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The Action of Formaldehyde on the Cystine Disulphide Linkages of Wool

2. THE CONVERSION OF SUBFRACTION A OF THE COMBINED CYSTINE INTO COMBINED LANTHIONINE AND DJENKOLIC ACID AND SUBFRACTION B INTO COMBINED THIAZOLIDINE-4-CARBOXYLIC ACID

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Fraction (A + B) of the combined cystine of wool reacts with $NaHSO_3$ to give cysteine and *S*-cystinesulphonate side-chains: those derived from subfraction A revert to disulphide when the bisulphited wool is rinsed with water, whereas the cysteine and *S*-cysteinesulphonate side-chains derived from subfraction B are water-stable (Elsworth & Phillips, 1941; Middlebrook & Phillips, 1942*a*). When wool is treated at 70° with solutions of formaldehyde at a pH between 5 and 7, half of the sulphur of subfraction B is converted into combined thiazolidine-4-carboxylic acid (Middlebrook & Phillips, 1942*b*). Since subfraction B reverts to disulphide when this formalized wool is boiled with H_3PO_4, it is possible that the formaldehyde condenses with the thiol group produced by the hydrolysis of the disulphide group (—SS— $\rightleftharpoons$ —SH + —SOH), but leaves the sulphenic acid group unchanged. The sulphenic acid group may condense with an adjacent imino group forming an *iso*thiazole ring (McClelland & Warren, 1930; Middlebrook & Phillips, 1942*b*), but may be liberated when the wool is boiled with H_3PO_4.

Stoves (1943) boiled keratin fibres with solutions of formaldehyde and from an examination of their load-extension curves suggested that some of the cystine cross-linkages had been converted into djenkolic cross-linkages (:$CHCH_2SCH_2SCH_2CH$:). We have treated wool under similar conditions to those used by Stoves (1943), but find that it does not contain combined djenkolic acid; subfraction A is converted into lanthionine and subfraction B into thiazolidine-4-carboxylic acid. On the other hand, when fraction (A + B) is reduced with thiolacetic acid (Middlebrook & Phillips, 1942*b*), and the reduced wool is treated with formaldehyde, combined djenkolic acid arises from subfraction A, whilst subfraction B is converted into combined thiazolidine-4-carboxylic acid.

When wool in which fraction (A + B) has been reduced is treated with methylene di-iodide, only subfraction A is converted into combined djenkolic acid; both subfractions are, however, cross-linked, when the reduced wool is treated with ethylene dibromide.

METHODS

Materials and analytical methods. The wool used (80's quality; total-S, 3·65; disulphide-S, 3·06%) was in the form of a knitted fabric which had been cleaned with soap, rinsed in water, extracted with ethanol and finally rinsed in water.

Unless otherwise stated, the treated and untreated wools were hydrolyzed under reflux in 5N-HCl for 4 hr. The

disulphide and thiol-S contents were determined by Shinohara's methods (1935*a*, *b*, *c*), using a Spekker absorptiometer. The cystine and djenkolic, lanthionine and thiazolidine-4-carboxylic acids were determined qualitatively by the paper chromatographic technique of Consden, Gordon & Martin (1944, 1946). The presence or absence of tyrosine and other amino-acids was noted. For use as standards, djenkolic acid (du Vigneaud & Patterson, 1936) and thiazolidine-4-carboxylic acid (Ratner & Clarke, 1937) were synthesized, whilst lanthionine was isolated from alkali-treated wool. Thiazolidine-4-carboxylic acid was determined from the colour increase due to the slow decomposition of the acid to cysteine by the $NaHSO_3$ used in the disulphide-S determinations (Middlebrook & Phillips, 1942*b*). Total S was determined by the Benedict-Denis method (Barritt, 1934) and formaldehyde by distilling the wools with 0·1 M-H_3PO_4, the formaldehyde being trapped in a solution of $NaHSO_3$ (Nitschmann & Hadorn, 1941). All the wools, described as conditioned, had been exposed to a controlled atmosphere of 21° and 70% R.H. until they attained constant weight. The analyses are all expressed as a percentage of the weight of the anhydrous wool.

RESULTS

Reaction between wool and formaldehyde at different pH *values*

In each experiment the wool was treated, under reflux, with 50 times its weight of 1 % (w/v) formaldehyde. Acid, neutral and alkaline solutions of formaldehyde were used as follows: (i) at 100° and pH 1·00 for 1 hr., in dilute HCl containing Na acetate in 0·2 M concentration; (ii) at 100° and pH 6·65 for 1 hr. with 0·02 M-Na_2HPO_4 as buffer; (iii) at 70° and pH 6·7 for 48 hr., the same buffer being used; (iv) at 100° and pH 10·12 (falling during the treatment to pH 9·40) for 1 hr. with 0·05 M-borax and NaOH as buffer.

(*a*) *Acid and neutral treatments* (*i*), (*ii*) *and* (*iii*). These caused closely similar changes in the wool, the disulphide-S falling in each experiment to 2·10 %. The 'colour increases', due to decomposition of the thiazolidine-4-carboxylic acid by the $NaHSO_3$ added in the disulphide-S determination, were equivalent to from 0·60 to 0·54 % cysteine-S, corresponding to approximately half the observed loss of disulphide-S (0·96 %). Thiazolidine-4-carboxylic acid was found in all three hydrolysates by one-dimensional paper chromatograms using collidine: it gave a yellow 'spot' with ninhydrin which changed to purple, like the spot given by proline. The acid had a high R_F value which exceeded that of tyrosine. By a two-dimensional paper chromatogram, the three hydrolysates were found to be free from lanthionine and tyrosine.

Samples (0·5 g.) of the pH 1·0-treated and pH 6·65-treated wools were distilled with 0·1 M-H_3PO_4 until the volume of the residual acid in the flask was 10 ml. The formaldehyde evolved was equal to 0·70 % of the pH 1·0-treated wool and to 1·45 % of the pH 6·65-treated. The two acid-treated wools were rinsed in water, conditioned and analyzed. In each case, their disulphide-S contents were found to be very close to 3·06 %, indicating that the formaldehyde reaction had been reversed completely. This was confirmed by a one-dimensional chromatogram of the hydrolysate of the formaldehyde-free pH 1·0-treated wool which showed the absence of thiazolidine-4-carboxylic acid: a two-dimensional chromatogram showed the absence of lanthionine.

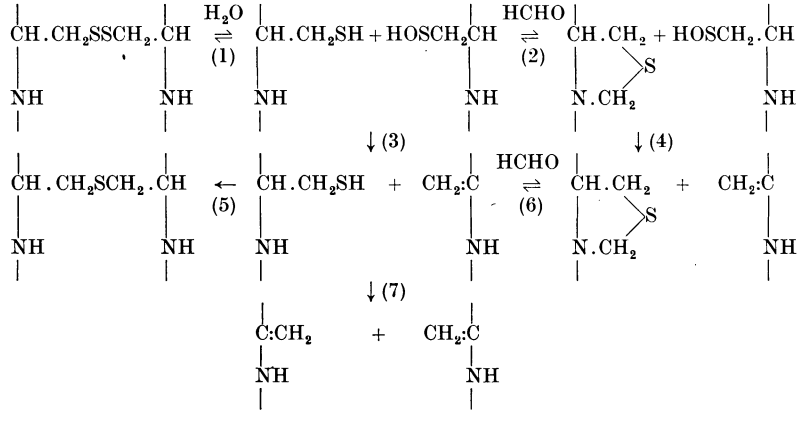
(*b*) *Alkali treatment.* During treatment, the total S of the wool decreased by 1·0 % and the disulphide-S to 0·2 %. The hydrolysate of the wool gave a 'colour increase' corresponding to 0·54 % cysteine-S. Since, when one S atom of a disulphide group reacts with formaldehyde, the other is converted into a sulphenic acid group, the total disulphide-S reacting with formaldehyde was 1·08 %, leaving 1·94 % disulphide-S which lost 1·0 % S to give 1 % lanthionine-S. A one-dimensional chromatogram confirmed the presence of thiazolidine-4-carboxylic acid, whilst the presence of lanthionine and the absence of cystine and tyrosine was confirmed by a two-dimensional chromatogram.

Although the significance of the numerical values of these determinations is decreased because of small, but unknown, losses of wool substance during the treatments, the results show that the reaction between cystine-S and alkaline formaldehyde differs markedly from that between cystine-S and either acid or neutral formaldehyde. In boiling acid or neutral solutions of formaldehyde, subfraction B undergoes reactions (1) and (2) (see below) yielding combined thiazolidine-4-carboxylic acid. In the absence of formaldehyde, boiling wool in a solution of pH 10·12 converts fraction (A + B) into lanthionine by reactions (1), (3) and (5), and by reactions (1), (3) and (7) partially converts fraction (C + D) into combined α-aminoacrylic acid (Lindley & Phillips, 1945). The present results suggest that in boiling alkaline solution at pH 10·12, even in the presence of formaldehyde, fraction (A + B) mainly undergoes the normal alkaline decomposition and yields lanthionine. Some combined thiazolidine-4-carboxylic acid is also produced and may arise from subfraction B by reactions (1) and (2) or from fraction (C + D) by reactions (1), (3) and (6).

If thiazolidine-4-carboxylic acid is produced from subfraction B in this manner, then alkaline decomposition of the sulphenic acid radical may occur by reaction (4) yielding combined α-aminoacrylic acid: the end-products of the reaction may therefore be the same as those arising from fraction (C + D) by reactions (1), (3) and (6).

Since we have found that the cystine-S of wool formalized under neutral and acid conditions can be regenerated by distilling the wool with 0·1 M-H_3PO_4, we conclude such treatment reverses reactions (2)

and (1). This provides a method of detecting whether reactions (4) and (6) take place when wool is treated with alkaline formaldehyde, since hydrolysis of the product of these reactions cannot yield cystine, but may yield combined cysteine, or alternatively, combined lanthionine, if the hydrolysis is followed by reaction (5).



The wool which had been formalized in alkaline solution was therefore distilled with 0·1 M-H_3PO_4. It yielded 2·32 % formaldehyde and when hydrolyzed subsequently was found to contain 0·54 % cystine-S, but no thiol-S. A one-dimensional chromatogram of the hydrolysate showed the absence of thiazolidine-4-carboxylic acid and a two-dimensional chromatogram the presence of lanthionine: the cystine spot was very faint.

During the distillation with 0·1 M-H_3PO_4, 0·34 % cystine-S (0·54–0·20) was reformed so that 0·37 % of cysteine-S (0·54–0·17) is unaccounted for. This may have been lost from subfraction B, when the formalized wool was hydrolyzed, owing to the occurrence of reaction (4) during the alkaline treatment and subsequent formation of lanthionine by reversal of reaction (6) followed by reaction (5). Alternatively, the missing thiol originated from fraction (C + D) and was converted into lanthionine during the hydrolysis, again by reversal of reaction (6) followed by reaction (5).

Reaction between reduced wool and formaldehyde before and after supercontraction

Before supercontraction. To determine whether djenkolic acid cross-linkage could be produced in wool in which fraction (A + B) had been reduced to thiol groups (Middlebrook & Phillips, 1942*b*), a wool was reduced by treating it at 37° for 17 hr. with 50 times its weight of M-thiolacetic acid titrated with NaOH to pH 5·2 (Patterson, Geiger, Mizell & Harris, 1941). The reduced wool was rinsed thoroughly with distilled water, then with ethanol and finally with water. After conditioning, it contained 2·28 % thiol-S and 0·78 % disulphide-S.

As in the previous experiment (i) with ordinary wool, it was treated with formaldehyde at pH 1·0. On analysis it was then found to be free from thiol-S, to contain 0·76 % disulphide-S and to give a 'colour increase' equivalent to 1·32 % thiol-S. In the previous experiments, a 'colour increase' equivalent to 0·60 % thiol-S corresponded with the reaction of formaldehyde with 0·96 % disulphide-S or the formation of thiazolidine-4-carboxylic acid from 0·48 % thiol-S. The observed 'colour increase' of 1·32 % is therefore equivalent to 1·06 % S combined as thiazolidine-4-carboxylic acid, making with the 0·76 % disulphide-S remaining unchanged, 1·82 % S accounted for out of the original 3·06 %, so that 1·24 % S has become linked in some other manner.

The treated wool was distilled with 0·1 M-H_3PO_4 to remove the combined formaldehyde and then hydrolyzed when it was found to contain 0·59 % thiol-S and 0·71 % disulphide-S. In addition the hydrolysate gave a 'colour increase' equivalent to 0·49 % thiol-S, indicating that the removal of the formaldehyde had been incomplete. A two-dimensional chromatogram of the acid hydrolysate showed that it contained djenkolic acid, so that this acid may therefore account for the missing 1·24 % S.

Djenkolic acid was distilled with 0·1 M-H_3PO_4 until the volume of the residual acid was 10 ml. This residual acid was free from thiol and disulphide-S and gave no 'colour increase' on standing with $NaHSO_3$ at pH 5. Although stable to 0·1 M-H_3PO_4, djenkolic acid was decomposed when heated with concentrated H_2SO_4 into formaldehyde, cysteine and cystine.

After supercontraction. When wool fibres are treated with reagents that break the disulphide cross-linkages of fraction (A + B), they almost invariably assume, when boiled with water, a length about 30 % less than their normal length. This contraction was called supercontraction by Astbury & Woods (1933), and has been assumed to be accompanied by molecular reorientation (Speakman, 1936) which may alter the relative spacial positions of the

two S atoms and hence restrict the types of reaction in which they can participate. Since we have found that thiolacetic acid-reduced wool supercontracted when boiled for 5 min. in dilute acetic acid (pH 2·8), we have compared the reactions of the reduced, and the reduced and supercontracted wool with formaldehyde. The two wools were each treated at 50° for 48 hr. in 50 times their weight of 1 % formaldehyde, buffered to the pH values indicated in Table 1. Before supercontraction the reduced wool contained thiol-S, 2·28 and disulphide-S, 0·78 %: after supercontraction its thiol-S content was 2·06 and its disulphide-S content 0·75 %. Apart from the two major differences, indicated as footnotes to Table 1, the two wools, after treatment with formaldehyde, gave the same analyses.

Table 1. *Analyses of reduced and supercontracted wools after treatment with* 1 % *formaldehyde at different* pH *values*

pH of formaldehyde solution	Thiol-S (%)	Disulphide-S (%)	Thiol-S calc. from 'colour increase' (%)
1·0	0·1	0·77	1·33
6·6	0·1	0·89	1·48*
8·0	0·05	0·96†	1·47

* Reduced and supercontracted wool gave 1·37 %.
† Reduced and supercontracted wool gave 0·76 %.

The identity in chemical analysis of the two treated wools persisted after they had both been distilled with 0·1 M-H_3PO_4; the reduced wool contained 0·62 % thiol-S, 0·97 % disulphide-S and its acid hydrolysate gave a 'colour increase' of 0·38 %, the corresponding analyses for the reduced and supercontracted wool being 0·51, 0·92 and 0·35 %.

The reaction between reduced wool and methylene di-iodide and ethylene dibromide

A fully reduced wool (Patterson *et al.* 1941) was treated for 4 hr. at 50° with 0·0005 mol. methylene di-iodide in 0·1 M-phosphate buffer (20 ml.) at pH 8·0–7·2 and then rinsed thoroughly and conditioned. The treated wool contained 1·24 % thiol-S and 0·98 % disulphide-S, and the hydrolysate gave no colour increase in the presence of $NaHSO_3$. The analysis of the wool was unchanged (thiol-S, 1·30 %; disulphide-S, 1·02 %) by distillation with 0·1 M-H_3PO_4. A one-dimensional chromatogram of the hydrolysate of this H_3PO_4-treated wool confirmed the presence of cystine and djenkolic acid and the absence of thiazolidine-4-carboxylic acid.

In order to demonstrate that the thiol-S in the reduced wool which did not react with methylene di-iodide would react with formaldehyde, reduced wool which had been treated with methylene di-iodide was heated under reflux with 1 % formaldehyde at pH 1·0 for 1 hr. When analyzed, it was found to be free from thiol-S, its disulphide-S was unchanged (0·99 %), but its hydrolysate in the presence of $NaHSO_3$ showed a 'colour increase' corresponding to 1·30 % thiol-S. The thiol groups in the reduced wool which failed to react with methylene di-iodide had therefore reacted with formaldehyde to give thiazolidine-4-carboxylic acid; hence, it is probable that they were produced mainly from subfraction B.

A comparison was then made between the reaction of reduced wool with methylene di-iodide and ethylene dibromide. A purified virgin Cape wool (Middlebrook & Phillips, 1942*a*) was reduced in 100 times its weight of M-thiolactic acid for 17 hr. at pH 5·0 and 32°. After rinsing in several changes of water, it contained 2·07 % thiol-S. Samples (*c.* 0·5 g.) of this reduced wool were treated, as set out in Table 2, in 50 ml. of buffer (M-Na_2HPO_4 with H_3PO_4 for pH 8·0 and M-Na acetate with acetic acid for pH 5·4) to which different quantities of the dihalides had been added. The thiol-S contents of the treated wools were then redetermined.

Table 2. *The reaction between reduced wool* (c. 0·5 *g.*) *and methylene di-iodide and ethylene dibromide*

Dihalide (g.)	Buffer (pH)	Treatment: Temp. (°)	Treatment: Time (hr.)	Residual SH, as S (%)
Methylene di-iodide				
0·3	8·0	32	17	0·69
0·3	8·0	32	48	0·74
0·6	8·0	50	18	0·65
0·3	5·4	32–55	66	0·53
Ethylene dibromide				
0·2	5·4	32–55	66	0·17
0·2	8·0	32	17	0·17

From Table 2 it will be seen that whereas a portion of the thiol groups in the reduced wool failed to react with methylene di-iodide, practically all the thiol groups reacted with ethylene dibromide.

The greater ease with which the thiol groups derived from subfraction A, as compared with those of subfraction B, can be cross-linked with methylene di-iodide, provides a method of comparing the relative rates at which subfractions A and B are reduced by thiolacetic acid. Samples of virgin Cape wool (Middlebrook & Phillips, 1942*a*) were reduced with 100 times their weight of M-, 0·2 M- and 0·05 M-thiolacetic acid at pH 5·0 for 18 hr. at 32°. After they had been rinsed to remove thiol and dithiolacetic acid, part of each sample was hydrolyzed to determine the thiol-S content and part (*c.* 0·5 g.) treated in 50 g. of M-phosphate buffer at pH 8·0 and 32° containing 0·3 g. methylene di-iodide for 22 hr. Their thiol-S contents were then redetermined and the

loss of thiol-S calculated and returned as djenkolic acid-S (Table 3). On the assumption that the thiol-S which did not react belongs to subfraction B and the thiol-S which was converted into djenkolic acid-S to subfraction A, the results suggest that the two subfractions are reduced at rates roughly dependent on the relative proportions of the two subfractions in the wool.

Table 3. *Reaction between fully and partially reduced wools and methylene di-iodide*

Reduced wools	Reduced wools after CH_2I_2	
S as SH (%)	S as SH (%)	S as djenkolic-S (%)
2·07	0·68	1·39
1·39	0·46	0·93
0·58	0·32	0·26

DISCUSSION

The simplest explanation of the observed differences in the reactivities of subfractions A and B would appear to be that when the S atoms of fraction (A + B) are separated by chemical reaction, those of the disulphide groups of subfraction A do not move so far apart as do those of subfraction B. This would explain why subfraction A can be transferred into lanthionine more readily than subfraction B (Lindley & Phillips, 1945) and why it yields djenkolic acid with methylene di-iodide and formaldehyde, whereas subfraction B reacts with ethylene dibromide but not with methylene di-iodide, and yields thiazolidine-4-carboxylic acid with formaldehyde.

Other observations on oxidation reactions depending on the availability of two thiol groups in close proximity suggest that some of the thiol groups of reduced wool, which have arisen from fraction (A + B), can move outside reactive distance (Blackburn, Middlebrook & Phillips, 1942). For example, the thiol groups which persist after treatment of reduced wool with methylene di-iodide in a buffer at pH 5 do not give the nitroprusside reaction. Further, about 0·7% of cysteine-S remains unoxidized when reduced wool, immersed in a buffer at pH 8, is treated with oxygen. On the other hand, all such thiol groups react readily with methyl iodide (Harris, Mizell & Fourt, 1942). In addition, a bisulphited and rinsed wool, which contains thiol and *S*-cysteinesulphonate groups derived from subfraction B, can be readily methylated (Blackburn, Consden & Phillips, 1944).

The variations in the ease with which free thiol groups in wool can be cross-linked or oxidized is allied to the phenomenon of the 'unmasking' of thiol groups during the denaturation of cystine-containing proteins (Neurath, Greenstein, Putman & Erickson, 1944). Neurath (1938) has suggested that this 'unmasking' takes place because the protein assumes a more flexible configuration which permits closer approach of the thiol groups of adjacent chains and thus facilitates their oxidation to disulphide groups.

The evidence that thiol groups exist in the native proteins is not so definite as with reduced wool. Anson (1940) showed that those of native egg albumin, although unreactive towards nitroprusside, would react with iodoacetamide, but Rosner (1940) could not cause them to react with iodoacetate until the albumin had been denatured. Iodoacetamide and iodoacetate do not, however, react with equal ease with the thiol groups of proteins, since Dr H. Lindley, in these laboratories, has found that whereas at pH 6 all the thiol groups of fraction (A + B) will react with iodoacetamide, only a portion will react with iodoacetate.

SUMMARY

1. Subfraction B of the combined cystine of wool, defined as the subfraction which gives water-stable thiol and *S*-cysteinesulphonate groups with $NaHSO_3$, reacts with formaldehyde at pH values of 1·0, 6·7 and 10·0 at 70–100° to give combined thiazolidine-4-carboxylic acid.

2. Subfraction A of the combined cystine of wool, defined as the subfraction which gives water-labile thiol and *S*-cysteinesulphonate groups with $NaHSO_3$, does not react with formaldehyde at pH values of 1·0, 6·7 and 10·0 at 70–100°, but at pH 10 is converted into combined lanthionine.

3. Wool in which fraction (A + B) has been reduced to thiol groups reacts with formaldehyde, subfraction A being converted into combined djenkolic acid and subfraction B into combined thiazolidine-4-carboxylic acid. When this reduced wool is treated with methylene di-iodide only subfraction A is cross-linked and converted into combined djenkolic acid.

4. Wool in which fraction (A + B) has been reduced to thiol groups and has then been caused to supercontract reacts with formaldehyde in the same manner as normal, reduced wool.

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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