ON THE *IN VITRO* INHIBITION OF CATALASE BY AMINO ACIDS

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The discovery, made by HARGREAVES AND DEUTSCH, of the anticatalase activity *in vitro* of boiled tumor extracts[1], seemed to provide a new and easy tool for investigating the problem of the reduction of the liver catalase activity observed in tumor-bearing organisms. Therefore a preliminary study was undertaken to test the specificity of the inhibition principle. No specificity could, however, be observed, because normal organ extracts often showed a sharper inhibiting action than tumors[2,3]. Similar conclusions were reached independently by some Japanese scientists[4]. Although the problem lost much of its cancerological interest, it was considered important, from a more general point of view, to investigate the substance responsible for the phenomenon. Interest in this research was increased by the fact that this principle, although resistant to heat and acids, seemed to be difficult to isolate[1]. Attempts were first made to separate the active substance by paper electrophoresis, paper and cellulose column chromatography; as the results of these experiments directed our attention towards amino acids, pure amino acids were studied for their anticatalase activity.

From the literature it appeared that, among amino acids, cysteine had already been rather extensively studied for its anticatalase activity[5–8].

Active organ extracts were analysed for their content of the more inhibiting amino acids and finally the activity of combined amino acid solutions, in proportions and concentrations comparable to those of organs extracts, was tested.

EXPERIMENTAL

Methods and materials

The organ extracts were prepared according to HARGREAVES AND DEUTSCH[1], by the method that involves the purification of boiled aqueous extracts by alcohol precipitation. A detailed description has been given previously[3]. The catalase activity was measured by the method of VON EULER AND JOSEPHSON[9] after two hours incubation at 0°C with increasing amounts of the samples to be tested for their inhibiting activity. For the test a beef liver crystalline catalase of 29,500 Kat.f. ("Katalase fähigkeit") was used; it was prepared by a modification of the Sarkar and Sumner method, described elsewhere[10].

Human gastric mucosa from ulcer patients was used for most of the present experiments because of the high catalase-inhibiting properties of its extracts; some human tumors were also investigated.

Fractionation of extracts

Paper electrophoresis, paper chromatography and column chromatography on cellulose powder indicated that the inhibitory action was distributed among all amino acid fractions, no matter which of the three methods was used. These results clearly in-

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dicated a plurality of inhibiting agents and strongly suggested the implication of amino acids in the process. An analysis of the anticatalase activity of the single amino acids was therefore undertaken.

Anticatalase activity of pure amino acids

Twenty-three of the more common amino acids were tested. The amino acids were dissolved in water. For some of them, because of their slight solubility at neutral pH, it was necessary to add some $N/10$ NaOH or HCl. The solutions were then neutralized and diluted immediately to the chosen concentration with phosphate buffer 0.01 M at pH 6.8. In order to test the possible interference of salts, formed during neutralization, on the catalase activity, some experiments were performed with 1% solutions of NaCl and 0.1 M phosphate buffer at pH 6.8. No influence on the catalase activity was noticed, under these conditions.

The different amino acids were tested at $M/100$ concentration, under the same experimental conditions used for the organs extracts[3]. To 1 ml of freshly prepared catalase solution, diluted so that it has a K_0 of $600 \cdot 10^{-4}$, 0.2 and 0.4 ml of the solutions of the single amino acids were added, and the mixture incubated for exactly two hours in a refrigerator at 0°C. It was then diluted with 30 ml of 0.01 N H_2O_2 in phosphate buffer $1/150$ M at pH 6.8. The H_2O_2 not destroyed was determined at zero time and after 3, 6, 9 and 12 minutes at 0°C by a 0.005 M solution of $KMnO_4$, in the presence of 30 ml of M H_2SO_4.

Several amino acids showed a distinct inhibiting activity on catalase. At a $M/100$ concentration the inhibitions were about 60% for cysteine and tyrosine, 50% for tryptophan and 45% for glutamic acid and cystine. Histidine, methionine, ornithine, lysine and leucine showed inhibitions ranging from 25 to 15%. For the other amino acids tested inhibition was 10% or less. This sequence of the various amino acids for inhibiting activity was found to be constant; however, the absolute values showed variations according to the different batches of catalase used. In fact several months ageing of the lyophilized catalase even when stored under vacuum in a refrigerator at 0°–2°C, apparently enhances its sensitivity toward inhibiting agents (Fig. 3).

The curves obtained by addition of increasing amounts of amino acid solutions to the catalase have the same shape as that obtained with increasing amounts of organ extracts (Fig. 1 and 2), that is, after a rather rapid increase, they tend to flatten out. Inhibition in connection with incubation time at 0°C, was also studied for tyrosine and for a mixture of amino acids and the curve obtained was compared with that given by organ extracts. In this case also we observed identical behaviour: the increase in inhibition is sharp; it reaches its maximum in about one hour and then remains constant (Fig. 3).

Analysis of extracts of gastric mucosa and of several tumors for their content of the more strongly inhibiting amino acids

The inhibiting activity of several amino acids on catalase *in vitro*, was clearly proved by the experiments described. It still had to be investigated whether the content of amino acids in these organ extracts was sufficiently high to account for all the inhibition observed, or whether this could be ascribed to some other factor as well. Therefore the organ extracts were analysed for their content of the more inhibiting amino acids, namely: cysteine, tyrosine, tryptophan, glutamic acid and cystine.

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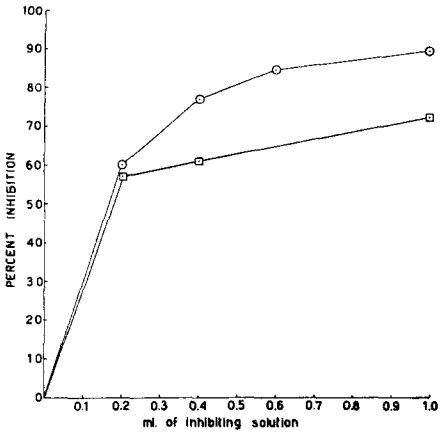

Fig. 1.

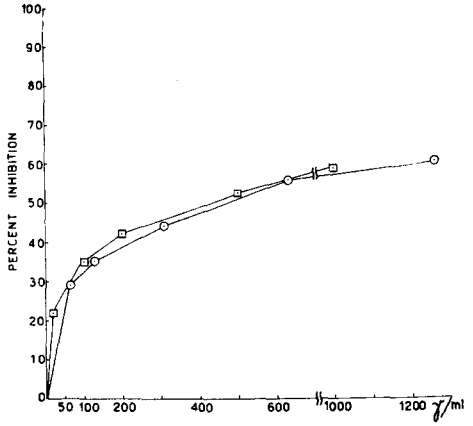

Fig. 2.

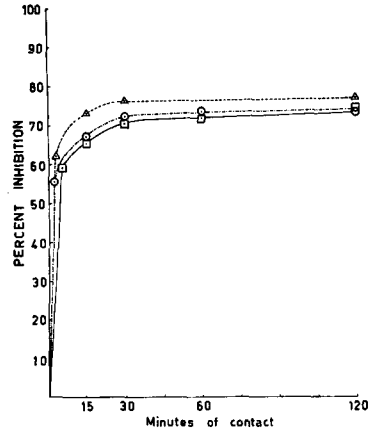

Fig. 3.

Fig. 1. Per cent inhibition of catalase activity for increasing amounts of an extract of human gastric mucosa (○) and of a mixture of amino acids (□) corresponding to the amounts determined in the extract itself (cysteine 125 γ, tyrosine 140 γ, tryptophan 21 γ, glutamic acid 1300 γ, cystine 59 γ per ml).

Fig. 2. Per cent inhibition of the catalase activity for increasing amounts of cysteine (○) and of tyrosine (□).

Fig. 3. Inhibition of catalase as a function of the time of contact with the inhibiting agents: ○ extract of human gastric mucosa: 0.6 ml; □ tyrosine 600 γ; △ amino acid mixture: 1 ml containing cysteine 165 γ, tyrosine 200 γ, tryptophan 29 γ, cystine 45 γ, glutamic acid 1470 γ. The sensitivity to inhibitors of the batch of catalase used for the experiments was high (lyophilized catalase stored in refrigerator for several months).

Methods. The SH and SS groups were determined by potentiometric titration with silver nitrate according to the method of CECIL AND MCPHEE[12].

For tyrosine the GERNGROSS reaction with α-nitroso-β-naphthol[13] was worked out to a quantitative method which gave excellent results.

A detailed description of this method will be given elsewhere.

The determination of tryptophan was carried out by means of the WINKLER reaction[14], according to the method of SHAW AND MCFARLANE[15].

A modification had to be introduced because in the case of organ extracts the color changes, during boiling, to a brownish red. The color is therefore allowed to develop for two hours at room temperature; under these conditions the sensitivity is lower, but the brown shade is sufficiently reduced to allow semi-quantitative evaluations.

We found the most convenient method for glutamic acid determination to be that based on absorption on an alumina column according to the Darling procedure[16]. After elution with acetic acid, glutamic acid is determined by nesslerization. Repeated control assays with 25 to 100 γ of pure glutamic acid always gave quantitative recovery.

With the above-mentioned methods, the amino acids under investigation were determined in eleven extracts from human gastric mucosa, two from intestinal carcinoma, one from intestinal mucosa and one from ovary carcinoma. The results obtained, together with the anticatalase activity, are tabulated in Table I.

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TABLE I

	Cysteine γ per ml of extract	*Tyrosine γ per ml of extract*	*Tryptophan γ per ml of extract*	*Cystine γ per ml of extract*	*Glutamic acid γ per ml of extract*	*Total nitrogen γ per ml of extract*	*Percent inhibition per 1 ml of extract*	*Percent inhibition per 1 ml of the corresponding amino acid mixtures*
Human gastric mucosa	12	80	12	36	930	860	78	54
Human gastric mucosa	48	104	—	34	635	1080	73	63
Human gastric mucosa	45	175	32	39	505	1120	83	50
Human gastric mucosa	62	120	20	38	970	1120	87	64
Human gastric mucosa	56	105	32	42	1170	1160	63	59
Human gastric mucosa	80	215	40	51	2670	1760	88	64
Human gastric mucosa	172	200	22	56	888	1380	92	63
Human gastric mucosa	125	140	21	59	1300	990	84	72
Human gastric mucosa	165	100	12	69	975	940	87	63
Human gastric mucosa	156	160	18	53	1060	1050	82	60
Human gastric mucosa	165	200	29	45	1470	1320	90	72
Intestinal carcinoma	118	88	25	51	750	880	60	60
Intestinal carcinoma	149	60	10	66	715	880	64	63
Intestinal mucosa	106	120	17	—	—	880	60	—
Ovary carcinoma	110	80	14	57	840	760	68	62

Cysteine, tyrosine, tryptophan, cystine, glutamic acid and total nitrogen content of several extracts of human gastric mucosa from ulcer patients, of intestinal carcinoma, of intestinal mucosa near the tumor, and of carcinoma of the ovary.
The per cent inhibition of the catalase activity for 1 ml of the same extracts is recorded and compared with the inhibitions observed with 1 ml of the corresponding mixtures of amino acids.

In the single extracts also the total nitrogen was determined by nesslerization (Table I).

An ultraviolet spectrum between 230 and 320 mμ was obtained for each extract, after dilution with $N/10$ NaOH to a concentration corresponding to 20 mg/ml of wet organ. The shapes of the curves obtained were identical for all the extracts studied, and showed a sharp maximum at 260 mμ. The intensity of absorption at this wavelength, however, differed very greatly from one extract to the other, independently of their anticatalase activity. The shape of the curve, the position of its maximum and the low content in aromatic amino acids, especially considering the high dilutions used, suggest that this absorption is due to purine and pyrimidine bases.

Action of amino acid mixtures on the catalase activity

The analysis of the different organ extracts as to their content in cysteine, tyrosine, tryptophan, cystine and glutamic acid, enabled us to reconstitute partially these extracts with pure amino acids, in order to test their anticatalase activity.

The mixtures were made with neutralized amino acid solutions and were diluted with phosphate buffer 0.01 M at pH 6.8.

The inhibition data obtained by addition of 1 ml of the different mixtures to the catalase solution are compared with those exibited by the same amount of the corresponding extracts (Table I). As can be seen from the table, the inhibition obtained with the amino acid mixtures generally reaches about 60%, that of the corresponding extracts about 80–85%.

However, the reconstitution of the extracts was only partial because only five amino acids were taken into account. There are many other less-inhibiting amino acids which could also be present in rather large amounts, as the high total nitrogen content of the extracts seems to indicate.

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DISCUSSION

It is rather difficult at present to explain how different amino acids may exert an analogous inhibiting activity on catalase.

In the case of cysteine, glutathione and the ovomucoid[16] the action has been attributed to the presence of the SH group. Inhibition of catalase has in fact also been observed with H_2S, which combines with the haem group [17], with thioglycolic acid and with thiourea[8]; it is likely that this action is due to the formation of an inactive complex. The formation of this complex, as shown by activity measurements of catalase after addition of cysteine, at first proceeds rapidly, then the curve flattens out and is dependent on the ratio of cysteine to catalase[8]. We observed the feature on adding cysteine as well as the other inhibiting amino acids to catalase.

It seems therefore reasonable to assume that each of these amino acids may form an inactive complex with catalase. Competitive equilibria may exist among the various amino acids for the point of attachment to the enzyme molecule, depending both on their different affinities and on their different concentrations.

The interference between catalase and some amino acids could have some bearing on the metabolism of the latter; in fact, as has been demonstrated very recently[18,19], catalase enters into the metabolism of tyrosine by making possible, in conjunction with another unidentified protein, the transformation of *p*-hydroxyphenylpyruvic to homogentisic acid.

SUMMARY

The nature of the factor, present in tumors and normal organs extracts, that inhibits catalase *in vitro* has been investigated. Evidence has been presented for the plurality of the inhibiting agents. These have been identified as several amino acids by direct determination of the anticatalase activity of amino acids, by analysis of extracts and by the partial reconstitution of these extracts with pure amino acids. The mechanism of this interference between amino acids and catalase and its possible significance have been briefly discussed.

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