The Absorption of Methionine by the Skin of the Guinea Pig

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The use of compounds labelled with isotopic sulphur is a well-established method for tracing the origin of the sulphur-containing proteins of hair and skin. Tarver & Schmidt (1939, 1942) fed or injected rats with methionine labelled with ^{35}S and showed that the radioactive sulphur appeared in the hair and tissues as cystine. Du Vigneaud, Kilmer, Rachele & Cohn (1944) labelled methionine with ^{34}S and with ^{13}C in the β and γ positions and fed the amino acid to rats in a diet otherwise deficient of sulphur-containing amino acids. They isolated cystine from the hair of the rats and found by isotopic analysis that about 80% of the sulphur but no significant amount of the carbon had been derived from the methionine. More recently, Williamson & Fromm (1953) have shown that wounded rats, injected with [^{35}S]methionine, accumulate the radioactive sulphur in the wound tissue mainly as cystine, even when the ^{35}S content of the other tissues is decreasing.

The object of the present investigation was to determine whether methionine, rubbed into the skin of the guinea pig, could be absorbed and incorporated into the protein of growing hair. The animals used were all healthy adults, kept on M.R.C. compressed diet no. 18, supplemented by cabbage leaves so that there would be no deficiency of methionine or vitamins essential to the normal growth of the hair. The methionine was labelled with ^{35}S , and evidence was obtained that this was used in forming hair cystine, and appeared in the internal organs even when precautions were taken against the accidental contamination of the food by radioactive material.

EXPERIMENTAL

Mode of application of [^{35}S]methionine

The methionine used in this work was DL-[^{35}S]methionine (supplied by The Radiochemical Centre, Amersham, Bucks). It had a specific activity of 0.127 mc/mg. on the initial assay and was applied in the form of an aqueous solution (0.175 mg./ml.). The solution was dispensed from a dropping bottle fitted with a hypodermic needle ground flat at the tip so that one drop of solution (0.0120 ± 0.0002 ml.) was equivalent to 2.10 $\mu\text{g.}$ of [^{35}S]methionine.

From time to time a drop of the solution was evaporated to dryness on aluminium foil and counted to check the activity. It was found that the observed activity/drop was

always within $\pm 2\%$ of the theoretical value calculated from the known half life of ^{35}S . This showed that (i) there was no appreciable loss of material from solution by adsorption on the walls of the container, and (ii) the drop volume remained constant over the period of the investigation.

A single drop of solution delivered in this manner was a convenient unit for application to the guinea pig in the following studies.

In order to compare the relative efficiencies of absorption of the amino acid by intramuscular injection, topical application and feeding, the experimental animals were divided into three groups.

Group I were injected in the thigh with 4 units or 20 units of labelled methionine. Hair clippings were taken at the time of injection and at further weekly intervals until the activity was too small to be measured accurately.

Group II were rubbed daily on the previously prepared area of the back with 1, 4, 10 or 15 units for 4 consecutive days each week and hair clippings taken on the seventh day. This procedure was continued for 4 weeks, after which the application of methionine was discontinued but weekly clippings were taken for a further period until the ^{35}S was eliminated from the growing hair.

Group III were fed with 15 units daily for four consecutive days each week and hair clippings were taken on the seventh day. The feeding was continued for 4 weeks only but clippings were taken every week until no further ^{35}S could be detected.

In all groups the area selected for clipping was marked out on the back of the guinea pig and was, as nearly as could be judged, equivalent to one-tenth of the total hairy surface of the animal. Clippings were taken as close to the skin as possible so that each week's yield represented the total growth of hair over that area for the preceding seven days. It was observed that the growth rate averaged about 0.8 g./week over the whole surface so that the area under investigation usually yielded about 80 mg. weekly, sufficient for the quantitative estimation of ^{35}S content.

The methionine was applied to the animals of group II over the clipped area by measuring the appropriate number of units on to the back of the guinea pig and gently massaging the solution into the skin until moisture was no longer visible. Massage was done with the operator's forefinger encased in a rubber glove, and although there was a certain amount of mechanical loss of radioactive material retained by the glove it was estimated that this was not serious and did not exceed 10% of the amount applied each day. Care was taken that no radioactive material should be allowed to contaminate the food of animals in this group. Excreta and loose hairs were removed from the cages before feeding and the cages were monitored from time to time to check the presence of any stray radioactivity that might otherwise be ingested.

In addition to that area of the back where the labelled methionine was applied, the animals of group II were clipped weekly over an area of equal size on the underbelly, as far as possible from the area of application. This was done because, although all hair samples were given a standard washing treatment before counting, there was the possibility that some of the [^{35}S]methionine might be so firmly adsorbed by the hair that a spuriously high count might be obtained on the samples taken from the back. The clippings from the underbelly, however, would be less liable to contamination, and their ^{35}S content would probably be more representative of the animal as a whole. This was partly confirmed by treating unlabelled hair clippings with various amounts of [^{35}S]methionine and then going through the standard washing treatment to remove the adsorbed amino acid. It was found that hair contaminated at the 10 and 15 units levels sometimes retained as much as 0.1 μg . [^{35}S]methionine/g. whilst the 1 and 4 units levels could retain up to about 0.05 μg /g. Any difference of this order between the activities of hair samples drawn from the back and belly of animals nos. 5–10 during the first 5 weeks could not therefore be interpreted as evidence that the methionine was more readily incorporated into the hair protein immediately adjacent to the area of application.

The measurement of relative activity in guinea pig hair labelled with ^{35}S

For the accurate assay of ^{35}S it is usual to oxidize all sulphur-containing samples to SO_4^{2-} and to precipitate as the barium or benzidine salt before counting under standardized conditions (cf. Henriques, Kistiakowsky, Margnetti & Schneider, 1946). As nearly all the measurements in this investigation were estimates of the relative activity of hair samples, a procedure involving a direct count on the hair itself was adopted; sufficiently reproducible results could rapidly be obtained in this manner without the need for a large number of time-consuming oxidations.

The average length of hair in the weekly clippings taken from each experimental animal was usually about 2 mm. and it was found that a circular pad of hair, formed in a manner analogous to the preparation of the asbestos-fibre pad in a Gooch filter, was uniform enough to give a reproducible count under equivalent geometrical conditions.

The filter assembly used in forming the pad was similar in principle to that described by Comar, Hansard, Hood, Plumlee & Barrentine (1951). The hair clippings were first examined, and any hairs substantially longer than 2 mm. were cut down to this length. The sample was then stirred at 60° for 1 hr. with 1 l. 50% (v/v) aqueous ethanol, filtered,

suspended in 0.3 l. 95% ethanol and stirred at 60° for a further 30 min. The ethanolic suspension was filtered on to a 2 cm. Whatman no. 1 paper in the brass filter assembly; the pad of hair was dried under the infrared lamp, mounted on the counting block and placed in a square lead castle (E.R.D. Engineering Co., Ltd., Slough, Bucks) fitted with a type E.H.M.2 end-window counter (General Electric Co. Ltd.). The counts were made with a Panax Model 44a combined amplifier, scaler, register and power unit (Panax Equipment Ltd., 173, London Road, Mitcham, Surrey).

All observed counts were compared with a [^{14}C]polymethylmethacrylate reference standard and corrected for background (10 counts/min.), resolving time (about 450 μsec .) and radioactive decay, using 87.1 days for the half life of ^{35}S . Where necessary the observed counts were converted into 'infinite thickness' counts by means of a weight-correction curve constructed from the results given in Table 1.

The relative activity of the hair sample could be expressed as a function of the concentration of labelled methionine by hydrolysing hair of known activity and comparing the activity of the hydrolysate with that of a hydrolysate prepared from unlabelled hair containing a known amount of added [^{35}S]methionine.

Thus, 0.3 g. of hair, having an observed relative activity of 510 counts/min. was hydrolysed with 2 ml. 10N-HCl for 12 hr. at 110° and, after removal of excess HCl, the amino acid hydrochloride solution was made up to 5 g. total weight with water; 0.076 g. of this solution, evaporated to dryness

Fig. 1. Distribution of radioactivity along a paper chromatogram of [^{35}S]methionine. For details see text.

Table 1. Self-absorption correction for three samples of guinea pig hair (see text)

A		B		C	
Wt. of hair (mg.)	Counts/min.	Wt. of hair (mg.)	Counts/min.	Wt. of hair (mg.)	Counts/min.
6	14.1	6	38.4	8	125
12	27.6	11	58.2	17	205
17	40.3	19	95.2	23	297
27	55.8	27	131	31	342
37	63.0	45	159	40	376
48	70.0	58	164	50	389
60	72.0	67	169	63	398
73	72.5	85	171	75	405
97	72.4	100	169	90	410

on aluminium foil, had an activity of 204 counts/min. A further 0.3 g. of unlabelled hair was hydrolysed in the same manner in the presence of 0.447 μ g. of added [35 S]methionine. After removing excess HCl and making up to 5 g., 0.0725 g. of this solution, evaporated to dryness on aluminium foil, had an activity of 307 counts/min.

The weight of dry amino acid hydrochlorides in these activity measurements was of the order 2–3 mg./cm.², low enough to make the self-absorption factor negligible so that the observed count could be taken as proportional to the 35 S content. Then a hair sample of relative activity 1 count/min. would have a [35 S]methionine concentration of

$$\frac{204 \times 0.0725 \times 0.447}{307 \times 0.0760 \times 0.3 \times 510} = 0.00185 \mu\text{g./g. hair,}$$

and from this factor the equivalent weight of [35 S]methionine combined in the hair could be calculated for all samples.

Paper chromatography of the amino acids from labelled hair

The combined sulphur in hair is distributed between the methionine and cystine residues of the keratin molecule and, in round figures, the hair hydrolysate contains about 10% of cystine to 1% of methionine (Block & Bolling, 1951). If any of the 35 S introduced as methionine is converted into cystine before incorporation in the hair protein, the [35 S]cystine should be detectable in the cystine spot after separation of the amino acids by paper chromatography.

A trial paper chromatogram of the original [35 S]methionine solution, using approx. 0.1 μ g. of amino acid, was cut into small sections and the distribution of radioactivity along the axis was determined by counting the relative activity of each section. It was found that over 90% of the radioactivity was confined to a narrow band (R_F , 0.44–0.54 with a peak at 0.49); about 4% was situated in a secondary band (R_F , 0.07–0.29 with a peak at 0.23); the remainder was distributed between these two bands (Fig. 1). The solvent consisted of diethylamine, 10 ml.; *n*-butanol, 50 ml.; ethanol, 50 ml.; water, 25 ml. R_F values of methionine and cystine control spots were 0.5 and 0.2 respectively. Downward displacement on Whatman no. 1 paper was used for all chromatograms.

A sample of labelled hair taken from animal no. 10 at the end of the fifth week, and containing nearly 1 μ g. [35 S]methionine/g., was hydrolysed with 10N-HCl in the usual manner and run on a paper chromatogram at the highest practical concentration over a wide front. This was done to increase the amount of radioactive material that could be eluted from each section of the paper and hence increase the sensitivity of the assay. The peak activity, determined after elution from each strip, was found to be concentrated around R_F 0.2; a very small secondary peak was found at R_F 0.6. The R_F values for methionine and cystine hydrochlorides, using the same solvent as before, were 0.6 and 0.2 respectively.

A control hydrolysis of unlabelled hair with added [35 S]methionine gave a chromatogram with the main peak at R_F 0.6 and a secondary peak at 0.3. The main peak corresponded to methionine hydrochloride and the secondary peak was probably an oxidation product.

The distribution of activities is shown in Fig. 2.

Precautionary measures taken during topical applications

The possibility that animals receiving topical application might ingest the labelled methionine by licking their hair or by direct absorption through lesions in the skin was given careful attention throughout these experiments. The reasons for accepting these results as evidence of the topical absorption of methionine may be summed up as follows:

(i) Radioactive material was applied only to an area of the back difficult if not impossible for the animal to reach with its mouth or feet. Control animals anointed with a solution of methyl violet in glycerol over an equivalent area could just reach the fringe of the area to lick the coloured glycerol. Here, however, the sensation of moistness on the back and the pleasant taste of the glycerol caused the animals to make a prolonged effort to decontaminate themselves, whereas after the normal methionine rub the back dried almost immediately and the animals exhibited no interest in the contaminated area.

(ii) Each animal had a cage to itself and the cages were frequently cleaned to remove any material that might have been rubbed off the backs of the animals during confinement. The washings were monitored and found to contain very little radioactive material, never more than 5% of the total application.

(iii) In dummy experiments in which the whole of the methionine was applied to the cage itself, the amount of radioactivity in the weekly hair growth was barely detectable, indicating that the animals were not in the habit of licking the wires of the cages.

(iv) None of the experimental animals were ever observed to lick their hair near the area of application.

(v) The animals were fed from clean containers under observation and excess food was removed afterwards to avoid possible contamination with radioactive excreta. The excreta showed some radioactivity after the first week but at such a low level that a very large amount would have to have been eaten to account for the observed activities in the subsequent hair growth.

(vi) When an equivalent dose of radioactive methionine was fed directly to the animals the greater part was elimi-

Fig. 2. Distribution of radioactivity along paper chromatograms of hydrolysates of labelled hair (solid line) and of unlabelled hair (hydrolysed in the presence of labelled methionine) (broken line).

nated during the next few weeks and only about 2% of the ^{35}S found its way into the growing hair. This would mean that about half the topically applied methionine would have to have been licked off to give the observed results if none were absorbed through the skin.

(vii) Hair was removed at the beginning of each week by means of electric clippers producing a closely cropped area of skin free from lesions. Direct absorption into the blood stream via cuts or scratches was thereby avoided.

(viii) The rough proportionality of the radioactive content of the hair at the fourth week to the total amount of methionine applied is further evidence in favour of the observed results. Accidental intake by random licking would have produced a less consistent set of results.

RESULTS

The ^{35}S contents of the weekly hair clippings from the fifteen experimental animals, expressed in terms of the original [^{35}S]methionine, are recorded in Tables 2-4, together with an approximate estimate of the percentage recovery of ^{35}S in the hair, based on the assumption that the amount recovered in the clipped area at the end of any one week is one tenth of the total amount combined in the growing hair during that week. Whilst it would have been more satisfactory to have collected the whole hair growth from each animal at the end of every week, it was felt that this treatment was too severe and might have interfered with the health of the guinea pigs. As it was, the animals appeared quite healthy and, with the exception of no. 11 which died at the fourth week, and no. 12 which died during the seventeenth week, all survived the experiments. The lack of hair growth on the clipped underbelly of no. 14 was caused by rubbing against the bottom of the cage; when a different type of cage was used this animal had a normal growth of hair from the eighth week onwards.

DISCUSSION

[^{35}S]Methionine rubbed into the skin of guinea pigs is readily absorbed, and the radiochromatographic analysis of the hair hydrolysate demonstrates quite clearly that the ^{35}S is combined in the hair protein as cystine. This rules out the possibility of chance contamination of hair by physically adsorbed [^{35}S]methionine in any quantity sufficient to confuse the issue. It is interesting to note that, whatever the daily level of methionine applied to the skin (from 1 to 15 units), the overall recovery in the hair is the same in each case, about 1%, and the peak activity of the hair at the end of the fourth week is roughly proportional to the daily level of application. This indicates that the absorption by the skin was directly proportional to the amount applied at these relatively low concentrations. When the same amount was fed (cf. animals nos. 12, 15 with no. 10) about 2.5 times as much ^{35}S was incorporated into the hair. An even higher proportion found its way into the hair protein when a small dose was injected intramuscularly.

On the basis of these experiments there is no reason to suppose that the mechanism of the utilization of the methionine is any different whether the amino acid is introduced into the system by rubbing, injection or *per os*. A large percentage of the topically applied methionine in group I was washed off the subsequent hair clippings, so that although the gross recovery figure was 1% for this series it is likely that the net recovery was of the same order as in group II. Animal no. 9, killed at the end of the fourth week, was dissected and the liver, spleen and heart removed, dried and powdered. The relative activity of these organs was in the ratio 111:42:198, respectively, on a dry matter basis, indicating that

Table 2. Content of ^{35}S of hair from injected animals

Animal no. ...	1	2	3	4
Units injected ...	4	4	20	20
Total [^{35}S]methionine ($\mu\text{g.}$)	8.4	8.4	42	42
	[^{35}S]Methionine equiv. of weekly hair growth ($\mu\text{g./g.}$)			
Weeks				
1	0.120	0.107	0.333	0.268
2	0.139	0.178	0.568	0.443
3	0.137	0.142	0.305	0.296
4	0.045	0.037	0.137	0.120
5	0.031	0.026	0.109	0.104
6	0.037	0.011	0.070	0.057
7	0.004	0.002	0.043	0.039
8			0.015	0.013
9			0.002	0.001
Total recovery as [^{35}S]-methionine in hair ($\mu\text{g./animal}$)	0.41	0.40	1.27	1.07
Percentage recovery	4.9	4.8	3.0	2.6

Table 3. Content of ³⁵S of hair from rubbed animals

Animal no. ... Units rubbed/day Total [³⁵ S]methionine rubbed in 4 weeks (μg.)	5		6		7		8		9		10		13		14	
	B	U	B	U	B	U	B	U	B	U	B	U	B	U	B	U
1	0.009	0.006	0.006	0.017	0.067	0.011	0.216	0.069	0.383	0.067	0.155	0.080	0.196	0.030	0.213	
2	0.076	0.052	0.043	0.022	0.259	0.205	0.202	0.118	0.500	0.161	0.540	0.352	0.716	0.231	0.740	
3	0.148	0.120	0.091	0.080	0.605	0.203	0.460	0.311	0.718	0.285	0.748	0.774	1.257	0.815	0.980	
4	0.098	0.054	0.080	0.130	0.361	0.426	0.671	0.410	0.918	0.735	0.852	0.845	1.156	0.910	1.340	No growth
5	0.091	0.113	0.102	0.100	0.374	0.264	0.500	0.367	Killed		0.927	0.963	0.855	0.769	0.686	
6	0.041	—	0.052	—	0.278	—	0.420	—	—	—	0.790	—	0.760	0.729	0.832	
7	0.009	—	0.009	—	0.142	—	0.122	—	—	—	0.481	—	0.324	0.296	0.695	
8	0.001	—	0.001	—	0.019	—	0.018	—	—	—	0.209	—	0.318	0.400	0.374	
9	—	—	—	—	0.002	—	0.002	—	—	—	0.046	—	0.242	0.289	0.320	0.294
10	—	—	—	—	—	—	—	—	—	—	0.009	—	0.163	0.262	0.224	0.206
11	—	—	—	—	—	—	—	—	—	—	0.001	—	0.130	0.224	0.157	0.159
12	—	—	—	—	—	—	—	—	—	—	—	—	0.178	0.294	0.100	0.192
13	—	—	—	—	—	—	—	—	—	—	—	—	0.074	0.111	0.072	0.100
14	—	—	—	—	—	—	—	—	—	—	—	—	0.113	0.116	0.068	0.170
15	—	—	—	—	—	—	—	—	—	—	—	—	0.028	0.033	0.072	0.050
16	—	—	—	—	—	—	—	—	—	—	—	—	0.009	0.007	0.033	0.031
17	—	—	—	—	—	—	—	—	—	—	—	—	0.003	0.004	0.010	0.008
18	—	—	—	—	—	—	—	—	—	—	—	—	0.001	0.001	0.003	0.001
Total recovery as [³⁵ S]-methionine in hair (μg./animal)	0.35		0.32		1.46		1.78		1.50		3.72		4.82		5.60	
Percentage recovery	1.0		0.9		1.1		1.3		0.5		1.1		1.1		1.3	

B, hair taken from back; U, hair taken from underbelly.

Table 4. *Content of ^{35}S of hair from fed animals*

Animal no.	...	...	11	12	15
Units fed/day	...	...	15	15	15
Total [^{35}S]methionine fed in 4 weeks ($\mu\text{g.}$)			441	441	441
Weeks	[^{35}S]Methionine equiv. of weekly hair growth ($\mu\text{g./g.}$)				
1	0.141	0.282	0.706		
2	0.886	0.581	1.85		
3	1.48	2.13	2.25		
4	Died	3.04	2.85		
5	—	2.30	2.42		
6	—	1.85	1.29		
7	—	1.052	0.820		
8	—	0.958	0.760		
9	—	0.460	0.346		
10	—	0.394	0.425		
11	—	0.389	0.292		
12	—	0.302	0.220		
13	—	0.235	0.185		
14	—	0.263	0.111		
15	—	0.172	0.075		
16	—	0.150	0.027		
17	—	Died	0.007		
Total recovery as [^{35}S]methionine in hair ($\mu\text{g./animal}$)	2.00	11.64	11.71		
Percentage recovery	0.5	2.6	2.7		

the ^{35}S was distributed throughout the system in the same manner as if it had been fed or injected (cf. Tarver & Schmidt, 1939). It seems likely that once the methionine reaches the blood stream the demethylation proceeds in the usual manner irrespective of the method of introduction. This does not preclude the possibility that a small proportion of the topically applied methionine might be converted into cystine locally in the root cells, but to demonstrate such a mechanism a more detailed investigation, possibly involving tissue-culture techniques, would be necessary.

The Effect of Ammonia on the Oxidation of Glucose by *Zygorhyncus moelleri*

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During an investigation of the respiration of filamentous fungi it was observed that when glucose was given to carbohydrate-starved suspensions of several species 2–3 hr. elapsed before the rate of oxygen consumption became constant. This behaviour is markedly different from glucose oxidation by many other micro-organisms under similar conditions, e.g. yeast and the green alga *Chlorella*,

in which the addition of glucose to starved cells results in a constant respiratory rate being attained within 10–15 min.

Induction periods have been noticed by other workers in the oxidation of acetate by both yeast and by bacteria. Winzler (1940), investigating acetate oxidation by suspensions of baker's yeast, found that preparations starved for 2 hr. required

SUMMARY

1. [^{35}S]Methionine was applied to the skin of the guinea pig; this resulted in incorporation of ^{35}S in the protein of the growing hair, largely as cystine. This was shown by the distribution of the radioactivity in a paper chromatogram of the hair hydrolysate. The ^{35}S content of the hair was similar whether the specimen was from the area of application of methionine or from a remote area. About 1 % of the applied ^{35}S was incorporated in the hair.

2. The amount of ^{35}S absorbed was proportional to the amount rubbed into the skin over the concentration range investigated.

3. If the same amount of [^{35}S]methionine was fed to the guinea pig, about 2.5 times as much ^{35}S was incorporated into the hair.

4. Injection of [^{35}S]methionine intramuscularly was relatively more efficient, as much as 5 % of ^{35}S being incorporated in hair protein; larger doses were utilized to a lesser degree.

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