relatively small deviations between the parallel readings indicate a promising accuracy of the described technique.

The operation of the four-column system imposed no technical difficulty, and the total assay of the five ingredients can be performed by an experienced operator in about 2 hours.

Preliminary experiments for some further simplification of the procedure are now in progress; the results will be reported in a subsequent publication.

SUMMARY

The mechanism of separation of some pharmaceutical derivatives of phenothiazine from mixtures with aspirin, phenacetin, caffeine, and itobarbital has been investigated.

A modified partition chromatography procedure was developed which facilitates a simultaneous quantitative assay of the five active ingredients with satisfactory accuracy.

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Absorption and Incorporation of Methionine-S^{35} into Hair

By A. P. INTOCCIA, J. M. WALSH, and R. L. BOGNER

Administration of methionine-S^{35} to guinea pigs by topical, oral, and intramuscular routes resulted in the incorporation and utilization of the S^{35}-radioactivity by the growing dorsal and ventral hair. Oral or intramuscular administration of methionine-S^{35} led to utilization by the growing hair of between 13 and 21 per cent of the total amount administered. Metabolic utilization by these routes was confirmed by the fact that roughly twice as much nonmethionine-S^{35} as methionine-S^{35} was found. The amount utilized was approximately proportional to the amount administered. A greater amount of S^{35}-radioactivity was detected in the dorsal hair than in the ventral hair even after allowing for the greater rate of hair growth in the dorsal area. When the methionine-S^{35} was applied topically to the dorsal area, approximately 10 per cent of the total amount applied was utilized by the total body hair. At the site of topical application, two-thirds of the S^{35} in the hair in the area appeared to be derived locally and the other one-third systemically.

THE CHEMISTRY and physiology of hair growth has been investigated to gain insight into the factors that control growth and differentiation of hair follicles and formation of keratin. Of special interest is the amino acid composition of and uptake into the proteins and other components of the variety of cells that constitute keratinizing structures and keratin.

This report describes the results of radiotracer investigations initiated to study the influence of various routes of administration on the incorporation of amino acids into hair protein. The amino acid methionine, indispensable for all animals, was chosen for study because it is incorporated into proteins and is converted, after demethylation, into cysteine and cystine which are vital components of keratinized structures.

EXPERIMENTAL

Hartley strain male guinea pigs, adequately adjusted to laboratory conditions and diet, were employed. The diet consisted of pregnant rabbit chow supplemented with fresh lettuce and water *ad libitum*. The animals were housed in individual wire mesh cages that were cleaned daily to prevent the accumulation of any radioactive excreta which could possibly be ingested or adsorbed to the hair of the animal. Prior to the administration of the labeled compound, an area of 2 sq. in. was shaved from the dorsal and ventral surfaces of each animal with an electric clipper. Care was taken in this and all subsequent clippings to prevent abrasions of the skin.

The animals were separated into three groups of six animals. Group *A* received methionine by topical application, group *B* by oral administration, and group *C* by intramuscular injection. Three of the animals in each group received a total dose of 500 mcg. of methionine-S^{35}, and three of the animals received a total dose of 250 mcg.

Radioactive *dl*-methionine-S^{35} was obtained from the Radiochemical Centre, Amersham, England, with a specific activity of 55.9 millicuries per millimole (mc./m*M*). The labeled methionine, shown by paper chromatographic methods to be of radiochemical homogeneity, was diluted with sufficient nonlabeled *dl*-methionine so that the animals at the 250-mcg. level were exposed to a total of 52 μc. and

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TABLE I.—METHIONINE EQUIVALENTS IN HAIR OF GUINEA PIGS (VALUES IN mcg. METHIONINE EQUIVALENTS PER GM. OF HAIR)

Route	Topical				Oral				Intramuscular			
Dosage	250 mcg.		500 mcg.		250 mcg.		500 mcg.		250 mcg.		500 mcg.	
Area	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral
Wk. 1	2.86	0.19	4.14	0.48	0.95	0.53	1.18	0.83	1.57	0.803	1.22	0.60
2	4.46	1.24	10.20	2.32	3.94	3.16	6.69	5.86	3.83	2.78	5.05	4.28
3	4.91	0.85	5.20	1.47	3.47	2.21	7.23	6.36	4.59	3.05	5.19	4.16
4	3.61	1.96	6.48	2.08	2.52	1.66	7.88	4.30	3.83	2.85	6.16	5.16
5	1.97	1.16	5.28	3.31	2.38	2.24	6.50	2.28	3.32	1.36	8.09	6.07
6	1.24	0.65	2.32	1.53	1.17	1.06	4.37	2.96	1.58	1.69	2.93	2.39
7	0.94	0.75	1.85	2.23	0.49	0.53	1.96	1.33	0.73	1.03	1.30	1.35
8	0.50	0.42	1.15	1.12	0.66	0.85	1.73	1.31	0.79	1.10	1.36	1.16
16	0.24	0.25	0.31	0.22	0.31	0.33	0.69	0.83	0.46	0.512	0.63	0.80

the animals at the 500-mcg. level received 128 μc. during the experiment.

The doses administered by topical application were dissolved in a mixture of 18 amino acids in denatured ethyl alcohol[1] to yield solutions that contained methionine concentrations of 250 and 500 mcg./ml. Fifty microliters of these solutions were applied to the shaved areas on the dorsal surfaces of the animals and massaged into the skin with the operator's forefinger encased in a rubber finger cot. Loss of radioactive material by adsorption to the finger cot did not exceed 10% of the daily administered dose.

The oral doses were prepared by dissolving nonradioactive methionine and methionine-S^{35} in distilled water to give the appropriate concentrations of 12.5 and 25.0 mcg./ml. The animals were administered 1 ml. of these solutions intragastrically by means of a 1-ml. syringe and an 18-gauge oral blunted needle.

The intramuscular doses were prepared to contain 125 and 250 mcg./ml. in distilled water; group *C* animals were injected daily with 0.1 ml. of the appropriate solution.

Radioactive methionine preparations were administered daily for 5 consecutive days of each week for 4 weeks. Hair clippings from both the dorsal and ventral surfaces of all animals were collected on the seventh day of each week during the period of methionine-S^{35} administration and for 12 additional weeks after administration was discontinued.

For radioactivity measurements, it was necessary to free the hair of any adsorbed radioactivity prior to S^{35}-measurements. Preliminary tests showed that over 99% of the adsorbed S^{35}-radioactivity on the dorsal hair from the animals treated topically could be removed by washing the hair with 50% ethanol made 1 *N* with hydrochloric acid. Accordingly, 20-mg. samples of the hair from all animal groups were washed, dried, and dissolved in 3 ml. of 1 *M* hydroxide of hyamine 10X. A toluene phosphor containing 0.4% 2,5-diphenyloxazole (PPO) and 0.1% 1,4-bis-2-(5-phenyloxazole)-benzene (POPOP) was added, and the samples were counted in a liquid scintillation spectrometer. Counting efficiency was determined with internal standards. The disintegrations per minute (d.p.m.) were converted to methionine equivalents by assuming that all of the radioactivity in a sample was present as methionine of the same specific activity as that of the administered methionine-S^{35}.

[1] Supplied by Beecham Products, Inc., Clifton, N. J., as Pure Silvikrin which contained ordinary methionine in a concentration of 100 mcg./ml.

For paper chromatography, approximately 20 mg. of the washed hair samples were acid hydrolyzed; the residue was dissolved in distilled water. Aliquots of these solutions were applied to Whatman No. 1 filter paper for chromatography in a solvent of phenol saturated with 6.3% sodium citrate and 3.7% dibasic potassium phosphate. The developed chromatograms were air-dried and cut into segments for liquid scintillation counting or for counting on flat cards in a thin end-window gas-flow proportional counter. Counting in this manner revealed two peaks of radioactivity in the positions of cysteic acid, cystine, and/or glutathione (R_f 0.24) and methionine (R_f 0.80). The dorsal and ventral hair from one animal in each of the three groups for 5 weeks was subjected to chromatographic analysis in the above manner to determine the relative concentrations of these amino acids in the hair.

RESULTS

The "methionine equivalents" of S^{35}-radioactivity of washed dorsal and ventral hair are presented in Table I; each value represents the average of three animals. The data indicated that the hair from the animals that received 250 mcg. of methionine-S^{35} contained approximately half as much activity at peak levels as those that received 500 mcg. The dorsal hair of the animals receiving methionine-S^{35} by topical application in doses of 250 and 500 mcg. showed the highest average S^{35} levels at the third and second week, respectively. Thereafter, the levels decreased gradually up to the point where administration was discontinued after 4 weeks. The average S^{35} levels of the ventral hair of these animals was considerably lower than those found in the dorsal hair. In general, the S^{35} persisted in the hair at least 1 week after discontinuation of administration before the concentration declined more sharply.

The difference between the dorsal and ventral hair of the topical group was not of the same magnitude in the oral and intramuscular groups. The higher values found in the dorsal hair of these latter two groups suggested preferential incorporation of systemically administered methionine-S^{35} by dorsal hair of the guinea pig.

In addition, the peak levels of radioactivity in both oral and intramuscular groups appeared later than did the peaks in the topical group; the peak value in the intramuscular group receiving 500 mcg. methionine-S^{35} did not appear until 1 week after administration was discontinued.

Examination of the dorsal hair data indicated that all routes of administration were equally efficient.

TABLE II.—METHIONINE EQUIVALENTS (mcg.) OF TOTAL HARVESTED HAIR AND TOTAL BODY HAIR

	250-mcg. Dose			500-mcg. Dose		
	Dorsal Harvested Area	Ventral Harvested Area	Total Body	Dorsal Harvested Area	Ventral Harvested Area	Total Body
Oral	1.65 ± 0.40	0.72 ± 0.31	31.92	4.56 ± 0.34	1.70 ± 0.48	84.68
Intramuscular	2.83 ± 0.69	1.05 ± 0.40	52.50	5.45 ± 1.47	2.09 ± 1.05	101.91
Total Topical	3.77 ± 0.84	0.53 ± 0.12	28.51	6.38 ± 1.25	0.79 ± 0.12	43.15
Local	2.39	...		4.33	...	
Systemic	1.38	0.53		2.05	0.79	

However, it was seen that S^{35}-uptake by the ventral hair of the oral and intramuscular groups was similar, but the level of incorporation into the ventral hair of the topical group was significantly lower.

Table II shows the actual weights of hair harvested from the dorsal and ventral surfaces of the animals multiplied by the radioactivity concentrations to give the actual average "methionine equivalents" laid down in the area of harvest. From the weights of hair harvested, the rate of hair growth in the dorsal area averaged 2.16 times the rate of growth in the ventral areas. On the other hand, the average "methionine equivalents" harvested from the animals of the orally administered and intramuscularly injected groups indicated that 2.6 times more radioactivity was recovered from the dorsal areas than from the ventral areas. Since the radioactivity ratio is greater than the growth rate ratio, it was concluded that a preferential uptake of systemic methionine-S^{35} or its S^{35}-labeled conversion products occurred in the dorsal area over and above that due to the higher rate of hair growth.

The values of the ventral areas of the topically applied groups were multiplied by the factor 2.6, previously found to reflect the directional influence of the systemic distribution of S^{35} between the dorsal and ventral hair of the oral and intramuscular groups. These calculated values represented the apparent systemic utilization or incorporation of the S^{35} in the dorsal hair of the topically treated groups and are included in Table II. Local utilization of the S^{35} in the dorsal hair of the topically treated animals was obtained by difference. No local utilization was assumed in the ventral hair of the topically treated groups and it was also assumed that the actual uptake of S^{35} by the animals in the oral and intramuscular groups reflected true systemic utilization.

Groups receiving the methionine-S^{35} by topical application showed high local dorsal utilization but lower systemic utilization. The conclusion was that the topical application of methionine-S^{35} to the small defined areas of the back yielded higher recoveries of S^{35} in the hair from these areas than the recoveries from the other two routes of administration, since higher recoveries were presumably due to local utilization of the methionine-S^{35} superimposed upon a smaller incorporation from penetration of the skin, systemic distribution, and delivery of the S^{35} to the same site. Approximately two thirds of the radioactivity in this area was due to local utilization and one-third was provided by systemic utilization.

Total utilization of the S^{35} over the entire body (Table II) was calculated by multiplying the quantities for the uptake in the areas of harvest by factors which integrated them in terms of total body hair growth. These factors were obtained by determining the weight of the harvested hair both dorsally and ventrally and the weight of the remaining dorsal and ventral hair of three test animals. It was then possible to arrive at factors relating the S^{35} in the harvested area to the remainder of the hair for both the dorsal and ventral areas of each animal. These factors were 14.17 for dorsal hair and 11.81 for ventral hair. It should be noted that the results of the oral and intramuscular administration were not dissimilar, although the intramuscular route appeared to be the more efficient of the two routes.

TABLE III.—RATIO OF NONMETHIONINE-S^{35} TO METHIONINE-S^{35}

Route	Level, mcg.	Dorsal Area	Ventral Area
Oral	250	2.4	3.3
	500	2.3	3.4
Intramuscular	250	2.5	1.5
	500	2.3	1.6
Topical	250	1.4	1.8
	500	1.5	1.7

From the data for the total "methionine equivalents" in the total body hair, estimations could be made of the percentages of the total dose utilized. It was found that the total utilization by either the oral or intramuscular route was between 13–17 and 20–21%, respectively, of the total S^{35} administered. The topical route led to a total incorporation of approximately 10% of the applied S^{35}, which represented 89 and 54% of that utilized by the more efficient oral and intramuscular routes, respectively, at the 250-mcg. dose and 51 and 42% at the 500-mcg. dose. Table III presents the average ratios of total nonmethionine-S^{35} to total methionine-S^{35} in the dorsal and ventral areas for the first 5 weeks of the experiments. Nonmethionine-S^{35} activity was not resolved chromatographically into cysteic acid, cystine, or glutathione. It is difficult to interpret the increased ventral ratio by the oral route and the increased dorsal ratio by both the oral and intramuscular routes. Since the S^{35}-radioactivity arrived systemically, similar results were expected in each case. The low ratio in the dorsal area by the topical route suggested local utilization of methionine *per se*.

DISCUSSION

Earlier studies (1) demonstrated that oral feeding of methionine-S^{35} to rats resulted in the appearance of cystine-S^{35} in hair and tissues. Similar results were obtained in guinea pigs by topical application, oral feeding, and intramuscular injection of methionine-S^{35} (2). However, no evidence of local utilization of topically applied methionine has been reported prior to the studies described here.

Several investigators have demonstrated the intense localization of sulfur-containing radioactive

amino acids in the keratogenous zone of hair. Belanger (3) noted localization of methionine-S^{35} and cystine-S^{35} after subcutaneous injections into rats. Fleischer, *et al.* (4), injected S^{35}-yeast protein hydrolysate containing cystine-S^{35}, methionine-S^{35}, and methionine sulfoxide-S^{35} intravenously into rats and found a rapid incorporation of S^{35} into hair, particularly over the length of the intensely vascularized part of the hair. Ryder (5) and Harkness and Bern (6) also found a rapid and marked uptake of S^{35} in the keratogenous zone of anagen follicles after injecting cystine-S^{35}; the latter investigators also noted uptake of C^{14} in the bulb and keratogenous zone after injecting C^{14}-labeled algal protein hydrolysate of undefined amino acid composition into mice.

Bern, *et al.* (7), have pointed out that transport of cystine from the circulatory system *via* absorption through the hair bulb is conceivable, but that a lateral transport from the capillaries around the follicle shaft (8) is also possible.

Apparently, S^{35}-amino acids are at least as equally utilized for hair protein synthesis as for plasma or tissue protein synthesis. The S^{35} specific activity of rabbit hair keratin was found (9) similar to that of the S^{35} activity of tissue proteins following intravenous injections of S^{35}-labeled yeast protein hydrolysate. A much higher specific activity of the cystine-S^{35} in wool than in plasma was noted following the intravenous administration of cystine-S^{35} to sheep (10). The latter concluded cystine is incorporated into wool as a free amino acid.

Only traces of cystine and methionine as free amino acids have been found in aqueous extracts of the roots (bulb, prekeratinized fiber portion, and attached inner root sheath) of keratinized structures, although more methionine is present in the protein of the inner root sheath of hair follicles, as detected by administering S^{35}- and C^{14}-labeled methionine to rats (11) and large quantities of cystine occur in the hair protein itself. According to Rogers (12), the pool of free amino acids in the hair roots is presumably available for keratin synthesis and the small concentration of cystine may be related to a high demand for cystine by the growing fiber and to mechanisms for maximum utilization of the sulfur-containing amino acids. Recently, it has been reported that other amino acids, H^3-labeled alanine and leucine and C^{14}-labeled serine, were incorporated into the growing hair of guinea pigs in the same pattern as cystine-S^{35} after intraperitoneal injection (13); however, no other studies of local utilization following topical application of amino acids by growing hair have been reported.

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Degradation of Phenylephrine Hydrochloride in Tablet Formulations Containing Aspirin

By A. E. TROUP and H. MITCHNER†

The breakdown of aspirin in tablet formulations containing phenylephrine was found to result in a concurrent loss of phenylephrine activity. Specific functional group analysis of the secondary amine function on phenylephrine was necessary to follow degradation in tablet formulations. Analyses with methods based on the phenolic function of phenylephrine did not show similar activity loss. With the use of thin-layer chromatography and comparative chromatograms with synthetic acetylated phenylephrine derivatives, three acetylated phenylephrine degradation products could be identified in tablet formulations. At room temperature the primary degradation pathway was the acetylation of the secondary amine function, but at elevated temperatures, acetylation was found to have progressed to phenylephrine's phenolic and alcoholic groups.

PHENYLEPHRINE HYDROCHLORIDE is frequently incorporated in multi-ingredient pharmaceutical preparations. Analytical assay methods for phenylephrine in such mixtures have been directed both to the development of specific molecular procedures and to methodology involving the separation of the phenylephrine followed by its analysis. Assay problems have been further complicated in the endeavor to evaluate degradation during stability testing where the

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