and move upwards to the shoot system some accumulation might take place in the roots of decapitated plants, provided that precursors were present in sufficient amount. However, no accumulation was observed in the roots of decapitated plants, at least during the period of one week after decapitation. The higher concentration of alkaloids in the roots of plants in the resting stage could be attributed either to a downward movement from the shoot system at the end of the growing season or, if synthesis takes place to any extent in the roots, to a restriction in upward movement to the leaves. The possibility that the root is an active site of synthesis cannot be ruled out, for small amounts of γ -coniceine and coniine are present in the roots of young plants, and traces of an unknown compound (R_f 0.21) which gave a red colour with nitroprusside were detected in bleeding sap. If upward transport of alkaloid from roots to shoot were vigorous the concentration of alkaloids in the root system may remain very low even if active synthesis is taking place in the root. Nevertheless, the conclusion is reached that in *C. maculatum* alkaloid synthesis appears to take place more readily in the tissues of the shoot than in the root.

SUMMARY

1. The alkaloids of *Conium maculatum* have been separated by the method of paper chromatography.
2. Methods for the micro-estimation of the individual alkaloids have been described.
3. These methods have been used for a study of the distribution of the alkaloids in the tissues of the plant during the phases of growth and reproduction.
4. The major alkaloid of *C. maculatum* in the vegetative state is γ -coniceine. In flowers and immature fruits the content of coniine and *N*-methylconiine progressively increases and the con-

tent of γ -coniceine decreases during development. In mature fruits the major alkaloid is *N*-methylconiine.

5. The significance of these results is discussed in relation to the synthesis of the alkaloids in the plant.

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Investigations on Rat Serum Albumin Marked with Tritium-Labelled Leucine

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Limitations that have been found in the use of isotopes for the investigation of protein metabolism include the low sensitivity of the techniques of measurement of stable isotopes and, with radio-active isotopes, the difficulty and expense of preparing labelled protein of sufficiently high specific activity.

Although ^3H -containing hydrogen of high specific activity has been available for some time, at comparatively low cost, its use as a label has not been extensive, owing to the lack of a convenient method of assay. However, routine assay is now possible by the combustion-bomb method of production of gas for counting (Payne & Done, 1954), followed by

proportional counting (White, Campbell & Payne, 1950). This combination of techniques gives a method which is at least as sensitive, accurate and convenient as those usually employed for the assay of other isotopes in biological materials.

Tritium was introduced into leucine, since blood albumin contains a considerable proportion of this amino acid and a convenient method of synthesis is available. The specific activity of serum albumin obtained from rats to which this labelled amino acid had been fed was high enough to permit accurate assays to be made on small samples after high dilutions.

Serial measurements were made on samples from an individual rat taken at times of up to 400 hr. after injection of only 0.11 μC of labelled albumin. It was also possible to assay similar samples from a malnourished rat on a low protein diet. Studies have been made of the effects of some of the conditions of paper electrophoresis on rat serum albumin and also of the specific activities of leucine released from the albumin during acid hydrolysis.

The determination of body water of rats with the aid of tritium is described.

EXPERIMENTAL

Tritium assay

Samples were burnt in oxygen under pressure in a metal bomb (Payne & Done, 1954); the gas mixture for counting was generated in the bomb by the action of aluminium carbide on the water produced by the combustion. For counting, the gas was pumped into a type GA 26 tube (20th Century Electronic Ltd., London) to a pressure of 20 cm. Hg. The tube was operated in the proportional region as described by White *et al.* (1950). Two 1300 v corona stabilizers (Nucleonic and Radiological Developments, Ltd., London) were used in series to supply an anode voltage of 2600 v.

Preparation of gas for counting. Two modifications have been made of the combustion-bomb technique (Payne & Done, 1954): (i) immediately before introduction of the oxygen, the lower part of the bomb is cooled, first by tap water, then by immersion in solid CO_2 -propanol to a depth of 2 in.; this counteracts a tendency of the water formed during combustion to get into the valve when the oxygen is released; (ii) it has been found necessary to change the time of heating the bomb to suit each batch of carbide: heating times of 30, 40 and 60 min. have been used. Insufficient heating gives rise to spurious counts, owing, apparently, to unchanged water vapour in the counting gas.

Selection of suitable aluminium carbide. Samples of aluminium carbide which contain large amounts of free or combined water cannot be used for the generation of the gas for counting. A loss of sensitivity will result from dilution of the active water of the samples to be assayed. Samples of Al_4C_3 from three sources have been tested and one of these (Baird Chemical Corporation, New York) proved to be suitable. This material was supplied as fused lumps containing a high proportion of yellow crystals. It was crushed, ground to a fine powder (approx. 180 mesh), and stored in

air-tight jars. When heated to redness in a tube, no visible water vapour was produced, there was no odour, and no loss of weight occurred. The other two samples were pale brown to green powders; on gentle heating these samples yielded a large amount of water vapour and gas with a strong odour. The loss of weight was 30%.

A portion (3.0 g.) of one of the latter samples was reacted with 0.3 ml. of standard active water in a combustion bomb. The count rate with this filling was 4.8 counts/sec. When replaced by a filling generated from the same standard active water and 3.0 g. of the first carbide sample, the count rate recorded was 33.7 counts/sec. Subsequently, a series of 15 assays of dilutions of standard active water, with this carbide, gave count rates which were reproducible to within 1% (Payne & Done, 1954).

Preparation of samples for combustion. Water volumes of 250–300 μl . are convenient for the preparation of gas for counting.

Procedure A. For samples of the order of 10^{-5} $\mu\text{C}/\text{mg.}$, a known amount of filter paper of known isotope dilution was used as a carrier. Pieces of Whatman no. 3MM paper, approx. 8 cm. $\times$ 3 cm., were dried to constant weight in a stream of hot air from an industrial blower (Type AM, Bylock Electric Ltd., Enfield, Middlesex) and the dry weights recorded. The papers were folded, wrapped round the filament of the combustion bomb, and secured with a loop of tungsten wire. Solutions of active materials were pipetted on the papers, which were again dried with hot air before combustion. Papers mounted on a filament as described, and having 150 μl . of water pipetted on them, were dried to constant weight in 3 min. The dimedone derivative of isovaleraldehyde was found to be slightly volatile, and a loss of activity of 15% was observed after drying with hot air. A cool air stream was accordingly used, and a correction made for the moisture retained by the paper.

Procedure B. Samples of specific activity of the order of 10^{-7} $\mu\text{C}/\text{mg.}$ were enclosed in cigarette paper in powder form (Payne & Done, 1954).

Calculation of specific activities. Active water formed by combustion of the sample is diluted by water from the paper. Also, the counter filling is only a fraction of the total gas produced. Thus, if m is mass of active substance; M is mass of diluting material; f_m is water equivalent of m ; f_M is water equivalent of M ; W is water equivalent of gas in counter tube; x is count rate; A is total counts in sample, and S is specific activity of sample, then the fraction of the sample in the counter tube is given by $W/(mf_m + Mf_M)$ and

$$A = \frac{x(mf_m + Mf_M)}{W} \quad (1)$$

In procedure A, $mf_m \ll Mf_M$ and equation (1) becomes $A = (x/W) Mf_M$, i.e. $S = xMf_M/mW$. For a series of samples of constant organic composition, xM/m is proportional to specific activity, since f_M and W are constant. In procedure B, mf_m is of the same order of magnitude as Mf_M . The values of both f_m and f_M are required. The value of f_M for the cigarette paper used to hold the larger samples in position on the heater filament was determined by combustion of known weights of papers after the addition of standard active water to the bomb (Payne & Done, 1954). The value f_m may be determined by performing two combustions with different weights of sample and paper. Thus if m_1 and m_2 are the weights of the two samples, M_1 and M_2 are the weights of

the two papers, and x_1 and x_2 are the count rates recorded, from (1) we have

$$WS = x_1(m_1f_m + M_1f_M)/m_1 = x_2(m_2f_m + M_2f_M)/m_2$$

$$\text{i.e. } f_m = f_M \frac{x_2 \left(\frac{M_2}{m_2} \right) - x_1 \left(\frac{M_1}{m_1} \right)}{x_1 - x_2}.$$

Synthesis of DL-[^3H]leucine

DL-[^3H]Leucine was prepared by adaptation of the method of Albertson & Archer (1945). Whereas in the original method ethyl 2-acetamido-2-ethoxycarbonyl-4-methyl-4-pentenoate (I) in ethanol was hydrogenated at 40 lb./in.² pressure with Raney nickel catalyst, the hydrogenation with ^3H was carried out at atmospheric pressure with Adams catalyst in order to minimize excess hydrogen. Chloroform was used as solvent in view of the exchangeable OH-H of ethanol. A 50 ml. flask containing (I) (2 g.), Adams catalyst (100 mg.) and chloroform (5 ml.) was immersed in solid CO_2 -propanol, and connected to a vacuum line, Töpler pump and reservoir containing active gas.

The flask and connecting tubing were alternately evacuated and flushed with hydrogen several times. The solid CO_2 -propanol was removed. When the apparatus reached room temperature, the contents of the flask were stirred magnetically and active gas from the reservoir was introduced. The pressure was kept at about 1 atm. In 3 hr. the catalyst flocculated and gas absorption had apparently ceased. Stirring was continued for 0.5 hr. and then the apparatus was flushed with hydrogen. The product was transferred to another 50 ml. flask through a filter stick, and the Pt washed with a few drops of chloroform. Chloroform was removed by evaporation at reduced pressure, and then hydrolysis and isolation of leucine were brought about by the procedures of Albertson & Archer (1945), the product being washed with chilled water. Leucine (0.78 g.) was obtained [yield 83 %, based on (I)]. The specific activity was $3.35 \pm 0.046 \mu\text{C}/\text{mg.}$ (determined by procedure A above). The activity was quantitatively recovered after addition of inactive DL-leucine followed by recrystallization, oxidation with ninhydrin or paper chromatography in butanol-acetic acid (Partridge, 1948). An earlier batch of (I) converted into DL-leucine by the above procedure but by the use of inactive hydrogen gave 0.74 g. of leucine (yield 80 %, N (micro-Kjeldahl) 10.7 %. $\text{C}_6\text{H}_{13}\text{O}_2\text{N}$ requires 10.6 %.

Labelled isovaleraldehyde from DL-[^3H]leucine

The procedure of Virtanen, Laine & Toivonen (1940) was modified so that a Markham still (Markham, 1942) could be used. Steam was passed through the outer jacket of the still, and 155 mg. of ninhydrin was introduced into the inner chamber with the minimum of hot water. A portion (20 mg.) of the recrystallized mixture of DL-[^3H]leucine and inactive DL-leucine (see Table 3) was dissolved in 20 ml. of hot water, together with 2 g. of KH_2PO_4 and 3 g. of NaCl, and the cooled solution introduced into the inner section. After a few bubbles of steam had been passed through the reaction mixture for stirring, the jacket was left open for 0.5 hr. Steam distillation for 0.5 hr. then followed. The receiving flask was immersed in solid CO_2 -propanol mixture throughout the experiment. The distillate was warmed to room temperature and treated with 75 ml. of dimesone solution (Virtanen *et al.* 1940) containing 1 drop of piperidine. The mixture was left overnight and filtered. The product was

recrystallized from aqueous ethanol [yield 53.5 mg., m.p. 156° (uncorr.), unchanged when mixed with an authentic sample].

Animal experiments

Male albino rats (Wistar strain) were used. The animals, fed on a stock diet (cube diet supplied by Oxo Ltd., London) were given, once a week, about 10 g./100 g. body wt., a mixture of fresh liver (1 part) and skimmed milk powder (1.5 parts).

'Donor' animal. Rat T16 (wt. 329 g.) was fasted for 24 hr. and then fed with 4 g. of chopped fresh liver, mixed with 117 mg. ($326.4 \mu\text{C}$) of the labelled leucine.

The animal was placed in a box through which a current of air was drawn by means of a water pump. Air from the box was passed through a U-tube immersed in a solid CO_2 -propanol trap. Samples of water collected in the trap were removed at intervals. A sample of the expired water, collected after 2 hr., had a specific activity of $1.4 \times 10^{-2} \mu\text{C}/\text{ml.}$ Water trapped from the sixth to the twenty-second hr. had an activity of $0.65 \times 10^{-2} \mu\text{C}/\text{ml.}$ During this period water was given *ad lib.*, and one feed consisting of 12 g. of the stock diet was given at the fifth hr.

After the twenty-fourth hr. the animal was anaesthetized with 15 mg. of Nembutal given by intraperitoneal injection, the abdomen opened and the abdominal artery cannulated. A sample (7–8 ml.) of blood was collected and the animal then perfused with saline via the right femoral vein, the perfusate also being collected. The blood was allowed to clot and the serum removed and stored frozen.

Experiments with labelled albumin in a rat on stock diet. The animal (rat T23, wt. 365 g.) was given 15 mg. of Nembutal by intraperitoneal injection. The right femoral vein was exposed and 1.15 ml. of a solution of albumin containing $0.11 \mu\text{C}$ injected. The wound was then closed and stitched. Blood samples (about 0.4 ml.) were taken from the tail, at intervals up to 430 hr., and the specific activities of the albumin determined.

Experiments with labelled albumin in a rat on cassava-flour diet. The diet consisted of cassava flour with 1 % (w/w) of NaCl, cooked each day with about 2.5 times its weight of water, fed *ad lib.* Vitamin D (4 i.u.) in 0.2 ml. of arachis oil was given once a week. The cassava flour used contained 0.28 % of N (micro-Kjeldahl). Bigwood & Adriaens (1954) have evaluated the amino acid composition of cassava meal and found it to have a low proportion of essential amino acids. The history of this animal (rat T19) is as follows, figures in parentheses being body weights in g.: weaned on to stock diet (37); 102nd day, transferred to cassava diet (298); 170th day (203); 190th day (194); 202nd day (187), intravenous injection of active albumin ($0.06 \mu\text{C}$ in 1.3 ml.; 120 hr. later serum albumin concentration was 2.10 g./100 ml.); 207th day, returned to stock diet (190); 225th day (285); 258th day (282). The animal was one of four male litter mates, all of which showed similar changes of body weight when fed on the cassava diet.

Body water determinations

Preparation of water samples from rat blood containing $^3\text{H}_2\text{O}$. Blood samples (about 0.5 ml.) required for determinations of body water were collected in polythene containers (5 mm. $\times$ 35 mm.) from a small cut at the end of the tail. The clot was separated from the side of the tube and the tube with the sample was inserted, open end first, into the bulb of

Table 1. Activity of water from blood after intraperitoneal injection of active water

Time (min.)	Specific activity (counts/sec./ml.)
15	378
60	448
90	448
180	445

a Thunberg tube. The tube was connected to the bulb, and the apparatus evacuated slightly to move most of the blood from the polythene container into the bulb. The bulb and the end of the tube section were then cooled in solid CO_2 -propanol and the apparatus was pumped to a pressure of about 10^{-2} mm. Hg. The end of the tube was kept in solid CO_2 -propanol until most of the water from the blood had collected there (about 2 hr.).

Equilibration of injected $^3\text{H}_2\text{O}$. A solution of standard $^3\text{H}_2\text{O}$ (0.986 ml. containing $1.9\mu\text{C}$) was injected intraperitoneally into a rat (248 g.) fed on stock diet. Blood samples (0.5 ml.) were taken at intervals from the tail and distilled *in vacuo* as described above. The water obtained was assayed for tritium. Table 1 shows the specific activity of samples taken at various times after injection. The figures show that equilibration is complete after 1 hr., and, for the purposes of body water determination in experimental animals, samples have been taken at 1.5 hr. The total body water is calculated as the ratio activity injected: specific activity of 1.5 hr. sample; e.g. in the experiment described above, injected activity was 7.03×10^4 counts/sec. Thus total body water was $7.03/448 \times 10^4$ g., or 157 g. (63%).

Paper electrophoresis

Electrophoresis of samples was carried out in a horizontal paper electrophoresis cell (Shandon Scientific Co., London) maintained at a temperature of 2° . Veronal buffer (pH 8.6, 0.01M) was employed, and a potential gradient of 4.6 v/cm. was maintained across the strips.

Preparation of labelled albumin

Labelled albumin was prepared as required by electrophoresis of up to $8 \times 84\mu\text{l}$. amounts of blood serum from the donor animal on strips (8 cm. wide) of Whatman no. 3MM paper. A small quantity of bromophenol blue in veronal buffer was added to the serum for location of the albumin fraction. After 16 hr. the strips were removed from the cell and laid on a sheet of thin polythene. Each blue-stained area of paper, and the polythene under it, were cut out, rolled and placed on special glass supports in centrifuge tubes. After centrifuging for 2 min. the protein solutions were removed and combined.

Separation of serum albumin from DL-[^3H]leucine during paper electrophoresis

Normal serum (50 μl .) with 5.5 μg . of DL-[^3H]leucine containing 208 counts/sec. was treated by the paper-electrophoresis procedure described for the isolation of albumin. The paper region containing the albumin gave 0.13 count/sec. above background.

Assay of labelled albumin in blood samples

Blood samples of 0.4 ml. were collected from the tail. They were allowed to clot and the serum was separated. Albumin for assay of radioactivity was prepared by electrophoresis of 84 μl . of serum on filter-paper strips as previously described, the albumin fraction migrating about 3 cm. After electrophoresis the strips were dried at 100° for 30 min. and placed for 10 min. in a saturated solution of naphthalene black in solution I described below. The strips were then washed for 10 min. in solution I, then for 30 min. in solution II, and finally they were dried in air.

Estimation of nitrogen in protein fractions obtained by paper electrophoresis

Reagents. Nessler reagent: 200 ml. of 2.5N NaOH (A.R., CO_2 -free) was added to 50 ml. of Nessler reagent (Umbreit, 1945) immediately before colour development. Solution I: methanol 9 vol., acetic acid 1 vol. Solution II: solution I, 2 vol., 0.03N-HCl 1 vol.

Samples (6 μl .) were used in duplicate for electrophoresis on Whatman no. 1 paper, and 0.01M veronal buffer, pH 8.6. The strips were dried on a horizontal rack at 100 – 105° for 15 min. Buffer salts were removed by washing for 15 min. in solution I, followed by three 5 min. washes in solution II. After a rinse in solution I, the papers were immersed for 10 min. in a 0.02% solution of bromophenol blue in solution I. After a final rinse in solution I, the papers were dried at room temperature. The paper was folded so that the albumin 'spot' could be cut out, together with an equivalent area of paper from in front of the albumin. The albumin spot and corresponding blanks were each placed in 50 ml. Kjeldahl flasks. A volume (0.5 ml.) of H_2SO_4 (N-free) was added together with two drops of H_2O_2 (100 vol.) and two glass beads. The flask was heated for 20 min. so that the acid refluxed gently, then cooled. Two drops of H_2O_2 (100 vol.) were added when the temperature was such that a gentle reaction occurred. The flasks were then heated for 0.5 hr., with the acid boiling, then cooled.

Nesslerization. Glass-distilled water (20 ml.) was added to each flask, followed by 15 ml. of freshly mixed Nessler reagent. Optical densities were read at $\lambda = 490\text{ m}\mu$. with a Coleman Junior Spectrophotometer. Calibration curves were plotted from readings of standard solutions of NH_4Cl (A.R.) dried at 105° .

Estimations of the N content of standard solutions of NH_4Cl and of leucine by this method at different times were all within 5% of the theoretical amount. Volumes (6 μl .) of rat serum were pipetted on to 0.5 in.² pieces of Whatman no. 1 paper which were then dried and treated by the routine heating, staining and washing technique. The results corresponded to 6.80 ± 0.19 g. of protein/100 ml. (protein = $N \times 6.25$). When further portions of the serum were digested directly, the results corresponded to 7.25 ± 0.10 g. of protein/100 ml. Thus in view of the presence of non-protein nitrogen, the results indicate that errors due to the method of treatment of the protein on paper were small. Portions of serum treated by the staining and washing procedure gave the same results as those which had not been stained. Thus no appreciable contamination with nitrogen occurred during the staining. The duplicate 'paper blanks' gave consistent readings, amounting to about 20–30% of the reading for the paper containing protein.

Estimation of amino nitrogen

Free NH_3 was removed from 17 μl . portions of the hydrolysates (Fowden, 1951), and these were then treated with ninhydrin reagent (Cocking & Yemm, 1954; Yemm & Cocking, 1955). Optical densities were compared with those from standard solutions of leucine.

Leucine estimation

Leucine was separated from other amino acids in the hydrolysates by paper chromatography with *tert*.-pentanol-water in the presence of diethylamine vapour (Work, 1949). Samples were selected by which leucine could be obtained 'ahead' of all other 'spots' in 30 hr. at 32°. The procedures described by Fowden (1951) were followed for location of the areas of the paper containing the amino acid and removal of NH_3 from the paper before treatment with ninhydrin. Colour development was by the method of Cocking & Yemm (1954).

Estimations of standard amounts of leucine showed losses of 20–30%. No marked improvement was obtained after purification of the solvent by steam distillation and washes with aqueous solutions of ferrous sulphate and ethylenediaminetetraacetic acid. A solution was prepared containing known amounts of labelled leucine and inactive leucine. Similar amounts of amino acid were found in the leucine 'spots' on estimation by either ninhydrin colour or radioactivity (Table 2), i.e. the specific activity was constant. Thus corrections for losses of leucine with this solvent could be made after determination of the losses in leucine standards included on each chromatogram.

RESULTS

Stability of tritium in the DL-[^3H]leucine

The results given in Table 3 show that no appreciable loss of activity occurred when the labelled leucine, together with inactive leucine, was recrystallized from aqueous ethanol. No loss of activity could be detected after paper chromatography with butanol-acetic acid-water (Partridge, 1948). A decrease in activity could have been detected if an appreciable amount of the tritium had been present in impurities, or in the carbonyl or amino groups, where the hydrogen equilibrates with the hydrogen of water. The quantitative recovery of the activity in isovaleraldehyde confirms the other results.

Table 2. Recovery of leucine after chromatography in *tert*.-pentanol-water

Leucine added (μg .)	Leucine found (μg .)	
	Ninhydrin estimation	Tritium estimation
19.7	16.5	16.7
9.83	8.5	8.4
4.92	3.7	3.9

Table 3. Recovery of activity after different treatments of DL-[^3H]leucine

Treatment	Recovery (%)
20.45 mg. of DL-[^3H]leucine + 501 mg. of inactive DL-leucine crystallized from water-ethanol	98.8
7.05 mg. of DL-[^3H]leucine + 459 mg. of inactive DL-leucine crystallized from water-ethanol	98.5
Above mixture recrystallized from water-ethanol	99.1
Paper chromatography in butanol-acetic acid-water	99.1
Oxidation by ninhydrin to isovaleraldehyde, isolated as the dimerone derivative	99.0

Tritium-labelled serum albumin

Paper electrophoresis. A volume (84 μl .) of a solution of active albumin was subjected to electrophoresis and stained with naphthalene black. The bromophenol blue which had been added to the albumin solution migrated considerably further than the albumin. The strip was cut into sections, which were assayed for radioactivity. The results are shown in Fig. 1; 72% of the total activity was found in the albumin. No detectable activity was found in the area containing the bromophenol blue dye.

The experiment was repeated with the addition of an equal quantity (84 μl .) of inactive normal rat serum; 70% of the total activity was in the albumin, and no separation of the bromophenol blue was noticed.

The effect of distance migrated on the recovery of total activity in the albumin fraction is shown in Fig. 2.

It may be seen that high recoveries of albumin were observed when the distance of migration was 3.0 cm. or less. In view of the lower recoveries found when the distances of migration were greater, it was of interest to investigate further the stability of the tritium in the albumin.

Hydrolysis. Albumin was isolated from six 84 μl . amounts of serum. One volume of 10% trichloroacetic acid was added to the solution that had been centrifuged from the paper strips as described. The precipitate was collected by centrifuging and washed with three small amounts of ethanol-ether (1:2, v/v), and once with ether. The dry product was heated in a sealed tube with 4.61 g. of 6.8N-HCl for 15 min. and then cooled. Weighed portions were heated for further periods. Hydrochloric acid was removed from the hydrolysates by drying *in vacuo* over KOH. Water (2 ml.) was added to each sample.

Fig. 1. 'Trailing' in paper electrophoresis of 84 μ l. of labelled albumin solution. (a) Activity distribution along the paper strip. (b) Contact print of paper strip dyed by naphthalene black. The blocked areas in (a) represent the percentage of the total activity per cm. of the regions marked (i), (iii), (iv) and (v) in (b). Area (ii), position of bromophenol blue after electrophoresis.

Fig. 2. Recovery of albumin activity after paper electrophoresis for different distances. O, 84 μ l. of labelled albumin solution; Δ , 84 μ l. of labelled albumin solution plus 84 μ l. of inactive rat serum.

Fig. 3. Hydrolysis of labelled albumin in 6.8N-HCl at 103°. Δ , Total amino nitrogen; O, free leucine; ●, leucine activity.

The activity released during hydrolysis, and appearing in the position normally occupied by leucine after chromatography on paper by the method described, was measured. Total amino nitrogen, free leucine and leucine activity were estimated by the methods given. Fig. 3 shows the values obtained after the different periods of hydrolysis. A considerable amount of peptides would be present after the shorter periods of hydrolysis, and it was not established that the

leucine was completely separated from such impurities. However, the curves for the activity and the ninhydrin reaction of the leucine 'spots' and for the free amino nitrogen all have the same general form. This is consistent with the liberation of active leucine from the albumin by hydrolysis. The final activity of the leucine released by hydrolysis after allowance for the loss in chromatography corresponds to 80% of the activity of the albumin.

Animal experiments

Labelled albumin in a rat on stock diet (rat T23). The curve showing the time relationship of albumin specific activity (Fig. 4) has the same general form as the curves obtained for labelled serum proteins by other workers. Initially there is a more rapid decrease in activity followed by a later period of less rapid, approximately logarithmic, decrease. It has generally been assumed that in the initial period equilibration takes place between intra- and extra-vascular pools. However, since this period lasted 4-5 days, the above assumption would imply a very low rate of movement of albumin in view of the small size of the animal.

Labelled albumin in a rat on a cassava flour diet (rat T19). The diet is one of a number of low protein

Fig. 4. Rat T23. Specific activity of serum albumin versus time.

Table 4. *Loss of albumin activity of rat T19, and ratios of albumin dilutions of rat T23 to albumin dilutions of rat T19*

For details see text. Figures in parentheses are the ratios calculated per g. dry weight.

Time (hr.)	Specific activity of albumin of rat T19 (counts/sec./mg.)	Relative albumin dilution
2.0	7.48	2.86 (1.06)
17.5	4.23	2.96 (1.10)
23.6	3.14	2.80 (1.03)
120.0	1.18	2.86 (1.06)

diets being investigated in this Unit. Because of the small size and low serum albumin of the animals it was considered necessary to determine whether the techniques which have been described could be applied. The serum albumin fraction from these animals was particularly interesting, since it has been found, during electrophoresis on paper, to have an apparently greater mobility than the corresponding fraction from control animals on stock diet.

For purposes of comparison, blood samples were taken from rat T19, at the same times as those from rat T23, until 120 hr., when the experiment had to be terminated owing to shortage of the cassava diet.

A comparison of the observations on the two animals may be made in the following manner: thus if S_1^t and S_2^t are the specific activities of albumin in blood samples taken from the two animals at a time t , and A_1 and A_2 are the initial injected activities, then

$$\frac{D_1}{D_2} = \frac{A_1}{S_1^t} / \frac{A_2}{S_2^t}$$

may be regarded as the ratio of the effective dilutions in the two animals at the time t . In Table 4, column 2 shows the specific activity of the serum albumin from rat T19 at various times after the injection of activity. Column 3 shows the dilution ratios D_{T19}/D_{T23} . In column 4 the ratios are calculated per unit dry wt. of animal. Estimations of total body water were made on litter mates of the two experimental animals. The body water of the rat fed on stock diet (399 g.) was 59.1%; that of the rat fed on cassava diet (175 g.) was 70.6%.

The constant ratio of the effective dilutions indicates that the dynamics of the change in albumin specific activity are similar in the two animals over the period from 2 to 120 hr. The figures in column 4 indicate that the effective dilutions per g. dry wt. for the two animals were approximately equal.

DISCUSSION

The difficulties associated with the assay of tritium arise from the very low energy of the β -particles emitted. In practice, these difficulties are usually overcome by the incorporation of the tritium from the sample into some suitable compound which can then be dispersed over the sensitive volume of the detector. Thus the requirements for the routine assay of tritium in labelled materials are the combination of a rapid and simple method for accomplishing the first stage and a stable and trouble-free detection apparatus.

The apparatus for estimation of tritium by proportional counting described by White *et al.* (1950) has now been in use for seven years, and its performance shows that high-stability amplifiers and

corona stabilizers make proportional counters at least as stable and trouble-free as Geiger-Müller counters. Individual counter tubes have been used for several years and counts of tritium standards are constant over long periods.

The apparatus does not require a highly purified gas-filling, and in combination with the method of preparation of gas for counting described by Payne & Done (1954), completely fulfils the requirements listed above.

The high sensitivity of the counting method has made possible the use of labelled albumin fractions obtained by electrophoresis on paper, which could be measured after a dilution of the order of 10^4 . Studies have also been made of total body water, and of water equilibration and turnover rates in experimental animals with a high degree of accuracy, by the use of about $1.5 \mu\text{C}$ of active water/kg. of body wt. For example, the doses used for rats and human subjects were 0.4 and $100 \mu\text{C}$ respectively. For determination of total body water only, even lower doses of the order of $0.2 \mu\text{C}/\text{kg}$. of body wt. have been used.

The method of synthesis of DL-leucine described by Albertson & Archer (1945) is very suitable for the preparation of DL- $[\text{}^3\text{H}]$ leucine, since tritium is attached in stable positions to carbon atoms distant from the amino and carboxyl groups, tritium may be introduced quantitatively by hydrogenation, and most of the preparative work does not involve the handling of radioactive materials. A useful synthesis of L- $[\text{}^3\text{H}]$ leucine would be the hydrogenation of a suitable derivative of β -methallylglycine (Goering, Cristol & Dittmer, 1948).

The high proportion of the albumin activity found in leucine released after hydrolysis is interesting since the active amino acid was fed in the DL-form. This may be due to the incorporation of a considerable amount of the activity of the D-enantiomorph. Ratner, Schoenheimer & Rittenberg (1940) found that, when rats were given a diet containing deuterium-labelled D-leucine, a considerable amount of the deuterium was found in leucine isolated from the animals' proteins. There is good evidence that four major components occur in human albumin (Saifer & Corey, 1954). The possibility of a similar heterogeneity of rat albumin might also have to be taken into account in the interpretation of measurements of the rate of loss of albumin activity *in vivo*, particularly when results are expressed as 'turnover rates'. An objective comparison of the results from the two experimental animals was made, on the basis of the relative albumin activity at different times. It was found that, throughout the experimental period, the rate of decrease of albumin activity for the animal on very low protein diet was strikingly close to that of the animal on stock diet. Thus, so far

as the albumin fraction is uniform in both animals, it could be considered that while on the cassava flour diet the protein metabolism of the animal must have been adjusted in many respects in response to the low protein content of the diet without loss of capacity to utilize the more adequate protein diet given later.

The highest dilution of the active serum albumin after which accurate assays could be made was of the order of 10^4 times. The measurement of exceptionally small amounts of tritium-labelled material is theoretically possible, since the pure isotope could be introduced into the initial material. Leucine could be made with specific activity as high as $0.47 \text{ C}/\text{mg}$.; of this, $10^{-8} \mu\text{g}$. could be accurately assayed. However, the amount of labelled leucine which could be incorporated into proteins by an animal is limited by the effects of the radiation of the isotope. Brues, Stroud & Rietz (1952) found a 30-day median lethal dose of about $1 \text{ mc}/\text{g}$. of body wt. for mice. If a rat could tolerate 300 mc of labelled leucine, serum albumin obtained from it should then be measurable after dilution of the order of 10^7 , by using 2 mg. samples for assay, or 10^9 by using 200 mg. samples.

SUMMARY

1. Details are given of the assay of tritium by the combustion-bomb technique of preparation of gas for the proportional counter.
2. A convenient synthesis of DL- $[\text{}^3\text{H}]$ leucine is described.
3. Labelled rat albumin was prepared by feeding labelled leucine. The activity released by hydrolysis of the albumin was studied.
4. The albumin activity of the animal on stock diet was assayed during 400 hr. after injection of labelled albumin.
5. The rates of loss of labelled albumin from the blood were similar for an animal on a very low protein diet and for an animal on a stock diet.
6. It is suggested that the activity of protein labelled with tritium and assayed as described could be measured after dilutions of about 10^9 .

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Fixation of Carbon Dioxide by Extracts of the Strict Autotroph *Thiobacillus denitrificans*

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Chemosynthetic bacteria have in common with photosynthetic organisms the ability to grow upon carbon dioxide as the sole source of carbon. In recent years much progress has been made towards the elucidation of the mechanism by which carbon dioxide is fixed during photosynthesis. Benson *et al.* (1950) and Fager, Rosenberg & Gaffron (1950) demonstrated that phosphoglyceric acid is the first stable fixation product; later, Calvin & Massini (1952) and Bassham *et al.* (1954) obtained evidence which suggested that ribulose 1:5-diphosphate is the carbon dioxide acceptor. Recently an enzyme has been found in extracts of *Chlorella* (Quayle, Fuller, Benson & Calvin, 1954) and spinach (Weissbach & Horecker, 1955) which carboxylates ribulose 1:5-diphosphate according to the equation:

The formation of ribulose 1:5-diphosphate from ribulose 5-phosphate and adenosine triphosphate (equation 2) has been shown by Weissbach, Smyrniotis & Horecker (1954a) and Hurwitz (1955):

Phosphoglycerate is reduced to triose phosphate, which is then converted into hexose diphosphate (HDP) by aldolase. Hexose monophosphate is formed by dephosphorylation of HDP.

The pathway by which pentose phosphate is regenerated from hexose monophosphate and triose phosphate without loss of fixed carbon dioxide

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involves the enzymes transaldolase and transketolase, which catalyse reactions (3)–(5) (Horecker & Smyrniotis, 1953, 1954, 1955; Horecker, Smyrniotis & Klenow, 1953; Horecker, Gibbs, Klenow & Smyrniotis, 1954; Racker, de la Haba & Leder, 1954; de la Haba, Leder & Racker, 1955).

Pentose phosphate isomerase catalyses the inter-conversion of ribulose 5-phosphate and ribose 5-phosphate (Horecker, Smyrniotis & Seegmiller, 1951; Axelrod & Jange, 1954).

In this paper the formation of phosphoglycerate from pentose phosphates and some aspects of the metabolism of sugar phosphates by extracts of a strict chemosynthetic autotroph are reported. The organism studied, *Thiobacillus denitrificans*, grows on a simple bicarbonate-mineral medium utilizing the energy obtained from the oxidation of thio-sulphate or sulphur, and can be cultivated on a