nucleic acid polymerase was used and much less is known about the effects of ethidium bromide on bacteria; Richmond (1959) found that the synthesis of a lytic enzyme by *Bacillus subtilis* was inhibited 50% by this drug at a concentration of 350 µg./ml. B. A. Newton (unpublished work) has observed that the growth of *E. coli* is inhibited by the drug at a concentration of 35–75 µg./ml.

The shift in the absorption spectrum of ethidium bromide when mixed with DNA observed in the present work shows that this compound is capable of forming a complex with DNA, but there is no evidence that the action of the drug is related to this capability. However, the biological action of actinomycin is believed to be related to its ability to form a complex with DNA (Rauen, Kersten & Kersten, 1960; Kawamata & Imanishi, 1961).

SUMMARY

1. The action of a number of antibiotics and other drugs on deoxyribonucleic acid polymerase of *Escherichia coli* has been studied by a modified assay procedure.

2. Actinomycin D inhibits at relatively high concentration, whereas the phenanthridine drug, ethidium bromide, is a powerful inhibitor at growth-limiting concentrations. The absorption spectrum of ethidium bromide shows a marked shift on mixing with DNA, showing complex-formation between the two, although it is not known whether the inhibitory effect of the drug is related to this capability. Neither ethidium bromide nor actinomycin D causes irreversible destruction of DNA primer.

3. The trypanocidal drug, antrycide, inhibits DNA synthesis but this is probably due to simple physical removal of DNA primer by precipitation.

A range of other antibiotics tested had no significant effect on the enzyme system.

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Biochem. J. (1963) **86**, 567

Relation of Structure to the Inhibitory Activity of Purines against Urate Oxidase

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In a previous paper (Bergmann, Ungar-Waron & Kwietny-Govrin, 1963) on the interaction of thiouric acids with mammalian urate oxidase it was reported that the position of the sulphur atom has a decisive influence. The sequence of decreasing affinity is as follows: 6,8-dithiouric acid, 8-thiouric

acid, 6-thiouric acid, 2-dithiouric acid. These results made it desirable to determine separately the binding power of the thiocarbonyl group in each of the three available positions in the purine molecule in order to evaluate their contribution to the formation of the enzyme-inhibitor complex. Such

studies may help to characterize the binding groups in the active centre of the enzyme and to elucidate the structure of the enzyme-substrate complex of urate oxidase.

MATERIALS AND METHODS

Purines. The following compounds were prepared by known methods: 2- and 8-hydroxypurine and 6,8-dihydroxypurine (Albert & Brown, 1954); 2,8-dihydroxypurine (Kalmus & Bergmann, 1961); 1-methyl-2-oxopurine (Johns, 1912); 3-methylxanthine (Bredereck, Schuh & Martini, 1950); 6-hydroxy-3-methyl- and 3-methyl-2-oxopurine (Bergmann, Levin, Kalmus & Kwietny-Govrin, 1961); 1- and 3-methyluric acid (Bergmann & Dikstein, 1955); 7-methyluric acid (Dikstein & Bergmann, 1955).

2-Hydroxy-6-methylpurine was the commercial product obtained from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.

Thiopurines. The following compounds were prepared by known methods: 8-hydroxy-6-mercaptopurine (Bergmann & Kalmus, 1961); 8-hydroxy-6-methylmercaptopurine and 6-hydroxy-8-mercaptopurine (Robins, 1958); 6,8-dimercaptapurine (Bergmann & Kalmus, 1962); 2- and 6-thioxanthine (Beaman, 1954); 8-hydroxy-2-mercaptopurine (Noell & Robins, 1959*a*); 2-, 6- and 8-thiouric acid (Bergmann *et al.* 1963); 2,6- and 2,8-dithiouric acid (Noell & Robins, 1959*b*); 6,8-dithiouric acid (Bergmann & Kalmus, 1962); 6,8-dihydroxy-2-methylmercaptopurine (Noell & Robins, 1959*a*); 3-methyl-6-thioxanthine and 3-methyl-2-thiouric acid (Bergmann *et al.* 1961); 3-methyl-2-thioxanthine (Elion, 1957); 7-methyl-6-thioxanthine (Prasad & Robins, 1957); 3-methyl-6-thiouric acid (Levin, Kalmus & Bergmann, 1960); 3-methyl- and 1,3-dimethyl-8-thiouric acid (Beilstein, 1937); 4,5-diamino-6-thiouracil (Levin *et al.* 1960).

6-Mercaptopurine was the commercial product obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.

2-Hydroxy-8-mercaptopurine was synthesized by F. Bergmann & A. Kalmus (unpublished work).

8-Azapurines. 8-Aza-6-hydroxypurine and 8-azaxanthine were prepared by the method of Roblin, Lampen, English, Cole & Vaughan (1945), and 8-aza-2-hydroxypurine as described by Bergmann, Levin & Kwietny (1959).

New compounds. (i) 2,8-Dihydroxy-6-methylmercaptopurine (Ia). 6-Thiouric acid (1 g.) (Levin *et al.* 1960) was dissolved in 10 ml. of 0.5N-sodium hydroxide and stirred with 1 g. of methyl iodide at room temperature for 1 hr. Acidification of the reaction mixture with acetic acid gave 0.9 g. (84%) of 2,8-dihydroxy-6-methylmercaptopurine. Crystallization from water gave needles decomposing above 300°, λ_{max} (in buffer, pH 8.0) 331 m μ , R_F [descending method with 95% (v/v) ethanol-acetic acid-water (17:1:2, by vol.) as solvent] 0.46 (Found: C, 36.2; H, 3.2; N, 28.1. $\text{C}_8\text{H}_8\text{N}_4\text{O}_5\text{S}$ requires C, 36.4; H, 3.0; N, 28.3%).

(ii) 8-Hydroxy-3-methyl-6-methylmercapto-2-oxopurine (Ib). 3-Methyl-6-thiouric acid (Levin *et al.* 1960) was methylated as described above. 8-Hydroxy-3-methyl-6-methylmercapto-2-oxopurine, obtained in 93% yield, crystallized from water in clusters of needles, decomp. above 330°, λ_{max} (in buffer, pH 8.0) 228 and 338 m μ , R_F (chromatography as described above) 0.56 (Found: C,

40.0; H, 4.1; N, 26.0. $\text{C}_7\text{H}_8\text{N}_4\text{O}_5\text{S}$ requires C, 39.6; H, 3.8; N, 26.4%).

(iii) 2,6-Dihydroxy-8-methylmercaptopurine was prepared in an analogous manner from 8-thiouric acid by the use of 1.0 equiv. of methyl iodide. The compound, obtained in 65% yield by recrystallization from water, was microcrystalline, decomp. above 310°, λ_{max} (in buffer, pH 8.0) 291–292 m μ , R_F (chromatography as described above) 0.47 (Found: C, 34.9; H, 3.0; N, 26.5. $\text{C}_8\text{H}_8\text{N}_4\text{O}_5\text{S}, \frac{1}{2}\text{H}_2\text{O}$ requires C, 34.8; H, 3.4; N, 27.0%). By the same method Biltz & Beck (1928) obtained 8-methylmercaptopurine, apparently by the use of excess of methyl iodide.

(iv) 2-Hydroxy-6-methylmercaptopurine resulted in 76% yield from the methylation of 6-thioxanthine. It crystallized from 50% (v/v) ethanol in rods decomposing above 300°, λ_{max} (in buffer, pH 8.0) 318 m μ , R_F (chromatography as described above) 0.6 (Found: C, 36.4; H, 3.9; N, 28.2. $\text{C}_8\text{H}_8\text{N}_4\text{O}_5\text{S}, \text{H}_2\text{O}$ requires C, 36.0; H, 4.0; N, 28.0%).

Pteridines. The pteridines used in the present study were a gift of Professor A. Albert, National Australian University, Canberra (Albert, Brown & Cheeseman, 1951; Albert, Lister & Pedersen, 1956).

Enzymic methods. In all experiments, 50 $\mu\text{g.}/\text{ml.}$ of urate oxidase ('uricase' of Leo Pharmaceuticals Inc., Ballerup, Denmark) was used. This concentration degrades, at 29° and pH 9.3, 4.6 $\mu\text{m.}$ -moles of uric acid/ml./min., when the substrate concentration is 71 $\mu\text{M.}$ The disappearance of uric acid was measured by the decrease of extinction at 293 m μ . Reactions were carried out in 10 mm. silica cells at a temperature of 29 $\pm$ 0.5°, which was maintained by a thermostat. Enzyme and inhibitor were preincubated for 30 min. during which period the refractoriness of each new compound towards urate oxidase was checked. Then 12 $\mu\text{g.}$ of uric acid/ml. was added. The concentration of inhibitor was varied so as to cover the range of 50% inhibition. The I_{50} value was derived from a plot of percentage inhibition against log (inhibitor concentration).

Buffers. For pH 9.0–11.0, 67 mM-glycine-sodium hydroxide was used; 0.05M-phosphate (NaH_2PO_4 – Na_2HPO_4) served for the pH range 5.8–8.5.

RESULTS

Importance of the oxygen atoms of uric acid for binding to urate oxidase. The contribution of each carbonyl group in uric acid to the overall affinity of this substrate may be evaluated from the inhibitory activity of the isomeric monohydroxypurines. Table 1 reveals the following descending order: 2-, 8-, 6-hydroxypurine. In accordance with these results are the effects of combination of two oxygen atoms in the isomeric dihydroxypurines. With the latter the order of decreasing inhibitory power is: 2,8-, 2,6-, 6,8-dihydroxypurine (Table 1).

Thiopurines as inhibitors of urate oxidase. The order, established for hydroxypurines, is disturbed when a sulphur substituent is introduced (Table 2). Replacement of oxygen by sulphur at C-2 greatly reduces the affinity for the enzyme. Thus 2-thioxanthine is only 10% as effective as xanthine, and similar ratios were found for the pairs 6-thio- and

2,6-dithio-uric acid, and 8-thio- and 2,8-dithio-uric acid. 8-Hydroxy-2-mercaptapurine possesses only about 1% of the affinity of 2,8-dihydroxypurine.

At C-6 the effect of oxygen-sulphur exchange depends on the other substituents present. In combination with a 2-carbonyl group, the 6-thio-carbonyl group augments the affinity for urate oxidase. Thus 6-thioxanthine is nearly 7 times as effective as xanthine. Similarly, the affinity of 6-thiouric acid, as expressed by its K_m (1.8 μM), is about 7 times as great as that of uric acid [K_m , 12 μM ; Bergmann *et al.* (1963)]. Finally, the inhibitory activity of 6,8-dithiouric acid is approximately 13 times as great as that of 8-thiouric acid.

On the other hand, in the absence of oxygen at C-2, i.e. when combination with the enzyme can occur only at position 8, the oxygen-sulphur exchange at C-6 increases the affinity only slightly, if

at all. Thus, as inhibitors, 8-hydroxy-6-mercaptapurine is nearly as effective as 6,8-dihydroxypurine, and 6,8-dimercaptapurine is only 1.5 times as effective as 6-hydroxy-8-mercaptapurine. 6-Mercaptapurine itself is only about one-third as effective as hypoxanthine.

Finally, replacement of oxygen by sulphur at C-8 decreases the inhibitory activity, when only one other substituent is present, but increases it when both positions 2 and 6 are substituted. This is clearly seen in Table 2 by comparing first 2,8- and 6,8-dihydroxypurine with their respective 8-mercapto derivatives and then comparing 2- or 6-thiouric acid with the corresponding dithiouric acids, bearing the second sulphur atom at C-8.

Influence of methylmercapto groups on the affinity of inhibitors for urate oxidase. The above findings indicate a specific relationship between oxygen at C-2 and sulphur at C-6 in the purine ring system. The pronounced enhancement of inhibitory activity which is observed when both substituents are present suggests that the 2-carbonyl group mediates binding of the purine ring in such a manner as to place sulphur at C-6 near the copper atom of urate oxidase and thus enables it to join the co-ordination sphere of the metal atom. Bivalent copper has only a weak tendency to form complexes with thio-carbonyl derivatives, such as thiourea, or with thioethers, unless chelation is possible. However, very stable complexes of Cu^+ ion with either type of sulphur derivative are known (Sidgwick, 1950). In the 6-thiopurines tested, tautomerism may occur ($\text{HN}\cdot\text{C}:\text{S}\rightleftharpoons\text{N}:\text{C}\cdot\text{SH}$). The sulphydryl form could reduce the copper atom to its univalent state, in which complex formation with another molecule of thiopurine could take place. Such a process can be barred by converting the thiopurines into their *S*-methyl ethers.

The results with some thioethers are summarized in Table 3. Though 2- or 8-methylmercapto derivatives are less effective than the corresponding thiopurines, the situation is more complex for 6-thiocarbonyls. Thus 6-thioxanthine is a better inhibitor than its 6-methylmercapto derivative. On the other hand, combination of a 8-hydroxyl with a 6-thioether group is more effective than with a 6-thiocarbonyl group. The greatest enhancement is attained when both C-2 and C-8 bear oxygen. The strongest inhibitor so far found is 2,8-dihydroxy-6-methylmercaptapurine (Ia), of which as little as 0.03 $\mu\text{g./ml.}$ produces 50% inhibition of urate oxidase under the standard conditions. A similar increase in affinity is observed when 3-methyl-6-thiouric acid is converted into its *S*-methyl ether (Ib). On this basis the 6-*S*-methyl derivative of 6,8-dithiouric acid should exhibit an extraordinarily high inhibitory activity. However, attempts to synthesize this compound have not

Table 1. *Inhibitory activity of mono- and di-hydroxypurines against urate oxidase*

Enzyme (50 $\mu\text{g./ml.}$) was incubated with various concentrations of inhibitor for 30 min. in the presence of 67 mm-glycine-sodium hydroxide buffer, pH 9.3, before uric acid (12 $\mu\text{g./ml.}$) was added. The disappearance of the substrate was measured at 293 m μ . The concentrations producing 50% inhibition, I_{50} , were obtained from a plot of percentage inhibition against log (inhibitor concentration). The inhibitory effect of xanthine has also been studied by van Pilsun (1953).

Purine derivative	pK	I_{50} (μM)
Monohydroxypurines		
Hypoxanthine	8.8	220
8-Hydroxypurine	7.9	110
2-Hydroxypurine	8.5	12
Dihydroxypurines		
2,8-Dihydroxypurine	7.5	5.2
Xanthine	7.7	18
6,8-Dihydroxypurine	8.0	66

Table 2. *Inhibitory effect of thiopurines on urate oxidase*

The experimental conditions were as described in Table 1.

Purine derivative	I_{50} (μM)
Monosubstituted purines	
6-Mercaptapurine	~ 700
Disubstituted purines	
6-Thioxanthine	2.7
2-Thioxanthine	190
8-Hydroxy-6-mercaptapurine	70
6-Hydroxy-8-mercaptapurine	~ 500
6,8-Dimercaptapurine	370
8-Hydroxy-2-mercaptapurine	500
2-Hydroxy-8-mercaptapurine	12
Trisubstituted purines	
2-Thiouric acid	250
6-Thiouric acid	14
8-Thiouric acid	5
2,6-Dithiouric acid	150
2,8-Dithiouric acid	80
6,8-Dithiouric acid	0.4

Table 3. *Inhibitory activity of methylmercaptapurines against urate oxidase*

The experimental conditions were as described in Table 1. *R* represents the ratio of the I_{50} value of methylmercaptapurine to that of the corresponding thiopurine.

Purine derivative	I_{50} (μ M)	<i>R</i>
6,8-Dihydroxy-2-methylmercaptapurine	> 500	> 2
2,6-Dihydroxy-8-methylmercaptapurine	38	7
2-Hydroxy-6-methylmercaptapurine	6	2.2
8-Hydroxy-6-methylmercaptapurine	32	0.5
2,8-Dihydroxy-6-methylmercaptapurine (Ia)	0.13	0.01
8-Hydroxy-3-methyl-6-methylmercapto-2-oxopurine (Ib)	7	0.017

yet been successful. The enzyme-inhibitor complex of compound (Ia) must be only very slightly dissociated. The character of the combination of

a: R = H; b: R = CH₃

compound (Ia) with the enzyme was tested by the method of Dixon & Webb (1958). Fig. 1 reveals that this compound, in contrast with all other inhibitors studied, exhibits a mixed, i.e. partly competitive and partly non-competitive, behaviour. The related compound (Ib) is a purely competitive inhibitor (Fig. 1).

Importance of the purine skeleton for inhibitory activity. Replacement of the purine skeleton by analogous heterocyclic structures decreases or abolishes inhibitory activity (Table 4). Uracil, which carries the same substituents in the pyrimidine moiety as xanthine, has no effect on the enzyme. Even 4,5-diamino-6-thiouracil, which lacks only the carbon atom at position 8 of 6-thioxanthine, is a very feeble inhibitor. Absence of the imidazole ring thus decreases the affinity for the enzyme considerably. 2-Hydroxypteridine is completely inactive at the highest concentration that could be tested (30 μ g./ml.), and 2,4-dihydroxypteridine exhibits only 6% of the inhibitory activity of the related xanthine. Replacement of the imidazole ring by the pyrazine ring thus greatly weakens the binding power to the active centre. 2,4,6-Trihydroxy- and 2,4,6,7-tetrahydroxy-pteridine, which are both related to uric acid, possess only faint inhibitory activity. However, replacement of the imidazole moiety by the isosteric triazole ring enhances affinity, as was observed for 8-azaxanthine by Norris & Roush (1950) and is further substantiated here by comparison of 8-aza-2-hydroxy- and 8-aza-6-hydroxy-purine

Fig. 1. Reciprocal rate of oxidation of uric acid as a function of the reciprocal molar substrate concentration. The incubation mixtures contained uric acid and urate oxidase, 50 μ g./ml., with the following additions: O, none; ●, 8-hydroxy-3-methyl-6-methylmercapto-2-oxopurine, 1 μ g./ml.; □, 2,8-dihydroxypurine, 0.5 μ g./ml.; △, 2-hydroxy-6-methylmercaptapurine, 1 μ g./ml.; ▲, 2,8-dihydroxy-6-methylmercaptapurine, 0.01 μ g./ml. All incubations were carried out at pH 9.3 and 29°. Enzyme and inhibitor were incubated for 30 min. before the addition of uric acid. Rates were measured as decreases of extinction at 293 m μ . All concentrations refer to final values in the complete reaction mixture. For the competitive inhibitors, the inhibitor constants, K_i , were derived as described by Dixon & Webb (1958). The intercept on the x -axis = $1/K_p$, where $K_p = K_m[1 + (i/K_i)]$. Here K_m denotes the Michaelis-Menten constant of uric acid and i expresses the constant inhibitor concentration used with the different substrate concentrations. The K_i values are as follows: 2,8-dihydroxypurine, 0.53 μ M; 2-hydroxy-6-methylmercaptapurine, 0.49 μ M; 8-hydroxy-3-methyl-6-methylmercapto-2-oxopurine, 1.3 μ M.

(Table 4) with the corresponding purines (Table 1). Purine itself lacks any measurable inhibitory action. It can be supposed that this ring system places the substituents into proper positions relative to the binding groups of the active surface of the enzyme. However, with suitable substituents in the pyrimidine moiety the imidazole ring plays an important part in binding. Finally, thiourea and its *S*-methyl derivative are devoid of inhibitory activity.

Role of free NH groups in the formation of enzyme-substrate and enzyme-inhibitor complexes. Free NH groups are essential for the attachment of uric acid to the enzyme (Table 5). Thus 1- and 3-methyluric acid are neither substrates nor inhibitors at concentrations of up to 100 $\mu\text{g./ml.}$, though degra-

dation of the 1-methyl derivative by urate oxidase has been claimed by Paul & Avi-Dor (1954). These methylated uric acids have the same pK_1 (5.75) as uric acid itself (Bergmann & Dikstein, 1955). Therefore, at the optimum pH of the reaction (9.3), they are all present as anions. The unfavourable influence of *N*-methylation extends to other purines. Thus the 3-methyl derivatives of xanthine and hypoxanthine are without effect on urate oxidase. 3-Methyl-6-thiouric acid is 30 times, and 3-methyl-6-thioxanthine 60 times, less active as inhibitor than the non-methylated mother substance. Methylation in the imidazole moiety of the purine ring also has an adverse effect. 7-Methyluric acid and 7-methyl-6-thioxanthine are almost inactive. These examples again stress the contribution of the imidazole ring to the binding of inhibitor and enzyme. On the other hand the 3-methyl derivatives of 2-thioxanthine and 2-thiouric acid are about twice as active as their mother compounds.

Bergmann, Kwietny, Levin & Brown (1960) demonstrated that, in substrates of xanthine oxidase, free NH groups are often essential for a tautomerization process that occurs in the enzyme-substrate complex. Similar transformations may take place when purines form complexes with urate oxidase. The role of free NH groups has been studied more thoroughly with 2-hydroxypurine (IIa), which combines with xanthine oxidase in its tautomeric form (IIIa) (Bergmann *et al.* 1960). Neither the 3-methyl derivative (IIb) nor the 1-methyl derivative (IIIb) of 2-hydroxypurine, in both of which one of the possible tautomers is fixed, exerts any inhibitory effect on urate oxidase. On the other hand 2-hydroxy-6-methylpurine is almost as active as the non-methylated mother substance (see Table 5).

a: R = H; b: R = CH₃ a: R = H; b: R = CH₃

pH-dependence of inhibitory activities. The above experiments suggest that the purine skeleton places the various substituents into proper relation to the binding groups of the enzyme. If the three hydroxyl groups in uric acid combine with three different components of the active surface, they may be expected to react differently to variations of pH. Table 6 summarizes the changes of inhibitory activity with pH, taking pH 9.3 as the reference

Table 4. *Inhibitory activity of various heterocyclic compounds against urate oxidase*

The experimental conditions were as described in Table 1. —, No inhibitory effect.

Purine derivative	I_{50} (μM)
Uracils	
Uracil	—*
4,5-Diamino-6-thiouracil	38
Pteridines	
2-Hydroxypteridine	—†
2,4-Dihydroxypteridine	300
2,4,6-Trihydroxypteridine	150
2,4,6,7-Tetrahydroxypteridine	(~500)‡
8-Azapurines	
8-Aza-6-hydroxypurine	47
8-Aza-2-hydroxypurine	1.6
8-Azaxanthine	5.9
Purines	
Purine	—*

* No activity up to 100 $\mu\text{g./ml.}$

† The highest concentration that could be attained (30 $\mu\text{g./ml.}$) gave no inhibition.

‡ Because of limited solubility (the highest concentration tested was 30 $\mu\text{g./ml.}$) the I_{50} value was obtained by extrapolation of the plot of percentage inhibition versus log (inhibitor concentration), by assuming a linear relationship.

Table 5. *Inhibitory effects of N-methylpurines on urate oxidase*

The experimental conditions were as described in Table 1. *R* represents the ratio of the I_{50} value of the methylated purine to that of its non-methylated mother substance. —, No inhibitory effect.

Purine derivative	I_{50} (μM)	<i>R</i>
Uric acids and thiouric acids		
1-Methyluric acid	—	.
3-Methyluric acid	—	.
7-Methyluric acid	—	.
3-Methyl-2-thiouric acid	150	0.6
3-Methyl-6-thiouric acid	~400	~29
3-Methyl-8-thiouric acid	190	38
1,3-Dimethyl-8-thiouric acid	—	.
Hypoxanthine and xanthines		
3-Methylhypoxanthine	—	.
3-Methylxanthine	—	.
3-Methyl-2-thioxanthine	100	0.5
3-Methyl-6-thioxanthine	160	59
7-Methyl-6-thioxanthine	~1000	~400
2-Hydroxypurine		
1-Methyl-2-oxopurine	—*	.
3-Methyl-2-oxopurine	—*	.
2-Hydroxy-6-methylpurine	17	1.4

* Because of limited solubility, the highest concentration tested was 30 $\mu\text{g./ml.}$

Table 6. *Influence of pH on the inhibitory activity of purines against urate oxidase*

Urate oxidase (50 $\mu\text{g./ml.}$) and sufficient inhibitor to produce about 60% inhibition at pH 9.3 were incubated in various buffers for 30 min. Then uric acid in the same buffer was added to give a final concentration of 12 $\mu\text{g./ml.}$ The rate of disappearance of substrate was determined at 293 $\text{m}\mu$. Simultaneously, the degradation of uric acid in the absence of inhibitor was observed in the same buffer. The temperature was 29°. The relative inhibitory activity expresses the inhibitory effect at a given pH as percentage of the value at pH 9.3.

Purine derivative	Relative inhibitory activity (%)			
	pH 6.5	pH 7.8	pH 9.3	pH 11.0
Hypoxanthine	62.5	71	100	125
2-Hydroxypurine	62.5	71	100	125
8-Hydroxypurine	100	100	100	100
Xanthine	76.5	82.5	100	100
2,8-Dihydroxypurine	64	77	100	77
6,8-Dihydroxypurine	63	74	100	63
6-Thioxanthine	100	100	100	73.5
8-Hydroxy-6-mercaptopurine	220	200	100	62.5
2-Hydroxy-6-methylmercaptopurine	70	100	100	80

point. Though 8-hydroxypurine (pK 7.9) shows no variation of affinity within the pH range 6.5–11, both hypoxanthine (pK 8.8) and 2-hydroxypurine (pK 8.5) manifest a steady increase of inhibitory activity with rising pH, suggesting that their hydroxyl groups may be attached to the same constituent of the active surface. This is not possible for a combination of these two hydroxyl groups as in xanthine. Indeed, the inhibitory effect of xanthine varies with pH in a different manner: it remains constant throughout the extreme alkaline range, but decreases below pH 9. On the other hand, 2,8- and 6,8-dihydroxypurine exhibit a maximum of activity at pH 9.3.

A 6-mercapto substituent introduces new phenomena. Thus 8-hydroxy-6-mercaptopurine resembles the other 8-substituted derivatives only on the alkaline side, where its activity declines steadily; with decreasing pH it becomes more inhibitory. 6-Thioxanthine manifests the reverse behaviour of xanthine. Its affinity remains unchanged throughout the acid range but declines at alkaline pH. The corresponding 2-hydroxy-6-methylmercaptopurine exhibits a broad maximum over the pH range 7.5–9.5, with a slow fall on both sides of the maximum.

DISCUSSION

Several features in the purine structure make important contributions to the formation of a stable enzyme-inhibitor complex. The purine skeleton as such serves as basic framework for the correct placing of polar substituents. This indicates that the active surface is specifically adapted to the dimensions of the purine system. Accordingly, closely related molecules such as the pyrimidines or pteridines exhibit only low affinity to urate oxidase, whereas 8-azapurines are more effective inhibitors than the corresponding purines themselves.

The following facts suggest that the oxygen at C-2 is bound through a hydrogen bridge to a OH or NH group in the active centre. Sulphur at C-2, presumably because of its feeble capacity to form a hydrogen bond, decreases the inhibitory power drastically. A methyl substituent at N-1 or N-3 seriously interferes with the attachment of the 2-carbonyl group. On the other hand, a N^3 -methyl group augments the binding power of 2-thiopurines, presumably by van der Waals forces, thus indicating that a 2-thiocarbonyl group is not associated with any specific component of the active surface.

Of all the possible linkages between enzyme and purine, the attachment of the 2-hydroxyl group through a hypothetical hydrogen bridge provides the best 'fit'. In the absence of this bond, the position of the purine ring relative to the active centre is apparently not well determined. Thus the identical variations of the inhibitory effects of hypoxanthine and 2-hydroxypurine with pH suggest that the same constituent of the enzymic surface is involved in the binding of either hydroxyl group, although in xanthine or uric acid this is impossible. Indeed, the I_{50} of xanthine is greater than that of 2-hydroxypurine, and the K_m of uric acid exceeds the K_i of 2,8-dihydroxypurine, indicating that here the contribution of the oxygen at C-6 to binding is insignificant or that this substituent may even have an adverse influence.

The presence of oxygen at C-2 is a prerequisite for the proper binding of a 6-thiocarbonyl group. The contribution of the latter to the affinity of a purine for urate oxidase is incomparably greater than that of oxygen in the same position. It is therefore probable that sulphur at C-6 can join the copper complex of the enzyme. This firm coordinate bond of the 6-thiocarbonyl group may be responsible for the very slow degradation of 6-thiouric acid (Bergmann *et al.* 1963).

Bivalent copper forms only weak complexes with sulphur derivatives, unless chelation is possible. Indeed, Mahler, Hübscher & Baum (1955) have shown that only after treatment with a reducing agent (hydroxylamine) can the enzyme bind certain inhibitors such as thiocyanate. On the other hand, sodium diethyldithiocarbamate chelates directly with urate oxidase, but does not inhibit the catalytic activity. Our observation that 6-methylmercapto derivatives may surpass by far the inhibitory power of 6-thiopurines argues against the necessity of reduction of the central copper atom to its univalent state before it can form a complex with a thio compound, unless one admits that the two types of sulphur derivatives may be fixed differently by urate oxidase. The strength of the co-ordinate linkage of the thio ethers finds an expression in the fact that compound (Ia) is a partly non-competitive inhibitor, although its 3-methyl homologue (Ib) exhibits purely competitive binding. Direct complex-formation of either sulphur derivative with the copper atom in its bivalent state appears probable, but the flat structure of the purine ring makes it unlikely that sulphur at C-6 together with, for example, an oxygen atom at C-2 can cause chelation. Another possibility, namely chelation of bivalent copper with the sulphur atom at C-6 and N-7, is suggested by the observation that methylation at N-7 reduces the inhibitory power almost to zero. However, it is also conceivable that copper in the form of a metalloprotein is endowed with unusual binding properties.

Finally, the present study clearly shows that the formation of a strong complex with the active surface does not in itself make enzymic attack possible. Thus both 6-thiouric acid and 2,8-dihydropurine are bound much more tightly than uric acid. Yet the former is attacked only very slowly and the latter not at all, although in both cases there is the possibility of the same distribution of negative charge by resonance as was proposed for uric acid by Bentley & Neuberger (1952).

SUMMARY

1. The inhibitory effect of purines and related heterocyclic compounds on the degradation of uric acid was used to measure their affinity for urate oxidase.

2. Of the three oxygen atoms of uric acid, that of the 2-carbonyl group possesses the greatest binding power for the active centre. Replacement of this oxygen atom by sulphur greatly diminishes the inhibitory activity.

3. Combination of a 2-carbonyl group with sulphur at C-6 enhances inhibitory activity considerably.

4. In certain purine derivatives, a 6-methylmercapto substituent is more effective than a 6-thiocarbonyl group. 2,8-Dihydroxy-6-methylmercaptapurine is the most potent inhibitor of urate oxidase known so far.

5. Replacement of the imidazole moiety of the purine ring by triazole enhances affinity, whereas introduction of the pyrazine ring, as in pteridines, greatly decreases it.

6. Free imino groups are essential for the attachment of purines to urate oxidase, as *N*-methylation weakens or abolishes the inhibitory effect. On the other hand, in 2-thiopurines methylation at N-3 increases the inhibitory power.

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The Amino Acid Composition of Human Collagens From Adult Dura Mater and Post-Menopausal Uterus

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The present results are an introduction to studies on the differences in amino acid composition between collagens from various normal and diseased human tissues. Adult dura mater represents a source of fairly pure collagen. The reasons for selecting uterus for study are: First, the shrinkage temperature and fluorescence of both pre- and post-menopausal uterine collagen have been shown (Brown, Consden & Glynn, 1958) to correspond to those of non-uterine collagen from a foetus or very young child. Secondly, uterus exhibits far greater synthesis and turnover of collagen than other tissues, and this process even continues into old age (Kao, Hilker & McGavack, 1961*a, b*). The third and major reason is interest in the rapid build-up during pregnancy and even more rapid resorption of uterine collagen during post-partum involution (Harkness & Harkness, 1954; Harkness & Moralee, 1956; Woessner, 1962; Morrione & Seifter, 1962).

Differences in the compositions of collagens from different tissues of one species have been established, e.g. the high hydroxylysine and low lysine contents of tendon collagen compared with skin and bone (Eastoe, 1955; Leach, 1957). However, collagens with greater metabolic activity, as in uterus, and those from rheumatic tissues have not yet been analysed completely for amino acids. Consden, Glynn & Stanier (1953) found no significant differences in the hydroxyproline and tyrosine contents of collagen extracted from rheumatic-fever nodules by autoclaving.

METHODS

Materials

Extractions were performed at room temperature unless otherwise stated. The tissues were extracted with approximately 20 times their wet weight of each solution.

Dura mater. A sample of dura mater from a 72-year-old woman was washed in water, freed from veins and extracted successively with (i) 0.9M-NaCl for 3 hr. (two changes) and (ii) citrate buffer, pH 3.7 (containing 0.1M-citric acid and 0.05M-sodium citrate), for 18 hr. The citrate buffer was shown by hydroxyproline determinations to have extracted less than 0.05% of the collagen. The extracted residue was dried under vacuum, giving material D₁. This contained impurities (see Results) and was therefore treated further with (a) trichloroethylene for 2 days, (b) ether-ethanol (3:1, v/v) for 2½ hr., (c) 3% (w/v) Na₂HPO₄ soln. for 2 days at 4°, and (d) saturated KCl soln. for 3 days at 4°. The residue was washed free of chloride and agitated vigorously twice with water and once with ethanol. It was then washed once in ethanol and twice in ether. The ether was decanted off and the fibres were spread in a flat glass dish and left overnight to equilibrate with atmospheric moisture. This material is D₂. The salt solutions extracted less than 0.05% by weight of the final residue, as shown by hydroxyproline determinations.

Uterus. The white outer layer of a uterus from a 59-year-old woman was removed and the remainder finely shredded in a food macerator and washed in water. It was then extracted with 0.9M-NaCl for 2 days, then ether-ethanol (3:1, v/v) for 2½ hr., 0.9M-NaCl for 3 hr., citrate buffer (pH 3.7) for 18 hr., saturated Na₂HPO₄ solution for 2 days at 4° and saturated KCl solution for 3 days at 4°. The residue was washed free of chloride and dried with ethanol and ether and equilibrated as for dura mater, giving the material U₁. This material contained non-collagenous proteins, probably elastin (see Results). Gelatin was extracted as follows: 0.28 g. of U₁ was suspended in 80 ml. of water. Sulphur dioxide was passed in until the pH fell to 2.5, and added for a further 2 hr. to maintain this pH. The beaker was then covered by polythene sheeting overnight (final pH 2.7). The supernatant was decanted and replaced by water. Sulphur dioxide was again passed in to lower the pH to 2.5. The beaker was then placed in a water bath at 80° for 2½ hr. After centrifuging, the residue was washed in water, ethanol and ether, equilibrated in air and weighed. The solution was warmed with about 0.2 g. of animal char-