Concentrations of Free Glucogenic Amino Acids in Livers of Rats Subjected to Various Metabolic Stresses

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1. The concentrations of alanine, aspartate, glutamate, glutamine and serine plus threonine have been measured by enzymic methods in 'quick-frozen' livers from normal, starved, alloxan-diabetic and phlorrhizin-treated rats. 2. The hepatic concentrations of alanine and serine plus threonine were decreased in rats starved for 48 hr. Treatment of these rats with phlorrhizin resulted in a rapid fall (within $2\frac{1}{2}$ hr.) in the concentrations of all the glucogenic amino acids except serine plus threonine, which increased. The pattern for alloxan-diabetic rats was similar to that for phlorrhizin-treated animals, except that here serine plus threonine also decreased in concentration. 3. The effects of anoxia on the hepatic concentrations of the glucogenic amino acids are reported. 4. Inhibition of glutamate-pyruvate transaminase *in vivo* by L-cycloserine resulted in the accumulation of alanine in situations involving high rates of gluconeogenesis from endogenous amino acids. 5. Measurements of the concentrations of the reactants of the glutamate-pyruvate transaminase and glutamate-oxoglutarate transaminase systems in various metabolic states suggest that they are both at or near equilibrium in rat liver. 6. New enzymic methods are described for the determination of serine plus threonine and alanine.

Little information is available on the concentrations of free glucogenic amino acids in rat liver *in vivo*, apart from a comparison of livers from normal and alloxan-diabetic rats carried out by Kirsten, Kirsten, Hohorst & Bücher (1961). Consequently, alanine, aspartate, glutamate, glutamine and serine plus threonine have been measured by enzymic methods in 'quick-frozen' livers (Wollenberger, Ristau & Schoffa, 1960) from rats subjected to various metabolic stresses, such as alloxan-diabetes, starvation, starvation plus treatment with phlorrhizin or starvation followed by a load of glycerol or dihydroxyacetone. These situations were chosen so as to obtain wide variations in the rates of extrahepatic protein catabolism and of hepatic gluconeogenesis from endogenous amino acids. The data obtained are discussed in relation to possible mechanisms for the control of the concentrations of individual amino acids in rat liver.

MATERIALS AND METHODS

Animals. Male rats of the Wistar strain, weighing 120–180 g., were used. Alloxan-diabetes was induced by intravenous injection of alloxan monohydrate (70 mg./kg. in 0.9% NaCl) under ether anaesthesia. The alloxan-treated animals were allowed free access to food and water. One

group of rats was killed 48 hr. after injection with alloxan (alloxan-diabetic, no insulin) and another group was given 2 units of protamine-zinc-insulin subcutaneously/day for 7 days and then killed 48 hr. after the last injection of insulin. (alloxan-diabetic, insulin withdrawal). Phlorrhizin (75 mg. suspended in 1.0 ml. of arachis oil) was administered intramuscularly to rats from which food had been withheld for 48 hr.

Diet. The rats were given a balanced diet of commercial rat cubes containing 15% of protein, 3% of fat and the remainder consisting mainly of carbohydrate plus mineral and vitamin supplements.

Enzymes and chemicals. Glutamate-oxaloacetate transaminase, glutamate, malate and lactate dehydrogenases were obtained from Boehringer Corp., London, W.5. Hydroxybutyrate dehydrogenase was prepared by the method of Williamson, Mellanby & Krebs (1962) and purified further on DEAE-Sephadex according to Bergmeyer, Gawehn, Klotzsch, Krebs & Williamson (1967). Alanine dehydrogenase was obtained from extracts of *Bacillus subtilis* essentially according to Yoshida & Freese (1964), except that chromatography on columns of calcium phosphate gel was omitted, and the protein solution from the initial fractionation with $(\text{NH}_4)_2\text{SO}_4$ was chromatographed directly on DEAE-Sephadex. After elution of inactive protein with 0.05 M-phosphate buffer, pH 7.4, and 0.05 M-phosphate buffer, pH 7.4, containing 0.2 M-NaCl, the enzyme was eluted with the same buffer containing 0.3 M-NaCl. The active fractions were combined and the protein was

precipitated by addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 90% saturation. Since at this stage the preparation contained no appreciable lactate or glutamate dehydrogenase activity, further purification was not attempted.

L-Cycloserine (4-amino-3-isoxazolidone) was obtained from Calbiochem, Los Angeles, Calif., U.S.A. NAD and NADH_2 were supplied by Boehringer Corp., London, W.5.

Treatment of livers. The rats were killed by cervical fracture; a portion of the liver was rapidly removed and clamped between metal blocks previously cooled in liquid N_2 (Wollenberger *et al.* 1960). The average time elapsing between dislocation of the neck and deep-freezing the tissue was 10 sec., although the transfer of the liver sample to the metal blocks took a considerably shorter time (approx. 1 sec.). The liver was then deproteinized as described by Berry, Williamson & Wilson (1965) and Williamson, Lund & Krebs (1967), except that the Florisil step was omitted.

Isolation of amino acid fraction. The enzymic method used for the determination of serine plus threonine required the preliminary separation of the amino acids from interfering substances. This was achieved by absorption of the neutral deproteinized liver extract (5.0 ml.) on a column (1 cm. $\times$ 5 cm.) of Amberlite IR-120 (H^+ form). The column was then washed well with distilled water and the amino acids were quantitatively eluted with aq. 2 N- NH_3 soln. (20 ml.). The eluate was dried *in vacuo* at 50° and the residue dissolved in distilled water.

Determination of serine plus threonine. The sum of serine plus threonine was determined by a new method in which these amino acids were oxidized with periodate at pH 7.0 and the glyoxylate formed was reduced to glycolate with NADH_2 and lactate dehydrogenase. The determination was carried out as follows.

The amino acid sample (containing 0.020–0.20 μmole of serine plus threonine), phosphate buffer, pH 7.0 (100 μmoles), and distilled water to a final volume 3.0 ml. were added to a 1 cm. spectrophotometer cuvette. Sodium periodate (0.020 ml.; 4 μmoles) was then added and the cuvette contents were mixed immediately. After the cuvette had been standing at room temperature for exactly 5 min., glycerol (0.050 ml.; 50 μmoles) was added and the contents were again mixed. The glycerol removes the excess of periodate and the oxidation products, formic acid and formaldehyde, do not interfere with the enzymic determination of glyoxylate. Ten minutes later NADH_2 was added (0.1 ml.; 0.6 μmole) and the initial extinction read at 340 m μ . The enzymic reaction was commenced by addition of lactate dehydrogenase (0.020 ml.; 100 $\mu\text{g.}$) After about 30 min. the reduction of glyoxylate was complete and the final extinction was then read. Although glyoxylate is oxidized by periodate to formate and CO_2 , under the conditions described above this reaction was slow and recovery of glyoxylate from known amounts of serine (or threonine) was consistently found to be 88–90%. The preliminary isolation of the amino acid fraction ensures the specificity of the method since no other amino acid apart from serine and threonine is oxidized by periodate to glyoxylate. However, this method does not distinguish between the optical isomers of the two amino acids. Theoretically it should be possible to differentiate threonine from serine by subsequent determination of the acetaldehyde formed from the former with alcohol dehydrogenase (Flavin, 1963). However, this refinement was not attempted in the present work.

Determination of L-alanine. L-Alanine was determined by oxidative deamination with NAD and alanine dehydrogenase isolated from *B. subtilis* (see above). Quantitative oxidation was achieved by a low H^+ ion concentration (pH 10), inclusion of a hydrazine trap for the pyruvate formed in the reaction and the virtual exclusion of NH_4^+ ions from the assay system. The determination was carried out as follows.

The amino acid sample (containing 0.020–0.20 μmole of L-alanine), NAD (0.2 ml.; 2.5 μmoles), hydrazine-tris, pH 10 (0.5 ml.; composition: 1 ml. of hydrazine hydrate, 4.0 ml. of 0.2 M-tris, 10 mg. of EDTA and 15 ml. of distilled water, adjusted to pH 10 with N-HCl) and distilled water to a final volume 3.0 ml. were added to a 1 cm. spectrophotometer cuvette. The initial extinction was read at 340 m μ and then the enzymic reaction was started by the addition of alanine dehydrogenase (0.010 ml.; activity: 15 i.u./ml. under above assay conditions). The increase in extinction at 340 m μ was followed until the reaction ceased (about 40 min.). The recovery of known amounts of L-alanine was 98–99%. A similar method, omitting the hydrazine trap and using larger amounts of NAD (20 μmoles), has been described by Yoshida (1965), but this method requires prior removal of pyruvate.

Determination of other amino acids. L-Aspartate was determined by the method of Pfeleiderer (1963) and L-glutamate by the method of Bernt & Bergmeyer (1963). The sum of glutamate plus glutamine was obtained after preliminary acid hydrolysis of the glutamine to glutamate (2 hr. at 100° in N-HCl.)

Choice of method for determination of amino acids. Enzymic methods of analysis were chosen, because they are the most specific and convenient techniques available for the particular amino acids under study. Another reason for the choice was the controversy existing between the results of Kirsten *et al.* (1961) and those of Roberts & Simonsen (1962) (see the Discussion section), who used ion-exchange chromatography and paper chromatography respectively for the determination of the amino acids.

Other determinations. Acetoacetate and β -hydroxybutyrate were determined according to Williamson *et al.* (1962). Glucose was determined by the method of Bergmeyer & Bernt (1963a). Oxoglutarate (Bergmeyer & Bernt, 1963b), pyruvate (Bücher, Czok, Lamprecht & Latzko, 1963) and lactate (Hohorst, 1963) were determined by standard methods.

Calculation and expression of results. Concentrations of amino acids and other metabolites are expressed as $\mu\text{moles/g.}$ wet wt. of liver. In the calculation of the concentrations it was assumed that the water content of rat liver was 80%. No correction has been made for possible contribution to the amino acid concentrations by blood contained in the livers, since this is likely to be small.

RESULTS

Concentrations of glucogenic amino acids in livers of fed, starved, alloxan-diabetic and phlorrhizin-treated rats. The concentrations of aspartate, glutamate and glutamine were remarkably similar in livers from fed and starved rats (Table 1), although the total weight of liver decreased by about 35% on starvation. However, the concentration of alanine fell by about 65% on starvation

Table 1. *Concentrations of certain glucogenic amino acids in livers from fed, starved and alloxan-diabetic rats*

For information on the alloxan-diabetic animals, see Table 2. The amino acid concentrations are expressed in $\mu\text{moles/g.}$ fresh wt. of liver and the results are mean values ($\pm$ S.D.). The number of animals in each group is shown in parenthesis.

State of animal	Aspartate	Serine + threonine	Glutamate	Glutamine	Alanine
Normal fed (15)	0.74 $\pm$ 0.23	1.27 $\pm$ 0.34	2.41 $\pm$ 0.29	4.35 $\pm$ 0.88	1.23 $\pm$ 0.64
Starved 48 hr. (30)	0.76 $\pm$ 0.32	0.93 $\pm$ 0.27	2.46 $\pm$ 0.71	4.43 $\pm$ 1.02	0.46 $\pm$ 0.36
Alloxan-diabetic, insulin-withdrawal (10)	0.51 $\pm$ 0.12	0.49 $\pm$ 0.13	2.79 $\pm$ 0.63	2.61 $\pm$ 0.46	0.61 $\pm$ 0.68
Alloxan-diabetic, no insulin (12)	0.35 $\pm$ 0.16	0.93 $\pm$ 0.36	0.92 $\pm$ 0.26	2.15 $\pm$ 0.52	0.47 $\pm$ 0.32

Table 2. *Concentrations of glucose and ketone bodies in blood of the alloxan-diabetic rats*

Alloxan monohydrate (70 mg./kg. in 0.9% NaCl) was injected intravenously under ether anaesthesia. One group of rats was killed 48 hr. after injection (alloxan-diabetic, no insulin). Another group was given 2 units of protamine-zinc-insulin subcutaneously/day for 7 days and then killed 48 hr. after the last insulin injection (alloxan-diabetic, insulin-withdrawal). The second group maintained their initial body wt. during the period of insulin treatment; the first group lost up to 20% during the 48 hr. before death. The results are mean values ($\pm$ S.D.) with the number of animals shown in parenthesis.

Group	Total ketone bodies (mm)	Blood sugar (mm)
Alloxan-diabetic, no insulin (10)	9.3 $\pm$ 3.3	46 $\pm$ 14
Alloxan-diabetic, insulin- withdrawal (10)	2.0 $\pm$ 1.6	30 $\pm$ 3

and that of serine plus threonine was significantly lower (about 25%; $P < 0.005$) (Table 1). In contrast with the livers from fed and starved rats, those from both alloxan-diabetic groups showed considerably lower values for all the amino acids determined, except glutamate in the insulin-withdrawal group (Table 1). This difference between the two groups of alloxan-diabetic rats may be partly explained by differences in the severity of the diabetic state (Table 2). Kirsten *et al.* (1961) found extremely variable results for hepatic glutamate in their alloxan-diabetic rats (four animals: range 0.89–4.90; mean 2.78 $\mu\text{moles/g.}$ fresh wt.).

The above results suggest that only when the rates of gluconeogenesis are abnormally high, e.g. in alloxan-diabetes, is there a general marked distortion of the concentration pattern of the free glucogenic amino acid pool. Confirmation of this was obtained by treatment of starved rats with phlorrhizin, which lowers the renal threshold for glucose. This causes an immediate increase in the demand for glucose, which can only be satisfied in the starved animal by an increased rate of glucose

synthesis from endogenous precursors, e.g. lactate, glycerol and amino acids. Shortly (2½ hr.) after administration of phlorrhizin there were decreases (about 35–40%) in the hepatic concentrations of alanine, aspartate, glutamate and glutamine, but contrary to expectations the serine plus threonine concentration increased by about 60% (Table 3).

An indication that the various metabolic stresses resulted in increased amino acid utilization via transamination was obtained with alanine by the use of L-cycloserine, a powerful competitive inhibitor of glutamate-pyruvate transaminase (Otto, 1965). Administration of cycloserine to fed, starved and phlorrhizin-treated rats caused an increase in the hepatic concentration of alanine, but the increase was about fivefold in starved rats and about 25-fold in phlorrhizin-treated rats compared with that in fed rats (Table 4). Cycloserine did not significantly affect the glutamate concentration in any of the situations tested,

Effects of anoxia on the concentrations of glucogenic amino acids. No studies have been reported of changes in amino acid concentrations during short periods of anoxia (1–2 min.). It is known that there is an immediate and large increase in the reduction state of the NAD–NADH₂ system of the liver cell, with a concomitant decrease in the amounts of pyruvate and oxaloacetate (Hohorst, Kreutz & Reim, 1961). These factors, together with a rapid increase in the rate of glycolysis, might be expected to cause changes in the amino acid concentrations. It was therefore surprising to find that the concentrations of aspartate, serine plus threonine, glutamate and glutamine did not alter significantly during a 2 min. period of anoxia, irrespective of the nutritional state of the animal (Table 5). However, the concentration of alanine increased two- to three-fold during the same period, although the absolute increase was less in starved rats.

Concentrations of the reactants of the glutamate-pyruvate and glutamate-oxaloacetate transaminase systems. Since alanine showed the greatest sensitivity to changes in metabolic state, it was important to establish whether the glutamate-pyruvate

Table 3. *Effect of phlorrhizin administration on the concentration of certain glucogenic amino acids in livers from starved rats*

Rats which had been starved for 48 hr. were given an intramuscular injection of either (a) 1.0 ml. of arachis oil or (b) 75 mg. of phlorrhizin suspended in 1.0 ml. of arachis oil. The rats were killed 2½ hr. after injection and the livers treated as described in the text. The results are expressed in μ moles/g. fresh wt. of liver ($\pm$ s.d.) and the number of animals in each group is given in parenthesis.

	Aspartate	Serine + threonine	Glutamate	Glutamine	Alanine
(a) Controls (15)	0.76 $\pm$ 0.32	0.93 $\pm$ 0.27	2.46 $\pm$ 0.71	4.43 $\pm$ 1.02	0.46 $\pm$ 0.36
(b) Phlorrhizin-treated (14)	0.49 $\pm$ 0.14	1.53 $\pm$ 0.65	1.63 $\pm$ 0.63	2.66 $\pm$ 0.63	0.20 $\pm$ 0.16

Table 4. *Effects of L-cycloserine on the hepatic concentrations of alanine and glutamate in fed, starved and phlorrhizin-treated rats*

The cycloserine-treated rats received an intraperitoneal injection of 5 mg. of L-cycloserine in 1.0 ml. of 0.9% NaCl approx. 1 hr. before killing. The control animals of each group were similarly injected with 1.0 ml. of 0.9% NaCl. The dose of phlorrhizin and method of administration are given under Table 3. The results are expressed in μ moles/g. fresh wt. of liver and are mean values for two or three rats in each group.

State of animals	(a) Controls		(b) Cycloserine-treated		Ratio of concn. (b/a)	
	Alanine	Glutamate	Alanine	Glutamate	Alanine	Glutamate
Fed	1.57	2.04	5.21	2.63	3	1.3
Starved 48 hr.	0.34	2.54	4.80	2.54	14	1.0
Phlorrhizin-treated (2½ hr.)	0.08	1.75	5.23	1.80	65	1.0
Phlorrhizin-treated (16 hr.)	0.15	1.38	12.02	1.28	80	0.90

Table 5. *Changes in the concentrations of glucogenic amino acids in rat liver during anoxia*

Portions of liver were removed at timed intervals after death of the animal and deep-frozen. The liver was left in the abdominal cavity, but was severed from the blood supply. The results are expressed in μ moles/g. fresh wt. of liver and are from a representative experiment.

State of animal	Time after death (sec.)	Aspartate	Serine + threonine	Glutamate	Glutamine	Alanine
Normal fed	10	0.77	1.91	3.51	3.21	0.63
	60	0.79	2.21	3.62	3.33	0.84
	120	0.74	1.70	3.07	3.53	1.40
Starved 48 hr.	10	0.66	0.98	2.71	3.27	0.21
	60	0.76	1.03	2.76	3.74	0.33
	120	0.72	0.98	2.56	3.82	0.72

transaminase system was at equilibrium. Consequently, the concentrations of the reactants of this system were measured in livers from fed and starved rats, and starved rats which had been given a load of a glucose precursor (glycerol or dihydroxyacetone) (Table 6). Calculation of the apparent equilibrium constant:

$$K_{app} = \frac{[\text{alanine}][\text{oxoglutarate}]}{[\text{pyruvate}][\text{glutamate}]}$$

gave values ranging from 0.39 to 0.64 (mean 0.53). The agreement between the values in the various experimental conditions indicates that the reactants

of the transaminase are in a steady state. Krebs (1953) found a value 1.5 for the apparent equilibrium constant in a homogeneous system, which might indicate that the transaminase is not at thermodynamic equilibrium in intact liver. However, a more likely explanation for the discrepancy between the two values is compartmentation of the reactants *in vivo*. For example, if within the liver cell there are two compartments in which glutamate-pyruvate transaminase establishes equilibrium, but where the absolute concentrations of the reactants are different, then measurement of the overall concentrations of the reactants in the liver will result in apparent disequilibrium.

Table 6. *Concentrations of the reactants of the glutamate-pyruvate transaminase system and the lactate/pyruvate ratio in rat liver under various experimental conditions*

Rats starved for 48 hr. were given an intramuscular injection of: (a) 1.0 ml. of 0.9% NaCl; (b) 1.0 ml. of 40% (w/v) glycerol; (c) 1.0 ml. of 40% (w/v) dihydroxyacetone. The animals were killed 40 min. after injection and the livers treated as described in the text. Values for normal fed rats are included for comparison. The results are expressed as $\mu\text{moles/g. fresh wt. of liver } (\pm \text{S.D.})$ and the number of animals in each group is given in parenthesis.

The calculated $K_{\text{app}} = \frac{[\text{alanine}][\text{oxoglutarate}]}{[\text{pyruvate}][\text{glutamate}]}$ for the glutamate-pyruvate transaminase system is also included.

State of animals		Alanine	Oxoglutarate	Glutamate	Pyruvate	Lactate	$\frac{\text{Lactate}}{\text{Pyruvate}}$	K_{app}
Starved 48 hr.								
(a) NaCl	(21)	0.56 ± 0.52	0.086 ± 0.032	2.64 ± 0.65	0.047 ± 0.024	0.78 ± 0.40	17	0.39
(b) Glycerol	(12)	1.34 ± 0.74	0.055 ± 0.024	2.21 ± 0.45	0.052 ± 0.024	2.05 ± 0.71	39.5	0.64
(c) Dihydroxyacetone	(13)	1.73 ± 0.67	0.174 ± 0.65	2.50 ± 0.55	0.229 ± 0.077	2.63 ± 0.64	11.5	0.53
Normal fed	(6)	1.23 ± 0.65	0.141 ± 0.13	2.41 ± 0.29	0.125 ± 0.043	1.62 ± 0.33	13	0.57

Table 7. *Concentrations of the reactants of the glutamate-oxaloacetate transaminase system in livers from fed and alloxan-diabetic rats*

Sources of the values given in this Table: oxoglutarate (Williamson *et al.* 1967); oxaloacetate (Wieland & Löffler, 1963; Kirsten *et al.* 1961); aspartate and glutamate (this paper, Table 1). The results are mean values expressed in $\mu\text{moles/g. fresh wt. of liver}$. The equilibrium constant $K_{\text{app}} = \frac{[\text{aspartate}][\text{oxoglutarate}]}{[\text{oxaloacetate}][\text{glutamate}]}$ was calculated from the concentrations of the reactants.

State of animals	Aspartate	Oxoglutarate	Glutamate	Oxaloacetate	K_{app}
Fed	0.74	0.145	2.41	0.006	7.2
Alloxan-diabetic, no insulin	0.35	0.045	0.92	0.002	8.5

Values for the concentrations of the reactants of the glutamate-oxaloacetate transaminase system in livers from fed and alloxan-diabetic rats are given in Table 7. The calculated apparent equilibrium constant:

$$K_{\text{app}} = \frac{[\text{aspartate}][\text{oxoglutarate}]}{[\text{oxaloacetate}][\text{glutamate}]}$$

for the two metabolic states (7.2 and 8.5; mean 7.8) is in agreement with a value of 6.7 found by Krebs (1953).

DISCUSSION

It is clear from the present work that the concentrations of free glucogenic amino acids in rat liver rapidly respond to changes in the metabolic state of the liver. This finding is in direct contrast with the conclusions of Roberts & Simonsen (1962) that the steady-state concentrations of free amino acids in tissues are remarkably stable in animals subjected to various stresses, e.g. starvation and alloxan-diabetes. However, these authors relied on paper-chromatographic separations and measurement of the ninhydrin colour for the quantitative determination of the amino acids. In general, the depletion of glucogenic amino acids reported here

in livers from alloxan-diabetic rats confirm the results of Kirsten *et al.* (1961).

The question arises as to the significance of the changes and how the concentrations are regulated. It has been suggested by Kirsten *et al.* (1961) that they are controlled by the amounts of the respective keto acids, pyruvate, oxaloacetate and oxoglutarate which in turn depend on the redox state of the NAD-NADH₂ system (see also Hohorst, Arese, Bartels, Stratmann & Talke, 1965). This hypothesis requires that the concentrations of alanine, aspartate and glutamate should move in a parallel direction. The finding that alanine concentration is not necessarily dependent on the lactate/pyruvate ratio, an indicator of the cytoplasmic NAD/NADH₂ ratio (Table 6), and that alanine decreases in livers from starved rats when aspartate and glutamate are unchanged (Table 1), indicate that other control mechanisms must operate.

It is more likely that the concentrations are controlled within the liver by the rate of entry and exit; the rate of utilization (for gluconeogenesis or protein synthesis) and the rate of formation (via transamination or protein catabolism). In the metabolic situations studied here alterations in the rate of utilization of amino acids for gluconeogenesis is probably the primary reason for the changes in

their concentration. Other specific mechanisms can contribute to the control of the concentrations of individual amino acids and these are briefly discussed below.

Glutamate. The relative constancy of the glutamate values under most conditions may reflect its catalytic function in transamination reactions.

Serine and threonine. The decrease in serine plus threonine concentration in starvation and alloxan-diabetes can probably be directly attributed to the increase in hepatic serine dehydrase activity in these conditions (Freedland & Avery, 1964). A possible explanation for the raised serine plus threonine concentration in liver after phlorrhizin treatment (Table 3) is the inability of the dehydrase to deal with increased influx of these amino acids into the liver (K. Bojanowska & D. H. Williamson, unpublished work).

Alanine. Since the reactants of the glutamate-pyruvate transaminase system are at or near equilibrium in rat liver (Table 6), a change in the availability of pyruvate should alter the alanine concentration. Thus when pyruvate is directed towards glucose synthesis and its other endogenous precursors (lactate and serine) are in limited supply, for example in starved or phlorrhizin-treated animals, a fall in the alanine concentration occurs. The increase in alanine during anoxia (Table 5) is presumably due to the increased supply of pyruvate via anaerobic glycolysis. The amino group may arise from free NH_4^+ via glutamate dehydrogenase and glutamate-pyruvate transaminase. Hathway, Mallinson & Akintonwa (1965) have recently drawn attention to the role of alanine in the disposal of free NH_4^+ in rat brain. The source of the amino group of alanine during anoxia and a possible role of glutamate-pyruvate transaminase in regulating the hepatic NH_4^+ concentration requires further study.

Aspartate. The decrease in aspartate in livers from alloxan-diabetic and phlorrhizin-treated rats may reflect a lower oxaloacetate concentration in these livers, since the latter is directly related to aspartate via the glutamate-oxaloacetate transaminase reaction. As measurements show that the transaminase system is at equilibrium in rat liver (Table 7), a change in the oxaloacetate concentration will alter that of aspartate, assuming a constant glutamate/oxoglutarate ratio. A lower oxaloacetate concentration could result from its increased removal for gluconeogenesis via phosphoenolpyruvate carboxykinase. The data reported here for aspartate supports the suggestion of Krebs (1966) that there is decreased availability of oxaloacetate in conditions associated with abnormally high rates of ketogenesis (e.g. alloxan-diabetes and phlorrhizin treatment). The failure of aspartate, unlike alanine, to rise in anoxia can be attributed to the

fact that there is no ready source of oxaloacetate in this condition, because pyruvate carboxylation cannot proceed owing to lack of ATP, and formation from malate is precluded by the high NADH_2 concentration.

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