The Inhibition of Accumulation of [1-¹⁴C]Glycine and its Incorporation into Protein of Ehrlich Ascites-Carcinoma Cells by DL-Methionine

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1. By incubation of Ehrlich ascites-carcinoma cells *in vitro* with [1-¹⁴C]glycine the relation between the uptake of glycine and its incorporation into protein was examined. 2. With DL-methionine as a competitive inhibitor, there was not only a decrease in uptake of this amino acid, but also inhibition of its incorporation into protein. 3. It is only in its initial stage that the increase in incorporation is accompanied by increase in intracellular concentration of free glycine. Further increase in the amino acid pool has no effect on protein synthesis. 4. Even with a high cell concentration of glycine, methionine produces a decrease both in the uptake and its incorporation. This suggests that the inhibition of incorporation of glycine by methionine is due, not only to decrease in its intracellular concentration, but also to changes in other processes responsible for protein synthesis.

In spite of intensive research into the relation between amino acid pools and protein synthesis (the results of which were surveyed by Segal, 1964) this problem still remains unsolved. It has been observed that in many cases where there is an increase in protein synthesis there is also an increase in the concentration of free amino acids. This occurs in normal growth, in malignancy or in response to hormones (Riggs & Walker, 1958; Christensen & Riggs, 1952; Debons & Pittman, 1962). Halvorson & Cohen (1958) found, however, that the kinetics of incorporation of amino acids into protein of bacteria are independent of the velocity with which amino acids enter the intracellular pool. Riggs & Walker (1963) found no correlation between active transport of free amino acids and their incorporation into protein, except when using low, limiting, concentrations. Apart from other inhibitors of active transport, they also used DL-methionine. They found that this competitive inhibitor decreased the accumulation of the amino acids studied (valine, which behaves like glycine, being one), but did not affect their incorporation into protein. Johnstone & Scholefield (1959) obtained similar results. However, Klavins, Kinney & Kaufman (1963), Kaufman, Klavins & Kinney (1960), Hardin & Hove (1951) and Schreier (1962) found that methionine, if given in large amounts in food, leads to a disturbance of protein metabolism, resulting in histopathological changes, weight loss and malnutrition. This induced the present author to use methionine as an inhibitor in dealing with the problem.

MATERIALS AND METHODS

Ehrlich mouse ascites-carcinoma cells were used, obtained by syringe 7–10 days after intraperitoneal transplantation. The cells were separated from ascites fluid by centrifuging at 250g for 7 min. and the 'packed-cell' volume (0.8–1.6 ml./mouse) was measured. The cells were then suspended in Ca²⁺-free Krebs–Ringer bicarbonate solution containing: NaCl (145 mm), KH₂PO₄ (1.5 mm), MgSO₄ (1.5 mm), KCl (5.8 mm), NaHCO₃ (25 mm) and glucose (10 mm), pH 7.4. The dilution ranged between 1:12 and 1:25. The suspension was transferred to eight 25 ml. Erlenmeyer flasks, each flask containing 3 ml. of suspension with 0.1–0.2 ml. of cells. The inhibitor was then added.

After shaking the cell suspension for 5–7 min. at 37° a solution of [1-¹⁴C]glycine (1 mc/m-mole; obtained from the Institute for Nuclear Science 'Boris Kidrič', Vinča, Yugoslavia) was added. All the experiments were carried out under aerobic conditions in an atmosphere of O₂ + CO₂ (95:5). The cell suspension was shaken at 70 cyc./min. with an amplitude of 8 cm. No agglutination of cells was observed.

After incubation the suspension was centrifuged at 950g for 10 min. The supernatant was removed and the interior of the tubes dried with filter paper. No washing was carried out, since this procedure decreases the intracellular concentration of amino acids by as much as 10% (Tenenhouse & Quastel, 1960). To the sedimented cells was then added 3 ml. of 95% (v/v) ethanol; the mixture was thoroughly homogenized and a minimum period of 30 min. was allowed for completion of the extraction of the free glycine. Re-extraction was done twice with 2 ml. of 95% ethanol. The extract thus prepared (0.5 ml.) was dried on aluminium planchets and the radioactivity was then measured. Since glycine suffers metabolic changes it can be assumed that the radioactivity values obtained do not refer to glycine alone.

However, the work of Riggs & Walker (1963), who separated pure glycine by Moore & Stein column chromatography, has shown that 95% of the cell radioactivity thus extracted belongs to glycine after 2 hr. of incubation.

Then 1 ml. of medium was diluted to 10 ml. with acetone to sediment protein traces and kept at 4° for about 2 hr. with subsequent centrifugation; 0.5 ml. was again dried on the planchet and measured for radioactivity.

The ethanol-insoluble fraction, representing proteins (Johnstone & Scholefield, 1959), was extracted twice with 3 ml. of 95% ethanol, once with 3 ml. of acetone and once with 2 ml. of ether. It contained 1–2% of free glycine, as determined by adding $[1-^{14}\text{C}]$ glycine to cells after the 95% ethanol. The precipitate (1.5–2 mg. dry wt.) was suspended in acetone and then placed on aluminium planchets, 0.2 ml. of acetone–water (1:1, v/v) being added just before evaporation of acetone to ensure uniform distribution of material. Counting was carried out with a gas-flow Geiger–Müller counter (Nuclear–Chicago Corp. model D-47) with helium + butane (98.7:1.3) and with a thin Micromil window (150 $\mu\text{g./cm}^2$). The background was about 16 counts/min. and the efficiency was about 40%. The counting time was always sufficient to keep the error below 5%. No correction of self-absorption was made as the amount of material placed on the planchet for test and control was always the same.

RESULTS

Effect of methionine on accumulation and incorporation of $[1-^{14}\text{C}]$ glycine. To examine the effect of a

decreased glycine pool on its incorporation into protein, DL-methionine (10 mM) was added to the medium containing 2 mM- $[1-^{14}\text{C}]$ glycine (1 $\mu\text{C}/3\text{ml.}$).

The results presented in Figs. 1 and 2 reveal

Fig. 2. Inhibition of $[1-^{14}\text{C}]$ glycine (2 mM) incorporation into protein by DL-methionine (10 mM). ○, Control (without inhibitor); ●, test (with inhibitor).

Fig. 1. Inhibition of $[1-^{14}\text{C}]$ glycine accumulation by DL-methionine. ○, Control (without inhibitor); ●, test (with inhibitor). $[1-^{14}\text{C}]$ Glycine accumulation is expressed by plotting the distribution ratio (counts/min./ $\mu\text{l.}$ of cellular water: counts/min./ $\mu\text{l.}$ of medium) against time, as shown.

Fig. 3. Relation between intracellular concentration of free glycine and its incorporation into protein. The cells were incubated for 90 min. in medium with increasing concentrations of $[1-^{14}\text{C}]$ glycine. The distribution ratio in all cases was over 20. ○, Radioactivity due to free intracellular $[1-^{14}\text{C}]$ glycine; ●, radioactivity incorporated into protein.

considerable decrease in [$1\text{-}^{14}\text{C}$]glycine accumulation (reaching 65%) and in its incorporation into proteins (reaching as much as 50%).

Relation between intracellular free glycine concentration and its incorporation into protein. Cells were incubated for 90 min. in the medium with increasing [$1\text{-}^{14}\text{C}$]glycine concentration (0.4–4.0 mM) and observations were made of free glycine concentra-

tions and protein radioactivity. As shown in Fig. 3, protein radioactivity increases only at lower free glycine concentrations, soon reaching a plateau, whereas free glycine increase remains without any appreciable effect. This is in agreement with the work of Riggs & Walker (1963) and Eagle & Piez (1962).

Effect of DL-methionine on accumulation and incorporation of [$1\text{-}^{14}\text{C}$]glycine at different intracellular glycine concentrations into protein. The results presented in Figs. 4 and 5 indicate that accumulation and incorporation are inhibited at all concentrations (0.4–8.0 mM) of glycine studied.

DISCUSSION

Christensen, Riggs, Fisher & Palatine (1952) were the first to observe the existence of competitive inhibition in the active transport of some amino acids. Later studies (Riggs, Coyne & Christensen, 1954; Johnstone & Scholefield, 1959; Riggs & Walker, 1963) showed that active transport of a neutral amino acid (e.g. glycine) can be inhibited only by neutral amino acids (methionine, valine, serine). The present work has confirmed that methionine is an inhibitor of glycine accumulation. In the present attempt to examine the effect of a decrease in the amino acid pool on protein synthesis, observations were made on the incorporation of [$1\text{-}^{14}\text{C}$]glycine in the presence of methionine at different concentrations of glycine. The results are not in agreement with those of Johnstone & Scholefield (1959) and Riggs & Walker (1963), who did not find any inhibition of [$1\text{-}^{14}\text{C}$]glycine incorporation in the presence of 5–10 mM-DL-methionine. However, the results agree with those of others (e.g. Klavins *et al.* 1963; Kaufman *et al.* 1960; Hardin & Hove, 1951; Schreier, 1962) showing that methionine given to rats and men in large amounts results in appreciable histopathological changes, weight loss and malnutrition. This phenomenon can only be explained as an effect of a disturbance of protein metabolism. Similar observations have been made with other amino acids (particularly hydroxyproline; Winitz, 1962), but Schreier (1962) concluded on the basis of studies on human material that methionine has the strongest toxic effect of all amino acids.

The chief purpose of the present research, however, was to prove the existence of a correlation between the accumulation of amino acids and their incorporation into proteins. As shown by the results (Fig. 3), such correlation exists only with low, limiting, concentrations of amino acids. It was expected that inhibition of [$1\text{-}^{14}\text{C}$]glycine incorporation by methionine would not take place at very high intracellular concentrations, or, if so, that it would be considerably less than inhibition at low

Fig. 4. Effect of DL-methionine (5 mM) on the intracellular concentration of free [$1\text{-}^{14}\text{C}$]glycine at different concentrations of glycine in the medium. Incubation was for 90 min. $\circ$, Control (without inhibitor); $\bullet$, test (with inhibitor).

Fig. 5. Inhibition of [$1\text{-}^{14}\text{C}$]glycine incorporation into protein by DL-methionine. Experimental conditions were the same as in Fig. 4. $\circ$, Control (without inhibitor); $\bullet$, test (with inhibitor).

concentrations. The experiments conducted show that, even where the intracellular concentration of glycine was much greater, the inhibitory effect of methionine was almost the same. For these reasons the inhibitory effect cannot be explained by a decrease in the free glycine concentration alone.

The experimental results suggest that imbalance in the concentration of amino acid produces an appreciable effect on protein synthesis. It can therefore be supposed that when cancer cells, which possess a strong ability to accumulate amino acid, find themselves close to normal cells they cause, among other things, such imbalance, which leads to damage and destruction of cells. Some authors (Wiseman & Ghadially, 1958; Quastrel & Bickis, 1959) consider this to be one of the main processes responsible for the infiltration of malignant neoplasm and subsequently of cachexy of the organism.

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