9. Sever, J. L., Castellano, G. A., Pelon, W., Huebner, R. J., and Wolman, F. J., Lab. Clin. Med. 64, 983 (1964).

10. Kaplan, M. M., Wiktor, T. J., Maes, R. F., Campbell, J. B., and Koprowski, H., J. Virol. 1, 145 (1967).

11. Murphy, F. A. and Fields, B. N., Virology 33, 625 (1967).

Received Nov. 27, 1967. P.S.E.B.M., 1968, Vol. 127.

Fat Storage, De-etherification and Elimination of Quinestrol in the Rat as Influenced by Route of Administration (32865)

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Quinestrol (ethynylestradiol-3-cyclopentyl ether; EECPE) is a potent oral estrogen in animals and human beings (1–4). Unlike its parent compound, ethynylestradiol (EE), EECPE is stored in and slowly released from body fat and brain of rats (5, 6). Storage is enhanced when a lipid rather than an aqueous vehicle is used (7). That portion of the dose which is not stored is metabolized to glucuronide and sulfate conjugates and rapidly eliminated in the bile and urine (8, 9). Using doubly labeled EECPE, it was shown that considerable cleavage of the cyclopentyl ether linkage occurs with elimination of the cyclopentyl label in the urine in excess of the steroid label (8). The site of de-etherification was unknown but was presumed to be either the gut or the liver. If the gut were the major site of de-etherification, then iv administered EECPE should undergo very little cyclopentyl group cleavage. Similarly, if the liver were the major site of de-etherification, then EECPE administered ip or po should initially undergo more extensive de-etherification than that injected iv by virtue of being absorbed entirely via the hepatic portal system. In the present experiments, doubly-labeled EECPE was administered by various routes and fat storage and excretion of the label was followed in the hope of clarifying the site of removal of the cyclopentyl group. In addition, the excretion of radioactivity following administration of labeled cyclopentanol was compared with that following EECPE labeled in the cyclopentyl ring.

Material and Methods. A. General. The chromatographically homogeneous radiosteroids used were ethynylestradiol-6,7-^{3}H-3-cyclopentyl ether (specific activity 1 μC/μg) and ethynylestradiol-3-cyclopentyl-1′-^{14}C ether (specific activity 0.037 μC/μg).[1] Doses were in general prepared to contain ^{3}H and ^{14}C in a ratio of approximately 10:1. Specific doses are indicated in subsequent sections and in the tables. Cyclopentanol-1-^{14}C (New England Nuclear Lot No. 161-275A, specific activity 0.023 μC/μg) was administered at a dose of 0.1 μC (4.3 μg) in aqueous vehicle.

In designing the various experiments, it was assumed that the concentration of radioactivity in body fat 24 hours after dosing would be proportional to the body store of unmetabolized EECPE. This assumption is supported by previous data which showed that: EECPE is rapidly absorbed from the gut when administered as an aqueous suspension (7); it is stored unaltered in body fat and brain (6); its transformation products are not so stored (7, 9); the fat levels remain relatively constant from hours 8 to 48 (7).

B. Influence of dose and vehicle of administration on body fat and brain storage. These experiments were performed to determine if excessive doses of EECPE would retard absorption or overwhelm metabolic or storage sites. Variable amounts of unlabeled EECPE were added to a constant amount (8.5 μC) of tritiated material. Doses varying from 20 μg to 12.5 mg were administered orally either as oily solutions or aqueous suspensions to male albino rats 300–400 gm of body weight. The animals were killed 24 hours after administration and radioactivity in perirenal fat and brain extracted and determined

[1] Radioactive steroids synthesized by Mr. E. Merrill, Radioisotope Safety Officer, WLRI.

by liquid scintillation counting as described previously (7, 8). In one experiment, radioactivity in urine and feces was also determined (8).

C. Influence of route of administration on body fat and brain storage and plasma levels. These experiments were designed to reveal the effects of the gut and liver on the amount of EECPE reaching the fat depots. Either intact or 15 days postcastration female albino rats, 180–200 gm of body weight, were used. Castrated animals received by either oral, intraperitoneal, subcutaneous, or intravenous route, 12.5 μC (20 μg) of tritiated EECPE suspended in 0.2 ml of aqueous vehicle. The animals were killed 24 hours after administration and blood, perirenal fat, and brain were obtained and radioactivity was determined as described previously (7, 8). Plasma samples were diluted 1:5 with water and shaken 3 times with 1 vol. of ether to give a "free" (ether) and "conjugated" (water) fraction (8, 9).

Intact animals received by either oral or intraperitoneal route, 5 μC (20 μg) of tritiated EECPE either suspended in 0.2 ml of aqueous vehicle or dissolved in 0.2 ml of sesame oil. The experiment was performed as that described above with the exception that subcutaneous instead of perirenal fat was analyzed.

D. Influence of route of administration of doubly-labeled EECPE on excretion of radioactivity in bile and urine. These experiments were designed to reveal the effects of the gut and liver on the pattern of metabolites appearing in the bile and urine. Doses of EECPE-^{3}H-^{14}C were suspended in 0.2 ml of an aqueous vehicle and administered by stomach tube or into the exposed jugular vein in 300–350 gm male rats which had been prepared with bile fistulas (9). Bile was collected at 2.5, 5, and 24 hours and urine was collected as a single 24-hour specimen. The distribution of ^{3}H and ^{14}C between the free, glucuronide, and sulfate fractions of bile and urine was determined as previously described (9) using ether extraction without and with β-glucuronidase hydrolysis and solvolysis with ethylacetate at pH 1 after saturation with NaCl.

E. Excretion of ^{14}C in bile and urine of rats treated with cyclopentanol-^{14}C or EECPE-^{14}C. These experiments were performed to compare the excretion of the cyclopentyl ether label of EECPE with that of cyclopentyl alcohol, a likely product of de-etherification of EECPE. The EECPE-^{14}C (0.5 μC/20 μg) or cyclopentanol-1-^{14}C (0.1 μC/4.3 μg) were administered orally or intravenously in 0.5 ml of aqueous vehicle. These doses provided approximately the same weight of cyclopentyl alcohol per rat. Bile samples and urine were collected and processed as in Section D.

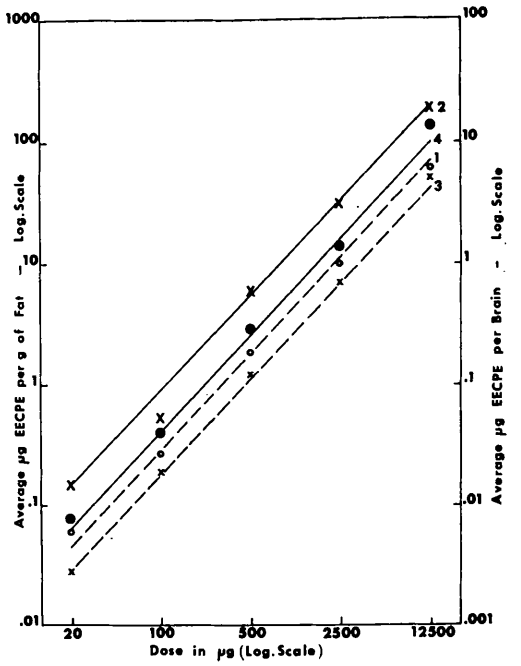

FIG. 1. Influence of dose and vehicle of administration on fat and brain storage of EECPE. 1, Brain from EECPE-oil-treated rats; 2, fat from EECPE-oil-treated rats; 3, brain from EECPE-SV-treated rats; 4, fat from EECPE-SV-treated rats. $b = 1.1463$; $s = 0.1754$; $\gamma = 0.1530$.

Results. A. Influence of dose and vehicle of administration on body fat and brain storage of EECPE. Whether the EECPE was administered orally in sesame oil or as an aqueous suspension, increasing dose resulted in increasing storage in fat and brain (Fig. 1). The slopes of the various dose-storage curves did not differ significantly from each other; however, the common slope was significantly greater ($p<0.01$) than 1 indicating that the storage increment was greater than the dose

TABLE I. Distribution of Dose in Fat, Urine, and Feces of Rats Treated Orally with EECPE.[a]

Dose[b] (μg)	26.4	137	685	3420	17100
Fat[c]	22.1 ± 4.0	22.0 ± 3.7	31.6 ± 4.2	31.8 ± 5.9	60.7 ± 3.3
Urine	4.3 ± 0.6	2.6 ± 0.3	1.8 ± 0.2	1.4 ± 0.3	0.6 ± 0.05
Feces	18.0 ± 2.2	11.4 ± 2.3	10.9 ± 1.7	10.0 ± 2.5	6.9 ± 1.1
Totals	44.4	36.0	44.3	43.2	68.2

[a] Values = % found.
[b] Each dose level was labeled with 9.8 μC of EECPE-^{3}H.
[c] Assumed 75 gm of fat/rat (10).

TABLE II. Influence of Route of Administration on Body Fat and Brain Storage, and Plasma Levels Following Tritiated EECPE (12.5 μC/0.2 ml of suspending vehicle/animal) in Castrated Female Rats.

Route of administration	No. of animals	Perirenal fat (dpm/gm)	Brain (dpm)	Plasma free (dpm/ml)	Plasma total conjugates (dpm/ml)
Subcutaneous	5	258,000 ± 37,000[a]	58,000 ± 5200[a]	1900 ± 210[a]	4100 ± 370[a]
Intravenous	5	316,000 ± 28,000	32,000 ± 2000	2000 ± 120	5400 ± 260
Oral	5	122,000 ± 15,000	19,000 ± 1500	1000 ± 20	3400 ± 120
Intraperitoneal	5	354,000 ± 93,000	41,000 ± 4900	1900 ± 190	5200 ± 370

[a] Average ± SE.

increment (i.e., a 5-fold increase in dose was accompanied by a 7-fold increase in the amount of estrogen stored).

As previously observed (7), a larger amount of radioactivity was found in body fat and brain when the EECPE was administered in oil rather than aqueous suspension. In one experiment, the excretion of ^{3}H in urine and feces was observed under conditions of increasing doses of EECPE in aqueous suspensions. The ^{3}H in perirenal fat was also measured and the total amount of EECPE stored in body fat was estimated by assuming 75 gm of fat per rat (10). As dose was increased, the percentage of dose stored in fat increased while decreasing percentages of the dose were found in urine and feces (Table I).

B. Influence of route of administration of EECPE on body fat and brain storage and plasma levels. The concentration of radioactivity found in perirenal fat or brain was significantly lower ($p<0.05$-0.01) when EECPE-^{3}H was administered orally rather than intravenously, subcutaneously or intraperitoneally (Table II). Plasma free and "conjugated" (water soluble) radioactivity was likewise lower when the oral rather than parenteral routes were used (Table II). Because of possible direct uptake of i.p.-injected EECPE into perirenal fat, the experiment was repeated comparing radioactivity in subcutaneous fat following oral or i.p. administration

TABLE III. Influence of Route of Administration on Body Fat and Brain Storage and Plasma Levels Following Tritiated EECPE (5 μC/0.2 ml per animal) in Intact Female Rats.

Route of administration	No. of animals	Vehicle	Subcutaneous fat (dpm/gm)	Brain (dpm)	Plasma free (dpm/ml)	Plasma total conjugates (dpm/ml)
Intraperitoneal	9	Aqueous	80,900 ± 12,000[a]	4600 ± 780[a]	479 ± 69[a]	1819 ± 214[a]
Oral	9	Aqueous	41,200 ± 3,100	2700 ± 430	312 ± 58	1262 ± 105
Oral	9	Sesame oil	61,400 ± 5,000	4530 ± 670	377 ± 40	1142 ± 75

[a] Average ± SE.

TABLE IV. Excretion of ^{3}H and ^{14}C Following Administration of EECPE-^{3}H-^{14}C by Oral or Intravenous Route.

Route	No. of rats	Percentage of administered dose excreted in:							
		Bile 0–2.5 hour		Bile 2.5–5 hour		Bile 5–24 hour		Urine 0–24 hour	
		^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
p.o.	4	3.6 ± 0.9	2.7 ± 0.8	13.4 ± 3.7	9.1 ± 2.8	31.9 ± 7.5	23.4 ± 5.5	2.1 ± 0.5	13.8 ± 2.8
i.v.	6	13.2 ± 1.8	8.2 ± 0.9	27.5 ± 2.4	18.6 ± 1.8	55.7 ± 2.9	36.5 ± 2.2	2.6 ± 0.6	16.1 ± 2.2

of aqueous suspensions of the estrogen (Table III). Again, radioactivity in fat was significantly higher ($p<0.05$) following the ip injection route. However, when the EECPE was administered orally in oil, rather than aqueous suspension, storage of radioactivity did not differ significantly from that observed with the i.p. injections (Table III).

C. Influence of route of administration of doubly-labeled EECPE on excretion of radioactivity in bile and urine. Intravenous injection of EECPE was followed by appearance of higher levels of ^{3}H and ^{14}C in bile than after oral administration (Table IV). However, similar amounts of both isotopes were found in 24-hour urine collections regardless of route of administration (Table IV). The distribution of biliary and urinary ^{3}H and ^{14}C between free, glucuronide, and sulfate fractions was virtually identical for the two routes of administration (Table V). In each case, most of the urinary ^{14}C remained nonextractable following application of hydrolytic procedures.

D. Excretion of ^{14}C in bile and urine of rats treated with cyclopentanol-^{14}C or EECPE-^{14}C. Very little ^{14}C appeared in the bile of rats treated orally or intravenously with cyclopentanol-^{14}C (Table VI). In contrast, 18–36% of the ^{14}C dose appeared in 24-hour bile collections from EECPE-treated animals (Table VI). Urinary excretion of ^{14}C was equal or greater in rats treated iv or orally with the labeled cyclopentanol than in the steroid-treated animals (Table VI).

Very little radioactivity could be extracted from bile of cyclopentanol-treated animals even after β-glucuronidase hydrolysis or solvolysis (Table VII). In contrast, these hydrolytic procedures liberated considerable quantities of ^{14}C from bile of EECPE-treated rats (Table VII). There were no significant differences in the free, glucuronide, or sulfate fractions of urine of cyclopentanol versus EECPE-treated rats (Table VII). It was noted, however, that considerable quantities of ^{14}C were extracted from the urine by solvolysis.

Fat samples obtained from the cyclopentanol-treated rats contained an estimated 0.9% of the administered radioactivity. This value is in contrast to the 20–30% found in fat of EECPE–treated animals (see Table I).

Discussion. When increasing doses of EECPE were administered orally either in sesame oil or in aqueous suspension, the percentage of the dose stored in body fat increased at a rate greater than that anticipated on the basis of the dose increment. No fat saturation point was reached, even at the enormous dose of 12.5 mg/rat. These findings suggest that some rate-limited metabolic alteration may occur enroute between the stomach and the vascular beds of the adipose tissue. Thus, as dose is increased, a greater percentage of the compound may escape this metabolic alteration. This interpretation is supported by the second experiment in which it was observed that with increasing dosage, decreasing percentages of the dose were excreted in the urine and feces while increasing amounts were again found in body fat. Previous experiments showed that absorption of EECPE from aqueous suspensions is extremely rapid and complete (7). Furthermore, in the present experiments, the slopes of the dose-storage curves did not differ whether the compound was administered in oil or in aqueous suspension although more radioactivity appeared in fat and brain at every dose interval when the oil vehicle was used. These findings make it appear unlikely that rate of passage through the liver has much influence

TABLE V. Distribution of Radioactivity in Bile and Urine of Rats Treated with EECPE-^{3}H-^{14}C by Oral or Intravenous Route.

		Percentage of radioactivity in sample			
		Oral route (4 rats)		Intravenous route (6 rats)	
Sample	Fraction	^{3}H	^{14}C	^{3}H	^{14}C
Bile 0–5 hour	Free	1.5 ± 0.1	4.7 ± 1.5	2.0 ± 0.2	4.4 ± 0.8
	Glucuronide	39.0 ± 3.4	35.1 ± 2.6	41.6 ± 0.8	35.5 ± 3.5
	Sulfate	26.8 ± 1.7	19.0 ± 1.2	27.6 ± 2.5	20.5 ± 2.3
	Total extracted	67.3	58.8	71.2	60.4
Bile 5–24 hour	Free	2.1 ± 0.1	4.6 ± 1.2	2.8 ± 0.6	3.2 ± 0.7
	Glucuronide	30.4 ± 1.9	25.7 ± 1.4	32.8 ± 0.6	22.6 ± 2.0
	Sulfate	29.9 ± 2.3	21.9 ± 1.4	30.6 ± 2.9	20.8 ± 3.3
	Total extracted	62.4	52.2	66.2	46.6
Urine	Free	10.9 ± 1.7	1.2 ± 0.1	11.3 ± 0.8	1.8 ± 0.2
	Glucuronide	14.4 ± 2.1	2.0 ± 0.3	12.8 ± 0.9	2.0 ± 0.3
	Sulfate	33.3 ± 4.5	26.2 ± 2.6	38.1 ± 3.3	28.6 ± 2.4
	Total extracted	58.6	29.4	62.2	32.4

TABLE VI. Excretion of ^{14}C in Rats Treated with Cyclopentanol-^{14}C in Comparison with EECPE-^{14}C.

			Percentage of dose excreted in:			
Treatment	Route	No. of rats	Bile 2.5 hour	Bile 5 hour	Bile 24 hour	Urine 24 hour
Cyclopentanol-^{14}C	po	4	0.7 ± 0.1	2.1 ± 0.2	5.2 ± 0.7	26.6 ± 1.7
EECPE-^{14}C	po	4	2.7 ± 0.8	9.1 ± 2.8	23.4 ± 5.5	13.8 ± 2.8
Difference			2.0 ± 0.8	7.0 ± 2.8	18.2 ± 6.0	−12.8 ± 3.3
Cyclopentanol-^{14}C	iv	1	0.9	1.7	4.5	19.8
EECPE-^{14}C	iv	6	8.2 ± 0.9	18.6 ± 1.8	36.5 ± 2.2	16.1 ± 2.2
Difference			7.3	16.9	32.0	−3.7

TABLE VII. Distribution of ^{14}C in Bile and Urine of Rats Treated Orally with Cyclopentanol-^{14}C in Comparison with EECPE-^{14}C.

Sample	Collection period (hours)	Fraction	Cyclopentanol-^{14}C (4 rats) ^{14}C in sample (%)	EECPE-^{14}C (4–7 rats) ^{14}C in sample (%)	Significant ($p < 0.05$) difference
Bile	5	Free	2.7 ± 1.1	4.7 ± 1.5	None
		Glucuronide	3.1 ± 1.2	35.1 ± 2.6	32.0 ± 2.9
		Sulfate	6.3 ± 1.0	19.0 ± 1.2	12.7 ± 1.6
		% Extracted	12.1	58.8	
Bile	24	Free	3.0 ± 1.5	4.6 ± 1.2	None
		Glucuronide	2.1 ± 0.9	25.7 ± 1.4	23.6 ± 1.7
		Sulfate	8.1 ± 0.4	21.9 ± 1.4	13.8 ± 1.4
		% Extracted	13.2	52.2	
Urine	24	Free	1.7 ± 0.4	1.2 ± 0.1	None
		Glucuronide	2.0 ± 0.2	2.0 ± 0.3	None
		Sulfate	19.9 ± 1.1	26.2 ± 2.6	None
		% Extracted	23.6	29.4	

on rate of metabolic alteration. The relative importance of the liver and the gut as sites for metabolic transformation of EECPE is not clear from the data. When the compound (in aqueous suspension) was injected intravenously, intraperitoneally, or subcutaneously, significantly more radioactivity was found in fat, brain, and blood than that observed following oral administration. On the other hand, oral administration of EECPE in sesame oil resulted in the same fat storage pattern as ip injection of an aqueous suspension. With the exception of bypassing the gastrointestinal tract, intraperitoneally administered steroids appear to follow the same pathway of transport as do orally administered ones (11). One interpretation of these data is that when EECPE is administered orally in water, a portion of the dose is metabolized in the gut. This is avoided when the compound is injected intraperitoneally, or when it is administered orally in a protective oil solution. Against this interpretation was the finding of a more rapid excretion of metabolites in bile of rats in which EECPE was injected iv despite the fact that more of the compound was also stored in body fat of similarly-treated rats. Furthermore, the pattern of conjugated metabolites in bile and urine was identical for the oral and intravenous routes. If a major site of metabolic transformation were the gut, one would have expected differences in the metabolic pattern between orally and intravenously injected EECPE. The problem of accounting for variations in fat storage with route of administration remains unsolved. However, a considerable portion of an oral dose of EECPE is bound in the gut wall before eventual absorption, and this may help to account for the differences observed (to be published).

Our data likewise do not indicate the site of removal of the cyclopentyl group. Apparently, it is not the gastrointestinal tract since iv injection resulted in the same excess excretion of the ^{14}C label in the urine as was observed with oral administration. The results obtained when cyclopentanol-^{14}C itself was administered make it clear that the pattern of urinary ^{14}C observed following EECPE administration is due entirely to cleaved cyclopentyl group derivatives. The failure of cyclopentanol-^{14}C to accumulate in bile supports the previous contention that biliary ^{14}C represents doubly-labeled metabolites in which the cyclopentyl ether linkage is intact. This reasoning is further supported by the observation that glucuronidase hydrolysis and solvolysis failed to liberate ^{14}C from bile of rats treated with cyclopentanol whereas these procedures resulted in the extraction of considerable quantities of ^{14}C from bile of EECPE-treated animals.

Summary. The influences of dose level and

route of administration on fat storage and excretion of ethynylestradiol-3-cyclopentyl ether (EECPE) were studied in rats. The EECPE was tagged with ^{3}H in the steroid nucleus and with ^{14}C in the cyclopentyl group in order to be able to trace the de-etherification process. Over a range of oral doses of 20 μg to 12.5 mg, there was a proportional increase in the concentration of compound in body fat and brain. The storage increment exceeded the dose increment suggesting that with increasing dose more of the compound escaped metabolic alteration. Nearly twice as much radioactivity was found in fat, brain, and blood of rats in which EECPE had been injected parenterally (iv, ip, sc) rather than administered orally. Evidence was sought but not found that EECPE is metabolized by the gastrointestinal tract; in fact, orally and intravenously administered EECPE were excreted in qualitatively similar patterns in bile and urine. Excretion of radioactivity following treatment of rats with cyclopentanol-^{14}C led to the conclusion that EECPE follows two basic metabolic pathways: (i) de-etherification with urinary excretion of cyclopentanol derivatives; and (ii) conjugation and biliary excretion of metabolites in which the ether linkage is still intact.

1. Falconi, G., Endocrinology **71**, 657 (1962).
2. Giannina, T., Steinetz, B., and Meli, A., Intern. J. Fertility **12**, 142 (1967).
3. Epstein, J. A., Intern. J. Fertility **12**, 239 (1967).
4. Roland M., Clyman, M., Decker, A., Ober, W., and Bronstein, S., Fertility Sterility **17**, 531 (1966).
5. Meli, A., Wolff, A., and Honrath, W. L., Steroids **2**, 417 (1963).
6. Meli, A., Steinetz, B., Beach, V., Wolff, A., Giannina, T., Proc. Soc. Exptl. Biol. Med. **119**, 602 (1965).
7. Steinetz, B., Meli, A., Beach, V., and Giannina, T., Proc. Soc. Exptl. Biol. Med. **123**, 163 (1966).
8. Steinetz, B., Meli, A., Giannina, T., Beach, V., and Manning, J., Proc. Soc. Exptl. Biol. Med. **124**, 111 (1967).
9. Steinetz, B., Meli, A., Giannina, T., and Beach, V., Proc. Soc. Exptl. Biol. Med. **124**, 1283 (1967).
10. Mickelson, O. and Anderson, B., J. Lab. Clin. Med. **53**, 282 (1959).
11. Meli, A., Endocrinology **72**, 715 (1963).

Received Nov. 27, 1967. P.S.E.B.M., 1968, Vol. 127.

Incorporation of Glycine-1-^{14}C into Liver Glutathione in Zinc Deficient Rats (32866)

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Du Vigneaud, *et al.* in 1931 (1) reported that glutathione (GSH) can inactivate insulin *in vitro*. Recent studies by Tomizawa and his associates (2–4) clearly indicate that the destruction of insulin in the liver is due primarily to the enzyme, glutathione insulin transhydrogenase. This enzyme degrades insulin by promoting reductive cleavage of the insulin molecule by GSH into its A and B chains. Thus, alterations of liver GSH content may bear a significance in the regulation of insulin-degrading activity. Since crystallization of insulin requires traces of zinc (5), and since the plasma insulin level is decreased in zinc deficient rats (6), it is of interest to determine the role of zinc on GSH synthesis. The present communication describes some experiments concerning incorporation of radioactivity from glycine-1-^{14}C into liver GSH and tissue proteins from normal and zinc deficient rats.

Materials and Methods. Male rats 21–24 days of age weighing 40–60 gm of McCollum strain from our own colony were randomly divided into two groups. The first group received a diet low in zinc. Its percentage composition was as follows: sucrose, 65.97; dried egg white, 15.0; casein hydrolyzate (salt free) 3.0; corn oil, 10.0; zinc free salt mixture, 5.74;[1] and vitamin supplements, 0.49.[2] The zinc content of the diet was 2 to 3 ppm as determined by the Jarrell Ash direct reading spectrometer. The second control group re-