Further, the ζ_1 -tocopherol behaves on gas-chromatographic columns as the higher homologue of ϵ -tocopherol; therefore both compounds must have a similar structure. The relationship between log (relative retention volumes) and the influence of the group separation factor, as shown in Fig. 5, permits us to predict the relative retention volumes of lower homologues of tocotrienols, should they be found in Nature.

SUMMARY

1. The separation of tocol, methylated tocols and tocotrienols by gas-liquid chromatography has been investigated.

2. A number of tocopherols could be resolved from one another that were separable only with great difficulty by two-dimensional paper chromatography. Thus 5-methyltocol, ϵ -tocopherol and η -tocopherol were separated, as was ζ_1 -tocopherol from ζ_2 -tocopherol.

3. Acetylation of tocopherols yielded esters that gave more reproducible results than non-esterified compounds.

4. On the silicone polymer SE-30, separation factors, calculated for methyl groups and for double bonds, permitted the prediction of relative retention volumes for tocopherols.

5. These separation factors, with silicone polymers as stationary phases, were additive, whereas in paper chromatography the effects of a

methyl group and the trienyl grouping cancel one another.

6. The gas-chromatographic behaviour of ϵ - and ζ_1 -tocopherols is in keeping with their trienyl structure.

7. As little as 0.2 μ g. of tocopherol could be assayed either in mixtures or in a natural source. The assay of the latter gave results that agreed with those obtained by paper chromatography.

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Utilization of Serum-Protein-Bound Glycine in the Formation of Globin and Haem

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The nature, significance and extent of the re-utilization of protein-bound amino acids in the formation of new protein (and other metabolites) have been a subject of interest for many years (Whipple, 1956; Loftfield & Harris, 1956; Walter *et al.* 1957; Walter, 1960). Thus estimates of the contribution by proteins of amino acid residues to the formation of other specific protein have ranged from minor (Cohen, Holloway, Matthews & McFarlane, 1956) to major (Loftfield & Harris, 1956), and the suggested intermediates have in-

cluded amino acids, peptides and activated amino acids (Loftfield & Harris, 1956; Whipple, 1956; Walter & Mahler, 1958).

In the present work we attempted to ascertain whether free amino acids were intermediates in the transfer of serum-protein-bound glycine to globin and haem. We also obtained results on the relative utilization of free and protein-bound glycine in the biosynthesis of globin and haem and investigated the percentage of the total glycine required in globin synthesis that is supplied by the serum proteins.

Haemoglobin was chosen for these experiments because it is a protein which, contained in the red

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cell, has a life-span of about 55 days in the rat (Levy, Walter & Sass, 1959). Thus over a reasonably short period no haemoglobin formed is then broken down. This fact makes interpretation of results obtained in these experiments less complicated, since only uptake of glycine into globin, not loss of glycine from globin, need be considered.

EXPERIMENTAL METHODS

Labelled proteins. Barium [^{14}C]carbonate of high specific activity was obtained from Oak Ridge National Laboratory and converted into [^{14}C]glycine by the Isotopes Specialties Co., Burbank, Calif., U.S.A. The glycine obtained had a specific activity of 4.2 mc/m-mole. In a typical experiment 20 mg. of the labelled glycine was injected intraperitoneally into a 300 g. rat. About 22 hr. later the animal was exsanguinated by heart puncture. The serum was diluted 1:4 with aq. 0.9% sodium chloride and the globulins were repeatedly salted out by the addition of an equal volume of neutralized saturated ammonium sulphate. The albumin in the supernatant solution was separated from non-protein [^{14}C] by dialysis. Since some glutathione, containing [^{14}C]glycine, might be bound to the protein by dithio bonds (Lee, Anderson, Miller & Williams, 1951), all protein preparations were treated with an excess of cysteine (i.e. about 4 mg. of cysteine/20 mg. of protein) at pH 8.5 for 2 hr., followed by exhaustive dialysis against aq. 0.9% sodium chloride. Serum proteins prepared in this way had an activity of about 65 $\mu\text{mc}/\text{mg}$.

Injection of rats. Male rats of the Sherman strain weighing 300–400 g. were injected intravenously via the femoral or tail vein. Rats received 20 mg. of the labelled serum globulin or serum albumin described above, or 0.25–0.75 mg. of [^{14}C]glycine containing 10–40 μC .

Preparation of plasma proteins, globin and haemin. At various times after the injection of the above labelled materials animals were exsanguinated by heart puncture and the blood was mixed with heparin. The red blood cells were centrifuged and the supernatant solution (plasma) was frozen. The red cells from each animal were subsequently washed six times with aq. 0.9% sodium chloride, the cells being spun and the supernatant solution carefully drawn off and discarded. Thereafter the globin and haem were prepared by the acid-acetone procedure of Anson & Mirsky (1930), and the haemin was precipitated by the addition of water. Dry powders of the globin and plasma proteins were prepared according to methods described by Walter *et al.* (1957). The haemin was suspended in water and transferred directly to a planchet, spread evenly and dried.

Preparation of dinitrophenylglycine from globin. Samples of the globin of rats injected with serum albumin, serum globulin or glycine were hydrolysed in 6N-hydrochloric acid for 24 or 48 hr. Since on the same sample the two times of hydrolysis yielded similar molar activity ratios (see 'Methods for calculating results' section below), the 24 hr. hydrolysis time was generally used. DNP-amino acids were prepared from the globin hydrolysates by the method of Levy (1954) and Levy & Chung (1955), and the DNP-glycine was subsequently isolated by the method of Perrone (1951). The purity of the DNP-glycine was checked by paper chromatography on Whatman no. 4

saturated with phthalate buffer, pH 6.0, and developed in 'tert.-amyl alcohol' (Fisher Scientific Co., N.Y., reagent grade) saturated with the same buffer (Blackburn & Lowther, 1951).

Percentage of activity due to glycine in injected serum proteins. To determine the percentage of activity due to glycine in the injected serum proteins, 150 mg. of carrier glycine and 100 mg. of carrier rat serum protein (albumin or globulin) were mixed with 1–2 mg. of the corresponding labelled serum protein (i.e. albumin or globulin), and the protein was then hydrolysed as above. DNP-glycine was prepared and isolated in a manner similar to that employed with globin. The count obtained for a portion of the DNP-glycine was recalculated for the amount of total free glycine originally added and expressed as a percentage of the total activity of the serum protein analysed. The percentage of [^{14}C]glycine in the precursor proteins used was found to be 31 for rat serum globulin and 29 for rat serum albumin.

Preparation of samples for counting. Samples (30 mg.) of dry protein powders of either globin or plasma-protein samples were weighed into planchets, spread evenly and counted on an Automatic Sample Changer (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.) with a D-47 counter with a window of Micromil [a conductive, coated, Formvar derivative (about 120 $\mu\text{g}/\text{cm}^2$) for window-counting].

Haemin was suspended in water, transferred to a tared planchet, dried, weighed and counted. When the weights differed from 30 mg. the samples were corrected to the thickness represented by that weight by using a self-absorption correction curve. Walter *et al.* (1957) found that portions of the same sample counted as a powder or dried directly into a planchet (from a volume of 1 ml.) differ in counts by an empirical value of 1.5, the dried sample giving the higher count. This factor has been employed throughout, by multiplying the counts obtained for the powder samples, so that comparisons between dried and powder samples could be made. Further, the haemin and the DNP-glycine samples were counted on the above-mentioned counter without the Micromil window to increase the number of impulses counted. The number of counts obtained on our instrument without the window is 2.04 times those obtained with the same sample with the window in place. Powders cannot be counted automatically without the window as the flow-gas blows them through the chamber, contaminating the counting wire. The factor for window and windowless counting has also been taken into account so that samples counted by either of the two methods could readily be compared. Samples were counted for a time sufficient to bring them within 5% standard error.

Methods for calculating results. The results in the Tables and Figs. are presented in one of three ways:

(a) To compare the amount of an injected isotopically-labelled precursor in haemin or globin in rats we expressed our results as relative specific activity, defined (Walter *et al.* 1957) as: (counts/min./100 mg. of sample)/(counts/min. injected/g. body wt.). This method has the advantage of reducing animal-weight differences to a common denominator.

(b) To compare the relative extent of incorporation of precursor into haemin and globin glycine in a given rat, we expressed our results in terms of molar activity ratios. These have been defined (Muir, Neuberger & Perrone,

1952) as: (counts/min./mg. of haemin $\times$ mol. wt. of haemin)/(counts/min./mg. of globin glycine $\times$ mol. wt. of glycine).

(c) To examine the relative content of [^{14}C]glycine and of [^{14}C]serine (Drabkin & Wise, 1960) at various times after injection of protein or glycine precursors in the globin of animals we expressed our results as: (counts/min./mg. of globin glycine)/(counts/min./mg. of globin).

RESULTS AND DISCUSSION

Shemin (1956) showed that the α -carbon atom of glycine was utilized in the biosynthesis of haem. Neuberger and his group (Muir *et al.* 1952) further showed that, when free glycine is injected into rats or rabbits, the molar activity ratio of haemin to globin glycine is about 8, from 1 day after the injection onward. Since 8 glycine α -carbon atoms are used in the biosynthesis of haem (Shemin, 1956), these results were interpreted by Muir *et al.* (1952) to mean that globin and haem are synthesized at the same rate and from the same amino acid pool. In Table 1 we present the results of an experiment in which the molar activity ratio of haemin to globin glycine was determined at various times after the injection of rat serum globulin containing [2- ^{14}C]glycine. The molar activity ratio is again approximately 8. A likely explanation for this finding is that free amino acids are intermediates in the transfer of serum-protein-bound amino acids to globin and haem.

In Fig. 1 the uptake of radioactivity is shown from injected free [^{14}C]glycine and from injected serum [^{14}C]globulin into globin and haem of rats over a 15-day period. The results are expressed in terms of relative specific activity (see above) and each of the points represents the average of four similarly treated animals. When free glycine is injected [Fig. 1 (a)], the relative specific activity reaches a maximum in a few days and then remains at essentially that level over the life-span of the red blood cell. When a labelled serum protein is the precursor, however, the uptake is slower and reaches the maximum level after about 15 days. This is because the labelled amino acids originating in the injected protein are incorporated into globin and haem only as they are released from the injected protein. The labelling of the globin and haemin becomes, therefore, dependent on the half-life of the injected serum protein.

We wished to calculate from our results the relative utilization of injected free glycine and of serum-protein-bound glycine in the formation of haem and globin. In preliminary experiments the half-life of rat serum globulin was found to be 3.5 days and that of rat serum albumin 3 days. Since on this basis practically all of the serum-protein-bound glycine has been released for utilization by the 15th day after injection, this time was chosen for our calculation.

In Table 2 we present a preliminary calculation for haem of this sort. The relative specific activity of haemin was found to be 0.38 in animals injected with serum [^{14}C]globulin and 1.2 in those injected with [^{14}C]glycine. To make a comparison of glycine utilization from the protein and from glycine, we have multiplied the determined relative specific activities by a factor which corrects the differences in total initial glycine available from each of the injected precursors. The percentage of the total activity due to glycine in the injected serum globulin was 31 (see Experimental Methods section above); yet, when free glycine was injected, all of the injected activity was due to glycine. Thus the corrected relative specific activities for haemin are about 1.2 for rats injected with serum globulin as well as for animals injected with free glycine.

Table 1. *Molar activity ratio of haemin to globin glycine in rats killed at different times after intravenous injection of rat serum-globulin-bound [2- ^{14}C]glycine*

Rats were injected with 20 mg. of rat serum [^{14}C]globulin. Other details are given in the text.

Day killed	Molar activity ratio (haemin:globin glycine)
1	8.1
3	8.6
6	6.5
9	8.5
12	8.5
15	6.4

Fig. 1. Uptake of radioactivity into globin (Δ , $\blacktriangle$) and haem ($\circ$, $\bullet$) in rats injected with (a) [2- ^{14}C]glycine or (b) rat serum [^{14}C]globulin.

Table 2. *Relative utilization of free glycine and of serum-globulin-bound glycine in the formation of haem*

The calculations were made from results obtained for animals which had been injected 15 days before being killed.

Injected material	Haem rel.sp.act. determined	Percentage of precursor activity due to glycine	Correction factor	Corrected rel.sp.act. of haem
Serum [^{14}C]globulin	0.38	31	3.2	1.2
[2- ^{14}C]Glycine	1.2	100	—	1.2

Fig. 2. Relative content of [^{14}C]glycine and [^{14}C]serine in globin at various times after the injection of [^{14}C]glycine (O), serum [^{14}C]globulin (□) or serum [^{14}C]albumin (Δ).

We consider this result to be only preliminary because the calculation does not take into account the effect of the labelled serine injected as part of the serum protein. The conversion of labelled serine into glycine (Shemin, 1946), as the serum protein is broken down, would raise the value of the effective injected dose of serum-protein-bound glycine, thereby decreasing the corrected relative specific activity of haemin.

Fig. 2 shows the results of an experiment designed in an attempt to estimate the effect of labelled protein-bound serine on the above calculation. The results are presented in terms of the ratio of the specific activity of globin glycine to that of globin in rats injected with labelled serum globulin, serum albumin or glycine. This ratio is quite different for the serum proteins as compared with glycine but remains constant over the period of the experiment for any one given precursor. Since globin contains about 5% of glycine (Spector, 1956) a ratio of 20 (see Fig. 2) would mean that the total globin radioactivity is due to glycine. Thus the curve referring to animals injected with glycine (O) indicates that approx. 60% of the radioactivity of globin is due to [^{14}C]glycine. The curve referring to animals injected with serum globulin (□) shows that 33% of the globin activity is due to [^{14}C]

glycine; and the curve referring to rats injected with serum albumin (Δ) corresponds to a percentage of 28. It thus appears that, when injected free glycine is incorporated into globin, 40% of it is converted into and incorporated as serine, since serine is the only other labelled amino acid found (Drabkin & Wise, 1960). When serum globulin is injected one might expect by analogy that the [^{14}C]glycine it contains would also be incorporated in a similar 3:2 glycine:serine ratio. Since 31% of the radioactivity of the injected serum globulin is in its glycine residues, 19% of the initial activity would be incorporated as glycine. However, as 33% of the globin activity is in the glycine residues when serum globulin is injected (Fig. 2), the difference between the determined 33% and the calculated 19% must be attributed to the conversion of serum-globulin-bound serine into glycine. The effective injected dose of serum-globulin-bound glycine is thereby raised from 31% to $(33/19) \times 31\%$, i.e. 54%. The corrected relative specific activity for haemin in rats injected with serum [^{14}C]globulin (Table 2) is thus 0.70 instead of 1.2.

Whether or not the assumptions on which the calculation correcting for the conversion of protein-bound serine into glycine is based are made the indication is that serum proteins are either as efficient in supplying glycine to haem and globin as injected free glycine, or less efficient. This finding in a system in which free amino acids appear to be intermediates in the transfer of amino acids from one protein to another is in contrast with that reported for other systems (Loftfield & Harris, 1956; Walter & Mahler, 1958), where proteins are used more efficiently but in which the intermediates involved are not established (Walter, 1960). In the latter systems the efficiency of transfer of amino acids from one protein to another [e.g. liver proteins to ferritin (Loftfield & Harris, 1956)] may be due either to the breakdown of the donor protein and the synthesis of the recipient protein at proximal sites or to the occurrence of amino acid derivatives (activated amino acids), rather than free amino acids, acting as intermediates in this process.

The percentages of serum-globulin and serum-albumin glycine re-utilized in globin synthesis can

be calculated from our results. We have also determined the percentage of the total glycine required for globin synthesis that has its origin in serum-protein glycine. The results are shown in Table 3, and a sample calculation of the type used in arriving at these values is presented below (Walter & Zipper, 1961). The results in Table 3 refer to individual rats and the range of values obtained is due to animal variation.

Sample calculation. The calculation is based on the counts/min./mg. of globin glycine found at time t after injection; the total effective activity of serum-protein-bound glycine injected into the rat, which is 54 % for injected rat serum globulins and 47 % for injected rat serum albumin (see above); the half-life of the injected serum protein (3.5 days for serum globulins and 3 days for serum albumin); the assumption that the total packed-red-cell volume corresponds to 3.4 % of the body weight, that there are 314 mg. of haemoglobin/ml. of packed cells (Creskoff *et al.* 1949) and that the life-span of red cells is 55 days in the rat (Levy *et al.* 1959). Further, the glycine content of globin is assumed to be 4.8 % (Spector, 1956), that of serum globulin 4.3 % and that of serum albumin 1.6 % (Spector, 1956; Owen, 1957); the molecular weight of haemoglobin is taken as 68 000 and that of globin as 65 400 (Haurowitz, 1950). The sample calculation is one which is based on a rat injected with labelled rat serum globulin and killed 6 days later. The results are typical (see Table 3) of those obtained in rats injected with serum globulin and killed at any time up to 15 days after injection.

(1) Total glycine radioactivity released on breakdown of injected labelled serum globulin from time 0 to time t ($t = 6$ days): (counts/min. injected) $\times$ (percentage of effective activity in glycine) $\times$ (fraction degraded at time t) = $196\,500 \times 54\% \times 69\% = 73\,216$.

(2) Total mg. of glycine released/day on breakdown of serum globulins: [rat wt. (g.)] $\times$ (ml. of

serum/g. body wt.) $\times$ (mg. of serum globulin/ml.) $\times$ (percentage of glycine in serum globulin) $\times$ (fraction of serum globulin degraded/day) = $206 \times 0.034 \times 32.2 \times 4.3\% \times 18\% = 1.75$.

(3) Total mg. of glycine required in globin synthesis/day: [rat wt. (g.)] $\times$ (ml. of packed-red-cell vol./g. body wt.) $\times$ (mg. of haemoglobin/ml.) $\times$ (mol. wt. of globin/mol. wt. of haemoglobin) $\times$ (percentage of glycine in globin) $\times$ (1/life-span of red cells) = $206 \times 0.034 \times 314 \times (65\,400/68\,000) \times 4.8\% \times (1/55) = 1.85$.

(4) Total radioactivity incorporated into total globin glycine from time 0 to time t ($t = 6$ days): (counts/min./mg. of globin glycine) $\times$ (total mg. of globin glycine) = (counts/min./mg. of globin glycine) $\times$ (mg. of glycine required for globin synthesis/day) $\times$ (life-span of red cells) = $5.8 \times 1.85 \times 55 = 590$.

(5) Percentage of serum-globulin glycine incorporated into globin glycine: (total counts/min. in globin glycine)/(total glycine counts/min. released from serum globulin) $\times 100 = (590/73\,216) \times 100 = 0.81$.

(6) Wt. of serum-globulin glycine incorporated into globin glycine over this 6 day time-period: (mg. of serum-globulin glycine released/day) $\times$ (days) $\times$ (fraction of serum-globulin glycine counts/min. incorporated into globin) = $1.75 \times 6 \times 0.0081 = 0.085$.

(7) Percentage of total glycine required in globin synthesis which has its origin in serum-globulin glycine: (wt. of serum-globulin glycine incorporated into globin/total mg. of glycine incorporated into globin in 6 days) $\times 100 = [0.085/(1.85 \times 6)] \times 100 = 0.77$.

Thus in this rat about 0.75 % of the total glycine required in globin synthesis has its origin in serum globulin and approx. 0.8 % of serum-globulin glycine is utilized for globin synthesis. The corresponding values (see Table 3) for animals injected with serum albumin are 0.25 and 0.7 %.

Table 3. *Re-utilization of serum-protein-bound glycine in globin biosynthesis as calculated from individual rats killed at various times over a 15-day period*

Details of the method of calculation are given in the text.

Rats injected with serum globulin		Rats injected with serum albumin	
Percentage of globulin-bound glycine incorporated into globin glycine	Percentage of total glycine required in globin synthesis which has its origin in serum globulin	Percentage of albumin-bound glycine incorporated into globin glycine	Percentage of total glycine required in globin synthesis which has its origin in serum albumin
0.34	0.33	0.66	0.26
1.96	1.91	0.57	0.21
0.81	0.77	0.29	0.11
0.42	0.40	0.54	0.21
0.63	0.59	1.22	0.46
0.50	0.48	—	—

Since serum proteins constitute only 2% of the rat's total proteins this serum contribution of about 1% of the total glycine required in globin synthesis appears to be significant.

SUMMARY

1. Rats were injected intravenously with rat serum globulin or serum albumin obtained from donor animals injected with [2-¹⁴C]glycine. The incorporation of serum-protein-bound glycine into haem and globin was followed for 15 days.

2. Serum-protein-bound glycine is utilized efficiently both as a source of glycine by the protein globin and as a precursor in the formation of the non-protein moiety, haem.

3. The specific activities of haem and globin glycine indicate that the injected protein is broken down to free amino acids before the incorporation of glycine into globin takes place.

4. On breakdown of serum protein, the extent of utilization of serum-protein-bound glycine in the formation of globin and haem is less than or equal to that of injected free glycine, a finding in contrast with those previously reported for other systems in which free amino acids may not be the intermediates involved.

5. About 1.4% of serum-protein-bound glycine is utilized as a source of globin glycine. This represents about 1% of the total glycine required in globin synthesis. Since serum proteins constitute only 2% of the rat's total proteins, this contribution of serum-protein-bound glycine to globin biosynthesis appears to be significant.

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L-Threonine Ethanolamine Phosphate: A Compound Newly Isolated from Fish

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Preliminary observations on the distribution of both stereoisomers of serine ethanolamine phosphate (2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate, I) have been reported (Rosen-

berg & Ennor, 1961). In further experiments, a hitherto undescribed ninhydrin-positive phosphorus-containing compound was detected in protein-free extracts of fish tissues. This compound, initially detected as a spot on a two-dimensional paper chromatogram, has been isolated and positively identified as L-threonine ethanolamine phosphate (2-aminoethyl L-2-amino-2-carboxy-1-methylethyl hydrogen phosphate, II).

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