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The Rates of Glutathione Metabolism in Brain and Liver Studied with Glycine-2-C^{14}

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THE RATES OF GLUTATHIONE METABOLISM IN BRAIN AND LIVER STUDIED WITH GLYCINE-2-C^{14}

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY G. Wilbur Douglas

ENTITLED The Rates of Glutathione Metabolism in Brain and Liver Studied with Glycine-2-C^{14}

BE ACCEPTED AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science in Biochemistry

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Determinations of the rates of glutathione (GSH) metabolism have been among the numerous investigations of this biologically important compound. The metabolism rates of this substance have been studied primarily by the use of isotopes—C^{14}, S^{35}, and N^{15}. Results of such studies in a number of tissues have been reported. These tissues include the liver but not the brain. Waelsch and Rittenberg (1), using N^{15} glycine, made the first estimate in the liver. Using an intact rat, they found a GSH half-life of 2 to 4 hours. With cystine-S^{35}, Anderson and Mosher (2) reported a 3 hour half-life in the liver of the intact rat. More recently, studies of Henriques, et al. (3) on live rabbits have yielded a 7 to 9 hour half-life for liver and a 10 to 13 hour value for muscle, using glycine-2-C^{14} as the label. In vitro studies by Kinsey and Merriam (4) on rabbit lens GSH have yielded a half-life of 29 hours using both glycine-1-C^{14} and glycine-2-C^{14}. Work in this laboratory with glycine-2-C^{14} by Mortensen et al., (5) has yielded a 65 hour half-life for the GSH of mature rat erythrocytes in vivo. Human studies done by Dimant, et al., (6), using N^{15} glycine have shown a 4 day half-life in the mature erythrocyte.

The present work was undertaken primarily to obtain information on GSH turnover in the brain of the intact rat. It seemed of interest, however, to study the liver at the same time, inasmuch as previously

reported estimations of the rate of GSH metabolism in that organ had been based on other methods.

In this present study, the experimental method consisted of measuring the decline in radioactivity of the glycine moiety after a preliminary labeling of the tripeptide by injecting glycine-2-C^{14}. A chromatographic technique was employed to isolate the glycine component.

The turnover rate thus found for GSH in the liver agreed well with the value deduced by Waelsch and Rittenberg. The turnover in the brain proved to be the slowest so far reported for any tissue of the rat.

Experimental

For the liver experiments, male Sprague-Dawley rats were placed on Purina Laboratory Chow as weanlings and were allowed to feed ad libitum until they weighed between 200-300 grams, at which time the experiment was begun. During the experimental period, food was denied the rats for one hour before injection, was returned one hour after injection, and finally removed five hours before sacrifice.

Glycine-2-C^{14} (Tracerlab), with a specific activity of 1.34 mc. per mmole, was injected intraperitoneally in a single dose of 3 μc. per 100g. of body weight. At intervals of 6 to 18 hours following injection, the animals were killed by stunning and decapitation, and the livers quickly excised, minced, and frozen with dry ice.

One gram samples of the liver were homogenized with 25 mg. of carrier GSH in 5 ml. of 10 per cent trichloroacetic acid (TCA) in a Potter-Elvehjem glass homogenizer. The resulting solution was centrifuged, and the supernatant decanted and filtered. The precipitate was washed once with 4 ml. of 10 per cent TCA and centrifuged. The supernatant from this washing was decanted, filtered and combined with the original decantate.

For the brain, equal numbers of male and female Sprague-Dawley rats, weighing between 225 and 375 grams, were used. They were allowed to feed ad libitum on Laboratory Chow before and during the experiment.

Brain GSH was labeled by injecting glycine-2-C^{14} into the cisterna magna. The rats were anesthetized with Nembutal and placed on a support with heads elevated as described by Jeffers and Griffith (7). While in this position, each animal was given 0.2 μc. of the labeled glycine per 100 gm. of body weight. The concentration (in physiological saline) was such that a total volume of 0.01 ml. would be introduced into a 300 gm. rat. The solution was administered with a Brooks blood sedimentation pipette (Fisher Scientific Company), the capillary of which had been calibrated with mercury to deliver known volumes. A 0.5 inch No. 26 hypodermic needle was placed on the ground tip of the pipette, and the labeled glycine solution was drawn up through the needle into the lower portion of the calibrated capillary. The stopcock was then closed and the needle wiped free of adherent solution and inserted into the cisterna magna. When entrance into the cistern had been achieved, as indicated by a small, sharp rise of the fluid level in the capillary, the stopcock was opened and the calculated volume of the glycine solution expelled by gentle pressure. The injection time was ordinarily about 15 seconds.

At forty eight hours and succeeding time intervals following injection, the rats were killed by decapitation, and the brains removed as a whole and frozen with dry ice. One gram samples of minced brain were homogenized with 25 mg. of carrier GSH in a manner similar to that for the liver.

The GSH from both the brain and the liver was isolated as the cuprous mercaptide, essentially as described by Waelsch and Rittenberg (8). Briefly, the technique employed was as follows: to the combined liver

or brain decantate was added a six per cent cadmium chloride solution equal in volume to one-fourth of the volume of the decantate. A few drops of brom-cresol green indicator were used. Normal sodium hydroxide was added dropwise, with shaking, until the yellow color of the solution faded. One molar sodium carbonate was then added until the solution turned light blue. The resulting precipitate was allowed to settle for one hour in a refrigerator. At the end of the hour, the solution was centrifuged, and the precipitate was twice washed with water, triturated and centrifuged. The washed precipitate was dissolved in the least possible amount of 2 N sulfuric acid (ca. 20 drops) and 10 ml. of 0.5 N sulfuric acid was added. Small quantities of an aqueous cuprous oxide suspension were added to this acid solution with considerable stirring. Each portion of cuprous oxide was added only after the red color from the previous addition had completely disappeared. This process was continued until the resulting cuprous mercaptide precipitate began to dissolve. Air was then bubbled through the solution for ten minutes. At the end of this time, the precipitate was washed, triturated and centrifuged in the following order: three times with 0.5 N sulfuric acid, four times with distilled water, three times with 50 per cent alcohol. It was then transferred for plating with absolute alcohol.

Carbon dioxide expiration studies were made to estimate tissue levels of free labeled glycine. Anesthetized male rats weighing about 300 grams were injected intracisternally or intraperitoneally using a dosage of 1.5 μc. per 100g. of body weight, and placed in a glass tube in an atmosphere of CO_2-free air. The air was drawn through the collecting apparatus (Fig. 1) by water aspiration at a rate of 500 ml. per minute. The expired CO_2 was absorbed in 200 ml. of 1N NaOH in a gas collecting bottle for time intervals of 15 minutes to 2 hours over a period of 12 hours. Ten milliliter

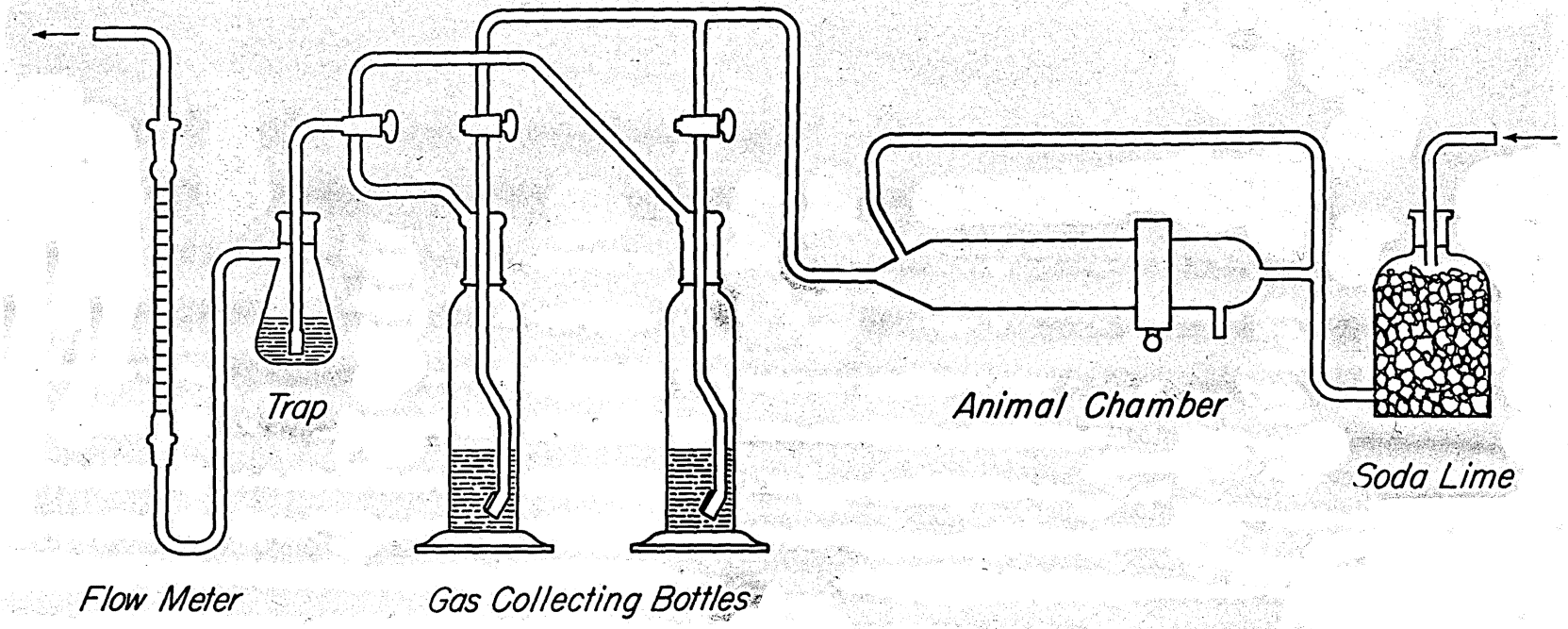

Fig. 1. Carbon dioxide collecting apparatus.

aliquots of the NaOH solution were used for the isolation of the CO_2 as $BaCO_3$ (9).

Both the glutathione copper mercaptide and the $BaCO_3$ were plated on aluminum planchets, using the method and equipment described by Hutchens, et al., (9). The plates were counted to a standard error of less than 4 per cent in a gas-flow Geiger-Muller tube and were corrected to infinite thinness using curves for wax and $BaCO_3$ (10).

Quantitative GSH determinations on brain and liver were made by means of the nitroprusside method of Grunert and Phillips (11).

Portions of the copper mercaptide plates from each time interval were combined to give a total of 50 mg. and were suspended in water and the copper precipitated by passing in a stream of H_2S. The resulting copper sulfide was washed twice with water and the combined filtrate was freed of residual H_2S by a stream of nitrogen. The GSH solution was hydrolyzed with 6N HCL for 12 hours, and the 2, 4-dinitrophenyl derivatives of the component amino acids prepared and chromatographed on silicic acid columns (5).

An acetone solution of the isolated DNP-amino acids was evaporated on lens paper in aluminum cups and counted in a gas-flow counter. Quantitative determinations of the isolated derivatives were made from an acetone aliquot in a Beckman Model B Spectrophotometer. Corrections were made for the amount lost in preparing the derivatives and for chromatographic losses.

Results and Discussion

Glycine Disappearance in Liver GSH.—A preliminary study was performed on 10 rats, using times of from 4 to 26 hours after injection. It was found that after 6 hours the radioactivity declined in a manner that approximated first order kinetics.

The final results are plotted in Figs. 2 and 3. Specific activities are expressed as counts per minute per γ/g. of liver GSH. Each point represents the average of from four to six rats. A straight line was assumed and was plotted by the method of least squares from the averages shown. Fig. 2 represents the disappearance of labeled GSH, while Fig. 3 gives the disappearance of the labeled glycine component of the isolated GSH.

It will be noticed that the glycine curve has a slightly steeper slope than the GSH curve. This seems to be due to a synthesis of radioactive glutamic acid and cysteine from labeled glycine, and incorporation of these into the GSH. It was found on analysis of the component amino acids that beginning at 10 hours after injection, the radioactivity of both non-administered amino acids steadily increased, so that at 18 hours the specific activities of the glutamic acid and cysteine, were respectively 5.9 and 9.4 per cent of the total (Table 1).

From the slope of the line in Fig. 3, the biological half-life of the glycine in liver GSH was calculated to be 4.8 hours. After the completion of this study, the work of Henriques, et al., (3) was published, which included a study of the disappearance of glycine from liver GSH of the rabbit. Their value of a 7 to 9 hour half-life of glycine was obtained by correcting the results for any uncombined radioactive glycine present during the same interval. This correction amounted to approximately 13 per cent. Applying a similar correction to our results would yield a 4.1 hour half-life. Waelsch and Rittenberg (1), using N^{15} glycine have reported a half-life of 4 hours or less.

Glycine Disappearance in Brain GSH—Preliminary investigation showed that no reliable curve could be obtained prior to 48 hours after injection. Considering this, the first animals were killed 48 hours after glycine

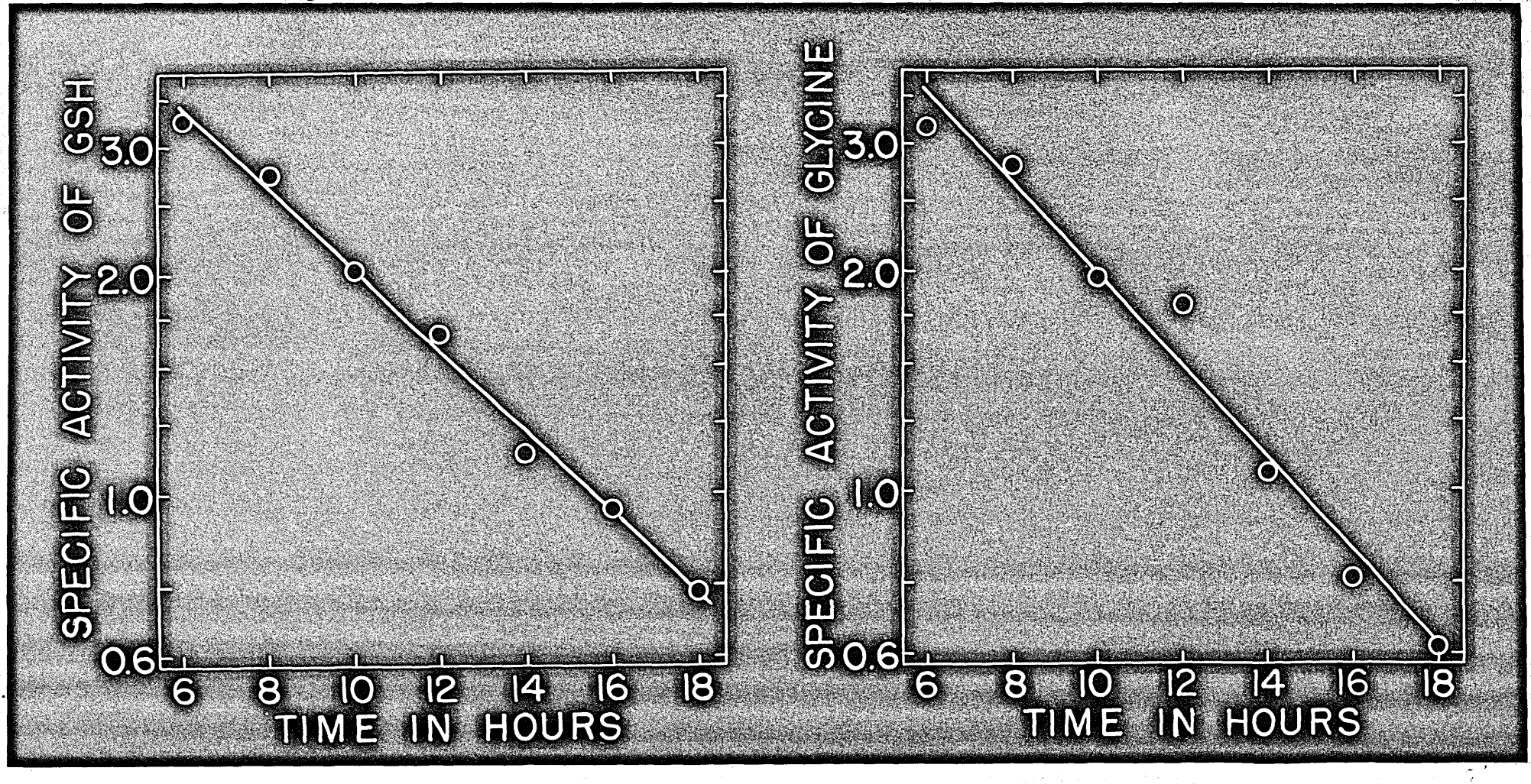

Fig. 2. Specific activity of liver glutathione. The rats were given a single intraperitoneal injection of glycine-2-C^{14} at zero time. Each point represents the average of 4 to 6 animals. Specific activities are in terms of counts per minute per μg of liver glutathione.

Fig. 3. Specific activity of the glycine obtained by hydrolysis of the pooled liver glutathione samples. Values are in counts per minute per μg of liver glutathione, after corrections for losses incurred in preparing and chromatographing the DNP-glycine.

Incorporation of C^{14} into
Cysteine and Glutamic acid Moieties
of Liver Glutathione

Time (hr.)	Cysteine*	Glutamic Acid*
6	—	—
8	—	—
10	1.8	1.2
12	3.1	1.4
14	6.1	2.1
16	6.6	3.6
18	9.4	5.9

*Results expressed as percentage of total specific activity

administration and every 48 hours thereafter, up to and including 240 hours.

Each point in Fig. 4 represents the average of from 4 to 7 animals. The specific activity is in cpm per γ g. of brain GSH. The isolation and analysis of the component amino acids of each GSH sample yielded no radioactivity in any but the glycine moiety for the entire period of 43 to 240 hours. Apparently the central nervous system does not synthesize appreciable amounts of cysteine and glutamic acid from the methylene carbon of glycine; although a slight conversion of glycine-2-C^{14} into glutamic acid in rat brain homogenates has been reported (12). Because of this lack of conversion, the specific activities of the GSH could be taken as those of the glycine component. Since the former

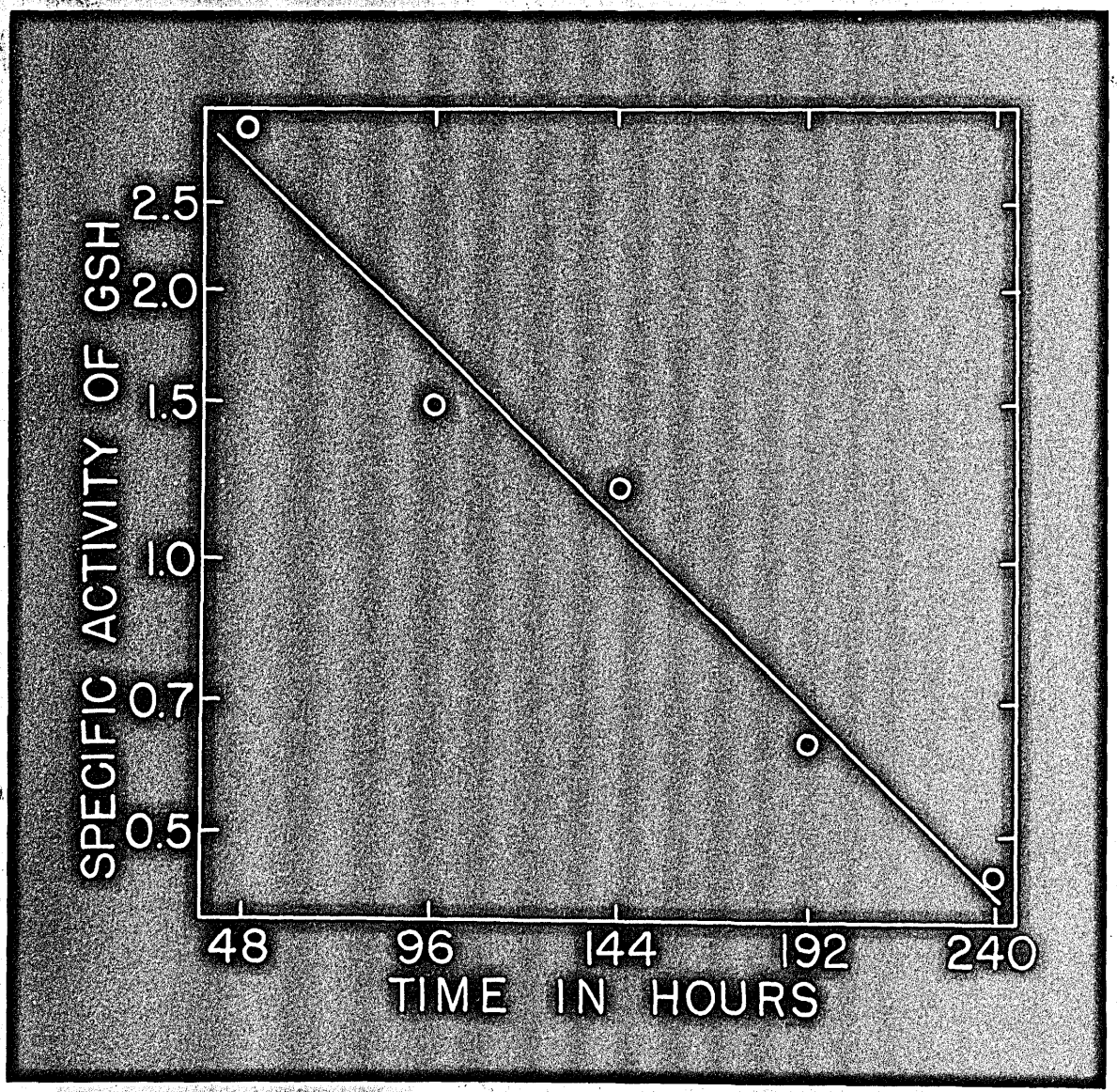

Fig. 4. Specific activity of brain glutathione. The rats were given a single intracisternal injection of glycine-2-C^{14} at zero time. Each point represents the average of 4 to 7 animals. Specific activities are in terms of counts per minute per μg. of brain glutathione.

values were subject to less experimental error, they were used in constructing the specific activity-time curve. The half-life of brain GSH calculated from the curve (Fig. 4) is 71 hours.

It was thought that this long turnover time could be caused by a high concentration of free radioactive glycine remaining in brain tissue and cerebrospinal fluid during the experimental time and being continually incorporated into the GSH. This was suggested by the slowness of diffusion of the amino acid through the blood-brain barrier (13). This may or may not be true of diffusion in the reverse direction. Also, in vitro experiments have shown that rat brain homogenates do not oxidize glycine-2-C^{14} to radioactive CO_2 in any detectable amount (12).

Carbon dioxide expiration studies were carried out in order to obtain a rough estimate of the amount of free glycine-C^{14} remaining at the start of the experiment. No attempt was made to determine free labeled glycine levels. Two rats were injected intracisternally and two intraperitoneally, and the CO_2 collected over a 12 hour period. Fig. 5 shows the resulting curves, each curve being the average of the two rats. The specific activity is cpm per γg. of carbon in the expired CO_2. The intraperitoneal curve is presented for comparison. In 12 hours the level of expired $C^{14}O_2$ was only 18 per cent of its maximum in the intracisternally injected rats. The total amount released during this period was very nearly the same in both the intraperitoneal and intracisternal injections, being about 18 per cent of the injected dose. This figure is in good agreement with the results obtained by others who have done similar studies with glycine-2-C^{14} —25 per cent of the injected dose in 17 hours after stomach intubation of the radioactive material (14), and 27 per cent in 18 hours after intravenous injection (15). A separate CO_2 collection was taken from intracisternally injected rats at 48 hours (not shown). The specific

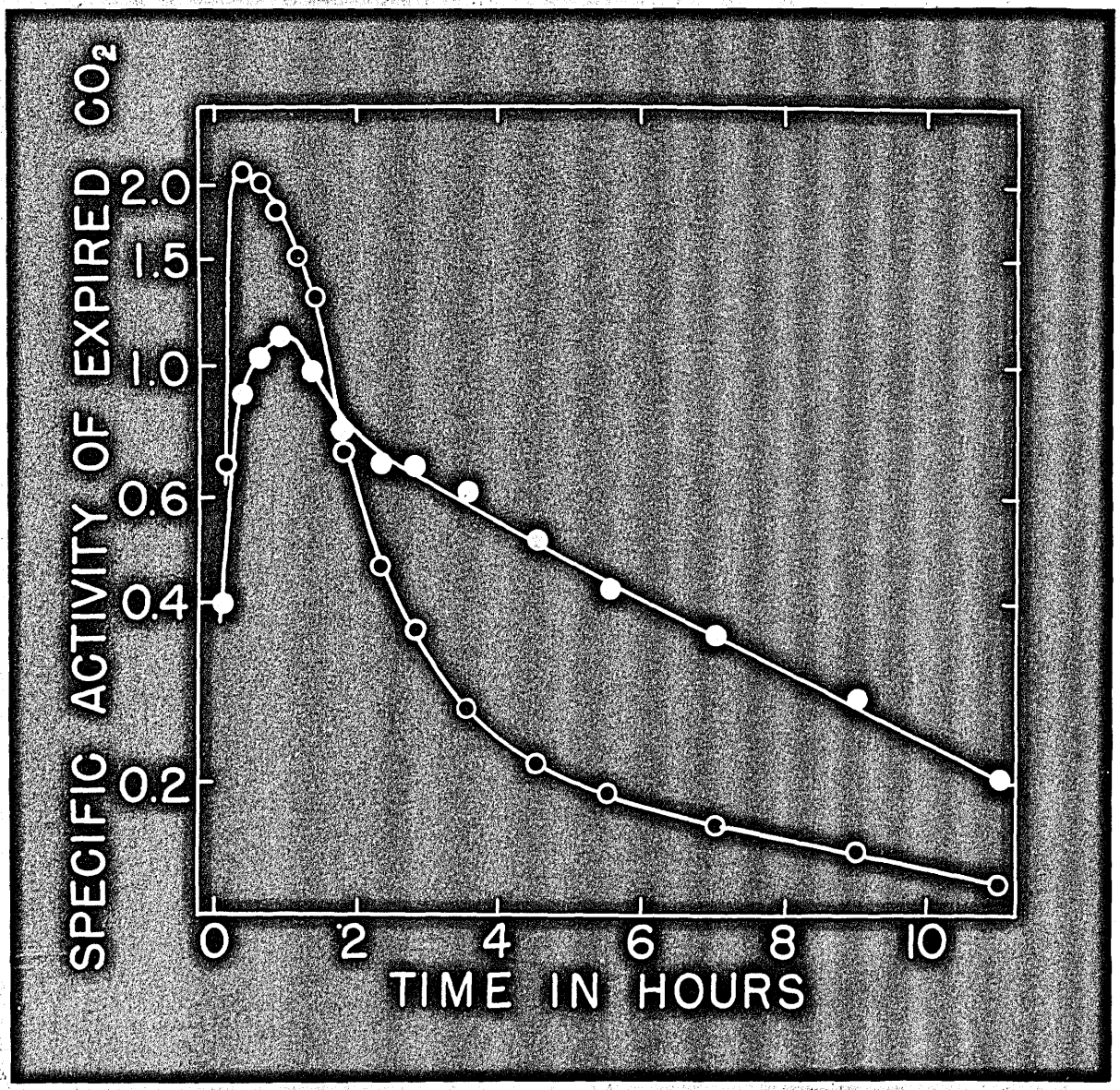

Fig. 5. Specific activity of expired CO_2 following a single injection of 1.5 μc. of glycine-2-C^{14} per 100 gm. of body weight. ● , intracisternal injection; ○ , intraperitoneal injection. All points represent the averages of 2 animals. Specific activities are in counts per minute per μg. of carbon in the expired CO_2.

activity at this time was only 3 per cent of the maximum. These levels of $C^{14}O_2$ expiration are probably due to oxidation of not only any free glycine remaining but also of other compounds labeled during this time, and, therefore, the radioactivity is at a higher level than would be obtained from the oxidation of glycine alone.

From these data it was assumed that any free radioactive glycine remaining at 48 hours, when the GSH measurements were begun, would not appreciably increase the apparent half-life of the GSH glycine.

Summary

Using glycine-2-C^{14} as the label, the half-life of the glycine moiety of liver and brain glutathione in the intact rat has been determined and found to be 4.8 and 71 hours, respectively.

The component amino acids of the labeled GSH were analysed for possible labeling of the non-administered moieties. In liver GSH, 5.9 per cent of the total specific activity was found in the glutamic acid and 9.4 per cent in the cysteine 18 hours after injection. In the brain GSH, no detectable activity was present in any but the glycine component throughout the experiment.

Carbon dioxide expiration studies were made with glycine-2-C^{14} on rats for a 12 hour period after intracisternal or intraperitoneal injection. Approximately 18 per cent of the injected dose was recovered during this time from both groups of animals. After 48 hours, the activity of the CO_2 from the intracisternally injected rats was 3 per cent of the maximum.

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