ON THE MECHANISM OF INCORPORATION OF [^{35}S]CYSTINE INTO WOOL

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Summary

A comparison was made of the rate of incorporation of ^{35}S into the plasma proteins and the wool of a sheep after an intravenous injection of DL-[^{35}S]cystine. The cystine of wool attained a much higher specific activity than that of the plasma proteins, suggesting that cystine is not incorporated into wool by any process involving a direct modification of the plasma proteins. It is also suggested that there is a metabolic "pool" of material containing cystine associated with the wool follicles themselves which is turned over with a half-time of about 3 days.

I. Introduction

Many advances in our understanding of protein synthesis have been made as a result of isotopic tracer experiments. For example, in a recent review of the biosynthesis of milk proteins Barry (1958) stated: "Ten years ago, it was generally concluded from arterio-venous measurement that they (milk proteins) are largely formed from plasma proteins; now it is generally concluded from experiments with tracers that they are formed largely, at least, from the free amino-acids of plasma". Other workers have concluded that proteins in general are synthesized from free amino acids without significant participation of peptide or protein residues (Loftfield 1957). Thus the hypothesis put forward by Madden and Whipple (1940) that plasma proteins may possibly be converted more or less directly to other proteins in the body has had to be rejected in most cases. However, ovalbumin seems to be an exception, since evidence for the synthesis of this protein by the oviduct from derivatives of serum proteins or intermediate peptide pools has been presented (Sankar and Theis 1959). There seems to be little evidence one way or the other on this aspect of wool biosynthesis. Ryder (1959) has shown that radioactivity is present in the wool follicles of a lamb soon after an injection of DL-[^{35}S]cystine but this evidence does not rule out the possible participation of plasma proteins in keratin synthesis. Fleischer, Lietze, Walter, and Haurowitz (1959), however, have found that hair keratin in the rat is probably synthesized from amino acids or very small peptide fragments.

Much of the evidence on the biosynthesis of milk proteins was obtained by injecting labelled essential amino acids into lactating animals and comparing the specific activities of the amino acids incorporated in casein and in the plasma proteins. During the first few hours after each injection the amino acid had a much higher specific activity in the casein and β-lactoglobulin than in the plasma proteins. These results suggested that a significant part of the essential amino acids of the

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milk proteins had come from the free amino acids of blood and not from the plasma proteins. The possibility that the radioactive amino acids had been rapidly incorporated into a small fraction of the plasma proteins in the liver before being absorbed by the mammary gland was excluded since the same results were obtained when an isolated mammary gland was perfused with blood (Barry 1958).

It is difficult to measure changes in the specific activity of an amino acid in wool over periods of only a few hours. Fleischer, Vidaver, and Haurowitz (1959) have measured changes in the optical density along radioautograms of rat hairs labelled with ^{35}S, but have not shown whether the absolute value of the specific activity can be determined accurately by this technique. In the present work it has been possible to show that the cystine in wool attained a much higher activity than the cystine of the plasma proteins when a sheep was given an intravenous dose of DL-[^{35}S]cystine. Cystine, therefore, is probably not incorporated in wool by a process involving a direct modification of the plasma proteins. However, the possibility that one or more minor components of the plasma proteins are precursors of wool keratin has not been definitely excluded.

II. Material and Methods

A Corriedale ewe (52 kg body wt.) was injected intravenously with DL-[^{35}S]cystine (21·7 mg, 829 μc, obtained from the Radiochemical Centre, Amersham, England) as the hydrochloride in 10 ml 0·9% NaCl. The cystine was shown to be radiochemically pure within experimental error ($\pm 2\%$) by carrier dilution analysis. Samples of blood and wool were taken at intervals in order to compare the maximum activity attained by the cystine in the plasma proteins and the wool protein. To determine the time at which the maxima occurred, the specific activities of the proteins themselves were measured first. The cystine was then isolated from hydrolysates of representative samples of the mixed plasma proteins and of the wool, and its specific activity measured. To build up a more complete picture of the fate of cystine in the sheep, samples of plasma were also analysed for total ^{35}S and for ^{35}S as free cystine and sulphate.

(*a*) *Measurements of Specific Activity*

Except for the wool, all samples were counted at "infinite" thickness on 1 cm^2 polythene disks (Popjàk and Beeckmans 1950) or the results corrected to the infinite thickness value where necessary from an empirically constructed correction curve. A thin end-window G.M. counter (G.E.C. type EHM2S) and an Ekco Scaler (N529) were used, at least 2500 counts being taken for each sample. Resolving time and background corrections were made and the net counting rates were further corrected for decay and for possible sensitivity changes in the counter by counting [^{35}S]cystine standards with each batch of unknowns. Material with a specific activity of 1 μc/g gave 730 counts/min.

(*b*) *Analysis of the Blood Samples*

The blood samples were centrifuged at 2700 *g* for 30 min at 5°C, and the plasma either analysed immediately or frozen until required.

(i) *Total ^{35}S Content.*—Plasma samples (1 ml) were evaporated to dryness under an infrared lamp, ground to a powder, and the specific activity measured.

(ii) *Trichloroacetic acid-precipitable ^{35}S.*—Plasma samples (1 ml) were added to trichloroacetic acid (TCA) (4 ml 10% w/v), allowed to stand at room temperature for 2–3 hr, and the precipitated proteins centrifuged, washed twice with TCA (10 ml 5% w/v), once with ethanol (95%), once with ethanol–ether (1 : 1 v/v), and finally with ether. The dried, finely ground TCA precipitates contained both the [^{35}S]cystine incorporated in the protein chains and that bound to the proteins through disulphide bonds. The amount of bound ^{35}S was found to increase considerably *in vitro* during the first few hours after taking the early blood samples.

(iii) *Protein ^{35}S.*—To remove the bound cystine other samples (1 ml) of the plasma were mixed with aqueous $NaHSO_3$ (0·2 ml 10%), or with mercaptoethanol (Lee *et al.* 1951) for 5 min at room temperature before the TCA precipitation. The ^{35}S in the product of this treatment was considered to be "protein-^{35}S", and the amount of bound ^{35}S was calculated as the difference between the TCA-precipitable ^{35}S and the protein ^{35}S.

(iv) *^{35}S as Free Cystine.*—The "carrier" technique was used because of the low concentration of cystine in plasma. Samples of plasma (1 ml) were added to TCA (5 ml 10%) containing non-radioactive DL-cystine (40 mg). The mixtures were allowed to stand overnight before isolating the TCA precipitates as described above. The TCA filtrate and washings were extracted twice with ether to remove most of the TCA and the pH adjusted to 5 with dilute NH_4OH to precipitate the cystine. An equal volume of ethanol was added to aid the precipitation and the cystine was filtered, washed, dried, and its specific activity measured. From the specific activity and the known mass of cystine added the amount of ^{35}S in the plasma as free cystine was calculated.

(v) *^{35}S Sulphate.*—The carrier technique was also used. The filtrate and washings from the cystine precipitation (Section II(*b*) (iv)) were recovered quantitatively and mixed with carrier sulphate ions in the form of aqueous K_2SO_4 (10 ml 0·0236M). Benzidine hydrochloride (4 ml 2%) and ethanol (final concentration 50%) were then added and the precipitate of benzidine sulphate filtered, washed well with aqueous ethanol, dried, and its specific activity measured.

(*c*) *The Wool Samples*

(i) *Preparation of the Wool.*—The wool was clipped from the sheep as closely as possible about 18 hr before the injection. Four areas on the skin were then defined by tattooing. Area 1 (18×44 cm) was shaved at intervals after the injection (cf. Fig. 3). Areas 2 (17×29 cm), 3 (13×37 cm), and 4 (26×27 cm) on the other side of the sheep were clipped as closely as possible 1, 2, and 3 days after the injection. Each of these three areas was then clipped every 3 days to give successive 3-day wool samples. Twenty days after the injection the four areas and the rest of the fleece were clipped. Since the clipped wool gave substantially the same results as the shaved wool during the first 20 days, one area only (area 1) was clipped at intervals for a further 70 days.

The wool was isolated from the soapy suspension resulting from the shaving of area 1 by centrifugation at 100 *g* for about 10 min. Most of the supernatant solution was decanted and the residue filtered and washed well with distilled water, ethanol, and ether. The clipped wool from the other areas was transferred directly to a filter paper and washed well with ether, ethanol, and water.

(ii) *Measurement of the Specific Activity of the Wool.*—Infinitely thick samples of the wool, cut into lengths of a few millimetres if necessary, were spread as evenly as possible on 1-in. dia. aluminium planchets, dried in an oven at 110°C, allowed to cool in a desiccator, and counted for no more than 10 min after being removed from the desiccator. This method gave sufficiently accurate results. For example, 10 such samples prepared from the same batch of wool gave the following counting rates: 1180, 1160, 1130, 1130, 1110, 1090, 1150, 1210, 1140, and 1180 counts/min; mean 1150 counts/min with a standard deviation of 32 counts/min. Some of the same wool was oxidized (Walkenstein and Knebel 1957) and the ^{35}S assayed as benzidine sulphate on 1-cm^2 polythene disks. It was calculated from these results and those from benzidine sulphate prepared by oxidizing samples of [^{35}S]cystine that wool with a specific activity of 1 μc/g counted directly on the aluminium planchets gave 2300 counts/min.

(*g*) *Isolation of Cystine from the Wool and Plasma Proteins*

Samples of the plasma proteins and of wool (about 1 g) were hydrolysed by refluxing with HCl (200 ml 6N) for 16 hr. The bulk of the HCl was removed by vacuum distillation and the hydrolysate added to a column of "Amberlite IR120" (2·2 by 150 cm) and eluted with HCl (Hirs, Moore, and Stein 1954). The fractions containing cystine were combined, evaporated to dryness *in vacuo*, and dissolved in a few ml water. This solution was filtered and the pH adjusted to 5 with NH_4OH to precipitate cystine, which was filtered, washed with water and ethanol, dried, and its specific activity measured. Paper chromatograms showed that cystine isolated in this way usually contained traces of leucine or isoleucine or both. However, removal of these impurities by reprecipitation of the cystine produced a negligible change in its specific activity.

The results for cystine were confirmed by measuring the specific activity of the cysteic acid isolated from samples of wool which had been oxidized with peracetic acid (Corfield, Robson, and Skinner 1958) or from plasma proteins oxidized with performic acid (Bidmead and Ley 1958). The cysteic acid was isolated by elution from columns of "Amberlite IR120" as described above. Under these conditions cysteic acid was eluted first, free of contamination by any other amino acid. The cysteic acid fraction was evaporated to dryness *in vacuo*, dissolved in water, filtered, and diluted with ethanol. The crystals which separated were filtered, washed with ethanol, dried, and the specific activity measured. Recrystallization from aqueous ethanol did not alter the specific activity.

III. Results

The radiochemical composition of the plasma from the blood samples taken during the first day after the dose of DL-[^{35}S]cystine is shown in Figure 1. Initially

the total and TCA-precipitable radioactivity were measured. However, the specific activities of the TCA precipitates from the first few samples were higher than expected; that is, they did not produce a curve rising from zero to a maximum. Moreover, although stored in the frozen state, each time the samples were thawed and analysed, the specific activities of the first two TCA precipitates (from the blood

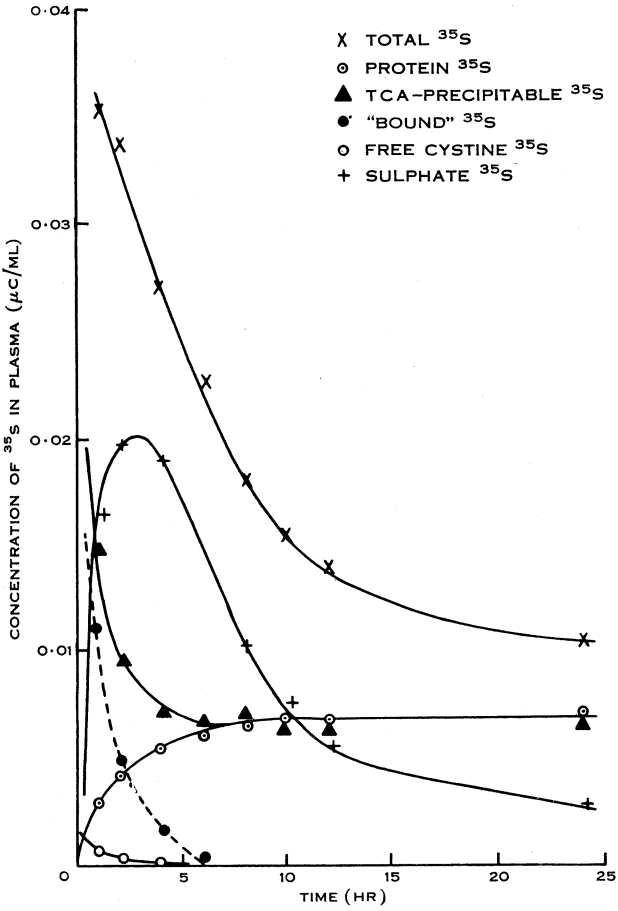

Fig. 1.—Amounts of ^{35}S present in various forms in the plasma from blood samples taken during the first day after the intravenous injection of DL-[^{35}S]cystine into a sheep. The results were obtained at the time of analysis for sulphate -^{35}S by which time the bulk of the non-peptide cystine had become bound to the proteins by disulphide bonds. The amount of bound ^{35}S was calculated by subtracting the protein -^{35}S from the TCA-precipitable -^{35}S.

samples taken 1 and 2 hr after the injection) had increased, whereas the later samples were unchanged. These anomalous results were shown to be due to the binding of cystine to the plasma proteins by disulphide bonds. Separate experiments, in which DL-[^{35}S]cystine was added to plasma *in vitro*, showed that a large proportion of the cystine added to plasma becomes bound and that the bound cystine could be removed with a reducing agent. Eight weeks after the samples were taken the distribution of ^{35}S was as shown in Figure 1.

After about 6 hr the protein -^{35}S and TCA-precipitable-^{35}S curves were identical, showing that a negligible amount of the ^{35}S was present as non-protein cystine after that time. A large proportion of the cystine -^{35}S was converted to sulphate-^{35}S, the peak concentration of which occurred after about 3 hr. The bulk of the radioactivity (more than 90%) was thus accounted for as protein, sulphate, and in the early stages, free and protein-bound ^{35}S (presumably still cystine).

The specific activity of the mixed plasma proteins reached a maximum value about 10 hr after the injection and then decreased exponentially with a half-time of about 20 days (Fig. 2). This figure also shows that the ratio of the specific activities of the dried plasma and plasma proteins did not become constant for about 3 days. After this time the bulk of the circulating ^{35}S was evidently present in the proteins, most of the sulphate-^{35}S having been excreted.

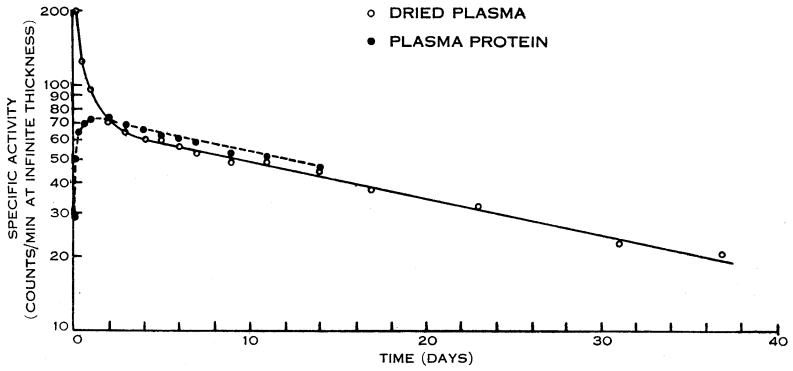

Fig. 2.—Changes in specific activity of the dried plasma and plasma proteins following an intravenous dose of DL-[^{35}S]cystine. The slope of the linear part of the curves indicates that the plasma proteins were turned over with a half-time of about 20 days.

The maximum counting rate of the plasma proteins was 73 counts/min, which corresponded to 0·10 μc/g. Assuming a plasma volume of 3 l. and a protein content of 7%, about 2·5% of the dose was incorporated into the plasma proteins.

The counting rates of the wool samples from area 1 are shown in Figure 3. Radioactivity was first detected in the third day's shavings. A rapid rise to a maximum specific activity on the sixth day was followed at first by a rapid and then a more gradual decline. The declining portion of the specific activity–time curve was resolvable into three exponential curves, as shown in Figure 3, with half-times of about 26, 3, and 1 days respectively.

Substantially the same results were obtained for the wool clipped from the other tattooed areas. Since the average specific activity of the wool grown during the first 20 days after the dose was 0·66$\pm$0·02 μc/g, and the total weight of wool collected was 119 g, it may be calculated that at least 9·5% of the radioactive dose appeared in the fleece during this period. The true value must be somewhat higher because the wool was not clipped from the head and legs of the animal. A similar calculation showed that the wool grown from the 20th to the 90th day contained a further 1·9% of the dose.

The specific activities of the cystine and cysteic acid isolated from various samples of wool and plasma proteins are shown in Table 1, together with those for benzidine sulphate prepared from completely oxidized wool. To compare the results more easily the specific activity of the *sulphur* in each sample was calculated and is shown in the table. Since the bulk of the sulphur in wool is present as cystine (Simmonds 1954) and since the specific activity of the sulphur in the

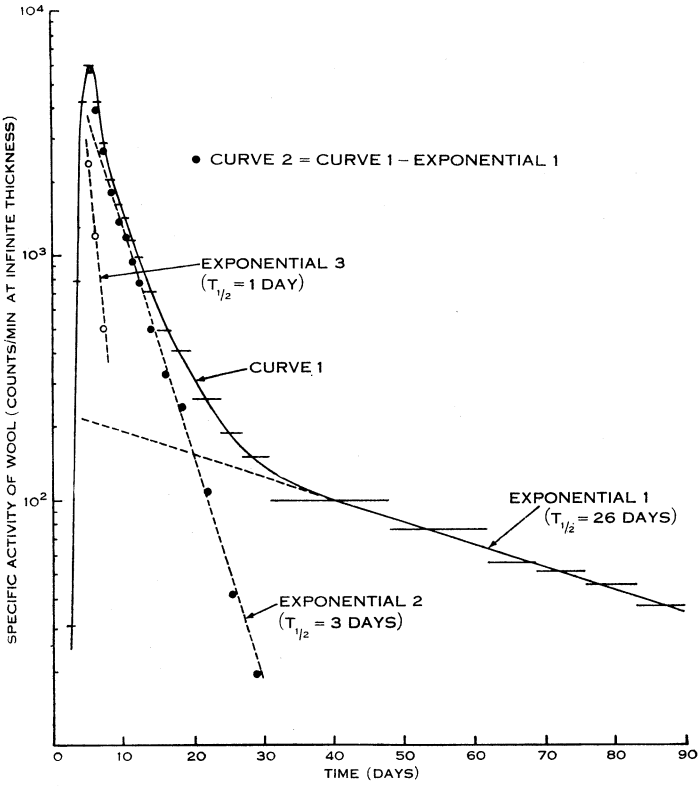

Fig. 3.—Graph showing the specific radioactivity of wool clipped at intervals from the same region of a sheep after an intravenous dose of DL-[^{35}S]cystine. The horizontal lines represent the average net counting rates, corrected for decay, of infinitely thick wool samples grown during periods of time indicated by the length of each line. The descending portion of the curve has been resolved into three exponentials, which are discussed in the text.

cystine isolated from the first 20 days wool was slightly higher than that of the total sulphur from the same wool, it follows that substantially all the ^{35}S in this wool was accounted for as cystine. Similarly all the ^{35}S in the plasma proteins examined was evidently present as cystine, since no other ^{35}S was observed in the effluent from the ion-exchange separations of the protein hydrolysates and since a reasonable value (3·3%) for the percentage of cystine in plasma proteins could be calculated from the results on the assumption that all the ^{35}S was present as cystine.

The maximum observed specific activity of the cystine in the wool was about seven times higher than the maximum for the cystine in the plasma proteins. Similar results have been obtained in two other experiments.

IV. Discussion

The results (Table 1) show that the cystine in the wool reached a much higher specific activity than the maximum attained by the cystine in the plasma proteins —about seven times higher. Radioautographic studies of the labelled wool fibres

Table 1

Specific activities of various samples isolated from wool and plasma proteins

Individual values given for benzidine sulphate are for separate oxidations; those for cystine and cysteic acid are for different portions of the effluent containing these compounds from each ion-exchange separation

Compound Assayed	Source	Specific Activity (μc/g) of:	
		Compound Assayed	Sulphur in Compound Assayed (calc.)
Cystine	Material injected	38,200	143,000
Benzidine sulphate	First 20 days' wool	1·96, 2·11 } 2·03	17·9
Benzidine sulphate	First 20 days' wool oxidized with peracetic acid	2·02, 2·07 } 2·05	18·1
Cystine	First 20 days' wool	5·07, 4·75, 5·03, 5·36 } 5·05	19·0
Cysteic acid	First 20 days' wool oxidized with peracetic acid	3·56, 3·88 } 3·72	19·6
Cystine	Wool grown during the 5th, 6th, and 7th day	18·2, 18·5 } 18·3	68·5
Cystine	Plasma proteins isolated from blood collected 6 days after dose	2·83, 2·87, 3·02 } 2·91	10·9
Cysteic acid	Plasma proteins isolated from blood collected 4 days after dose, then oxidized with performic acid	1·94, 1·98 } 1·96	10·3

have shown that the region of maximum specific activity corresponds to less than 1 day's wool growth (Downes and Lyne 1959). Therefore, the true maximum specific activity of the wool cystine must have been much higher than the observed value because the latter was the average for a 24-hr period, not including all of the most radioactive segments of the fibres.

This comparison suggests that a large proportion of the cystine in wool keratin is incorporated, not by any process involving a modification of plasma proteins, but probably directly as the free amino acid. The alternative possibility is that the labelled cystine was concentrated in a component of the plasma proteins with a higher specific activity than was observed in the cystine of the wool. Although insufficient plasma was available to examine this point, fractionations of plasma proteins similarly labelled with [^{35}S]cystine in subsequent experiments have since been carried out as described by Campbell and Work (1952). Radioactivity was present in the fibrin, globulin, and albumin fractions of the plasma obtained 6 and 30 days after an intravenous dose, and their specific activities did not differ by more than a factor of about 2 (Downes, unpublished data). All the radioactivity is therefore not concentrated into a minor component of the plasma proteins. A more detailed study of the labelling patterns of the various plasma proteins is now being made.

Tarver (1954) has pointed out that the non-peptide binding of radioactive amino acids by proteins can be especially troublesome with labelled cystine, and other authors have referred specifically to the binding of cysteine by plasma proteins (Lee *et al.* 1951; Samarina, Kritzman, and Konikova 1956). Cysteine can evidently be bound as half-cystine residues by disulphide bonds and removed by reducing agents such as mercaptoethanol. However, as may be seen from Figure 1, there was a negligible amount of radioactive free or bound cystine in the plasma from about 6 hr after the injection. After this time, pretreatment with a reducing agent did not alter the specific activity. The plasma protein samples used to measure the specific activity of the incorporated cystine were prepared from blood taken from the sheep at least 4 days after the dose, by which time practically all the circulating ^{35}S must have been present as cystine incorporated into protein chains by peptide bonds. This is evident from Figure 2 which shows that the ratio of the specific activity of the plasma proteins to that of the dried plasma was constant from approximately 3 days after the injection. Such proteins must have contained some bound cystine but the error introduced would have been small since other work (Downes, unpublished data) has shown the mass of the bound cystine to be less than 5% of that of the peptide-linked cystine.

There is therefore little doubt that the [^{35}S]cystine was incorporated in peptide linkage in the plasma proteins referred to in Table 1. Similarly, all the labelled cystine found in the wool keratin must have been incorporated in the protein chains otherwise the oxidation with peracetic acid would have removed some of the radioactivity.

There are two observations which suggest that at least some of the cystine in wool is incorporated during the final keratinization and the present results support this hypothesis. Firstly, radioautographic studies in the lamb (Ryder 1959) and mouse (Harkness and Bern 1957) showed an intense concentration of radioactivity in the keratogenous zone soon after the administration of an intraperitoneal dose of labelled cystine; and secondly, cystine occurs in hair roots in much lower concentrations than in fully keratinized hair (Rogers and Simmonds 1958). In view of the exceptional role played by cystine in keratinization, the conclusion that the

cystine in wool keratin is largely derived from the free amino acid may not be applicable to the other amino acids.

Finally, what is the significance of the three exponential curves shown in Figure 3? The graphical analysis of a specific activity curve in the way shown may be taken as an indication of the number of metabolic "pools" with which the substance being studied equilibrates (see, for example, Matthews 1957 for the application of this method to labelled plasma proteins). Since wool is an excretion product which is formed at a steady rate we may presume that changes in its specific activity reflect changes in the specific activity of the material from which it is synthesized. A straight line could reasonably be drawn through the points representing the mean specific activities of the wool samples grown from the 35th to the 90th day. This line, extrapolated back to zero time on the graph presumably indicates the results that would have been obtained if the injected cystine had been mixed instantaneously with all the other cystine in the animal, excluding that in the wool already grown. Evidently equilibrium was not reached until about the 35th day, after which time the specific activity fell exponentially with a half-time of about 26 days. This half-time, which is of the same order as that for the plasma proteins, probably indicates the average turnover half-time of cystine in the sheep.

If free cystine in the plasma were the only direct precursor of the cystine in wool and if it had been possible to measure the instantaneous specific activity of the cystine in wool continuously, then one would have expected to find a high specific activity in the cystine incorporated in the wool immediately after the injection followed at first by a rapid fall during the first day or so and subsequently by the slow exponential disappearance with a half-time of 26 days. In practice, with daily shavings as a poor substitute for instantaneous measurements, the first part of such a curve would have been masked by the delay of a few days taken for the radioactive portions to emerge above the skin and by the fact that these portions do not all emerge at the same time. Nevertheless, in these circumstances one would have expected the descending portion of the specific activity curve to be resolvable into two exponentials only—a rapid drop which would be a reflection of the ascending portion of the curve and would therefore have indicated an apparent half-time of only a day or so; and the slow component representing the overall turnover of cystine in the whole animal.

The presence of the line with an intermediate half-time of about 3 days suggests that there is an intermediate metabolic pool between the free cystine in plasma and the cystine in wool. As pointed out above, a half-time of 3 days seems much too long to be associated with the free cystine in the plasma. Free amino acids in plasma are known to have turnover half-times of only a few minutes and unpublished experiments carried out here have shown that the exchange between free and bound cysteine in plasma occurs with a half-time of about 1 hr. This is confirmed by Figure 1 which shows that there was a very small amount of ^{35}S circulating as free or bound cystine about 6 hr after the dose. Provided there were no large differences in the maximum specific activities of the cystine in the individual plasma proteins, the specific activity of the free plus bound cystine would have been less than the maximum specific activity of the cystine in the plasma proteins,

after this maximum was attained. This follows from the relationship between the specific activity of a product and its precursors (Zilversmit, Entenman, and Fishler 1943). The maximum specific activity of the cystine in the plasma proteins was reached in 24 hr at the latest and must have been about 3 μc/g (Table 1), but the specific activity of the cystine in the wool did not fall to this value until about the 15th day after the injection. Allowing for the average delay of about 6 days taken for the radioactivity to appear above the skin surface it may be concluded that the specific activity of the wool cystine was higher than that of the free cystine of the plasma during the first 9 days.

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