magnesium protoporphyrin monomethyl ester by intact wild-type cells of *R. spheroides* indicates that the divergence takes place at a stage between this compound and magnesium vinyl phaeoporphyrin a_5. The detection by Tait & Gibson (1961) of an enzyme system in *R. spheroides* that methylates magnesium protoporphyrin supports this view, although these authors do not report the specificity of this enzyme with regard to side chains on the porphyrin nucleus.

In low-iron media the synthesis of bacteriochlorophyll was decreased, but there was increased production of magnesium protoporphyrin monoester (Fig. 2). Although compounds of the magnesium vinyl phaeoporphyrin a_5 and phaeophytin *a* type would be extracted into ether under the conditions used (O. T. G. Jones, unpublished work), none was detected and bacteriophaeophytin was absent from the iron-deficient media. This suggests that iron may be required for the enzymic transformations of the side chains of magnesium protoporphyrin monoester in addition to its activity during the early stages of porphyrin biosynthesis, where it has been shown that δ-aminolaevulate synthase is inhibited by Fe^{2+} ions (Brown, 1958; Burnham, 1962).

SUMMARY

1. An ether-soluble pigment secreted into the medium by intact cells of *Rhodopseudomonas spheroides* has been purified by chromatography on a powdered sucrose column.

2. The spectroscopic and chromatographic properties of this purified material and its derivatives produced by acid treatment were studied and the material was identified as magnesium protoporphyrin monomethyl ester.

3. The ester was not detected in whole cells or in acetone–methanol extracts of whole cells.

4. In low-iron media there was increased secretion of this ester into the medium and bacteriochlorophyll production decreased.

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Substrate Competition in the Respiration of Animal Tissues

THE METABOLIC INTERACTIONS OF PYRUVATE AND α-OXOGLUTARATE IN RAT-LIVER HOMOGENATES

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A readily oxidizable substrate—an intermediate or a starting material—often inhibits the oxidation of other substrates when added to respiring material (Krebs, 1935; Edson, 1936). In terms of enzyme chemistry this means that oxidizable substrates and intermediates derived from them compete with each other for the joint pathway of electron transport to molecular oxygen or for a shared co-factor. The present investigation is concerned with the detailed study of the competitive and other interactions of pyruvate, α-oxoglutarate and endogenous substrates in respiring rat-liver homogenates.

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EXPERIMENTAL

Chemicals. Sodium pyruvate was prepared from technical pyruvic acid as described by Bartley & Davies (1954). The crystalline product was 100% pure by enzymic assay and showed no trace of parapyruvate on chromatography of the 2,4-dinitrophenylhydrazone derivative.

All ^{14}C-labelled substrates were obtained from The Radiochemical Centre, Amersham, Bucks. On arrival sodium [1-^{14}C]- and sodium [2-^{14}C]-pyruvate were dissolved in sufficient 0·5M-pyruvic acid, prepared from pure sodium pyruvate by ion exchange with Amberlite IR-120 (H^+ form), to give final specific activities of 50 μc/m-mole. Stored at −18° in this form the labelled pyruvates were stable for several months and no parapyruvate was formed. Immediately before use samples were neutralized and if necessary diluted with further unlabelled sodium pyruvate to give an appropriate specific activity.

Pure monosodium α-oxoglutarate was prepared as described by Krebs, Eggleston & D'Alessandro (1961). Neutral solutions of the disodium salt were prepared from this when required. On arrival α-oxo[5-^{14}C]glutarate was dissolved in sufficient unlabelled 0·2M-disodium α-oxoglutarate to give a final specific activity of 50 μc/m-mole and this solution was stored at −18°.

α-Oxo[1-^{14}C]glutarate was prepared from DL-[1-^{14}C]-glutamate and unlabelled α-oxoglutarate by an isotope-exchange reaction catalysed by glutamate–aspartate transaminase (Nisonoff, Barnes, Enns & Schuching, 1954; Jenkins & Sizer, 1959). A mixture of 10 μmoles of DL-[1-^{14}C]glutamate (30 μc) and 50 μmoles of α-oxoglutarate, dissolved in 4·0 ml. of water, plus 0·6 ml. of 0·1M-tris buffer, pH 8·5, was incubated for 2–3 hr. at room temperature with 0·1 ml. of glutamate–aspartate transaminase (2 mg./ml.; C. F. Boehringer und Soehne, Mannheim, Germany). The reaction mixture was then added directly to Whatman no. 4 papers (0·5 μmole of α-oxoglutarate/cm.) for chromatographic separation of the α-oxo[1-^{14}C]glutarate formed from the D-[1-^{14}C]glutamate and L-glutamate. Before use the papers were washed with water to remove an unidentified inhibitor of tissue respiration. The pentan-1-ol–aq. 5N-formic acid solvent system of Buch, Montgomery & Porter (1952) was used. When the papers were developed in a descending direction a complete separation was achieved in 6 hr. (R_F of glutamic acid, 0·05, and R_F of α-oxoglutaric acid, 0·48; Buch *et al.* 1952). The α-oxo-[1-^{14}C]glutaric acid was located by radioautography, eluted with water, freeze-dried to remove traces of pentanol and formic acid, and dissolved in 0·2M-disodium α-oxoglutarate to give a final specific activity of 12·5 μc/m-mole. This solution was stored at −18° and samples were neutralized immediately before use. The radioactivity of the α-oxoglutarate on the chromatograms was only slightly less than that of the glutamate (mostly residual D-glutamate), indicating that isotopic equilibrium between the L-glutamate and α-oxoglutarate had been reached during the incubation period.

Preparation of homogenates. Male Wistar albino rats weighing 250–350 g. and fed *ad libitum* on an Oxoid diet [Herbert C. Staples (Bewdley) Ltd.] based on 'diet 41' of Bruce & Parkes (1949) were used in all experiments. The animals were killed by stunning followed by decapitation. Immediately after removal from the animals, the livers were immersed for 2 min. in ice-cooled 0·154M-KCl and then minced in a chilled Fischer mincer (Jouan, Paris, France). The weighed minced tissue was homogenized in 2–3 vol. of cold (0°) 0·154M-KCl in a stainless-steel Potter–Elvehjem homogenizer. For each gram of tissue taken were added: 1·25 ml. of 0·1M-sodium phosphate buffer, pH 7·4, 0·5 ml. of 0·02M-$MgCl_2$ and sufficient 0·154M-KCl to bring the volume to 7·5 ml. In some experiments 0·154M-$KHCO_3$ (0·5 ml./g. of tissue) was included in each 7·5 ml. of homogenate. All solutions used were at 0°.

Incubations. The homogenates were incubated aerobically at 30° in conical Warburg flasks. Substrates were usually added to the vessels as 0·2M-solutions of the sodium salts. In all incubation vessels 3 vol. of homogenate was added to 1 vol. of ice-cooled solution containing substrates, any other additions and 0·154M-KCl to volume, so giving a final tissue concentration of 10% (w/v). Warburg flasks with approximate volumes of 20 ml. or 50 ml. were used containing respectively 4 ml. or 8 ml. of 10% homogenate in the main compartments. The vessel contents were kept at 0° until the zero time of the incubation. The gas space of the Warburg flasks contained O_2. The incubations were terminated by tipping 2N-HCl from the side arms after 10–60 min. according to the nature of the experiment. The centre wells of the Warburg flasks contained CO_2-free 2N-NaOH and, when it was desired to collect $^{14}CO_2$ for assay, shaking was continued after the addition of acid from the side arms until no further change in gas pressure occurred (approx. 1 hr.). The acidified tissue suspensions were stored at −18° until analysed.

Chemical analyses. Pyruvate and α-oxoglutarate were determined as described by Holzer & Holldorf (1957). It was not necessary to precipitate the protein with perchloric acid. A portion (0·5 ml.) of acidified tissue suspension was neutralized with N-KOH and mixed immediately with 0·1M-tris buffer, pH 7·4, to give a final volume of 3·0 ml. This suspension was centrifuged in a M.S.E. bench centrifuge until clear (approx. 5 min. at 2400*g*) and the supernatant was used for the analysis of pyruvate and of α-oxoglutarate. This technique gave results identical with those obtained by the use of perchloric acid. $NADH_2$, lactate dehydrogenase and glutamate dehydrogenase were obtained from C. F. Boehringer und Soehne, Mannheim, Germany.

Lactate was determined enzymically by the method of Lehmann (1938) as modified by Wieland (1957) and Williamson & Krebs (1961). Glutamate plus glutamine and aspartate were usually determined manometrically (Krebs, 1948; Krebs & Bellamy, 1960). Alternatively, glutamate, aspartate and alanine were determined by the ninhydrin reaction (Meyer, 1957) after chromatographic separation by the two-dimensional system described below. Acetate was determined by the microdiffusion technique of Serlin & Cotzias (1955) followed by automatic titration of the diffused acetic acid (with a Radiometer TTT 1b titrator). Acetoacetate was determined either manometrically as described by Edson (1935) or colorimetrically as described by Walker (1954), β-hydroxybutyrate by the method of Greenberg & Lester (1944) as described by Kulka, Krebs & Eggleston (1961), acetone as described by Thin & Robertson (1952), citrate as described by Taylor (1953), succinate as described by Rodgers (1961) and the sum of fumarate and malate as described by Nossal (1952). *cis*-Aconitate and isocitrate were not determined but were assumed to amount together to 10% of the citrate present (Krebs, 1953*a*).

Chromatographic analysis. The main non-volatile products of the metabolism of labelled pyruvate (and α-oxoglutarate) were identified by two-dimensional chromatography and radioautography, followed by co-chromatography of the isolated radioactive materials with pure known compounds. The chromatograms were developed in a descending direction successively with phenol–formic acid–water (500:13:167, w/v/v) as described by Kornberg (1958) and with butan-1-ol–propionic acid–water (47:22:31, by vol.; Benson *et al.* 1950). The details of the chromatography, radioautography and sprays for carboxylic acids and amino acids were as described by Large, Peel & Quayle (1961).

General radioactive-tracer techniques. The $^{14}CO_2$ trapped in 2N-NaOH in the centre wells of Warburg flasks was converted into $Ba^{14}CO_3$ and plated on filter-paper disks as described by Sakami (1955). The planchets were assayed for radioactivity with a Panax counter and a mica end-window Geiger–Müller tube enclosed in a lead castle. Counting was continued until at least 10 000 counts were recorded. The count was corrected for background, variations in the count of a standard source, dead time, and for self-absorption. The specific activities of labelled substrates were estimated by their conversion into $^{14}CO_2$, which was trapped in 2N-NaOH, plated as $Ba^{14}CO_3$ and counted as above. This was achieved by combustion by the Van Slyke–Folch method as described by Sakami (1955) and by manometric decarboxylation for [1-^{14}C]pyruvate and for α-oxo[1-^{14}C]glutarate. [1-^{14}C]Pyruvate was decarboxylated with yeast carboxylase and α-oxo[1-^{14}C]glutarate with ceric sulphate (Krebs & Johnson, 1937).

The radioactivity on chromatograms was also assayed with a mica end-window Geiger–Müller tube. At least 2000 counts were recorded and the count was corrected for background and variation in the count of a standard source. The specific activities (counts/min./μmole) of the radioactive substrates on Whatman no. 4 paper were approximately determined from the counts of known amounts of the substrates or their metabolic products.

Determination of specific activities of residual α-oxo acids. Both the dilution of labelled α-oxo acids by unlabelled material and the incorporation of ^{14}C into unlabelled α-oxo acids were determined by the isolation, counting and chemical determination of the residual α-oxo acids as the 2,4-dinitrophenylhydrazones. Acidified tissue suspension (0·5 ml.) was mixed with 0·5 ml. of 0·66N-H_2SO_4 and 0·5 ml. of 10% (w/v) sodium tungstate. After standing for 10 min. the suspension was centrifuged for 5 min. at 2400*g* in a M.S.E. bench centrifuge. The clear supernatant was incubated for 30 min. at 30° with an excess (1–2 ml.) of 0·2% (w/v) 2,4-dinitrophenylhydrazine in 2N-HCl. The mixture was then extracted with 5 ml. of ethyl acetate, which in turn was extracted with 5 ml. of M-Na_2CO_3 to give a solution of the acid 2,4-dinitrophenylhydrazones. No attempt was made to make the extraction procedures quantitative at any stage. The M-Na_2CO_3 was neutralized with cold conc. HCl and the hydrazones were extracted into ethyl acetate, which was concentrated to 0·1–0·2 ml. by evaporation at 35° in a stream of air. In this form the α-oxo acid 2,4-dinitrophenylhydrazones were applied to the origins of paper chromatograms (Whatman no. 4 paper) and developed in either an ascending direction with butan-1-ol–ethanol–aq. 0·5N-NH_3 (7:1:2, by vol.; El Hawary & Thompson, 1953) or in a descending direction with toluene–acetic acid–water (4:3:1, by vol.; Bush & Hockaday, 1960). The second solvent system was used in studying the incorporation of labelled pyruvate into α-oxoglutarate as it clearly separated α-oxoglutaric acid 2,4-dinitrophenylhydrazone (R_F 0·51) from that of parapyruvic acid (R_F 0·20), which is frequently a trace contaminant of labelled pyruvates. In the butan-1-ol–ethanol–aq. 0·5N-NH_3 solvent system the 2,4-dinitrophenylhydrazones of both α-oxoglutaric acid and parapyruvic acid have R_F values of 0·18–0·27. Each α-oxo acid 2,4-dinitrophenylhydrazone spot was eluted with water and extracted into ethyl acetate, which was concentrated to about 0·1 ml. and added drop by drop to a ground-glass disk 1·7 cm. in diameter. As the ethyl acetate evaporated, the hydrazone was spread as evenly as possible. Since there was never more than 0·2–0·3 mg. on a glass plate, no correction was necessary for self-absorption and the recorded count, assayed as above, should have been directly comparable with that of $Ba^{14}CO_3$ plates corrected to infinite thinness, assuming that no important change in plate geometry or back-scattering was involved. This assumption was verified by the finding that the specific activities of the original labelled substrates, as measured by plating of the 2,4-dinitrophenylhydrazones, were almost identical with those obtained by methods involving the preparation of $Ba^{14}CO_3$ planchets. The 2,4-dinitrophenylhydrazones were assayed spectrophotometrically by dissolving the material on the glass plates in a mixture of 2 parts of M-Na_2CO_3 to 1 part of 2N-NaOH, measuring the extinction at 500 mμ, and comparing the readings with standard curves based on the pure derivatives of pyruvic acid and of α-oxoglutaric acid. The specific activities of the α-oxo acids were derived from the radioactivity and chemical assays of the isolated 2,4-dinitrophenylhydrazone samples. The total count incorporated into pyruvate or α-oxoglutarate, or lost from them during the incubation, was calculated from the specific activities of the residual α-oxo acids and their total amounts measured enzymically.

The specific activity of residual [1-^{14}C]pyruvate was also determined by decarboxylation with yeast carboxylase. The $^{14}CO_2$ was collected in 2N-NaOH in the centre well of a Warburg flask and determined as described above.

RESULTS

General considerations

When either pyruvate or α-oxoglutarate was added alone to rat-liver homogenates the oxygen consumption increased by up to 50% (Table 1). The stimulation of oxygen uptake caused by both substrates added together was similar to that caused by either alone, indicating that competition had occurred for a rate-limiting step involved in the oxidation of both substrates. At initial substrate concentrations of 10 mM the addition of pyruvate decreased the removal of α-oxoglutarate by 12–35%, and α-oxoglutarate diminished the removal of pyruvate by 50–65%. These mutual inhibitions suggest that the competition was between the initial oxidative decarboxylations of the two α-oxo acids. However, determination of

Table 1. *Intermediates formed from pyruvate, α-oxoglutarate and pyruvate plus α-oxoglutarate in rat-liver homogenates*

The results refer to 8 ml. of homogenate, containing added bicarbonate (7·75 mM), incubated for 60 min. in large (50 ml.) Warburg vessels. Each vessel contained 210 mg. dry wt. of tissue. The initial concentration of each added substrate was 10 mM.

	Metabolic changes (μmoles)			
Vessel no.	1	2	3	4
Substrates added	None	Pyruvate	α-Oxoglutarate	Pyruvate + α-oxoglutarate
Expt. 1				
O_2	−43·9	−64·8	−59·1	−60·4
Pyruvate	+ 0·3	−66·6	+ 1·2	−26·9
α-Oxoglutarate	0·0	+ 7·7	−36·0	−26·3
Lactate	+ 3·8	+ 7·6	+ 2·4	+ 3·5
Glutamate + glutamine	− 0·4	− 4·4	+ 6·8	− 1·2
Aspartate	+ 0·5	+ 0·5	+ 0·5	+ 0·5
Acetoacetate	+ 1·3	+ 3·1	+ 0·1	+ 2·2
Citrate + *cis*-aconitate + isocitrate	− 0·4	+10·5	+ 1·5	+ 6·1
Succinate	− 0·1	+ 0·1	+ 1·5	+11·0
Fumarate + malate	− 1·8	+ 1·1	+18·4	+ 9·5
Sum of tricarboxylic acid-cycle intermediates	− 2·3	+19·4	−14·6	+ 0·3
Expt. 2				
O_2	−53·5	−67·4	−64·6	−68·2
Pyruvate	0·0	−69·7	+ 2·3	−33·0
α-Oxoglutarate	+ 0·7	+ 6·7	−39·2	−33·2
Lactate	− 1·3	− 2·1	− 4·3	− 2·1
Acetoacetate	+ 0·2	+ 2·9	+ 1·2	+ 4·3
Citrate + *cis*-aconitate + isocitrate	0·0	+10·8	+ 3·7	+11·4
Succinate	—	− 0·2	+ 7·4	+ 3·4
Fumarate + malate	+ 2·8	+ 8·1	+24·1	+15·9
Sum of tricarboxylic acid-cycle intermediates	+ 3·5	+25·4	− 4·0	− 2·5

the metabolites present in the homogenates after incubation with pyruvate, α-oxoglutarate or pyruvate plus α-oxoglutarate (Table 1) indicated that the α-oxo acids may also interact in several other ways.

When pyruvate was the sole added substrate at least 1 mole of pyruvate disappeared per mole of oxygen absorbed. Thus pyruvate was not completely oxidized. The nature of the products of incomplete oxidation depended on, among other factors, whether or not bicarbonate was added. In the absence of bicarbonate ketone bodies were an important product (see below). In homogenates with added bicarbonate (Table 1) there was a large accumulation of tricarboxylic acid-cycle intermediates, particularly citrate, malate and α-oxoglutarate. This was mainly due to the formation of dicarboxylic acids by the fixation of carbon dioxide (Utter & Wood, 1951; Utter & Keech, 1960), but some (23 % in Expt. 1) was accounted for by the removal of endogenous glutamate, which was converted into α-oxoglutarate by transamination with the added pyruvate (as confirmed by subsequent observations). When α-oxoglutarate was the sole added substrate its oxidation was also incomplete. In Expt. 1 (Table 1) 78 % of the α-oxoglutarate removed could be accounted for by the formation of the intermediates of the tricarboxylic acid cycle, especially fumarate and malate, and of glutamate. In Expt. 2, 90 % was accounted for by the formation of the intermediates. The changes in the total amounts of acids of the tricarboxylic acid cycle present, which occurred on incubating pyruvate or α-oxoglutarate alone with homogenates, were not additive when both substrates were included (Table 1). This may be explained by an inhibition by α-oxoglutarate (or one of its products) of the formation of dicarboxylic acids from pyruvate through the fixation of carbon dioxide (see Tables 4 and 6).

The accumulation of glutamate formed from α-oxoglutarate was prevented by the addition of pyruvate (vessels 3 and 4, Table 1). Subsequent experiments showed that α-oxoglutarate diminished the formation of alanine from pyruvate (Table 4), and both these results can be attributed to mass action in the presence of a highly active glutamate–alanine transaminase. This inhibition of the formation of glutamate by pyruvate can account for 78 % (8·0 out of 10·3 μmoles) of the decrease in the removal of α-oxoglutarate in the presence of pyruvate in Expt. 1, but a lesser proportion was attributable to this effect in other experiments (see, for example, Table 2).

The formation of lactate from pyruvate varied from 0 to 11 % of the total pyruvate removed and was not consistently affected by α-oxoglutarate. In one experiment (Expt. 2, Table 1) there was a

Table 2. *Conversion of α-oxo[5-^{14}C]glutarate into pyruvate and the contribution of this reaction to the inhibition by α-oxoglutarate of the removal of pyruvate*

The results refer to 4 ml. of homogenate, with added bicarbonate, incubated with pyruvate in the presence and absence of α-oxo[5-^{14}C]glutarate in Warburg vessels. Each vessel contained 112 mg. dry wt. of tissue. The specific activity of α-oxo[5-^{14}C]glutarate was 50 μc/m-mole (8630 counts/min./μmole as the 2,4-dinitrophenylhydrazone plated at infinite thinness).

Incubation time	10 min.		20 min.		30 min.		40 min.	
Vessel no.	1	2	3	4	5	6	7	8
Additions (μmoles)								
Pyruvate	40·9	40·9	40·9	40·9	40·9	40·9	40·9	40·9
α-Oxo[5-^{14}C]glutarate	—	40·4	—	40·4	—	40·4	—	40·4
Metabolic changes (μmoles)								
O_2	− 8·9	− 8·9	−19·4	−19·0	−25·9	−29·5	−36·0	−35·7
Pyruvate	−17·0	− 6·1	−27·2	−11·1	−35·2	−16·3	−39·7	−19·9
α-Oxoglutarate	+ 0·6	− 7·9	+ 1·6	−13·5	+ 2·3	−16·6	+ 2·7	−21·1
$^{14}CO_2$	—	+ 0·33	—	+ 0·85	—	+ 1·42	—	+ 2·08
Specific activity of residual pyruvate (counts/min./μmole)	—	73	—	117	—	213	—	268
α-Oxo[5-^{14}C]glutarate incorporated into residual pyruvate (μmoles)	—	0·59	—	0·81	—	1·22	—	1·30
Calculated α-oxo[5-^{14}C]glutarate metabolized further than pyruvate (μmoles)	—	0·05	—	0·18	—	0·36	—	0·54
Total α-oxo[5-^{14}C]glutarate converted into pyruvate (μmoles)	—	0·64	—	0·99	—	1·58	—	1·84
Apparent inhibition of the removal of pyruvate due to formation from α-oxoglutarate (%)	—	4	—	4	—	4	—	5
Total observed inhibition of the removal of pyruvate (%)	—	64	—	59	—	54	—	50

decrease in the endogenous lactate (from 13·0 to 10·9 μmoles) even in the presence of pyruvate.

As the two α-oxo acids are interconvertible in rat liver it is feasible that the apparent reciprocal inhibitions of the removal of substrate are partly or entirely due to increases in their rates of formation from each other. For example, most of the observed inhibition by pyruvate of the removal of α-oxoglutarate could be accounted for by the formation of the amount of α-oxoglutarate accumulating on incubation with pyruvate alone (vessel 2), as well as by the observed inhibition of the formation of glutamate. The accumulation of pyruvate when α-oxoglutarate was the sole substrate was small, but this does not rule out the possibility of appreciable formation of pyruvate, followed by further reactions of the pyruvate. Labelled substrates were used to measure the extent of the interconversions of pyruvate and α-oxoglutarate.

Effects of α-oxoglutarate on the metabolism of pyruvate

The conversion of α-oxoglutarate into pyruvate was studied with α-oxo[5-^{14}C]glutarate, and the effects of α-oxoglutarate on the oxidation and carboxylation of pyruvate were examined with [1-^{14}C]pyruvate.

Incorporation of α-oxo[5-^{14}C]glutarate into pyruvate. The conversion of α-oxo[5-^{14}C]glutarate into pyruvate via the tricarboxylic acid cycle and decarboxylation of oxaloacetate should yield [1-^{14}C]-pyruvate of half the specific activity of the original α-oxoglutarate, if the randomization of ^{14}C between the two carboxyl groups of malate and oxaloacetate is complete. α-Oxo[5-^{14}C]glutarate and unlabelled pyruvate were added to homogenate, with (see Table 2) and without bicarbonate, and the formation of [1-^{14}C]pyruvate was measured. The [1-^{14}C]-pyruvate was estimated both by direct counting of the isolated 2,4-dinitrophenylhydrazone and by the assay of the [^{14}C]carbon dioxide liberated by decarboxylation with yeast carboxylase. The latter method gave slightly higher values for the formation of [1-^{14}C]pyruvate especially at the later stages of incubations, a difference probably due to the partial liberation by carboxylase of [^{14}C]carbon dioxide from α-oxo[1-^{14}C]glutarate formed from α-oxo[5-^{14}C]glutarate through one turn of the cycle. The value of the labelled pyruvate formed was corrected for losses due to the metabolic reactions of pyruvate. These losses were calculated by graphical integration of the removal rates of labelled pyruvate, which were obtained from the rates of the removal of pyruvate and the specific activities of pyruvate at different times.

Both in the presence (Table 2) and absence of bicarbonate, and throughout 40 min. incubation periods, the conversion of α-oxoglutarate into pyruvate was relatively slow. It produced less than a 5% apparent inhibition of the removal of pyruvate, i.e. less than 10% of the observed inhibitory effect of α-oxoglutarate. Thus over 90% of the decrease in the removal of pyruvate was a real inhibition. These results were borne out by the low rate of formation of [^{14}C]carbon dioxide from α-oxo-[5-^{14}C]glutarate. The maximum rate of conversion of α-oxoglutarate into pyruvate is twice the rate of formation of [^{14}C]carbon dioxide from α-oxo-[5-^{14}C]glutarate.

Production of pyruvate from endogenous sources. For the interpretation of later results it was necessary to know to what extent labelled pyruvate was diluted by pyruvate produced endogenously. In the experiment described in Table 3 there was, throughout a 60 min. incubation period, no significant dilution of [1-^{14}C]pyruvate by unlabelled material. In fact after the first 10 min. the [^{14}C]carbon dioxide released from the residual α-oxo acids by carboxylase was consistently slightly greater than the amount of residual pyruvate. This was probably due to decarboxylation of α-oxo-[1-^{14}C]glutarate formed from the [1-^{14}C]pyruvate.

Metabolism of [1-^{14}C]pyruvate. The oxidation of [1-^{14}C]pyruvate to acetyl-CoA involves a direct release of [^{14}C]carbon dioxide, whereas carboxylation to oxaloacetate followed by condensation with acetyl-CoA to form citrate causes a release of [^{14}C]carbon dioxide in the decarboxylations of isocitrate and α-oxoglutarate. The extent to which α-oxo[1-^{14}C]glutarate is formed depends on the degree of exchange of ^{14}C between [1-^{14}C]malate and fumarate, which in the calculations below is assumed to be complete. However, if the pyruvate carboxylase of Utter & Keech (1960) rather than the malic enzyme is mainly responsible for the formation of dicarboxylic acids and if the oxaloacetate is not in equilibrium with fumarate, all or most of the

Table 3. *Dilution of [1-^{14}C]pyruvate by unlabelled pyruvate from endogenous sources*

The results refer to 4 ml. of homogenate, without added bicarbonate, incubated in Warburg flasks with [1-^{14}C]-pyruvate for different time-intervals. Each vessel contained 132 mg. dry wt. of tissue. The specific activity of the original [1-^{14}C]pyruvate was 2·5 μc/m-mole.

Time of incubation (min.)	Total residual pyruvate (μmoles)	α-Oxo-glutarate formation (μmoles)	$^{14}CO_2$ liberated from residual α-oxo acids by carboxylase (μmoles)
0	38·0	0·0	37·9
10	27·3	0·4	27·2
20	19·9	0·9	20·7
30	13·8	1·2	14·8
40	7·3	1·2	7·8
50	2·6	1·2	3·1
60	0·7	0·9	1·7

[^{14}C]carbon dioxide will be released in the oxidation of the isocitrate, but this does not affect the validity of the conclusions drawn below. Any succinate, malate or fumarate formed oxidatively from [1-^{14}C]pyruvate via the tricarboxylic acid cycle is free from ^{14}C, so that any labelled dicarboxylic acid found must have been formed reductively from [1-^{14}C]pyruvate.

Table 4 shows the metabolic changes that occurred on the incubation of 10 mM-[1-^{14}C]pyruvate with homogenates in the presence and absence of 7·75 mM-bicarbonate and 10 mM-α-oxoglutarate. Bicarbonate stimulated the removal of pyruvate by 40–50 % both with and without α-oxoglutarate present, and α-oxoglutarate inhibited the removal of pyruvate by 57 % both in the presence and absence of bicarbonate. In this experiment the formation of [^{14}C]carbon dioxide represented 55–60 % of the total removal of pyruvate in each vessel. This formation of [^{14}C]carbon dioxide is equivalent to the oxidation of pyruvate to acetyl-CoA subject to the following three conditions.

First, the dilution of the [1-^{14}C]pyruvate by unlabelled material formed from endogenous sources (see Table 3) or other added substrates must be negligible, whether it is by net formation of pyruvate (e.g. during glycolysis) or by exchange of ^{14}C (e.g. with endogenous lactate). The conversion of α-oxoglutarate into pyruvate has already been examined (Table 2) and was too slow to affect appreciably the formation of [^{14}C]carbon dioxide from [1-^{14}C]pyruvate provided that the [1-^{14}C]-pyruvate concentration did not fall below 2 mM.

Secondly, the fixation of [^{14}C]carbon dioxide liberated from [1-^{14}C]pyruvate into tricarboxylic acid-cycle intermediates must be negligible. This will be the case if the carboxylation of pyruvate is inhibited or if the [^{14}C]carbon dioxide becomes sufficiently diluted by unlabelled carbon dioxide. The retention of fixed [^{14}C]carbon dioxide in intermediates has been calculated for vessels 2 and 4 of Table 4 (Table 5). As these calculations involve the assumption that the [^{14}C]carbon dioxide produced equilibrates with all the carbon dioxide plus bicarbonate of the system before fixation into dicarboxylic acid, the values obtained are approximations. Table 5 shows that the retention of fixed [^{14}C]carbon dioxide equalled 16 % of the [^{14}C]-carbon dioxide collected in the absence and 20 % in the presence of bicarbonate.

Thirdly, the formation of [^{14}C]carbon dioxide from the oxidation of isocitrate and of α-oxoglutarate must be negligible. This quantity was calculated (see Table 5) for vessels 2 and 4 of Table 4 and equalled 15 and 27 % respectively of the [^{14}C]carbon dioxide collected. The calculation assumed that all of the acetyl-CoA entering the system was derived from pyruvate. It so happens that the fixation of [^{14}C]carbon dioxide into tricarboxylic acid-cycle intermediates tends to cancel out the extra formation of [^{14}C]carbon dioxide from the oxidation of isocitrate and α-oxoglutarate, so

Table 4. *Effects of α-oxoglutarate and of bicarbonate on the metabolism of* [1-^{14}C]*pyruvate*

The additions and metabolic changes refer to 8 ml. of homogenate incubated for 30 min. in large (50 ml.) Warburg vessels. Each vessel contained 226 mg. dry wt. of tissue. The specific activity of [1-^{14}C]pyruvate was 12·5 μc/m-mole. The results under 'chromatographic analysis' represent the actual counts recorded after chromatography of one-fortieth of the vessel content.

Vessel no.	1	2	3	4	5
Additions (μmoles)					
[1-^{14}C]Pyruvate	0	80·7	80·7	80·7	80·7
α-Oxoglutarate	0	0	79·0	0	79·0
$KHCO_3$	0	0	0	62·0	62·0
Metabolic changes (μmoles)					
O_2	−33·8	−38·3	−38·5	−46·8	−48·2
Total CO_2	+21·3	+43·4	+44·0	+39·0	+42·8
$^{14}CO_2$	—	+27·4	+10·9	+36·4	+15·9
Pyruvate	0·0	−46·1	−18·9	−65·5	−28·0
α-Oxoglutarate	+ 0·1	+ 2·6	−31·3	+ 3·8	−36·2
Alanine	—	+ 6·1	+ 4·8	+ 6·4	+ 5·0
Acetate	0·0	+ 1·2	+ 1·2	+ 0·8	+ 1·2
Acetone	+ 1·8	+ 3·6	+ 1·1	+ 2·6	+ 2·4
Acetoacetate	—	+ 1·63	+ 0·51	+ 1·18	+ 0·89
β-Hydroxybutyrate	—	+ 3·33	+ 2·70	+ 1·85	+ 3·15
Citrate + *cis*-aconitate + isocitrate	—	+ 3·8	+ 7·8	+10·1	+14·9
Succinate	—	0·0	+ 2·1	0·0	+ 1·7
Fumarate + malate	—	+ 4·4	+20·0	+ 9·7	+18·1
Chromatographic analysis (counts/min.)					
Alanine	—	176	138	184	160
Malate	—	114	<10	175	42
Citrate	—	46	<10	225	40

that on incubation of [1-^{14}C]pyruvate without added bicarbonate the calculated oxidation of pyruvate was almost equal to the [^{14}C]carbon dioxide collected in this experiment. In the presence of added bicarbonate the calculated oxidation of pyruvate amounted to 93% of the [^{14}C]carbon dioxide collected. In subsequent experiments the formation of [^{14}C]carbon dioxide from [1-^{14}C]pyruvate incubated with homogenates in a medium lacking added bicarbonate was taken as the best available index of the oxidation of pyruvate.

The results presented in Table 6 show that most of the pyruvate metabolized was accounted for by three reactions: oxidation to acetyl-CoA plus carbon dioxide, carboxylation to dicarboxylic acids and conversion into alanine, all of which were inhibited by α-oxoglutarate, though to different extents. In this experiment 10 mM-α-oxoglutarate inhibited the oxidative decarboxylation of 10 mM-pyruvate by about 60% in the absence of bicarbonate and about 50% in its presence. The inhibition of the carboxylation of pyruvate was

Table 5. *Calculation of the oxidation of pyruvate to acetyl-CoA in vessels 2 and 4 of Table 4*

All results are expressed as μmoles.

Vessel no.	2	4
Substrates added	Pyruvate	Pyruvate + $KHCO_3$
Fixation of $^{14}CO_2$ into intermediates		
Accumulated tricarboxylic acid-cycle intermediates (i.e. fixed CO_2)	10·8	23·6
Fixed CO_2 liberated in the formation of α-oxoglutarate	1·3	1·9
Hence fixed CO_2 retained in intermediates (x)	9·5	21·7
$^{14}CO_2$ collected (a)	27·4	36·4
Total CO_2 formed (b)	43·4	39·0
Measured total $CO_2 + HCO_3^-$ ion in vessel at zero-time (c)	14·9	70·0
Hence $^{14}CO_2$ retained in intermediates (R) = $xa/(b+c)$	4·5	7·2
$^{14}CO_2$ released in the oxidation of isocitrate and of α-oxoglutarate		
Total $^{14}CO_2$ formation ($a+R$)	31·9	43·6
$^{14}CO_2$ formation accounted for by the accumulation of:		
α-Oxoglutarate (1) from the oxidation of pyruvate	2·6	3·8
(2) from the oxidation of isocitrate	1·3	1·9
Acetate	1·2	0·8
Acetone	7·2	5·2
Acetoacetate	3·3	2·4
β-Hydroxybutyrate	6·7	3·7
Citrate + *cis*-aconitate + isocitrate	3·8	10·1
Total accounted for	26·1	27·9
$^{14}CO_2$ formation not accounted for by the above metabolites	5·8	15·7
Oxidation of citrate and of α-oxoglutarate will contribute half the formation of $^{14}CO_2$ not accounted for	2·9	7·9
Thus total formation of $^{14}CO_2$ from oxidation of isocitrate and of α-oxoglutarate	4·2	9·8
Oxidation of pyruvate to acetyl-CoA ($^{14}CO_2$ collected plus $^{14}CO_2$ retained in intermediates after fixation of CO_2 minus $^{14}CO_2$ released in oxidation of isocitrate and of α-oxoglutarate)	27·7	33·8

Table 6. *Effects of α-oxoglutarate and of bicarbonate on the metabolism of pyruvate (calculated from Tables 4 and 5)*

In vessels 2 and 4 the formation of dicarboxylic acids from pyruvate was taken as equal to the increase in total tricarboxylic acid-cycle intermediates. In vessels 3 and 5 it was calculated from the count incorporated into malate and citrate (corrected for equilibrium amounts of fumarate, *cis*-aconitate and isocitrate), but may be slightly higher than this, as no allowance was made for any α-oxoglutarate formed entirely from pyruvate. Under the conditions of the experiment 1·0 μmole of [1-^{14}C]pyruvate or one of its products in the incubation mixture was equivalent to 29 counts/min. on the chromatograms. 'Pyruvate not accounted for' may represent lactate which was not determined in this experiment.

Vessel no.	2	3	4	5
Substrates added	Pyruvate	Pyruvate + α-oxoglutarate	Pyruvate + bicarbonate	Pyruvate + α-oxoglutarate + bicarbonate
Metabolic changes (μmoles)				
Pyruvate to acetyl-CoA + CO_2	27·7	10·9	33·8	15·9
Pyruvate to dicarboxylic acids	10·8	<1·0	23·6	3·4
Pyruvate to alanine	6·1	4·8	6·4	5·0
Total pyruvate accounted for	44·6	15·7	63·8	24·3
Pyruvate not accounted for	1·5	3·2	1·7	3·7

greater but its exact extent was difficult to determine. The chromatographic analysis (Table 4) shows that α-oxoglutarate reduced the ^{14}C incorporated into malate and citrate by at least 80%. Radioactive fumarate and α-oxoglutarate were not identified on the chromatograms in this experiment because the specific activity of the [1-^{14}C]pyruvate used, though suitable for study of the formation of [^{14}C]carbon dioxide, was too low for metabolites produced in small quantities to be identified. Comparison of the incorporated count with the chemical analysis of the total amounts of malate and citrate formed suggests that about 20% of the malate and 15% of the citrate were unlabelled on the incubation of pyruvate in the presence of added bicarbonate (Table 7). This material was probably derived partly from dicarboxylic acids that had passed once through the tricarboxylic acid cycle and so lost their radioactivity (Table 5) and partly from endogenous glutamate. Table 6 gives the best estimates of the conversion of pyruvate into dicarboxylic acids that can be made with the available results. α-Oxoglutarate inhibited the formation of alanine by about 20% both in the presence and absence of bicarbonate.

The addition of bicarbonate altered the relative proportions of pyruvate metabolized by the different pathways. When pyruvate was the only added substrate, 7·75 mM-bicarbonate stimulated the oxidation of pyruvate to acetyl-CoA plus carbon dioxide by 20% and the formation of dicarboxylic acids from pyruvate by over 100%, changing the ratio of oxidation to carboxylation from 2·5 to 1·4 and decreasing the removal of pyruvate accounted for by the formation of ketone bodies from 38 to 18% (Tables 6 and 8). The inhibition of the carboxylation of pyruvate by α-oxoglutarate was much more potent than the stimulation by bicarbonate. Hence in the presence of α-oxoglutarate bicarbonate stimulated the oxidation of pyruvate more (in terms of μmoles of pyruvate metabolized) than the carboxylation of pyruvate. This may explain the fact that, when α-oxoglutarate was present, bicarbonate increased the accumulation of ketone bodies, which appears to depend on the balance between the rates of formation of acetyl-CoA and of oxaloacetate. Though α-oxoglutarate decreased the formation of ketone bodies from pyruvate in the absence of bicarbonate, it increased it with bicarbonate present, despite the marked inhibition of the oxidation of pyruvate. Thus the carboxylation of pyruvate in the presence of bicarbonate was a better source of oxaloacetate for condensation with acetyl-CoA than the oxidation of α-oxoglutarate, probably because α-oxoglutarate inhibits the oxidation of malate, as suggested by the accumulation of the latter in the presence of α-oxoglutarate. Considerable amounts of acetone appeared when pyruvate was added but it is not clear whether this was formed by the decarboxylation of acetoacetate during the incubation or during the subsequent period of collection of [^{14}C]carbon dioxide; but in either case the ratio of acetoacetate to β-hydroxybutyrate formed from pyruvate was markedly decreased by α-oxoglutarate in both the presence and absence of bicarbonate.

Effects of pyruvate on the metabolism of α-oxoglutarate

The inhibition of the removal of α-oxoglutarate by added pyruvate was studied by the use of α-oxo[1-^{14}C]glutarate and of [2-^{14}C]pyruvate. In addition the formation of glutamate was measured. The object was to determine the relative contributions of the inhibition of the formation of glutamate, the inhibition of the oxidation of α-oxoglutarate and the conversion of pyruvate into α-oxoglutarate.

Inhibition of the formation of glutamate. The inhibition of the formation of glutamate from α-oxoglutarate on the addition of pyruvate was somewhat variable but often complete (Tables 1, 9 and 10). In the experiment shown in Table 9 the contribution of the decrease in the formation of

Table 7. *Incorporation of* [1-^{14}C]*pyruvate into malate and citrate in vessels* 2 *and* 4 *of Table* 4

The total malate present was calculated from the manometric determination of fumarate plus malate by using a value of 3·54 for the malate:fumarate ratio at 30° (Krebs, Smyth & Evans, 1940). The radioactive malate and citrate were calculated in μmoles from the chromatogram counts, by using an equivalent specific activity for [1-^{14}C]pyruvate of 29 counts/min./μmole obtained from the chemical determination and chromatogram count of alanine; the estimated error was ±0·5 μmole.

	Metabolic changes (μmoles)			
Vessel no.	2		4	
Substrates added	[1-^{14}C]Pyruvate		[1-^{14}C]Pyruvate + $KHCO_3$	
	Malate	Citrate	Malate	Citrate
Total metabolite	+3·4	+3·5	+7·6	+9·2
Radioactive metabolite	+3·9	+1·6	+6·0	+7·8

Table 8. *Analysis of the metabolic reactions in vessels 2 and 4 of Table 4*

Metabolite formed	To form 1 μmole of metabolite from pyruvate: O_2 required (μmoles)	To form 1 μmole of metabolite from pyruvate: Pyruvate required (μmoles)	Vessel 2. Pyruvate added: O_2 required (μmoles)	Vessel 2. Pyruvate added: Pyruvate required (μmoles)	Vessel 2. Pyruvate added: % of total removal of pyruvate accounted for	Vessel 4. Pyruvate + bicarbonate added: O_2 required (μmoles)	Vessel 4. Pyruvate + bicarbonate added: Pyruvate required (μmoles)	Vessel 4. Pyruvate + bicarbonate added: % of total removal of pyruvate accounted for
α-Oxoglutarate	+1	2	+2·6	5·2	11	+3·8	7·6	12
Alanine	0	1	0·0	6·1	13	0·0	6·4	10
Acetate	+0·5	1	+0·6	1·2	3	+0·4	0·8	1
Acetone and acetoacetate	+1	2	+5·2	10·5	23	+3·8	7·6	12
β-Hydroxybutyrate	+0·5	2	+1·7	6·7	15	+0·9	3·7	6
Citrate + *cis*-aconitate + isocitrate	+0·5	2	+1·9	7·6	16	+5·1	20·2	31
Succinate	+1·5	2	0·0	0·0	0	0·0	0·0	0
Fumarate + malate	−0·5	1	−2·2	4·4	10	−4·9	9·7	15
Total uptake of O_2 and removal of pyruvate accounted for			9·8	41·7	90	9·1	56·0	86
Observed uptake of O_2 and removal of pyruvate			38·3	46·1	—	46·8	65·5	—
Uptake of O_2 not accounted for			28·5	—	—	37·7	—	—
Removal of pyruvate not accounted for			—	4·4	—	—	9·5	—

Table 9. *Conversion of* [2-^{14}C]*pyruvate into α-oxoglutarate and the contribution of this reaction to the apparent inhibition by pyruvate of the removal of α-oxoglutarate*

The results refer to 4 ml. of homogenate incubated with α-oxoglutarate in the presence and absence of [2-^{14}C]pyruvate in Warburg vessels. Each vessel contained 111 mg. dry wt. of tissue. The specific activity of [2-^{14}C]pyruvate was 50 μc/m-mole (8210 counts/min./μmole as the 2,4-dinitrophenylhydrazone plated at infinite thinness).

Incubation time	20 min.				40 min.			
Vessel no.	1	2	3	4	5	6	7	8
Additions (μmoles)								
α-Oxoglutarate	39·2	39·2	39·2	39·2	39·2	39·2	39·2	39·2
[2-^{14}C]Pyruvate	—	28·8	—	28·8	—	28·8	—	28·8
$KHCO_3$	—	—	31	31	—	—	31	31
Metabolic changes (μmoles)								
O_2	−15·2	−14·3	−18·6	−19·6	−33·1	−31·3	−37·6	−36·9
α-Oxoglutarate	−14·9	−11·3	−17·8	−12·8	−26·6	−18·2	−23·2	−17·9
Pyruvate	+ 0·7	− 7·3	+ 1·3	−10·0	+ 1·1	−12·9	+ 1·5	−19·1
Glutamate + glutamine	+ 2·3	+ 0·7	+ 2·8	+ 1·5	—	—	—	—
Specific activity of residual α-oxoglutarate (counts/min./μmole)	—	960	—	1140	—	2030	—	1690
[2-^{14}C]Pyruvate incorporated into residual α-oxoglutarate (μmoles)	—	3·26	—	3·67	—	5·18	—	4·40
Calculated [2-^{14}C]pyruvate oxidized further than α-oxoglutarate (μmoles)	—	0·72	—	0·82	—	2·72	—	2·34
Total [2-^{14}C]pyruvate converted into α-oxoglutarate (μmoles)	—	3·98	—	4·49	—	7·90	—	6·74
Apparent inhibition by pyruvate of the removal of α-oxoglutarate (μmoles)	—	3·6	—	5·0	—	8·4	—	5·3

glutamate to the total inhibition of the removal of α-oxoglutarate was 44% in the absence and 26% in the presence of bicarbonate. Rather variable contributions of about 25–50% were found in several incubations lasting 10–30 min. The decrease in the accumulation of glutamate caused by pyruvate was associated with the formation of alanine but no strict stoicheiometry was observed between the two changes (Table 10). However, as the equilibrium constant of glutamate–alanine transaminase is close to unity (Krebs, 1953*b*) the effect of pyruvate on the formation of glutamate from equimolar α-oxoglutarate is probably due to mass action.

Inhibition of the oxidation of α-oxoglutarate. The formation of [^{14}C]carbon dioxide from α-oxo-[1-^{14}C]glutarate was used as a measure of the oxidation of α-oxoglutarate, an assumption which is valid so long as the dilution of the α-oxo[1-^{14}C]-glutarate by unlabelled α-oxoglutarate formed through the tricarboxylic acid cycle and by ^{14}C exchange with endogenous glutamate is relatively small. This was the case in incubations of 30 min. or less, when less than half the added α-oxoglutarate was removed.

Though pyruvate diminished the removal of α-oxoglutarate by 31%, the formation of [^{14}C]-carbon dioxide, and therefore the oxidation of α-oxoglutarate, was inhibited by only 9% (Table 10). The formation of [^{14}C]carbon dioxide represented 56% of the α-oxo[1-^{14}C]glutarate removed in the absence and 73% in the presence of pyruvate. Thus the fraction of the total decrease in the removal of α-oxoglutarate which could be attributed to an inhibition of the oxidation of α-oxoglutarate was small—less than 20%. The 9% inhibition by pyruvate of the oxidation of α-oxoglutarate contrasted with a 56% inhibition by α-oxoglutarate of the oxidation of pyruvate in the same system (Table 10). The increase in the percentage of the removal of α-oxoglutarate by oxidation produced by the addition of pyruvate can be accounted for by the inhibition of the formation of glutamate.

In the experiment shown in Table 10, 30% of the α-oxoglutarate removed cannot be accounted for either by oxidation (formation of [^{14}C]carbon dioxide) or the formation of glutamate plus glutamine. Chromatographic analysis revealed the existence of at least two radioactive products of α-oxo[1-^{14}C]glutarate metabolism besides glutamate and glutamine. One of these, accounting for about one-third of the missing α-oxoglutarate, was tentatively identified as α-hydroxyglutarate by co-chromatography with authentic material in the butan-1-ol–propionic acid solvent system. The other, present in similar amounts, was chromatographically identical with citrate in this solvent system. Labelled citrate can be formed from α-oxo[1-^{14}C]glutarate only by reversal of the tricarboxylic acid cycle or via the intermediate fixation of [^{14}C]carbon dioxide. Less ^{14}C was found in glutamate than in glutamine, which was identical with the citrulline-like material described by Derks & Grisolia (1958). There was some variation in the proportions of α-oxoglutarate metabolized by different pathways, as in some experiments

Table 10. *Effects of unlabelled pyruvate on the metabolism of α-oxo[1-^{14}C]glutarate and of unlabelled α-oxoglutarate on the metabolism of [1-^{14}C]pyruvate*

The results refer to 4 ml. of homogenate, without added bicarbonate, incubated for 20 min. in Warburg vessels. Each vessel contained 116 mg. dry wt. of tissue. The specific activity of [1-^{14}C]pyruvate and of α-oxo[1-^{14}C]-glutarate was 12·5 μc/m-mole.

Vessel no.	1	2	3	4
Additions	[1-^{14}C]Pyruvate (37·2 μmoles)	[1-^{14}C]Pyruvate (37·2 μmoles) + unlabelled α-oxoglutarate (39·7 μmoles)	α-Oxo[1-^{14}C]-glutarate (39·6 μmoles)	α-Oxo[1-^{14}C]-glutarate (39·6 μmoles) + unlabelled pyruvate (38·1 μmoles)
Metabolic changes (μmoles)				
O_2	−13·9	−13·3	−14·2	−14·5
Pyruvate	−11·9	− 4·4	+ 1·0	− 6·0
α-Oxoglutarate	+ 0·7	− 8·9	−13·3	− 9·2
$^{14}CO_2$	+ 8·9	+ 3·9	+ 7·4	+ 6·7
Alanine	+ 1·90	—	− 0·20	+ 0·60
Glutamate + glutamine	− 1·42	—	+ 1·66	− 0·16
$\frac{^{14}CO_2 \text{ formed}}{\text{Labelled substrate removed}} \times 100$	75	89	56	73
Inhibition by unlabelled substrate of the removal of labelled substrate (%)	—	63	—	31
Inhibition by unlabelled substrate of the formation of $^{14}CO_2$	—	56	—	9

(Table 1) up to 90% can be accounted for by the accumulating tricarboxylic acid-cycle intermediates.

Conversion of pyruvate into α-oxoglutarate. The conversion of pyruvate into α-oxoglutarate was determined by measuring the incorporation of [2-^{14}C]pyruvate into α-oxoglutarate (Table 9). α-Oxoglutarate was added to homogenates with and without [2-^{14}C]pyruvate and the removal of the α-oxo acids was determined in incubations lasting 20 and 40 min. The [2-^{14}C]pyruvate incorporated into the residual α-oxoglutarate was determined and corrected for losses due to the further metabolism of the labelled α-oxoglutarate formed (as described above for the conversion of α-oxo[5-^{14}C]-glutarate into [1-^{14}C]pyruvate). The total amount (μmoles) of [2-^{14}C]pyruvate converted into α-oxoglutarate equalled within narrow limits the apparent decrease in the removal of α-oxoglutarate caused by the pyruvate, both in the presence and absence of bicarbonate. This suggests that, subject to the following two conditions, the whole of the apparent inhibition of the removal of α-oxoglutarate could be attributed to the formation of α-oxoglutarate from pyruvate. First, only 1 mol.-prop. of [2-^{14}C]pyruvate and 1 mol.prop. of oxaloacetate derived from α-oxoglutarate must be involved in the formation of 1 mol.prop. of α-oxoglutarate. This was the case, as the inhibition by α-oxoglutarate of the carboxylation of pyruvate makes the formation of appreciable amounts of α-oxoglutarate from 2 equiv. of pyruvate impossible in the presence of α-oxoglutarate. Secondly, in the absence of added pyruvate no α-oxoglutarate must be regenerated by oxidation through the tricarboxylic acid cycle, the acetyl-CoA required being derived from endogenous sources or the α-oxoglutarate itself. That this condition was not met was suggested by the fact that at least 50% of the effect of pyruvate on the removal of α-oxoglutarate can be accounted for by the inhibitions of the formation of glutamate and of the oxidation of α-oxoglutarate described above. Further, small quantities of citrate accumulated when α-oxoglutarate was the sole added substrate (Table 1) suggesting that some acetyl-CoA was available. It can be concluded that the conversion of pyruvate into α-oxoglutarate was sufficient to account for that part of the decrease in the removal of α-oxoglutarate not attributable to the inhibitions of the formation of glutamate and of the oxidation of α-oxoglutarate.

Oxidation of endogenous substrates in the presence of pyruvate

Apart from competing with other added substrates pyruvate may also compete with endogenous oxidizable materials. Information on the extent of this competition can be derived from the results on the amounts of pyruvate removed, intermediates formed and oxygen taken up. The quantities of oxygen and pyruvate required to form intermediate metabolites from pyruvate are listed in the second and third vertical columns of Table 8. The values given hold if the intermediates arise solely from the added pyruvate and not from endogenous sources, and if fumarate and malate are only formed reductively. On the basis of these assumptions about 90% of the removal of pyruvate and about 20–25% of the oxygen used can be accounted for by the metabolites that were determined. If it is assumed that the remaining 10% of pyruvate was completely oxidized it would still leave much of the oxygen uptake unaccounted for (46% in vessel 2 and 30% in vessel 4). This is more than can be attributed to analytical errors and thus leads to the conclusion that a substantial fraction of respiration in the presence of pyruvate was due to the oxidation of endogenous substrates. The same conclusion is reached if it is assumed that the fumarate and malate are formed not reductively but oxidatively via the tricarboxylic acid cycle.

Information on the extent to which metabolites are formed according to the assumptions made in Table 8 can be obtained from the results of the chromatographic analysis given in Table 4. The specific activity of the original [1-^{14}C]pyruvate in terms of counts/min./μmole on paper can be derived from the analysis and count of alanine, and from these values it can be calculated (Table 7) that most of the malate and citrate appearing on incubation with [1-^{14}C]pyruvate alone was derived directly from the pyruvate. Only a relatively small proportion (15–20% in vessel 4) was unlabelled. These unlabelled tricarboxylic acid-cycle intermediates may have been formed from endogenous glutamate or from [1-^{14}C]pyruvate by more than one turn of the cycle, as the ^{14}C present in citrate (formed from [1-^{14}C]pyruvate, carbon dioxide and acetyl-CoA) is lost during the oxidation of citrate to succinate. Thus 2 mol.prop. of [1-^{14}C]pyruvate can form 1 mol.prop. of unlabelled malate (oxygen uptake 2 mol.prop.), and 3 mol.prop. of pyruvate can form 1 mol.prop. of unlabelled citrate (oxygen uptake 3 mol.prop.). That pyruvate passes through more than one turn of the cycle is in fact indicated by the formation of [^{14}C]carbon dioxide from added [2-^{14}C]pyruvate, especially on prolonged incubation. In the experiment recorded in Table 11, 35% of the ^{14}C of the [2-^{14}C]pyruvate removed appeared as [^{14}C]carbon dioxide within 1 hr. when bicarbonate was not added to the medium, and 21% appeared in the presence of bicarbonate. This lower value reflects the accumulation of larger pools of intermediates in the presence of bicarbonate and

Table 11. *Formation of* [^{14}C]*carbon dioxide from* [2-^{14}C]*pyruvate*

The results refer to 8 ml. of homogenate incubated for 60 min. in large (50 ml.) Warburg vessels. Each vessel contained 250 mg. dry wt. of tissue. The specific activity of [2-^{14}C]pyruvate was 5 μc/m-mole.

Vessel no.	1	2	3
Additions (μmoles)			
[2-^{14}C]Pyruvate	81·3	81·3	81·3
α-Oxoglutarate	—	—	78·1
$KHCO_3$	—	62·0	62·0
Metabolic changes (μmoles)			
O_2	−96·3	−110	−113
$^{14}CO_2$	+22·5	+16·4	+ 0·8
Pyruvate	−64·1	−78·3	−44·9
α-Oxoglutarate	+ 2·8	+ 2·3	−54·8
$\frac{^{14}CO_2 \text{ formed}}{\text{Pyruvate removed}} \times 100$	35	21	2

supports the assumption that the metabolites undergoing oxidation mix freely with the accumulating pools of intermediates. This is further supported by the observation that the addition of 10 mM-α-oxoglutarate decreased the formation of [^{14}C]carbon dioxide from 21 to 2 % of the [2-^{14}C]-pyruvate removed.

Thus, although there are several uncertainties in the calculations made in Table 8, these do not affect the main conclusion that the inhibition of endogenous respiration by pyruvate was only a partial one. The nature of the endogenous substrates is still an open question.

DISCUSSION

Effects attributable to competition between pyruvate and α-oxoglutarate have been described by a number of workers. Evans (1940) found an accumulation of α-oxoglutarate on incubation of a suspension of minced pigeon liver with pyruvate alone. When both substrates were added, pyruvate and α-oxoglutarate appeared to inhibit the removal of each other, and the oxygen uptake was not additive. Emmelot & Bos (1961) found that aerobic incubation of α-oxoglutarate with homogenates of ascites-tumour cells caused a marked rise in the concentration of pyruvate. On the other hand, Tyler (1960) stated that the total utilization of pyruvate and of α-oxoglutarate, when added together to tissue homogenates, was equal to the sum of the individual rates or may be even greater.

The earlier observations (Evans, 1940) indicated that interactions between α-oxoglutarate and pyruvate occur but, as they were limited to measurements of the rates of the removal of α-oxo acids and of oxygen consumption, they did not supply sufficient information on the nature of the interaction. The use of ^{14}C-labelled substrates in the present work has made it possible to establish that α-oxoglutarate inhibits the oxidative decarboxylation of pyruvate and is therefore preferentially oxidized by rat-liver homogenates. The determination of the intermediates that accumulated in the presence of pyruvate plus α-oxoglutarate has shown that the α-oxo acids also interact through the reaction catalysed by the liver glutamate–alanine transaminase and in the carboxylation of pyruvate.

Thus 10 mM-α-oxoglutarate can cause 80 % inhibition of the formation of dicarboxylic acids from pyruvate. The enzymic reaction which is inhibited and the identity of the inhibiting compound, whether α-oxoglutarate or a product derived from it, are still uncertain. The inhibited enzyme may be either the malic enzyme or the carbon dioxide-fixing enzyme (pyruvate carboxylase) of Utter & Keech (1960). According to Stickland (1959) 1 mM-L-malate can inhibit the action of the malic enzyme on 10 mM-pyruvate by 50 % and, as the concentration of malate which was reached in tissue homogenates in the presence of 10 mM-α-oxoglutarate was higher than 2 mM, the inhibitory effect of α-oxoglutarate on the carboxylation of pyruvate could be attributed to an inhibition of the malic enzyme by malate. However, no information is as yet available on the relative activities of the two enzymes in rat liver, or on the effect of dicarboxylic acids on the pyruvate carboxylase.

Effect of bicarbonate on the formation of ketone bodies. It is known (Lehninger, 1946; Krebs, 1960) that in the absence of oxaloacetate pyruvate yields acetoacetate as a major end product in respiring liver preparations. The present work indicates that the bicarbonate concentration affects the formation of ketone bodies from pyruvate, probably by stimulating the formation of oxaloacetate. The addition of 7·75 mM-bicarbonate to homogenates increased the formation of dicarboxylic acids by over 100 % and halved the yield of ketone bodies. Analogous effects have been described by Bartley (1953, 1954) with kidney particles, where bicarbonate increases the formation of phosphoenolpyruvate from pyruvate and decreases the accumulation of acetate.

Effect of α-oxoglutarate on the oxidation of pyruvate. Although pyruvate and α-oxoglutarate are oxidized at similar rates when added alone to rat-liver homogenates, in the presence of both substrates at 10 mM concentrations α-oxoglutarate is oxidized preferentially. This effect may result from competition between the oxidations of pyruvate and of α-oxoglutarate for a component of the electron-transport chain or for a co-factor involved in the oxidative decarboxylation of both substrates. Alternatively a competitive inhibition of pyruvate oxidase at the level of the active centre of the enzyme may occur.

The preferential oxidation of α-oxoglutarate may be part of a regulatory mechanism inhibiting the formation from pyruvate of surplus acetyl-CoA, which would otherwise appear in the form of ketone bodies. The inhibition of the production of ketone bodies from pyruvate by α-oxoglutarate, which occurred in the absence of bicarbonate, can be better explained by the observed decrease in the oxidation of pyruvate to acetyl-CoA than by the formation of oxaloacetate from the α-oxoglutarate.

It must be emphasized that the present results apply to liver only. In brain, for example, pyruvate is oxidized in preference to α-oxoglutarate (R. J. Haslam & H. A. Krebs, unpublished work).

SUMMARY

1. The addition of pyruvate or of α-oxoglutarate increased the rate of respiration of rat-liver homogenates by about 20–30% but the oxygen uptake in the presence of both substrates was not additive. The addition of α-oxoglutarate decreased the removal of pyruvate by 50–65%, and the addition of pyruvate decreased that of α-oxoglutarate by 12–35%.

2. Experiments with [1-^{14}C]pyruvate showed that pyruvate was metabolized by three main reactions: (*a*) 50–75% of the pyruvate removed was oxidized to acetyl-CoA, which was partly converted into ketone bodies and partly entered the tricarboxylic acid cycle; (*b*) 15–40% was carboxylated to dicarboxylic acids; (*c*) 10–15% was converted into alanine. The proportion of (*b*) was increased within the above range by the addition of 7·75 mM-bicarbonate, and this was associated with a decreased accumulation of ketone bodies. While pyruvate remained in the homogenate little was completely oxidized and the main products of pyruvate metabolism were intermediates of the tricarboxylic acid cycle, ketone bodies and alanine.

3. The addition of α-oxoglutarate inhibited all three primary reactions of pyruvate although to different degrees: 10 mM-α-oxoglutarate inhibited the oxidative decarboxylation of 10 mM-pyruvate by 50–65%, the carboxylation by about 80% and alanine formation by about 20%.

4. When α-oxoglutarate was the sole added substrate, 55–90% of the α-oxoglutarate removed underwent decarboxylation, followed by a further oxidation of the succinate formed, and 10–20% was converted into glutamate plus glutamine. Apart from carbon dioxide, malate, fumarate, glutamate and glutamine were the main end products. The existence of other minor pathways was demonstrated by experiments with α-oxo[1-^{14}C]glutarate, which formed two radioactive products tentatively identified as α-hydroxyglutarate and citrate.

5. The inhibition of the removal of α-oxoglutarate by pyruvate was due to three factors. About 25–50% of the effect resulted from a decreased yield of glutamate owing to transamination of glutamate with pyruvate. The second factor was the roughly 10% inhibition by pyruvate of the decarboxylation of α-oxoglutarate, which accounted for not more than 25% of the effect of pyruvate on the removal of α-oxoglutarate. The third factor accounting for the remainder of the effect was the conversion of pyruvate into α-oxoglutarate.

6. Pyruvate (10 mM) suppressed the oxidation of endogenous substrates by roughly 50%.

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The Biochemistry of Rumen Protozoa

6. THE MALTASES OF *DASYTRICHA RUMINANTIUM*, *EPIDINIUM ECAUDATUM* (CRAWLEY) AND *ENTODINIUM CAUDATUM**

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The digestion of starch is an important feature of the rumen fermentation, and a number of micro-organisms which decompose starch have been found in rumen contents. Several types of protozoa are active in this decomposition, and the amylases of the following species have been examined in detail: mixed *Isotricha intestinalis* plus *I. prostoma*, and *Dasytricha ruminantium* (Mould & Thomas, 1958), *Epidinium ecaudatum* (Crawley) (Bailey, 1958) and *Entodinium caudatum* (Abou Akkada & Howard, 1960). The amylases of these organisms are all of the α-type, and yield maltose as the main product of amylose hydrolysis. With the exception of the *Isotricha* species, these protozoa also produce maltase; some of the properties of this enzyme in the three species concerned are described here.

MATERIALS AND METHODS

Enzymes. The dialysed and freeze-dried extracts of *Dasytricha ruminantium* and *Entodinium caudatum* were prepared by the methods of Howard (1959) and Abou Akkada & Howard (1960) respectively. The freeze-dried powders each contained about 9% of N. The extracts of *D. ruminantium* contained amylase, invertase, cellobiase, β-glucosidase and pectinase in addition to maltase (Mould & Thomas, 1958; Howard, 1959; Abou Akkada & Howard, 1961). The extracts of entodinia contained amylase, proteinase and peptidase in addition to maltase (Abou Akkada & Howard, 1960, 1962).

Epidinia were disrupted, and water and phosphate buffer extracts made, as described by Bailey, Clarke & Wright (1962). The extracts were freeze-dried. Three such extracts, I, II and III, were used, obtained from three batches of protozoa. All corresponded to 'solution D' (phosphate buffer extract) of Bailey *et al.* (1962), except that in the preparation of I and II the second water-extraction step ('solution B') was omitted. The three freeze-dried powders contained respectively 1·1, 0·5 and 0·6% of N. The presence in these materials of carbohydrase activities other than maltase was investigated. Enzyme powder (5 mg.) and carbohydrate (2–5 mg.) were incubated for 24 hr. in citrate buffer, pH 6·0 (0·4 ml.). The solutions were examined by paper chromatography before and after incubation.

Carbohydrates. These were from the usual commercial sources except for the following compounds. Commercial maltose was freed from traces of glucose and maltotriose by fractionation on charcoal–Celite columns. Maltodextrins were isolated from a partial acid hydrolysate of amylose (Whelan, Bailey & Roberts, 1953). Maltitol and malto-

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