CXLIX. THE ACTION OF POLYHYDRIC PHENOLS ON UREASE; THE INFLUENCE OF THIOL COMPOUNDS.

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It has been reported by the writer [1932, 1], in a discussion on enzymes, that urease is poisoned by exceedingly minute quantities of polyhydric phenols and is completely protected from such poisoning by thiol compounds. This paper is concerned with a description of these phenomena.

Technique.

The experimental method adopted was that described in a previous communication [Quastel, 1932, 2] on the effects of dye-stuffs on urease. Briefly it consisted in exposing urease, prepared from soya bean or jack bean, to a dilute solution of a polyhydric phenol, at 45° and p_H 7·4, for 1 hour. Urea was then added to the mixture, and the ammonia formed at the termination of a subsequent hour's incubation at 45° was estimated by a suitable aeration method. The amount of enzyme usually taken was just sufficient to decompose 0·03 g. urea in 1 hour at 45° in the presence of phosphate buffer at an initial p_H of 7·4, *i.e.* the amount of $N/10$ ammonia liberated by the enzyme under these circumstances was 10 cc. Preliminary experiments were performed to determine the activity of any urease preparation, and the appropriate quantity was taken to fulfil the above conditions.

Urease from soya bean was prepared by shaking 1 part of soya bean meal with 9 parts of water for 30 minutes, centrifuging and diluting the centrifugate until the preparation had the desired enzymic activity. On certain occasions the enzyme was purified by passing CO_2 through the preparation so that proteins *etc.* were precipitated. Urease from jack bean was prepared in a similar manner, the amount of dilution of centrifugate being much greater (approximately 15-fold) to obtain a preparation of the same activity. A solution of crystalline urease was obtained from jack bean by Sumner's method. Solutions or suspensions of the enzyme were stored at 0° and frequently examined for change of activity. Fresh preparations were made as soon as any appreciable loss of activity occurred.

In a typical experiment 1 cc. of the urease preparation was added to 2 cc. $M/5$ phosphate buffer solution p_H 7·4. The phenol and any other substance[1] under investigation were added, and the volume was made up to 9 cc. with distilled water. The mixture was incubated at 45° for 1 hour. 1 cc. 3 % urea was then added and the incubation allowed to proceed at 45° for a further hour. At the

[1] All solutions of substances added to the enzyme were brought to p_H 7·4 at the commencement of the experiment.

end of this period the free ammonia was estimated on 1 cc. and compared with that obtained from urease treated under similar conditions but in the absence of the phenol (*i.e.* with a control experiment).

The toxic action of polyhydric phenols.

Representative results showing the effects of a number of polyhydric phenols, each at a final concentration of 1/10,000, on the activity of jack bean urease are recorded in Table I. The percentage inhibitions of the activity of the enzyme are given.

Table I. *Percentage inhibitions of activities of urease (jack bean) by polyhydric phenols 1/10,000.*

Catechol	98
Resorcinol	2
Quinol	99
Pyrogallol	96
Phloroglucinol	0
Gallic acid	96
Adrenaline	94
Protocatechuic acid	51
Protocatechuic aldehyde	15

The following facts are of note:

(1) Of the three dihydric phenols, resorcinol exhibits no inhibition of urease activity (within the experimental error). Catechol and quinol, on the other hand, are extremely toxic.

(2) Phloroglucinol is inactive, but pyrogallol is highly toxic.

(3) The inhibitory action of catechol is decreased by the introduction into the molecule of a —COOH group (protocatechuic acid) or a —CHO group (protocatechuic aldehyde).

Examination of Table II, in which the percentage inhibitions of the activity of jack bean urease by various concentrations of polyhydric phenols are noted, shows that catechol and quinol exert highly toxic actions at a concentration of 1 part in a million. Pyrogallol proves to be less toxic than catechol or quinol.

Table II. *Percentage inhibitions by various concentrations of polyhydric phenols (jack bean urease).*

	$1/10^4$	$1/10^5$	$1/10^6$
Catechol	98	96	80
Quinol	99	95	80
Resorcinol	2	1	0
Gallic acid	96	57	10
Protocatechuic acid	51	11	0
Pyrogallol	96	91	31

The behaviour of adrenaline is of some interest. This substance is highly toxic to urease, but the toxicity varies greatly with the purity of the enzyme. For example, whereas adrenaline at a concentration of 1/10,000 effected 92 % inhibition of the activity of a jack bean preparation of urease, it only produced 40 % inhibition of a soya bean preparation of the same enzymic activity. The latter preparation contained a greater quantity of impurities in the form of proteins *etc.* than the jack bean preparation.

Similarly adrenaline at a concentration of 1/20,000 effected 96 % inhibition of the activity of a crystalline urease preparation and 36 % inhibition of activity of a jack bean preparation of the same enzymic strength.

This behaviour is very similar to that of many dyestuffs [Quastel, 1932, 2], whose toxicities towards urease are less in the presence of proteins than in their absence.

Catechol and quinol, however, even at a concentration of 1/100,000 are as toxic to soya bean as to jack bean preparations of the same enzymic content, which indicates the very powerful affinity of certain chemical groupings in urease for these dihydric phenols.

The influence of thiol compounds on the toxicity of polyhydric phenols.

If a thiol compound be added to a mixture of urease and a toxic phenol (at p_H 7·4), the inhibitory action of the latter is either diminished or eliminated. Such thiol compounds are cysteine, glutathione, thiolacetic acid and H_2S. Sodium hydrosulphite is also very effective. Illustrative results are shown in Table III.

Table III. *Percentage inhibitions of urease activity (jack bean) by catechol (1/10,000) in presence of various sulphur compounds (at* p_H *7·4).*

Sulphur compound	Concentration %	Percentage inhibition
None	—	95
Cysteine	0·05	0
Glutathione	0·05	33
Thiolacetic acid	0·05	0
Dithiodiglycollic acid	0·05	93
Sodium hydrosulphite	0·02	18
H_2S water*	—	0

* Prepared by passing H_2S through distilled water for 5 minutes. 1 cc. of this was added to the mixture of enzyme and catechol, the final volume being 10 cc.

The percentage inhibition of urease activity effected by catechol or quinol decreases with increasing concentration of thiol compound present. Typical results are shown in Table IV.

Table IV. *Variation in percentage inhibitions of urease (jack bean) activity by catechol or quinol in the presence of varying concentrations of thiolacetic acid*.*

Concentration of catechol *M*	Concentration of thiolacetic acid *M*	% inhibition of activity
$1·8 \times 10^{-5}$	—	84
,,	$1·1 \times 10^{-5}$	66
,,	$2·7 \times 10^{-5}$	45
,,	$5·4 \times 10^{-5}$	12
Concentration of quinol *M*		
$1·8 \times 10^{-5}$	—	97
,,	$1·1 \times 10^{-5}$	76
,,	$2·7 \times 10^{-5}$	60
,,	$5·4 \times 10^{-5}$	22

* Present as the Na salt.

Similar results to those given in Tables III and IV may be obtained with soya bean urease and with a crystalline preparation of the enzyme. The toxicities of 1/10,000 concentrations of adrenaline, protocatechuic acid and gallic acid as well as those of catechol and quinol are entirely eliminated by 0·05 % thiolacetic acid. The oxidised form of this thiol compound—dithiodiglycollic acid—has no detoxicating action (Table III).

The action of potassium cyanide and of amino-acids.

It is well known that urease is poisoned by traces of metals, the toxicity being entirely removed by the presence of cyanide. Sumner has shown that the decrease in activity of crystalline urease in aqueous solutions is to be attributed to traces of metals (probably copper) in the water—the addition of a trace of cyanide to the water restores the full activity of the enzyme. Jacoby [1933] has recently made a study of the toxic actions of metals on urease and of the reactivating effects of cyanide and thiol compounds.

It seemed conceivable that the toxic effects of polyhydric phenols might be due to the presence of traces of metals. Recently the writer [1932, 2] has shown that hydroxylamine is highly toxic to urease but that the toxicity is entirely removed by the presence of 0·01 % potassium cyanide. Probably the hydroxylamine was contaminated with metals.

Investigations with catechol or quinol failed to show any reactivating action of potassium cyanide when this was added to a mixture of urease and the phenol. For example the activity of a crystalline urease preparation was destroyed to the extent of 97 % by the presence of 1/10,000 catechol. On addition of 0·01 % potassium cyanide the inhibition of activity became 96 %. When a much more dilute solution of catechol was used a very slight protective action of the cyanide was found, but with quinol as the toxic agent the writer has failed to find any protective action of cyanide (see Table V).

Table V. *Percentage inhibition of urease (jack bean) activity by catechol and quinol in presence of KCN and amino-acids.*

	Catechol 1/500,000	Quinol 1/100,000
Control	89	100
KCN (0·01 %)	78	100
Glycine (0·1 %)	61	100
Aspartic acid (1 %)	35	100

It appears very unlikely from these results that metallic impurities are responsible for the toxicity of catechol or quinol—a conclusion supported by the fact that purification of catechol by repeated sublimation failed to show an appreciable reduction in toxicity.

It has been shown by the writer [1932, 2] that amino-acids and amines afford protection to urease against the toxic effects of many dyestuffs. With very dilute solutions of catechol such protection can also be observed with glycine or aspartic acid, but the degree of protection is far smaller than in the case of the dyestuffs. Typical results are shown in Table V.

With a concentration of catechol and quinol of the order of 1/100,000 no protective action by amino-acids has been observed. Methylamine ($M/30$) which protects urease against the toxic activity of brilliant green (1/50,000) failed to show any protective action against 1/500,000 catechol.

These results show that the affinity of the toxic dihydric phenols for urease is far greater, under comparable conditions, than those of the most toxic dyestuffs yet investigated. This particularly high affinity is shown also in the fact that the presence of proteins such as those of egg-white or serum fail to protect urease from the toxic action of catechol (1/10,000).

It is, however, a fact of some interest that whereas egg-white does not protect urease (jack bean) against catechol, boiled egg-white is very effective.

E.g. % inhibition of urease by 1/500,000 catechol in presence of 1 cc. 10 % egg-white[1] is 89; % inhibition of urease by 1/500,000 catechol in presence of 1 cc. 10 % boiled egg-white is 7.

The most likely explanation of this phenomenon in view of the protective effects of thiol compounds already described is that the action of boiled egg-white is due to the liberated thiol groups.

Mode of action of catechol or quinol.

If the toxicities of catechol or quinol are due to their hydroxyl groups it is difficult to understand the mechanism of action of thiol compounds or of sodium hydrosulphite in producing a reactivation. Since the possibility of metallic compounds being responsible for the toxicity of the dihydric phenols is remote, it seems likely that the toxicity may be due to the presence of oxidation products of the phenols, these oxidation products being reduced by thiol compounds or sodium hydrosulphite to inert compounds. Two oxidation products suggest themselves: (*a*) hydrogen peroxide, (*b*) the quinone corresponding to the dihydric phenol.

(*a*) *Toxicity of hydrogen peroxide.* Hydrogen peroxide has a powerful inhibitory effect on the activity of urease, but a comparison of the toxicities of hydrogen peroxide and catechol at equivalent concentrations shows the latter to be the more toxic agent (see Table VI).

Table VI. *Percentage inhibition of urease (jack bean) activity by catechol, hydrogen peroxide and "hyperol".*

	Concentration *M*	% inhibition
Catechol	$1{\cdot}8 \times 10^{-5}$	85
Hydrogen peroxide	$1{\cdot}7 \times 10^{-4}$	28
,, ,,	$4{\cdot}4 \times 10^{-4}$	52
"Hyperol"	$2{\cdot}1 \times 10^{-4}$	39

"Hyperol" (an equimolecular compound of urea and hydrogen peroxide) is also less toxic than catechol. It follows that the toxicity of the latter cannot be due to the presence of traces of hydrogen peroxide in the aqueous solution of the dihydric phenol.

(*b*) *Toxicity of quinone.* On comparing the toxicities of quinone and quinol at equivalent concentrations it was found that the former had the greater inhibitory action (see Table VII).

Table VII. *Percentage inhibitions of urease activity by quinol and quinone.*

	Concentration	% inhibition
Quinol	1/2,500,000	64
Quinone	1/2,500,000	98
,,	1/5,000,000	57

Quinone reduced the activity of urease (jack bean), under the experimental conditions given, over 50 % at a concentration of 1 part in 5 millions. Considering this very high activity of quinone it is not unreasonable to assume that the toxicity of aqueous quinol solutions may be due to the presence of traces of quinone. Quinol, even at very high dilutions, may well give rise to concentrations of quinone of the order of 1 part in 5 millions, since exposure of the solutions

[1] Prepared by adding 1 cc. fresh egg-white to 9 cc. water.

to atmospheric oxygen takes place freely under the experimental conditions employed. Experiments under anaerobic conditions have been carried out with a view to determining whether quinol was less toxic under these conditions, but the results were not satisfactory and mostly negative, owing, it is believed, to the difficulty of removing all traces of oxygen and of obtaining a specimen of quinol which could be assumed at the outset to be free from traces of quinone.

If the toxicity of quinol or catechol is due to the presence of the corresponding quinone in the aqueous solution and the protective action of thiol compounds is due to reduction of the quinone, it would follow:

(*a*) That the toxicity of a mixture of catechol and hydrogen peroxide should be greater than the sum of the individual toxicities owing to the production (by interaction of the peroxide with catechol) of a quinone more toxic than the hydrogen peroxide.

(*b*) That the amount of thiol compound necessary to protect urease from the toxic action of quinone should be greater than that necessary to protect urease from the toxic action of an equivalent concentration of quinol.

These predictions were verified by experiment. The following are typical results.

1. *Toxicity of a mixture of catechol and "hyperol" towards soya bean urease (purified by CO_2 precipitation).*

(*a*) Percentage inhibition of activity due to 1/5,000,000 catechol 30

(*b*) Percentage inhibition of activity due to 1/50,000 "hyperol" 5

(*c*) Percentage inhibition of activity due to a mixture of 1/5,000,000 catechol and 1/50,000 "hyperol" ... 87

2. *Comparison of the protective actions of thiolacetic acid against the toxic effects of quinol and quinone (jack bean urease).*

(*a*) Percentage inhibition of activity due to 1/10,000 quinol 99

(*b*) Percentage inhibition of activity due to a mixture of 1/10,000 quinol and 1/20,000 thiolacetic acid[1] ... 12

(*c*) Percentage inhibition of activity due to 1/10,000 quinone 100

(*d*) Percentage inhibition of activity due to a mixture of 1/10,000 quinone and 1/20,000 thiolacetic acid 100

On increasing the thiolacetic acid concentrations in (*b*) and (*d*) to 1/5000 the percentage inhibitions fell to 3 % in (*b*) and 14 % in (*d*).

Discussion.

The evidence as a whole appears to be in favour of the view that the high toxicities of catechol and quinol towards urease are due to the presence of the corresponding quinones in the aqueous solutions of the dihydric phenols. The powerful protective effects of thiol compounds can then be explained as due to reduction of the quinones to the dihydric phenols[2]. Presumably the toxicities of other polyhydric phenols are explicable on a similar basis.

The results are of interest in indicating the existence of substances, other than the metals, which are exceedingly toxic to urease and whose effects can be

[1] Present as Na salt.

[2] Baudisch and Dyer [1933] have recently shown that *o*-quinone is reduced by cysteine to catechol.

eliminated by the presence of thiol compounds. The fact that the dihydric phenols and their corresponding quinones are widespread in the biological kingdom lends biological significance to these results and points to the possibility that tissue extracts may well contain natural inhibitors to enzymes—quite apart from metals—whose effects are diminished or eliminated by sulphydryl compounds.

SUMMARY.

1. Of the three dihydric phenols, catechol and quinol are exceedingly toxic to urease but resorcinol is without action. Quinol at a concentration of 1 part in 2 millions will remove over 50 % of the activity of urease under the experimental conditions employed.

2. Adrenaline, protocatechuic acid, protocatechuic aldehyde, gallic acid and pyrogallol are toxic to urease but less so than catechol. Phloroglucinol is without action.

3. The toxicity of catechol or quinol at concentrations as low as 1/100,000 is not affected by the presence of potassium cyanide or amino-acids. This distinguishes the toxicity of catechol or quinol from that of metals or that of dyestuffs. The presence of protein (serum or egg-white) does not protect urease from catechol. Boiled egg-white has a protective action (due probably to liberated thiol groups).

4. Thiol compounds (cysteine, glutathione, thiolacetic acid, H_2S) and sodium hydrosulphite diminish or eliminate the toxicity of polyhydric phenols to urease.

5. Hydrogen peroxide and "hyperol" though very toxic to urease are less so than catechol.

6. Quinone is more toxic to urease than quinol.

7. Evidence is given to show that the toxic effects of catechol and quinol are probably due to the presence of the corresponding quinones in the aqueous solutions of these dihydric phenols, and that the protective action of thiol compounds is due to reduction of the quinone to the corresponding dihydric phenol.

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