Table 2. *Splitting of radioactive adenosine triphosphate with 'heavy' meromyosin to determine the fraction of ^{32}P that is in the γ -phosphate group*

The incubation mixture contained in 2.1 ml.: 1 μ mole of ATP; 5 μ moles of Ca^{2+} ion; 47 μ moles of tris buffer, pH 7.8; 0.05 ml. of a solution of 'heavy' meromyosin ($E_{280\text{m}\mu}^{1\text{cm.}} = 7.6$; see Mueller & Perry, 1962). Incubation was at 25°. After the incubation, 0.5 ml. of ammonium molybdate [2.5% (v/v) in 3 N- H_2SO_4] was added and the mixture was extracted three times with 2.5 ml. portions of isobutanol.

Sample	Fraction of radioactivity left in aqueous phase after extraction with isobutanol (%)
Labelled inorganic phosphate	0.02
ATP incubated for 20 min. with 'heavy' meromyosin	2.68
ATP incubated for 60 min. with 'heavy' meromyosin	1.77
ATP incubated for 25 min. with 'heavy' meromyosin, then 1 μ mole of unlabelled ATP added and the incubation continued for a further 65 min.	1.34

both β - and γ -phosphate groups, merely by the addition of adenylate kinase, and perhaps ADP, to the basic reaction mixture described above. On

treatment with myosin the doubly-labelled ATP should yield ADP of high specific activity labelled in the β -phosphate group.

SUMMARY

1. A method is described for preparing ^{32}P -labelled ATP of high specific activity by an exchange reaction.

2. The method is simple and requires only substrates and enzymes that are available commercially.

3. Hydrolysis of the labelled ATP with 'heavy' meromyosin indicates that 98–99% of the ^{32}P is in the γ -phosphate group.

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Inhibition of Gluconeogenesis by α -Oxo Acids

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During tests for the formation of glucose from DL-methionine in kidney-cortex slices it was noted that the addition of this amino acid to the medium inhibited the formation of glucose from endogenous substrates and from added lactate or fumarate. No inhibition was observed with L-methionine and the inhibitory effect was traced to the α -oxo derivative of methionine formed in the kidney from the D-isomer by D-amino acid oxidase. The further investigation of this effect is reported in the present paper. Several but not all α -oxo acids were found to inhibit gluconeogenesis.

EXPERIMENTAL

The general procedure and the analytical methods were as described by Krebs, Bennett, de Gasquet, Gascoyne & Yoshida (1963). Checks were made to test whether the

added α -oxo acids interfered with the determination of glucose by the glucose-oxidase method. When the samples contained 4 μ moles of α -oxo acid and about 0.1–0.2 μ mole of glucose, inhibitions of colour formation of up to 20% were observed, with the standard extinction curve remaining a straight line. As the quantity of α -oxo acid present was usually much smaller the error arising from this inhibition could be neglected in most experiments. In some cases the standard sample included approximately the same amount of α -oxo acid as the unknown sample. The glucose-oxidase method was checked in several cases by the hexokinase method as described by Slein (1963) but no major difference was found.

Some α -oxo acids were prepared with the help of the D-amino acid oxidase described by Sentheshanmuganathan & Nickerson (1962). The organism was grown according to the instructions of these authors in 500 ml. of medium in 2 l. flasks. These were inoculated with two 5 ml.-test-tube cultures and incubated on a rotary shaker for 3 days at 30°. The centrifuged and washed cells (yield about 15 g.

wet wt. from 1500 ml. of medium) were suspended in 0.1 M-potassium phosphate buffer, pH 8.0, and exposed to ultrasonic vibrations in a M.S.E. 50w disintegrator (15 min.; 19 kcyc./sec.). The material was centrifuged and the clear supernatant containing the enzyme was dialysed for 18 hr. against 0.01 M-KCl. This preparation was stored in the deep-freeze. For the preparation of solutions of the α -oxo acids, 0.8 m-mole of DL-amino acids was mixed with 4 ml. of 0.05 M-phosphate buffer, pH 8.0, in the main compartment of a conical Warburg flask. The side arm contained 1 ml. of the cell-free extract, the centre well 0.2 ml. of 2 N-NaOH and the gas space O_2 . The manometers were shaken at 30° until the rates of O_2 consumption fell to very low values (3–5 hr.). The initial rates of O_2 consumption (μ moles/5 min.) were, for example: with DL-methionine, 25; with DL-phenylalanine, 24; with DL- α -aminobutyrate, 20. At the end of the incubation the contents of the flask were transferred to a centrifuge tube with 2 ml. of 25% metaphosphoric acid. After centrifuging the supernatant was poured into a Kutscher-Steucler extractor and extracted at a rapid rate with ether for 2–3 hr., with 'silicone MS antifoam A' (Hopkin and Williams Ltd.) as antifoam agent. With most α -oxo acids this proved sufficient for complete extraction. Water (2 ml.) was added to the ethereal extract and the ether was evaporated. The aqueous phase was transferred to a measuring cylinder with small quantities of water. The concentration of α -oxo acids was determined with the manometric ceric sulphate method of Krebs & Johnson (1937). The yield was 60–75%. For use the solutions were neutralized with M-Na₂CO₃. This method was used for preparing the α -oxo acids corresponding to aminobutyric acid, valine, norvaline, leucine, isoleucine, norleucine, methionine, phenylalanine and tyrosine. A sample of phenylpyruvic acid prepared according to the method of Herbst & Shemin (1943) was made available by Dr D. J. Morton.

In experiments where the O_2 uptake was to be measured a saline medium low in bicarbonate and buffered by phosphate was used. The phosphate concentration was kept low to prevent precipitation of Ca²⁺ ions, the presence of which in previous work had proved to be essential for glucose synthesis. The phosphate-saline medium consisted of: 116 ml. of 0.154 M-NaCl, 4 ml. of 0.154 M-KCl, 3 ml. of 0.11 M-CaCl₂, 1 ml. of 0.1 M-MgSO₄, 1 ml. of 0.154 M-KH₂PO₄ and 6.5 ml. of 0.1 M-sodium phosphate buffer, pH 7.4.

The experimental animals were Wistar rats fed either on a standard rat-cube diet, or for short periods (5–7 days) on a low-carbohydrate diet consisting of 3 parts of casein, 1 part of margarine, and vitamin-mineral supplements.

RESULTS

Effect of DL-methionine on glucose formation. The observation that formed the starting point of the present work is shown in Table 1. DL-Methionine

inhibited endogenous glucose formation as well as glucose formation in the presence of lactate and fumarate. L-Methionine (not recorded in Table 1) had no effect. Since the D-amino acid oxidase of rat kidney oxidizes D-methionine very readily to the α -oxo acid—more readily than any other D-amino acid (Krebs, 1935)—the findings suggested that the difference in the behaviour of DL- and L-methionine were connected with the formation of the α -oxo acid from methionine. This was borne out by the following experiments.

Inhibition of glucose formation from lactate by α -oxo acids. Marked inhibitions of glucose formation from lactate are found at low concentrations of the α -oxo acids corresponding to methionine, phenylalanine and tyrosine (Table 2). The inhibition by 0.4 mM-phenylpyruvate was 72% and by 0.2 mM-*p*-hydroxyphenylpyruvate 62%. A slight inhibition occurred with β -methyl- α -oxopentanoate and γ -methyl- α -oxopentanoate, and no inhibition occurred in the presence of 2 mM- α -oxobutyrate, β -methyl- α -oxobutyrate, α -oxopentanoate and α -oxohexanoate.

Effect of α -oxo acids on glucose formation and oxygen uptake in the presence of various glucose precursors. According to Table 3, inhibition of glucose formation occurred with all substrates tested on the addition of phenylpyruvate, but the extent of the inhibition was considerably greater (85–95%) with lactate and pyruvate as substrates than with α -oxoglutarate, glutamate or fumarate (41–57%). The effect on respiration was nil or slight, except with pyruvate where the inhibition was 32%. Similar results on the specific inhibition of gluconeogenesis were obtained with γ -methyl- α -oxobutyrate (Table 4). There was a slight inhibition of the oxygen uptake (8%) by 2.5 mM-inhibitor whereas gluconeogenesis was inhibited 87%.

Effect of α -oxo acids on lactate removal. The removal of lactate was inhibited by phenylpyruvate and *p*-hydroxyphenylpyruvate to the same extent as the glucose formation from lactate. Hence the amounts of lactate removed that were not accounted for by the formation of glucose were about the same with and without the α -oxo acids (Table 5). They were equivalent to about one-third of the amounts of oxygen consumed, values that suggest that lactate was the main substrate of respiration under the test conditions.

Effects of other phenolic compounds on gluco-

Table 1. *Effect of DL-methionine on gluconeogenesis in rat-kidney-cortex slices*

A well-fed rat on the standard diet was used. The incubation time was 60 min.

Concn. of DL-methionine (mM)	...	...	0	20	0	10	20	0	10	20
Concn. of L-lactate (mM)	...	...	0	0	10	10	10	0	0	0
Concn. of fumarate (mM)	...	...	0	0	0	0	0	10	10	10
Glucose formed (μ moles/g. dry wt./hr.)	...	19.3	0.9	91	61	43	138	104	66	

neogenesis. Several compounds related to phenylpyruvate, namely DL-phenyl-lactate, phenylacetate, cinnamate, benzoate and salicylate, also inhibited gluconeogenesis from lactate at 1 mM concentration without affecting the oxygen uptake. Phenylalanine, on the other hand, had no effect at 10 mM (Table 6).

α -Oxo acids as precursors of glucose. As α -oxo acids are intermediates in the conversion of amino acids into carbohydrate, some α -oxo acids, e.g. those corresponding to glutamate, aspartate, alanine, valine, isoleucine, threonine and norleucine, are expected to be gluconeogenic. That this is the case is shown in Table 7 (and in Table 3 of Krebs

Table 2. *Inhibition of gluconeogenesis from lactate by α -oxo acids in rat-kidney-cortex slices*

Rats fed on the low-carbohydrate diet were used. Incubation was under standard conditions in bicarbonate-saline for 60 min. in the presence of 10 mM-L-lactate.

Parent amino acid	α -Oxo acid added	Concn. of α -oxo acid (mM)	Glucose formed (μ moles/g. dry wt./hr.)		Inhibition (%)
			Without α -oxo acid	With α -oxo acid	
Methionine	γ -Methylthio- α -oxobutyrate	2	217	74	66
		4	217	55	75
Alloisoleucine	β -Methyl- α -oxopentanoate	3.5	226	168	25
Leucine	γ -Methyl- α -oxopentanoate	2	215	190	12
Phenylalanine	Phenylpyruvate	0.4	230	64	72
		0.4	276	74	73
		0.4	198	58	71
		2.0	174	33	81
Tyrosine	<i>p</i> -Hydroxyphenylpyruvate	0.1	198	151	24
		0.2	208	79	62

Table 3. *Effect of phenylpyruvate in glucose formation and oxygen uptake in the presence of various substrates*

Rats fed on the low-carbohydrate diet were used. Washed rat-kidney-cortex slices were suspended in phosphate-saline. The substrate concentrations were 10 mM.

Concn. of phenylpyruvate (mM)	...	Glucose formed (μ moles/g. dry wt./hr.)		Inhibition by phenylpyruvate (%)	O ₂ used (μ moles/g. dry wt./hr.)		Inhibition by phenylpyruvate (%)
		0	1		0	1	
Substrate added							
None		10.4	5.4	48	950	900	0
L-Lactate		167	23	86	1650	1600	0
		220	35	84	1980	1790	10
L-Glutamate		76	38	50	2440	2040	16
		126	55	57	2360	2000	15
α -Glycerophosphate		45	21	53	1005	1080	0
Pyruvate		302	16	95	1790	1200	32
α -Oxoglutarate		417	244	41	2060	1740	16
Fumarate		445	229	48	2240	1750	22

Table 4. *Effect of γ -methylthio- α -oxobutyrate on glucose formation and oxygen uptake in rat-kidney-cortex slices*

A rat fed on the low-carbohydrate diet was used. Slices were suspended in phosphate-saline. Incubation was for 60 min. in the presence of 5 mM-L-lactate.

Concn. of γ -methylthio- α -oxobutyrate (mM)	...	Glucose formed (μ moles/g. dry wt./hr.)			O ₂ used (μ moles/g. dry wt./hr.)		
		0	2.5	5.0	0	2.5	5.0
Substrate added							
None		20	15	3	950	880	890
L-Lactate		146	19	9	1660	1530	1295

et al. 1963). Where tested (see Table 7) some α -oxo acids at 2.5 mM gave higher rates of glucose formation than at 5 and 10 mM, a result indicating that α -oxo acids act in two ways: as precursors supplying the carbon skeleton of carbohydrates and as inhibitors interfering with the process of gluconeogenesis.

The α -oxo acids corresponding to phenylalanine, tyrosine and methionine did not increase glucose formation. There was the small effect with the α -oxo acid corresponding to leucine (Table 6). The

nature of this effect is probably the same as that of acetoacetate (Weil-Malherbe, 1938).

Gluconeogenesis from L- and D-amino acids. As may be expected from the preceding paragraph and the well-established ready oxidation of D-valine, D-isoleucine and D-alanine, these three amino acids are gluconeogenic in kidney-cortex slices (Table 8). The L-forms, on the other hand, failed to give more glucose than the control. Thus the rate at which the L-forms were converted into the α -oxo acids must have been relatively slow.

Table 5. *Effect of α -oxo acids on lactate utilization in rat-kidney-cortex slices*

A rat fed on the low-carbohydrate diet was used. Incubation was in bicarbonate-saline for 60 min. in the presence of 10 mM-L-lactate.

α -Oxo acid	Concn. of α -oxo acid (mM)	Glucose formed (μ moles/g. dry wt./hr.)		Lactate removed (μ moles/g. dry wt./hr.)		Lactate removed not accounted for by glucose formation (μ moles/g. dry wt./hr.)	
		With		With		With	
		Control	α -oxo acid	Control	α -oxo acid	Control	α -oxo acid
Phenylpyruvate	0.4	230	64	1430	938	970	810
	0.4	198	58	1070	718	674	602
<i>p</i> -Hydroxyphenylpyruvate	0.1	198	151	1070	1006	674	704
	0.2	208	79	915	786	499	628

Table 6. *Effect of compounds related to phenylpyruvate on gluconeogenesis and respiration of rat-kidney-cortex slices in the presence of L-lactate*

The conditions of incubation were as given in Table 3. The L-lactate concentration was 10 mM. The concentration of other substances added was 1 mM except that of L-phenylalanine which was 10 mM.

Added substances (other than lactate)	Glucose formed (μ moles/g. dry wt./hr.)	Inhibition (%)	O ₂ used (μ moles/g. dry wt./hr.)	Inhibition (%)
None	220	—	1980	—
DL-Phenyl-lactate	143	47	1740	12
Phenylacetate	127	58	2045	0
Cinnamate	116	35	2005	0
Benzoate	104	53	1710	14
Salicylate	78	65	1970	0
L-Phenylalanine	212	0	2040	0

Table 7. *Glucose formation from α -oxo acids in rat-kidney-cortex slices*

Rats fed on the low-carbohydrate diet were used. Incubation was in bicarbonate-saline for 60 min. The results are given as means $\pm$ S.E.M. with numbers of observations in parentheses.

Parent amino acid	α -Oxo acid added	Concn. of α -oxo acid (mM)	Glucose found (μ moles/g. dry wt./hr.)	
			Control	With α -oxo acid
Threonine	α -Oxobutyrate	5	20	26
		2.5		49
Valine	β -Methyl- α -oxobutyrate	2.5	17.6 $\pm$ 3.3 (2)	60.5 $\pm$ 3.6 (2)
		5		42.8 $\pm$ 7.4 (4)
Norleucine	α -Oxohexanoate	10	16.6 $\pm$ 1.5 (2)	101.9 $\pm$ 8.2 (2)
Isoleucine	β -Methyl- α -oxopentanoate	2.5	20.3 $\pm$ 3.3 (3)	43.8 $\pm$ 3.0 (3)
		5		38.8 $\pm$ 3.5 (3)
		10		31.6 $\pm$ 2.8 (4)
L-Leucine	γ -Methyl- α -oxopentanoate	5	19	28

Table 8. *Gluconeogenesis from L- and D-amino acids in rat-kidney-cortex slices*

Rats fed on the low-carbohydrate diet were used. Incubation was in bicarbonate-saline for 60 min.; the substrate concentration was 10 mM. The results for valine and isoleucine are given as means $\pm$ S.E.M. with the numbers of observations in parentheses.

Isomer used	Glucose formed (μ moles/g. dry wt./hr.)		
	From valine	From isoleucine	From alanine
None	22.7 $\pm$ 1.8 (3)	21.4 $\pm$ 2.2 (3)	14
L	23.1 $\pm$ 2.0 (3)	20.2 $\pm$ 1.8 (2)	11
D	51.5	42.6 $\pm$ 3.2 (2)	133

DISCUSSION

Mode of action of α -oxo acids. The inhibition of gluconeogenesis by α -oxo acids at low concentrations is a relatively specific effect in as much as respiration, anaerobic glycolysis, the synthesis of urea from ammonium salts and the synthesis of glutamine from ammonium glutamate in animal tissues is not affected by concentrations of α -oxo acids, e.g. phenylpyruvate, which strongly inhibit gluconeogenesis (unpublished work). The cause of the inhibition is unknown. Since gluconeogenesis from various types of precursors, e.g. C_3 acids, C_4 dicarboxylic acids and α -glycerophosphate, is inhibited, though to various degrees, the site of action must be a reaction common to the gluconeogenesis from the various precursors. Shared steps are the aldolase reaction and the reactions leading from fructose 1,6-diphosphate and glucose, but tests indicate that these reactions are not inhibited by α -oxo acids.

Physiological effects of the inhibition of gluconeogenesis by α -oxo acids. Toxic effects of DL-methionine and of other D- and L-amino acids in the intact animal have been described by Gullino *et al.* (1956), Winitz, Gullino, Greenstein & Birnbaum (1956), Kinsell *et al.* (1949), Singh, Barclay & Cooke (1954), Greenstein, Winitz, Gullino, Birnbaum & Otey (1956) and Phear, Ruebner, Sherlock & Summerskill (1956). It is not likely that the toxic effects observed by Gullino *et al.* (1956) and by Greenstein *et al.* (1956) were connected with the inhibition of gluconeogenesis by α -oxo acids because in general the toxicity of the L- and D-isomers were of the same order, although the L-isomers do not form α -oxo acids as readily as the D-isomers. Moreover, the toxicity was relieved by arginine which accelerates the removal of ammonia and suggests that the latter was the toxic agent. Toxic effects of DL-methionine in oral doses of 8–10 g./day given to human subjects were prevented or decreased by the antibiotic chlortetra-

cycline (Phear *et al.* 1956), which has been taken to indicate that the toxic principle was formed by intestinal bacteria.

It is unlikely that the brain damage in phenylketonuria is connected with the inhibition of gluconeogenesis by phenylpyruvate. According to Jervis (1952) the concentration of phenylpyruvate in the blood of phenylketonurics is between 0.02 and 0.11 mM, which is hardly sufficient to bring about a major inhibition of gluconeogenesis. As gluconeogenesis does not occur in brain the effect of any inhibition of gluconeogenesis would be expected to show itself in a lowered blood sugar concentration, and there are no reports of hypoglycaemia in phenylketonurics.

SUMMARY

1. DL-Methionine inhibited the synthesis of glucose from lactate and fumarate in rat-kidney-cortex slices. The inhibitory agent proved to be the α -oxo acid corresponding to methionine (γ -methylthio- α -oxobutyrate) formed by D-amino acid oxidase.

2. Marked inhibitions were found with the α -oxo acids corresponding to phenylalanine and tyrosine. No inhibition occurred in the presence of α -oxobutyrate, β -methyl- α -oxobutyrate, α -oxopentanoate and α -oxohexanoate.

3. Concentrations of α -oxo acids which strongly inhibited gluconeogenesis (0.2–4 mM) had no major effect on the rate of the oxygen uptake.

4. Phenyl-lactate, phenylacetate, cinnamate, benzoate and salicylate (all at 1 mM) also inhibited gluconeogenesis from lactate without affecting respiration. L-Phenylalanine had no effect at 10 mM.

5. The α -oxo acids corresponding to glutamate, aspartate, alanine, valine, isoleucine, threonine and norleucine formed glucose in rat-kidney-cortex slices, as expected from the known glucogenic action of the parent amino acids.

6. D-Valine, D-isoleucine and D-alanine formed glucose in kidney-cortex slices whereas the L-forms did not. These differences are presumed to be due to differences in the rate of the conversion of the D- and L-amino acids into the α -oxo acids.

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Lipids of Sheep Lymph

TRANSPORT FROM THE INTESTINE

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The depot fats of ruminants are characterized by their relatively high content of saturated fatty acids (in particular of stearic acid) compared with the amounts present in the depots of other herbivorous animals such as the horse and the rabbit (for detailed information see Hilditch, 1956; Garton, 1960*a*). To account for the presence of these 'stearic acid-rich' triglycerides Hilditch & Sleight-holme (1931) and Banks & Hilditch (1932) suggested that preformed oleo-glycerides underwent hydrogenation in the tissues of the ruminant animal. Though this possible mechanism cannot be excluded it is rather to events in the rumen that attention has been directed. Studies such as those of Reiser (1951), Shorland, Weenink, Johns & McDonald (1957) and Garton, Lough & Vioque (1961) have shown that unsaturated fatty acids present in the lipids of the feed can be hydrogenated under the influence of the micro-organisms (mainly bacteria) that inhabit the rumen. Stearic acid is the final product of hydrogenation of the C₁₈ unsaturated fatty acids (oleic acid, linoleic acid and linolenic acid) of which the lipids of pasture grasses and other herbage are rich sources (Weenink, 1959, 1961; Garton, 1960*b*). It has also been demonstrated that the feeding to oxen and goats of oils having a high content of C₁₈ unsaturated fatty

acids (linseed oil, cottonseed oil) leads to the deposition of enhanced amounts of stearic acid in the depot fats (Willey, Riggs, Colby, Butler & Reiser, 1952; Reiser & Reddy, 1956). Further, when linseed oil was given to a sheep via a duodenal cannula, thereby by-passing the rumen, Ogilvie, McClymont & Shorland (1961) found that the perinephric fat contained much more linoleic acid and linolenic acid than are normally found in sheep depot fat, thus supporting the indirect evidence that the high stearic acid content of ruminant tissue lipids results from the intestinal absorption and subsequent assimilation of stearic acid produced in the rumen by microbial hydrogenation of unsaturated C₁₈ fatty acids.

To obtain further information on this topic it was decided to examine the lipids present in the feed, the alimentary tract and, in particular, the lymph draining the sheep's intestine, with special reference to stearic acid. An account of these experiments was communicated to the International Conference on Biochemistry of Lipids, Birmingham, August 1962 (see Lough, Feliński & Garton, 1963).

EXPERIMENTAL

Animals and diet

The sheep used were four young adult animals, weighing 70–100 lb.; two (sheep 1 and 2) were castrated Scottish Blackface males, sheep 3 was a castrated Shetland male and sheep 4 was a Shetland ewe. Sheep 1 and 2 had previously been fitted with rumen fistulae and for several

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