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The Action of Alloxan and Related Compounds on Alkaline Phosphatase

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The action of alloxan in producing degranulation and degeneration in the β -cells of the pancreatic islets is unique in many ways. The toxic action is apparently irreversible after a few minutes in which time the earliest signs of degeneration appear (Bailey, Bailey & Hagan, 1944; Goldner, 1945; Weinglass, Frame & Williams, 1945). The lesion is very specific in that the α -cells are almost wholly spared and only with larger doses do renal and hepatic necrosis appear. Goldner & Gomori (1944) showed that insulin injection did not protect the pancreas against alloxan, unlike diabetes due to anterior pituitary extracts (Young, 1937).

As general cytotoxic factors can clearly be excluded by the very specificity of the lesion, it would seem reasonable to suppose that alloxan might interfere with cell enzymes, possibly those involved in insulin synthesis. An approach to the problem could thus be made by studying the action of alloxan and the related compounds that are diabetogenic (Brückmann & Wertheimer, 1945, and others) on enzyme systems both isolated and in tissue slices.

Alkaline phosphatase has been studied here because we found early that it was strongly inhibited by alloxan itself, and it has the advantage that it can

be studied quantitatively in tissue sections by a modification of the Gomori (1939) histochemical technique. Further, Cloetens (1942, 1944) showed that Zn was the prosthetic group of alkaline tissue phosphatase and Zn appears to be an integral part of the insulin molecule.

METHODS

Hog kidney phosphatase, purified by kaolin adsorption and elution into aqueous pyridine, was used for the *in vitro* experiments with a phenylphosphate substrate buffered at pH 9.4. The liberated phenol was estimated by the method of Theis & Benedict (1924). In tissue sections Gomori's method (Gomori, 1939) was used but incubation was carried out usually for 15–30 min. only and all the slides in a series were treated simultaneously so that the comparative depth of the black cobalt sulphide precipitated was a roughly quantitative index of the phosphatase activity. The CaCl_2 used by Gomori tended to precipitate some of the inhibitors and was advantageously replaced by the magnesium salt.

RESULTS

Table 1 summarizes the results obtained with four diabetogenic ureides and ten related compounds. Alloxan, dialuric acid, and 1-methylalloxan were all

Table 1. Action of alloxan and related compounds on alkaline phosphatase

	Phosphatase inhibition	Diabetogenic activity
1. Alloxan	+++	+
2. Dialuric acid	+++	+
3. 1-Methylalloxan	+++	+
4. Alloxantin	++	+
5. <i>iso</i> Dialuric acid	+	-
6. Alloxanic acid	+	-
7. Mesoxalic acid	-	-
8. Violuric acid	-	-
9. Uracil	-	-
10. Thiouracil	-	-
11. Barbiturates	-	-
12. Allantoin	-	-
13. Urea	-	-
14. Thiourea	-	-

diabetogenic and strong inhibitors of phosphatase in concentrations down to 0.001M (Fig. 1). Alloxantin was also diabetogenic but only about two-thirds as powerful an inhibitor of phosphatase. Alloxanic acid and *isodialuric acid* were not diabetogenic and they had a weaker inhibitory action on phosphatase; at a concentration of 0.001M they inhibited less than 10%. The inhibition with these two compounds was almost linear with concentration (Fig. 1), unlike the previous four compounds which gave almost cubic expressions.

Fig. 2 shows the chemical relationships of the compounds tested. All four diabetogenic and strongly phosphatase inhibiting compounds are

Fig. 1. Inhibition of kidney phosphatase by alloxan and related ureides.

closed ring ureides with a hydroxyl or a reactive keto group in the 5-position. Compounds such as uracil and barbituric acid without this group are inactive; if this group is blocked as by oxime formation in violuric acid all activity is lost. Alloxantin

Fig. 2. The chemical relationships of the compounds tested.

illustrates this in that, as a result of the taking up of one 5-hydroxyl group in an ether linkage, only one group is free for the two rings and the phosphatase inhibitory action per ring is somewhat less than half that of alloxan. The diabetogenic activity, however, is unchanged. On opening the ureide ring as in alloxanic acid the anti-phosphatase activity declines and the power to produce diabetes is lost; complete hydrolysis to mesoxalic acid and urea removes the rest of the activity against phosphatase. *iso*Dialuric acid which has a carboxyl group replaced by a hydroxyl group is isomeric with dialuric acid and behaves like alloxanic acid.

This inhibition of phosphatase has been demonstrated in kidney sections and these have shown an inhibition comparable to that in the test tube. Plate 3 shows that with increasing concentrations of alloxan there is a progressive inhibition of both nuclear and cytoplasmic phosphatase in the kidney tubule. This inhibition was reversible as shown by washing the section and then incubating in substrate alone, when a normal amount of phosphatase appeared. The same phenomenon has been shown *in vitro* by incubating the concentrated enzyme with 0.06M-alloxan buffered at pH 9.4 at 37° for 1 hr., then diluting with buffer and incubating with substrate, giving a final concentration of 0.0003M-alloxan. The enzyme did not differ in activity from an uninhibited enzyme-alloxan mixture prepared in the same way and was only 5% less active than enzyme incubated with buffer alone. It is clear that alloxan does not destroy phosphatase.

Goldner (1945), Goldner & Gomori (1945) and Archibald (1945) suggest that alloxan but not dialuric acid or alloxantin are very rapidly destroyed at neutral or alkaline pH. But alloxan and dialuric acid behave similarly in their action, which is more marked than that of alloxanic acid, their supposed end-product. We have found the inhibitory action of alloxan unaffected by the presence of plasma or whole blood.

The inhibition of kidney phosphatase was next tested *in vivo* by injecting rats and rabbits with alloxan and assaying their kidney phosphatase both quantitatively and histochemically a day or more later. In every case islet degeneration was present in the pancreas. The assays revealed:

Phosphatase content of kidneys of control rats
= 41 ± 3 King-Armstrong units/g.

Phosphatase content of kidneys of rats given 100 mg. alloxan/kg.

= 39 ± 5 King-Armstrong units/g.

These differences are not significant.

No difference was to be seen in the kidneys of the control and treated rats. In the pancreas the degenerated islets had less phosphatase than the normal, which was not surprising in view of the

degree of shrinkage and degeneration the islets had undergone.

We have shown further that the inhibition by alloxan was independent of the concentration of Mg^{++} ions present.

Fig. 3. Enzyme-substrate dissociation constants plotted according to the method of Lineweaver & Burk.

The dissociation constant of enzyme-substrate complex has been studied by Lineweaver & Burk's (1934) modification (Fig. 3) of the Michaelis-Menten (1913) method. In the presence of 0.024M-alloxan the maximum velocity of the reaction was reduced from 1.09 μ mol. hydrolyzed/min. to 0.42 μ mol./min., whereas the slope which represents the ratio of dissociation constant K_s to the maximum velocity V_{max} only changed from 5.1×10^6 to 7.0×10^6 . These findings indicate that the inhibitory action of alloxan is mainly non-competitive.

DISCUSSION

Alloxan and three other diabetogenic ureides have been shown to be strong inhibitors of alkaline phosphatase in the concentrations required to produce diabetes. This is quite independent of the power of alloxan in concentrated solutions to deaminate and decarboxylate amino-acids, as in the low concentrations used in these experiments alloxan has no such action. Brückmann & Wertheimer (1945) tested ninhydrin and sodium β -naphthoquinone sulphonate both of which also oxidatively deaminate and decarboxylate α -amino-acids and found them not to be diabetogenic. Man, Kartin, Durlacher & Peters (1945) found that alloxan combines with the sulphhydryl group of enzymes, but again only in high concentrations. The action of alloxan and related compounds on phosphatase described in this paper is a reversible non-competitive inhibition, the nature

of which might conceivably be due to interaction with the zinc prosthetic group of the enzyme. Perhaps some interference with zinc transfer in insulin synthesis may account for the action of alloxan on the β -cells of the pancreatic islets.

SUMMARY

1. Four diabetogenic ureides were examined for their power to inhibit alkaline phosphatase and were all highly active. Of ten related compounds examined, two were poor inhibitors of phosphatase

although not diabetogenic and non-inhibiting for phosphatase. The anti-phosphatase activity seems to reside in a free 5-hydroxyl group.

2. This action on phosphatase has been demonstrated in the presence of plasma and blood and in tissue sections. It is reversible, non-competitive and unaffected by magnesium.

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The Estimation of Histidine In Urine and its Excretion by Normal and Pregnant Women

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The urinary excretion of histidine has been a matter of interest since Voge (1929) demonstrated an increase in the excretion during normal pregnancy. This finding has been confirmed (Kapeller-Adler, 1934; Niendorf, 1940; Racker, 1941; Langley, 1941; O'Brien & Quelch, 1943; Dawson, 1944), together with the fact that the excretion drops in pre-eclamptic toxæmia. These results have been obtained by examination of a few urine samples from a considerable number of individuals, but in studying the metabolism of histidine it is of interest to examine the excretion for the duration of the pregnancy in order to ascertain whether any definite trends in histidine excretion are observed. The results obtained in the course of two pregnancies are presented in this paper together with some observations on the excretion of histidine by normal women at different times during the menstrual cycle. The latter work was undertaken as a result of a suggestion by Boxer & Kapeller-Adler (1937) that the gonadotrophic hormones inhibited the activity

of liver histidase thus giving rise to the increased excretion of histidine during pregnancy. This conception would be strengthened if it could be demonstrated that normal women showed a rise in histidine excretion at the time of greatest gonadotrophic hormonal activity in the menstrual cycle, although Boxer & Kapeller-Adler found the rise in excretion occurred approximately at the time of the first occurrence of a positive Ascheim-Zondek test which suggested that a minimal level of circulating gonadotrophic hormone was essential to affect the histidine excretion.

The method of estimation of urinary histidine is based on a colour reaction described by Knoop (1908) and consists of a bromination of the histidine, removal of excess bromine and heating the product at 100° when a characteristic colour develops. The method as applied by Armstrong & Walker (1932) and Kapeller-Adler (1934) has been criticized on the grounds of interference by the urea, urates, phosphates and pigments of the urine (Racker, 1940;