Thiouric Acids as Substrates and Inhibitors of Mammalian Urate Oxidase

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In spite of numerous investigations, the intricate mechanism of the oxidative degradation of uric acid by urate oxidase (urate-oxygen oxidoreductase, EC 1.7.3.3) has not been completely elucidated. The liver enzyme is a copper-protein complex (Mahler, Huebscher & Baum, 1955) which is activated by cysteine (Scheer & Scheer, 1943; Santagati & Topi, 1953). Cyanide, thiocyanate and hydroxylamine inhibit the enzyme, presumably by combination with the metal in its univalent state (Mahler *et al.* 1955). By the use of [6-¹⁴C]uric acid, Bentley & Neuberger (1952) showed that C-6 appears as the carbon dioxide molecule, liberated according to the schematic equation of Keilin & Hartree (1936):

However, allantoin is only one of the products of a complex reaction, the primary step of which is still unknown (Klemperer, 1945). In the present experiments, the relationship of urate oxidase to thiouric acids has been studied. Because of the high affinity of copper for sulphur, the thiouric acids may enter the metal complex of the enzyme and thus serve either as substrates or as inhibitors. If the thio analogues were susceptible to enzymic degradation, the sulphur atom would introduce a useful label for the identification of reaction intermediates. On the other hand, refractory thiouric acids may inhibit the enzyme and thus lead to a definition of the specific features responsible for attachment of purines to the active surface of urate oxidase. In this way one may indirectly obtain valuable information on the binding groups of the active centre.

MATERIALS AND METHODS

Thiouric acids. 6-Thiouric acid and its 3-methyl derivative were prepared according to Levin, Kalmus & Bergmann (1960). 2- and 8-Thiouric acid were synthesized by the method of Loo, Michael, Garceau & Reid (1959), and 6,8-dithiouric acid by condensation of 4,5-diamino-6-thiouracil with thiourea (F. Bergmann & A. Kalmus, unpublished work).

Enzymes. Urate oxidase was the commercial uricase of Leo Pharmaceuticals Inc., Ballerup, Denmark. The contents of one ampoule (about 5 mg. of powder), when

diluted with 100 ml. of buffer, pH 9.3, containing 71 μM -uric acid, degraded 0.46 μmole of the substrate/min. at 29°. Enzyme concentrations were calculated on the assumption that each ampoule contained 5 mg. of powder. The rates of uric acid oxidation were linearly proportional to enzyme concentration. Therefore relative rates were calculated by reference to the rate of urate oxidation at the same enzyme dilution. All experiments were carried out at pH 9.3, unless otherwise stated.

The enzyme catalase (Worthington Biochemical Corp.) was added to urate oxidase in some experiments at a concentration of 1 unit/ml., i.e. an amount which, if added to 1.5 mM-hydrogen peroxide, would decompose 1 mg. of hydrogen peroxide/ml./min.

Buffers. For all enzyme assays, 0.05 M-phosphate buffers (NaH_2PO_4 , Na_2HPO_4), pH 5.8–8.5, and 0.067 M-glycine-sodium hydroxide buffers, pH 9.0–11.0, were used. We have established that, at pH 8.5–9.0, both buffers give identical enzymic rates, so that the left- and right-hand parts of the pH-activity curves in Fig. 4 are commensurable.

For the determination of the effect of pH on the wavelength of ultraviolet-absorption maxima of uric acid and 6-thiouric acid the following buffers were used: pH –3, 13.5 N- H_2SO_4 ; pH –1, 5 N- H_2SO_4 ; pH 0–3, perchloric acid; pH 4–6, 0.1 M-acetate; pH 6–8, 0.1 M-phosphate; pH 8–11, 0.1 M-borate adjusted with NaOH; pH 11–14, sodium hydroxide.

Enzymic methods. The disappearance of uric acid was measured by the decrease of extinction, E , at 293 $m\mu$ (Praetorius, 1948a) and of 6-thiouric acid by the decrease in E_{347} . Although the wavelengths of the absorption maxima vary with pH (Bergmann & Dikstein, 1955), degradation of a substrate can always be determined at the same wavelength provided that changes in the molar extinction coefficient are taken into account.

All solutions were equilibrated with air. Reactions were carried out in 10 mm. silica cells at a temperature of $29 \pm 0.5^\circ$ which was maintained with a thermospacer. The rate of oxidation was derived from the initial, linear, portion of the curve, representing E as function of time. For each experiment, the 'standard activity' of the enzyme was determined by incubating 12 $\mu\text{g.}$ of uric acid/ml. with 50 $\mu\text{g.}$ of urate oxidase/ml. and measuring the rate of oxidation. These standard activities did not vary by more than 5% from one ampoule to another.

Inhibitors. Inhibitors were either incubated with the enzyme 30 min. before the start of the reaction or were added simultaneously with the substrate. Because of the high extinction of some inhibitors at 293 $m\mu$, it was necessary to include the inhibitor in the blank cell.

Paper chromatography. The descending method was used on Whatman paper no. 1, with 95% (v/v) ethanol-water-

acetic acid (17:2:1, by vol.) as solvent. Spots were located by their fluorescence under a Mineralight ultra-violet lamp, emitting light of about 255 m μ .

RESULTS

6-Thiouric acid as a substrate of urate oxidase

The only thio analogue susceptible to attack by uricase is 6-thiouric acid. At a concentration of 3 μ g./ml., and in the presence of 400 μ g. of enzyme, it is degraded at a uniform rate of 0.485 μ mole/l./min. under the conditions of Fig. 1 (curve A). Thereafter the reaction slows down progressively. This reaction can be compared with the degradation of uric acid in presence of 50 μ g. of enzyme (curve B) where the corresponding rate is 4.6 μ -moles/l./min. The activity of urate oxidase towards 6-thiouric acid is thus

$$(100 \times 0.485)/(8 \times 4.6) = 1.3 \%$$

of the activity towards uric acid.

These observations should be considered together with Fig. 2, which relates velocity of oxidation at pH 9.3 to substrate concentration. At low concentrations of 6-thiouric acid, the decline is obviously due to consumption of substrate. However, different results are obtained at higher substrate concentrations. Uric acid, in the range 12–35 μ g./ml., reacts at its maximum velocity, which remains constant until less than 12 μ g./ml. is left. On the other hand, although an increase in the concentration of 6-thiouric acid from 3 to 23 μ g./ml. leaves the initial rate unchanged, it leads to premature retardation of the reaction. Thus in Fig. 1, curve C, where 10 μ g. of 6-thiouric acid/ml. is used, the rate decreases to about one-half after the oxidation of only 0.5 μ g./ml., and then remains constant at the lower level for another hour.

The premature decline in the rate of oxidation of 6-thiouric acid at higher substrate concentrations may be due to the formation of an intermediate with inhibitory properties. In those cases where the enzymic degradation went to completion, on the basis of disappearance of ultraviolet absorption, it was impossible to isolate from the reaction mixture by paper chromatography any component fluorescing under ultraviolet light. After complete degradation of 6-thiouric acid, full enzymic activity remained. Thus when uric acid was added to enzyme which had been incubated for 24 hr. with 6-thiouric acid, the rate of uric acid oxidation was identical with that of a control with fresh enzyme. Therefore any inhibitory intermediate that might have been formed was ultimately destroyed.

However, when the initial spectral changes were observed in the presence of catalase, which prolongs the life of the unstable intermediates in uric

acid oxidation (Praetorius, 1948b), there appeared to be a small increase in extinction on both sides of the absorption peak of 6-thiouric acid, especially in the region 270–290 m μ , but not at the maximum itself, indicating the formation of a primary degradation product with higher absorption in these regions than the starting material (Fig. 3). However, this unknown intermediate vanished rapidly and the reaction then proceeded in the same way as in the absence of catalase.

If the oxidation of 6-thiouric acid passes through the thio analogues of any of the intermediates proposed by previous authors (Klemperer, 1945; Praetorius, 1948b; Bentley & Neuberger, 1952), the 6-thio group should appear at some stage as a

Fig. 1. Oxidation of uric acid and 6-thiouric acid by urate oxidase. A, $\bigcirc$, 6-Thiouric acid, 3 μ g./ml.; enzyme, 400 μ g./ml.; readings at 347 m μ . B, $\bigcirc$, Uric acid, 12 μ g./ml.; enzyme, 50 μ g./ml.; readings at 293 m μ . C, $\bullet$, 6-Thiouric acid, 10 μ g./ml.; enzyme, 200 μ g./ml. The ordinate for curve C represents $E - 1$.

Fig. 2. Activity of urate oxidase as a function of substrate concentration at pH 9.3. $\bigcirc$, Uric acid; enzyme, 50 μ g./ml. (inner ordinate). $\bullet$, 6-Thiouric acid; enzyme, 67 μ g./ml. (outer ordinate). The temperature was 29°.

thiocarboxylic group, which should react with hydroxylamine to form a stable hydroxamic acid (Feigl, 1954). Since hydroxylamine inhibits urate oxidase, these experiments were carried out with 3.5 mM-hydroxylamine which produced about 30% inhibition. However, all attempts to isolate an intermediate in this way were unsuccessful. The presence of an inhibitory intermediate in the initial stages of the reaction could not be tested against uric acid as substrate because 6-thiouric acid itself is a very effective inhibitor of urate oxidase (see below).

Kinetic constants for urate oxidase

To derive the Michaelis-Menten constants of the enzyme, the results of Fig. 2 were replotted according to Dixon (1953). The value obtained for 6-thiouric acid is about one-seventh of the corresponding value for uric acid (Table 1), thus demonstrating that the lower rate of oxidation of the former cannot be attributed to a low affinity for the enzyme.

Fig. 3. Initial spectral changes during the oxidation of 6-thiouric acid in the presence of catalase at pH 7.3. The concentrations were: substrate, 30 $\mu\text{g./ml.}$; urate oxidase, 100 $\mu\text{g./ml.}$; catalase, 1 unit/ml. The temperature was 29°. ●, 270 $\text{m}\mu$; ○, 285 $\text{m}\mu$.

pH-dependence of reactions catalysed by urate oxidase

The pH-activity curves for uric acid and its 6-thio derivative (Fig. 4) both exhibit a similar unsymmetrical shape, but the curve for 6-thiouric acid is shifted to lower pH. The divergence may reflect a different state of ionization. The pK values of uric acid are 5.75 and 10.3 (Bergmann & Dikstein, 1955), whereas the corresponding figures for 6-thiouric acid, derived from Fig. 5, are 3.4 and 9.9. These values establish that, at the optimum pH of enzymic oxidation, both substrates are present predominantly as monoanions. However, for elucidation of the reaction mechanism and for an understanding of the slow rate of oxidation of 6-thiouric acid, it is important to localize the dissociation processes within the molecule. The ionization of uric acid involves first N-9, then N-3 (Bergmann & Dikstein, 1955). A similar study for 6-thiouric acid has not yet been undertaken. The only N-methylated derivative known, namely 3-methyl-6-thiouric acid (Levin *et al.* 1960), possesses a single pK value of 10.6 (Fig. 5). The lack of a dissociation corresponding to pK 3.4 of the mother substance suggests that in 6-thiouric acid ionization takes place first at N-3. Because of this result, the second dissociation step in 6-thiouric acid may involve N-1.

Thiouric acids as inhibitors of urate oxidase

Neither 2- nor 8-thiouric acid, nor 6,8-dithiouric acid, is attacked by the enzyme, but each acts as an inhibitor. Though 2-thiouric acid inhibited only slightly, the 8-isomer proved very effective, its K_i being 0.39 μM (Table 1). Fig. 6 demonstrates competitive inhibition, as the curves in the presence and absence of 8-thiouric acid intersect at the ordinate (Dixon & Webb, 1958). The formation of

Table 1. Michaelis-Menten constants and inhibitory activity of various uric acids

The K_m values were derived by replotting the results of Fig. 2 in the form of s/v as a function of s (Dixon, 1953). The straight lines so obtained intersect the abscissa at a point defined as $-K_m$. The concentrations of inhibitors producing 50% inhibition, I_{50} , of uric acid oxidation were determined from a plot of percentage inhibition against inhibitor concentration. For these experiments the conditions were: uric acid, 12 $\mu\text{g./ml.}$; urate oxidase, 50 $\mu\text{g./ml.}$; temp., 29°; pH 9.3. Uric acid and various concentrations of 6-thiouric acid were added at the same time as the enzyme. With the other thio analogues, urate oxidase was incubated with the inhibitor for 10 min. before the addition of the substrate. The inhibitor constants K_i were obtained from Fig. 6, according to Dixon & Webb (1958).

Compound	$\lambda_{\text{max.}}$ at pH 8 ($\text{m}\mu$)	$\log \epsilon_{\text{max.}}$	K_m (μM)	I_{50} (μM)	K_i (μM)
Uric acid	293	4.03	12	—	—
6-Thiouric acid	347	4.32	1.8	14	2.6
2-Thiouric acid	275	4.19	—	250	—
	308	4.07			
8-Thiouric acid	260	3.97	—	5	0.39
	307	4.18			
6,8-Dithiouric acid	266	4.24	—	0.38	—
	369	4.46

Fig. 4. pH-activity curves for urate oxidase. ○, Uric acid, 12 $\mu\text{g./ml.}$; enzyme, 50 $\mu\text{g./ml.}$ ●, 6-Thiouric acid, 10 $\mu\text{g./ml.}$; enzyme, 200 $\mu\text{g./ml.}$ The temperature was 29°. The buffers used were: for pH 5.8–8.5, 0.05M-phosphate; for pH 9.0–11.0, 0.067M-glycine-sodium hydroxide.

Fig. 5. Absorption maxima as a function of pH. ●, 6-Thiouric acid. ○, 3-Methyl-6-thiouric acid. The following buffers were used: pH -3, 13.5N- H_2SO_4 ; pH -1, 5N- H_2SO_4 ; pH 0 to 3, perchloric acid; pH 4 to 6, 0.1M-acetate; pH 6 to 8, 0.1M-phosphate; pH 8 to 11, 0.1M-borate, adjusted with NaOH; pH 11 to 14, sodium hydroxide.

the enzyme-inhibitor complex is practically instantaneous, as no difference in the rate of uric acid degradation could be detected whether 8-thiouric acid was added simultaneously with uric acid or 30 min. earlier. 6,8-Dithiouric acid was even more effective than the 8-thio derivative.

These observations led us to test 6-thiouric acid as an inhibitor. To prevent the possible interference of (hypothetical) inhibitory intermediates, formed during its degradation (see above), a mixture of uric acid and its 6-thio analogue was added to the enzyme and the rate extrapolated to zero time. 6-Thiouric acid is a competitive inhibitor (Fig. 6). Within experimental error, its K_i is close to its K_m (Table 1). The latter thus measures essentially the equilibrium between free and bound substrate.

pH-dependence of the inhibitory activity of 6-thiouric acid

When a compound can serve both as substrate and inhibitor, the first step in the formation of a complex with the enzyme must be identical for both actions. Therefore we compared the influence of pH on the inhibitory activity of 6-thiouric acid with the influence on the rate of its catalysed oxidation (Fig. 4). Discrepancies between these two functions may shed light on the question whether the rapid decline of the rate of oxidation on either side of the pH optimum is due to differences in the affinity of various ionic forms of the substrate for the active surface or to inability of the enzyme to attack a specific structure bound in the enzyme-substrate complex. The results in Table 2 show that the two functions do not closely parallel

Fig. 6. Reciprocal rate of oxidation of uric acid as a function of the reciprocal of the substrate concentration. Each system contained uric acid and urate oxidase, 50 $\mu\text{g./ml.}$ ○, No inhibitor; ●, in the presence of 6-thiouric acid, 1 $\mu\text{g./ml.}$; ▲, in the presence of 8-thiouric acid, 0.5 $\mu\text{g./ml.}$ Inhibitor and substrate were added simultaneously with the enzyme. The temperature was 29°, and the pH 9.3.

Table 2. Influence of pH on the rate of enzymic oxidation and on the inhibitory activity of 6-thiouric acid

Relative rates represent the ratio of the reaction velocity at a given pH to the optimum rate at pH 9.3. The conditions were: urate oxidase, 200 $\mu\text{g./ml.}$; substrate, 10 $\mu\text{g./ml.}$, temp., 29°. The rate was measured at 347 m μ . The relative inhibitory activity expresses the inhibitory effect at a given pH as a percentage of its maximal value at pH 8.0-9.3. The inhibition was measured at 29° by adding a mixture of 3 $\mu\text{g.}$ of 6-thiouric acid/ml. and 12 $\mu\text{g.}$ of uric acid/ml. to an enzyme solution containing 50 $\mu\text{g./ml.}$ The following buffers were used: pH 5.75-8.5, 0.05M-phosphate; pH 9.0-11.0, 0.067M-glycine-sodium hydroxide.

pH	Relative rate of oxidation of 6-thiouric acid	Relative inhibitory activity of 6-thiouric acid	Percentage of monoanion	Percentage of bisanion
5.75	37	46	99	—
6.8	55	82	100	—
8.0	80	100	99	1
8.6	95	100	95	5
9.3	100	100	80	20
9.8	63	74	56	44
10.2	25	56	33	67
10.6	—	48	17	83

each other. Both above and below pH 9.3, the inhibitory activity of 6-thiouric acid decreases much more slowly than its enzymic rate. Comparison with Fig. 4 indicates that in the alkaline range of pH the affinities of urate oxidase for both uric acid and its 6-thio derivative decline in parallel, whereas on the acid side the binding power for the former decreases much more rapidly.

DISCUSSION

Bentley & Neuberger (1952) have proposed a mechanism for the enzymic breakdown of the monoanion (I) of uric acid, in which the negative charge is placed by resonance also at C-5 (II). Abstraction of 2 electrons by the enzyme reverses the sign of the charge, and the resulting carbonium ion (III) now attacks the NH-1 group in an electrophilic substitution process. Since the first dissociation step involves N-9 (Bergmann & Dikstein, 1955), the original scheme of Bentley & Neuberger must be slightly modified as shown, although this

change does not alter the basic hypothesis of these authors. In the bisanion of uric acid, the same type of charge transfer can still take place, although the second negative charge (at N-3) probably diminishes the positive charge of the carbonium ion at C-5 and thus may be responsible for the decline of the enzymic rate in alkaline media.

Application of the above mechanism to 6-thiouric acid stresses the importance of localization of the dissociation processes within the molecule. If the first ionization step involves N-3, a charge transfer to C-5 is again feasible. As the rate of oxidation of this substrate in the alkaline range declines much faster than its inhibitory activity, it may be concluded that its bisanion, in contrast with that of uric acid, cannot be attacked by the enzyme. Thus, at pH 9.8, 6-thiouric acid solution contains approximately 56 % of mono- and 44 % of bis-anions, and its rate of degradation is reduced to about 63 % (Fig. 4 and Table 2). A similar relationship does not hold for uric acid, which at pH 10.8, for example, still reacts with about 60 % of its maximal speed, although its solution now contains 24 % of mono- and 76 % of bis-anions. The latter, although inferior to the monoanionic form, are thus still susceptible to enzymic attack. The hypothesis that the bisanions of 6-thiouric acid are refractory towards urate oxidase may also explain the slight shift of the pH optimum of this substrate to the acid side (see Fig. 4), as at pH 9.3 already 20 % of the inactive, double negatively charged, form is present. This discrepancy may be attributed to a different localization of the second negative charge. If N-1 is indeed involved in the second dissociation step of 6-thiouric acid, then resonance with the 6-thiocarbonyl group:

creates a negatively charged region which prevents placement of a negative charge at the neighbouring C-5. A similar effect does not arise in the bisanion of uric acid, because here both negative charges exert a synergistic influence with respect to the charge transfer to C-5.

In the acid range of pH, the enzymic rate with uric acid declines much faster than that with 6-thiouric acid. At pH 7.0, where the solution of uric acid contains 95 % of monoanions and 5 % of neutral molecules, oxidation is slowed down to 36 % of the peak value at pH 9.3 (Fig. 4). A similar situation is encountered with 6-thiouric acid. At pH 7.0, the thio acid is oxidized at 57 % of the maximal rate (at pH 9.0), although the contribution of the monoanionic form is still higher than 99 %. Since the decline of reaction velocities below pH 9 is thus not due to changes in the ionization of the substrate, it may be related to structural alterations in the active surface or in the enzyme-substrate complexes. The fall in reaction rate in the acid range occurs at a higher pH for uric acid than for its 6-thio analogue, presumably because the latter forms with urate oxidase a complex of higher stability, as evidenced by its lower K_m value. These conclusions are also supported by the observation that the rate of oxidation of 6-thiouric acid in the acid range declines considerably faster than its inhibitory power. Acid conditions thus impair the oxidative mechanism of urate oxidase before they weaken its binding power for purines.

These considerations do not account for the low rate of degradation of 6-thiouric acid. Probably the critical step in the scheme of Bentley & Neuberger (1952), namely the electron transfer, involves the copper atom at the active centre. The metal complex may become stabilized by inclusion of a thiocarbonyl group, as evidenced by the low K_m and K_i values with 6-thiouric acid, and thus may be less easily reduced. Although 8-thiouric acid exhibits high affinity to urate oxidase, it is completely refractory to enzymic attack. An answer to this problem may be sought in its ionization sequence which is entirely unknown at present. An even more intricate problem is posed by 2-thiouric acid. It appears possible that its low affinity for the enzyme arises from the position of the sulphur atom. This question may be approached by studying the influence of the 2-thiocarbonyl group on the binding power of purines to urate oxidase. The present results provide clear indications that the effect of a substituent depends on its location in the heterocyclic ring system and invites a more thorough investigation of the relation between structure and inhibitory activity in the purine series. Such experiments may furnish information on the specific groups participating in

the attachment of substrates or inhibitors to the active surface.

Finally, it should be recalled that 6-thiouric acid had been isolated from the urine of mice (Elion, Bieber & Hitchings, 1954) and of humans (Hamilton & Elion, 1954) after injection or administration by mouth of 6-mercaptopurine. Excretion of the metabolite by mice appears surprising since these animals degrade uric acid completely. It may be due to the very slow rate of oxidation of 6-thiouric acid which permits part of the compound to escape enzyme action. On the other hand, one would then expect also strong diminution by competitive inhibition of the turnover of uric acid. These considerations suggest that application of thiouric acids to such animals may cause the appearance of uric acid in their urine.

SUMMARY

1. Of the thiouric acids, only the 6-thio analogue is oxidized by liver urate oxidase and at a rate about 1 % of the breakdown of uric acid.
2. The pH-activity curve of 6-thiouric acid is similar to that of uric acid, but is shifted towards the acid side. It appears that the bisanion of the 6-thio analogue is refractory towards urate oxidase, in contrast with the bisanion of uric acid.
3. Thiouric acids inhibit urate oxidase in the descending order: 6,8-dithio-, 8-thio-, 6-thio-, 2-thio-uric acid.
4. The inhibitory effect of 6-thiouric acid is influenced less by pH changes than is the rate of its enzymic oxidation.
5. The differences between uric acid and its 6-thio analogue are tentatively explained by the assumption that dissociation in the latter involves first N-3, then N-1, whereas the ionization of uric acid follows the sequence N-9 and N-3.

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Nucleic Acid Metabolism of Mammalian Spermatozoa

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The absence of ribonucleic acid from spermatozoa has been reported by several workers (Brachet, 1933; Mauritzen, Roy & Stedman, 1952; White, Leslie & Davidson, 1953; Mann, 1954; Daoust & Clermont, 1955; Bhargava, Bishop & Work, 1959a). RNA is considered to be an essential component of all protein-synthesizing systems. Therefore the demonstration that suspensions of spermatozoa can incorporate radioactive amino acids into their proteins (Bhargava, 1957; Bhargava, Bishop & Work, 1959b) was of interest. Martin & Brachet (1959), on the basis of micro-radioautographic studies, concluded that this incorporation did not actually take place in the spermatozoa but occurred in the cell debris or bacteria contained initially in the semen. Recent work (Abraham & Bhargava, 1963; P. T. Iype, K. A. Abraham & P. M. Bhargava, unpublished work) has, however, demonstrated that the above-mentioned incorporation occurs in the spermatozoa and represents synthesis of spermatozoal proteins; this work has also afforded an explanation for the negative findings of Martin & Brachet (1959). It therefore appeared to be of interest to re-investigate the reported absence of RNA from spermatozoa.

In most of the earlier experiments, the RNA estimations were made on small quantities of semen. In the investigation by Bhargava *et al.* (1959a) the lower limit of detection of RNA in bull spermatozoa was 0.1% of the deoxyribonucleic acid, or 0.033% of the dry weight of the cells. It therefore seemed possible that spermatozoa might contain a small amount of RNA, which could have escaped detection in earlier investigations with conventional analytical techniques. The presence of even traces of RNA in spermatozoa would be significant from the point of view of protein syn-

thesis by these cells. Recent investigations suggest that the code for the determination of the sequence of amino acids in a protein molecule is carried from the DNA in the form of a short-lived RNA, the 'messenger RNA' (Brenner, Jacob & Meselson, 1961; Gros *et al.* 1961). Both the 'messenger RNA' and the 'soluble RNA' (which is believed to carry the activated amino acids to the site of protein synthesis), are unlikely to represent more than a small fraction of the total RNA content of somatic mammalian cells.

This paper reports studies on the incorporation of radioactive precursors of nucleic acids into the total nucleic acid fraction of bovine spermatozoa; the effect of a few inhibitors on this incorporation has also been studied. These studies show the presence of minute quantities of a metabolically active RNA in mature bovine spermatozoa. A preliminary communication based on this work has already appeared (Abraham & Bhargava, 1961).

EXPERIMENTAL

Materials

Semen. The semen was obtained from two normal, fertile buffaloes (BB1 and BB2), and from five normal, fertile bulls (CB1, CB2, CB3, CB4 and CB5). Semen was collected by the artificial-vagina technique (Walton, 1945) under conditions which minimized bacterial contamination; precautions were also taken to avoid contamination of the semen with talc from the rubber. The semen was collected, on an average, once a week from each bull. The samples were checked, just before use, under a phase-contrast microscope for motility and for the presence of possible contaminants such as bacteria and cell debris. The semen of these animals appeared quite normal in all respects, except for the relative abundance of cytoplasmic droplets in the semen of bull CB3. Usually, the spermatozoa collected after an interval of more than 3 weeks from the date