4. After intravenous injection the concentrations of ^{85}Sr and ^{140}Ba in the soft tissues closely follow those in the blood, and except in the submaxillary gland the $^{85}\text{Sr} : ^{140}\text{Ba}$ ratio is always greater than 1. The submaxillary gland concentrates both ^{85}Sr and ^{140}Ba from the serum, but the concentration of ^{140}Ba is always greater than that of ^{85}Sr . The uptake of both ^{85}Sr and ^{140}Ba in soft tissues is generally in proportion to the calcium content of the tissues.

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Turnover of Liver Glutathione in Trauma

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Previous studies (Beck & Linkenheimer, 1952; Beck, Kalser & Alexander, 1961) have shown that mice subjected to tourniquet trauma exhibit a marked depletion of liver glutathione and also exhibit a decreased incorporation of tracer amounts of L- ^{35}S cysteine into liver glutathione. The latter finding suggested, as a working hypothesis, that the decrease in liver glutathione induced by trauma was due to an inhibition of glutathione synthesis rather than to a facilitation of glutathione degradation. Additional evidence for this concept was obtained in glutathione repletion (net synthesis) experiments (Kalser & Beck, 1962). After administration of large amounts of L-cysteine, a rapid repletion of liver glutathione occurred when the depletion of glutathione had been obtained by the administration of bromobenzene, but not when the depletion had been secured by tourniquet trauma. In the present experiments, turnover-

time and turnover-rate studies (Zilversmit, Entenman & Fishler, 1943) have been applied to the problem of whether trauma affects the synthesis or degradation or both of liver glutathione *in vivo*. Both short-term and long-term labelling experiments with L- ^{35}S cysteine have been performed. In the short-term experiments turnover is calculated from the rate of appearance of ^{35}S in liver glutathione. In such experiments the incorporation rate presumably approaches the rate of synthesis, since little labelled glutathione is available at these periods for degradation processes to influence the results. In long-term experiments turnover is calculated from the rate of disappearance of ^{35}S from the liver glutathione that has already become labelled. Although the initial impetus to this problem was to study glutathione synthesis in trauma, a far more important and widespread application resulted from the finding that the two types of experimental approaches generally used in turnover studies can lead to widely divergent results in certain situa-

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tions. Thus the appearance of radioactivity in a product and the calculation of its turnover from the change in specific activities of precursor and product can, in some cases, show a large discrepancy with the turnover calculated by the disappearance of radioactivity from a product after it has become labelled. The two approaches have given markedly different turnover values for glutathione in control mice. The factors responsible for this discrepancy are considered.

MATERIALS AND METHODS

L-[³⁵S]Cysteine. L-[³⁵S]Cystine, obtained from Schwarz BioResearch Inc., Mount Vernon, N.Y., U.S.A., was electrolytically reduced to L-[³⁵S]cysteine in a model 1930 desalter (Research Specialties Co., Berkeley, Calif., U.S.A.).

The specific activity of this amino acid varied from 8.9×10^5 to 74.2×10^5 counts/min./mg. and was diluted, as necessary, to give an injected dose of approx. 10^7 counts/min./kg. when injected at a dose rate of 5 ml./kg. body wt.

Treatment of animals. Male mice (21–40 g.) of the Carworth Farms Webster strain, maintained on Rockland Rat Diet, were used. All mice were injected rapidly by tail vein (injected in 4–7 sec.) with the quantity of L-[³⁵S]cysteine described above. In the entire series of experiments, all mice were killed between 3.00 and 4.00 p.m. to avoid diurnal variations in the liver non-protein thiols (Beck, Rieck & Duncan, 1958). The labelling period was the time, in minutes, from the intravenous injection of the L-[³⁵S]cysteine to the death of the animal. Labelling periods ranged from 1 to 120 min. in the present series of experiments. In experiments in which the labelling period was less than 5 min., the mice were killed by dropping them into liquid nitrogen to obtain a very rapid cessation of metabolic processes. The livers were dissected out carefully and completely while frozen. In experiments in which the labelling period was longer than 5 min., the mice were killed by cervical dislocation and the entire livers quickly removed and frozen between two slabs of solid carbon dioxide.

Mice receiving trauma had rubber-band ligatures placed high on both hind legs while under light ether anaesthesia (Rosenthal, 1943). The ligatures were left in place for 3 hr.; the mice were killed at 1 or 2.5 hr. after removal of the ligature. This post-tourniquet period is referred to as the 'trauma period'. The groups of traumatized mice were injected at various times after removal of the ligatures such that the trauma period minus the labelling period marked the time of injection after removal of the ligatures. For example, an animal having a labelling period of 10 min. and a trauma period of 60 min. would have been injected 50 min. after the removal of the ligatures.

Preparation of liver extract. The frozen livers from an entire group, consisting of 7–9 mice, were pooled and weighed. A 10% (w/v) trichloroacetic acid extract was prepared, tissue being ground successively with 1, 0.5 and 0.5 ml. of trichloroacetic acid/g. of liver. The pooled non-protein extract was then adjusted to a final volume of 25 ml. with 10% (w/v) trichloroacetic acid. The following analyses were made on the pooled non-protein liver extract.

Determination of the specific activity of glutathione. This

was carried out by counting the radioactivity, by using liquid-scintillation counting, of a known weight of copper glutathionate precipitate isolated by the method of Waelsch & Rittenberg (1941). About 16–20 ml. of extract was used for this procedure. All thiols in the extract were initially precipitated by the addition of cadmium chloride. Since this procedure, as performed, was found to precipitate 94–105% of added glutathione or cysteine or both, any radioactivity remaining in the supernatant was attributed to non-thiol compounds. The cadmium precipitate, after several washes with water, was dissolved in acid and the glutathione precipitated as its copper salt by the slow addition of cuprous oxide. The precipitation was not carried to completion in order to avoid contamination with excess of cuprous oxide. Because of the small quantities of glutathione that could be isolated, it was not practicable to dissolve and reprecipitate the glutathione to check its purity by means of a constant specific activity. However, its purity was ascertained by two means. First, combustion of the glutathionate and analysis of its ash for copper oxide in seven randomly selected samples showed the excess of cuprous oxide to be only $1.72 \pm 1.60\%$ (s.d.). Secondly, it was found that of the various thiols that might be present in liver extract (cysteine, ergothionine, coenzyme A, glutamylcysteine) none co-precipitated with glutathione by more than 1–3% even when present in concentrations as high as that of the glutathione itself.

The copper glutathionate precipitate was then washed twice each with acid, water, ethanol and ether. After the ether had been driven off, the precipitate was dried overnight in a desiccator over anhydrous calcium sulphate. The specific activity of the glutathionate was then determined by dissolving a known weight of material (0.5–5.0 mg., weighed on a Cahn electrobalance; Fisher Scientific Co., Pittsburgh, Pa., U.S.A.) with 1 ml. of 2 mM-EDTA in 10% (w/v) trichloroacetic acid. Then 1 ml. of 1 M-Hyamine hydroxide (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) and 8.0 ml. of P611 counting mixture [containing the primary phosphor, diphenyloxazole, and the secondary phosphor, 1,4-bis-2-(5-phenyloxazolyl)benzene] were added to the sample (Davidson & Feigelson, 1957). The sample was counted for 30 min. in a Packard Instrument Co. Tri-Carb liquid-scintillation spectrometer, with a voltage setting of 1040 and a discriminator setting of 10–50. After the initial counting, 100 μ l. of a ³⁵S standard, containing a known number of counts, was added to each sample, the sample recounted, and a correction made for internal quenching.

Determination of the specific activity of cysteine. This gave only approximate results, since cysteine was present in quantities too small for actual isolation. An upper and lower limit was placed on the possible specific activities of cysteine, and both values were used in all calculations involving the specific activity of cysteine. The lower limit was obtained by assuming that the radioactive material precipitated by cadmium chloride (which precipitated total non-protein thiols) but not by cuprous oxide (which precipitated only glutathione) existed as cysteine. An upper limit was obtained by assuming that all of the activity existing in the non-protein fraction, except that activity known to be present in glutathione, was present as cysteine. The radioactivity of this solution was counted in the same solvent mixture as described for glutathione except that 1.0 ml. of the 10% (w/v) trichloroacetic acid extract to be

counted was used in place of the 1.0 ml. of 2 mM-EDTA in 10% (w/v) trichloroacetic acid. This activity was then divided by the 'cysteine' concentration determined as described below.

Determination of the concentration of total non-protein thiols. The colorimetric method of Grunert & Phillips (1951), with nitroprusside, was employed.

Determination of the concentration of glutathione. This was determined by an isotope-dilution technique. Samples (1 ml.) of the liver extract were diluted with known quantities of non-radioactive glutathione and the precipitation for copper glutathionate was carried out as described above. The specific activities of these diluted samples were then compared with the specific activity of the glutathione isolated from a sample to which no carrier glutathione was

added, and calculation of the concentration was made by using the equation:

$$A_0 X = A_a(X + a)$$

i.e.

$$X = aA_a/(A_0 - A_a)$$

where A_0 is the specific activity of glutathione isolated with no carrier glutathione added, A_a is the specific activity of glutathione isolated with a mg. of carrier glutathione added, a is the amount (mg.) of carrier glutathione added, and X is the amount (mg.) of glutathione in the sample of filtrate used.

Determination of the concentration of 'cysteine' (including cystine). This was carried out by the colorimetric method with naphthaquinonesulphonate (Sullivan & Hess, 1931) as modified by Fodor, Miller & Waelsch (1953).

Table 1. *Effect of trauma on the incorporation of ^{35}S in mouse liver after short-term labelling with $\text{L-}^{[35}\text{S}]\text{cysteine}$*

Experimental details are given in the text. C, Control animals; T1, animals subjected to 1 hr. of trauma; T2.5, animals subjected to 2.5 hr. of trauma. The specific radioactivity of cysteine is given as: (a) radioactivity of non-protein extract - (radioactivity of non-thiol S + radioactivity of glutathione)/average concn. of cysteine in liver; (b) (radioactivity of non-protein extract - radioactivity of glutathione)/average concn. of cysteine in liver. The average concn. of cysteine (in mg. of glutathione equivalents/g. of liver) was: Expt. 46C, 0.1361; Expt. 46T2.5, 0.1333; Expt. 47C, 0.1029; Expt. 47T1, 0.1030. The average concn. of glutathione (in mg./g. of liver) was: Expt. 46C, 1.217; Expt. 46T2.5, 0.712; Expt. 47C, 1.240; Expt. 47T1, 0.941.

Expt. no.	Time (min.)	Radioactivity (counts/min./g. of liver)			Specific radioactivity (counts/min./mg.)		
		Non-protein extract	Non-thiol S	Glutathione	Cysteine		Glutathione
					(a)	(b)	
46C	1	8 008	1669	1115	38 383	50 646	842
	2	11 576	3928	2644	36 767	65 628	2258
	4	11 777	3498	4472	27 972	53 673	3875
46T2.5	1	15 943	4276	1318	77 637	109 715	1757
	2	16 559	6951	2413	53 976	106 122	3333
	4	18 016	7918	4313	43 400	102 799	6515
47C	1	7 813	3014	1280	34 198	63 489	882
	2	8 265	2961	1689	35 131	63 907	1418
	4	9 662	3962	2849	27 707	66 210	2643
47T1	1	14 774	8256	1199	51 641	131 796	1367
	2	12 576	6785	1792	38 825	104 699	1842
	4	10 627	5593	2621	23 427	77 728	2694

Table 2. *Turnover times and turnover rates for glutathione in the livers of control and traumatized mice, as shown by short-term labelling with $\text{L-}^{[35}\text{S}]\text{cysteine}$*

The glutathione turnover rates and turnover times were calculated from the results given in Table 1 by using the equations of Zilversmit *et al.* (1943). The turnover time is taken to be: (area under cysteine specific-radioactivity curve - area under glutathione specific-radioactivity curve)/average change in glutathione specific radioactivity during 4 min. period. The turnover rate is taken to be: average concn. of glutathione (in mg./100 g. of liver)/turnover time; the average concn. of glutathione is given in Table 1. The values listed under (a) and (b) were calculated from the cysteine specific-radioactivity values (a) and (b) (given in Table 1) respectively.

Expt. no.	Integrated area under sp.-radioactivity curves for 0-4 min. period		Glutathione	Average change in sp. activity of glutathione during 4 min. period (counts/min./mg.)	Turnover time for glutathione (min.)		Turnover rate for glutathione (mg./100 g. of liver/min.)	
	Cysteine				(a)	(b)	(a)	(b)
	(a)	(b)						
46C	125 150	207 160	8 340	3808	30.7	52.2	3.96	2.33
46T 2.5	211 900	374 480	13 284	6584	30.2	54.9	2.36	1.30
47C	116 150	227 180	5 612	2760	40.0	80.3	3.10	1.54
47T 1	139 420	364 420	6 864	3064	43.3	116.7	2.17	0.81

RESULTS

Mortality from trauma. The ligature trauma produced a 50% mortality at 4.5 hr. after removal of the ligature, and at 24 hr. the mortality had increased to 75%. However, at the longest trauma period used in these experiments, 2.5 hr., the mortality was only 8%. At this time, the mice showed severe swelling of both hind legs, prostration and marked indifference to stimuli.

Short-term labelling experiments. By use of liver samples excised 1, 2 or 4 min. after the injection of L-[^{35}S]cysteine, the results given in Table 1 were obtained. These results permitted determination of the specific activity of the product, glutathione, and approximate estimation between upper and lower limits of the specific activity of the precursor, L-cysteine. The equations of Zilversmit *et al.* (1943) were then employed to estimate upper and lower limits for glutathione turnover times and turnover rates, as indicated in Table 2. For each of the two experiments performed, the turnover times for controls and group of traumatized mice did not differ greatly. However, since the glutathione pool was appreciably less for the group of traumatized mice than for the control group, the turnover rate or the estimated rate of glutathione synthesis was appreciably less for the group of traumatized mice than for the control group in each experiment.

If the formalized equations of Zilversmit *et al.* (1943) are disregarded, a decreased rate of glutathione synthesis is still evident when interpreted as in Fig. 1. In each of three experiments and at every time-interval, the percentage incorporation (percentage of radioactivity present in the total non-protein fraction which has been incorporated into glutathione) was appreciably higher for livers of control than of traumatized mice. For such short time-intervals, when little labelled glutathione is yet available for degradation, the chief factors governing the incorporation will be the rate of glutathione synthesis and the concentration of the precursor, L-cysteine. L-Cysteine concentrations were nearly the same for livers of both control groups and groups of traumatized mice; hence the incorporation should become a valid indicator of the rate of glutathione synthesis.

Long-term labelling experiments. Fig. 2 shows the turnover times calculated from the rate of disappearance of labelled glutathione from the liver according to the equations of Zilversmit *et al.* (1943). The specific activities of the glutathione were corrected to a common injected dose for all experiments. In the determination of the disappearance slopes, all the points are not used since the early points presumably represent the condition before equilibrium has been established between original and newly formed glutathione. Only those points

occurring after maximum labelling had been obtained are used in the calculation of the disappearance of label. As shown by the slopes of the curves and by the calculated turnover times listed below Fig. 2, the turnover time for the group of traumatized mice was very much shorter than for the control group. It also seems apparent that the turnover calculated in this manner agrees with that calculated for the short-term labelling experiments only for the groups of traumatized mice. The turnover time calculated by the rate of disappearance of label from glutathione is much slower for the control groups than that calculated in the short-term labelling experiments.

Fig. 1. Percentage incorporation of ^{35}S from non-protein extract into glutathione in the livers of control and traumatized mice. Experimental details are given in the text. The percentage incorporation is calculated as the percentage of the total radioactivity in the non-protein extract present in the glutathione of that fraction (as determined by the glutathione specific radioactivity $\times$ concentration). $\circ$, $\square$, $\triangle$: Groups of traumatized mice; $\bullet$, $\blacksquare$, $\blacktriangle$: control groups. $\circ$, $\bullet$: Expt. 38, 60 min. of trauma; $\square$, $\blacksquare$: Expt. 47, 60 min. of trauma; $\triangle$, $\blacktriangle$: Expt. 46, 150 min. of trauma.

Fig. 2. Calculation of turnover times from the rate of disappearance of $[^{35}\text{S}]$ glutathione from the livers of control and traumatized mice. The specific radioactivity of $[^{35}\text{S}]$ glutathione was corrected to a common injected dose of L- $[^{35}\text{S}]$ cysteine for all groups. $\circ$, $\square$: Groups of traumatized mice; $\bullet$, $\blacksquare$, $\blacktriangle$: control groups. The trauma period was 150 min. for all traumatized mice. The turnover time, t_t , was calculated as $-1/\text{slope}$, where the slope equals $(\ln b - \ln a)/(t_b - t_a)$; b is the specific radioactivity at time t_b , and a is the specific radioactivity at time t_a . The turnover rate is the concn. of glutathione (in mg./100 g. of liver)/ t_t . The calculated turnover times and turnover rates are as follows:

Symbol	Expt. no.	Turnover time for glutathione (min.)	Turnover rate for glutathione (mg./100 g. of liver/min.)
$\blacktriangle$	41C	441	0.296
$\bullet$	43C	164	1.010
$\blacksquare$	45C	317	0.461
$\circ$	31T	32	2.388
$\square$	44T	51	1.182

DISCUSSION

Cysteine appears to be incorporated into glutathione at extremely rapid rates. At intervals as short as 1 min. after an intravenous injection, 15% of the activity found in the non-protein fraction was already in the form of glutathione. Because of the rapidity of the reaction, the question arises whether the reaction represents true glutathione synthesis or merely cysteine exchange in preformed glutathione. It is known that the label of $[^{14}\text{C}]$ -glycine can appear in glutathione not only as a result of synthesis but also through an enzyme-catalysed exchange reaction (Snoke & Bloch, 1955). However, the entrance of significant amounts of labelled L-cysteine into glutathione by an exchange reaction *in vivo* is unlikely. Cysteine occupies the central position in the tripeptide and enters the glutathione molecule only as a result of two successive enzyme-catalysed synthetic reactions, both of which require ATP (Bloch, 1949; Yanari, Snoke & Bloch, 1953):

In the short-term labelling experiments, for each particular incorporation period used (1, 2 or 4 min.) much less L- $[^{35}\text{S}]$ cysteine was incorporated into the liver glutathione of traumatized mice than into that of control mice. Decreased incorporation owing to lack of substrate is apparently ruled out by the findings that: (a) total radioactivity present in all non-protein compounds was generally somewhat higher for livers of traumatized mice than for those of control mice, showing that trauma did not interfere appreciably with the passage of labelled L-cysteine from the tail vein to the liver; (b) total liver cysteine was nearly the same for traumatized mice as for control mice. The trauma-induced decrease in incorporation of L- $[^{35}\text{S}]$ cysteine into liver glutathione in the short-term experiments must therefore be attributed to a true decrease in the rate of glutathione synthesis. This interpretation is emphasized by the values obtained for glutathione synthesis rates by using the turnover concept and appropriate equations. Calculated liver-glutathione synthesis rates were about half as great for traumatized mice as for comparable control mice.

Traumatized mice gave similar values for liver-glutathione turnover times and turnover rates, whether these values were calculated from results obtained in short-term or long-term labelling experiments. On the other hand, the turnover times were much shorter and the turnover rates much higher for control mice of short-term labelling experiments compared with long-term labelling experiments. If we take the turnover-time and

turnover-rate values literally, we are confronted by two discrepancies: (a) trauma inhibits glutathione synthesis if we use the short-term labelling approach, but accelerates glutathione synthesis if we use the long-term labelling approach; (b) in control mice, when ^{35}S is entering liver glutathione, the rate of glutathione synthesis is far greater than when ^{35}S is leaving liver glutathione. One plausible explanation may be that intracellular glutathione pools of different size and location are involved in the results seen after short-term compared with long-term labelling studies. Two groups of workers (Edwards & Westerfield, 1952; Barford & Eden, 1956) have presented evidence for the existence of what they have termed 'stable' and 'labile' glutathione. The 'labile' glutathione is the portion depleted by special dietary regimens leading to a decrease in liver non-protein thiols; the 'stable' fraction is extremely difficult to deplete by such dietary procedures. They agree that the major portion of the liver glutathione is present in the soluble supernatant (after centrifuging at 30 000g for 90 min.) and that it is this fraction which is rapidly depleted during deprivation of protein. The 'stable' fraction, which may amount to 10–40 % of the total glutathione and exists in the nuclear and mitochondrial fractions, is not depleted by these procedures.

Measurements obtained in the short-term experiments will undoubtedly be most influenced by the turnover rate of the rapidly metabolizing pool, and in this pool the turnover times for traumatized and control animals are similar. However, in the long-term experiments, the more slowly metabolizing pool will also influence the rate of disappearance of the label. In control animals newly synthesized glutathione may pass from the 'labile' pool to the 'stable' pool, whereas in the traumatized animals there may be a metabolic fault in this transfer. Hence, in the trauma experiments, whether short- or long-term labelling is studied the turnover times will be similar, since the newly synthesized glutathione will not enter into the 'stable' pool and consequently this pool will not participate in the rate determination.

SUMMARY

1. Rates of glutathione synthesis in the liver were studied in control and traumatized mice. The quantities of injected L- ^{35}S cysteine found as liver

glutathione after periods of 1–4 min. in short-term studies and 5–120 min. in long-term studies were measured.

2. In the short-term studies, turnover times for groups of both control and traumatized mice were similar. The groups of traumatized mice had a smaller quantity of glutathione present, and therefore the turnover rate for the groups of traumatized mice was only about half that of the control groups.

3. Results from the long-term and short-term labelling studies showed similar turnover times in the groups of traumatized mice. The turnover times of control groups were much longer when measured by long-term than by short-term labelling procedures. From this discrepancy, it is postulated that one of the faults in glutathione synthesis in trauma may be in the transfer of newly formed glutathione from a 'labile' to a 'stable' form.

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