Amino Acid Incorporation into Protein of Hepatic Tissue in Cloudy Swelling.* (28660)

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Hepatic cloudy swelling, a cell degeneration easily obtainable by various procedures (1), is characterized by an increase in cell protein content(2). In studying the metabolism of rat tissues affected by this degeneration we found that the incorporation rate of labeled amino acids into protein is faster in liver with toxin-induced cloudy swelling than in normal liver(3). This result could be explained either by an enhanced protein synthesis or by an accelerated amino acid transfer across the cell membrane of the swollen hepatocyte. Indeed swelling increases the rate of amino acid transport across the cell membrane(4). This fact could account for the faster labeling of protein if one assumes that the intracellular amino acid pool has the same size in normal and swollen liver and that this pool is functionally homogeneous with respect to protein synthesis. However, measurements of concentration of intracellular amino acids in liver with toxin-induced cloudy swelling have not been made and the role of the intracellular amino acid pool as an homogeneous precursor pool for protein synthesis has been seriously questioned, as a result of recent studies on bacteria(5), yeasts (6,7,8), molds(9) and mammalian cells(10, 11). The question then arises as to whether the specific activity of the intracellular amino acid precursor, available for protein synthesis in experiments with labeled amino acids, is different in normal and swollen liver and, therefore, whether the faster amino acid transfer found in swollen liver actually affects the rate of appearance of amino acids into protein.

The purpose of this work was to study the incorporation of labeled amino acids into protein of normal and swollen liver, selecting experimental conditions in which size and specific activity of the pool for the precursor amino acid are similar in either tissue and in which the transport of amino acid across the cell membrane is not involved. Evidence suggesting that the faster rate of amino acid incorporation into protein of swollen liver reflects an enhanced protein synthesis will be presented.

Methods. Male Wistar rats weighing 160-180 g were injected with *S. typhimurium* toxin to produce cloudy swelling(1). After a 12-hour fast, control and injected animals were decapitated and their livers removed. Incubation of liver slices was carried out in open 100 ml flasks in Dubnoff metabolic shaker at 12° and 38°C. The basic incubation medium has been described(4); unless otherwise specified, the following substances were added: unlabeled glycine and glycine-1-C^{14} (s.a. 0.33 mc/mmole) 3 mM; DL-leucine-1-C^{14}, 0.26 or 2 mM; L-proline, 15 mM; inulin, 0.5 mg/ml of medium. Final volume was 15 to 30 ml. Tissue slices were added in amounts corresponding to an intracellular to extracellular fluid volume ratio of approximately 1:50. Wet weight was obtained by a torsion balance after careful blotting of the slices on acid-hardened Whatman filter paper. Total water and extracellular spaces were determined as previously described(4). Glycine extraction was carried out with a saturated solution of picric acid(12,13). Medium and extraction fluid samples were assayed for total free glycine† according to Christensen *et al*(14) and plated for radioactivity measurements as described(4). All determinations were performed in duplicate. Chromatograms of the extracts showed that

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† The term 'free glycine' is used to indicate the total quantity of the amino acid which can be extracted from the tissue under conditions in which macromolecules are not degraded into subunits. It does not imply any conclusion as to the true state of the intracellular amino acid.

90-95% of the radioactivity was contained in one spot which coincided with glycine. Breakdown of labeled glycine during paper chromatography was minimized by a suitable proceduce(15). Intracellular glycine concentration and radioactivity of the intracellular amino acid pool were calculated by correcting for the material trapped in the extracellular space. Liver proteins were purified(16), plated and counted beneath a gas flow G.M. counter fitted with a mylar window. Counting error was less than 2%. Plasma free glycine was determined as described by Christensen *et al*(17).

Experimental procedure. Liver slices were collected in Ringer-phosphate and allowed to stand for 30 minutes at 12°C in the same medium, sometimes supplemented with proline (first phase). Slices were then transferred to Ringer containing labeled or unlabeled glycine and incubated for 60 minutes at 12°C (second phase). Finally, the slices were transferred into a plain- or labeled glycine-containing Ringer preheated to 38°C and were incubated at 38°C for 30 minutes (third phase). In some additional experiments, after standing at 12°C for 0, 30 and 60 minutes, liver slices were transferred directly into a medium containing labeled amino acids and incubated for 60-90 minutes at 38°C.

Results. Measurements of glycine concentration in plasma and liver tissue of normal rats and rats with swollen liver provide the following results. Plasma glycine: 0.42 ± 0.04 μmoles/ml (normal rats) and 0.42 ± 0.07 μmoles/ml (toxin-injected rats). Liver glycine: 3.87 ± 0.18 μmoles/g of wet tissue (normal rats) and 5.36 ± 0.20 μmoles/g of wet tissue (toxin-injected rats). These figures, means of 5 to 6 experiments, indicate that glycine concentration is higher in swollen than in normal liver and that no differences are detectable in plasma of normal and toxin-injected animals. Values obtained in normal rats agree well with those reported by others (18,19).

Fig. 1 provides data on intracellular concentration of glycine in liver slices kept in Ringer-phosphate-proline at 12°C and subsequently incubated at 12° and 38°C in a Ringer-phosphate containing labeled glycine. An outflow of the amino acid, somewhat accelerated by the presence of proline in the medium(20), takes place during the first phase, leading to low concentrations of intracellular glycine in both normal and swollen liver. During the second phase, when slices are incubated at 12°C, intracellular glycine concentration increases reaching similar values in normal and swollen liver; in these conditions, if slices are incubated at 38°C, intracellular glycine concentration increases more rapidly than at 12°C and approaches a constant value after 45 minutes. The lower curves in Fig. 1 refer to intracellular concentration of labeled glycine, *i.e.*, to the concentration of glycine which has the same specific activity of the tracer in the medium. The rate of labeled glycine penetration is slightly lower than that of total glycine accumulation. At 12°C, the intracellular labeled glycine concentration does not exceed its concentration in the medium; on the contrary, a net concentrative transfer occurs at 38°C.

Intracellular concentration of glycine and rate of glycine incorporation into protein of normal and swollen liver slices transferred to a Ringer-phosphate equilibrated at 38°C (third phase), are shown in Fig. 2, 3 and 4. When slices have been incubated with labeled or unlabeled glycine at 12°C and when labeled glycine is present in the incubation medium at 38°C (Fig. 2 and 3), the concentration of intracellular total and labeled glycine increases at a rate slightly faster in swollen than in normal liver. The apparent rate of glycine incorporation into protein proceeds as a linear function of time in slices preloaded with labeled glycine and as a curvilinear function in slices preloaded with unlabeled glycine. In both cases, swelling markedly enhances the incorporation of the tracer into protein. When slices have been incubated with labeled glycine at 12°C and no glycine is present in the incubation medium at 38°C (Fig. 4), intracellular concentration of total glycine does not change appreciably and concentration of labeled glycine decreases slightly. Concentration values and rate of changes are similar in normal and

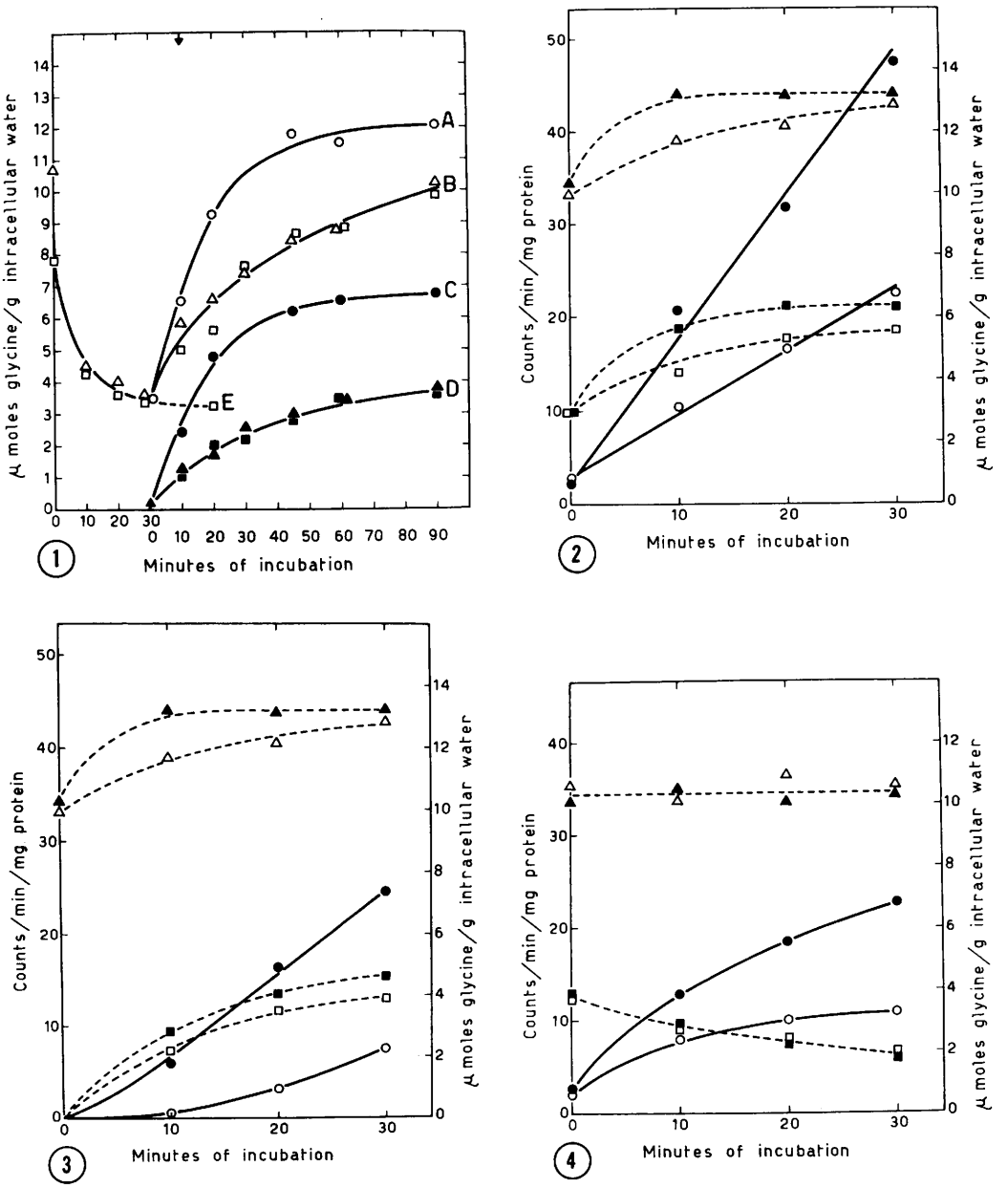

FIG. 1. Glycine concentration in intracellular water of normal and swollen liver slices. Slices incubated at 12°C in glycine-free Ringer (Curve E) then transferred (arrow) into a Ringer containing C^{14}-glycine (initial concentration, 3 mM). Curves A and C represent total (○) and labeled (●) intracellular glycine in normal liver slices incubated at 38°C. Curve B represents total intracellular glycine in normal (□) and swollen (△) liver slices incubated at 12°C. Curve D represents labeled intracellular glycine in normal (■) and swollen (▲) liver slices incubated at 12°C. Each point is the mean of 3 experiments. Points at 0 time (1st phase) do not represent true values of intracellular glycine since no correction has been made for plasma glycine trapped in the extracellular space.

FIG. 2. Glycine concentration in intracellular water and C^{14}-glycine incorporation into protein of normal and swollen liver slices incubated at 38°C in presence of C^{14}-glycine (3mM). Slices preloaded with C^{14}-glycine (3 mM) at 12°C (see text). Legends: Glycine incorporation into protein of normal (○) and swollen (●) liver; total intracellular glycine in normal (△) and swollen (▲) liver; labeled intracellular glycine in normal (□) and swollen (■) liver.

TABLE I. Effect of Preliminary Incubation at 12°C on Subsequent Incorporation of Amino Acids into Protein of Normal Liver Slices Incubated at 38°C.

Preincubation* at 12°C (min)	Incorporation into protein (cpm/mg protein) † Glycine-1-C^{14}‡	Glycine-1-C^{14}§	DL-leucine-1-C^{14}‖
0	335 ±34 (2)	50 ± 7 (4)	678 ±14 (2)
30	351 ±11 (2)	60 ±10 (4)	587 ±26 (2)
60	347 ± 3 (2)	53 ± 2 (4)	650 ± 8 (2)

* Preincubation starts after a 'slicing' period of 10-12 min during which slices are kept in Ringer solution at 12°C. Slices are first incubated in amino acid-free Ringer and then transferred to the amino acid-containing medium preheated to 38°C. Incubation lasts 60 min.

† Mean values ± S.E. No. of experiments in parentheses.

‡ Initial concentration, .5 mM; s.a. 2 mc/mmole.

§ Initial concentration, 3.0 mM; s.a. .33 mc/mmole.

‖ Initial concentration, .26 mM; s.a. 3.8 mc/mmole.

swollen liver. Also in this case the incorporation of labeled glycine into protein is faster in swollen than in normal liver slices.

The effect of preliminary incubation of liver slices at 12°C on subsequent incorporation of labeled amino acids into protein is presented in Table I. The analysis of variance applied to the sets of data from which the mean values have been calculated, shows that, in our experimental conditions, preincubation at low temperature does not affect amino acid incorporation ($P > 0.05$).

When the incorporation of C^{14}-leucine into protein was investigated, the following results have been obtained. Normal liver: 78.7 ± 6.6 counts/min/mg protein; swollen liver: 189.3 ± 18.2 counts/min/mg protein. These figures are means of 6 experiments ± S.E. In these experiments slices were incubated for 60 minutes in the presence of 2 mM labeled leucine (s.a. 0.33 mc/mmole). In agreement with the results obtained with glycine(3), the incorporation of leucine into protein is markedly enhanced in swollen liver.

Discussion. At 12°C the concentration ratio of the tracer between intracellular and extracellular water approaches unity in normal and swollen liver, showing that, under these experimental conditions, labeled glycine is able to penetrate approximately the whole of the tissue water of liver in the absence of an active process of concentration. However, the intracellular glycine pool is larger and rate of accumulation of total glycine is slightly faster than that of labeled glycine penetration, suggesting that in the liver a considerable fraction of intracellular glycine is not readily exchangeable with extracellular glycine and that at least a minor portion of intracellular glycine originates from intracellular sources. The decrease of glycine concentration to a constant value when slices are incubated at 12°C in plain Ringer (Fig. 1), the lack of correspondence between rates of C^{14}-glycine penetration and total glycine accumulation at 38°C (Fig. 1) and the decrease of specific activity of the intracellular pool, in C^{14}-glycine preloaded slices incubated in the absence of extracellular precursor (Fig. 4), give support to this interpretation. Studies on rate of protein breakdown and endogenous glycine synthesis are needed to clarify this point.

The incubation of liver slices at 12°C allows one to modify the intracellular amino acid pool and to obtain normal and swollen liver slices with almost identical intracellular concentrations of total and labeled glycine, without appreciable labeling of proteins and without impairment of synthetic processes. Indeed, incorporation of glycine into protein at 12°C does not exceed 5% of its incorporation at 38°C (results not shown) and incor-

Each point is the mean of 3 experiments; values of total glycine have been averaged with those from experiments presented in Fig. 3, making a total of 6 experiments.

FIG. 3. Glycine concentration in intracellular water and C^{14}-glycine incorporation into protein of normal and swollen liver slices incubated at 38°C in presence of C^{14}-glycine (3 mM). Slices preloaded with unlabeled glycine (3 mM) at 12°C (see text). Legends as in Fig. 2. Each point is the mean of 3 experiments; values of total glycine have been averaged with those from experiments presented in Fig. 2, making a total of 6 experiments.

FIG. 4. Glycine concentration in intracellular water and C^{14}-glycine incorporation into protein of normal and swollen liver slices incubated at 38°C in a glycine-free Ringer. Slices preloaded with C^{14}-glycine (3 mM) at 12°C (see text). Legends as in Fig. 2. Each point is the mean of 3 experiments.

poration of the tracer into protein at 38°C is substantially identical in non-incubated and in liver slices first incubated at 12°C (Table I). This latter finding indicates also that the outflow of amino acids occurring in the cold phase does not lower the intracellular concentration of any amino acid below the critical level at which it would become the limiting factor for protein synthesis in subsequent incubation at 38°C.

In all our experiments the rate of glycine incorporation into protein was faster in swollen than in normal liver slices, although the intracellular glycine pool and its specific activity had been rendered similar in both tissues. This result is consistent with the hypothesis that an enhancement of protein synthesis occurs in swollen liver.

The fact that the size of the glycine pool increases faster in swollen than in normal liver slices when they are transferred to a 38°C medium in the presence of the labeled precursor (Fig. 2), is not at variance with the above interpretation since the specific activity of glycine pools does not vary appreciably throughout the incubation period in either condition. Furthermore, the constant rate of C^{14}-glycine incorporation into protein indicates that, in both tissues, the glycine pool relevant to protein synthesis already had reached equilibrium with the extracellular amino acid in the cold phase.

The curvilinear pattern of C^{14}-glycine incorporation into protein of slices preloaded with unlabeled glycine (Fig. 3) may reflect the rate of equilibration of glycine pool with the extracellular labeled precursor. However the possibility that more than one pool for amino acids exists within the liver cell makes this interpretation uncertain. Indeed, Kipnis *et al*(10) suggested a functional compartmentalization of the intracellular amino acid pool for protein synthesis and reported an extremely rapid equilibration of this pool with extracellular precursors. If this were the case, the lag of glycine incorporation into protein found in our experiments could be tentatively explained by the fact that, in liver slices, labeled glycine which enters the cell is likely to be diluted by unlabeled glycine trapped in the extracellular space. In turn, a dilution effect decreasing with time may explain the apparent parallelism of the rates of glycine penetration in normal and swollen hepatocytes when measured from the appearance of the labeled precursor into the cell and may justify the lack of correspondence between increase of intracellular total glycine and increase of labeled glycine concentration as the incubation proceeds. Whether or not this speculation is correct, comparison of the rates of C^{14}-glycine penetration and total glycine accumulation indicates that, in this experimental condition, the marked increase of glycine incorporation into protein of swollen liver slices occurs in a situation in which the specific activity of total glycine pool may actually increase at a slower rate than in normal liver slices.

When slices preloaded with C^{14}-glycine are incubated at 38°C in the absence of the amino acid (Fig. 4), intracellular glycine concentration approaches a transitory steady-state. Since the specific activity of the glycine pool decreases, the steady-state condition for total glycine must be maintained by glycine deriving from intracellular sources. The faster incorporation of C^{14}-labeled glycine into protein of swollen liver slices under conditions in which glycine pool and its specific activity are similar in normal and swollen liver and in which no appreciable *inward* transfer of amino acid takes place, indicates that a real stimulation of protein synthesis occurs in swollen liver. Results obtained using C^{14}-leucine as amino acid precursor confirm this conclusion. This interpretation does not exclude the possibility that the reported acceleration of amino acid transport across the cell membrane of swollen hepatocytes(4) *per se*, may enhance the incorporation of amino acids into protein, perhaps facilitating the access of these precursors to the site of protein synthesis. Indeed both phenomena could conceivably be responsible for the increase in cell protein content found in liver with toxin-induced cloudy swelling(2).

Summary. Incorporation of labeled amino acids into protein of normal rat livers and of livers with toxin-induced cloudy swelling has been studied *in vitro*, under experimental conditions in which size and specific activity

of the intracellular pool for the precursor amino acid in the 2 tissues had been rendered similar by a suitable preincubation procedure. Under these conditions, swelling markedly enhances the rate of incorporation of C^{14}-glycine into protein, and this increase is still present when no appreciable inward transfer of amino acid across the cell membrane takes place. A faster incorporation rate of C^{14}-leucine into protein of swollen liver slices has also been observed. These results indicate that a real stimulation of protein synthesis occurs in swollen liver and suggest that this stimulation may explain, at least in part, the increase in cell protein content found in liver with cloudy swelling.

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Uremic Toxicity Correlated with Unidentified Anions. (28661)

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It is known that experimental administration of urea, creatinine or uric acid does not reproduce the clinical manifestations of the uremic syndrome, even though blood levels of these nitrogenous wastes often parallel uremic toxicity. The identity of the "uremic toxin" is still not clear. Seligson(1) and Hyman(2) analyzed serum and dialysis fluid from patients with acute renal failure: clinical signs and symptoms of uremia were present in these persons when an organic anion moiety was increased in their body fluids. The data suggested a pathogenic role of unidentified organic anions in uremia.

Ten patients with terminal chronic uremia underwent enterostomy here, to be reported elsewhere. After the enterostomy had become established, the fluid discharged from it sufficed to maintain water balance; it was helpful in controlling serum cation levels; for weeks at a time the discharge contained enough urea and other non-protein nitrogen to achieve nitrogen equilibrium. Critical deficits of blood volume, hemoglobin or plasma proteins were absent; liver function was well maintained. When the patients suffered from distressing symptoms or signs these could not be explained by "pseudo-uremic overlay" (such as overhydration)(3). Representative data from 2 patients support earlier tentative conclusions of other authors(1,2).

Case reports: J. B., a 40-year-old man,