Inactivation of Guanosine 5′-Phosphate Reductase by 6-Chloro-, 6-Mercapto-, and 2-Amino-6-mercapto-9-β-D-Ribofuranosylpurine 5′-Phosphates*

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ABSTRACT: 6-Chloro-9-β-D-ribofuranosylpurine 5′-phosphate (Cl-IMP) in high dilution rapidly inactivates guanosine 5′-phosphate (GMP) reductase purified from *Aerobacter aerogenes;* the process is slowed by GMP but not by reduced nicotinamide–adenine dinucleotide phosphate (NADPH) (the second substrate) unless GMP is present. Enzyme preparations which required glutathione (GSH) for activity were inactivated by Cl-IMP only when GSH was present. 6-Chloropurine nucleoside did not inactivate the enzyme; iodoacetamide inactivated reversibly with respect to GMP but was much less potent than Cl-IMP. When GSH is absent, the 6-mercapto analogs of IMP (inosine 5′-phosphate) and GMP strongly inactivate GMP reductase; 6-thioguanosine was *ca.* 20 times less effective. Inactivations by the 6-thio analogs were retarded by GMP and reversed by GSH.

Inhibition by IMP was reversed by GMP but unaffected by GSH. The 6-chloro and 6-mercapto analogs appear to inactivate by forming thioether and disulfide bonds, respectively, with a sulfhydryl group at the GMP site. These inactivations of GMP reductase could play a part in inhibition of cell growth by the antineoplastic agents 6-chloropurine, 6-mercaptopurine, and 6-thioguanine which are anabolized to the nucleotide analogs of the present study. Inhibition of GMP reductase by adenosine 5′-triphosphate (ATP) is probably involved in cellular regulation of purine nucleotide interconversion; this inhibition appears to be exerted at a site other than the GMP and NADPH sites since NADPH did not reverse ATP inhibition and since strongly inhibitory levels of ATP did not retard inactivation of the enzyme by Cl-IMP.

The reductive deamination of GMP[1] to IMP (eq i) by GMP reductase (Mager and Magasanik, 1960) is analogous to the conversion of IMP to XMP (eq ii) by

$$GMP + NADPH + H^+ \rightarrow IMP + NADP^+ NH_3 \quad (i)$$
$$IMP + NAD^+ + H_2O \rightarrow XMP + NADH + H^+ \quad (ii)$$

IMP dehydrogenase (Magasanik *et al.*, 1957). Both reactions are apparently irreversible, utilize pyridine nucleotide coenzymes, are promoted by aliphatic thiols, and involve interchange of hydrogen and a nucleophile at C-2 of a purine ring. Previous studies (Hampton, 1963; Hampton and Nomura, 1967) have shown that an additional property of IMP dehydrogenase is its facile inactivation under assay conditions by 6-chloro and 6-mercapto analogs of IMP and GMP and that the loss of catalytic function probably results from covalent reactions between the analogs and the IMP site of the enzyme. The present communication reports that GMP reductase of *Aerobacter aerogenes* also is inactivated by the above IMP analogs, and in a fashion strikingly similar to that of IMP dehydrogenase from the same organism. This and other inhibition studies presented here indicate that a sulfhydryl group is located at the GMP site within one or two bond distances of C-6 of GMP in the GMP–enzyme adduct.

The antileukemic drugs 6-chloropurine, 6-mercaptopurine, and 6-thioguanine and a variety of other purine analogs are believed to inhibit biological systems as a consequence of their anabolism to ribonucleoside 5′-phosphates (Brockman and Anderson, 1963; Elion and Hitchings, 1965). A number of enzymes involved in purine nucleotide synthesis and interconversions are known to be inhibited by the nucleoside 5′-phosphate derivatives of 6-chloropurine, 6-mercaptopurine, and 6-thioguanine (reviewed by Brockman and Chumley,

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[1] Abbreviations and trivial names used: GMP reductase, reduced NADP:GMP oxidoreductase (deaminating), EC 1.6.6.8; IMP dehydrogenase, IMP:NAD oxidoreductase, EC 1.2.1.14, NAD (in equations, NAD^+) nicotinamide–adenine dinucleotide; NADH, reduced NAD; NADP (in equations, $NADP^+$), nicotinamide–adenine dinucleotide phosphate, NADPH, reduced NADP; AMP, IMP, GMP, and XMP, adenosine, inosine, guanosine, and xanthosine 5′-phosphates; ATP and GTP, adenosine and guanosine 5′-triphosphates; 6-thio-IMP, 6-thio-GMP, 6-chloropurine nucleotide (Cl-IMP), 6-mercapto-, 2-amino-6-mercapto-, and 6-chloro-9-β-D-ribofuranosylpurine 5′-phosphates; PMB, *p*-mercuribenzoate; GSH, reduced glutathione.

TABLE I: Purification of GMP Reductase.

Step	Fraction	Vol. (ml)	Total Protein (mg)	Total Act. (Units)	Sp Act. (units/mg of protein $\times 10^3$)	Purifcn Factor
1	Crude extract[a]	497	2982	*b*		
2	Protamine sulfate	508	2286	10.2	4.5	1.0
3	Ammonium sulfate	35	788	26.3	33.4	7.4
4	Phosphocellulose	84	71	15.2	212	47.5

[a] From 60 g of washed packed cells. [b] No GMP reductase activity was detected in the crude extract.

1965; Elion and Hitchings, 1965). It is not yet clear, however, whether inhibition at any of these loci is critical for biological growth inhibition.[2] The present findings indicate that GMP reductase is now to be included among the above enzymes, particularly since it occurs in mammalian systems (Hershko *et al.*, 1963) as well as in the bacterial sources initially discovered by Mager and Magasanik (1960).

Methods and Materials

Chemicals. Sodium *p*-mercuribenzoate, iodoacetamide, and iodoacetate were from Calbiochem and NADPH was from the Sigma Chemical Co. and Calbiochem. Streptomycin sulfate was generously provided by Merck & Co. The 2-amino-6-chloro-9-β-D-ribofuranosylpurine (Robins, 1960) was a gift from Dr. R. K. Robins. The sources of other chemicals were as described previously (Hampton, 1963) except for 6-Cl-IMP, a new synthesis of which is given below.

GMP Reductase. The source of *A. aerogenes*, strain P-14, was as described previously (Hampton and Nomura, 1967). The cells were disrupted sonically by the procedure of Mager and Magasanik (1960) except that the operation was carried out in the presence of 1 mM β-mercaptoethanol instead of 5 mM glutathione. After removal of cell debris, the cell extract was treated with one-tenth its volume of 5% streptomycin sulfate, the precipitate was removed by centrifugation, and the supernatant (referred to hereafter as fraction A) was stored at −20°. Dialysis of the cell extract prior to this streptomycin treatment, described by Mager and Magasanik (1960), was not carried out since it led to extensive loss of GMP reductase activity.

The methods for purification of GMP reductase by the scheme of Table I were the same as those for purification of IMP dehydrogenase (Hampton and Nomura, 1967). Step 2 contained the same buffer as fraction A above, but unlike fraction A it contained no added thiol and contained protamine sulfate in place of streptomycin sulfate. Step 4 fractions were pooled and stored at −20° in polyethylene bottles. Enzyme solutions were thawed at room temperature just prior to use and thereafter were kept at 4° and not refrozen.

Assay. The method was based on that of Mager and Magasanik (1960). The decrease in optical density at 340 mμ due to NADPH oxidation was followed at 23–24° in 1.0-cm light-path cuvets with a Cary Model 15 spectrophotometer or a Gilford spectrophotometer. In several cases mixtures contained relatively high concentrations of 6-thio-GMP (λ_{max} 340 mμ) and the enzymic reaction was then conveniently followed from the decrease in optical density at 285 mμ due to the conversion of GMP to IMP. The rate of decrease in optical density remained constant for at least 10 min. Final concentrations in assay mixtures (1 ml) were: Tris-HCl buffer (pH 7.5) (40 mM), NADPH (0.2 mM), GMP (0.2 mM), and GSH (2 mM). The reaction could be started equally well by addition of GMP and/or NAPDH or enzyme. The control cuvet lacked GMP in order to correct for any contaminating NADPH oxidase activity. One unit of GMP reductase is defined as the amount giving an optical density change of 1.00/min at 340 mμ.

Results

Preparation of 6-Chloro-9-β-D-ribofuranosylpurine 5′-Phosphate. The procedure was based on that developed by Robins (1963) for the conversion of 6-thioinosine to 6-chloropurine ribonucleoside. A suspension of 50 mg of barium 6-thioinosinate (Hampton and Maguire, 1961) in 2 ml of methanol was cooled to below −10° in an acetone–solid CO_2 bath with exclusion of moisture. Chlorine gas was bubbled slowly into the mixture for 15 min during which the temperature was maintained below −5°. The suspended material dissolved within 7 min. The yellow solution was kept for an additional 15 min at the same temperature, after which cooling was continued and nitrogen was bubbled through to remove excess of chlorine. Saturated methanolic ammonia, precooled to −10°, was added to adjust the pH of the solution to 7. A heavy white precipitate formed; water was added to dissolve it (final volume of the mixture, 8 ml) and 0.5 ml of 1 M barium acetate was added. After 2 hr at 4° the solution was centrifuged to remove small amounts of insoluble material. Ethanol (32 ml) was added and the mixture was stored at 4° overnight. The white precipitate was collected by cen-

[2] Studies of Bennett and Smithers (1964) indicate that feedback inhibition of purine biosynthesis may play a part in growth inhibition of mammalian cells in culture by some purine analogs.

trifugation and washed once with 8 ml of aqueous 80% ethanol. The product was dried over NaOH *in vacuo*, then dissolved in 2 ml of water, and applied to a column containing about 20 equiv of Dowex 50 (Na^+) ion-exchange resin. The column was washed with water and 10 ml of effluent was collected. The spectral characteristics of this solution in 0.05 M acetate buffer (pH 4.8) were λ_{max} 264 mμ, λ_{min} 226.5 mμ; absorbancy ratio $A_{264}:A_{226.5} = 4.3$, $A_{250}:A_{260} = 0.76$, and $A_{280}:A_{260} = 0.19$. The values reported for 6-chloro-9-β-D-ribofuranosylpurine 5′-phosphate by Hampton and Maguire (1961) are 263 and 226 mμ and absorbancy ratios 4.0, 0.82, and 0.175, respectively. Chromatography of the solution in 1-butanol–acetic acid–water (5:3:2), isopropyl alcohol–1% $(NH_4)_2SO_4$ (2:1), and isoamyl alcohol–0.3 M phosphate (pH 6.9) (1:1) showed one fluorescent spot with the same R_F as Cl-IMP (Hampton and Maquire, 1961). The yield of 6-chloropurine nucleotide by the present procedure was determined spectrophotometrically to be 92%. The barium salt of Cl-IMP was precipitated from the solution of the sodium salt with barium acetate and ethanol in the usual manner. When dried over P_2O_5 for 3 hr at 0.1 mm and 100° it showed λ_{max} 264 mμ (ε 8260) at pH 4.8 (reported previously, ε 8400 at 263 mμ); this extinction coefficient corresponds to the anhydrous form of the barium salt of this nucleotide, and this form is known to be produced by the above drying conditions (Hampton and Maguire, 1961).

Purification of GMP Reductase. The GMP reductase of step 4 (Table I) was eluted with 0.25 M KCl and contained only traces of IMP dehydrogenase which was eluted with 0.5 M KCl. The elution diagram has been presented previously (Hampton and Nomura, 1967) and was essentially the same whether chromatography was carried out at pH 6.5 or 7.4. At least 90% of the loss in total activity between steps 3 and 4 occurred during dialysis of the ammonium sulfate fraction prior to column chromatography. In one case activity totally disappeared during dialysis of 15.1 units of the step 3 fraction, but 8.3 units were subsequently eluted from the phosphocellulose with 0.25 M KCl. During purification of one batch of enzyme, activity was present in steps 1, 2, and 4 but not in step 3, and the respective purification factors were 1, 9.7, and 300. The increase in total activity during the first two steps (Table I) was always observed.

A very active GMP-independent NADPH oxidase activity was present prior to step 4. In crude extracts the decrease in optical density at 340 mμ brought about by this enzyme(s) was three to ten times greater than that brought about by the GMP reductase. Almost all of the NADPH oxidase activity appeared in the breakthrough peak of the phosphocellulose column.

The crude GMP reductase preparations contained an enzyme or enzymes capable of hydrolyzing 6-chloropurine nucleoside and 6-chloropurine nucleotide to inosine and IMP, respectively. This dechlorinase activity, which was not adsorbed on the phosphocellulose, was evidenced by a shift in absorption maxima from 264 to 248.5 mμ accompanied by an increase in optical density which corresponded to that expected from the known extinction coefficients of 6-chloropurine nucleoside and inosine and the related nucleotides. The hydrolyses were analyzed by paper chromatography in butanol–acetic acid–water (5:3:2) and isopropyl alcohol–1% ammonium sulfate (2:1). The 6-chloropurine nucleotide was attacked more slowly than its nucleoside; *e.g.*, about 0.5 mg of step 3 protein catalyzed approximately 50% conversion of 0.1 μmole of nucleotide (R_F values 0.36 and 0.65, respectively) to IMP (R_F values 0.15 and 0.54) within 40 min at pH 7.5. In a similar experiment with 0.07 μmole of 6-chloropurine nucleoside, conversion to inosine was complete within 30 min as judged by the above spectral shift and by paper chromatography. Adenosine deaminase activity was detected in the same enzyme preparation. The step 4 fraction contained no detectable amounts of NADPH oxidase or dechlorinase activity.

Properties of GMP Reductase. Fraction A (described under Methods), which contained 0.9 mM β-mercaptoethanol, did not require additional thiol for maximum activity. The step 4 enzyme fraction (Table I), in contrast to the purified preparation of Mager and Magasanik (1960), did not exhibit an absolute requirement for a thiol, although a thiol was required for maximal activity with almost all the step 4 preparations. GSH, cysteine, or β-mercaptoethanol was equally effective at a concentration of 2 mM. The amount of activity observed in the absence of a thiol usually varied from 10 to 75% of maximal (2 mM thiol) from one step 4 preparation to another. GTP (tested at 200 μM) did not increase the activity of the preparations. Since inhibition by PMB and the 6-thio nucleotides is suppressed by thiols, these effects (Table II, Figures 4 and 5) were studied with a step 4 preparation which had no GSH requirement and which was freshly obtained by phosphocellulose chromatography.

Fractions A and 1 lost about 25% of their activity within 4 days at 4°. Fractions A and 2–4 were stable for at least 4 months at −20°. The step 4 preparation lost about 25% of its activity within 2 weeks at 4°; upon

TABLE II: Inhibition of GMP Reductase by PMB.[a]

PMB Concn (μM)	GSH Concn (mM)	Rel Act.
0	2.0[b]	100
0	0	62
1	0	47
2.5	0	16
10	0	0
10	2.0[c]	91

[a] The PMB was added to the complete assay mixture (GSH omitted) 5 min after the reaction was started. Each mixture (1 ml) contained 50 μl of the step 4 enzyme preparation used in the experiments of Figures 4 and 5. [b] GSH included in assay mixture prior to addition of PMB. [c] GSH added to PMB-inhibited system 5 min after PMB addition.

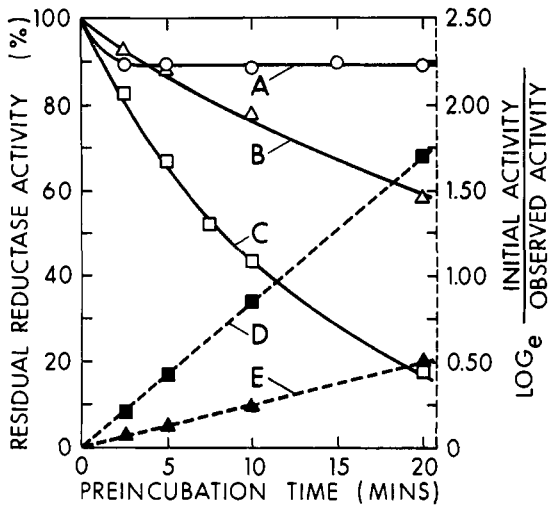

FIGURE 1: Inactivation of GMP reductase by 500 μM iodoacetamide or iodoacetate. Step 4 enzyme (25 μl) was preincubated at 23° with the iodo compounds in assay mixtures which lacked GMP and/or NADPH, as indicated. Curve A: effect of iodoacetamide or iodoacetate in the presence of 2 mM GMP (mixtures assayed after addition of NADPH). Curve B: effect of iodoacetate in the absence of GMP. Curve C: effect of iodoacetamide in the absence of GMP. Curve D: logarithm plot of the data of curve C. Curve E: logarithm plot for curve B. In the absence of inhibitors the decrease in optical density at 340 mμ was no less than 0.06 in 10 min.

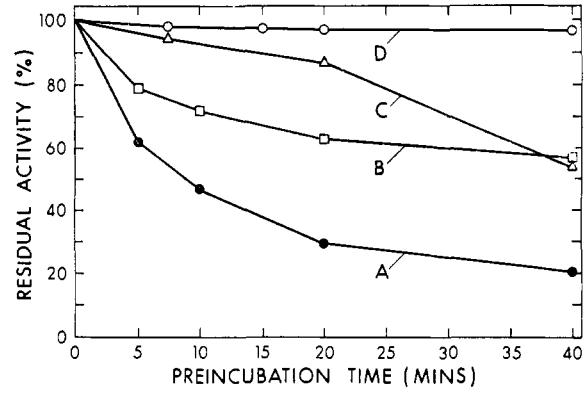

FIGURE 2: Inactivation of GMP reductase by 10 μM 6-Cl-IMP. The enzyme (25 μl of step 4 fraction) was exposed at 23° to the nucleotide in the assay system. Plot A, preincubation conditions as follows: (a) GMP and NADPH omitted, (b) GMP omitted and 600 μM NADPH included, or (c) NADPH omitted and 50 μM GMP included. Plot B, NADPH omitted and 200 μM GMP included. Plot C, 600 μM NADPH and 50 μM GMP included and enzyme activity measured after addition of more GMP (final concentration, 200 μM). Plot D, NADPH omitted and 1 mM GMP included.

thawing, the activity exhibited in the absence of thiol disappeared after 1 or 2 days at 4°, but full activity could still be elicited upon addition of thiol. The step 4 enzyme was less stable if diluted with the assay buffer.

In the presence or absence of thiol the reaction velocity was proportional to the step 4 protein concentration over the range examined (15–75 μg/ml). Assays ordinarily employed 25–50 μg/ml of protein.

6-Thio-GMP (62 μM) was tested as a substrate of the step 3 preparation in the standard assay mixture; the control cuvet lacked 6-thio-GMP. No change in absorption at 305 mμ occurred during 8 min. The extinction coefficient of 6-thio-IMP at 305 mμ and pH 7.6 is relatively high (*ca.* 15,000) and more than three times greater than that of 6-thio-GMP (Fox *et al.*, 1958) and under the above conditions would have permitted detection of an enzymatic reaction proceeding at 2% the rate of the conversion of GMP to IMP. 6-Thio-GMP (400 μM) was tested as a substrate also with the step 4 enzyme preparation, but no change in optical density at 310 mμ occurred during 45 min.

Effect of Reagents for Sulfhydryl Groups. In the absence of thiol the enzymic reaction was stopped by the inclusion of 10 μM PMB (Table II). With this and the lower levels of PMB the reaction rate decreased abruptly (within 30 sec) and remained constant for at least 30 min. Addition of 2 mM thiol almost completely restored the original rate with similar rapidity. Increasing the GMP concentration to 5 mM and/or increasing the NADPH concentration to 0.6 mM did not afford protection. Omission of either GMP or NADPH during a 10-min preincubation of the enzyme with PMB did not affect the degree of inhibition. If addition of thiol to the PMB-inhibited system was delayed for more than 30 min less activity was recoverable.

Iodoacetamide and iodoacetate inhibited GMP reductase progressively during preincubations (Figure 1). When 2 mM GMP was present the inactivation did not exceed 11% during the time shown. NADPH (600 μM) did not protect the enzyme. These iodo compounds react with the GSH present in the assay mixture and it was calculated from published data (Smythe, 1936) that the half-lives for the concentrations involved would be 25–35 min for iodoacetamide and 65–75 min for iodoacetate. Incubation of the alkylating agents with GSH for as long as 30 min prior to addition of enzyme did not alter the rate of inactivation. The inactivations appear to follow a first-order rate equation with a half-life for enzymatic activity of about 8.5 min with iodoacetamide and 25 min with iodoacetate (Figure 1).

The conversion of Cl-IMP (initial concn, 60 μM) to a 6-alkylmercapto derivative by nonenzymatic reaction with GSH (2 mM) at pH 7.6 and 24° was followed from the increase in absorption at 290 mμ; after 1 hr less than 5% of the Cl-IMP had reacted. Under the same conditions, at least 50% of iodoacetate reacts with GSH.

Inhibition by 6-Chloropurine Nucleotide. GMP reductase progressively lost activity when preincubated with Cl-IMP. Under the conditions of Figure 2, GMP at a level of 1 mM effectively protected the enzyme from

TABLE III: Inhibition and Inactivation of GMP Reductase by Purine Nucleotides.[a]

Inhibitor	Enzyme Fraction	Concn (μM)		Pretreatment of Enzyme[b]		Inhibn (%)
		Inhibitor	GMP	Components Present	Time (min)	
6-Cl-IMP	A[c]	92	200	Cl-IMP, NADPH	5	47
	A[c]	92	200	Cl-IMP, NADPH	10	86
	3	110	200	Cl-IMP, GSH	10	80
	4	100	200	Cl-IMP, GSH	35	95
	4	50	5,000	Cl-IMP, GSH	10	80
	4	50	5,000	400 μM ATP, Cl-IMP, GSH	10	82
	4	50	5,000	400 μM ATP, Cl-IMP, GSH, NADPH	10	80
	4	50	5,000	1 mM GTP, Cl-IMP, GSH	10	78
ATP	4	400	200			92
	4	400	2,000			38
	4	400	4,000			0
ADP	4	2,000	200			85
	4	2,000	2,000			11
GTP	4	200	200			0
	4	2,000	200			20
6-Chloropurine nucleoside	4	4,000	200	Inhibitor, GSH, NADPH	10, 30	22
	4	20,000	200	Inhibitor, GSH, NADPH	10	60
2-Amino-6-chloropurine nucleoside	4	4,000	200	Inhibitor, GSH, NADPH	10	11
6-Thio-GMP	A[c]	9	100	Thio-GMP	15	56
	A[c]	9	100	Thio-GMP	30	81
	A[c]	100	200	NADPH, GMP	0.25–0.5	25[d]
6-Thio-IMP	A[c]	320	200	NADPH, GMP	0.25–0.5	75[d]
	A[c]	320	2,000	NADPH, GMP	0.25–0.5	10
Purine ribonucleoside 5′-phosphate	3	625	200			0
IMP	2[e]	1,000	200			44
	2[e]	1,000	2,000			15
	4[f]	1,000	200			32
6-Thio-IMP	4[f]	1,000	200			30
Inosine 2′-phosphate	4[f]	2,000	200			0
AMP	4	1,000	200			20
Adenosine 2′-phosphate	4	1,000	200			48

[a] Unless stated otherwise, assays employed 2 mM GSH and 200 μM NADPH and sufficient enzyme to produce a decrease in optical density at 340 mμ of 0.15–0.20 in 10 min in the absence of an inhibitor. [b] If the inhibitor is not listed it was the last component added. [c] Assay mixtures for fraction A contained 150 μl of enzyme preparation, no GSH, and were 130 μM with respect to β-mercaptoethanol. [d] Value after 15 min; no inhibition was observed initially. [e] GSH did not stimulate enzyme activity and was omitted. [f] Assay employed 100 μM NADPH.

inactivation; however, the inactivation, once established, was not reversed by 1 mM GMP. NADPH (600 μM) did not influence the rate of inactivation, nor did IMP (200 μM). However, 600 μM NADPH in combination with 50 μM GMP (Figure 2, plot C) markedly slowed inactivation, whereas the same concentrations of either NADPH or GMP singly (Figure 2, plot A) had no effect on inactivation. The loss in activity was

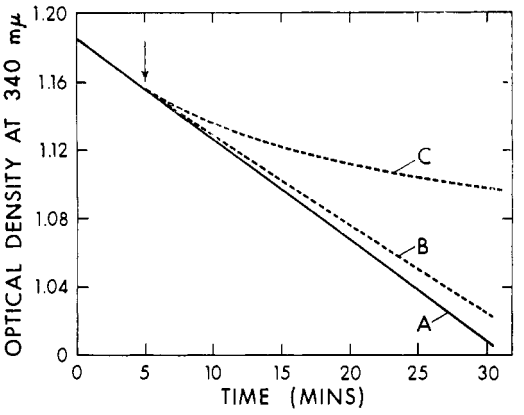

FIGURE 3: Inactivation of GMP reductase by 6-Cl-IMP. Step 4 enzyme (25 μl) was employed and nucleotide was added (arrow) to the standard assay mixture. Curve A: no inhibitor added. Curve B: 10 μM 6-Cl-IMP. Curve C: 190 μM 6-Cl-IMP.

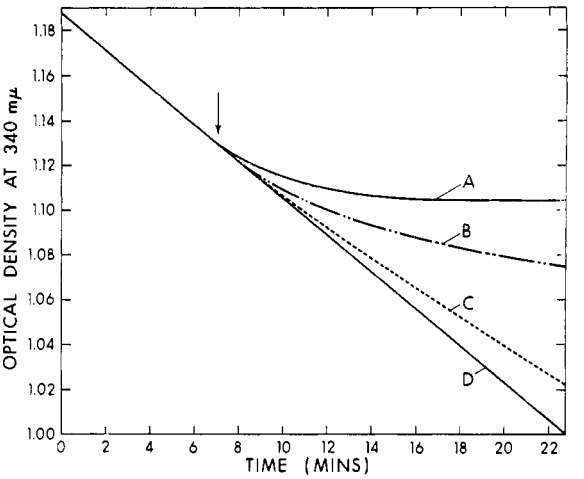

FIGURE 4: Inactivation of GMP reductase by 15 μM 6-thio-GMP. The step 4 enzyme preparation employed (50 μl/assay) was not stimulated by GSH. The thio-GMP was added (arrow) to assay mixtures which lacked GSH and contained the following initial concentrations of GMP: curve A, 100 μM; curve B, 500 μM; and curve C, 2 mM. Curve D was obtained under conditions A–C in the absence of 6-thio-GMP.

virtually complete when relatively high concentrations of Cl-IMP were employed (see Table III). The less purified preparations of the enzyme, *e.g.*, fraction A and the step 3 preparation, were inactivated in the same manner and to the same extent as was the phosphocellulose-purified step 4 (Table III). Figure 3 shows the progressive inhibition by Cl-IMP of the enzymatic conversion of GMP to IMP. These experiments provide further indication that the inactivation is retarded more effectively by GMP and NADPH than by GMP alone. Inhibitor (10 μM) inactivated 32% of the enzyme in 15 min in the presence of 200 μM GMP (Figure 2), whereas when 200 μM NADPH was also present (Figure 3) the inhibition was 15% after 15 min.

A step 4 enzyme preparation which had no activity in the absence of GSH was treated for 20 min in the assay buffer with 340 μM Cl-IMP; upon addition of 1 mM GMP and the assay amounts of NADPH and GSH no inactivation or inhibition by the Cl-IMP was detectable. In the presence of GSH the same enzyme preparation was extensively inactivated when treated as above with Cl-IMP. In the absence of GSH the activity (40% of maximal) of another step 4 fraction was progressively reduced by 10 μM Cl-IMP at *ca.* the same rate as in the experiments of Figure 2. The reactivity of Cl-IMP toward IMP dehydrogenase varies in the same manner in the presence and absence of GSH (Hampton and Nomura, 1967).

The inhibitory effects of AMP and ATP given in Table III are similar to those reported by Mager and Magasanik (1960) for GMP reductase partially purified by a procedure different from that of Table I. The degree of inhibition by ATP in the present studies was unaffected by increasing the level of NADPH to 600 μM. Table III shows that the rate of inactivation by Cl-IMP during preincubation was unaffected by ATP at a level (400 μM) strongly inhibitory to the enzymic reaction; inclusion of NADPH did not affect the result. GTP (1 mM) likewise did not influence inactivation by Cl-IMP. In further experiments GSH was omitted during treatment of step 4 enzyme with 10 μM Cl-IMP and omitted also in the subsequent enzyme assays (200 μM NADPH and 4 mM GMP added), but 500 μM ATP had no effect on the extent of inactivation at either 5, 10, 20, or 40 min.

Inhibition by 6-Thiopurine Derivatives. In the absence of an added thiol, 6-thio-GMP at low concentrations brought about a progressive inhibition of GMP reductase. The rate of the progressive inhibition was reduced by GMP (Figure 4) but was unaffected by NADPH (tested at 600 μM). When 2 mM thiol was added to the inhibited systems almost all of the maximal activity was regained within 5 min. When 2 mM GSH was present under the conditions of Figure 4, a nonprogressive inhibition of 5–10% occurred.

6-Thioguanosine also caused progressive inhibition when a thiol was absent. The time for 50% inhibition was about 8.5 min in the presence of 15 μM 6-thioguanosine and about 0.5 min with the same concentration of 6-thio-GMP. The progressive inhibition could be prevented or reversed by a thiol and reduced in rate by additional GMP, in a similar manner to the inhibition by 6-thio-GMP.

6-Thio-IMP gave a similar pattern of progressive inhibition which was retarded by GMP (Figure 5) and reversed by 2 mM thiol. Under the normal assay conditions (2 mM GSH present) 6-thio-IMP inhibited conversion of GMP to IMP moderately and to approximately the same extent as did IMP itself (Table III). Inhibition by IMP under these conditions was at least partly competitive with respect to GMP and occurred to about the same extent with either crude or relatively

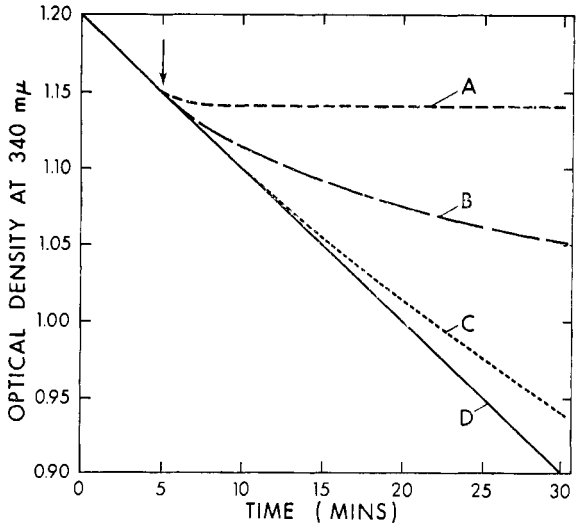

FIGURE 5: Inactivation of GMP reductase by 2.5 μM 6-thio-IMP. Conditions were as in Figure 4. Initial concentrations of GMP were: curve A, 50 μM; curve B, 250 μM; and curve C, 2 mM. Curve D was obtained in the absence of 6-thio-IMP.

purified enzyme preparations (Table III). Inosine 2′-phosphate inhibited less strongly than IMP, whereas adenosine 2′-phosphate inhibited more strongly than AMP (Table III); these data suggest that IMP acts at the GMP site in preference to the NADPH site.

A crude enzyme preparation (fraction A) was affected by the 6-thio nucleotides in the same way as was the step 4 preparation, although to a lesser degree, presumably because of the presence of β-mercaptoethanol in fraction A. Table III shows that fraction A was extensively inactivated when pretreated with a low level (9 μM) of 6-thio-GMP or when a higher level (100 μM) was added to the assay mixture under conditions similar to those of Figure 4. Fraction A was also subject to progressive inhibition by 6-thio-IMP which was slowed by GMP (Table III).

Discussion

The most active preparation of GMP reductase (step 4, Table I) was free of NADPH oxidase activity, of all but traces of IMP dehydrogenase, and of an enzyme which converts 6-Cl-IMP to IMP and which, as discussed previously (Hampton and Nomura, 1967), is probably an AMP deaminase. The purification tended to labilize the ability of the reductase to function unimpaired in the absence of aliphatic thiols but did not influence other properties including the interactions of the reductase with IMP and its 6-thio and 6-chloro analogs.

6-Chloropurine shows selective antitumor toxicity both clinically (Ellison *et al.*, 1959) and in experimental mouse neoplasms (Clarke *et al.*, 1954; Tarnowsky and Stock, 1957; Sartorelli and Booth, 1960a). The drug is converted by Sarcoma 180 mouse ascitic cells to 6-Cl-IMP by an intracellular purine nucleotide pyrophosphorylase for which 6-chloropurine is a substrate (Hampton and Paterson, 1966). That 6-Cl-IMP inhibits the conversion of IMP to XMP (Hampton, 1963; Anderson and Sartorelli, 1967) but not that of IMP to AMP (Hampton, 1962) in cell-free systems is consistent with the ability of 6-chloropurine to inhibit biosynthesis of guanine nucleotides but not of adenine nucleotides in various mouse neoplasms (Sartorelli and Booth, 1960b; Sartorelli *et al.*, 1960). The present finding that 6-Cl-IMP powerfully inhibits GMP reductase whereas the corresponding nucleoside does not suggests that GMP reductase is a second potential locus for the growth inhibitory action of 6-chloropurine. The reductase does occur in mammalian tissues (Hershko *et al.*, 1963) and levels of 6-Cl-IMP in tumor cells can be high enough (50 μM was attained under nonoptimized conditions; Hampton and Paterson, 1966) to inactivate significant amounts of IMP dehydrogenase and GMP reductase. 6-Cl-IMP inhibits conversion of IMP to XMP with equal effectiveness when the enzyme is from Sarcoma 180 as when it is from *A. aerogenes* (A. C. Sartorelli and J. H. Anderson, private communication) and IMP dehydrogenase of *A. aerogenes* is almost totally inactivated under physiological conditions by approximately 8 mol equiv of 6-Cl-IMP (L. W. Brox and A. Hampton, unpublished data). The present findings indicate a similar order of sensitivity for GMP reductase.

6-Mercaptopurine and 6-thioguanine, like 6-chloropurine, are clinically useful antineoplastic agents which are anabolized in susceptible biological systems to their ribonucleoside 5′-phosphates, *i.e.*, to 6-thio-IMP and 6-thio-GMP. In this form, probably, they inhibit synthesis and interconversion of purine mononucleotides (reviewed by Brockman and Chumley, 1965; Elion and Hitchings, 1965); in addition, 6-thio-GMP strongly inhibits conversion of GMP to GDP (Miech *et al.*, 1967). It is not clear which, if any, of the known metabolic hindrances is necessary for inhibition of growth; however, should depletion of adenine nucleotides be involved, the ability of 6-thio-IMP and 6-thio-GMP to inhibit conversion of GMP to IMP would tend to exacerbate the deficiency by restricting utilization of GMP derived from XMP or from exogenous guanine and IMP–GMP pyrophosphorylase. Normally, the conversion of GMP to adenine nucleotides tends to be stimulated when the level of ATP drops. The present studies show that 6-thio-IMP and 6-thio-GMP could powerfully inhibit GMP reductase in intact cells provided the immediate environment of the enzyme was not too strongly reducing. It is of interest that the highly cytotoxic 2-fluoroadenosine, at the nucleotide level, interferes with GMP reductase activity (A. Bloch, personal communication).

ATP inhibits GMP reductase of *A. aerogenes* reversibly with respect to GMP and thereby can help regulate cellular conversion of guanine nucleotides to adenine nucleotides (Mager and Magasanik, 1960; Magasanik and Karibian, 1960). GMP reductase purified by the

present procedure was inhibited by ATP to the same extent and ADP and AMP were considerably less effective. ATP at concentrations which virtually prevented conversion of GMP to IMP had no effect on the rate at which 6-Cl-IMP inactivated the enzyme (Figure 2), showing that ATP and 6-Cl-IMP inhibit GMP reductase at separate loci. 6-Cl-IMP probably inhibits at the GMP site (see below), whereas ATP probably does not because GTP, which should bind more strongly to the GMP site, is less inhibitory than ATP. NADPH did not reverse inhibition by ATP and hence ATP evidently acts at a site separate from the two substrate sites.

AMP deaminase of rat tissues is inhibited by GTP and activated by ATP (Setlow *et al.*, 1966). This suggested that GMP reductase of *A. aerogenes* which is inhibited by ATP might in reciprocal fashion be activated by GTP, but no such effect could be detected.

Low concentrations of 6-Cl-IMP progressively inactivated GMP reductase about as readily as IMP dehydrogenase (Hampton, 1963; Hampton and Nomura, 1967). Inactivation of the reductase further resembled that of the dehydrogenase: (a) it was slowed markedly by the mononucleotide substrate (*i.e.*, GMP) (Figure 2) but not by the pyridine coenzyme (NADPH) alone, (b) 6-chloropurine nucleoside at 1000-fold higher concentration was weakly inhibitory and did not cause inactivation, (c) unsubstituted purine ribonucleotide did not inactivate, and (d) the extent of inactivation, even after prolonged exposure to 6-Cl-IMP, was limited by the degree of activity of the enzyme preparation in the absence of GSH. Thus, GMP reductase fractions with an absolute requirement for GSH were not appreciably inactivated by 6-Cl-IMP in the absence of GSH, whereas under the same conditions enzyme fractions which were not stimulated by GSH were extensively inactivated. The specificity of 6-Cl-IMP for alkylation of GMP reductase is further shown by the finding that iodoacetamide, which is a more powerful alkylating agent, inactivated the enzyme much more slowly. Moreover, GMP could only partially protect the enzyme from the action of iodoacetamide (Figure 1), possibly as a consequence of alkylation at sites other than that for GMP.

In the absence of GSH, GMP reductase was rapidly inactivated by concentrations of 6-thio-GMP and 6-thio-IMP as low as one-tenth the estimated Michaelis constant of GMP (Figures 4 and 5). The process was retarded by GMP and reversed by GSH. 6-Thioguanosine was considerably less effective than 6-thio-GMP. IMP inhibited the enzyme reversibly with respect to GMP (Table III) but in contrast to the effects of 6-thio-IMP the degree of inhibition was essentially unaffected by addition of GSH. The 6-thio analogs exert similar effects on IMP dehydrogenase and, in addition, the action of IMP on the reductase is paralleled by the action of GMP on IMP dehydrogenase (Hampton, 1963; Hampton and Nomura, 1967).

Inactivation of GMP reductase, in analogy to that of IMP dehydrogenase (Hampton, 1963), is concluded to involve binding of the 6-chloro and 6-mercapto nucleotide analogs to the GMP site followed by formation of thioether and disulfide linkages, respectively, with an adjacent enzymic sulfhydryl group. Recently, direct spectrophotometric evidence has confirmed that inactivation of IMP dehydrogenase by 6-Cl-IMP is accompanied by formation of a 6-alkylthio purine derivative (L. W. Brox and A. Hampton, unpublished data).

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Enzymatic Hydrolysis of Calf Thymus Deoxyribonucleic Acid Adsorbed on Diethylaminoethylcellulose*

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ABSTRACT: A procedure is described for the enzymatic hydrolysis of calf thymus deoxyribonucleic acid (DNA) adsorbed on DEAE-cellulose. After nuclease inactivation, stepwise elution with solutions of increasing NH_4HCO_3 concentrations yielded large oligomers. These compounds were characterized by base ratio and end-group chain-length determinations, centrifugation through sucrose density gradients, and column chromatography on DEAE Sephadex A-25 in 7 M urea. Applications of the procedure are discussed.

Studies on the determination of nucleotide sequences in DNA have lagged behind those concerned with RNA largely because (a) of the smaller size of RNAs, especially tRNAs, which allowed the elucidation of complete base sequences (Holley *et al.*, 1965; Zachau *et al.*, 1966; Madison *et al.*, 1966), and (b) because of the lack of specific DNases comparable to pancreatic RNase and RNase T_1. Such specific DNases, if available, would facilitate the preparation and isolation of large oligomers ($n = >10$), important for nucleotide sequence characterization. Known DNases such as micrococcal nuclease (EC 3.1.4.7), pancreatic DNase (EC 3.1.4.5), and *E. coli* endonuclease I do not have strict specificities and yield, in limit digests, oligomers of average chain length 2–8 (Roberts *et al.*, 1962; Tomlinson and Tener, 1963; Sinsheimer, 1952; Lehman, 1963) Chemical methods of DNA hydrolysis (Volkin *et al.*, 1951; Tamm *et al.*, 1952; Whitfeld, 1954; Dekker *et al.*, 1953; Burton and Petersen, 1960; Habermann *et al.*, 1963; Chargaff *et al.*, 1963; Hall and Sinsheimer, 1963) or combinations of enzymatic and chemical methods (such as DNA polymerase incorporation of ribonucleotides into newly synthesized DNA followed by alkaline hydrolysis; Berg *et al.*, 1964) yield large oligonucleotides from DNA. However, there is a need for additional methods for the preparation of large oligodeoxynucleotides, especially of those with mixed purine:pyrimidine ratios, because they yield more nucleotide sequence information than oligomers of chain length $n = 2$–8.

Enzymatic hydrolysis of high molecular weight RNA adsorbed on DEAE-cellulose was previously shown to yield large oligomers in good yields. Thus, after RNA of the *E. coli* phage MS 2 was bound to the adsorbent, hydrolyzed *in situ* with ribonucleases, and the enzymes were removed, stepwise elution with solutions of increasing salt concentrations yielded large oligomers in good yield. This was ascertained by end-group chain-length determinations and by column chromatography. With the RNase of *Bacillus subtilis* (Nishimura and Nomura, 1958), nucleotide composition of the oligomers so obtained was significantly different from that of MS 2 RNA itself (Rushizky *et al.*, 1966).

This report describes an extension of the above RNA procedure to the enzymatic hydrolysis of calf thymus DNA adsorbed on DEAE-cellulose. With both pancreatic DNase and micrococcal nuclease, oligomer fractions so obtained had average chain lengths (by end-group determination) ranging from $n = 27$ to 228. The oligomer fractions were also examined by column chromatography in 7 M urea on DEAE-Sephadex, by nucleotide composition, and by sedimentation through sucrose density gradients.

Experimental Procedure

Spectrophotometric measurements were made in cells of 1-cm light path and are expressed as absorbance at 260 mμ (A_{260}).

Enzymes. Micrococcal nuclease (EC 3.1.4.7) of 90% purity was a gift of Drs. H. Taniuchi and C. B. Anfinsen (Heins *et al.*, 1967). Alkaline phosphatase was isolated from *E. coli* C 90 as described by Neu and Heppel (1965). Recrystallized pancreatic DNase (EC 3.1.4.5) was obtained from Sigma.

Nucleic Acid Materials. Calf thymus DNA (highly polymerized, lot DNA 607) was obtained from Worthington. DNA from calf thymus was also prepared by extraction with 1 M NaCl–phenol (Colter *et al.*, 1962)

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