LXV. STUDIES ON THE CATALYTIC OXIDATION OF URIC ACID.

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WHILST numerous papers have been published on the oxidation of uric acid in the presence of metallic catalysts, such as copper, manganese, *etc.*, little work appears to have been done on the action of hydrogen acceptors on uric acid. In this paper the action of thioglycollic acid, cysteine and methylene blue will be described.

The results obtained with thioglycollic acid differ from those described in an earlier paper [Dobrowolska, 1928]; in this earlier research thioglycollic acid was found to catalyse the oxidation of uric acid. The earlier work was done with an old preparation of Kahlbaum's, which apparently contained some impurity catalysing the oxidation of uric acid, as Merck's pure thioglycollic acid in no case catalysed the reaction in question.

EXPERIMENTAL.

Kahlbaum's or Merck's pure or analytical reagents were taken. The solutions of thioglycollic acid were neutralised to p_H 7 with NaOH before final dilution. Uric acid solutions were prepared by dissolution in the minimal quantity of saturated lithium carbonate and adding water to the requisite dilution. Uric acid was in most cases determined colorimetrically, using Folin's reagents [Folin and Denis, 1913]. p_H was determined colorimetrically (Clark and Lubs's indicators) or potentiometrically, using a hydrogen electrode.

Thioglycollic acid.

The action of thioglycollic acid on uric acid was studied at various p_H values in the following way. A series of systems, each containing 10 cc. of 0.05 % thioglycollic acid, 0.2–0.6 % uric acid, and buffer solution (Sørensen's phosphates below p_H 8 and Palitzsch's borates above p_H 8), was set up, together with two control series, containing 10 cc. of water in place of thioglycollic or uric acid respectively. A separate control containing only buffers and thioglycollic acid was necessitated by the circumstance that this substance gives a coloration with Folin's reagents. Toluene or thymol crystals were added in order to ensure sterility of the systems. The intensity of coloration given by

every system was determined at the beginning of the experiment, and after various periods of time (the reaction temperature was 37°), and the sums of the two controls were compared with the values obtained for the experimental systems. The intensities of coloration are expressed as mg. of uric acid in Table I, from which it appears that at no p_H from 6.1 to 9.8 does any oxidation of uric acid take place. At the same time it is evident that the intensity of coloration of the systems is greater immediately after adding thioglycollic acid to uric acid than would correspond to the sum of that given by each constituent separately. Thus, at p_H 7.8, the intensity of coloration of the system corresponds to 39.2 mg. of uric acid, that of uric acid alone to 12.6, and that of thioglycollic acid to 6.0, giving a total of 18.6 mg., which is less than half of the value found for the whole system. The coloration given by thioglycollic acid alone rapidly diminishes in intensity with time, as a result of oxidation by atmospheric oxygen to dithioglycollic acid; at the same time the coloration given by the whole system approaches that given by uric acid alone, the velocity of this process being greater the higher is the p_H of the system.

Table I. *Action of thioglycollic acid on uric acid.*

No. of exp.	Time hrs.	p_H	Control systems			Apparent uric acid content mg.	Apparent change in uric acid content mg.
			Uric acid found mg. a.	Thio-glycollic acid* b.	Sum of a + b mg.		
1	0	7.8	12.6	6.0	18.6	39.2	20.6
	24	7.8	12.6	4.4	17.0	20.0	3.0
	48	7.8	12.6	3.2	15.8	16.7	0.9
	72	7.8	12.6	0	12.6	14.2	1.6
	84	7.8	12.6	0	12.6	14.1	1.5
2	0	9.2	12.6	0.4	13.00	20.0	7.00
	48	9.2	12.6	0.28	12.88	14.6	1.72
	72	9.2	12.6	0.16	12.76	12.6	0.16
	96	9.2	12.2	0.16	12.36	12.6	0.24
3	0	9.8	17.8	0.10	18.20	30.8	12.6
	24	9.8	17.8	0.28	18.08	20.0	1.92
	48	9.8	16.6	0.28	16.88	16.8	0.08
	72	9.8	15.4	0	15.40	16.0	0.6
4	0	8.0	6.4	2.4	8.80	14.6	5.8
	24	8.0	6.4	2.4	8.80	11.4	2.6
	48	8.0	6.4	0	6.40	6.0	0.4
	72	8.0	6.4	0	6.40	6.0	0.4
5	0	6.1	8.0	1.8	9.80	14.6	4.8
	24	6.1	8.0	0.8	8.8	8.0	0.8
	48	6.1	8.0	0	8.0	8.0	0

* Expressed as the number of mg. of uric acid which would give an equal intensity of coloration with the Folin-Denis reagent.

No explanation of the above phenomenon is evident; possibly an unstable double compound is formed between the two acids, of a higher reduction potential.

In systems the p_H of which was below 6 or above 10, uric acid crystallised out as free acid and as sodium salt monohydrate respectively—for this reason the action of thioglycollic acid was studied only within these p_H limits.

Action of thioglycollic acid in the presence of Fe and Cu salts.

Warburg and Sakuma [1923] found that the oxidation of sugars in the presence of cysteine depended on the presence of traces of iron or copper. That this is not the case with thioglycollic acid was shown by experiments in which iron (0.0001–0.0007 *M* ferrous ammonium sulphate) or copper (0.00005–0.0001 *M* CuSO₄, 5H₂O) was added to systems similar to those described above. The only effect observed was an intensification of the coloration given by thioglycollic acid with the Folin-Denis reagent at the beginning of the reaction with subsequent rapid disappearance of the coloration with conversion into dithioglycollic acid by atmospheric oxygen.

Verification of results obtained by the colorimetric method.

In view of the relative unreliability of the results obtained by the colorimetric method in such systems as the above, and of the possibility that the product of action of thioglycollic acid on uric acid also gives a coloration with the Folin-Denis reagent, it was thought desirable to verify them using the following methods.

(1) *Determination of CO₂ by Van Slyke and Cullen's method.* Should uric acid undergo decomposition to allantoin, the CO₂ content of the experimental systems should be greater than that of the component solutions taken separately. No difference was observed in systems of *p*_H 8.0–9.8, which were isolated from atmospheric CO₂.

(2) *Determination of NH₃ and of urea.* These were determined in the experimental and control systems by Folin's and by Marshall's methods. Only insignificant traces were found in all systems, indicating that decomposition of uric acid with production of NH₃ or urea does not take place under the given conditions.

(3) *Determination of uric acid by Krüger and Schittenhelm's method, and by iodimetric titration.* The results given by these methods similarly indicate that no oxidation of uric acid takes place in the presence of thioglycollic acid.

Cysteine.

Cysteine, prepared by reduction of Kahlbaum's cystine, was without action on uric acid, either alone or in the presence of Cu, at *p*_H 8.0–9.1.

Methylene blue.

Grubler's methylene blue (this will be designated in future as M.B.) was taken, in concentrations of 0.000077–0.0001 *M*. Such low concentrations were taken for two reasons, firstly, that the coloration due to the dye should not interfere with the colorimetric determination of uric acid, and secondly that at higher concentrations M.B. is precipitated at *p*_H 10.5–12.0 in the form of long violet-blue needles.

(1) *Action under aerobic conditions.* Preliminary experiments at p_H 8 indicated that in the presence of 0.00031 *M* M.B. over 12 mg. of uric acid are decomposed at 18° within 48 hours in the absence of antiseptics. Toluene was found to exert no inhibitory action at p_H values below 9.3; in more alkaline solutions M.B. is converted into a red modification, soluble in toluene, which extracts it from aqueous solution, and thus prevents its action on uric acid. As a result the velocity of reaction varies inversely with p_H and with the volume of toluene added. When only a small volume of toluene was added (2 drops) the results obtained were substantially the same as in its absence. Thymol acts similarly, except that velocity of reaction is greater at p_H 11.6 than at p_H 9.3; in this case M.B. accumulates on the surface of the crystals of thymol and then slowly diffuses into the interior. These results indicate that toluene in small quantity does not interfere with the reaction.

A number of systems was set up, containing 10 cc. of uric acid solution, 10 cc. of 0.00031 *M* M.B. or water and 10 cc. of Sørensen's or McIlvaine's buffers, 10 cc. of 0.008 or 0.016 *M* KCN or water and 2 drops of toluene. The potassium cyanide solution was previously brought to p_H 7 by addition of *N* HCl. Uric acid was determined colorimetrically after 14–24 hours at 18°. The results, given in Table II, show that, on the whole, oxidation is greater the higher the p_H of the system, and that cyanide has practically no effect at any p_H .

Table II. *Influence of p_H and KCN on oxidation of uric acid in presence of methylene blue.*

Time hrs.	p_H	Concentration of KCN	Mg. uric acid				
			In control	Found		Decomposed	
				Without KCN	With KCN	Without KCN	With KCN
24	6.11	<i>M</i> /500	18.8	18.8	18.8	0	0
	6.79		18.8	18.8	18.8	0	0
	7.47		18.8	14.6	15.8	4.2	3.0
	8.35		18.8	10.0	10.6	8.8	8.2
	9.37		18.8	11.4	11.4	7.4	7.4
	9.75		18.8	9.4	9.4	7.8	7.8
	11.20		17.2	9.4	9.4	7.8	7.8
	11.75		16.4	5.2	5.2	11.2	11.2
	6.30	<i>M</i> /500	10.8	9.6	10.4	1.2	0.4
15	7.02		10.8	8.8	8.8	2.0	2.0
	7.70		10.8	5.3	6.9	5.5	4.9
	8.60		10.8	4.4	4.9	6.4	5.9
	9.75		10.8	4.9	5.5	5.9	5.3
	10.15		10.8	4.6	5.0	6.2	5.8
	11.85		8.6	2.94	2.54	5.66	6.06
	12.30		8.6	1.32	1.16	7.28	7.44
14	5.75	<i>M</i> /250	13.2	10.4	10.4	2.8	2.8
	6.40		13.2	8.01	9.2	5.1	4.0
	7.00		13.2	6.7	7.1	6.5	6.1
	7.82		13.2	5.6	6.0	7.6	7.2
	8.84		13.2	4.04	5.6	9.16	7.6
	9.27		13.2	4.6	5.5	8.6	7.7
	10.75		12.4	3.14	3.14	9.26	9.26
	11.25		12.4	1.82	1.82	10.58	10.78

Oxidation augments sharply from p_H 6 to about 9.6, above which it falls to p_H 10, again rising steadily to p_H 12. In the presence of cyanide a break occurs in the oxidation- p_H curve at about p_H 8.6, above which value the number of mg. of uric acid oxidised increases at first slowly and then rapidly to attain at p_H 11–12 the same value as in the absence of cyanide.

The action of M.B. in the presence of Cu and Fe ions was next studied. Systems similar to the above were prepared, with the difference that copper sulphate or ferrous ammonium sulphate solution was added in place of KCN.

It was found that 0.0001 *M* ferrous ammonium sulphate did not influence the velocity of reaction between p_H 5.7 and 12.0.

Copper alone catalyses the oxidation of uric acid, in the absence of M.B.; where both are present together they exert a mutually antagonistic action, which is more marked at higher than at lower p_H . Thus, at p_H 12.1, 7.5 mg. are oxidised in the presence of copper alone, and 6.74 mg. in the presence of M.B. alone; in the presence of both M.B. and Cu only 6.34 mg. of uric acid are oxidised (Table III). The influence of Cu on the action of M.B. is probably due to the formation of a less active compound of the two substances, and, at certain p_H values (7–10), a deposit of dark blue needles was observed in the systems, with partial decoloration of the solution.

Table III. *Influence of 0.0001 M copper sulphate on oxidation of uric acid by methylene blue at 21°.*

		Mg. uric acid						
		In absence of CuSO ₄			In presence of CuSO ₄			
Time hrs.	p _H	In control	In system	De-composed	In control	In system	De-composed	Inhibition mg.
14½	5.87	11.2	11.2	0	11.2	11.2	0	0
	6.79	11.2	10.8	0.4	11.2	11.2	0	0.4
	7.47	11.2	8.1	3.1	10.2	10.2	0	3.1
	8.37	11.2	5.0	6.2	10.0	6.5	3.5	2.7
	9.55	11.2	6.3	4.9	10.0	7.5	2.5	2.4
	10.1	11.2	6.4	4.8	8.8	7.3	1.5	3.3
	11.8	9.0	4.05	4.95	3.65	4.6	0	4.95
	12.1	9.8	3.06	6.74	2.3	3.46	0	6.74
14	5.81	12.2	12.2	0	12.2	12.2	0	0
	6.65	12.2	11.6	0.6	12.0	12.0	0	0.6
	7.34	12.2	9.2	3.0	11.6	11.8	0	3.0
	8.14	11.8	7.4	4.4	11.8	8.8	3.0	1.4
	9.3	11.8	6.2	5.6	10.0	7.3	2.7	2.9
	9.8	10.6	4.8	5.8	9.2	6.3	2.9	2.9
	11.45	8.8	3.65	5.15	2.75	4.4	0	5.15
	12.0	9.6	2.3	7.3	2.88	3.35	0	7.3

It was observed that freshly prepared solutions of M.B. exerted a considerably smaller catalytic action than did aged solutions. Thus in one case, in a system containing 10 cc. of uric acid solution (18 mg.), 10 cc. of freshly dissolved M.B., and 20 cc. of buffers (p_H 8.2), 0.14 mg. of uric acid was decomposed during 24 hours at 23°, whilst in a similar system containing a 20-day old solution of M.B. 8.4 mg. were oxidised in 24 hours; after 20 days the activity of M.B.

solutions does not appear further to increase. This activation can be accelerated by alkalisng the M.B. solution, as is shown by the following experiment. Part of a solution of M.B. was alkalisng with NaOH to p_H 11.6, and left 3 days, when the p_H was reduced to 4.74 by addition of HCl. Two sets of systems were then set up, previously alkalisng M.B. being taken in one set, and ordinary M.B. in the other. The results, given in Table IV, indicate that an activation of about 20 % is attained in the above way.

Table IV. *Activation of methylene blue.*

Time hrs.	p_H	Mg. uric acid					% acti- vation
		In control	With ordinary methylene blue	De- composed	With methylene blue	De- composed	
22½	8.5	11.0	9.2	1.8	6.9	4.1	21
22	8.7	11.8	8.6	3.2	3.4	5.46	20

(2) *Action of M.B. under anaerobic conditions.* Systems containing uric acid, M.B. and buffers (p_H 6–11.4) were left in Schroetter fermentation vessels for 43–69 hours at 23°, atmospheric oxygen being excluded by sealing the side tube with mercury.

Under these conditions no oxidation of uric acid took place. That reaction was inhibited by exclusion of oxygen and not by the action of mercury on M.B. was shown by similar systems left in contact with the air in the presence of mercury; no retardation of reaction was observed in these circumstances.

DISCUSSION.

The inactivity of sulphhydryl compounds, and of methylene blue under anaerobic conditions indicates that uric acid cannot be oxidised by dehydrogenation under these conditions, but only by direct addition of oxygen. Methylene blue is probably itself inactive even under aerobic conditions—the actual catalyst is most probably some product of methylene blue, capable of combining with atmospheric oxygen, and of donating this oxygen to uric acid. Whether or not iron or copper enters into the composition of this catalyst it is not possible, on the basis of the above experimental results, to state with certainty; the fact that cyanide does not inhibit reaction is not evidence that metals are absent, as haemoglobin is capable of catalysing the oxidation of uric acid in the presence of cyanide [Gomolińska, 1928]. The absence of activation on addition of Cu or Fe salts similarly does not exclude the possibility that the presence of traces of metal may be essential to the action of M.B., as was found to be the case for cysteine [Warburg and Sakuma, 1923].

The nature of the red modification of M.B. obtained in alkaline solutions is similarly not clear. Commencing from p_H 9, an increasing proportion of a red, ether-soluble modification is found, and at p_H 12 practically the whole of

the dye is extractable from aqueous solution by organic solvents. The ether extract on shaking with distilled water loses its red coloration, and the aqueous layer becomes coloured blue, indicating that a labile equilibrium ordinarily exists between the two forms, the mutual proportions of which depend on the p_H . The increase in oxidation with p_H may be due to the increasing concentration of the red form.

The observation that the coloration given by mixtures of uric acid and sulphydryl compounds is greater than the sum of those given by each substance separately is of importance in the application of colorimetric methods to the determination of cysteine, ergothioneine, *etc.*, in the presence of uric acid, or *vice versa*. Unless Folin's isolation procedure is applied for the removal of uric acid the results obtained for most physiological fluids would be untrustworthy, and the same applies to the determination of uric acid in solutions in which processes of decay have taken place, with the production of hydrogen sulphide.

SUMMARY.

1. The sulphydryl compounds thioglycollic acid and cysteine, and the disulphide compounds dithiodiglycollic acid and cystine are without influence on the oxidation of uric acid, either in the presence or absence of salts of iron or copper.

2. The coloration given on the addition of the Folin-Denis reagent to a system containing uric acid and a sulphydryl compound is more intense than the sum of the colorations given by each constituent separately; this extra coloration diminishes with increasing p_H . A similar effect is observed in systems containing thioglycollic acid and copper sulphate.

3. Monohydrated sodium urate separates from systems containing uric acid and thioglycollic acid at p_H 10.5–11.5.

4. Methylene blue catalyses the reaction of oxidation of uric acid in aerobic but not in anaerobic conditions; the velocity of reaction varies with the p_H of the medium.

5. The reaction is inhibited to a varying extent, according to the p_H of the medium, by copper salts (0.0001 M copper sulphate).

6. The reaction is to a slight extent inhibited by 0.004 M potassium cyanide over the range p_H 6–10. At p_H 11 and 12 the cyanide undergoes hydrolysis, with consequent loss of inhibitory action.

7. Crystalline thymol, added as an antiseptic, retards the reaction, as a result of adsorption or solid solution of methylene blue.

8. The addition of toluene as an antiseptic similarly retards the reaction, as a result of the solubility in organic solvents of a red variety of methylene blue, formed in alkaline solution, p_H 10–12.

9. The catalytic activity of methylene blue rises considerably during the first few weeks after preparation of its dilute solutions; this process is completed more rapidly in alkaline than in other solutions.

10. The reaction of oxidation of uric acid in the presence of methylene blue appears to be one of catalytic oxidation, oxygen being in all probability activated by methylene blue.

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REFERENCES.

- Dobrowolska (1928). *Compt. Rend. Soc. Biol.* **99**, 1022.
Folin and Denis (1913). *J. Biol. Chem.* **14**, 95.
Gomolińska (1928). *Biochem. J.* **22**, 1307.
Warburg and Sakuma (1923). *Arch. ges. Physiol.* **200**, 203.