Enzyme Inhibition by Allicin, the Active Principle of Garlic

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Extracts of garlic (*Allium sativum*) have been used for medicinal purposes for several thousand years. Their use is described by Virgil in the *Second Idyll* as a treatment for snake bite and by Hippocrates for treating pneumonia and suppurating wounds. The use of garlic extracts for treating such ailments as gastric catarrh, dysentery, typhoid and cholera has continued in many parts of the World. Recently garlic extracts have been claimed to be more satisfactory than penicillin for the treatment of some throat infections (Fortunatov, 1952).

A scientific basis for the medicinal use of garlic extracts was established by Lehmann (1930), who demonstrated that the growth of *Eberthella typhosa* and *Escherichia coli* was abolished by the extracts. The true nature of the active principle, however, remained obscure until Cavallito & Bailey (1944), by steam distillation of ethanolic garlic extracts, obtained a colourless strong-smelling oil which possessed a powerful bactericidal action in concentrations as low as 1:85000, against a wide range of bacteria. This oil, called allicin, was diallylthiosulphinic acid (II) (Cavallito, Buck & Suter, 1944).

Stoll & Seebeck (1948), however, showed that the undamaged garlic bulb contained, not allicin, but a related substance, alliin, which was converted into allicin enzymically when the bulb was crushed. Alliin has no bactericidal action. Stoll & Seebeck (1951) showed by synthesis that alliin was *S*-allyl-L-cysteine *S*-oxide (I). Stoll & Seebeck (1949*a*) formulated its conversion into allicin as

$$\underset{\text{(I)}}{2R.SO.CH_2.CH(NH_2).CO_2H} \xrightarrow[\text{Alliinase}]{+H_2O} \underset{\text{(II)}}{R.SO.S.R} + 2CH_3.CO.CO_2H + 2NH_3$$

$$(R = -CH_2.CH{:}CH_2)$$

Garlic extracts inhibit alkaline phosphatase and invertase (Danilenko & Epshtein, 1953), urease and succinic dehydrogenase (Szymona, 1952), and blood peroxidase (Vinokurow, Bronz & Korsak, 1947), but do not affect carboxylase and cytochrome oxidase (Szymona, 1952), or amylase and pepsin (Vinokurow *et al.* 1947). Invertase activity of red-clover extracts is, however, stated to be increased by garlic extracts (Morozow, 1950). Allicin inhibits urease (Agarwala, Murti & Shrivastava, 1952), papain and amylase (Rao, Rao & Venkataraman, 1946). No study of the effect of alliin on enzymes appears to have been made.

The object of the present work has been, by the use of pure synthetic alliin, to establish whether enzyme inhibition observed with garlic extracts can be attributed to alliin or to allicin and to examine the possibility that the antibacterial action of allicin could be ascribed to enzyme inhibition. Twenty-eight enzymes have been tested, in the presence of various concentrations of alliin and allicin. The effect on a few enzymes of some substances with a structure related to allicin has also been examined, in an attempt to establish the mechanism of action of allicin.

EXPERIMENTAL

Materials

DL-*Alliin*. This synthetic product was a gift from Sandoz Ltd.

Allicin. This was prepared enzymically by incubating at 37° for 15 min. an alliin solution of the required strength with an equal volume of alliinase solution (see below). Allicin was synthesized as described by Small, Bailey & Cavallito (1947).

Diallyl sulphide (L. Light and Co. Ltd.). This was redistilled *in vacuo* before use.

Diallyl disulphide. This was synthesized from allyl chloride as described by Twiss (1914). It was distilled at 76° at 11 mm.

Diallyl sulphoxide. This was synthesized as described by Stoll & Seebeck (1948).

Enzyme preparations and activity measurements

The methods of preparation and estimation of activity for most of the enzymes used have been previously described (Wills & Wormall, 1950; Wills, 1954).

Alliinase. This enzyme was prepared by the method of Stoll & Seebeck (1949*a*) with the modification that the ammonia used for neutralization was replaced by NaOH since traces of ammonia interfere with the estimation of the enzyme activity. The enzyme extracted from 25 g. of garlic was finally made up in 25 ml. of 0·1M phosphate buffer, pH 6·0.

The ammonia produced by alliinase action was estimated in a Conway vessel with 0·25 M phosphate buffer, pH 6·0 (0·5 ml.), 0·02 M alliin solution (0·5 ml.), water (0·5 ml.) and alliinase solution (0·5 ml.) in the outer compartment and 1% (w/v) boric acid containing mixed indicator (1 ml.) in the inner compartment. The vessel was incubated at 37° for 15 min., when a saturated solution of K_2CO_3 (1 ml.) was run into the outer compartment. The vessel was incubated for a further 60 min. at 37° and the boric acid finally titrated with 0·0059 N-HCl.

Succinic dehydrogenase. This was estimated as previously described (Wills & Wormall, 1950) and also, with a 10% (w/v) homogenate of rat liver, by the colorimetric method of Kun & Abood (1949).

Papain. This was estimated by a milk-clotting method already described (Wills & Wormall, 1950), and as previously described for trypsin (Wills, 1954).

Xanthine oxidase. (*a*) *From milk.* Preparations used were untreated cream, a cream extract in phosphate buffer described by Horecker & Heppel (1949) and samples of stages in the preparation of crystalline xanthine oxidase, which were kindly supplied by Professor F. Bergel. These were samples M1, M2, M5 and M7 (crystalline enzyme) as described by Avis, Bergel & Bray (1955). (*b*) *From liver.* This was prepared from rat liver either as described by Axelrod & Elvehjem (1941), or by I. Lewin (private communication). The enzyme activity was estimated manometrically with hypoxanthine as substrate, as previously described (Wills & Wormall, 1950).

Triose phosphate dehydrogenase. The enzyme source and method of estimation were as described by Mackworth (1948).

Lactic dehydrogenase. A 5% (w/v) aqueous extract of acetone-dried powder of rabbit muscle was used. Enzyme activity was measured by the Thunberg method with 0·2 M phosphate buffer, pH 7·4 (0·5 ml.), water (0·5 ml.), 0·1% (w/v) coenzyme I (Co I) solution (0·5 ml.) and enzyme solution (0·5 ml.) in the main tube, and 0·005% (w/v) methylene blue (0·2 ml.) and 2% (w/v) sodium lactate (0·2 ml.) in the side bulb. The tube was evacuated, warmed to 37° and the contents were mixed. The time for decolorization of the methylene blue was observed.

Alcohol dehydrogenase. (*a*) *From yeast.* This was prepared from dried yeast by the first stages of the method described by Racker (1950). (*b*) *From liver.* This was prepared as described by Lutwak-Mann (1938). The Thunberg method was used for the estimation.

Alkaline phosphatase. Ox or rabbit serum was used as a source of this enzyme. It was estimated by the method of King (1951).

Adenosine triphosphatase. An extract of rat skeletal muscle or cardiac muscle was used and its activity was estimated by the method of Dubois & Potter (1943).

RESULTS

Alliinase action and allicin production

It has been previously shown that natural L-alliin is completely and rapidly converted into allicin, ammonia and pyruvic acid by alliinase. The enzyme was, however, optically specific and did not attack the D-isomer, so that only 50% of *S*-allyl-DL-cysteine sulphoxide was converted into allicin even after incubation for 80 min. (Stoll & Seebeck, 1949*b*).

These results have been confirmed with DL-alliin. When the alliinase preparation was mixed with an equal volume of 0·05 M alliin solution, 50% of the alliin was converted into allicin within 5 min. and no more allicin or ammonia was formed during periods up to 90 min. Allicin is unstable and was therefore prepared just before use by incubating at 37° for 15 min. the calculated quantity of alliin solution with an equal volume of alliinase. The preparation contained, in addition to allicin, unchanged D-alliin, alliinase, ammonia and pyruvic acid, so that for study of the effect of allicin on the enzymes, control experiments with equivalent or greater concentrations of alliin, alliinase, ammonia and pyruvic acid were necessary.

Effect of alliin, allicin and alliinase on enzymes

Alliin (0·0057 M) and alliinase (0·3 ml. of the standard preparation) when tested separately had no effect on the succinic oxidase system. When, however, they were incubated together the allicin produced powerfully inhibited the succinic oxidase system; the inhibition produced by 0·00029 M allicin was approximately equivalent to that produced by a solution obtained by extracting garlic with 50 times its weight of water (Fig. 1).

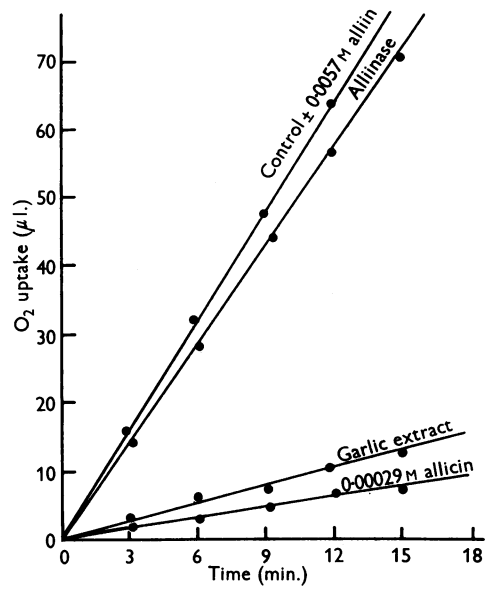

Fig. 1. Comparison of the effects of alliin, allicin, alliinase and crude garlic extracts on succinic oxidase. Alliinase: the preparation described in the text diluted 10 times. Garlic extract: a solution obtained by extracting garlic with 50 times its weight of water. Substrate: 0·11 M sodium succinate.

The inhibition of the succinic oxidase system was most likely due to inhibition of succinic dehydrogenase since cytochrome oxidase was unaffected, and succinic dehydrogenase activity, measured by the colorimetric method of Kun & Abood (1949), was strongly inhibited.

Neither alliin (0·002 M) nor alliinase affected to any measurable extent any other enzyme tested. The enzymes tested in presence of allicin, however, were divisible into the two groups of Table 1 depending on whether they were inhibited or not by 0·0005 M allicin. Table 1 shows that allicin inhibits sulphydryl enzymes, and that very few members of this class of enzyme tested were unaffected. Some enzymes listed as inhibited in Table 1 were only mildly inhibited by allicin; thus 0·001 M allicin caused only 45% inhibition of tyrosinase action. The three enzymes in Table 2 were, however, extremely sensitive to allicin in very dilute solutions. The concentration of allicin used in these experiments is comparable to that ($4·5 \times 10^{-5}$ M) found by Cavallito & Bailey (1944) to inhibit completely the growth of many bacteria.

Table 1. *Summary of the effect of* 0·0005 M *allicin on some enzymes*

Enzymes inhibited	Enzymes unaffected
*Succinic dehydrogenase	Cytochrome oxidase
*Urease	Lipase
*Papain	Rennin
†Xanthine oxidase (cream)	Pepsin
†Xanthine oxidase (liver)	Trypsin
*Choline oxidase	Invertase
*Hexokinase	α-Amylase
*Cholinesterase	Esterase (serum)
*Glyoxylase	D-Amino acid oxidase
*Triose phosphate dehydrogenase	Ascorbic acid oxidase
	Catalase
*Alcohol dehydrogenase	Carbonic anhydrase
Lactic dehydrogenase	*Carboxylase
Tyrosinase	*Adenosine triphosphatase
Alkaline phosphatase	*β-Amylase

* SH enzymes. † Possibly SH enzymes.

Table 2. *Enzymes strongly inhibited by allicin*

	Substrate concn. (M)	Allicin concn. (10^{-5} M)	Inhibition (%)
Succinic dehydrogenase	0·11	33·3	90
	0·11	14·3	85
	0·11	5·6	50
Triose phosphate dehydrogenase	0·01	10·0	100
	0·01	5·0	87
	0·01	2·0	40
Xanthine oxidase (in cream) (pH 6·4)	0·005	20·0	95
	0·005	10·0	71
	0·005	5·0	36

Effect of allicin on xanthine oxidase

The xanthine oxidase activity of cream was powerfully inhibited by allicin (Table 2). Xanthine oxidase may be an SH-enzyme (Mackworth, 1948; Harris & Hellerman, 1953) and thus fit into the general pattern. Inhibition of cream xanthine oxidase by allicin was, however, dependent on pH as shown in Fig. 2, which shows that 0·0001 M allicin completely inhibited the enzyme at pH 5·5 but had practically no effect at pH 8·2. This pH effect was the reverse of that observed with urease,

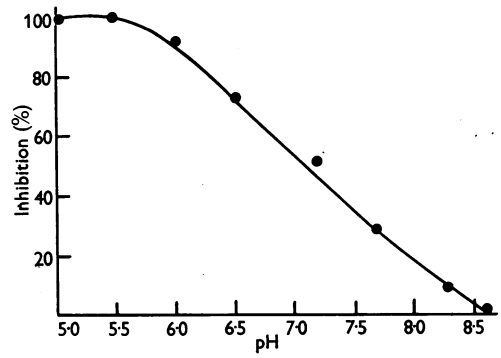

Fig. 2. Effect of pH on the inhibition of xanthine oxidase (cream) by 0·0001 M allicin. Substrate: 0·005 M hypoxanthine.

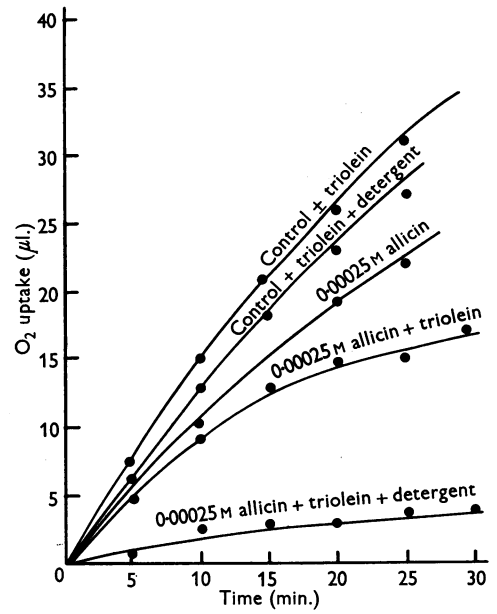

Fig. 3. Effect of allicin, triolein and a detergent on purified xanthine oxidase (Fraction M5). Triolein: 0·1 ml./3·0 ml. of total aqueous phase. Detergent: 3% (w/v) Lubrol M.O. Substrate: 0·005 M hypoxanthine.

when inhibition by allicin increased with increasing alkalinity (Fig. 5).

Although allicin inhibited oxygen uptake by xanthine oxidase it did not affect the dehydrogenase activity as measured by the methylene-blue technique. In addition, methylene blue protected xanthine oxidase against inhibition by allicin of oxygen uptake. Thus 0·0167 M methylene blue completely abolished inhibition by 0·0001 M allicin, and the protective action was still detectable with 0·0001 M methylene blue.

When cream extracted with phosphate buffer was used as a source of xanthine oxidase, inhibition was produced by allicin but the inhibitory effect was less than that observed with cream. The buttermilk (M 1), filtrate enzyme (M 2) and crystalline enzymes (M 7) of Avis *et al.* (1955) were practically unaffected by 0·0001 M allicin at any pH tested. This was due to the complete absence of fat in these preparations. The addition of a small quantity of triolein produced a marked increase in the inhibitory effect, and this was enhanced if the enzyme and triolein were first emulsified with a detergent before addition of the allicin (Fig. 3). The xanthine-oxidase activity of rat liver was also completely inhibited by 0·00025 M allicin.

Effect of allicin on yeast and tissue respiration

Yeast fermentation of glucose, the respiration of yeast in the presence of glucose and the endogenous respiration of rat-liver homogenate and rat-muscle homogenate were all inhibited by allicin but unaffected by alliin. Thus 0·00025 M allicin caused 43 % inhibition of yeast respiration and 50 % inhibition of the respiration of rat-muscle or liver homogenate.

Effect of garlic extracts on enzymes

Aqueous extracts of crushed garlic bulb were tested on urease, succinic oxidase, amylase, trypsin and lipase. Garlic was extracted with 5 times its own weight of water, and the juice was filtered. Succinic oxidase was completely inhibited by this extract diluted a further 10 times and urease was 67 % inhibited. Trypsin, amylase and lipase were unaffected. The pattern of the effect of crude garlic extract on these enzymes was thus parallel to the effect of allicin. These results are in agreement with those of Szymona (1952) and Vinokurow *et al.* (1947).

Protection of enzymes against inhibition by allicin

Various compounds were tested for their ability to protect succinic dehydrogenase and urease against inhibition by allicin. The sulphydryl compounds cysteine, glutathione and BAL (2:3-dimercaptopropanol) were good protective agents, and serum had a weak protective action. Alanine, glycine, glutamic acid, arginine, histidine, phenylalanine, gelatin, native or denatured ovalbumin, and serum albumin had no effect. Cysteine (0·01 M) completely protected succinic oxidase against 0·00025 M allicin if mixed with the allicin before addition of enzyme, and 0·001 M, but not 0·0001 M, cysteine completely protected against 0·00014 M allicin. Glutathione protected to an approximately equal extent when used in the same concentrations as the cysteine. If, however, the allicin had had prior contact with the enzyme for even a short time, cysteine or glutathione restored only a small fraction of the enzymic activity.

Combination of allicin with these sulphydryl enzymes was extremely rapid. Thus 0·00025 M allicin produced 81 % inhibition of succinic oxidase if added with the substrate, and 92 % inhibition if the enzyme was in contact with allicin for 14 min. before addition of substrate. Prolonged periods of contact between allicin and enzyme did not significantly increase the inhibition. Similar results were obtained for urease by Agarwala *et al.* (1952). Addition of glutathione or cysteine to succinic oxidase at different times after addition of allicin showed that some regeneration of enzyme activity was obtainable after short contact periods, but little activity was recoverable after enzyme and allicin had been in contact for 15 min. (Fig. 4).

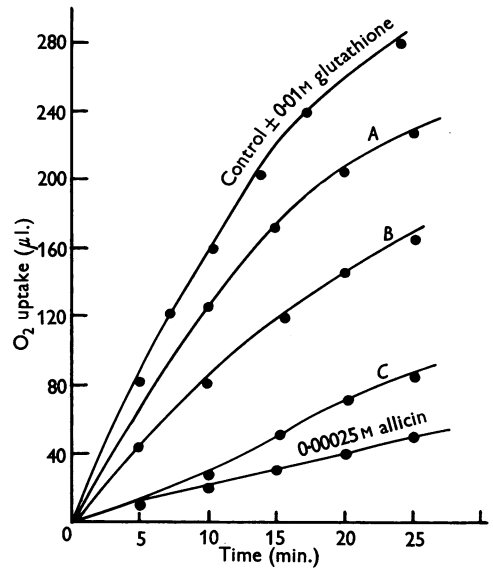

Fig. 4. Effect of glutathione (0·01 M) on the inhibition of succinic oxidase by allicin (0·00025 M). *A*, glutathione mixed with enzyme before addition of allicin; *B*, glutathione added after enzyme and allicin had been in contact for 5 min.; *C*, glutathione added after enzyme and allicin had been in contact for 15 min. Substrate: 0·11 M sodium succinate.

The effect of pH on the inhibition of urease by allicin and on the protective effect of 0·002 M cysteine was investigated. The extent of inhibition by 0·00025 M allicin decreased slightly as the pH decreased from pH 7·0 to 5·0, but the protection by 0·002 M cysteine, which was complete at pH values greater than 6·5, was much reduced at pH 5·0 (Fig. 5).

Effect of compounds related to allicin on enzymes

Urease and succinic oxidase were used for testing the effect of several compounds with a structure related to that of allicin. Results obtained with succinic oxidase (Table 3) showed that allicin was a very powerful inhibitor but that diallyl disulphide, diallyl sulphide or diallyl sulphoxide had no effect under the experimental conditions.

Synthetic allicin

Synthetic allicin inhibited urease and succinic oxidase to approximately the same extent as did allicin prepared enzymically.

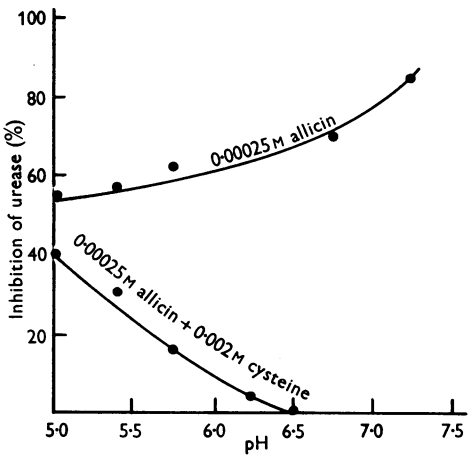

Fig. 5. Effect of pH on the inhibition of urease by 0·00025 M allicin and on the effect of the addition of 0·002 M cysteine. Cysteine and allicin were in contact for 5 min. at specified pH before addition of urease. Substrate: 0·004 M urea.

DISCUSSION

The experiments described leave little doubt that enzyme inhibition observed with crude garlic extracts is caused by allicin. Table 1 shows that with few exceptions SH-enzymes were inhibited and that most other enzymes were not affected. The cause of the inhibition of the enzymes which are not normally regarded as SH-enzymes, and the nature of the groups in the enzyme molecule attacked by allicin, are not yet clear. Rao *et al.* (1946) found that sweet potato β-amylase was 7% inhibited by 0·00031 M allicin after contact for 5 min. but that the inhibition increased to 26% after contact for 30 min. In the present work no inhibition of unpurified malt β-amylase by 0·0005 M allicin could be detected after contact for 15 min. A likely interpretation of these experiments is that the SH groups of β-amylase are much less accessible to or reactive with allicin than are those of succinic dehydrogenase, where maximum inhibition is rapidly obtained. A similar explanation is probable for the failure of allicin to inhibit carboxylase and adenosine triphosphatase, which are usually regarded as sulphydryl enzymes (Barron, 1951).

Cavallito & Bailey (1944) found that 1:125 000 ($\simeq 4{\cdot}5 \times 10^{-5}$ M) allicin completely inhibited the growth of fourteen out of fifteen strains of bacteria tested, but that half that concentration had no effect on any strain tested. In the present work powerful inhibition of sulphydryl enzymes was observed with $5{\cdot}6 \times 10^{-5}$ M allicin for the succinic oxidase system, 2×10^{-5} M for triose phosphate dehydrogenase and 5×10^{-5} M for xanthine oxidase. It is therefore clear that some vital oxidation enzymes are inhibited in a concentration that would adequately explain the bactericidal action of allicin.

Small *et al.* (1947) have, by the synthesis of a series of alkylthiosulphinates of general formula R–SO–S–R, demonstrated that the –SO–S– group was essential for bactericidal action. The results of Table 3 show that the –SO–S– grouping is also essential for inhibition of the succinic-oxidase system and that the –S–S–, –S– and –SO– groupings were not effective. Similar results were obtained

Table 3. *Comparison of the effect of allicin and related compounds on the succinic oxidase system*

Enzyme and compound in contact for 30 min. before start of experiment. Sodium succinate (0·11 M) was used as the substrate in each experiment.

	Formula (R = $-CH_2.CH{:}CH_2$)	Concn. (mM)	Inhibition (%)
*Diallyl sulphide	R.S.R	3·3	0
*Diallyl disulphide	R.S.S.R	3·3	0
Diallyl sulphoxide	R.SO.R	3·3	0
Alliin	$R.SO.CH_2.CH(NH_2).CO_2H$	4·0	0
Allicin	R.S.SO.R	0·14	84

* Used as a suspension.

with urease. There is thus a close relationship between the grouping essential for bactericidal action and that for enzyme inhibition. These authors also observed that increasing the length of chain in the R group increased the activity of the thiosulphinate against Gram-positive organisms, and thus, for example, the diallyl compound, allicin, was more effective than the dimethyl derivative. They suggested that the increased length of the R chain rendered the compound more lipid-soluble, and the thiosulphinate, partially dissolved in the lipid portion of a lipoprotein, would thus more effectively attack the protein SH groups. This hypothesis receives strong support from the results of the present experiments with xanthine oxidase. This enzyme was practically unaffected by allicin when purified and free from fat, but it was powerfully inhibited in the natural state when absorbed by the liposomes of the milk. The purified enzyme was inhibited when added to fat artificially emulsified. This is a rare example of an enzyme which is more powerfully inhibited in the crude than in the purified state. In the case of xanthine oxidase in cream the fat is likely to act by dissolving the allyl groups and bringing the allicin into close proximity with the enzyme.

Cavallito (1946) has classified bactericidal agents attacking SH compounds into three groups, depending on their specificity. Members of the first group, which attack nearly all sulphydryl compounds, react rapidly with the SH group of cysteine and cysteine peptides. Allicin is a member of this group. The second, and third group which includes penicillin, are more specific and only attack some SH groups. Cavallito *et al.* (1944) concluded that the main product of the action of cysteine on allicin was *S*-thioallylcysteine:

$$C_3H_5.S.S.CH_2.CH(NH_2).CO_2H.$$

Rao *et al.* (1946) state that *S*-thioallylcysteine is quantitatively formed. Similar combination of allicin might occur with enzyme SH groups, and the difficulty of reversing the inhibition is in accord with this possibility. On the other hand, the labile oxygen atom of allicin may simply oxidize the SH groups to –S–S–. The effect of pH on the inhibition of urease by allicin and the protective action of cysteine shows that inhibition of urease, although decreasing with increasing acidity, does so much less than does the protection by cysteine (Fig. 5). A probable interpretation of these results is that the SH group of cysteine is much less readily attacked than are the SH groups of urease as the pH is progressively lowered. The effect of pH on the inhibition of xanthine oxidase by allicin probably bears no relation to the case of urease, and is likely to be connected with the effect of pH on the state of the lipoprotein complex of the cream.

SUMMARY

1. All enzyme inhibitory effects observed with garlic extracts may be explained by the presence of allicin in them.

2. Alliin (0·002 M) did not inhibit any enzyme tested, but 0·0005 M allicin inhibited nearly all sulphydryl enzymes but very few non-sulphydryl enzymes tested. Some enzymes, such as triosephosphate dehydrogenase, were inhibited by 0·00005 M allicin.

3. Sulphydryl enzymes could be protected by cysteine or glutathione against inhibition by allicin, but only partial recovery of enzyme activity was obtainable after the allicin and enzyme had been in contact for a short period.

4. Inhibition of sulphydryl enzymes has been shown to be associated with the presence of the –SO–S– grouping and not the –SO–, –S–S– or –S– groups.

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Pathways of Glucose Catabolism in Rat Liver in Alloxan Diabetes and Hyperthyroidism

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A previous publication (Glock & McLean, 1955) reported striking alterations in levels of activity of enzymes of the hexose monophosphate oxidative pathway of glucose metabolism both in alloxan diabetes and hyperthyroidism. It was found that levels of activity of both glucose 6-phosphate and 6-phosphogluconate dehydrogenases were markedly reduced in the livers of diabetic rats and greatly increased after thyroxine treatment. In an attempt to substantiate these findings and to assess the relative importance of glycolytic and non-glycolytic pathways of carbohydrate metabolism, the utilization of [1-^{14}C]glucose and [6-^{14}C]glucose by liver slices from normal, diabetic and hyperthyroid rats has been determined by measuring the conversion of these labelled substrates into $^{14}CO_2$. In addition to these isotope measurements, the levels of activity of glucose 6-phosphatase and phosphoglucose isomerase have also been determined, since the former is one of the factors regulating the availability of glucose 6-phosphate, the common substrate for both metabolic pathways, whilst striking alterations in activity of phosphoglucose isomerase might be expected to influence the proportion of glucose 6-phosphate metabolized via the glycolytic route.

EXPERIMENTAL

Materials

D-*Glucose 6-phosphate* (*G 6-P*). This was a preparation of the heptahydrate of the barium salt obtained from Sigma Chemical Co.

6-*Phosphogluconate* (*6-PG*). This was a preparation of the barium salt used previously (Glock & McLean, 1953).

[1-^{14}C]*Glucose and* [6-^{14}C]*glucose*. [1-^{14}C]Glucose was obtained from the Radiochemical Centre, Amersham, and [6-^{14}C]glucose from Dr H. S. Isbell, National Bureau of Standards, Washington.

Methods

Estimation of G 6-P dehydrogenase and 6-PG dehydrogenase activities. These were determined spectrophotometrically by following the rate of reduction of triphosphopyridine nucleotide (TPN) at 340 mμ. in 1 cm. cells in a Hilger Uvispek spectrophotometer as described previously (Glock & McLean, 1953). In the assay of G 6-P dehydrogenase activity, the reaction mixture consisted of 0·5 ml. of 0·25 M glycylglycine (pH 7·6), 0·5 ml. of 0·1 M-$MgCl_2$, 0·1 ml. of 6-PG dehydrogenase prepared from rat liver by fractionation with $(NH_4)_2SO_4$, followed by dialysis (Glock & McLean, 1953), 0·1 ml. of liver supernatant and 0·2 mg. of TPN in a total volume of 2·4 ml. The reaction was started by the addition of 0·1 ml. of 0·05 M G 6-P to both cells, the blank being devoid of TPN. In the assay of 6-PG dehydrogenase activity, the $(NH_4)_2SO_4$ liver fraction was omitted, G 6-P was replaced by 0·1 ml. of 0·05 M 6-PG and the reaction was carried out in glycylglycine buffer at both pH 7·6 and pH 9·0. A unit of enzyme activity is defined as the quantity of enzyme which reduces 0·01 μmole of TPN/min. at 20°.

Estimation of glucose 6-phosphatase activity. This was determined according to Duve, Pressman, Gianetto, Wattiaux & Applemans (1955). The incubation mixture contained 0·04 M G 6-P, 0·007 M histidine, 0·001 M ethylenediaminetetraacetate (pH 6·5) and 0·2 ml. of 10% (w/v) liver homogenate in a total volume of 1 ml. After incubation for 10 min. at 37°, the reaction was stopped by the addition of an equal volume of 10% trichoroacetic acid, and inorganic P was determined in the filtrate by the method of Fiske & Subbarow (1925). A unit of enzyme activity is defined as the quantity of enzyme which hydrolyses 1 μmole of G 6-P/min. at 37°.

Estimation of phosphoglucose isomerase activity. This assay was based on that described by Slein (1955), for muscle phosphoglucose isomerase, fructose 6-phosphate formation being determined colorimetrically by the method of Roe (1934), which gives approximately 65% of the colour of free fructose. The incubation mixture contained 4 μmoles of G 6-P, 20 μmoles of glycylglycine (pH 7·6), and 0·1 ml. of 0·1% liver homogenate in a total volume of 0·5 ml. The homogenate was prepared in ice-cold isotonic KCl (0·15 M)