thermal stability of human plasma DBH[10]. It is important that the biochemical basis of these variations in thermal stability be elucidated. We have shown that the development of plasma DBH thermolability is dependent on the presence of molecular oxygen. This observation may serve as a basis for future studies of individual variations in the biochemical properties of the DBH molecule and may be applied to studies of the structural gene for DBH in man.

1 This work was supported in part by NIH grants HL 17487 and NS 11014.
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Effects of various inhibitors and 2,4-dinitrophenol on adenosine triphosphatase from Malpighian tubules of *Locusta migratoria* L.

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Summary. Both Mg^{2+}-ATPase and HCO_3^--stimulated ATPase activity were inhibited by sodium azide and to a lesser extent ethacrynic acid and amiloride. 1 mM DNP stimulated Mg^{2+}-ATPase activity by 22% and HCO_3^--stimulated ATPase activity by 7%.

Since Kasbekar and Durbin[1] and Sachs et al.[2] demonstrated the presence of a Mg^{2+}-dependent HCO_3^--stimulated ATPase in frog gastric mucosa, numerous other researchers have reported similar anion-sensitive ATPase activity in homogenates of various epithelia from a variety of different species. These include mammalian pancreas[3-5], submandibular gland[6,7], renal proximal tubule[8,9], K^+-transporting midgut of *Hyalophora cecropia*[10], rectum of *Schistocerca gregaria*[11] and the Malpighian tubules of *Locusta migratoria*[12]. Such anion-sensitive ATPase has been implicated in a variety of ion transport systems. For example, H^+/HCO_3^- transport[3-5], Na^+/H^+ exchange[8,9] and Cl^- transport[11]. Since ion transport is considered to be the 'driving force' in fluid secretion by insect Malpighian tubules, the nature of the HCO_3^--stimulated ATPase reported in this tissue[12] warrants further investigation. The present study has been carried out to determine the effects of 2,4-dinitrophenol (DNP), ethacrynic acid, amiloride and sodium azide on the Mg^{2+}-dependent ATPase of *Locusta* Malpighian tubules as a basis for future physiological studies and to permit comparison between this anion-sensitive enzyme and ATPases from other sources.

Materials and methods. Mature adult locusts, *Locusta migratoria* L., were used and these were taken from a population maintained under crowded conditions at 28 ± 0.5 °C and 60% relative humidity.

The methods of preparation of the membrane microsomal homogenate, of enzyme assay and of protein determination were essentially as described previously[12]. In determining enzyme activity, 3 incubation media, having the following composition, were used: 1. 2 mM magnesium chloride; 2. 2 mM magnesium chloride and 20 mM sodium chloride; 3. 2 mM magnesium chloride and 20 mM sodium bicarbonate, unless otherwise stated. Each medium contained 3 mM ATP (sodium salt) final concentration and 20 mM imidazole/HCl, pH 7.5. All tubes were thermoequilibrated for 15 min before starting the reaction by the addition of 0.5 cm^3 microsomal suspension. Incubations were carried out at 30 ± 0.1 °C. All solutions were made up in glass-distilled, deionized water. All inorganic salts were AnalaR grade or the best commercially available. Imidazole and ATP (di-sodium salt) were obtained from Sigma Chemical Co., sodium azide and 2,4-dinitrophenol from BDH Chemicals Ltd, Ethacrynic acid and amiloride were a gift from Merck, Sharp & Dohme Ltd.

Results. The inclusion of 1 mM sodium azide in the reaction media resulted in almost complete inhibition of Mg^{2+}-dependent ATPase ($\pm$ 20 mM NaCl) and Mg^{2+}-dependent HCO_3^--stimulated ATPase activity; Mg^{2+}-dependent ATPase activity being reduced from 315.6 ± 21.4 to 2.0 ± 0.3 nmoles Pi liberated/mg protein/min ($n = 3$) and from 322.0 ± 20.0 to 2.1 ± 0.4 nmoles Pi liberated/mg protein/min (+20 mM NaCl; $n = 3$) whilst the Mg^{2+}-dependent HCO_3^--stimulated ATPase activity was reduced from 526.3 ± 37.9 to 4.2 ± 0.3 nmoles Pi liberated/mg protein/min ($n = 3$). In contrast, amiloride had no effect on Mg^{2+}-dependent ATPase ($\pm$ 20 mM NaCl) over the concentration range 5×10^{-5} M to 5×10^{-8} M. There was, however, some slight inhibition of activity at the highest concentration of amiloride used (5×10^{-4} M); the inhibition of Mg^{2+}-

dependent ATPase activity being 12% and 7% in the presence and absence of NaCl respectively. Similar results were obtained with Mg^{2+}-dependent HCO_3^--stimulated ATPase activity; 5×10^{-4} M amiloride effecting about 15% inhibition.

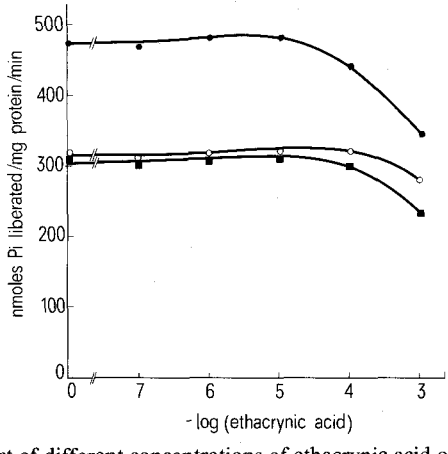
Ethacrynic acid did not affect the activity of either the Mg^{2+}-dependent ATPase (± 20 mM NaCl) or the Mg^{2+}-dependent HCO_3^--stimulated ATPase (figure) over the concentration range $0\text{-}10^{-5}$ M. Only at concentrations greater than 10^{-4} M was enzyme activity significantly inhibited. Thus, in the presence of 10^{-3} M ethacrynic acid, Mg^{2+}-dependent ATPase activity was 82.2 ± 3.8% (or 74.8 ± 3.6% in the presence of 20 mM NaCl) of the activity observed in its absence. The relative activity of Mg^{2+}-dependent HCO_3 stimulated ATPase was reduced to 74.7 ± 2.5% when 10^{-3} M ethacrynic acid was included in the reaction medium.

1 mM DNP stimulated Mg^{2+}-dependent ATPase activity to 121.9 ± 1.4% of the activity observed in its absence (118.5 ± 3.1% in the presence of 20 mM NaCl) (n = 4). However, this concentration of DNP did not significantly affect Mg^{2+}-dependent HCO_3^--stimulated ATPase activity; the activity in the presence of 1 mM DNP being 107.2 ± 5.6% of that observed in its absence (n = 4).

Discussion. In the present study, sodium azide was the most potent inhibitor of ATPase activity; almost total inhibition of Mg^{2+}-dependent and Mg^{2+}-dependent HCO_3^+-stimulated ATPase activity being effected by 1 mM inhibitor concentration. Similar results have been reported by other investigators. For example, Van Amelsvoort et al.[13] reported 90% inhibition of rabbit gastric mucosal Mg^{2+}-ATPase and Lambeth and Lardy[14] found that 0.1 mM azide inhibited beef heart mitochondrial ATPase by 65% and rat liver mitochondrial ATPase by 80%. In contrast, Van Amelsvoort et al.[15] reported only 5% inhibition of erythrocyte ATPase activity whilst Anada et al.[16] showed that 1 mM azide inhibited mitochondrial and microsomal Mg^{2+}-ATPase by 85% and 8% respectively.

Ethacrynic acid is known to inhibit Na^+/K^+-ATPase from a variety of tissues[17-19]. However, the effect of this drug appears to be relatively nonspecific since it affects a variety of cellular processes[20,21]. Furthermore, Wald et al.[22] have shown that ethacrynic acid preferentially inhibits Mg^{2+}-ATPase as opposed to Na^+/K^+-ATPase in microsomes of rat brain, submaxillary gland and kidney. 1 mM ethacrynic acid inhibited Mg^{2+}-dependent ATPase activity of *Locusta* Malpighian tubules by 18% (25% in the presence of 20 mM NaCl). The presence of 20 mM $NaHCO_3$ did not change the percentage inhibition significantly. Somewhat higher levels of inhibition have been reported by Wald et al.[22] who recorded 35-80% inhibition with microsomal Mg^{2+}-dependent ATPase from various tissues of rat, and kidney from a variety of different species. Ethacrynic acid and amiloride are known to markedly inhibit fluid secretion by insect Malpighian tubules[23,24]. However, similar concentrations of these 2 inhibitors did not substantially inhibit the activity of the Mg^{2+}-dependent ATPase or the Mg^{2+}-dependent HCO_3^+-stimulated ATPase of *Locusta* Malpighian tubules. It would seem, therefore, that the inhibitory action of ethacrynic acid and amiloride on Malpighian tubule fluid secretion is likely to be independent of this anion-sensitive ATPase.

Sachs et al.[25] and Anada et al.[16] report that DNP stimulates mitochondrial ATPase activity but not the activity of the microsomal ATPase. In the present study 1 mM DNP effected 22% stimulation of Mg^{2+}-dependent ATPase activity (7% in the presence of 20 mM $NaHCO_3$). This represents a very small response to DNP when compared with the data reported by Lambeth and Lardy[14] for pure mitochondrial ATPase from rat liver. These authors observed 100% stimulation of ATPase activity by DNP, whilst Ebel and Lardy[26] observed 470% stimulation with mitochondrial ATPase from the same tissue. On this basis, one might suppose that the present preparation was relatively insensitive to DNP and this might be considered evidence for its microsomal origin; a fact which is clearly important if this enzyme is to be implicated in plasma membrane transport processes. However, examination of the literature indicates considerable variability in the percentage stimulation of ATPase activity by DNP. Thus Santiago et al.[27] observed only 21% stimulation of rat liver mitochondrial ATPase which contrasts markedly with the values referred to above for the same tissue and species. It is not possible, therefore, to conclude on the basis of its response to DNP whether the Mg^{2+}-dependent HCO_3^+-stimulated ATPase of *Locusta* Malpighian tubules is mitochondrial or microsomal in origin. However, other studies[28] suggest that *Locusta* Malpighian tubule anion-sensitive ATPase is not exclusively mitochondrial in origin.



The effect of different concentrations of ethacrynic acid on ATPase activity in the presence of 2 mM Mg^{2+} (○), 2 mM Mg^{2+} + 20 mM NaCl (■) and 2 mM Mg^{2+} + 20 mM $NaHCO_3$ (●). Typical experiment representative of 4 experiments. Ordinate: ATPase activity as nmoles Pi liberated/mg protein/min. Abscissa: the negative logarithm of ethacrynic acid concentration (M).

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Purification of mouse liver thymidylate synthetase by affinity chromatography using 10-methyl-5,8-dideazafolate as the affinant[1]

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Summary. Thymidylate synthetase (methylenetetrahydrofolate: 2′-deoxyuridine-5′-monophosphate C-methyltransferase; EC 2.1.1.45) from neonatal mouse liver has been purified 714-fold by affinity chromatography on aminohexylsepharose bound 10-methyl-5,8-dideazafolate.

Thymidylate synthetase catalyzes the 5,10-methylenetetrahydrofolate-dependent conversion of deoxyuridinemonophosphate (dUMP) to thymidylate. The properties of this enzyme in mammalian systems are of particular interest because the reaction catalyzed is the lone de novo source of the thymidylate required for DNA synthesis. Therefore, the enzyme is the target for a number of cancer chemotherapeutic agents and has been the subject of extensive investigations[2].

Attempts to purify the enzyme from various sources by conventional means have been hampered by the innate instability and very low concentrations of the enzyme present in most tissues. However, limited amounts of purified enzyme have been secured using traditional multistep procedures[3–15]. Recently, affinity chromatography has been successfully utilized for the purification of thymidylate synthetase from several sources. The enzyme from *Lactobacillus casei* has been purified to apparent homogeneity with 2′-deoxyuridylate[16], 5-fluoro-2′-deoxyuridylate (FdUMP)[17], and N^{α}-[pteroyltetra-(γ-glutamyl)]-lysine[18] as affinity ligands. Successful application of these affinity matrices to the purification of the mammalian enzyme has not been reported. Affinity columns based on dUMP-dependent binding to immobilized tetrahydromethotrexate have been used to purify both the bacterial and the mammalian enzyme[19,20]. The major difficulty with this biospecific adsorbent is the instability of the reduced folate analog. The adsorbent cannot be stored for long periods and columns are typically used only once. Dolnick and Cheng have avoided this problem by using methotrexate as the affinity ligand[21]. Although the purification achieved on the methotrexate column appeared to involve both specific and hydrophobic interactions, the utilization of a stable affinant represents a significant advantage over previously reported methods.

Quinazoline analogs of folic acid are quite stable and have been found to be exceptionally effective inhibitors of thymidylate synthetase from a number of sources.[22–25]. Recently, the successful application of 10-formyl-5,8-dideazafolate-aminoethyl-sepharose to the purification of thymidylate synthetase from L1210 leukemia cells has been reported[26]. We report here the immobilization of a somewhat more potent inhibitor of both the L1210 enzyme ($I_{50} = 0.5 \times 10^{-7}$ M) and the enzyme from *L. casei* ($I_{50} = 4 \times 10^{-7}$ M)[25] and its application to the purification of thymidylate synthetase from neonatal mouse liver. Binding to the 10-methyl-5,8-dideazafolate column is dUMP-dependent and presumably is the result of reversible ternary complex formation.

Materials and methods. [6-^{3}H] FdUMP (17 Ci/mmole) and [5-^{3}H] dUMP (18 Ci/mmole) were purchased from Moravek Biochemicals. Tetrahydrofolate was prepared according to the method of Davis[27] and converted to the methylene derivative by addition of formaldehyde for storage at −70 °C. The 10-methyl-5,8-dideazafolate was synthesized as previously described[28] and was coupled to the amine group of a spacer on Sepharose 4B (Pharmacia) using 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide as

Purification of thymidylate synthetase from neonatal mouse liver

Purification stage	Protein (mg)	Volume (ml)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)
Crude homogenate	1450	98	1.4×10^{-5}	1.0	100
Streptomycin sulfate (1%)	1040	103	2.2×10^{-5}	1.6	100
Ammonium sulfate (30–55%)	365	9.5	3.4×10^{-5}	2.4	59
DEAE-cellulose	75.6	27	1.9×10^{-4}	13.4	34
Affinity column	0.56	8	1.0×10^{-2}	714	20