the tertiary bases are in most cases too insoluble at the pH of the test solution (pH 7.4) to be studied in detail.

The lipophilic character of the thiol part of the esters appears to be of importance for the inhibitory activity. This can be observed by small structural changes such as the replacement of methyl with ethyl groups at the quaternary nitrogen (*cf.* **6** and **9**). A remarkable increase in activity is obtained by the introduction of a benzyl group as can be seen within each series of esters in Table I (*cf.* **2** and **3**; **8** and **9**; **11** and **12**; **14** and **15**). Generally, it may be said that the benzyldiethylammonium thiol esters **3**, **9**, **12**, and **15** are two or three times more active than the corresponding diethylmethylammonium thiol esters **2**, **8**, **11**, and **14**. It seems likely that this increased activity is due to the higher lipophilicity of the benzyl derivatives.

The most active inhibitor of FSF hitherto described in the literature is monodansylcadaverine. In the bioassay used in this study, monodansylcadaverine can inhibit the cross-linking at a minimum concentration of 4.0 m*M*. Two of the thiol esters, **6** and **9**, in Table I have approximately the same inhibitory activity as monodansylcadaverine, whereas two other esters, *viz.* 2-benzyldiethylammonioethyl 3-phenylthiolpropionate (**12**) and 2-benzyldiethylammonioethyl 4-phenylthiolbutyrate bromide (**15**) can inhibit the cross-linking at concentrations of only 3.0 and 3.5 m*M*, respectively. These are the most active inhibitors of the fibrin-stabilizing factor so far described.

Experimental Section

General Comments. Melting points were determined with calibrated Anschütz thermometers in an electrically heated metal block. All crystallized compounds were characterized by elemental analyses (C, H, N), which were within ±0.4% of the theoretical values, and ir spectra, which were run for identification purposes on a Perkin-Elmer 237 spectrophotometer.

Synthesis. The thiol esters carrying a tertiary amino group, **1**, **4**, **7**, **10**, and **13**, were all prepared as described for **1**.[8] These were then quaternized using methyl iodide or benzyl bromide as described for **2**, yielding **2**, **3**, **5**, **6**, **8**, **9**, **11**, **12**, **14**, and **15**.

2-Diethylaminoethyl Thiolbenzoate (1). Benzoyl chloride (4.7 g, 33.4 mmol) in dry benzene (50 ml) was slowly added at room temperature to a solution of 2-diethylaminoethanethiol (4.7 g, 35.1 mmol) and triethylamine (3.6 g, 35.4 mmol) in benzene (30 ml). After 3 hr the triethylamine hydrochloride was filtered off and the filtrate evaporated yielding the title compound as an oil (7.0 g, 88%). The hydrochloride had mp 140–141° (from EtOH) (reported[9] 137–138.5°).

2-Diethylmethylammonioethyl Thiolbenzoate Iodide (2). Compound **1** (1.3 g, 5.5 mmol) in dry acetone was allowed to react with methyl iodide (2.5 g, 17.6 mmol) at room temperature for 48 hr. A crystalline compound (48%) was obtained, mp 118–119° (from EtOH). *Anal.* ($C_{14}H_{22}INOS$) C, H, N.

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Three New Adenosine Triphosphate Analogs. Synthesis and Effects on Isolated Gut

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ATP is known to induce relaxation of isolated mammalian gut preparations,[1-6] and evidence has been presented indicating that a purine nucleotide, probably ATP, is the transmitter released from nonadrenergic, noncholinergic inhibitory nerves to the gastrointestinal tract.[5-9] The pharmacological responses of ADP, AMP, and adenosine are substantially modified by purine ring or phosphate chain substitutions. For example, the platelet-aggregating properties of ADP are greatly enhanced by substitution of chloro or methylthio groups in position 2 of the purine ring and are abolished when the anhydride oxygen of the pyrophosphate moiety is replaced by a methylene group.[10] 2-Methylthio-AMP is a significantly more potent inhibitor of ADP-mediated platelet clumping than is AMP[11] and 2-chloroadenosine has more potent and longer lasting coronary and peripheral vasodilator effects than adenosine.[12] Mihich, *et al.*,[2] reported that 2-chloroadenosine was 2–3 times more active than adenosine in inhibiting spontaneous motility and tone in the isolated rabbit intestine. In order to compare the effects of analogous structurally modified derivatives of ATP on the isolated gut with those of ATP, we have synthesized 2-chloroadenosine 5'-triphosphate (2-chloro-ATP), 2-methylthioadenosine 5'-triphosphate (2-methylthio-ATP), and 2-chloro-5'-adenylyl methylenediphosphonate (2-chloro-AOPOPCP). We compared the relaxations elicited by these analogs and by ATP on the guinea-pig isolated taenia coli.

2-Chloro-ATP and 2-methylthio-ATP were synthesized in good yield by the general method of Moffat[13] from the 4-morpholine-*N*,*N*'-dicyclohexylcarboxamidinium salts of the appropriate adenosine 5'-phosphoromorpholidate analogs, the syntheses and characterization of which have been described elsewhere.[10,14] Preparations of 2-chloroadenosine 5'-phosphoromorpholidate contained 2-morpholinoadenosine 5'-phosphoromorpholidate as a minor contaminant, as previously noted,[10] resulting from nucleophilic substitution of chlorine by morpholine at the 2 position. Thus, although 2-chloroadenosine 5'-phosphoromorpholidate was chromatographically homogeneous in two different solvent systems and gave a satisfactory elemental analysis, it exhibited a bright blue fluorescence under ultraviolet light, in contrast to both 2-chloro-AMP and the 4-morpholine dicyclohexylcarboxamidinium ion; it also possessed an anomalous uv spectrum at pH 1, with a small extra peak at 300 nm. Replacement of 2-chloro-AMP by 2-chloroadenosine in the phosphoromorpholidate synthesis[10,14] resulted in the formation of a by-product, which was isolated in 45% yield after separation from unchanged starting material and characterized as the previously unreported nucleoside 2-morpholinoadenosine. Whenever the 2-chlo-

Table I. Molar Inhibitory Potencies of the ATP Analogs Relative to ATP[a] on the Guinea Pig Taenia Coli Preparation

Compound	Potency ratios
ATP	1.0
2-Chloro-ATP	3.1
2-Chloro-AOPOPCP	6.3
2-Methylthio-ATP	50

[a] ATP ED_{50} was 0.75 ± 0.034 μM.

Table II. Chromatographic, Electrophoretic, and Spectral Properties of ATP Analogs

Compound	R_f, solvent I	M_{AMP}	λ_{max} (0.1 N HCl), nm	ϵ ($\times 10^{-3}$)
ATP	0.37	1.88		
2-Chloro-ATP	0.36	1.83	265	12.4
2-Methylthio-ATP	0.45	1.73	268	14.5
2-Chloro-AOPOPCP	0.42	1.74	264	12.4

roadenosine 5′-phosphoromorpholidate was used for the synthesis of 5′-polyphosphates, each product in the reaction mixture was found to be accompanied by its fluorescent 2-morpholino counterpart. Although these could easily be removed by preparative paper chromatography, it must be noted that, as in the case of the previously described synthesis of 2-chloroadenosine 5′-diphosphate,[10] use of the 5′-phosphorimidazolidate derivative as the activated intermediate[15] would entirely eliminate the problem of formation of such side products. The synthesis of 2-chloro-AOPOPCP was accomplished by reaction of impure 2-chloroadenosine 5′-phosphoromorpholidate with di(tri-*n*-butylammonium) methylenediphosphonate in pyridine for 3 days. After DEAE column chromatography and separation from the 2-morpholino homolog by preparative paper chromatography, 2-chloro-AOPOPCP was obtained in good yield.

Each of the three new ATP analogs caused relaxation of the guinea-pig taenia coli. Dose–response curves were parallel to that of ATP, except for 2-chloro-AOPOPCP which had a somewhat steeper curve. Molar potency ratios of the compounds are listed in Table I. They show that all three analogs were more potent than ATP in causing inhibition of the guinea-pig taenia coli. The 2-methylthio-substituted analog was 50 times more potent than ATP in this action, reminiscent of the 30-fold enhancement of the platelet aggregating potency of ADP caused by 2-substitution of the methylthio group in ADP.[10] The 2-chloro-AOPOPCP analog was even more potent than 2-chloro-ATP, each being 6.3 and 3.1 times more potent than ATP, respectively. This result suggests that hydrolysis of the terminal phosphate of ATP or its analogs is not a prerequisite for inhibitory activity. The mechanism of action of ATP in causing relaxation of mammalian gut is not known. ADP is as active as ATP, and AMP and adenosine also elicit relaxation but at considerably lower dose levels.[4,6] More detailed studies of the inhibitory actions of these analogs and comparison with other ATP, AMP, and adenosine analogs are reported elsewhere.†

Experimental Section

Analytical chromatograms were run on Whatman No. 1 paper and preparative chromatograms on Whatman No. 3 MM paper; the solvent system (solvent I) was isobutyric acid–1 M NH_4OH (5:3). Paper electrophoresis was carried out on Schleicher and Scheull No. 2043 paper (4 × 41 cm) in 0.025 M citrate buffer pH 4.8[16] with a potential drop of 200 V for 5 hr. AMP was run as a reference marker, and mobilities are expressed as M_{AMP}, *i.e.*, distance of travel relative to that of AMP. Ultraviolet absorption spectra were obtained on a Perkin-Elmer 350 spectrophotometer. Chromatographic, electrophoretic, and spectral data of the nucleotide analogs are listed in Table II. Microanalyses were carried out by Dr. J. Fildes, Australian National University. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical value.

2-Chloroadenosine 5′-Triphosphate. 2-Chloroadenosine 5′-phosphoromorpholidate[14] (86 mg, 0.1 mmol) was allowed to react with tetra(tri-*n*-butylammonium) pyrophosphate in anhydrous DMSO for 3.5 days as described by Moffatt.[13] The mixture was then diluted with H_2O (20 ml), and the resulting solution was applied to a column of Whatman DE23 DEAE cellulose (2.5 × 20 cm, HCO_3^- form). The column was washed with water and eluted with a linear gradient of NH_4HCO_3 (0–0.4 M in 3 l.); 15-ml fractions were collected. Two peaks were obtained. The first (fractions 58–70) contained a mixture of 2-chloro-AMP and 2-morpholino-AMP. Tubes 81–120 containing the second peak were analyzed individually by chromatography in solvent I. Substances having the same R_f values as 2-chloro-ADP and 2-morpholino-ADP[10] were in fractions 81–95 and the desired product was located in fractions 96–120, together with (presumably) 2-morpholino-ATP in fractions 96–106. The 2-morpholino-substituted nucleotides exhibited a distinctive bright blue fluorescence under uv light and were well separated from the corresponding 2-chloro analogs by paper chromatography in solvent I.

Fractions 96–120 were combined and evaporated to yield 46.7 mg of a colorless glass which was stored for several months below 0° before final purification. Paper chromatography indicated that considerable breakdown to the mono- and diphosphates had occurred during storage. The material was purified by preparative paper chromatography in solvent I; the ultraviolet absorbing band at R_f 0.36 was cut out, washed with *i*-PrOH to remove ammonium isobutyrate, and eluted with H_2O. Lyophilization of the aqueous eluates gave 35.4 mg (53%) of 2-chloro-ATP. *Anal.* Calcd for $C_{10}H_{15}ClN_5O_{13}P_3 \cdot (NH_3)_3 \cdot 4H_2O$: C, 18.1; H, 4.8; N, 16.8; P, 14.0. Found: C, 18.7; H, 4.6; N, 16.1; P, 13.4.

2-Methylthioadenosine 5′-Triphosphate. 2-Methylthioadenosine 5′-phosphoromorpholidate[10] (79 mg, 0.1 mmol) was allowed to react with pyrophosphate and the reaction mixture was worked up as described above. The product was eluted from the DEAE cellulose column in fractions 96–125, which were pooled and evaporated yielding 45 mg of glassy solid, chromatographically homogeneous in solvent I. This material also degenerated after storage for several months and was purified by preparative paper chromatography in solvent I to give 33.4 mg (51%) of 2-methylthio-ATP. *Anal.* Calcd for $C_{11}H_{18}N_5O_{13}P_3S \cdot (NH_3)_3 \cdot 3H_2O$: C, 20.1; H, 5.0; N, 17.0; P, 14.1. Found: C, 20.6; H, 4.7; N, 16.6; P, 13.5.

2-Chloro-5′-adenylyl Methylenediphosphonate. 2-Chloroadenosine 5′-phosphoromorpholidate (61 mg, 0.07 mmol) and di(tri-*n*-butylammonium) methylenediphosphonate (0.28 mmol, prepared from 50 mg of methylenediphosphonic acid and 0.135 ml of n-Bu_3N) were separately rendered anhydrous by several evaporations of their solutions in dry pyridine, then combined in dry pyridine (1 ml), and allowed to react for 3 days. Solvent was removed by coevaporation with water and an aqueous solution of the residue was applied to the DEAE column, which was then washed and eluted in the usual manner. Two peaks were eluted, in fractions 55–66 (2-chloro-AMP and 2-morpholino-AMP) and fractions 84–108.

The second peak yielded, after preparative paper chromatography in solvent I as described above, 38.4 mg (86%) of pure 2-chloro-5′-adenylyl methylenediphosphonate as a white freeze-dried solid. *Anal.* Calcd for $C_{11}H_{17}ClN_5O_{12}P_3 \cdot (NH_3)_3 \cdot 2.5H_2O$: C, 20.78; H, 4.91; N, 17.63; P, 14.61. Found: C, 21.39; H, 4.39; N, 18.11; P, 14.24. The purified ATP analogs were stable on storage below 0°; electrophoresis after 12 months showed only a trace of decomposition.

Identification of the Fluorescent Contaminant in 2-Chloroadenosine 5′-Phosphoromorpholidate. 2-Chloroadenosine 5′-phosphoromorpholidate (2 mg) was dissolved in 0.1 N HCl (0.5 ml) and allowed to stand at room temperature. Aliquots were chromatographed at 5 and 10 min in solvent II. At 10 min no starting material remained and the uv-absorbing spot of 2-chloro-AMP had a bright blue leading edge, indicating the presence of an incompletely resolved contaminant. A reaction analogous to

†D. G. Satchell and M. H. Maguire, unpublished results.

that described for the preparation of 2-chloroadenosine 5′-phosphoromorpholidate was carried out in which 2-chloroadenosine was substituted for 2-chloro-AMP. Chromatography of the water-soluble reaction product in i-Pr_2O-EtOH-H_2O (25:10:saturated)[17] on a cellulose column separated 2-chloroadenosine and the fluorescent material. Evaporation of fractions containing the latter product gave a crystalline solid, 2-morpholinoadenosine, which was recrystallized from water as needles: mp 245–246°; λ_{max} (0.1 N HCl) 260 nm (ϵ 15,700), 299 (7800); λ_{max} (0.1 N NaOH) 261 nm (ϵ 13,000), 284 (8000). *Anal.* ($C_{14}H_{20}N_6O_5$) C, H, N.

Guinea Pig Taenia Coli Preparation. Strips of taenia coli were dissected and mounted in organ baths in modified Krebs solution containing hyoscine as described.[6] Muscle activity was recorded with an isotonic lever writing on a smoked drum. ATP and triphosphate analogs were added to the bath to give concentrations of 0.10–10 μM. Dose–response curves were plotted for six different concentrations over this range, each point representing the mean of at least four observations. Molar potencies were obtained from the antilog of the difference between the pD_2 values for the analog and ATP.[18]

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Methotrexate Analogs. 2. A Facile Method of Preparation of Lipophilic Derivatives of Methotrexate and 3′,5′-Dichloromethotrexate by Direct Esterification†,[1]

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Recent years have witnessed an expanding interest in the clinically advantageous pharmacological properties of "prodrugs" or drug derivatives exhibiting the phenomenon of latentiation.[2] The synthesis[3] of a series of lipophilic long-chain alkyl esters of the antitumor agent 1-β-D-arabinofuranosylcytosine (ara-C) with superior therapeutic properties as immunosuppressive and antileukemic agents in experimental animals[4] provides but one notable illustration of this approach to drug design. While latentiation was achieved in this instance *via* esterification of an alcohol function in the parent molecule, the converse strategy of esterifying a carboxy group with a long-chain alcohol appears to have received sparse attention in the literature. In the work reported here, some potential latent derivatives of the second type were prepared, in the form of a series of heretofore undescribed alkyl esters of 4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid (amethopterin, methotrexate, MTX), another cancer chemotherapeutic agent in widespread clinical use.[5,6] The structures of eight such MTX esters are shown in Table I, together with six others derived from 3′,5′-dichloro-4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid (3′,5′-dichloromethotrexate, DCM).‡ The latter were viewed with particular interest because of the possibility that their enhanced lipophilicity might favor passage across the blood-brain barrier and thereby lead to agents useful for the treatment of tumors of the central nervous system.

Esters of both MTX and DCM were obtained in excellent yield *via* a facile procedure for direct esterification adapted from the early work of Hutchings and coworkers[8] who esterified folic acid in 65% yield by reaction with 0.1 N HCl in anhydrous methanol at room temperature for 24 hr. In the present study, esterification was achieved *via* any of four modifications of this method, the choice of variant being dictated mainly by the chemical reactivity and water miscibility of the particular alcohol selected. Examples of each procedure are given in the Experimental Section and yields are presented in Table I. A surprising and significant aspect of the findings reported here is the apparent stability of the 4-amino group of MTX under moderately strenuous acid conditions.

Esterifications proceeded rapidly at room temperature when either MTX or DCM was stirred in low-molecular-weight primary alcohols containing 0.25 N HCl (methods A and C). Reaction mixtures tended to remain heterogeneous with MTX but became homogeneous almost immediately when DCM was used, in accord with the expected enhancement of lipophilicity by 3′,5′-dichloro substituents. Although uniform reaction times of 24 or 48 hr were generally employed for the sake of convenience, thin-layer chromatography revealed disappearance of the starting material after a much shorter time. For example, in one experiment involving the dibutyl ester **5**, work-up after only 6 hr produced a yield not significantly different from that obtained with other primary alcohols after longer intervals.

With secondary alcohols or long-chain primary alcohols such as 1-octanol, reactions at room temperature gave poor yields. Two procedural variants were developed in order to surmount this problem. In the first of these (method B), involving for example the diisopropyl ester **4**, water generated during the esterification was removed continuously by azeotropic distillation with benzene, the reaction being run in a Soxhlet apparatus containing molecular sieves in the thimble. This method was successful with 1-pentanol but failed with 1-octanol, apparently because hydrogen chloride is sparingly soluble in boiling mixtures of benzene and 1-octanol and therefore is easily lost. Accordingly, for the synthesis of esters **12** and **13**, another variant (method D) was devised which involved

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‡ A referee has kindly called the author's attention to a recent abstract describing MTX and DCM esters as substrates for liver aldehyde oxidase; see Wolpert, *et al.*[7]