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Received Mar. 6, 1969. P.S.E.B.M., 1969, Vol. 131.

Inhibition of the Binding of Cystine to Proteins by Glutamic Acid* (33985)

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Cystine in proteins may participate in peptide bonds, disulfide bonds, or both. The turnover of half-cystine bound to protein by disulfide bonds only is very much more rapid than is that bound by peptide bonds (1, 2). *In vivo,* this turnover of disulfide-bound half-cystine has been reported to be mediated by a disulfide–sulfhydryl interchange enzyme (3–5).

During the study of sulfur amino acid metabolism in regenerating wound tissue, we had shown that labeled peptide-bound half-cystine in the tissue protein of rats can be distinguished from that bound to the protein by disulfide bonds exclusively (1, 6). To verify these results *in vitro,* the uptake of cystine by proteins in fresh tissue homogenates was studied. Only a relatively small amount of labeled cystine became associated with the homogenate proteins by means of peptide bonds. In an attempt to increase peptide-bound ^{35}S-cystine incorporation, the homogenates were incubated with mixtures of amino acids—to no avail. Not only was there no increased incorporation of peptide-bound cystine, but the presence of the amino acids appeared to cause a diminution in the disulfide-bound half-cystine which became attached to the protein. This effect was soon traced to the presence of glutamic acid in the mixtures.

* Supported in part by the U. S. Army Medical Research and Development Command under Research Contract No. DA-94-193-MD-2493.

A few characteristics of the inhibition of the sulfhydryl–disulfide interchange reaction by glutamic acid are presented below. Other parameters of the reaction are presently under investigation.

Experimental Methods. In early experiments, we compared the uptake of labeled cystine by native and denatured tissue proteins. When once it had been established that the effect of the glutamic acid is nonenzymatic *in vitro,* much of the subsequent work was carried out on protein from heat-denatured homogenates. Inactivation of tissue proteins is of obvious importance to prevent metabolic structural changes when studying the inhibitory capacity of glutamic acid or related compounds.

Rat tissue homogenates were prepared in an all-glass homogenizer with about 2–4 vol of 0.05 *M* tricine buffer at pH 7.3–7.5. After diluting the homogenate with 30–50 vol of buffer, it was heated in a water bath at 80–85° for 5 min and then filtered several times through glass wool. The filtrate was diluted with buffer to contain approximately 0.7–1.0 mg of protein/ml.

Experiments on the binding of labeled cystine were carried out at room temperature by mixing 2.0 ml of diluted tissue homogenate with 2.0 ml of glutamic acid solution, adjusted to the appropriate pH, and 2.0 ml of buffer containing 0.01 μCi of ^{35}S-L-cystine. The control was a similar mixture, containing 2.0 ml of tricine buffer instead of the gluta-

mic acid solution. After incubation, the reaction mixtures were dialyzed against separate 200-vol quantities of 0.01 *M* buffer at 5° for 18–22 hr with shaking. Further dialysis against another 200 vol of buffer under the same conditions resulted in only a very small reduction in the amount of ^{35}S activity remaining in the dialysand, usually about 5%.

The ^{35}S activity in an aliquot of dialysand was measured with a thin end-window G-M counter. The protein content was determined in another aliquot of the dialysand (7). The protein content of the dialyzed homogenates varied between 250 and 350 μg/ml, depending upon the preparation.

Essentially all of the ^{35}S-cystine remaining in these preparations after dialysis was linked to protein by means of disulfide bonds only. This was shown by the fact that more than 90% of the ^{35}S activity remaining in the dialysands could be removed from the protein by dialysis overnight against 200 vol of 0.03 *M* Na_2SO_3 containing Cu^{2+} ions (6), or by treating the protein solution with 0.05 *M* mercaptoethanol and dialyzing against buffer for the same length of time (8).

Results and Discussion. The disulfide-bound half-cystine we measured becomes attached to proteins by the well-authenticated reaction shown below (9–13):

$$\begin{matrix} {}^{35}S\text{–Cys} \\ | \\ {}^{35}S\text{–Cys} \end{matrix} + \text{protein–SH} \rightarrow \text{protein–S–}{}^{35}S\text{–Cys} + \text{Cys–}{}^{35}SH.$$

The rate of binding of the disulfide-bound labeled half-cystine into liver and wound tissue proteins is shown in Fig. 1. The difference in the amount of ^{35}S activity found in the liver as compared to that in the wound tissue is most likely due to the difference in the number of free sulfhydryl groups available for reaction in these different congeries of proteins. The data in this plot suggest the possibility that the amount of ^{35}S-cystine which can be bound to the tissue protein in the presence or absence of glutamic acid, will eventually reach the same level. It might then be argued that the glutamic acid does not have an absolute inhibitory effect, but merely affects the *rate* of the reaction. Alternatively, there may be two different mechanisms by which the interchange reaction is accomplished, only one of which is inhibited by the presence of glutamic acid.

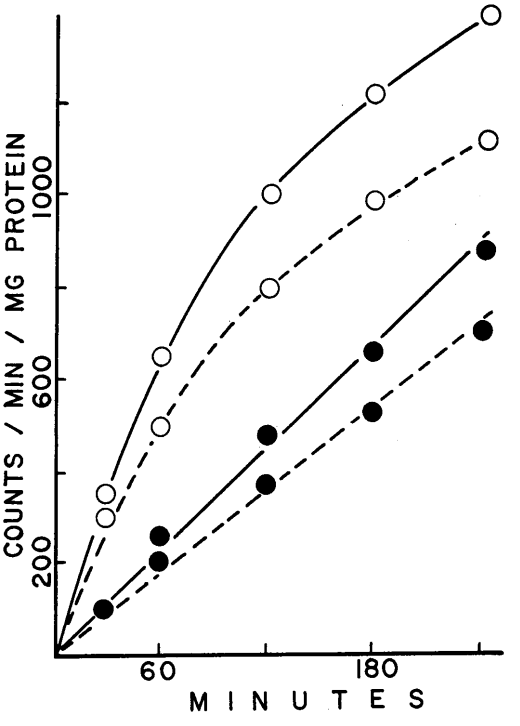

FIG. 1. Rate of incorporation of ^{35}S-cystine into the proteins of fresh tissue homogenates. At least 92% of the ^{35}S is bound to the protein by means of disulfide bonds only, since this amount could be removed from the protein by dialysis against 0.03 *M* Na_2SO_3. Protein concentration 270-295 μg/ml; ^{35}S-cystine, 0.0016 μCi/ml (sp act, 4.88 mCi/mmole); pH 7.2; temperature, 25–27°; solid line= liver homogenate; broken line = granulation tissue homogenate; open circles = control; solid circles = 0.004 *M* glutamic acid.

The same inhibitory effect of glutamic acid on the binding of cystine by both native and denatured proteins in homogenates of muscle and kidney was observed. Under our experimental conditions, the lowest concentration at which an unequivocal inhibitory effect by glutamic acid regularly could be identified was 0.004 *M*. The inhibition of cystine binding to purified proteins also can be obtained, as shown in Table I.

The structure of glutamic acid appears to

TABLE I. Inhibition of Disulfide–Sulfhydryl Interchange Reaction by Glutamic Acid.[a]

Protein	(counts/min/mg of protein) Control	Glutamic acid
Serum albumin	320 ± 25	183 ± 28
Egg albumin	415 ± 36	180 ± 23
Serum β-globulins	550 ± 28	245 ± 30

[a] Glutamic acid, 0.008 *M*; protein, 200 μg/ml; ^{35}S-cystine, 0.0016 μCi/ml (sp act, 4.65 mCi/mmole); reaction carried out at 22–26° for 1 hr at pH 7.4.

be quite unique for inhibiting the sulfhydryl–disulfide interchange reaction. This is illustrated by a comparison of the inhibitory activity of the free amino acid with various related compounds (Table II). Since glutamic acid derivatives which have a substituent attached to the γ-carboxylic acid group show no inhibitory activity, we may conclude that this carboxylic acid group is involved in the mechanism of obstructing the interchange reaction. The effect of blocking the α-carboxylic acid group, as with an ester or in peptides, indicates the importance of this group for inhibition. More than merely two free carboxylic acid groups are required in compounds which will be able to inhibit the interchange reaction. Thus, glutaric and α-ketoglutaric acids, as well as other dicarboxylic acids, are inactive. On the other hand, the presence of a primary amino group in the glutamic acid derivative is not essential for inhibitory activity; *N*-acetyl, DNP- and *N*-peptide derivatives of glutamic acid have the same activity as the parent compound. No amino acid, not even the closely related aspartic acid, seems to have any inhibitory effect on the interchange reaction when tissue proteins are involved. Only in the case of the reaction between bovine serum β-globulins and ^{35}S-cystine does aspartic acid show about 10% of the activity of glutamic acid, under the same conditions of pH and concentration. The apparent inhibitory effect of unlabeled cystine was found to be due to the dilution of the labeled cystine.

How pH and concentration of glutamic acid influence the interchange reaction is shown in Fig. 2. Of particular significance is the fact that glutamic acid shows a maximal inhibitory effect in the physiological pH range; considerably less inhibition occurs in either more acidic or more basic media. Increasing the glutamic acid concentration does not result in proportionately greater degrees of inhibition at any pH; each succeeding increment of concentration appears to give a smaller amount of inhibition.

Extrapolation of the curves in Fig. 2 results in their meeting at a common point on the abscissa, a plot typical for identifying substances which take part in a noncompetitive inhibition. This suggests that the glutamic acid does not enter into competition with either the labeled cystine or with the sulfhydryl groups of the protein. This, in turn, suggests that glutamic acid probably affects an intermediary compound required for the interchange reaction, or a reaction which

TABLE II. Effect of Glutamic Acid Derivatives and Related Compounds on the Sulfhydryl–Disulfide Interchange Reaction between Denatured Liver Proteins and ^{35}S-Cystine.

Compound[a]	No. of expts.	(cpm/mg of protein)
Tricine buffer (pH 7.4)	18	1012 ± 62
L-Glutamine	10	1052 ± 56
N-Acetyl-L-glutamine	4	1022 ± 45
L-Glutamic acid (γ) ethyl ester	5	1035 ± 49
L-Glutamic acid (α) methyl ester	4	1050 ± 62
L-Glutamic acid (α) benzyl ester	2	1002 ± 43
L-Glutamyl-L-alanine	2	985 ± 38
Glutaric acid	7	998 ± 55
α-Ketoglutaric acid	6	1045 ± 57
Citric acid	2	990 ± 35
Fumaric acid	2	964 ± 42
Succinic acid	4	992 ± 54
L-Aspartic acid	10	1068 ± 58
L-Glutamic acid	18	780 ± 60
N-Acetyl-L-Glutamic acid	8	795 ± 46
DNP-L-Glutamic acid	5	748 ± 46
L-Arginyl-L-glutamic acid	2	766 ± 37
Glycyl-L-glutamic acid	2	794 ± 26

[a] Concentration, 0.005 *M*; protein concentration, 250–350 μg/ml, depending on the experiment; ^{35}S-Cystine, 0.0016 μCi/ml (sp act, 6.85 mCi/mmole); reaction carried out for 2 hr at room temperature at pH 7.4 before dialysis.

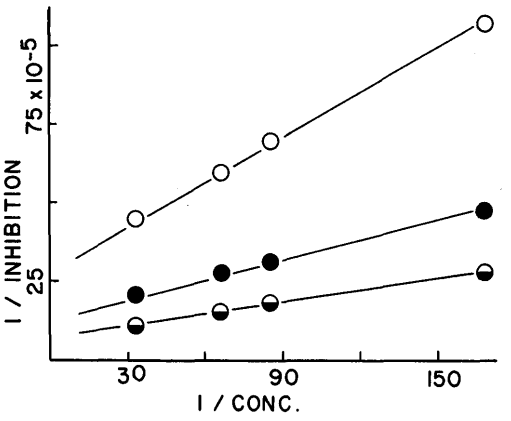

FIG. 2. Effect of pH and glutamic acid concentration on the inhibition of the binding of ^{35}S-cystine to the proteins in rat liver homogenate, plotted as the reciprocal of inhibition against the reciprocal of the molar concentration of glutamic acid. Inhibition is considered to be the difference in the amount of ^{35}S cystine which becomes bound to the protein in the presence and absence of glutamic acid, in terms of cpm/mg of protein. Denatured liver protein, 290 ± 10 μg/ml; ^{35}C-cystine, 0.003 μCi/ml (sp act, 6.85 mCi/mmole). All solutions used were adjusted to the appropriate pH prior to mixing; reactions were carried out for 2 hr at 21–23° before dialysis. Data indicated by open circles were obtained at pH 6.2; half-solid circles at pH 7.4; solid circles at pH 8.6.

obligatorily precedes the interchange reaction.

Not only is the inhibitory effect of glutamic acid greatest at pH 7.3–7.5, but also the largest amount of ^{35}S-cystine becomes bound to the protein (per unit time) at this pH. The interchange reaction was reported to increase with pH (9, 13), probably because dissociation of the sulfhydryl groups varies directly with pH. The reaction rate being greater at pH 7.4 than at 6.2 most likely is due to the greater dissociation of sulfhydryl groups at the higher pH. However, the greater rate shown at pH 7.4 over that at pH 8.6 infers that some compound, other than the cystine or the protein, but required for the interchange reaction, is less available in alkaline medium.

The significance of this inhibitory reaction may relate to its ability to regulate enzyme activity and thereby the rate of metabolic reactions. The fact that activation of many enzymes involves the sulfhydryl–disulfide interchange reaction is well known. It follows that substances which affect the interchange reaction also may be expected to affect the activity of these enzymes. The above data implies, therefore, that glutamic acid may regulate some phases of metablism by virtue of its effect on the activity of certain enzymes.

Summary. The reaction of ^{35}S-cystine with sulfhydryl groups of both native and denatured proteins is inhibited in the presence of low concentrations of glutamic acid. Identical inhibitory activity is displayed by *N*-substituted derivatives of this amino acid, but not by derivatives in which either carboxylic acid group is blocked. Aspartic acid does not interfere with this reaction. The effect of glutamic acid is greatest at pH 7.4 and decreases markedly in more acidic or more basic media. It is suggested that glutamic acid may regulate metabolic processes by affecting the activity of sulfhydryl-activated enzymes.

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Received Mar. 10, 1969. P.S.E.B.M., 1969, Vol. 131.