present results extend this view to livers perfused with glutamine or in the absence of a nitrogen donor. Further evidence to support the above are the findings that the redox state of the mitochondrial NAD couple with glutamine or alanine as substrates is similar, and in both instances closer to the redox state obtained with lactate than with pyruvate (Table 8). Thus glutamate dehydrogenase, as predicted by Braunstein & Asarkh (1945), seems to play a central role in amino acid catabolism.

Owing to the important role of glutamate dehydrogenase in nitrogen metabolism, attention has been paid to the control of its activity *in vivo*. Many workers have focused their attention on the possible regulatory role of the nicotinamide nucleotides. Although some, on the basis of their work with mitochondria, believe that glutamate dehydrogenase is an NADP-linked enzyme (De Haan *et al.*, 1967; Tager & Papa, 1965; Papa *et al.*, 1967),

others believe that, *in vivo*, glutamate dehydrogenase functions with NAD or NADP (Williamson *et al.*, 1967a; Krebs & Veech, 1969; Veech *et al.*, 1969). The present results, indicating that the redox potential of the glutamate and β -hydroxybutyrate couples show similar values and parallel changes (Table 8), strongly suggest that these two dehydrogenases share a common mitochondrial NAD pool. Williamson (1969) reached a similar conclusion in his work on perfused isolated liver by using for the calculations a hypothetical hepatic NH_3 concentration. A very active hepatic mitochondrial glutaminase (Guha, 1961) and a permeability barrier for glutamate (Ross *et al.*, 1967) led to a high intracellular glutamate concentration when livers were perfused with glutamine as substrate (Table 4). The similar values obtained for the mitochondrial $[\text{NAD}^+]/[\text{NADH}]$ ratio, when calculated with the β -hydroxybutyrate and glutamate dehydrogenase couples (Table 8), seems to indicate that the enormous intracellular accumulation of glutamate was evenly distributed throughout the cell. Chamalaun & Tager (1970) found that perfusion of livers with ammonia (as NH_4Cl) produced a more oxidized state of the free mitochondrial $[\text{NAD}^+]/[\text{NADH}]$ ratio only when calculated by using the glutamate dehydrogenase couple. This finding was used by Chamalaun & Tager (1970) to support the view that glutamate dehydrogenase reacts preferentially as an NADP-linked enzyme. This was the case when their experiments in which alanine and NH_3 were used as nitrogen donors were compared. Nevertheless, a different conclusion could be reached if one were to compare their experimental group in which NH_3 was added with the control group of 24h-starved rats. When calculated it can be seen that in relation to the control, both redox couples showed parallel changes in the presence of NH_3 and further, the value of the expression:

$$\frac{[\beta\text{-hydroxybutyrate}][\alpha\text{-oxoglutarate}][\text{NH}_3]}{[\text{acetoacetate}][\text{glutamate}]}$$

(obtained by dividing mass-action ratios of both dehydrogenases), was identical in both instances. When their reported values are compared with the control group they all showed parallel changes except for the glutamine group.

Under our experimental conditions the glutamate-pyruvate transaminase reaction appears to be near equilibrium (Table 7). If transamination is not a determinant for the net flux of nitrogen, the question arises as to the source of the nitrogen produced, especially during perfusion without a nitrogen donor. From the present reported measurements of some of the cell nitrogen pool constituents, the source of the nitrogen cannot be

determined. Results not reported here (R. Parrilla & M. N. Goodman, unpublished work) showed a moderate decrease in the liver adenine nucleotide pool during the perfusion period, which could not account for the negative nitrogen balance. The most likely mechanism by which the liver keeps pace with the maintenance of the nitrogen pools seems to be proteolysis. The experiments reported by Schimassek & Gerok (1965), showing that the amino acid release by the perfused isolated liver was not a passive 'washing out', as the intracellular content of the amino acids in liver is not diminished during perfusion, seem to support this conclusion. The removal of the liver from the animal and its perfusion in the absence of any fuel enhanced endogenous protein degradation as reflected by the rate of valine release into the perfusion medium (Table 6). Woodside & Mortimore (1972) focused their attention on this problem in a careful study on the role of amino acids in the regulation of the rate of proteolysis. Parrilla *et al.* (1972) also reported their findings on the hormonal control of hepatic proteolysis. The experiments reported in the present paper confirm those of Woodside & Mortimore (1972), in that the addition of amino acids to the perfusion medium arrests the rate of proteolysis. However, the addition of pyruvate to the perfusion medium also decreased the rate of valine release, by 40% (Table 6). This finding poses the question as to the relevance of the amino group for the suppression of proteolysis and suggests that the availability of fuel may be of prime importance. More studies are needed to elucidate the metabolic implications of this finding and its possible physiological significance.

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