Because the haemin carbon/haemoglobin carbon specific activity ratio was not measured in these patients and because the hippuric acid ^{14}C content may be lower than the ^{14}C content of the free plasma glycine, only general deductions can be made, and the calculations as made above on the effect of the persistence of labelled glycine on the isotopic content of the haemoglobin are only approximate. This does not, however, negate the general conclusion that there is a continual synthesis of labelled haemoglobin from labelled glycine, long after administration of the labelled glycine and that this continual synthesis can account for a large part and possibly all of the tail portion of the curve depicting the isotope content of haemoglobin.

SUMMARY

1. The specific activity of the glycine available for conjugation with benzoate has been determined in three persons after the administration of labelled glycine.

2. The rate of decrease of the specific activity of the hippuric acid can be described in terms of two exponential components having average half-times of 6.8 and 75 days.

3. The rates for these two components agree well with the rates of the two slowest components of the breath $^{14}\text{CO}_2$ excretion.

4. The observed specific activity of the hippuric acid was almost entirely confined to the α -carbon atom of the glycine moiety except during the period 85–106 days after administration.

5. It is concluded that the tail portion of the curve of the specific activity of haemin or haemoglobin could be due, in large measure if not entirely,

to the continual formation of red cells containing ^{14}C derived from persisting labelled glycine and is probably not due to a population of red cells having life spans far in excess of about 120 days.

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Preparation of ^{32}P -labelled Adenosine 5'-phosphate, Inosine 5'-phosphate and Ribose 5-phosphate

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In studies on the metabolism of adenosine and inosine phosphates it was desirable to use compounds labelled with ^{32}P in the α -phosphate group. Ostern & Terszakowec (1937), Ostern, Baranowski & Terszakowec (1938), Caputto (1951) and Kornberg & Pricer (1951) have synthesized adenosine 5'-phosphate (AMP) by incubating adenosine with adenosine triphosphate (ATP) in a yeast extract

(Lebedev juice), according to the reaction: adenosine + ATP $\rightarrow$ AMP + ADP (adenosine diphosphate). Experiments in this laboratory showed that the labile phosphate of ATP readily exchanges with inorganic [^{32}P]phosphate in Lebedev juice without added substrate. This has been utilized for the preparation of radioactive AMP (AM ^{32}P). The resultant AM ^{32}P , chromatographically homo-

geneous, has a very high specific radioactivity and can be easily deaminated to form inosine 5'-phosphate (IM³²P), or decomposed with dilute acid to form ribose 5-phosphate.

MATERIALS

The barium salt of ATP. This was prepared from rabbit muscle according to LePage (1949) and analysed by the methods of Eggleston & Hems (1952) and Krebs & Hems (1953). In solutions of the sodium salt prepared from it the percentage distribution of total P was ATP, 72.5; ADP, 13.5; inorganic phosphate, 14.0.

Radioactive phosphate. KH₂³²PO₄ was obtained in aqueous solution from the Atomic Energy Research Establishment, Harwell. It was diluted with distilled water before use.

Yeast extract. Fresh brewer's yeast from a local brewery (Wm. Stones Ltd.) was dried in a current of warm air (not above 30°). Dry powdered yeast (100 g.) was soaked in 300 ml. water for 4 days at 4° and then centrifuged. The supernatant (Lebedev juice) was stored at -15°; its activity showed no diminution after several months.

Low-phosphate yeast extract. To 6 ml. Lebedev juice were added 8 ml. 0.1 M-MgCl₂ and 4 ml. 0.2 M-NH₄Cl at 0° with the dropwise addition of approx. 2 ml. N-NH₃ until the pH was 7.1-7.2. After standing in ice for 60 min. the precipitate of NH₄MgPO₄ was separated by centrifuging at 0° and the supernatant stored at -15°. This treatment decreased the total P content by about 80%.

METHODS

Paper chromatography. This was carried out as described by Krebs & Hems (1953). It was usually necessary to dry comparatively large volumes of the solutions to be examined on to the starting line of the papers. In these instances the sample was transferred to the paper with a finely drawn-out Pasteur pipette. Drying was accelerated with hot air from a hair drier. In this way 0.1 ml. was dried on to an area of 1 cm.², and 1.5-2 ml. could be accumulated on to a 12 × 1 cm. band in 30-40 min. Trichloroacetic acid (TCA) extracts could be used directly in this way without neutralization. After completion of the chromatography the papers were dried in warm air and then examined under u.v. light from a Chromatolite lamp (Hanovia Ltd., Slough, Bucks). Absorbent areas of adenosine or inosine phosphates were ringed with pencil. The second of the two chromatography solvent-mixtures (isobutyric acid-aq. NH₃ containing ethylenediaminetetraacetic acid) was only used once, as it became contaminated with radioactivity from the small wick of paper dipping into it.

Phosphorus. Determinations of phosphorus were made according to Berenblum & Chain, as described by Eggleston & Hems (1952) and Bartley (1953).

Radioactivity measurements. These were made directly on the coloured isobutanol-ethanol solutions from the phosphorus determinations. In some cases extensive dilutions with ethanol were necessary. A β -counting tube (liquid type M6 of 20th Century Electronics) was used and was washed out successively with N-NaOH, water, N-H₂SO₄ and water.

Radioactive adenosine 5'-phosphate

When an untreated Lebedev juice was used AM³²P was formed and ATP disappeared, but the inorganic phosphate

Table 1. *Formation of ³²P-labelled AMP in an incubated yeast extract*

See text for experimental details. TCA extract (0.1 ml.) was dried on to an area of 1 cm.² on a chromatogram paper and separated by the method of Krebs & Hems (1953). Only AMP was visible in u.v. light.

Area on paper	P content (μ g.)	Radioactivity	
		Found (counts/min.)	As % of total
Inorganic phosphate	6.19	7 550 000	79.00
AMP	2.63	1 056 000	11.05
ADP	0.98	147 800	1.54
ATP	0.76	26 900	0.28
Rest of paper*	—	777 320	8.13

* Includes hexose, pentose and triose phosphates, and IMP.

content of the system was so high that the distribution of total radioactivity rarely exceeded 1.5% in the AMP. The proportion of total radioactivity in the AMP was greatly increased when the low-phosphate extract was used. The addition of acetate or pyruvate had no effect; fluoride was slightly inhibitory. The highest yields of AM³²P were obtained by using 5 ml. low-phosphate extract, 0.5 ml. 0.02 M ATP, 0.4 ml. 1% (w/v) adenosine, 5 ml. water containing 10 mc KH₂³²PO₄. This mixture was shaken for 4 hr. in air at 20° in an Erlenmeyer flask containing two glass beads. After deproteinization with 4 ml. 30% (w/v) TCA, the sediment was separated by centrifuging and washed with 5 ml. 5% (w/v) TCA. The supernatant extracts were combined (19.3 ml.) and stored at -15°. The result of the chromatographic analysis of a 0.1 ml. sample of a typical preparation is given in Table 1.

The specific radioactivity of the AM³²P (counts/min./ μ g. P) was usually found to be about 0.3-0.5 of that of the inorganic phosphate. This indicates that part of the AMP came from the added ATP and ADP, the stable P of which does not exchange appreciably with inorganic phosphate. In the preparation described in Table 1 the maximum AMP expected would have been 4.7 mg. from adenosine and 3.4 mg. from the ATP-ADP mixture. A total of 5.68 mg. AM³²P was found in the whole TCA extract with an activity of about 1 mc. Losses were probably due to activity of 5-nucleotidase and adenylic and adenosine deaminase.

For the isolation of the AM³²P 1.5-2 ml. samples of the TCA extract were separated as a band on paper chromatograms. After location in u.v. light the AM³²P band was cut out, washed in anhydrous ether, cut up into 12 equal pieces and stored as such. Each piece contained 33-55 μ g. AM³²P with an activity of 1.2-1.7 × 10⁶ counts/min. In studies on the reactions of nucleotides it was sufficient to drop directly into the reaction vessels one or more of these pieces, rather than make a preliminary elution of the AM³²P with water.

Radioactive inosine 5'-phosphate

Attempts to deaminate the AM³²P with Schmidt's adenylic deaminase, made according to the two procedures of Kalekar (1947), met with variable success. Unless the adenylic deaminase was very highly purified it was contaminated by 5-nucleotidase or some other phosphatase which led to the appearance of radioactive inorganic phosphate with low yields of IM³²P.

Deamination with nitrous acid proved more reliable. As the addition of NaNO₂ and acid to the AM³²P solution made the subsequent separation by paper chromatography impracticable owing to salt effects, the nitrous acid was supplied in gaseous form. Ten pieces of an AM³²P band (equivalent to 0.4 mg. AMP with an activity of 15×10^6 counts/min.) were placed in 10 ml. water in a test tube (125 × 16 mm.) with a ground-glass stopper. A fine stream of gas generated in a Thunberg tube was passed through the solution for 30 min. at room temperature. The Thunberg tube contained 1–2 g. solid NaNO₂ with 1 ml. 12N-HCl in the side arm, one drop of the HCl being tipped on to the NaNO₂ at intervals of a few minutes. The test tube was then stoppered and kept for 4 hr. at 45–50°. After filtration through a small cotton-wool plug, the paper pulp was washed with small amounts of water. The filtrates were combined and concentrated to dryness *in vacuo* over NaOH and excess P₂O₅. The drying was usually complete in about 3–4 hr., if the filtrates were in a shallow layer and if the P₂O₅ was renewed at least once. The dry material was taken up in a few drops of water and separated on paper chromatograms as a 5 cm. band. One spot of the IM³²P solution with added standard solutions was run simultaneously for analysis of radioactivity distribution. The IM³²P band was located in u.v. light, cut out and washed with anhydrous ether. The percentage distribution of total radioactivity on the papers was IM³²P, 80–95; AMP, 0.5–2; inorganic phosphate, 2–3; the rest of the radioactivity trailed down the paper, chiefly in the ribose phosphate area. As before, it was satisfactory to drop pieces of the IM³²P band directly into reaction vessels for enzyme experiments.

Radioactive ribose 5-phosphate

The quantities of AM³²P used were too small for the method of Marmur, Schlenk & Overland (1951) for the preparation of ribose 5-phosphate from AMP and separation by paper chromatography was not possible in the presence of H₂SO₄ or sulphate. Heating AMP in N-HCl at 100° was more satisfactory, although the yields of ribose 5-phosphate were not as high as those obtained by Marmur *et al.* (see Table 2). There was also a small loss of ribose 5-phosphate during the drying process *in vacuo*, but the final yields (60–70%) were adequate.

Table 2. *Decomposition of AM³²P by N-HCl at 100°*

Solutions (1.5 ml.) containing AM³²P (0.01M) and HCl (N) were heated in a boiling-water bath. Samples with a total radioactivity of approx. 10⁸ counts/min. were separated by paper chromatography.

Time at 100° (min.)	Distribution of radioactivity (%)		
	AMP	Inorganic P	Ribose 5-phosphate
0	98.6	0.8	0.6
10	17.3	3.2	79.5
20	13.0	7.6	79.4
30	14.8	21.5	63.7
60	1.4	39.8	58.8
10*	7.0	22.4	70.6

* N-HCl (5 ml.) containing AM³²P (10⁻⁴M) concentrated to dryness *in vacuo* before separation.

Five to ten pieces of an AM³²P band, equivalent to 0.2–0.4 mg. and $7\text{--}15 \times 10^6$ counts/min., were heated in 5 ml. N-HCl for 10 min. at 100°. After cooling and filtration through a small cotton-wool plug the solution was evaporated to dryness *in vacuo* over NaOH and excess P₂O₅. Rapid drying was essential to avoid the further breakdown of ribose 5-phosphate. With a shallow layer of solution and at least one renewal of P₂O₅, complete dryness was achieved in 2–4 hr. After dissolving in a few drops of water the material was dried on a chromatogram paper as a 3 × 1 cm. band in a stream of cold air from a hair drier. One spot of the same solution was run simultaneously with added standards for analysis of radioactivity distribution. Separations were made using only the second of the two solvent mixtures (isobutyric acid–aq. NH₃–ethylenediaminetetraacetic acid) of Krebs & Hems (1953), which was run with descending flow for 16–18 hr. at room temperature. The paper was dried in a current of cold air, then the strip containing the standards was cut off and heated for 30 min. at 80–100°. Under these conditions ribose and ribose 5-phosphate become visible as pale brown spots which give a yellow fluorescence in u.v. light. Absorbent areas of AMP and adenine could also be located in u.v. light. After marking all these areas with a pencil the strip was sprayed with a molybdate reagent to locate phosphate (Hanes & Isherwood, 1949). By means of this standard strip the position of the ribose 5-phosphate band, which was invisible in daylight or u.v. light, could be located on the unheated paper. The position of the band could also be located by using an end-window Geiger counter with a small aperture. There was usually a little trailing of radioactivity behind the ribose 5-phosphate band, but the majority of total activity was in the ribose 5-phosphate area indicated by the strip of standards.

The detection of ribose or ribose 5-phosphate as visible spots on the standard strips was due to prolonged heat in the presence of traces of constituents of the solvent mixture remaining in the paper. These constituents, each reacting with the sugar, were found to be ammonium isobutyrate and ethylenediaminetetraacetic acid. The formation of similar compounds, brown in daylight, fluorescent in u.v. light, obtained by heating sugars with compounds containing an amino (or ammonium) group, is already known (Chargaff, Levine & Green, 1948; Graham, Hsu & McGinnis, 1949; de Lange & Dekker, 1954). If insufficient solvent remained in the papers to give visible spots after heating, the standard strip was sprayed with 5% (NH₄)₂SO₄ in 50% (v/v) aqueous ethanol and reheated for 30 min. at 80–100°.

As the solvent dripped off the lower edge of the papers the movements of chromatographic zones are given relative to the movement of AMP as follows: ribose 5-phosphate 0.54, IMP 0.56, inorganic phosphate 0.66, ribose 0.87, AMP 1.00, adenine 1.44. It is obvious that AM³²P is preferable to IM³²P as the starting material for the preparation of ³²P-labelled ribose 5-phosphate by this method.

For use in metabolic experiments, pieces of the ribose 5-phosphate band could be added directly to reaction vessels, or if required could be eluted with water and concentrated *in vacuo*.

DISCUSSION

Although a system for the synthesis of AMP similar to that in yeast is known to exist in animal tissues (Caputto, 1951; Kornberg & Pricer, 1951), con-

siderable purification is required before it can be demonstrated. Brewer's yeast extracts, which require little further treatment, are therefore very convenient for the preparation of AM^{32}P . As dialysis results in inactivation of these extracts of yeast the method used here to decrease phosphate content is to be preferred, although treatment with such high concentrations of magnesium and ammonium chlorides may diminish the activity of the enzyme system.

The concentrations of adenosine ($1.4 \times 10^{-3} \text{ M}$) and ATP ($9.1 \times 10^{-4} \text{ M}$) used in the method described are respectively 7 and 1.8 times higher than those required for optimum activity of the enzyme system (Kornberg & Pricer, 1951), but to use less would introduce difficulties in the isolation of the final AM^{32}P which would contain a smaller proportion of the total radioactivity.

The chemical isolation of AM^{32}P from these extracts resulted in heavy losses even with added carrier AMP, but with the paper-chromatographic technique described here the recovery was quantitative and comparatively simple.

SUMMARY

1. A method is described for the preparation of ^{32}P -labelled adenosine 5'-phosphate in an extract of brewer's yeast. The product is quantitatively separated and is chromatographically homogeneous. It has an activity of approximately 0.2 mc/mg.

2. After deamination of this adenosine 5'-phosphate with nitrous acid, radioactive inosine 5'-phosphate, chromatographically pure, can be separated in a similar manner.

3. Radioactive ribose 5-phosphate is obtained by heating this adenosine 5'-phosphate in N hydrochloric acid and can be separated by paper chromatography.

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The Long-chain Paraffin Constituents of Rose-petal Wax

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In a series of papers dealing with the long-chain constituents of plant and insect waxes it has been shown, from evidence based largely on melting point and long crystal spacing data, that all the saturated, ketonic and hydroxy acids, as also the corresponding primary alcohols, contain an even number of carbon atoms, whereas the paraffins, ketones and secondary alcohols, which are probably formed from these respective acids by decarboxylation, contain an odd number of carbon atoms (Chibnall & Piper, 1934 (for main references); Blount, Chibnall & Mangouri, 1937; Chibnall, Piper, Mangouri, Williams & Iyengar, 1937). Such findings, in so far as they relate to the saturated

acids and primary alcohols, bring the wax constituents into line with those of somewhat shorter chain length present in glycerides and similar natural products. There is, indeed, an overlap between the two sets of components and collectively the complete series of even-numbered members from C_{10} to C_{36} is represented, suggesting that all of them can be regarded as manifestations of the same sequence of metabolic events.

The evidence with respect to the paraffins is much less widespread. Chibnall, Piper, Pollard, Williams & Sahai (1934) analysed the data then available, and concluded that in all cases other than the one mentioned below the products that had