results very close to those obtained with 12N-hydrochloric acid at 37°.

3. The amide contents of a series of deionized gelatins, ranging from pI 4.80 to 9.4, are related to their isoionic points by a curve of similar shape to the titration curve.

4. Certain gelatins and glues that show evidence of containing polysaccharide impurities give rise to larger quantities of ammonia on hydrolysis than would be expected from their isoionic points.

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Formation of Labelled Amino Acids by Exchange Transamination

By R. G. HILLER AND D. A. WALKER
 Queen Mary College (University of London), London, E. 1

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Glutamate-aspartate transaminase, which catalyses reaction (1)

is known to catalyse also the exchange of an amino group (reaction 2) between glutamate and its keto acid analogue (Nisonoff, Barnes, Enns & Schuching 1954; Jenkins, Yphantis &Sizer, 1959).

To the best of our knowledge the possible catalysis of the corresponding reaction (3), between aspartate and its keto acid analogue,

has not previously been investigated.

If such reactions occurred *in vivo* they would have a bearing on the interpretation of the results of $^{14}\text{CO}_2$ -feeding experiments. Accumulation of label in glutamate or aspartate would then not necessarily be indicative of synthesis in the usual sense, since these acids could be formed (in reactions 2 and 3) at the expense of their unlabelled counterparts. Clearly, however, much would depend upon the relative rates of the normal transamination and what we shall call 'exchange transamination' (reactions 2 and 3). The following experiments were

therefore primarily carried out to determine (a) whether or not $^{14}\text{CO}_2$ could be introduced into

aspartate in a model system by carboxylation of phosphopyruvate followed by exchange transamination of the resulting oxaloacetate, and (b) if the rate of incorporation was of the same order as that

of the corresponding system in which glutamate serves as the amino donor in the normal transamination.

MATERIALS AND METHODS

Phosphoenolpyruvate was from the Sigma Chemical Co., St Louis, Mo., U.S.A. L- ^{14}C Glutamic acid and sodium ^{14}C bicarbonate were obtained from The Radiochemical Centre, Amersham, Bucks. Castor beans were kindly given by the Baker Castor Oil Co., San Diego, Calif., U.S.A.

Enzymes. Mitochondria were prepared from the endosperm of 50 4-day castor-oil-bean seedlings (*Ricinus communis* var. Cimarron) according to the method of Beevers & Walker (1956) and washed three times in cold 0.5M-sucrose-0.1M-phosphate (NaH_2PO_4) adjusted to pH 7.1 by the addition of potassium hydroxide. They were then sus-

pended in 200 ml. of ice-cold water and left to stand in the cold for 30 min. After centrifuging at 33 000 *g* for 30 min. at 0°, spectrophotometric assay (Walker & Ranson, 1958) indicated that almost all the glutamate-aspartate transaminase was present in the supernatant. Two volumes of ice-cold saturated (NH₄)₂SO₄ were added to the supernatant and the mixture was left to stand in the cold room overnight. The precipitate which formed was removed (by centrifuging at 33 000 *g* at 0° for 10 min.) and stored in the deep-freeze in saturated (NH₄)₂SO₄ at -20°. For use the precipitate was again separated by centrifuging, redissolved in 10 ml. of 0.1% 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7.4, and dialysed in the cold for 72 hr. against 15 l. (3 × 5 l.) of the same buffer.

Cell-free extracts of *Kalanchoë* leaves were prepared as described by Walker (1957). The fraction of protein (150 mg.), which was precipitated between 30 and 60% saturation with (NH₄)₂SO₄, was redissolved in 25 ml. of tris buffer, pH 7.4, and dialysed in the cold for 72 hr. against the same buffer before use.

Chromatography. Samples were chromatographed in one dimension on Whatman no. 1 paper with butanol-acetic acid-water (74:19:50) as the developing solution.

Degradation of aspartate. The reaction mixture consisted of 2 ml. of eluted aspartate + added carrier (3.0 μ moles of aspartate), 0.2 ml. of 3M-acetate buffer, pH 4.9, and 0.1 ml. of pyruvate solution (made by dissolving 0.66 g. of sodium pyruvate in 10 ml. of 0.2M-acetate buffer, pH 4.9). After equilibration, 0.6 ml. of aspartate decarboxylase prepared from *Nocardia* (Crawford, 1958) was added. The vessel was incubated for 1½ hr. at 22° and was continually flushed with air. Residual radioactivity was counted with a thin end-window Geiger-Müller tube on aluminium planchets covered with lens tissue.

RESULTS

Introduction of ¹⁴CO₂ into aspartate

In a preliminary experiment, *Kalanchoë* extract was incubated with sodium [¹⁴C]bicarbonate and phosphoenolpyruvate together with the additions shown in Table 1. Samples were then chromatographed and radioautographs showed that more

than 99% of the activity was restricted to an amino acid with the same *R_F* as aspartate. The actual level of ¹⁴CO₂ fixed was measured with a Geiger-Müller end-window counter placed directly on the chromatograms and the values obtained are given in Table 1.

The small fixation in the absence of added amino acids is possibly attributable to the presence of traces of free amino acids in the enzyme solution. Evidently the fixation in the presence of added amino acids was approaching completion, if equilibrium had not actually been achieved, with the major part of the added ¹⁴CO₂ incorporated into aspartate. At this stage the fixation with aspartate as the amino donor (86%) was not greatly different from that with glutamate as the amino donor (83%).

Rate measurements of fixation of carbon dioxide

To obtain comparative measurements of the actual rates of incorporation of ¹⁴CO₂ into aspartate further experiments were carried out in which samples were taken at frequent time intervals (Fig. 1). In the experiment shown in Fig. 1 the initial rate with aspartate as the amino donor was approximately three times that with glutamate as the amino donor. In each of two additional experiments (with a second sample of enzyme) in which 2 μ moles and 1 μ mole of amino donor were added this rate was again approximately threefold (Fig. 2).

The radioactive amino acid which accumulated in the reaction mixtures to which aspartate had been added was eluted from the chromatograms and incubated with a cell-free extract of *Nocardia*. This preparation catalyses specifically the β -decarboxylation of L-aspartate (Crawford, 1958). After incubation less than 2% of the activity remained in the eluate, confirming that the fixation product was in fact L-[4-¹⁴C]aspartate. It has previously been shown that L-[4-¹⁴C]aspartate is

Table 1. *Incorporation of ¹⁴CO₂ into aspartate in reaction mixtures containing Kalanchoë enzyme, phosphoenolpyruvate and ¹⁴CO₂ together with the additions listed below*

In this experiment 0.3 ml. of tris buffer (20%, w/v; pH 7.4), 0.9 ml. of MgCl₂ (30 μ moles) and 3 ml. of *Kalanchoë* enzyme were added to each of four 8 ml. reaction vessels together with the additions shown below. At zero time 0.5 ml. of a solution containing potassium phosphoenolpyruvate (10 μ moles at pH 7.4) and sodium [¹⁴C]bicarbonate (2 μ moles, 20 μ C) was injected through the rubber cap of each vessel. After 40 min. the reactions were stopped by adding formic acid (1 ml.) and boiling for 2 min. The volume of each reaction mixture was then adjusted to 10 ml. and samples (0.025 ml.) were chromatographed (see Methods). Volume of each of the additions listed was 0.3 ml.

Additions	10 ⁻⁴ × Counts/min. in aspartate	10 ⁻⁴ × Counts/min. added*	Percentage of CO ₂ fixed
Water	0.44	146	0.3
NH ₄ Cl (30 μ moles)	0.32	146	0.2
Glutamate (30 μ moles)	121	146	83
Aspartate (30 μ moles)	126	146	86

* 146 × 10⁴ counts/min. = 10 μ C under conditions of measurements.

Fig. 1. Rate of incorporation of $^{14}\text{CO}_2$ into L-aspartate with aspartate or glutamate as the amino donor. At the start each reaction mixture (4 ml.) contained: *Kalanchoë* enzyme (3 ml.); MgCl_2 (30 μmoles) and O, aspartate (10 μmoles) or ●, glutamate (10 μmoles). Each reaction was started by the addition of 0.5 ml. of a solution containing phosphoenolpyruvate (10 μmoles), $\text{NaH}^{14}\text{CO}_3$ (10 μC , 1 μmole) and tris buffer (10%, w/v; pH 7.4). Samples (0.5 ml.) were taken at the times indicated, added rapidly to hot formic acid and boiled for 2 min. Radioactivity was counted directly on one-dimensional chromatograms.

Fig. 2. Rate of incorporation of $^{14}\text{CO}_2$ into aspartate with aspartate and glutamate as the amino donor. Reaction mixtures were as described for Fig. 1 but with a different sample of *Kalanchoë* enzyme and ●, aspartate (2 μmoles), O, glutamate (2 μmoles), ▲, aspartate (1 μmole), Δ, glutamate (1 μmole).

the major product of the corresponding system in which glutamate is the amino donor (Dearing & Walker, 1960).

Exchange transamination between glutamate and α -oxoglutarate

The exchange between glutamate and its keto acid analogue (reaction 2) has been previously demonstrated with crude (Nisonoff *et al.* 1954) and highly purified (Jenkins *et al.* 1959) preparations from pig heart, but it seemed desirable to repeat these experiments with an enzyme from a plant source. The route by which $^{14}\text{CO}_2$ is introduced into α -oxoglutarate and glutamate during the course of feeding experiments is not certainly known, but, at least in the dark, the reactions of the Krebs cycle are probably involved (see e.g. Kunitake, Stitt & Saltman, 1959). Accordingly an extract of mitochondria (see Methods) was used in the following experiment rather than the extract of *Kalanchoë* leaves. The actual site (within the leaf) of the *Kalanchoë* enzymes used in the previous experiment has not been determined but there is evidence that both phosphopyruvate carboxylase and glutamate-aspartate transaminase are present in chloroplasts (Rosenberg, Capindale & Whatley, 1958).

Fig. 3. Decline in radioactivity of $[^{14}\text{C}]$ glutamate and concomitant appearance of radioactivity in α -oxoglutarate in the presence of glutamate-aspartate transaminase from *Ricinus* mitochondria. At the start each reaction mixture (1.3 ml.) contained *Ricinus* enzyme (1 ml.), and Δ, ▲, oxaloacetate (10 μmoles), or O, ●, α -oxoglutarate (10 μmoles). Each reaction was started by the addition of glutamate (1.6 μC , 0.16 μmole). Samples (0.02 ml.) were taken at the times indicated and pipetted directly on to a spot of formic acid on chromatography sheets and dried with a hot-air blower. Radioactivity in glutamate and α -oxoglutarate was counted directly on the chromatograms after development in butanol-acetic acid (see Methods).

Reaction mixtures were prepared in which [^{14}C]glutamate was incubated with mitochondrial transaminase and either oxaloacetate or α -oxoglutarate. Samples were taken after suitable time intervals and chromatographed as before (Fig. 3).

As the reaction proceeded the level of radioactivity in glutamate fell and there was a concomitant rise in the level of activity in α -oxoglutarate. The initial rate of the exchange transamination (in which α -oxoglutarate was the amino acceptor) was about 1.5 times that of the normal transamination (in which oxaloacetate was the amino acceptor).

DISCUSSION

Partially purified extracts of *Kalanchoë crenata* leaves are known to contain phosphopyruvate carboxylase (Walker, 1957) and glutamate-aspartate transaminase (Walker & Ranson, 1958). In the presence of phosphopyruvate, $^{14}\text{CO}_2$ and glutamate such extracts catalyse the synthesis of L-[4- ^{14}C]-aspartate (Dearing & Walker, 1960) and it would seem clear that the carboxylation

and the transamination

are linked to bring about the overall reaction:

In the present experiments, when aspartate was substituted for glutamate, the only stable labelled product was again L-[4- ^{14}C]aspartate. It is known (Nisonoff *et al.* 1954; Jenkins *et al.* 1959) that glutamate-aspartate transaminase from pig heart can catalyse the transfer of an amino group from glutamate to its keto acid analogue (α -oxoglutarate) and we have demonstrated that this reaction occurred in the presence of *Ricinus* mitochondrial extracts. It is not unreasonable that the corresponding transamination between aspartate and its keto acid analogue (reaction 3) should also occur, and in fact our results with the *Kalanchoë* enzyme are consistent with the view that reaction (4) can be linked with reaction (3):

to bring about the overall reaction

According to Jenkins *et al.* (1959) 'exchange transamination' between α -oxoglutarate and glutamate (reaction 2) may proceed at a greater rate than the normal transamination (reaction 1)

between α -oxoglutarate and aspartate. This was also our experience with extracts of *Ricinus* mitochondria. Similarly the initial accumulation of label in aspartate in reaction (6) was about three times as rapid as the corresponding accumulation in reaction (5). If glutamate-aspartate transaminase catalyses both types of transamination then the relative rapidity of the exchange reaction may be a consequence of an alignment of the substrates on the enzyme surface such that the transfer of an amino group between carbon chains of equal length (reactions 2 and 3) proceeds more rapidly than the transfer between carbon chains of unequal length (reaction 1).

Our results affect the interpretation of feeding experiments with $^{14}\text{CO}_2$. Unless exchange transamination is completely inhibited by the substrates of the normal transamination some exchange transamination will occur whenever glutamate-aspartate transaminase and its substrates come together. In these circumstances $^{14}\text{CO}_2$ could appear in aspartate or glutamate either in the absence of synthesis (in the usual sense) or even during metabolic breakdown of these amino acids.

It would appear that the rate of the exchange transamination (as compared with the normal transamination) would not be the limiting factor in any accumulation of label by this mechanism.

When crassulacean plants such as *Kalanchoë crenata* are fed with $^{14}\text{CO}_2$ in the dark, label appears first in malate and aspartate [for a recent review see Ranson & Thomas (1960)] and subsequently in other compounds. Although there is a striking diurnal fluctuation in the malate content of such plants there does not appear to be any corresponding fluctuation in the level of amino acids (Lyndon, 1957). This anomaly could be explained by the present findings, since label could accumulate in aspartate (and possibly other amino

acids) in the absence of net synthesis. Our results would also explain the finding of Rosenberg *et al.* (1958) that fixation of carbon dioxide in the dark in aspartate by chloroplast extracts was increased

'when in addition to phosphoenolpyruvate either alanine, glutamine or aspartic acid was added to the reaction mixture'.

The appearance of $^{14}\text{CO}_2$ in certain amino acids during photosynthetic carbon dioxide fixation in a variety of organisms might also be attributed, at least in part, to exchange transamination. Work is at present being undertaken in this laboratory in an attempt to determine the contribution of exchange transamination to the accumulation of label in aspartate by *Chlorella* during photosynthesis and fixation of carbon dioxide in the dark.

SUMMARY

1. In the presence of phosphoenolpyruvate, L-aspartate and an extract of *Kalanchoë crenata*, $^{14}\text{CO}_2$ has been introduced into the β -carboxyl group of L-aspartate. The reaction is attributed to an initial carboxylation of phosphoenolpyruvate to yield oxaloacetate followed by an exchange transamination in which aspartate functions as the amino donor.

2. The rate of accumulation of ^{14}C in aspartate has been compared with that of the corresponding reaction, in which the initial carboxylation is followed by a normal transamination in which glutamate serves as the amino donor, and has been shown to be more rapid.

3. The rate of the normal transamination between glutamate and oxaloacetate has been compared with that of the exchange transamin-

ation between glutamate and α -oxoglutarate in reactions catalysed by an enzyme from *Ricinus mitochondria*.

4. The results have been discussed in relation to their bearing on $^{14}\text{CO}_2$ -feeding experiments.

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The Isolation of Bradykinin, a Plasma Kinin from Ox Blood

By D. F. ELLIOTT, E. W. HORTON AND G. P. LEWIS
National Institute for Medical Research, Mill Hill, London, N.W. 7

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Bradykinin belongs to a group of peptides which stimulate smooth muscle and have been given the generic name of plasma kinin; this name has been adopted by general consent of most workers in the field (see Lewis, 1958). The formation of bradykinin from ox-plasma proteins by the action of trypsin or the venom of the snake, *Bothrops jararaca*, was first observed by Rocha e Silva, Beraldo & Rosenfeld (1949). Blood and many other tissues are known to contain enzymes which can be activated under certain conditions and will then cause liberation of a plasma kinin from the plasma proteins (Frey, Kraut & Werle, 1950). Bradykinin is pharmacologically indisting-

uishable from other plasma kinins (Lewis, 1958; Gaddum & Horton, 1959; Werle, 1953; Holdstock, Mathias & Schachter, 1957; Mathias & Schachter, 1958; Hilton & Lewis, 1956).

The experiments described in this paper have led to the isolation of bradykinin, which is the first member of the plasma-kinin group to be obtained in the pure state.

EXPERIMENTAL

Materials. $(\text{NH}_4)_2\text{SO}_4$ was a commercial grade. Ammonium acetate was A.R. grade. Ammonium acetate buffer, pH 5, was prepared from stock solutions of 10M-