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The Synthesis of Tritiovaline and its Incorporation into Rat-Visceral Proteins

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There are certain investigations in which it is an advantage to label an amino acid with a radioactive isotope other than ^{14}C. Tritium can be employed as a tracer where double labelling is required and in detailed investigations of metabolic pathways (cf. Arnstein & Crawhall, 1957). It is of value when the synthesis of the corresponding ^{14}C compound is difficult (Pearlman, 1957), and it has great economical advantages in the biosynthetic preparation of labelled proteins where the efficiency of incorporation is low (Done & Payne, 1956).

The difference in mass between the isotopes of hydrogen gives rise to a greater 'isotope effect' than occurs with isotopes of an element of higher atomic number (Eidinoff, 1953). We thought that this effect might not be appreciable if the tritium label were attached to the amino acid molecule at a site remote from that at which enzyme action occurs. To test this hypothesis we have prepared [β-^{3}H]valine and have compared its rate of incorporation into rat-visceral proteins with that of [γ-^{14}C]valine. A preliminary account of some of this work has been published (Crawhall & Smyth, 1955).

EXPERIMENTAL

Radioactivity measurements

Thin-window Geiger counter. This consisted of an Ecko EHM2S Geiger–Müller tube coupled to a scaler and E.H.T. unit (Panax Ltd., Type 100C). A poly[^{14}C]methyl methacrylate disk (1 μc/g. supplied by the Radiochemical Centre, Amersham, Bucks) was mounted in a stainless-steel planchet (internal diameter 1·51 cm.). This had an activity of 1053 counts/min. (S.D. 1%) with a background count of 12/min.

Windowless flow-type counter. This instrument, described by Banks, Blow & Francis (1956*a*), was coupled to a scaler and E.H.T. unit (Panax Ltd., Type 100C). The same poly-[^{14}C]methyl methacrylate standard gave 3362 counts/min. (S.D. 1%) with a background of 15/min. The method of counting solid tritium samples was described by Banks, Crawhall & Smyth (1956*b*). It was necessary to use a tritium-labelled compound as a standard for tritium counting, as the windowless counter can show a greater daily variation of sensitivity to tritium emissions than to ^{14}C emissions. Certain compounds when mixed with graphite and mounted in planchets showed a greater decrease in count rate over a period of 3 months than could be accounted for by the natural rate of decay of the isotope. The 2:4-dinitrophenylhydrazone of [1:2-^{3}H]*iso*butyraldehyde, and [α-^{3}H]cystine (Arnstein & Crawhall, 1957) had a reproducible count rate over a period of 2 years and were used as standards. At the time of the experiments reported in this paper the standard deviation in count rate of the tritium standards was 7%. This is a maximum value and has subsequently been reduced with different anode wires and is now only 2%. All comparative counts are the mean of three determinations made on the same day (S.D. 2%).

DL-[$\alpha\beta$-^{3}H]*Valine*

Adams platinum oxide catalyst (100 mg.) was suspended in dry tetrahydrofuran (10 ml.) and reduced with hydrogen. 2-Phenyl-4-*iso*propylideneoxazol-5-one (1·0 g.) was then added and the apparatus (see Fig. 1) evacuated. The seal of an ampoule containing tritium (100 mc) was broken and the tritium transferred to the reaction vessel by using the gas burette as a Töpler pump. Inactive hydrogen was then added and mechanical stirring of the solution commenced. More hydrogen was added at intervals to maintain atmospheric pressure in the reaction vessel, and after 2 hr., when 120 ml. (theoretically 112 ml.) of hydrogen had been absorbed, no further absorption was observed. The catalyst was removed by centrifuging, and the solvent by distillation *in vacuo* at room temperature. 6N-Hydrochloric acid (50 ml.) was added to the residue and the solution heated under reflux for 24 hr. The solvent was removed by distillation *in vacuo*, water (10 ml.) was added and the solution again distilled *in vacuo* to remove excess of

hydrochloric acid. The white crystalline residue was dissolved in water (100 ml.), extracted with ether (20 ml.), the ethereal extract being discarded, and a saturated aqueous solution of silver acetate (84 mg.) was added. The resulting suspension was heated on a steam bath for 1 hr.; then the silver chloride was removed by filtration and washed with water (100 ml.). Hydrogen sulphide was passed into the combined filtrate and washings, and the precipitated silver sulphide removed by filtration. The solvent was removed from the filtrate by distillation *in vacuo*, leaving a white crystalline residue of valine (0·52 g., 88% yield, m.p. 280–285°, 1·99 mc/m-mole). This represents an overall recovery of isotope of 8·8%. Radioactivity measurements were performed on solid samples by the method of Banks *et al.* (1956*b*).

Degradation of [$\alpha\beta$-^{3}H]*valine*

Oxidation to iso*butyraldehyde.* [$\alpha\beta$-^{3}H]Valine (100 mg., 9·9 μc/m-mole) dissolved in water (10 ml.) was heated with ninhydrin (600 mg.) and the product removed by steam-distillation for 20 min. The distillate was collected in a solution of 2:4-dinitrophenylhydrazine (200 mg.) in ethanol (20 ml.) and conc. sulphuric acid (1 ml.). A thick yellow precipitate rapidly formed which, after two recrystallizations from ethanol, yielded *iso*butyraldehyde 2:4-dinitrophenylhydrazone (36·8 mg., 10·0 μc/m-mole, m.p. 182°).

Oxidation to iso*butyric acid.* [$\alpha\beta$-^{3}H]Valine (200 mg., 3·71 μc/m-mole) was oxidized with ninhydrin as described above, and the *iso*butyraldehyde was passed into a small excess of alkaline potassium permanganate solution. Manganese dioxide and excess of permanganate were removed by filtration, acidification with dilute sulphuric acid and addition of ferrous sulphate solution. The product was extracted with ether (3 × 10 ml.) and 0·5% ammonia solution (3 ml.) was added to the combined ethereal extracts. The aqueous layer was separated and added to an aqueous solution of silver nitrate (300 mg., 0·5 ml.). The precipitate of silver *iso*butyrate (80 mg., 0·73 μc/m-mole) was isolated by centrifuging.

Oxidation to acetone. [$\alpha\beta$-^{3}H]Valine (150 mg., 101 μc/m-mole) was boiled under reflux for 30 min. with chromium trioxide (0·3 g.) and acetic acid (5 ml.), and the product was steam-distilled into a solution of 2:4-dinitrophenylhydrazine (300 mg.) in ethanol (20 ml.) and conc. sulphuric acid (1 ml.). After four crystallizations from ethanol, acetone 2:4-dinitrophenylhydrazone (m.p. 124°, 58 mg., 2·87 μc/m-mole) was obtained.

DL-[β-^{3}H]*Valine*

[$\alpha\beta$-^{3}H]Valine (1·0 g., 4·1 μc/m-mole) was heated under reflux for 1 hr. with acetic anhydride (5 ml.) and acetic acid (20 ml.). The solvent was removed by distillation *in*

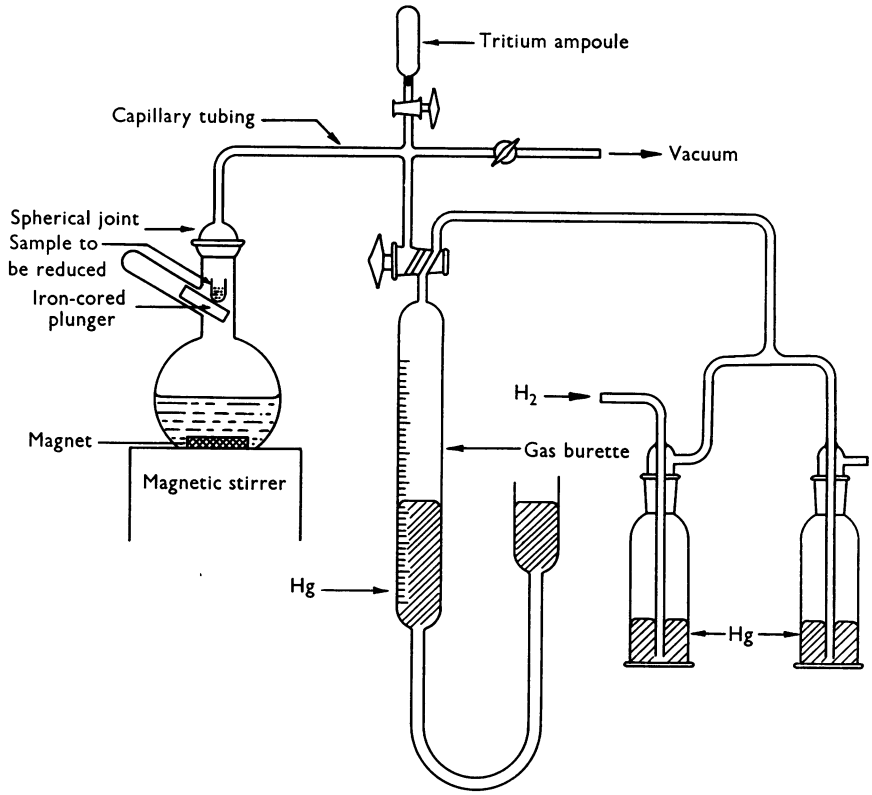

Fig. 1. Apparatus for catalytic hydrogenations with tritium. The tritium ampoule shown vertically in this diagram in fact lay horizontally behind the apparatus. The catalyst was first reduced with hydrogen and the plunger was then withdrawn by a magnet so that the sample dropped into the solvent.

vacuo, leaving a pale-yellow oil. 6N-Hydrochloric acid (20 ml.) was then added and the solution was heated under reflux for 3 hr. The acid was removed by distillation *in vacuo*, the solid residue was dissolved in ethanol (10 ml.) and [β-^{3}H]valine (0·6 g., 0·81 μc/m-mole) was precipitated by addition of aniline.

DL-[α-^{3}H]*Valine*

DL-Valine (2·4 g.) was heated under reflux with acetic anhydride (30 ml.) and tritiated water (1·4 ml., approx. 20 mc/ml.) for 1½ hr. The solvent was removed by distillation *in vacuo*. 6N-Hydrochloric acid (20 ml.) was added and the solution was heated under reflux for 6 hr. The acid was removed by distillation *in vacuo*, the solid residue was dissolved in ethanol (20 ml.) and [α-^{3}H]valine (1·6 g., 183 μc/m-mole) precipitated on addition of aniline.

Administration of DL-[β-^{3}H]- *and* DL-[γ-^{14}C]*valine*

Two female albino rats (combined wt. 580 g.) were starved for 24 hr., then were each fed with a mash containing twelve powdered food pellets mixed with 4·5 ml. of a solution (10 ml.) of DL-[β-^{3}H]valine (58·8 mg., 0·75 mc) and DL-[γ-^{14}C]valine (0·705 mg., 6·25 μc) which was obtained from the Radiochemical Centre, Amersham, Bucks. A portion (1 ml.) of this solution was retained as a control. The rats were killed 36 hr. later and were dissected immediately.

Extraction of amino acids from visceral proteins. Liver, kidney, heart, lungs, spleen and gonads from each rat were homogenized together in 95% ethanol, and the homogenate was washed twice with 95% ethanol (total vol. 250 ml.) by centrifuging. The residue was then washed with trichloroacetic acid (21% w/v, 3 × 50 ml.). Finally the residue was washed with ethanol and ether, and dried at 37°. The product was hydrolysed by heating under reflux for 18 hr. with 6N-hydrochloric acid (100 ml.) together with a trace of octan-2-ol to prevent frothing. The acid was removed by distillation *in vacuo* and the residue dissolved in water (200 ml.). The solution was approximately neutralized, and sodium carbonate solution (10%, w/v, 75 ml.) and mercuric acetate solution (25%, w/v, 75 ml.) were added simultaneously dropwise with stirring. Ethanol (400 ml.) was added and the mixture left at 0° overnight. The precipitate formed was separated by centrifuging, washed twice with ethanol (66%, w/v, 100 ml.) and suspended in water (200 ml.) and the suspension was saturated with hydrogen sulphide. Mercuric sulphide was removed by centrifuging, and the water by distillation *in vacuo*.

Separation of valine. The mixture of amino acids was dissolved in 1·5N-hydrochloric acid (10 ml.), and the solution was poured into an ion-exchange column (138 cm. × 3 cm.) packed with Zeo-Karb 225 ion-exchange resin (Permutit Co. Ltd., London, W. 4). The column was eluted with 1·5N-hydrochloric acid, and the effluent fractions (50 ml.) were collected on a Towers fraction-collector. The first 2 l. of eluate contained glycine, alanine, serine, glutamic acid and aspartic acid. No amino acids were present in the next four fractions; then valine exclusively was eluted. The fractions containing valine were combined, the solvent was removed by distillation *in vacuo* and valine hydrochloride separated from non-amino acid impurities by solution in ethanol. Valine (77·6 mg., 4·6 μc of ^{3}H/m-mole) was precipitated by the addition of aniline to the ethanolic solution.

RESULTS

The reduction of 2-phenyl-4-*iso*propylideneoxazol-5-one with hydrogen on Adams catalyst, followed by hydrolysis of the product, gave an 88% yield of valine, but the radioactive yield was only 9% of the nominal radioactivity of the sample of tritium (supplied by A.E.R.E., Harwell, Berks).

To test whether hydrogen atoms were being absorbed from the hydrogen–tritium mixture preferentially to tritium atoms, the hydrogen gas remaining in the reaction vessel at the end of the reduction was withdrawn, and a second reduction was carried out with this residual gas. Subsequently more inactive hydrogen was added until this reduction was complete. Valine isolated from this reduction contained only 1% of the radioactivity of that from the first reduction, which indicated that most of the tritium atoms were absorbed in the first reduction.

The oxidative decarboxylation of [$\alpha\beta$-^{3}H]valine with ninhydrin to *iso*butyraldehyde took place without loss of radioactivity. Analogous oxidations with alloxan, chloramine-T, hydrogen peroxide and acidified potassium permanganate each gave rise to *iso*butyraldehyde in which no loss of radioactivity had occurred. These results are summarized in Table 1.

It was shown by oxidation of [$\alpha\beta$-^{3}H]valine to *iso*butyric acid, which takes place with the loss of the α-hydrogen atom only, that the amount of tritium entering the α and β positions as a result of the reduction of the double bond of the *iso*propylideneoxazolone was not the same, and further that reductions carried out on different occasions gave rise to a different proportion of tritium entering the α and β positions. The oxidation of one sample of [$\alpha\beta$-^{3}H]valine is described in the experimental section. The ratios of tritium in the α and β positions as the result of three other reductions are shown in Table 2.

Table 1. *Comparison of specific activities of* [$\alpha\beta$-^{3}H]-*valine and the corresponding* iso*butyraldehyde dinitrophenylhydrazones derived by different oxidation procedures*

Method of oxidation	[$\alpha\beta$-^{3}H]Valine (μc/m-mole)	*iso*Butyraldehyde 2:4-dinitrophenylhydrazone (μc/m-mole)
Ninhydrin	9·9	10·0
Alloxan	9·3	9·1
Chloramine-T	27·1	27·1
Hydrogen peroxide	9·0	9·4
Acidified potassium permanganate	9·0	8·7

These results were confirmed by treatment of [$\alpha\beta$-^{3}H]valine with hot acetic anhydride and acetic acid, as under these conditions the α-hydrogen atom of an amino acid is made labile. Valine was reisolated from the reaction mixture and the loss of radioactivity corresponded to that expected to be in the α position in accordance with the above oxidation experiments. Repetition of the acetic anhydride treatment caused no further loss of activity.

Acetone 2:4-dinitrophenylhydrazone, obtained by oxidation of valine with chromium trioxide, contained about 3 % of the original radioactivity. This small amount could be accounted for by contamination of the product with active *iso*butyraldehyde dinitrophenylhydrazone, which might have been removed incompletely by recrystallization. Inactive acetone heated under reflux (under similar conditions) with chromium trioxide and a little tritiated water did not contain radioactivity when reisolated as its dinitrophenylhydrazone. This indicated that the hydrogen atoms of acetone were not labile under the conditions of this experiment and any tritium originally in the γ position of valine would have been detected in the acetone which was isolated from its oxidation. It seems probable that only a very small proportion, if any, of the radioactivity resided in the γ position of valine.

[β-^{3}H]Valine, prepared as described above, was mixed with [γ-^{14}C]valine and fed to two rats. Subsequently the valine was reisolated from the combined visceral proteins. The ^{14}C content of the isolated valine was measured on a thin-window Geiger counter. The combined ^{14}C and ^{3}H activities were measured on a windowless flow-type counter (Banks *et al.* 1956*b*). The contribution of ^{14}C to the total count in the windowless counter was determined by preparing a sample of [^{14}C]valine which was counted first on a thin-window counter and then on a windowless Geiger counter. The necessary correction was then applied to the count rate observed with the isolated valine, and the ^{3}H activity was determined by difference. The ratio of ^{14}C to ^{3}H in the prepared valine mixture was adjusted so that the contributions of ^{14}C and ^{3}H to the total count in the windowless counter would be of the same order. The ratio of ^{14}C to ^{3}H in the isolated valine was found to be the same as that in the administered valine (Table 3).

In a second experiment [$\alpha\beta$-^{3}H]valine alone was fed, and the ratio of tritium in the α and β positions of the isolated valine was compared with that in the administered valine. Whereas initially the ratio of tritium in the α and β positions was 1·96:1, the ratio in the isolated valine was 0·76:1.

Table 2. *Ratio of tritium attached to the α and β positions of valine obtained in four separate reductions of 2-phenyl-4-isopropylideneoxazolone*

Expt. no.	α:β ratio
1	2·16:1
2	2·08:1
3	3·85:1
4	4·14:1

DISCUSSION

Synthesis of DL-[$\alpha\beta$-^{3}H]*valine*

We wished to have available a tritium-labelled amino acid which would be suitable for preparing radioactively labelled antibodies and decided to prepare tritium-labelled valine for the following reasons. The method described here is simple and the isotope is introduced only one stage before the final product is obtained. The isolation of this amino acid by the original method of Stein & Moore (1950) is easy; further, valine is an essential amino acid and so is not diluted by endogenously synthesized material.

2-Phenyl-4-*iso*propylideneoxazolone is a readily available unsaturated precursor of valine and the reduction of this oxazolone with hydriodic acid and phosphorus (Adams & Tolbert, 1952) and by lithium aluminium hydride (Baltazzi & Robinson, 1953) has been reported. The reduction of 2-phenyl-4-*iso*propylideneoxazol-5-one in the presence of tritium with Adams catalyst gave a good yield of valine, but the radioactive yield was only 9 % of tritium nominally available. It has not been possible to improve this radioactive yield.

The possibility that hydrogen is being preferentially used for the reduction is not supported by the observation of Williams & Ronzio (1950) that reductions can take place slightly faster with pure tritium than with hydrogen. Further, we have used the residual gas from our first reduction in effecting an analogous reduction on a second specimen of

Table 3. *Comparison of the radioactivities of administered and isolated* ^{3}H- *and* ^{14}C-*labelled valine from rat-visceral proteins*

	Counts/min. on windowless counter from ^{14}C	Counts/min. on windowless counter from ^{3}H	Ratio ^{14}C:^{3}H
Administered valine	760	1472	1:1·94
Isolated valine	650	1321	1:2·06

*iso*propylideneoxazolone; the product was found to contain only a very small amount of radioactivity.

The hydrogen at position 4 of 4-alkyloxazolones is labile in certain circumstances (Neuberger, 1948), and there is a possibility that some tritium may have ionized before the ring was opened. All precautions were taken to keep the solvent anhydrous and the temperature low before hydrolysis took place, and more tritium was found in the α than in the β position in the valine synthesized. Attenburrow, Elliott & Penny (1948) have shown that catalytic reduction of an alkenyloxazolone can take place in the oxazolone ring as well as in the side chain, but the high yield of product when only 1 mole-equiv. of hydrogen was absorbed makes this possibility unlikely in our experiments. Pearlman (1957) also has observed an apparently poor incorporation of tritium during a catalytic reduction, though his yield of product was high; further investigation is being made to test the hypothesis that some of the tritium may have been absorbed on the glass of the ampoule which contains it.

Location of the tritium atoms in DL-[αβ-^{3}H]*valine*

Valine was allowed to react with ninhydrin under the conditions of Virtanen & Rautanen (1947). The *iso*butyraldehyde which was isolated had the same specific activity as its parent valine. This retention of activity was observed also when valine was oxidized by acid potassium permanganate, hydrogen peroxide, chloramine-T and alloxan. Thus the α-H atom of valine is not made labile during oxidation to the corresponding aldehyde, as is required by the mechanism of Wieland & Bergel (1924). Bergel & Bolz (1933) and Herbst & Clarke (1934) have shown that α-substituted α-amino acids can be oxidized under mild conditions, with the liberation of carbon dioxide and ammonia. Thus the presence of an α-hydrogen atom is not essential for the reaction. A preliminary communication discussing a possible mechanism for this oxidation has been published (Spenser, Crawhall & Smyth, 1956). Schoenheimer & Rittenberg (1935) drew attention to the lability of hydrogen atoms adjacent to carbonyl groups in aldehydes and ketones. There is much evidence for the lability of hydrogen atoms that are α with respect to keto groups (Halford, Anderson & Bates, 1934), when enolization can occur; however, our experiments indicate that the conditions used by us do not result in loss of hydrogen from the C-1 or C-2 positions of *iso*butyraldehyde.

In order to determine the distribution of tritium in the α and β positions of our synthesized valine, this *iso*butyraldehyde was further oxidized by alkaline potassium permanganate to *iso*butyric acid, thus removing the hydrogen atom on C-1 of the aldehyde. The specific activity of the silver salt of the *iso*butyric acid was determined, the difference between the specific activities of this salt and of the starting material representing the activity originally on the α position of the αβ-tritiated valine.

This distribution of tritium was confirmed by an alternative procedure. It seemed probable that the α hydrogen atom of valine would be removed under conditions which caused racemization of the amino acid (Neuberger, 1948). Attempts to remove the α-tritium atom from [αβ-^{3}H]valine by heating with alkali were unsuccessful, as valine appeared most resistant to racemization with alkali. Racemization of amino acids also occurs when an oxazolone derivative of the amino acid is prepared (Baltazzi, 1955). We found that the specific activity of [αβ-^{3}H]valine decreased when this compound was treated with acetic anhydride under the conditions of Bergmann & Köster (1926). On repeating this treatment no further loss of radioactivity occurred. The two methods gave results in close agreement. The ratio of tritium in the α and β positions was not unity, the isotope being concentrated in the α position. In all, four radioactive syntheses of this type have been carried out and the α:β ratios have not been constant. Table 2 shows that in two experiments the ratio was approximately 2:1, and in the others approximately 4:1. It is known that the energies of the C–^{3}H and C–^{1}H bonds are very different (Eidinoff, 1953), and further that the distribution of electrons about the C-4 atom of the oxazolone ring is different from that about the unsaturated carbon atom in the side chain. It is likely that the activation energy required to introduce a hydrogen isotope into one position is different from that of the other; thus more isotope might be expected to enter one position than the other. Possibly the catalyst operates in such a way that hydrogen atoms enter the extracyclic end of the double bond faster than tritium atoms do.

It is more difficult to find an explanation for the variation in ratio of incorporation. Four experiments are too few to establish a statistical relationship; but, in fact, a different specimen of catalyst was used on each occasion, and we observed that where a rapid reduction took place the subsequent isotopic distribution was approximately 4:1, whereas with slower reductions the distribution was approximately 2:1. It may be that isotopic selection was not as prominent when a relatively inactive catalyst was used. A possible case of unsymmetrical incorporation of tritium has been reported by Glascock & Reinius (1956). It has been shown (Clarke, Johnson & Robinson, 1949) that labilization of hydrogen on C-4 of oxazolones occurs after ring closure, and this could have some bearing on the α:β isotopic ratio observed in our valine syn-

thesis. That this ratio is greater than unity, and not less, suggests that very little migration of tritium from C-4 of the oxazolone takes place at the hydrolysis stage. After completion of the reduction and removal of the solvent by evaporation, concentrated hydrochloric acid is added and the solution is heated under reflux. These conditions must lead to a rapid opening of the ring, or much of the C-4 label would be lost, whereas once the ring is opened the α-hydrogen atoms are stabilized. This effect is demonstrated in experiments in which an α-labelled amino acid is obtained by treating an inactive amino acid with acetic anhydride and acetic acid labelled at the carboxyl hydrogen. [α-^{3}H]Valine was prepared in this manner in good radioactive yield.

Incorporation of labelled valine into rat-visceral proteins

The second isotope of hydrogen, deuterium, has been used in many investigations into biological processes, though the occurrence of an 'isotope effect' has been reported (Thorn, 1951). With the advent of tritium as a commercially available isotope, this 'isotope effect' has been more prominently demonstrated (Verly, Rachele, du Vigneaud, Eidinoff & Knoll, 1952; Glascock & Duncombe, 1952) and has tended to discourage the use of tritium as a biological label. However, tritium possesses advantages over ^{14}C in that it may be easier to incorporate into an organic molecule, that it is cheaper than ^{14}C, and that much greater specific activities can be obtained with tritium. A previous study of the methods of estimating tritium (Banks *et al.* 1956*b*) has shown that it can be estimated in the solid state as well as in the gaseous phase.

In spite of the considerable isotope effect which can occur with tritium, we thought it possible that, if the tritium were present in a molecule at a point remote from one exposed to enzymic activity, no isotope effect would occur. To investigate this we prepared [β-^{3}H]valine and compared its incorporation into proteins with that of [γ-^{14}C]valine.

It is known that valine can undergo transamination *in vivo* (Cammarata & Cohen, 1950) and also that D-valine, after de-amination and re-amination, can be utilized in protein synthesis (White, Fones & Sober, 1952). Each of these reactions results in loss of α-hydrogen, and therefore for our experiment we compared [β-^{3}H]valine with [γ-^{14}C]valine. The former was prepared from [αβ-^{3}H]valine by the acetic anhydride treatment described above.

When valine labelled with these two isotopes was fed to two rats and the visceral proteins were isolated after 36 hr., it was found that the ratio ^{3}H:^{14}C in the isolated valine was identical with the ratio ^{3}H:^{14}C in the ingested valine. Clearly, during the process of transference across the gut, and in the incorporation of valine into proteins, no significant isotope effect has occurred. [β-^{3}H]-Valine is therefore suitable for studying protein biosynthesis. An earlier experiment has been described in a preliminary communication (Crawhall & Smyth, 1955).

In a second experiment in which [αβ-^{3}H]valine was fed to rats, valine was isolated as in the first experiment. Some of the α-tritium still remained attached to valine after its incorporation into protein. Thus complete transamination of this amino acid has not occurred within the time interval of feeding and isolating isotopic valine. This result is in agreement with the observations of Sprinson & Rittenberg (1950) on a comparable experiment with deuterium-labelled leucine.

SUMMARY

1. The synthesis of [αβ-^{3}H]-, [β-^{3}H]- and [α-^{3}H]-valine is reported.

2. The distribution of tritium between the α and β positions of [αβ-^{3}H]valine in this synthesis was found to be unequal, and varied in successive preparations.

3. Oxidation of [αβ-^{3}H]valine with a variety of reagents to *iso*butyraldehyde takes place without loss of isotope, indicating retention of α-hydrogen during the reaction.

4. [β-^{3}H]Valine is incorporated into rat visceral proteins at the same rate as [γ-^{14}C]valine.

5. The α-tritium label of [αβ-^{3}H]valine is only partially removed during the incorporation of [αβ-^{3}H]valine into rat visceral proteins.

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ADDENDUM

The Biosynthesis of a Tritium-Labelled Antibody

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Antibodies labelled with a radioactive isotope were required for a biological investigation. Previously ^{14}C-labelled rabbit antibodies have been synthesized by Cohen, Holloway, Matthews & McFarlane (1956). In view of the very low isotopic yield in these biosynthentic preparations and the high cost of ^{14}C-labelled precursors, it was decided to biosynthesize tritium-labelled antibodies to bovine serum albumin in a rabbit by injection of DL-[$\alpha\beta$-^{3}H]valine.

A rabbit was immunized against bovine albumin by three intramuscular injections of the alum-precipitated antigen made according to the directions of Proom (1943); 16 days after the last of these injections, when its serum contained a high concentration of antibody, the rabbit was injected intraperitoneally with DL-[$\alpha\beta$-^{3}H]valine (200 mg.; 3 mc) and a sample of blood was withdrawn from the ear 7·5 hr. later, preliminary experiments having shown that the radioactivity of the serum proteins was at a maximum at this time interval after the administration of the radioactive valine. The blood was allowed to clot and the serum was dialysed against 0·9 % sodium chloride solution, containing about 0·2 % of valine, with daily changes for a week to remove any free valine that might have been circulating in the rabbit's plasma.

The total serum proteins were precipitated at an ethanol concentration of 80 % (v/v). The precipitate was washed with 95 % ethanol and then with ether, and dried *in vacuo*. γ-Globulins were separated by precipitation with sodium sulphate solution, one precipitation at 15 % (w/v), followed by one at 12 % (w/v) (cf. Kekwick, 1940). Sodium sulphate was removed from the precipitate by dialysis, and the γ-globulin was reprecipitated

Table 1. *Radioactivity of various protein fractions after injection of* DL-[$\alpha\beta$-^{3}H]*valine* (200 *mg.*, 3 *mc*)

Radioactivity measurements were made in a windowless flow-type counter by using 10 mg. samples mixed with 10 mg. of graphite, as described by Banks *et al.* (1956). The observed activity of 110 counts/min. (last row of table) has been corrected for the non-radioactive antigen content of the antigen–antibody complex. The precipitate was assumed to contain five-sixths of its weight of antibody, a fraction which is regularly found when the precipitates are formed in the equivalence zone in this system (Francis, G. E. & Hawkins, J. D., unpublished observations).

	Observed activity (counts/min.)	Specific activity (μc/g.)
Radioactivity of total serum protein	363	5·0
Radioactivity of γ-globulin fraction	244	3·4
Radioactivity of antibody to bovine albumin	110	1·5

with ethanol (final concn. 80%, v/v); the precipitate was washed with ethanol and with ether and dried *in vacuo*. For the isolation of the antibody the serum was roughly calibrated for antibody content and antigen was added in such amount that the precipitate was formed in or near the equivalence zone. The precipitate was washed with 0·9% sodium chloride solution and then with water, and dried *in vacuo*. The radioactivity measurements were made in a windowless flow counter as described by Banks, Crawhall & Smyth (1956). The results are shown in Table 1.

If the total circulating plasma volume of the rabbit (4·13 kg.) is assumed to be 140 ml. and the plasma protein concentration 7%, 9·8 g. of plasma protein will be circulating. Thus at 7·5 hr. the total radioactivity incorporated into the plasma proteins was 49 μc, or 1·6% of the radioactivity administered. The actual count rate of the antibody fraction can be increased more than 100-fold by converting the hydrogen of the samples into methane and measuring the radioactivity of this gas (Banks *et al.* 1956).

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The Use of Radioactive Isotopes in Immunological Investigations

13. THE ROLE OF LEUCOCYTES AND NON-PLASMA ANTIBODY IN THE REMOVAL OF ANTIGENIC PROTEINS FROM THE BLOOD STREAM OF IMMUNIZED RABBITS*

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In a previous paper (Francis, Hawkins & Wormall, 1957) the behaviour of intravenously injected trace-labelled native proteins in normal and immune rabbits has been described. It was shown that, provided that suitable doses of antigen were injected, the immune rabbits rapidly eliminated some or all of the injected protein from the blood stream by a first-order reaction whose half-life was of the order of 5–10 min. This rapid elimination was believed to be dependent on the reaction of the injected antigen with circulating antibody. It was therefore decided to compare the amount of antigen that can be eliminated rapidly from the blood stream of an immune rabbit with the amount of antigen that could be precipitated *in vitro* by all its serum, under conditions as nearly comparable as possible with those prevailing *in vivo*.

It was found that more antigen could be rapidly eliminated from the blood after intravenous injection than could be precipitated *in vitro* when the same amounts of antigen/ml. of serum were used in the two experiments. This suggested that the injected antigen might be reacting not only with circulating antibody, but also with additional antibody not present in the circulation but capable of being made available with great rapidity. However, it as also possible that these two determinations were made under such different conditions that a direct comparison between them was not valid. In order to decide between these two possibilities some similar experiments were carried out on rabbits 24 hr. or more after passive immunization, when all the antibody should be in the plasma or in fluids directly in equilibrium with it, and on rabbits at different stages during and after an immunization schedule.

A further possibility was that this non-plasma antibody might be released from lymphocytes circulating in the plasma and lymph, or from lymphoid tissue, since these cells are known to contain antibody.

Some immunized rabbits were therefore injected with cortisone, which has been reported to cause the liberation of considerable amounts of antibody from lymphocytes (Dougherty, Chase & White, 1945), to see if this treatment would have any effect on the relative amounts of antigen eliminated rapidly from the blood stream and precipitated *in vitro* by the animal's plasma. At the same time, both normal and immunized rabbits were injected with antigen, and total and differential leucocyte counts were made before and at intervals after antigen injection. The results of these experiments have confirmed and amplified those of Stavitsky, Stavitsky & Ecker (1949) and Stavitsky (1952).

* Part 12: Hawkins (1957).