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EFFECTS OF TUBERCIDIN AND ITS RIBONUCLEOTIDES ON VARIOUS METABOLIC PATHWAYS IN *SCHISTOSOMA MANSONI**

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Abstract—The onset of tubercidin's (7-deazaadenosine; Tu) effects upon *S. mansoni in vitro* is dose-related. At 10^{-5} M, Tu caused abnormal activity patterns and separation of the sexes within 6–9 hr. During this time, Tu did not appreciably affect the rate of glucose consumption, lactate production, or glycogen content of the worms.

Tu inhibited the utilization of radioactive adenosine for adenine nucleotide formation by greater than 50 per cent *in vitro*. This effect was more than just a simple competition between Tu and adenosine for entrance into the cell and the formation of their respective ribonucleotides. Yet, the rate of conversion of AMP to ADP or ADP to ATP was not influenced or only inhibited to a small extent. For the guanine nucleotides, Tu inhibited the utilization of radioactive adenosine for the formation of these nucleotides as well; however, this effect appears to be solely a simple competition between adenosine and Tu for entrance into the cell.

The synthesis of tubercidin-5′ mono-, di- and triphosphate, TuMP, TuDP and TuTP, respectively, was accomplished simultaneously by enzymes from the erythrocytes of the dog. TuDP was a much poorer substrate for schistosome adenylate kinase in comparison to ADP.

The particulate fraction of schistosome homogenates contained ATPase activity which was primarily Mg^{2+}-dependent, but also had a small component that was Na^+, K^+-dependent and ouabain-sensitive. Instability of the preparation precluded definitive investigation of the suitability of TuTP as a substrate, but TuTP was found to be a less efficient substrate than ATP for Na^+, K^+-dependent ATPase from rabbit kidney.

Supernatant fractions of schistosome homogenates did not contain detectable amounts of creatine phosphokinase (CPK) activity utilizing creatine phosphate as the phosphagen. However, TuDP was nearly as effective a substrate for CPK from rabbit muscle as was ADP.

TUBERCIDIN (7-deazaadenosine; Tu) was found to affect adversely the viability and egg-laying capacity of *Schistosoma mansoni* in mice with markedly reduced host toxicity when it was administered after absorption into the red cells of the host.[1] This adenosine analog was also active against *S. mansoni in vitro*, causing separation of paired adults, alteration of the muscular activity pattern, loss of ventral sucker function, and inhibition of egg-laying when present in a concentration as low as 10^{-7} M.[1]

The mode of action of the potent cytotoxic antibiotic in other systems has been investigated, and it was found that Tu after its assimilation by susceptible cells is anabolized to the 5′-mono-, di-, and triphosphate forms which can substitute for the

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corresponding adenosine nucleotides in a variety of enzymatic reactions. Acs *et al.*[2] have shown that Tu is incorporated into the RNA and DNA of mouse fibroblasts *in vitro*, with lethal consequences. In this case, cell death was preceded by inhibition of RNA, DNA as well as protein synthesis. On the other hand, the inhibitory action of Tu against the growth of *Streptococcus faecalis* could be reversed or prevented by pyruvate, suggesting in this case that Tu may be interfering with one or more aspects of phosphorylative glycolysis.[3]

Since *S. mansoni* seems to depend primarily if not exclusively upon preformed purines (especially adenine or adenosine) for its total purine nucleotide requirements,[1,4] and because in this species phosphorylative glycolysis constitutes the major, if not the exclusive, source of metabolic energy,[5] we were led to investigate the effects of Tu and its nucleotides on the uptake and anabolism of adenosine by *S. mansoni*, as well as on phosphorylative glycolysis and other ATP-requiring metabolic pathways.

MATERIALS AND METHODS

Organic reagents of the highest available purity which were used for thin-layer chromatography and liquid scintillometry were purchased from the Fisher Scientific Company, Medford, Mass., and the Packard Instrument Co., Dowers Grove, Ill.

Polyethyleneimine (PEI) cellulose thin-layer plastic sheets were purchased from Brinkmann Instruments, Inc., Westbury, N.Y.

Commercial sources of enzymes were: Calbiochem, Los Angeles, Calif. (creatine kinase, EC 2.7.3.2, 274 i.u./mg; glucose 6-phosphate dehydrogenase, EC 1.1.1.49, 160 i.u./mg; and hexokinase, EC 2.7.1.1., 140 i.u./mg); Sigma Chemical Co., St. Louis, Mo. (lactate dehydrogenase, EC 1.1.1.28, 600 i.u./mg and pyruvate kinase, EC 2.7.1.40, 400 i.u./mg); Boehringer Mannheim Corp., New York, N.Y. (myokinase, EC 2.7.4.3, 360 i.u./mg); Worthington Biochemical Corp., Freehold, N.J. (Glucostat). Diazyme (containing a 1,4-, 1,6- α-amyloglucosidase) was kindly provided by Miles Laboratories, Marshall Division, Elkhart, Ind.

Adenine and guanine nucleotides, NAD^+ and $NADP^+$ were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Adenosine-8-^{14}C (53 mCi/mole) was purchased from the New England Nuclear Corp., Boston, Mass. Tubercidin (Lot No. 8458-THP-65·5) was generously provided by Dr. G. B. Whitfield, Jr. of the Upjohn Co., Kalamazoo, Mich.

Synthesis of tubercidin nucleotides. Tubercidin nucleotides were synthesized in this way: approximately 14 ml of packed dog red cells were suspended by the addition of an equivolume of ice-cold doubly distilled water, and the suspension was sonicated at −15° for 30 sec with a Sonifer Cell Disrupter, model W185 (Heat Systems-Ultrasonics, Inc., Plainview, L.I., N.Y.) equipped with a microtip and set at a power output of 40 W. After centrifugation at 27,000 *g* for 20 min (4°), the supernatant was dialyzed against 1 l. of 50 mM Tris-acetate buffer, pH 7·4, with two changes of the buffer hourly for 3 hr. ATP, creatine phosphate, $MgSO_4$ and KF were added to the dialysate to yield final concentrations (in mM) of 0·1, 5·0, 0·4 and 100 respectively. Tu, previously dissolved in 0·5 ml of 100% dimethylsulfoxide (DMSO), was then added with stirring to yield a final concentration of 0·67 mM together with creatine phosphokinase at 3 i.u./ml. The reaction mixture (final volume 30 ml) was incubated at room temperature for 18–20 hr in the dark.

After incubation, the reaction mixture was cooled to 0°, and an equivolume of ice-cold 10% trichloroacetic (TCA) was added with stirring. After centrifugation at 27,000 *g* for 20 min (4°), the precipitate was discarded, and the TCA in the supernatant fraction was mostly removed by at least six extractions with 2 vol. of water-saturated diethyl ether until the pH of the solution was 5. The supernatant fraction, diluted to 100 ml with cold water, was subjected to DEAE column chromatography (4°) by the procedure of Miech *et al.*[6] Fractions of 10 ml were collected with the effluent monitored at 272 nm, and those containing nucleotides were concentrated by evaporation under reduced pressure. The residues were dissolved in water to yield concentrations varying from 1 to 4 mM, as measured spectrophotometrically. These samples were kept at −20° until used. Yields of Tu 5′-nucleotides were: Tu monophosphate (TuMP), 30%; Tu diphosphate (TuDP), 30%; and Tu triphosphate (TuTP), 15% based on total Tu. These concentrations were calculated using the molar absorbancy of Tu, 12,000 at pH 7,[7] assuming that phosphorylation of the nucleotide does not modify the molar absorbancy.[8,9]

To further ascertain the purity of particular Tu nucleotides contained in the collected fractions (see Fig. 1) after their identification by spectral pattern,* they were subjected to PEI cellulose thin-layer chromatography according to Randerath.[10] After spotting samples, plates were washed with 50% methanol (v/v) to remove salts,[11] dried, then developed with 1·0 M LiCl to a 10 cm solvent front. The R_f values for ATP, ADP and AMP were 0·06, 0·27 and 0·50 respectively. Those for TuTP, TuDP and TuMP were 0·04, 0·13 and 0·30 respectively. In this system, at least 0·5 nmole of adenine nucleotides can be detected. Chromatographic tanks were covered during elutions but not saturated, and were kept at room temperature. Elution time was abour 1 hr.

Incubation of schistosomes. Adult schistosomes were obtained from mice infected 8–9 weeks previously† in the same manner as described elsewhere.[12] For studies *in vitro*, groups of ten pairs of worms (unless otherwise indicated) were preincubated at 37° for 18–24 hr in 6 ml of Minimum Essential Medium Eagle with Hanks's balanced salt solution, supplemented with 2% fetal calf serum, 1% non-essential amino acids, streptomycin, 100 μg/ml, and benzyl-penicillin, 100 units/ml (Microbiological Associates, Inc., Bethesda, Md.). The incubation vessels were cell culture tubes, 10-ml capacity, and during the preincubation period they were slowly turned on a rotor drum. After the preincubation period, the medium was removed and replaced with 4 ml of fresh medium. The tubes were then transferred to a constant temperature module heater (Multi-Temp-Blok, Labline Instruments, Inc., Melrose Park, Ill.) and were incubated for an additional 30 min at 37° before timed experiments were begun.

Desired concentrations of Tu or non-radioactive adenosine were dissolved in 10% DMSO, and no more than 20 μl of each solution was introduced into each vessel containing 4 ml of medium. Thus the highest possible concentration of DMSO in contact with the worms was 1·0 per cent, and this amount was found not to affect any aspect of their metabolism, when compared with controls.

Identification of purine nucleotides in TCA-soluble fractions of schistosomes. After incubation of schistosomes in the presence of adenosine-8-^{14}C for varying times with

* The spectrums of the concentrated 1–4 mM solutions were recorded, and compared to that of authentic Tu.

† Only schistosomes of identical age were used in each individual experiment.

and without added Tu, the medium was removed to 25 μl and less by siphoning off and tube inversion over tissue paper for 15–30 sec drainage period. Ice-cold 10% TCA (0·5 ml) was added immediately to the medium-freed worms, and the tube rapidly vortexed for 15 sec. Each tube, so treated, was placed in ice. The worms were homogenized by transferral of the contents of each tube to a 3-ml conical-shaped tissue homogenizer in ice and homogenizing by eight passes or more of the pestle. The TCA homogenates were centrifuged for 15 min at 2000 rev/min in a refrigerated International centrifuge (4°). The supernatants were transferred to 12-ml conical centrifuge tubes, and TCA was removed by extraction with 10 vol. of water-saturated ether, five times. If the ether extracted fluid was not clear, it was centrifuged as above, and then stored at −30° until radioactive analyses.

The method of Crabtree and Henderson,[11] slightly modified, was used to resolve the purine nucleotides present in the acid-soluble fraction. By means of this method of PEI cellulose thin-layer chromatography, sharp separations of most nucleotides were obtained, the exceptions being GMP vs. XMP and AMP vs. NAD^+, which either overlapped or were closely adjacent. Careful analysis of the distribution of radioactivity in such couplets containing ^{14}C-labeled nucleotides indicated that most of the radioactivity was localized in either GMP or AMP.

Crabtree and Henderson[11] did not determine the position of $NADP^+$ in their system. It was found to elute between XMP and IMP with adequate separation. In this study, the PEI sheets were not pre-washed with formate buffer, and the 50% methanol wash was carried out for 5 hr first, then overnight. Under these conditions of washing, uric acid is not removed from the PEI sheet, but was located immediately above and close to IMP.

The PEI cellulose plastic sheet, bearing several concurrently chromatographed 20-μl samples of acid-soluble, ^{14}C-containing nucleotides was cut into strips, and rectangular sections containing the corresponding nucleotide were placed in 10 ml of scintillation fluid (composed of 4 g 2,5-diphenylozazole (PPO) and 0·1 g 2,2-*p*-phenylenebis(5-phenyloxazole) (POPOP)/l. of toluene) for liquid scintillometry. Radioactivity was counted by a Packard Tri-Carb liquid scintillation spectrometer model 3214, with a counting efficiency of 75 per cent. No greater than 10 per cent quenching was found in the counting of the PEI rectangular sections when checked by a channels ratio method described by Wang and Willis.[13]

Estimation of glucose consumption, lactate production and glycogen content of schistosomes. After varying periods of incubation, the worms and medium were separated by siphoning off, and the worms quickly brought to −20° until assayed for glycogen content, while aliquots of the medium were assayed for glucose and lactate. Glycogen levels in KOH digests of the worms[14] were measured spectrophotometrically by the coupled enzyme system (Diazyme, hexokinase, and glucose 6-phosphate dehydrogenase) described by Robinson *et al.*[15] The amount of glucose remaining in the medium was estimated by the Glucostat method[16] and the amount of lactate by the method of Barker and Summerson.[17]

Myokinase and creatine phosphokinase activity in schistosome homogenates. One hundred pairs of adult worms were placed in 2 ml of 25 mM glycylglycine buffer, pH 7·5, and sonicated at −15°, using two 30-sec exposures with a power output of 40 W. After centrifugation at 10,000 *g* for 20 min (4°), the supernatant was saved for assay. The assay for creatine phosphokinase was a modification of that of Rosalki,[18]

in that the final volume was 1 ml rather than 3·0 ml; 15 mM $MgCl_2$ was substituted for 30 mM $MgCl_2$; and 10 mM 2-mercaptoethanol was substituted for 5 mM cysteine-HCl.

The assay for myokinase was identical to that for creatine phosphokinase except that AMP and creatine phosphate were omitted.

Na, K-ATPase isolation and measurements. Approximately 500 pairs of adult worms were quickly frozen at −55°, and then allowed to thaw. They were immediately placed in a 5-ml Elvehjem-type homogenizer in ice to which was added 4 ml of cold 0·25 M sucrose and 5 mM EDTA in 20 mM Tris–HCl buffer, pH 7·4. The worms were homogenized by eight or more passes of the pestle. From this homogenate, Na, K-ATPase activity was received after processing it according to the procedure of Nakao *et al.*[19] for homogenates. The resultant 20,000 *g* pellet was suspended in the above sucrose, EDTA buffered solution instead of distilled water. Yet, this preparation was unstable to repeated freeze–thaw cycles, and lost all Na, K-ATPase activity within 1 week.

Na, K-ATPase was prepared from 250 mg of rabbit kidney parenchyma by the method of Nechay and Nelson.[20] The final volume of the microsomal suspension was 2 ml. Further purification of an aliquot of this preparation (1·5 ml) was achieved by the method of Nakao *et al.*,[19] and the final suspension of this purified Na, K-ATPase in 0·5 ml of the sucrose, EDTA buffered solution remained stable at −20° for 2–3 weeks despite repeated freeze–thaw cycles.

Two methods were used to measure the Na, K-ATPase activity in the particulate fractions of schistosomes and rabbit kidney. The first was that of Fiske and Subba-Row[21] which measures Pi released from ATP. The second was a modification of the coupled enzyme system of Schwartz *et al.*[22] which measures the oxidation of NADH, indicated by a decrease in absorbancy at 340 nm. In our procedure, the mixture of lactate dehydrogenase (LDH) and pyruvate kinase (PK) was prepared by combining suspensions of each, and after centrifugation, dissolving the pellet in 0·1% Fraction V bovine serum albumin (Armour), such that the final stock solution contained 60 i.u. of LDH and 44 i.u. of PK/ml. The 1-ml assay system contained 0·1 ml of this mixture. In addition, the final concentrations of NaCl and KCl were increased to 140 and 14 mM, respectively, and $MgSO_4$ was substituted for $MgCl_2$. Sodium, K-ATPase activity was calculated as the difference between the total ATPase activity and that remaining in the presence of 10^{-4} M ouabain.

RESULTS AND DISCUSSION

The onset of tubercidin's adverse effects upon *S. mansoni in vitro* is dose-dependent. When present in a concentration of 10^{-7} M, Tu causes the copulating worms to separate after 48 hr exposure; at 10^{-6} M, this and other effects can be seen after 24 hr; and at 10^{-5} M, after only 6 hr. The results of experiments discussed below were obtained when Tu was used in concentration of 10^{-5} M.

When groups of five pairs of schistosomes were incubated at 37° in the presence of 10^{-5} M of Tu for 9 hr, their rate of glucose consumption was unimpaired, despite the fact that from 6 hr onward, they exhibited abnormal muscle activity patterns and separation of the sexes (Table 1). Under our experimental conditions, the average rate of glucose consumption by five pairs of worms, obtained from mice infected 8–9 weeks previously, was 0·35 μmole (63 μg)/hr over a 9-hr period. According to

Radke *et al.*,[23] the mean dry body weight of a schistosome pair 8–9 weeks old is around 60 μg; thus five pairs would be equivalent to 300 μg dry wt. If their hourly rate of glucose consumption remained steady during 24 hr of incubation, these schistosomes would consume a total of 8·4 μmole or 1500 μg. This extrapolated value is consistent with the earlier finding of Bueding,[24] that in 24 hr *S. mansoni* consumed an amount of glucose 3–5 times greater than its dry wt.

TABLE 1. GLUCOSE CONSUMPTION, LACTATE PRODUCTION AND GLYCOGEN CONTENT OF FIVE PAIRS OF SCHISTOSOMES, 8–9 WEEKS OLD, AFTER INCUBATION IN 10 μM TUBERCIDIN*

	Incubation (hr)	Glucose consumption (μmoles) Average	(range)	Lactate production (μmoles) Average	(range)	Glycogen content (μg) Average	(range)
Experiment 1		Contr. 1·3	(1·1–1·6)	N.D.†		41	(34–52)
	3	Tu 1·5	(1·1–1·7)			37	(33–41)
		Contr. 1·9	(1·8–2·0)	N.D.		49	(44–55)
	6	Tu 2·2	(2·1–2·5)			38	(32–45)
		Contr. 3·0	(2·7–3·3)	N.D.		46	(43–48)
	9	Tu 3·2	(3·0–3·7)			41	(37–45)
Experiment 2		Contr. 2·8	(2·6–3·0)	5·8	(5·6–6·0)	N.D.	
	6	Tu 3·4	(3·2–3·4)	6·8	(6·2–7·0)		

* Groups of five pairs of schistosomes were preincubated in 3 ml of medium as described under Materials and Methods before timed experiments were begun. The averages and range of values represent the results of triplicate experiments.

† Not done.

Furthermore, Tu apparently did not modify largely schistosomal lactate production or glycogen content during the time it caused toxic manifestations *in vitro* (Table 1). The observed average rate of lactate production was 1·1 μmole (99 μg)/300 μg dry body wt./hr, a value similar to that found by Bueding.[24] The average glycogen content of 42 μg/300 μg dry wt. is again consistent with the earlier finding of Bueding and Koletsky[25] that glycogen accounts for 14–29 per cent of the dry wt. of adult male *S. mansoni* and 3–5 per cent of the dry wt of adult females.

It can therefore be concluded that the early adverse effects of Tu upon *S. mansoni* cannot be accounted for on the basis of inhibition of glucose uptake, phosphorylative glycolysis, or glycogen depletion.

The data in Table 2 indicate that 6 hr after groups of ten schistosome pairs were incubated in the presence of 20 μM adenosine-8-^{14}C (2 μCi), approx. 55 per cent of the total acid-soluble ^{14}C nucleotides was in the form of ATP, 14·5% as ADP and 10% as AMP. In the guanine nucleotide series, approx. 12·5 per cent of the total acid-soluble ^{14}C nucleotides was in the form of GTP, 5% as GDP and less than 1% as GMP. Little radioactivity was detected in $NADP^+$, IMP, or uric acid suggesting a small turnover of $NADP^+$, rapid conversion of IMP to either AMP or GMP, and a slow rate of uric acid formation or the rapid diffusion of it into the medium.

In the presence of Tu, conversion of adenosine into adenine nucleotides was reduced by greater than 50 per cent (Tables 2 and 3). When dilution of the radioactive population of adenosine in the medium by unlabeled adenosine or its analog, Tu, was tested, reduction of the incorporation of radioactive adenosine into adenine nucleo-

TABLE 2. EFFECT OF TUBERCIDIN ON THE INCORPORATION OF RADIOACTIVITY DERIVED FROM ADENOSINE-8-^{14}C INTO THE ACID-SOLUBLE FRACTION OF *S. mansoni**

Acid-soluble component	Counts/min × 10^3 in the acid-soluble extract of ten worm pairs			
	Controls (10 μM Ad-8-^{14}C (2 μCi), 10 μM adenosine)		Treated (10 μM Ad-8-^{14}C (2 μCi), 10 μM Tu)	
	Average	(range)	Average	(range)
ATP	41·1	(34·4–54·1)	16·3	(12·8–18·5)
ADP	10·9	(9·1–13·3)	5·0	(4·0–5·6)
AMP and NAD^+	8·4	(7·9–9·2)	3·7	(2·9–4·1)
GTP	9·4	(7·3–12·6)	12·3	(10·3–14·2)
GDP	3·9	(3·3–4·6)	4·1	(3·4–4·9)
GMP and XMP	0·5	(0·4–0·6)	0·5	(0·5–0·6)
NADP†	0·3	(0·1–0·6)	0·03	(0–0·1)
IMP	0·3	(0·2–0·4)	0·3	(0·1–0·4)
Uric acid	0·2	(0·1–0·4)	0·03	(0–0·1)

* Groups of ten pairs of schistosomes, 8–9 weeks old, were preincubated in 6 ml of medium as described under Materials and Methods before the timed experiments were begun. Incubation was for 6 hr. The average and range of values represent the results of triplicate experiments in which controls and Tu-treated worms were incubated simultaneously.

tides by Tu was still greater than 50 per cent in comparison to the incorporation in the presence of the added unlabeled adenosine (Table 3). Therefore, for the control of the experiment in Table 2, in which we were mainly interested in the influence of Tu on the phosphorylation of adenosine to form adenine nucleotides, we added 10 μM cold adenosine to emphasize the effects of Tu on the rate of conversion of adenosine into various nucleotides which are definitely different from those expected to occur if Tu acted alike adenosine in all respects (Table 3).

The above results indicate that the inhibitory effect of Tu (or Tu metabolites) on the utilization of adenosine by schistosomes for purine nucleotide formation cannot solely be due to simple competition between these two ribonucleosides for entrance into the cell, nor to the fact that Tu may simply be acting as another adenosine molecule. These results further suggest that Tu or its metabolites suppress mainly the phosphorylation of adenosine by adenosine kinase. If Tu only blocked uptake of adenosine into the cell, there should have been a proportional decrease in the rate of conversion of adenosine into guanine nucleotides. Likewise, if AMP formation resulted in part or wholly from the enzymatic conversions of adenosine to IMP via adenosine deaminase,* inhibition of this pathway by Tu (or its metabolites) should have lowered both guanine and adenine nucleotides and not solely adenine nucleotides.

The data of Table 2 demonstrate that Tu or its metabolites do not strongly inhibit the phosphorylation of AMP or ADP. If potent inhibition of either ATP or ADP formation did occur, a large change in the ratios of ATP/ADP or ADP/AMP should have occurred assuming that equivalent inhibition of all three kinases involved in

* This enzyme as well as adenosine kinase has been reported to be present in schistosomes.[26]

adenosine phosphorylation is unlikely.* However, there was only a small decrease (around 14 per cent) in the ratio of ATP–ADP between control and Tu-treated worms and none for the ADP/AMP ratio (Table 2).

Hakala *et al.*[27] have observed that Tu inhibited the uptake of adenosine into S-180 cells cultured in Eagle's medium. At 3 μM adenosine, a concentration of 18–23 μM of Tu was required to inhibit uptake by 50 per cent. With schistosomes maintained in a similar medium, a greater than 50 per cent inhibition of adenosine utilization was observed at 10 μM of adenosine and 10 μM of Tu. Thus, the inhibitory effect of Tu on the worm's utilization of adenosine is more potent than its effect on S-180 cells.

The toxic consequences of such competition of Tu and adenosine for identical pathways subserving uptake and anabolism in schistosome cells could depend upon a reduced intracellular concentration of vital adenosine nucleotides or the presence of tubercidin nucleotides if any of them exert an antimetabolic action. The muscle activity pattern of schistosomes is adversely affected when the worms are incubated in the presence of Tu. Therefore, the synthesis of Tu nucleotides was undertaken in order to test their interaction with several enzymes likely to be associated with worm muscle function.

TABLE 3. COMPARATIVE EFFECT OF TUBERCIDIN AND UNLABELED ADENOSINE ON THE INCORPORATION OF RADIOACTIVITY DERIVED FROM ADENOSINE-8-^{14}C INTO THE ACID-SOLUBLE FRACTION OF *S. mansoni**

Incubation conditions	Counts/min × 10^3 in the acid-soluble extract of ten worm pairs			
	Total adenine nucleotides Average	(range)	Total guanine nucleotides Average	(range)
10 μM Ad-8-^{14}C (2 μCi)	111	(96·2–127)	25·5	(23·5–27·5)
10 μM Ad-8-^{14}C (2 μCi), 10 μM adenosine	60·5	(54–66·2)	15·1	(13·1–17·1)
10 μM Ad-8-^{14}C (2 μCi), 10 μM tubercidin	12·9	(12·6–13·1)	16·6	(15·3–18)

* Conditions were the same as described in Table 2.

After the addition of Tu to the dog lysed red cell preparation, DEAE-chromatography of the supernatant fraction yielded four distinct peaks, three of them absorbing maximally at 272 nm (Fig. 1). The material in peaks 2, 3 and 4 exhibited spectral characteristics which were closely similar or identical to those of Tu.[7] However, the spectral characteristics of the material in peak 1 (λ_{max} 248 nm) were unlike those of Tu and closely resembled those of IMP.[8]

The peaks 2, 3 and 4 were identified as TuMP, TuDP and TuTP by inorganic phosphate (Pi) and enzyme analyses. Peaks 2, 3 and 4 yielded 1, 2 and 3 μmoles of total Pi/μmole of Tu respectively. However, peak 2 yielded no Pi upon heating at 100° for 7 min in 1 N H_2SO_4 whereas peaks 3 and 4 yielded 1 and 2 μmoles of Pi/μmole of Tu respectively. Peak 2 was hydrolyzed to a compound which co-chromatographed with Tu by phosphatases present in dog plasma;[28] peak 3 substituted on a molar basis for ADP in the coupled pyruvate kinase–lactate dehydrogenase system; and peak

* Dr. Crabtree and associates have found that the level of schistosomal nucleoside diphosphokinase is very high, greater than 100 μmole units/g of worms[26] (also Dr. J. W. Crabtree, personal communication).

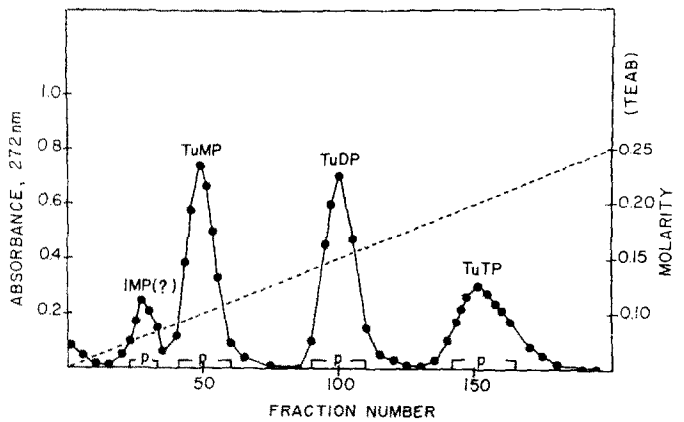

FIG. 1. DEAE–HCO_3 column chromatography of the reaction mixture for the preparation of Tu nucleotides. The diluted extract described in the Materials and Methods section was flowed on to a 2·5 × 12 cm DEAE cellulose column, washed with 100 ml of 0·05 TEAB, and subjected to a linear gradient of 0·05–0·25 M TEAB (dashed line) over a total volume of 2 l. Bracketed "P" indicates the factions pooled for each peak.

4 was hydrolyzed to peak 3 (determined chromatographically) by ATPases from rabbit kidney. Thin-layer chromatography (TLC) indicated that TuMP and TuDP were relatively free of adenosine nucleotides (less than 10 per cent; see Materials and Methods section), but the degree of purity of TuTP could not be determined by this method. However, appreciable contamination of TuTP with ATP was considered unlikely for two reasons: (1) the spectra of Tu and TuTP coincided, and (2) the first peak's total absorption whose spectral characteristics suggest it to be IMP indicated that most ATP was gradually converted to IMP during incubation.

These Tu nucleotides are considered to be 5′-nucleotides because: Tu was unreactive with a bacterial nucleoside phosphorylase and intestinal adenosine deaminase;[3] adenosine kinase is found present in the erythrocyte;[29] yeast adenosine kinase is found to specifically phosphorylate only the 5′-position of adenosine;[30] and Tu is reported to be a substrate for liver adenosine kinase.[31]

The particulate fraction of adult schistosome homogenates contained ATPase activity which was primarily Mg^{2+}-dependent, but which had a small component that was Na, K-dependent and ouabain-sensitive (complete inhibition in the presence of 10^{-5} M ouabain). The Na, K-ATPase activity was estimated to be 0·11 unit, a unit being defined as that amount of protein which catalyzed the hydrolysis of 1 m-mole of ATP/g wet wt. of tissue/hr at 37°. The small amount of Na, K-ATPase present in schistosome homogenates, the instability of the preparations, and the difficulty in routinely harvesting a sufficient number of worms for the efficient isolation of this enzyme precluded further definitive studies at this time.

Rabbit kidney parenchyma was a rich source of Na, K-ATPase. After the treatment of a Nechay–Nelson ATPase preparation by the method of Nakao *et al.*,[19] a particulate fraction rich in Na, K-ATPase (total units, 0·6) was achieved. The ratio of Na, K-ATPase to Mg^{2+}-dependent ATPase was 4:1. When an equimolar amount of TuTP was substituted for ATP as a substrate for this mammalian Na, K-ATPase, it was hydrolyzed at 50 per cent the rate of the latter as measured by the release of Pi and identification of the product by TLC. When the rabbit kidney ATPases were

isolated by the procedure followed for the schistosome homogenates, TuTP was hydrolyzed at a rate 75 per cent of that found for an equimolar amount of ATP utilizing the procedure of Schwartz *et al.*[22] to measure the rates. Thus for two different preparations of Na, K-ATPase, TuTP was a less effective substrate than ATP. The degree of suitability of TuTP as a substrate for schistosomal ATPases remains to be determined.

The intimate association of the hydrolysis and regeneration of ATP in muscle contraction and relaxation is universally recognized. The maintenance of ATP in vertebrates is mediated in part by the action of ATP:creatine phosphotransferase (EC 2.7.3.2; CPK), with creatine phosphate serving as the phosphogen. Most invertebrates use a related guanidinium compound, arginine phosphate, as a reservoir of high energy phosphate[32], but the comparative properties of their analogous guanidinophosphotransferases have not been studied extensively.[33] It is believed that all these analogous enzymes have similar properties and functions. Using creatine phosphate as the phosphogen, we attempted without success to detect CPK activity in supernatant fractions of schistosome homogenates despite the fact that our assay could detect 0·1 unit of activity/g wet wt. of tissue. Either CPK is lacking in schistosomes, is present in a small quantity, is unstable to the procedures utilized for its isolation, or requires a phosphogen other than creatine phosphate. We resorted to commercially available CPK from rabbit muscle in order to test the substrate activity of TuDP with this enzyme. When 10^{-4} M of TuDP was substituted for an equimolar amount of ADP, the former was phosphorylated at 80 per cent the rate of the natural substrate. The addition of an equimolar amount of ADP to the reaction mixture, initially containing only TuDP, nearly doubled the amount of phosphorylation indicating that not only could TuDP serve as a high energy phosphate acceptor, but also that both TuDP and TuTP did not greatly interfere with the phosphorylation of ADP by way of mammalian CPK.

Another enzyme that is believed to play an important role in the regulation of the rate of ATP regeneration is ATP:AMP phosphotransferase (EC 2.7.4.3; adenylate kinase; myokinase).[34] We have confirmed the finding of Crabtree *et al.*[26] that homogenates of *S. mansoni* possess adenylate kinase activity, and it has been estimated that the amount present in a high-speed (10^5 *g*) supernatant fraction was 60 μmole units/*g* wet wt. of worms.* One such unit is defined as that quantity of protein which catalyzes the conversion of 2 μmole of ADP to 1 μmole of AMP and 1 μmole of ATP/min at 30°.* When 4×10^{-4} M of TuDP was substituted for an equimolar amount of ADP, the former was converted to TuMP and TuTP at approx. 20 per cent† the rate determined with ADP. When ADP was added to the reaction mixture containing TuDP, the rate of conversion of ADP to its products was not significantly affected in any way. It would appear that TuDP is a much poorer substrate for schistosomal adenylate kinase than is ADP, a fact which might become significant if for some reason the availability of ADP were severely curtailed.

Thus, the indicated ability of Tu to suppress the utilization of adenosine for purine nucleotide formation in schistosome cells and the possibility that Tu is anabolized to its nucleotides within these parasites may result in both a reduced availability of

* Dr. J. W. Crabtree, personal communication.

† Average of rates found at low enzyme concentration (0·006 μmole units/ml and high enzyme concentration (4·0 μmole units/ml).

physiologically vital nucleotides and the presence of Tu nucleotide analogs with lower efficiency as substrates, cofactors or regulators of allosteric enzymes. This would seem to be a more likely explanation for the rapidly developing antischistosomal activity of Tu than the possibility that, as was observed by Acs *et al.*[2] for mouse fibroblasts, this adenosine analog could be lethal to schistosomes only after first being incorporated into RNA and DNA (especially RNA), subsequently inhibiting nucleic acid and protein synthesis. Acs *et al.*[2] found that actinomycin prevented the cytotoxicity of Tu without affecting incorporation of Tu into DNA. It would be interesting to determine whether actinomycin could act similarly in Tu-treated schistosomes.

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