Synthesis of Essential Amino Acids from Their α-Keto Analogues by Perfused Rat Liver and Muscle

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ABSTRACT Most essential amino acids can be replaced by their α-keto-analogues in the diet. These ketoacids have therefore been proposed as substitutes for dietary protein. In order to determine their fate in tissues of normal animals, isolated rat liver and hindquarter (muscle) preparations were perfused with ketoanalogues of valine, leucine, isoleucine, methionine, or phenylalanine. When perfused at 1.5–2.0 mM, all five compounds were utilized rapidly by the liver of 48-h starved rats, at rates varying from 49 to 155 μmol/h per 200 g rat. The corresponding amino acids appeared in the medium in significantly increased concentrations. Perfusion with phenylpyruvate also led to the appearance of tyrosine. Urea release was unaltered. Measurement of metabolite concentrations in freeze-clamped liver revealed two abnormalities, particularly at ketoacid concentrations of 5 mM or above: a large increase in α-ketoglutarate, and a moderate to marked decrease in tissue glutamine. This decrease was quantitatively sufficient to account for nitrogen appearing in newly synthesized amino acids.

Isolated hindquarters of fed rats were perfused with the same ketoacids at concentrations of 1.3–8.0 mM. All were utilized at rates varying from 1.4 to 7.0 μmol/h per g muscle perfused. The corresponding amino acids were released at greatly increased rates. Alanine and glutamate levels fell in some perfusions, but the principal nitrogen donor in muscle was not identified; the content of glutamine in tissue, and its rate of release into the perfusate remained constant.

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INTRODUCTION

It has been known for many years that most of the essential amino acids (all except lysine and threonine) can be replaced in the diet by their α-keto-analogues (see reference 1). For example, in rats fed a mixture of five of these ketoacids, Wood and Cooley (2) were able to demonstrate growth, albeit at a reduced rate, on a diet free of the corresponding amino acids. A possible therapeutic implication of these observations was suggested by Schloerb (3): feeding these compounds to subjects with chronic uremia might permit them to maintain nitrogen balance on much lower protein intake than otherwise required. Under these circumstances ammonia derived from intestinal ureolysis (4), which may be greatly accelerated in uremia (5, 6), could be utilized in the synthesis of essential amino acids. Observations in uremic subjects have lent support to this hypothesis (1, 7, 8). Another possible use of these compounds is in the treatment of hyperammonemia caused by hepatic failure or by hereditary deficiency of urea cycle enzymes.

Although the pathways of degradation of all of these ketoacids have been elucidated (9), little is known of their rates of degradation or transamination or of their effects upon intermediary metabolism in individual tissues. The present studies were undertaken to determine the metabolic fate and effects upon intermediary metabolism in isolated perfused rat liver and muscle of five

of these ketoacids, viz., the analogues of valine, isoleucine, leucine, methionine, and phenylalanine.

METHODS

Liver perfusion. Perfusions were carried out on female Wistar rats (180–220 g) obtained from Scientific Products Farm, Ash, Canterbury, England. The animals were starved for 48 h before the experiments. The technique of perfusion and the medium used were as described by Hems, Ross, Berry, and Krebs (10) except that albumin was dialyzed before use (Biebuyck, Lund, and Krebs [11]) and was present in the perfusion medium at a concentration of 2.6%. The initial volume of the perfusate was 150 ml. Sodium oleate (2 mM) was included in the medium in all experiments. The α-ketoacids were added as sodium salts, dissolved in 1 or 2 ml of water, approximately 10 min before the liver was connected to the circulation.

Samples of medium (4 ml) were taken at 0, 5, 20, 40, and 60 min into 0.2 ml 60% (wt/vol) perchloric acid, mixed, chilled, and centrifuged. A portion of supernate was neutralized with a measured volume of 30% (wt/vol) KOH to precipitate $KClO_4$.

At the end of the 60 min perfusion period, a piece of liver was quickly excised and pressed between metal clamps previously cooled in liquid N_2 (12). The frozen liver was pulverized in a mortar to a fine powder, with frequent additions of N_2. A portion (about 2 g) of the powder was transferred to a plastic centrifuge tube, cooled in liquid N_2, weighed, and homogenized with 8 ml of 3.6% (wt/vol) $HClO_4$. After centrifugation in the cold at 30,000 *g*, a portion of supernate was adjusted to pH 5–6 with a measured volume of KOH to precipitate $KClO_4$.

Hindquarter perfusion. Female Wistar rats (190–230 g), fed ad lib., were used. The perfusion technique and the composition of the 150 ml perfusion medium were as described by Ruderman, Houghton, and Hems (13). Ketoacids were added as in the liver perfusions. Duplicate samples of medium (2 ml) were collected at 5, 20, 35, and 50 min, placed in 4 ml of ice-cold 10% (wt/vol) perchloric acid, centrifuged, and neutralized as in the liver perfusions. At the end of each perfusion a portion of the musculature of one hindlimb was freeze-clamped *in situ,* deproteinized, and neutralized as above. Series I experiments were performed in Oxford and series II experiments in Boston.

Materials. Sodium α-ketoisovalerate and sodium α-keto-β-methylvalerate (the analogues of valine and isoleucine) were synthesized by Cyclo Chemical Corp., Los Angeles, Calif. α-Ketoisocaproic acid (the analogue of leucine), obtained from Fluka AG, Basel, Switzerland, was converted to the sodium salt and recrystallized. Sodium phenylpyruvate was obtained from Koch-Light Laboratories, Colnbrook, Buckinghamshire, England. Sodium α-keto-γ-methylthiobutyrate (the analogue of methionine) was synthesized in our laboratory, according to a modification[1] of the procedure described by Weygand, Steglich, and Tanner (14). Identity and purity of all five compounds were verified by elemental analysis, thin-layer chromatography, both as the free acids and as the 2,4-dinitrophenylhydrazone derivatives, infrared spectroscopy, high resolution mass spectroscopy, and nuclear magnetic resonance. α-Keto-β-methylvaleric acid exhibited an optical rotation of −36°, compared with a literature value of −32° (15).

[1] Coulter, A. W., S. Dighe, and M. Walser. 1973. An improved synthesis of 4-(methylthio)-2-oxybutyric acid, the α-oxo analogue of methionine. Submitted for publication.

Oleic acid was a product of Fluka AG, Basel, Switzerland. Purified enzymes and coenzymes were obtained from the Boehringer Mannheim Corp., New York. Glutaminase (grade IV or V) was obtained from the Sigma Chemical Corp., St. Louis, Mo.). Albumin was obtained from Pentex Biochemicals, Kankakee, Ill.

Determination of metabolites. Pyruvate was determined by the method of Hohorst, Kreutz, and Bücher (16) and α-ketoglutarate by the method of Bergmeyer and Bernt (17). The two metabolites could be determined in the same cuvette by successive additions of lactate and glutamate dehydrogenases. The α-ketoacids under study reacted to varying extents with lactate dehydrogenase; hence, reliable values for pyruvate could not be obtained. There was no reductive amination of ketoacids by glutamate dehydrogenase under the assay conditions. L-Glutamate was assayed by the method of Bernt and Bergmeyer (18) and glutamine according to Lund (19). Ammonia was assayed with glutamate dehydrogenase (20). Glucose was determined using hexokinase, NADP, and glucose-6-phosphate dehydrogenase (21). L-Alanine was measured with alanine dehydrogenase. The enzyme was prepared from *Bacillus subtilis* by Mr. R. Hems, using the method of Yoshida and Freese (22), as modified by Williamson, Lopes-Vieira, and Walker (23) or was purchased from Boehringer Mannheim Corp. (series II experiments). 3-Hydroxybutyrate and acetoacetate were determined according to Williamson, Mellanby, and Krebs (24). Urea was determined by the diacetyl monoxime-antipyrine method (25) in which citrulline also reacts. The content of citrulline in the perfusate was less than 0.005 μmol/ml. Because the diacetyl monoxime-antipyrine-urea color complex fades in light of near-UV wavelength (26), the samples were protected from sunlight and fluorescent light at all stages. Perchloric acid extracts of the last perfusate samples in each experiment, except in those of series II, were analyzed for valine, methionine, alloisoleucine, isoleucine, tyrosine, and phenylalanine, using a Beckman model 120C amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.). Analysis of 11 free amino acids in extracts from the last samples of perfusate in the experiments in series II was carried out with a Technicon amino acid analyzer (Technicon Instruments Corp., Tarrytown, N. Y.).

Total ketoacids were determined promptly on perchloric acid extracts. Samples containing approximately 0.1 μmol of ketoacid, in a volume of 1 ml, were mixed in conical centrifuge tubes with 0.2 ml of freshly prepared 10 mM 2,4-dinitrophenylhydrazine (recrystallized from ethyl acetate) in 2 N HCl. Samples containing only branched-chain ketoacids were allowed to stand at least 20 min; those containing α-keto-γ-methylthiobutyrate for at least 2 h, and those containing phenylpyruvate overnight at room temperature. 1 ml of a 1:1 mixture of diisopropylether and heptane was added. Extraction of hydrazones into the organic solvent layer was complete after 30 s of vigorous mixing. The aqueous layer was removed as completely as possible with a Pasteur pipette and discarded. After adding 3 ml of 10% Na_2CO_3, extraction was continued for 1 min. Under these conditions the hydrazones were completely extracted into the aqueous layer, but most of the unreacted dinitrophenylhydrazine was not. Optical density was read at 380 nm in a Zeiss PMQ II spectrometer (Carl Zeiss, Inc., New York), using a slit width of 0.035 mm or less. Results were calculated in relation to those obtained with freshly prepared standards of the particular ketoacid employed in each experiment; these were analyzed simultaneously. The optical density of the reagent blank averaged

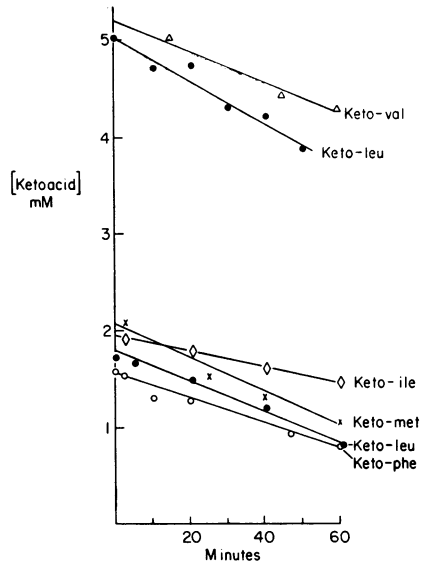

FIGURE 1 Disappearance of α-ketoacids from the medium during perfusion of isolated rat livers in representative experiments.

0.15 U. Recovery of each of the five ketoacids from blood was between 98 and 101%.

Solvents with a dielectric constant lower than the solvent employed here failed to extract the hydrazones completely from acid media; use of solvents with higher dielectric constants, such as ethyl acetate, led to incomplete extraction from the organic layer into the carbonate solution. In order to determine the applicability of this method to mixtures of these ketoacids, pure hydrazones of all five compounds were prepared by precipitation from water, washing with water, and recrystallization from ethanol: water. After drying, weighed portions were dissolved in ethanol and diluted to a concentration of 0.067 mM in 10% Na_2CO_3, and optical density was determined as above. All five compounds exhibit absorption maxima close to 380 nm. Molar absorptivities calculated from these data were as follows: keto-val,[2] 20,800; keto-leu, 21,100; keto-ile, 22,500; keto-met, 21,700; keto-phe, 20,850. For mixed perfusions a value of 21,400 was used in calculating the results.

This method also measures pyruvate and α-ketoglutarate, but does not measure acetoacetate or α-ketoglutaramate.[3] Disappearance rates of ketoacids could therefore be in error to the extent that pyruvate and α-ketoglutarate are released during the perfusion. Using the above method, liver perfusate concentrations of ketoacids in control experiments rose from 0.06±0.02 mM (SEM, $n=3$) to 0.12±0.02 mM (SEM, $n=4$) between 0 and 60 min. In one control hindquarter perfusion, an increment of 0.1 mM ketoacid occurred. No correction was made for these small increases in endogenous ketoacids.

Calculations. Freeze-clamping of tissue prevented an accurate determination of tissue weight. Rates of uptake or release were therefore divided by body weight, and expressed in relation to a 200 g rat. From previous experience, the liver weight of 200 g 48-h starved rats averages 6.5 g, whereas the estimated weight of muscle perfused in a hindquarter preparation is 33 g in a 200 g rat, or 16.5% body weight (13).

Disappearance of each ketoacid was calculated as the product of the slope of the linear decrease in perfusate concentration, the duration of the perfusion, and the midpoint perfusate volume. This rate (in micromoles per hour) was also corrected for the measured quantity of ketoacid remaining in the tissue at the end of the perfusion, and was normalized to a 200 g rat as indicated above.

The quantity of each measured amino acid released was calculated as the product of final perfusate concentration and midpoint perfusate volume. Amino acid within the tissue was measured in some experiments; in these, the total amount of amino acid present in perfusate plus tissue at the end of the experiment could be calculated. Amino acid release or total amino acid present was corrected for body weight as indicated above.

Glucose and urea release, in liver perfusions, was calculated as the product of the linear increases of concentration between 5 and 60 min, and midpoint perfusate volume, and normalized to a 200 g rat.

RESULTS

Liver perfusions

Ketoacid disappearance. Disappearance rates of ketoacids were linear, or nearly so, during the 60 min of observation (Fig. 1). No disappearance from the medium occurred until the liver was placed in the perfusion circuit. When tested individually, keto-ile, keto-leu, keto-met, keto-val, and keto-phe, at 1.5–2.0 mM, were removed by the perfused liver at rates of 49±13, 115±25, 155±22, 113±23, 91±19 μmol/h per 200 g rat, respectively (mean±SEM, $n=4$). When all five ketoacids were added to the perfusate, the total disappearance rate increased to 287±36 μmol/h, but was considerably less than the sum of the disappearance rates measured individually (524 μmol/h).

Release of essential amino acids. The concentrations of free amino acids in the medium at the end of the 60 min perfusions are presented in Table I. These values are similar to those found in previous reports (27, 28), when corrected for rat weight to 200 g and for perfusate volume to 150 ml. Perfusion with each of the five ketoacids at concentrations of 1.5–2.0 mM led to significantly increased release of the corresponding amino acid into the medium. Perfusion with phenylpyruvic acid led to the appearance of increased quantities of tyrosine as well as phenylalanine, owing to the activity of phenylalanine hydroxylase. Alloisoleucine was not detected. The amount of extra amino acid formed from each of the five ketoacids varied in the order methionine > leucine = phenylalanine plus tyrosine > valine > isoleucine. When keto-val or keto-leu was perfused at 5 mM in single experiments, valine or leucine release was

[2] *Abbreviations used in this paper:* keto-val, α-ketoisovaleric acid; keto-ile, L-α-keto-β-methylvaleric acid; keto-met, α-keto-β-methylthiobutyric acid; keto-leu, α-ketoisocaproic acid; keto-phe, phenylpyruvic acid.

[3] Kindly supplied by Dr. Thomas E. Duffy, New York Hospital-Cornell Medical Center, New York.

TABLE I

Formation of Essential Amino Acids from Their Keto-Analogues by Isolated Perfused Rat Liver

Addition	Perfusate concentration, mean ±SEM					
	Val*	Met*	Ile*	Leu*	Tyr*	Phe*
				μM		
None (3)	62 ±10	0	34 ±6	78 ±14	14 ±1	31 ±3
Keto-val, 2 mM (4)	93‡ ±7	1 ±1	36 ±2	81 ±7	16 ±2	28 ±2
Keto-val, 5 mM (1)	96	3	33	72	20	25
Keto-met, 2 mM (3)	64 ±7	278‡ ±77	30 ±4	72 ±5	10 ±1	21 ±1
Keto-ile, 2 mM (3)	58 ±3	4 ±2	56‡ ±1	69 ±4	16 ±3	27 ±2
Keto-leu, 2 mM (4)	57 ±6	3 ±1	28 ±3	256‡ ±29	10 ±1	24 ±2
Keto-leu, 5 mM (1)	56	0	24	305	11	30
Keto-phe, 2 mM (3)	54 ±13	5 ±3	25 ±6	60 ±17	128‡ ±30	112‡ ±19
Mixture of five ketoacids (4)	76 ±17	159‡ ±15	43 ±9	115 ±22	75‡ ±15	80‡ ±11

Free amino acid concentrations in perfusate (initial volume 150 ml) at the end of 60 min perfusion are shown; results have not been corrected for variations in liver weight.

* *Abbreviations used in this table:* val, valine; met, methionine; ile, isoleucine; leu, leucine; tyr, tyrosine; phe, phenylalanine.

‡ Significantly different from control perfusion ($P < 0.02$).

scarcely any greater, suggesting that the rate of examination was not limited by the supply of ketoacid at 2 mM. Perfusion of a mixture of five ketoacids, each at a concentration of 2 mM, led to increases in the appearance of all of the corresponding amino acids, but these changes were statistically significant only for methionine, tyrosine, and phenylalanine. The combined increments in all six amino acids were smaller during the mixed perfusion than when administered individually. This could be due to competition for a transaminase or to limitation in available nitrogenous precursors for transamination.

The ratio of amination to disappearance, which expresses the fraction of metabolized ketoacid appearing as an increment in release of the corresponding amino acid, varied from 5% for keto-val to 36% for keto-phe. For the mixture, it averaged 20%. Ketoacid disappearance not accounted for by release of free amino acid may represent degradation of the ketoacid or utilization of newly synthesized amino acid. The rate of this unaccounted for metabolism averages 84 μmol/h per 200 g rat for the five ketoacids perfused individually at 1.5–2.0 mM, and varied in the sequence keto-met = keto-val > keto-leu > keto-phe > keto-ile. During perfusion of all five compounds the rate increased to 236 μmol/h.

Tissue amino acids and ketoacids. Analysis of tissue extracts showed that there was no accumulation of newly synthesized free essential amino acids in the liver. Tissue/medium ratios were close to unity for valine (mean ±SEM = 1.33±0.15), leucine (0.89±0.12), isoleucine (1.00±0.13), tyrosine (0.89±0.23), and phenylalanine (0.90±0.14). Methionine was measured only in one experiment (a mixed perfusion); the tissue/medium ratio was 0.55. In all experiments the tissue/medium ratio for the α-ketoacids was less than unity (Table II).

Glucose and urea release. Appearance rates of glucose and urea were approximately linear between 5 and 60 min. None of the ketoacids singly or in combination caused statistically significant changes in the release of glucose (average 2 μmol/min per 6.5 g liver). This is somewhat surprising, since oxidation of keto-val or keto-ile gives rise to proprionyl-CoA, a glucogenic compound. Furthermore, keto-met and keto-phe, which are inhibitors of glucose synthesis (29) did not appear to

TABLE II

Effect of Ketoacids on Concentrations of Metabolites in Perfused Rat Liver

Addition	At the end of 60 min perfusion					Liver/medium ratio of ketoacid
	NH_4	Glut	αKG	Ala	ATP	
			μmol/g wet wt			
None	0.46 ±0.09	1.57 ±0.12	0.17 ±0.03	0.16 ±0.01	1.37 ±0.13	
Keto-val, 2 mM	0.45 ±0.07	1.96 ±0.29	0.31 ±0.05	0.16 ±0.01	1.38 ±0.06	0.44 ±0.04
Keto-val, 5 mM	0.28	3.15§	0.06	0.15	1.53	0.57
Keto-met, 2 mM	0.63 ±0.10	1.04 ±0.22	0.17 ±0.09	0.18 ±0.02	1.32 ±0.13	0.65 ±0.12
Keto-ile, 2 mM	0.59 ±0.08	1.61 ±0.09	0.23 ±0.03	0.18 ±0.02	1.48 ±0.38	0.53 ±0.04
Keto-ile, 5 mM	0.49	2.66§	0.57§	0.33§	1.50	0.37
Keto-leu, 2 mM	0.81 ±0.14	1.16* ±0.03	0.22 ±0.06	0.18 ±0.02	1.37 ±0.13	0.55 ±0.11
Keto-leu, 5 mM	0.38 ±0.11	1.31 ±0.06	0.71 ±0.29		1.47 ±0.28	0.42
Keto-phe, 2 mM	0.54 ±0.17	1.16 ±0.22	0.18 ±0.02	0.17 ±0.01	1.23 ±0.16	0.57 ±0.19
Mixture of five ketoacids	±0.05	±0.11	±0.26	±0.01	±0.09	±0.08

Number of observations as in Table I.

Abbreviations: NH_4^+, ammonium; Glut, glutamate, αKG, α-ketoglutarate; Ala, alanine.

* Significantly different from control perfusions ($P < 0.05$).

‡ Significantly different from control perfusions ($P < 0.01$).

§ Individual values lying more than 8 SD from the mean value in control perfusions.

inhibit endogenous gluconeogenesis at 1.5–2 mM. At 5 mM keto-val, keto-ile, and keto-leu there appeared to be a decreased rate of glucose release, but the differences were not significant statistically owing to considerable variability in control perfusions.

Urea release averaged 0.97±0.07 μmol/min per 200 g rat in controls. No significant change occurred after any of the ketoacids perfused individually or together.

Tissue metabolite concentrations. Analysis of freeze-clamped liver after 60 min perfusion with ketoacids was carried out to ascertain whether these compounds induce metabolic derangements at the concentrations employed. The metabolites were chosen to give some criterion of normal liver function: ATP (as an indication of the energy state), glutamate, α-ketoglutarate, and ammonia (because they allow calculation of the mitochondrial redox state) and alanine (which increases when respiration is inhibited; Brosnan, Krebs, and Williamson [30]). Because of uncertainties in the determination of pyruvate in the presence of the α-ketoacids under study, meaningful calculations of the redox state of the cytoplasm from concentrations of the reactants of lactate dehydrogenase were not possible. The concentration of α-ketoglutarate was not significantly affected by the individual α-ketoacids perfused at concentrations of 1.5–2.0 mM, but in tissues perfused with 5 mM keto-leu or keto-ile, somewhat higher values of α-ketoglutarate were found. When the mixture of five ketoacids was perfused, α-ketoglutarate increased markedly (from 0.17±0.03 μmol/g in controls to 1.69±0.26 μmol/g). Since there was no compensatory change in either glutamate or ammonia, which should occur because of the near-equilibrium that exists in the glutamate dehydrogenase system (31), the increase in α-ketoglutarate concentration probably occurs in the cytoplasm. This conclusion is supported by the finding that the redox state of the mitochondria, as calculated from the 3-hydroxybutyrate dehydrogenase system, was unchanged under these conditions (data not shown). Alanine and ATP concentrations were constant.

Source of α-amino nitrogen for formation of essential amino acids. Because the amounts of essential amino

TABLE III

Glutamine as a Source of α-Amino Nitrogen for the Synthesis of Essential Amino Acids from Their Keto-Analogues in Isolated Perfused Rat Liver

Substance added	Tissue glutamine at 60 min	N present as glutamine plus glutamate after 60 min perfusion			Amount of ketoacid aminated
		Medium	Liver	Total	
	μmol/g wet wt	*μmol N*	*μmol N*	*μmol N*	*μmol*
None	1.84 ±0.42	45.9 ±12.6 (5)	33.3 ±5.6 (5)	79.2 ±13.8 (5)	0
Keto-val, 2 mM	0.98 ±0.28	32.0 ±1.9 (3)	25.3 ±3.9 (4)	59.1 ±4.4 (3)	3.9 ±0.9 (4)
Keto-ile, 2 mM	0.91 ±0.29	24.7, 24.9 (2)	22.1 ±4.6 (3)	38.1, 48.9 (2)	2.9 ±0.3 (3)
Keto-leu, 2 mM	0.36* ±0.10	14.6* ±1.2 (3)	13.3* ±2.1 (4)	25.8* ±0.8 (3)	22.2 ±3.4 (4)
Keto-leu, 5 mM	0.02* ± 0.02	10.1, 11.3 (2)	8.0 6.2 (2)	18.1, 17.5 (2)	28.6 43.5 (2)
Keto-met, 2 mM	0.26* ± 0.05	6.4* ±0.8 (4)	6.6* ±3.1 (3)	13.0* ±2.4 (3)	35 ±10 (3)
Keto-phe, 2 mM	0.40* ±0.33	3.8, 7.0 (2)	13.3* ± 5.4 (3)	12.6, 14.1 (2)	24.7 ±5.9 (3)
Mixture of five ketoacids	0.03* ±0.01	6.1* ±0.4 (5)	8.6* ±0.7 (5)	14.5* ±0.9 (5)	56.7 ±5.8 (3)

* Significantly different from controls ($P < 0.01$).

acid formed were considerable, it was important to establish the source of the α-amino nitrogen. No free amino acids were initially present in the perfusion medium so that α-amino nitrogen derived from endogenous precursors was the only possible source. Of these, only glutamine is present in the liver in sufficient amount. Enzymic determination of glutamine in the tissue extracts after 60 min perfusion showed that glutamine was consistently decreased by the presence of α-ketoacids (Table III). Moreover the decrease was related to the amount of ketoacid aminated: when 5 mM ketoleu or the mixture of ketoacids was perfused, glutamine concentration decreased to < 0.1 μmol/g wet wt of tissue. Glutamine and glutamate were therefore also determined in the samples of perfusion medium taken at 60 min. From these data the total nitrogen disappearing as glutamine and glutamate could be calculated by subtraction from the total found in the control perfusions. The results of the calculations are given in Table III. There is a reciprocal relationship between the amount of nitrogen recovered in glutamine plus glutamate and the amount of ketoacid aminated. It is clear that glutamine is the ultimate source of nitrogen for the essential amino acids synthesized, whether via decreased formation or increased breakdown.

Muscle perfusions

General observations. Hindquarters perfused with ketoacids appeared grossly normal and did not exhibit disturbances in blood flow. They also did not have increased lactate release (data not shown) such as occurs when hindquarters are anoxic or ischemic (13).

Ketoacid disappearance. No disappearance of ketoacids from the medium occurred during a 35 min incubation with the medium alone. The rates of disappearance of all five ketoacids during perfusion of muscle were linear (Fig. 2). Expressed as μmol/h per 33 g muscle, the rates varied from 46 to 119 during perfusion with individual ketoacids at 1.3 mM. At 5 mM, the three branched-chain ketoacids each disappeared at a rate of about 230 μmol/h.

Release of essential amino acids. Concentrations of valine, methionine, leucine, tyrosine, and phenylalanine in the medium after 50 min perfusion were similar in the two control series (Table IV). These final concentrations cannot be interpreted as indicating rates of release because amino acid concentrations 5 min after starting the perfusions were not measured in these experiments, as they were in previous studies of this preparation (13). Each of the five ketoacids, perfused

TABLE IV

Formation of Essential Amino Acids from Their Keto-Analogues by Perfused Rat Hindquarters

Addition	Perfusate concentration at 50 min, mean ±SEM					
	Val*	Met*	Ile*	Leu*	Tyr*	Phe*
			μM			
Series I						
None (6)	36	10	14	27	15	25
	±6	±2	±2	±5	±2	±6
Keto-leu, 2 mM (2)	42	<5	8	184	20	20
Keto-leu, 5 mM (4)	30	11	8	515§	19	19
	±9	±4	±2	±29	±11	±5
Keto-leu, 8 mM (2)	49	6	16	735‡	16	44
Keto-val, 5 mM (2)	726‡	7	12	27	27	22
Keto-ile, 5 mM (3)	42	29	359§	24	11	21
	±12	±12	±28	±8	±5	±5
Series II						
None (5)	44	20	18	38	21	26
	±1	±1	±1	±1	±2	±1
Keto-met, 1.3 mM (4)	32§	435§	11§	21§	21§	18§
	±4	±28	±1	±3	±4	±1
Keto-phe, 1.3 mM (3)	45	14	19	37	23	140§
	±4	±2	±1	±5	±2	±17
Keto-leu, 1.3 mM (1)	30‡	8‡	8‡	193‡	27‡	21‡
Keto-val, 1.3 mM (1)	326‡	4‡	6‡	14‡	11‡	17

Free amino acid concentrations at the end of 50 min perfusion are shown. Values not corrected for variations in weight of tissue.

* Abbreviations as in Table I.

‡ Individual values lying more than 5 SD from the mean of control observations.

§ Significantly different from control means ($P < 0.01$).

alone, led to greatly increased release into the medium of the corresponding amino acid. Keto-phe led to the appearance of phenylalanine alone, instead of phenylalanine plus tyrosine, as in liver. The rate at which the individual amino acids were released, above control rates, when compared at equal ketoacid concentration, varied as follows: keto-met > keto-val > keto-leu > keto-ile = keto-phe. Higher concentrations of keto-val and keto-leu led to greater release of valine and leucine, respectively.

TABLE V

Effect of Keto-phe and Keto-met on Total Amount of Various Amino Acids Present in Muscle Tissue plus Perfusate after 50 min Perfusion

	Quantity present, μmol/33 g tissue, mean ±SEM										
	Gly*	Ala*	Val*	Met*	Ile*	Leu*	Tyr*	Phe*	Lys*	His*	Arg*
Controls (3)	109 ±14	75 ±11	9.0 ±1.0	3.3 ±0.2	3.6 ±0.2	6.9 ±0.6	4.6 ±0.3	5.1 ±0.6	58 ±8	22 ±4	21 ±2
Keto-phe (3)	111 ±19	62 ±1	7.6 ±0.0	2.3 ±0.3	3.2 ±0.1	6.1 ±0.2	4.9 ±0.9	26.3‡ ±2.5	38 ±6	26 ±5	15 ±2
Keto-met (1)	109	57	6.4	68.0	1.9	4.1	5.7	3.7	44	32	15

33 g tissue is the estimated weight of muscle perfused in a 200 g rat.

* Additional *abbreviations used in this table:* gly, glycine; lys, lysine; his, histidine; arg, arginine.

‡ Significantly different from controls ($P < 0.01$).

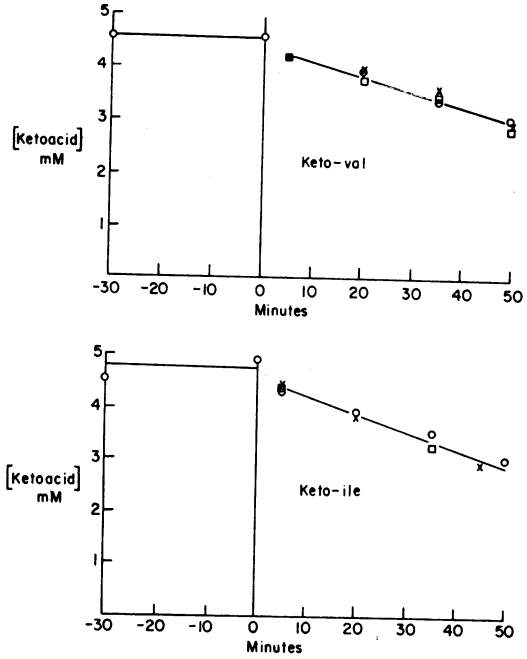

FIGURE 2 Disappearance of keto-val or keto-ile from the medium during perfusion of isolated rat hindquarters. No disappearance is seen before the hindquarter is connected to the perfusion circuit (at zero time). Results in individual experiments are represented by different symbols.

Tissue amino acids and ketoacids. In seven of the series II experiments, analysis of muscle extracts for 11 amino acids were carried out. The quantities of each amino acid present in tissue plus perfusate were calculated as explained in Methods. The results are shown in Table V. Large increases were observed in the amino acids corresponding to the ketoacids infused. In addition, the average concentration of each of the other essential amino acids fell. This change is statistically significant only in the case of methionine after keto-phe perfusion, but the consistency with which this change occurred suggests that it might have been generally significant with larger numbers of observations. Perfusate concentrations of valine, isoleucine, leucine, and phenylalanine were significantly reduced by keto-met (Table IV).

This effect of ketoacids on unrelated essential amino acids is apparently not attributable to an effect on amino acid transport across the cell membrane. As shown in Table VI, tissue/perfusate concentration ratios were not appreciably affected, even for those amino acids newly synthesized (phenylalanine and methionine). Possible explanations are transamination between essential amino acids and the administered ketoacids, increased protein synthesis, and decreased proteolysis.

Tissue metabolite concentrations. Acetoacetate and 3-hydroxybutyrate, normally absent from the perfusate, appeared during perfusion with keto-leu (Table VII). Approximately 10% of keto-leu disappearance could be accounted for in this way. Keto-val led to a large increment in the release of a substance which assayed as 3-hydroxybutyrate. Presumably this was 3-hydroxyisobutyrate, a normal intermediate in valine catabolism (9): acetoacetate was not found in these experiments. We were unable to obtain 3-hydroxyisobutyrate to confirm this hypothesis. Ketone bodies were also not detectable during perfusion with keto-ile. α-Ketoglutarate, measured in five experiments in series II, was unaltered (data not shown).

Source of α-amino nitrogen for synthesis of essential amino acids. Alanine and glutamine are the main vehicles for transport of nitrogen from muscle to other tissues. They account for about half of the amino acid released by the human forearm and the rat hindquarter even though they comprise less than 15% of muscle protein. Evidence from several sources indicates that both alanine and glutamine can be synthesized by the muscle cell; the extra nitrogen probably derives from catabolism of other amino acids (32–34).

TABLE VI
Effect of Keto-phe and Keto-met on Tissue/Medium Concentration Ratios for Various Amino Acids in Muscle after 58 min Perfusion

	Tissue/medium ratio, μmol/g/μmol/ml										
	Gly*	Ala*	Val*	Met*	Ile*	Leu*	Tyr*	Phe*	Lys*	His*	Arg*
Controls (3)	11 ±2	6 ±1	2.5 ±0.9	1.2 ±0.3	2.3 ±0.6	1.9 ±0.5	3.2 ±0.5	2.1 ±0.6	11 ±3	17 ±5	14 ±3
Keto-phe (3)	13 ±1	5 ±1	1.4 ±0.4	1.1 ±0.1	1.3 ±0.2	1.2 ±0.2	2.1 ±0.2	1.9 ±0.1	6 ±1	21 ±4	9 ±2
Keto-met (1)	15	4	1.2	1.7	1.8	1.1	1.8	1.7	8	25	8

* Abbreviations listed in Tables I and V.

TABLE VII

Effect of Ketoacids on Selected Metabolites in Perfused Rat Hindquarters

Addition	Release				Tissue concentration		
	Ala*	Glu·NH_2*	Acac*	3-HB*	Ala*	Glu·NH_2*	Glut*
	μmol/h/33 g muscle				*μmol/g wet wt*		
Series I							
None (9)	19 ±1	25 ±3	0	0	0.78 ±0.04		0.85 ±0.11
Keto-leu, 2 mM (2)	13	29	9	5	0.68		0.72
5 mM (4)	9‡ ±1	33 ±6	11‡ ±2	5‡ ±1	0.60 ±0.14		0.52 ±0.12
8 mM (2)	9	21	7	3	0.57		0.49
Keto-val 5 mM (3)	10‡ ±1	29 ±8	0	37§ ±14	0.48 (2)		0.54‖ ±0.05
Keto-ile, 5 mM (2)	11	9	0	0	1.06		0.38
Series II							
None (5)	20 ±1	24 ±4			1.13 ±0.26	2.93 ±0.30	0.83 ±0.10
Keto-met, 1.3 mM (4)	14‡ ±2	26 ±4			0.77 ±0.13	2.10 ±0.47	0.68 ±0.04
Keto-phe, 1.3 mM (3)	21 ±6	28 ±7			1.27 ±0.10	2.39 ±0.60	0.72 ±0.07
Keto-leu, 1.3 mM (1)	16	25			0.60	3.62	0.92
Keto-val, 1.3 mM (1)	18	34			0.24	2.48	0.64

Results of perfusate analyses at the end of 50 min perfusions are shown, corrected for variations in weight, and tissue concentrations.

* Additional *abbreviations used in this table:* glu·NH_2, glutamine; glut, glutamate, acac, acetoacetate; 3-HB, 3-hydroxybutyrate.

‡ Significantly different from control mean ($P < 0.01$).

§ Probably artifactual owing to the presumed presence of 3-hydroxyisobutyrate.

‖ Significantly different from controls ($P < 0.05$).

The effect of ketoacids on the release of alanine and glutamine was therefore examined. We reasoned that if the ketoacids received an amino group from glutamate, the concentration of glutamate would fall, and as a result, the transamination from pyruvate to alanine and the synthesis of glutamine might be inhibited.

In general the release of alanine and the tissue concentrations of both glutamate and alanine were somewhat reduced in tissues perfused with the keto-analogues of leucine, valine, or methionine (Fig. 3). These changes were not observed consistently with phenylpyruvate, the keto-derivative that was converted to its corresponding amino acid at the lowest rate. Glycine, histidine, arginine, and lysine also failed to fall sig-

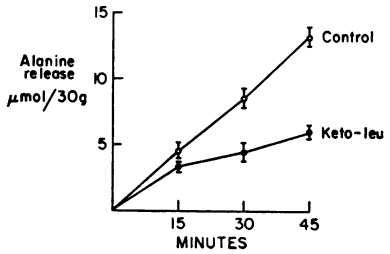

FIGURE 3 Alanine release during perfusion of isolated rat hindquarters with keto-leu, 5 mM. Compared with control perfusions, release of alanine is progressively reduced during keto-leu perfusion, but the quantity of nitrogen thus made available is not sufficient to account for all of the leucine synthesized.

nificantly during keto-phe perfusion (Table V), although the change observed in lysine is quantitatively sufficient to account for the phenylalanine synthesized. In no experiments was the release or the tissue concentration of glutamine significantly altered (Table VII).

Thus these experiments failed to identify the major source of the nitrogen used for aminating ketoacids in muscle, if indeed a single amino acid is the predominant source.

Tissue/medium concentration ratios for ketoacids in muscle were as follows: at 1.3–2.0 mM, keto-val, 0.60; keto-met, 0.99±0.34 (SEM, $n = 4$); keto-leu, 0.82 ($n = 2$); keto-phe, 1.32±0.29 (SEM, $n = 3$); at 5 mM, keto-val, 0.43±0.06 (SEM, $n = 3$); keto-ile, 0.42 ($n = 2$); keto-leu, 0.39±0.04 (SEM, $n = 4$).

DISCUSSION

These results establish that the keto-analogues of valine, leucine, isoleucine, methionine, and phenylalanine can be converted to their respective amino acids by normal liver and muscle. The only other conceivable source of the increased amounts of amino acids appearing in the perfusate is proteolysis, since there was no change in tissue/medium ratios of free amino acids. But proteolysis cannot produce large increments in the concentration of a single amino acid unless there also occurs synthesis of a polypeptide lacking this specific amino acid. This theoretical possibility seems too improbable to warrant serious consideration. Furthermore, isotopic evidence for conversion of keto-met to methionine by rat liver homogenate has been reported previously (35).

Although the enzymes capable of catalyzing these reactions have been demonstrated in tissue extracts (35, 36), evidence that similar reactions occur in vivo has been indirect. The ability of these ketoacids to substitute for the corresponding amino acids in the diet of rats (1) could be attributed to bacterial action in the intestinal tract. In ruminants, for example, most of the essential amino acids can be synthesized in the rumen from simple nitrogenous precursors such as urea (37). Data obtained in human subjects fed ketoacids plus ^{15}N-labeled ammonia, in which incorporation of ^{15}N into the corresponding amino acids has been shown (8), could be explained by bacterial activity or by exchange reactions. In addition, experiments in the intact organism are difficult to interpret quantitatively because of the high rates at which amino acids are transported between tissues, metabolized, or utilized for biosynthesis. The advantage of the isolated perfused organ is that the functional capacity of an individual tissue can be assessed in quantitative terms.

Another aspect of the work was the possible toxicity of the ketoacids. Since their therapeutic use in uremia and hyperammonemia has been advocated, safe upper limits of concentration are important to establish. In branched-chain ketoaciduria, keto-val, keto-leu, and keto-ile accumulate in body fluids, because of a defect in the enzyme catalyzing oxidative decarboxylation of these acids. Levels as high as 10 mM have been reported (38). These compounds inhibit pyruvate dehydrogenase and α-ketoglutarate dehydrogenase at concentrations of the order of 5 mM (39, 40). Keto-met and keto-phe at low concentration are inhibitors of gluconeogenesis from various precursors in kidney (29). The present data suggest that endogenous metabolism of the liver is not affected by these compounds at concentrations of 2 mM, but the presence of 2 mM oleate could have obscured an inhibitory action on gluconeogenesis. The increase in α-ketoglutarate that occurred at 5 mM or higher concentration could be attributed to inhibition of α-ketoglutarate dehydrogenase. The maintenance of ATP concentration under these conditions can be explained by β-oxidation of the added oleate or endogenous fatty acid.

Although the amounts of ketoacid aminated per unit weight were in general higher in the liver than in skeletal muscle, the greater mass of muscle may mean that it has a greater capacity for amination, especially for formation of branched-chain amino acids. However, the liver perfusions were performed on starved rats and the muscle perfusion in fed rats. It is also important to bear in mind that these rates were obtained in the absence of added substrate and were probably limited by the supply of endogenous amino acids as nitrogen donors. A third element of uncertainty derives from the possibility that catabolism of newly synthesized amino acids may have occurred, particularly in the cases of phenylalanine and methionine. The main pathways of degradation of these two amino acids do not involve transamination to their keto-analogues, so that flux through the synthetic pathway may have been greater than our measurements indicate. The branched-chain amino acids, on the other hand, are not catabolized to any extent by liver (41) but are degraded in muscle. Under the conditions of our experiments, it seems unlikely that much degradation of these amino acids could have occurred in muscle, either, in the face of far higher concentrations of the corresponding ketoacids.

The high rate of metabolism of these compounds observed here is also relevant to their therapeutic use. From the experiments in which tissues were perfused with 2 mM keto-leu, for example, one may infer that a 200 g starved rat metabolized about 2 μmol/min of the compound in the liver, whereas a 200 g fed rat metabolized 6 μmol/min in muscle (the latter value is based upon the observed rate of 2 μmol/min per 33 g muscle and an estimate of muscle weight as 45% of body weight). This high rate of utilization was confirmed in four additional experiments by infusing either keto-phe or keto-ile at a constant rate of 15 μmol/min i.v. in intact

unanesthetized starved rats: after 60 min perfusion, plasma concentrations were found to be approximately 1.5 mM, suggesting an even higher rate of utilization in vivo.[4]

If the disappearance of ketoacids from the liver perfusate not accounted for by appearance of newly synthesized amino acids represents catabolism, these compounds could be an important alternate fuel. For example, oxidation of 1 μmol of keto-leu requires approximately 5.5 μmol of O_2 (36). Catabolism of this compound by liver at 1.4 μmol/min by a 200 g rat could account for 40% of the oxidative metabolism of the liver (3 μmol/min per g [42]), at least as observed during perfusion.

Several reactions are known for the amination of the keto-analogues of the essential amino acids. These include (*a*) glutamine-α-ketoacid aminotransferase (9), (*b*) leucine aminotransferase (43) (*c*) branched-chain aminotransferase (9), and (*d*) nonspecific aminotransferases, distinct from glutamate oxaloacetate aminotransferase and glutamate pyruvate aminotransferase, in which glutamate or alanine (44) is the amino-group donor. Amination of the ketoacids by glutamate dehydrogenase (45) was highly unlikely in view of the nonreaction of the ketoacids with the purified commercial preparation of glutamate dehydrogenase from beef liver (see Methods).

Glutamine-α-ketoacid amino transferase occurs in liver, but not in skeletal muscle (46). Gordon (35) has shown that added glutamine increases the conversion of keto-met to methionine in supernate of rat liver homogenate. Cooper and Meister (47) have examined the specificity of the enzyme towards various ketoacids. The relative activities are keto-met > keto-phe > keto-leu; keto-ile is a very poor substrate, and keto-val does not react. The results of our liver perfusion experiments indicate participation of this enzyme in amination of the ketoacids, although direct amination by glutamate, derived from glutamine via glutaminase, cannot be ruled out. Whatever the mechanism, glutamine is the ultimate source of the amino groups under the experimental conditions, as shown in the following reaction schemes:

(*a*) glutamine aminotransferase reaction

glutamine + α-ketoacid ⇄ α-ketoglutaramate + amino acid

α-ketoglutaramate ⟶ α-ketoglutarate + NH_3

α-ketoglutarate + NH_3 ⇄ glutamate

sum: glutamine + α-ketoacid ⟶ glutamate + amino acid

(*b*) nonspecific aminotransferase reaction

glutamate + α-ketoacid ⇄ α-ketoglutarate + amino acid

glutamine ⟶ glutamate + NH_3

α-ketoglutarate + NH_3 ⇄ glutamate

sum: glutamine + α-ketoacid ⟶ glutamate + amino acid

[4] We are indebted to Dr. R. A. Hawkins for assistance with these experiments.

In skeletal muscle, branched-chain aminotransferase is present in very high activity relative to its activity in liver (48, 49). This enzyme would appear to be responsible for amination of the ketoacids in muscle even though the source of the required glutamate was not identified.

Diseases characterized by abnormal accumulations of the ketoacids studies here may also exhibit some of the secondary metabolic disturbances we have observed in the perfused liver. In phenylketonuria, for example, plasma glutamine is subnormal (50, 51). The earlier suggestion that this abnormality is connected with mental impairment in the disease has not been confirmed (52–55) and no hypothesis to explain the decrease in glutamine has been offered. In view of the high reactivity of glutamine transaminase with phenylpyruvate (47) and our observation of decreased liver glutamine following perfusion with phenylpyruvate, it would appear that this reaction is probably involved. In branched-chain ketoaciduria, no systematic study of glutamine levels has been reported, but isolated observations (56, 57) suggest that glutamine may be low in these patients.

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REFERENCES

1. Walser, M., A. W. Coulter, S. Dighe, and F. Crantz. 1973. The effect of keto-analogues of essential amino acids in severe chronic uremia *J. Clin. Invest.* **52**: 678.
2. Wood, J. L., and S. L. Cooley. 1954. Substitution of α-keto acids for five amino acids essential for growth of the rat. *Proc. Soc. Exp. Biol. Med.* **85**: 409.
3. Schloerb, P. R. 1966. Essential L-amino acid administration in uremia. *Am. J. Med. Sci.* **252**: 650.
4. Walser, M., and L. J. Bodenlos. 1959. Urea metabolism in man. *J. Clin. Invest.* **38**: 1617.
5. Walser, M. 1970. Use of isotopic urea to study distribution and degradation of urea in man. *In* Urea and the Kidney. B. Schmidt-Nielsen, editor. Excerpta Medica Foundation, Publishers, Amsterdam. 421.
6. Jones, E. A., R. A. Smallwood, A. Craigie, and V. M. Rosenoer. 1969. The enterohepatic circulation of urea nitrogen. *Clin. Sci. (Oxf.)*. **37**: 825.
7. Richards, P., B. J. Houghton, C. L. Brown, and E. Thompson. 1971. Synthesis of phenylalanine and valine by healthy and uraemic men. *Lancet.* **2**: 128.
8. Giordano, C., C. De Pascale, M. E. Phillips, N. G. De Santo, P. Fürst, C. L. Brown, B. J. Houghton, and P. Richards. 1972. Utilisation of ketoacid analogues of valine and phenylalanine in health and uraemia. *Lancet.* **1**: 178.

9. Meister, A. 1965. Biochemistry of the Amino Acids. Volume 1. Academic Press, Inc., New York.
10. Hems, R., B. D. Ross, M. N. Berry, and H. A. Krebs. 1966. Gluconeogenesis in perfused rat liver. *Biochem. J.* **101**: 284.
11. Biebuyck, J. F., P. Lund, and H. A. Krebs. 1972. The effects of halothane (2-bromo-2-chloro-1,1,1,-trufluoroethane) on glycolysis and biosynthetic processes of the isolated perfused rat liver. *Biochem. J.* **128**: 711.
12. Wollenberger, A., O. Ristau, and G. Schoffa. 1960. Eine einfache Technik der extrem schnellen Abkühlung größerer Gewebstücke. *Pfluegers Arch. Eur. J. Physiol.* **270**: 399.
13. Ruderman, N. B., C. R. S. Houghton, and R. Hems. 1971. Evaluation of the isolated perfused rat hindquarter for the study of muscle metabolism. *Biochem. J.* **124**: 639.
14. Weygand, F., W. Steglich, and H. Tanner. 1962. Eine neue Methode zur Umwandlung von α-Amino säuren in α-Ketosäuren. *Justus Liebigs Ann. Chem.* **658**: 128.
15. Meister, A. 1951. Studies on d- and l-α-keto-β-methylvaleric acids. *J. Biol. Chem.* **190**: 269.
16. Hohorst, H. J., F. H. Kreutz, and T. Bücher. 1959. Über Metabolitgehalte und Metabolit-konzentration in der Leber der Ratte. *Biochem. Z.* **332**: 18.
17. Bergmeyer, H. U., and E. Bernt. 1963. α-Oxoglutarate. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press, Inc., New York. 324.
18. Bernt, E., and H. U. Bergmeyer. 1963. L-Glutamate determination with glutamic dehydrogenase. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press, Inc., New York. 384.
19. Lund, P. 1970. Bestimmung mit Glutaminase und Glutamine Dehydrogenase. *In* Methoden der Enzymatischen Analyse. H. U. Bergmeyer, editor. Verlag-Chemie, Weinheim, Germany. 2nd edition. 1670.
20. Kirsten, E., C. Gerez, and R. Kirsten. 1963. Eine enzymatische Mikrobestimmung des Ammoniaks, geeignet für Extrakte tierischer Gewebe und Flüssigkeiten. *Biochem. Z.* **337**: 312.
21. Slein, M. W. 1963. D-Glucose determination with hexokinase and glucose-6-phosphate dehydrogenase. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press, Inc., New York. 117.
22. Yoshida, A., and E. Freese. 1964. Purification and chemical characterization of alanine dehydrogenase of *Bacillus Subtilis*. *Biochim. Biophys. Acta.* **92**: 33.
23. Williamson, D. H., O. Lopes-Vieira, and B. Walker. 1967. Concentration of free glucogenic amino acids in livers of rats subjected to various metabolic stresses. *Biochem. J.* **104**: 497.
24. Williamson, D. H., J. Mellanby, and H. A. Krebs. 1962. Enzymic determination of D (−) β-hydroxybutyric acid and acetoacetic acid in blood. *Biochem. J.* **82**: 90.
25. Cerriotti, G., and L. Spandrio. 1963. A spectrophotometric method for determination of urea. *Clin. Chim. Acta.* **8**: 295.
26. Perkins, J. R. 1971. Effects of light on determination of citrulline. *Clin. Chim. Acta.* **35**: 247.
27. Bloxam, D. L. 1972. Nutritional aspects of amino acid metabolism. Effects of starvation on hepatic portal-venous differences in plasma amino acid concentrations and on liver amino acid concentrations in the rat. *Br. J. Nutr.* **27**: 233.
28. Mallette, L. E., J. H. Exton, and C. R. Park. 1969. Effects of glucagon on amino acid transport and utilization in the perfused rat liver. *J. Biol. Chem.* **244**: 5724.
29. Krebs, H. A., and De Gasquet, P. 1964. Inhibition of gluconeogenesis by α-oxo acids. *Biochem. J.* **90**: 149.
30. Brosnan, J. T., H. A. Krebs, and D. H. Williamson. 1970. Effects of ischaemia on metabolite concentrations in rat liver. *Biochem. J.* **117**: 91.
31. Williamson, D. H., P. Lund, and H. A. Krebs. 1967. The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. *Biochem. J.* **103**: 514.
32. Ruderman, N., and P. Lund. 1972. Amino acid metabolism in skeletal muscle. Regulation of glutamine and alanine release in the perfused rat hindquarter. *Isr. J. Med. Sci.* **8**: 295.
33. Marliss, E. B., T. T. Aoki, T. Pozefsky, and G. F. Cahill, Jr. 1971. Muscle and splanchic glutamine and glutamate metabolism in postabsorptive and starved man. *J. Clin. Invest.* **50**: 814.
34. Felig, P., E. Marliss, T. Pozefsky, and G. F. Cahill. Jr. 1970. Alanine: key role in gluconeogenesis. *Science (Wash. D. C.)*. **167**: 1003.
35. Gordon, R. S. 1964. Metabolism of other *d*- and *l*-hydroxy acids. *Ann N. Y. Acad. Sci.* **119**: 927.
36. Meister, A. 1965. Biochemistry of the Amino Acids. Volume 2. Academic Press, Inc., New York.
37. Briggs, M. 1967. Urea as a Protein Supplement. Pergamon Press. Ltd., Oxford.
38. Patrick, A. D. 1961. Maple syrup urine disease. *Arch. Dis. Child.* **36**: 269.
39. McArthur, C. L., III, and J. A. Bowden. 1972. Metabolic diseases and mental retardation. II. The comparative effects of α-ketoisocaproic acid inhibition of pyruvate decarboxylation in the chick liver and brain. *Int. J. Biochem.* **3**: 193.
40. Bowden, J. A., E. P. Brestel, W. T. Cope, C. L. McArthur, D. N. Westfall, and M. Fried. 1970. α-Ketoisocaproic acid inhibition of pyruvate and α-ketoglutarate oxidative decarboxylation in rat liver slices. *Biochem. Med.* **4**: 69.
41. Miller, L. L. 1961. The role of the liver and non-hepatic tissues in the regulation of free amino acid levels in the blood. *Proc. Symp. Free Amino Acids 1961.* 708.
42. Ruderman, N. B., C. J. Toews, C. Lowry, I. Vreeland, and E. Shafri. 1970. Inhibition of hepatic gluconeogenesis and fatty acid oxidation by pent-4-enoic acid. *Am. J. Physiol.* **219**: 51.
43. Aki, K., K. Ogawa, and A. Ichihara. 1968. Transaminase of branched chain amino acids. IV. Purification and properties of two enzymes from rat liver. *Biochim. Biophys. Acta.* **159**: 276.
44. Rowsell, E. V. 1956. Transaminations to pyruvate and other α-ketoacids. *Biochem. J.* **64**: 246.
45. Drotman, R. B., W. R. Featherston, and R. A. Freeland. 1972. Reductive amination of branched chain keto acids by glutamate dehydrogenase from several animal sources. *Comp. Biochem. Physiol.* **41**: 171.
46. Langer, B. J., Jr. 1965. The biochemical conversion of 2-hydroxy-4-methylthiobutyric acid into methionine by the rat in vitro. *Biochem. J.* **95**: 683.
47. Cooper, A. J. L., and A. Meister. 1972. Isolation and properties of highly purified glutamine transaminase. *Biochemistry.* **11**: 661.
48. Ichihara, A., and E. Koyama. 1966. Transaminase of

branched chain amino acids. I. Branched chain amino acids-α-ketoglutarate transaminase. *J. Biochem.* (*Tokyo*). **59**: 160.
49. Taylor, R. T., and W. T. Jenkins. 1966. Leucine aminotransferase. II. Purification and characterization. *J. Biol. Chem.* **241**: 4396.
50. Efron, M. L., E. S. Kang, J. Visakorpi, and F. X. Fellers. 1969. Effect of elevated plasma phenyalanine levels on other amino acids in phenylketonuric and normal subjects. *J. Pediatr.* **74**: 399.
51. Hill, A., J. Macauley, and W. A. Zaleski. 1972. Laboratory note. Plasma glutamine in phenylketonuria. *Clin. Biochem.* **5**: 194.
52. McKean, C. M., and N. A. Peterson. 1970. Glutamine in the phenylketonuric central nervous system. *N. Engl. J. Med.* **283**: 1364.
53. Wong, P. W. K., J. L. Berman, M. W. Partington, M. E. O'Flynn, and D. Y. Y. Hsia. 1971. Glutamine in PKU. *N. Engl. J. Med.* **285**: 580.
54. Perry, T. L., S. Hansen, B. Tischler, R. Bunting, and S. Diamond. 1970. Glutamine depletion in phenylketonuria. A possible cause of the mental defect. *N. Engl. J. Med.* **282**: 761.
55. Colombo, J. P. 1971. Plasma glutamine in a phenylketonuric family with normal and mentally defective members. *Arch. Dis. Child.* **46**: 720.
56. Boisse, J., J.-M. Saudubray, P.-H Tung, C. Charpentier, M. Castets, A. Lemmonier, H. Jerome, and P. Mozziconacci. 1971. La variante intermittente de la leucinose. *Arch. Fr. Pediatr.* **28**: 161.
57. Gaull, G. E. 1970. Pathogenesis of maple-syrup-urine disease: observations during dietary management and treatment of coma by peritoneal dialysis. *Biochem. Med.* **3**: 130.