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Inhibition of macromolecular synthesis in *Escherichia coli*, L5178Y cells, and mitochondria by ethidium bromide, related compounds, and metabolites*

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THE TRYPANOCIDAL drug ethidium bromide (3,8-diamino-6-phenyl-5-ethylphenanthridinium bromide) inhibits growth and nucleic acid synthesis in the flagellate *Strigomonas oneopelti*,[1] in *Escherichia coli* and *Bacillus cereus*,[2] Ehrlich ascites carcinoma cells,[3] HeLa cells,[4] yeast mitochondria,[5] and animal viruses.[6] Ethidium bromide intercalates between DNA bases and causes uncoiling and reverse coiling of circular DNA,[7,8] inhibits exogenous but not *de novo* synthesized purines from being incorporated into nucleic acids,[9,10] inhibits cell-free aminoacylation,[11] and causes a specific inhibition of mitochondrial as opposed to nuclear DNA synthesis.[12] The present investigation reports the effects of three phenanthridinium compounds: 2-amino-6-*p*-carbethoxyaminophenyl-5-methylphenanthridinium sulfate (carbidium), 3,8-diamino-6-*p*-aminophenyl-5-methylphenanthridinium chloride (150C47), and 3-amino-8-(2-amino-6-methylpyrimidin-4-ylamino)-6-*p*-aminophenyl-5,1′-dimethylphenanthridinium bromide (prothidium), and ethidium on macromolecular synthesis in L5178Y mouse lymphoma cells, *E. coli*, rat liver mitochondria, and rat cerebral cortex mitochondria. Since it has recently been established[13] that the rat metabolizes these compounds to acetylated derivatives, the available metabolites and also the completely acetylated derivatives of ethidium, 150C47, and carbidium were tested for their effects on macromolecular synthesis.

Ethidium and prothidium were gifts of Dr. G. Woolfe of the Boots Pure Drug Co., Nottingham, England. Carbidium and 150C47 were gifts of Drs. L. P. Walls and R. A. Neal of the Wellcome Research Laboratories, Beckenham, Kent, England. The monoacetylated metabolites of 150C47, ethidium, and carbidium which have recently been found in rat bile and the totally acetylated derivatives were synthesized as previously described.[13,14] The products obtained as described[13] were dissolved in distilled water and the concentration of an aliquot was determined by the method of Bratton and Marshall,[15] after hydrolysis in 1 N HCl for 60–90 min at 90°.

L-Leucine-4,5-^{3}H (sp. act. 25 c/m-mole), D-glucosamine-6-^{3}H (2 c/m-mole), thymidine-methyl-^{3}H (15 c/m-mole), and uridine-5-^{3}H (10 c/m-mole) were obtained from New England Nuclear Corp. L5178Y cells (mouse lymphoma cell line) were grown in suspension culture in sealed containers in Fischer medium[16] with 10% horse serum and were utilized in the logarithmic growth phase. All cells were harvested by conventional methods in the logarithmic growth phase and utilized immediately. The rat liver mitochondria were isolated by a modification of the method of Schneider and Hogeboom[17] as previously described.[18] The intraneural mitochondria of the rat were prepared by a modification of the method previously described.[19] *E. coli* strain B were grown in nutrient broth (Baltimore Biological Lab.) supplemented with 5 g/l. of NaCl. All cells were used in the logarithmic phase of growth.

Synthesis of *E. coli* macromolecules was determined as previously described.[19,20] DNA, RNA and protein synthesis and the incorporation of glucosamine into acid-insoluble material were determined by the incorporation of radioactively labeled thymidine, uridine, leucine and glucosamine, respectively, into trichloroacetic acid, ether–ethanol-insoluble material. The complete incubation system included the following: 10 mM $MgCl_2$, 10 mM sodium phosphate (pH 7·6), 5 mM phospho(enol)-pyruvate, 20 μg of pyruvic kinase (rabbit skeletal muscle, 1 mg protein converts 430 μmoles of phospho(enol)pyruvate to pyruvate per min), 10 mM ATP, 2 mM EDTA, 22·5 μg/ml of a complete amino acid mixture minus leucine, 0·154 M KCl, 100 μl of the *E. coli* (between 0·3 and 0·7 mg protein) in 0·1 M Tris, pH 7·6, and 50 μl of 0·1 M Tris, pH 7·6, containing the required concentrations of drug. In addition, 10 μc of thymidine-methyl-^{3}H or uridine-5-^{3}H, 5 μc of L-leucine-4,5-^{3}H, or 5 μc of glucosamine-^{3}H were added in 10 μl. In all instances the final volume was 280 μl. After incubation at 37° for 20 min the macromolecular bound radioactivity was precipitated with 10% trichloroacetic acid and washed 3 times with 10% trichloroacetic acid and once with a mixture of ethanol and diethyl ether (2:1 by volume). In assays for the incorporation of leucine-4,5-^{3}H, the precipitate after the 10% trichloroacetic acid washes was heated in 3 ml of 10% trichloroacetic acid for 45 min at 90° before proceeding with the ethanol–diethyl ether washes. The resulting pellet was dissolved in 1·0 N NaOH, plated on a glass fiber filter, and the radioactivity was determined by counting in a liquid scintillation

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counter. It has been previously determined that lipopolysaccharide (LPS) was the major macromolecular class being labeled by the *E. coli* with the glucosamine-^{3}H.[20]

All incubations of L5178Y cells were carried out for the experiments described in the complete growth media for the L5178Y cells. Routinely, logarithmically harvested cells were resuspended at a concentration of 10^5 cells per ml in complete Fischer medium. One hundred μl of this suspension was then quickly pipetted into the desired number of 5-ml reaction tubes. Five-tenths μc L-leucine-4,5-^{3}H, uridine-5-^{3}H, thymidine-^{3}H or D-glucosamine-^{3}H was added to each tube in 10 μl of distilled water and either 50 μl of 0·1 M Tris buffer, pH 7·6, or 50 μl of the indicated amount of drug was added. The final volume was always 160 μl. The reaction tubes were then placed in a Dubnoff metabolic shaker (60 oscillations per min) at 37° for 1 hr. The reaction was terminated and radioactivity determined as given above.

Protein, DNA, RNA and glycoprotein synthesis in either rat liver or brain mitochondria were determined as previously described.[18,19] The complete system for either protein, DNA, RNA or glycoprotein synthesis was as given for the *E. coli* system above with mitochondria substituted for the *E. coli*. After incubation for 1 hr at 37° the reaction mixture was precipitated and radioactivity determined as given for the *E. coli* system above. Protein was determined with bovine serum albumin as the standard by the method of Lowry *et al.*[21]

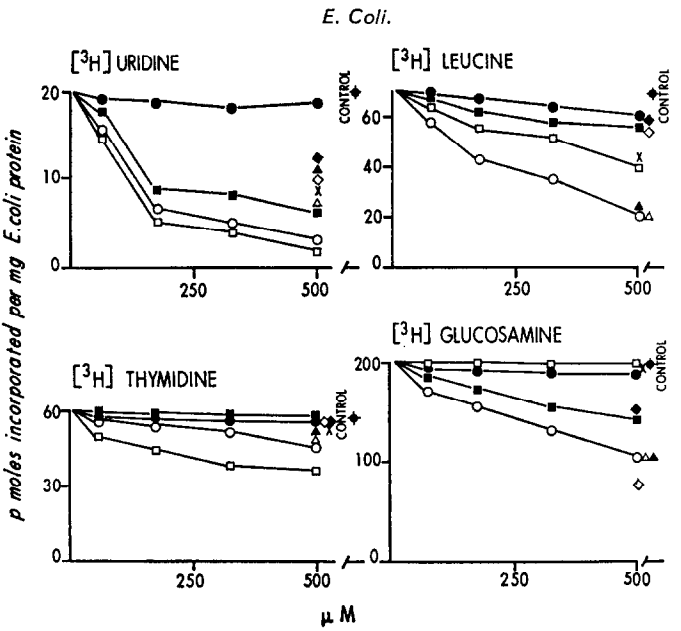

FIG. 1. Effects of phenanthridinium compounds and acetylated derivatives on the synthesis of macromolecules in *E. coli*. Experiments were performed as described. Each point is the mean of five or more independent experiments. Variability between experiments was less than 15 per cent. Activities of acetylated compounds were determined at 0·5 mM even though space on the graph may have necessitated offsetting the points.

○ Ethidium, □ Carbidium, ● Prothidium, ■ 150C47, × Acetylated carbidium, △ Mono-acetyl ethidium, ▲ Fully acetylated ethidium, ◇ Mono-acetyl 150C47, ◆ Fully acetylated 150C47

The data presented in Fig. 1 demonstrate that the phenanthridinium compounds except prothidium severely inhibited RNA synthesis in *E. coli*. The order of greatest inhibition was carbidium > ethidium > 150C47 ≫ prothidium. Ethidium and carbidium inhibited RNA synthesis more than 80 per cent at 0·5 mM. The phenanthridinium compounds were much less potent inhibitors of DNA synthesis. Prothidium and 150C47 were without effect, while carbidium was more inhibitory than ethidium (at 0·5 mM carbidium inhibited DNA synthesis about 40 per cent). Ethidium was the

most potent inhibitor of protein synthesis (about 70 per cent inhibition at 0·5 mM), while carbidium also produced inhibition (about 40 per cent inhibition at 0·5 mM). Prothidium and 150C47 inhibited protein synthesis slightly at 0·5 mM (Fig. 1). The incorporation of glucosamine into LPS was not appreciably affected by carbidium or prothidium, but 0·5 mM ethidium inhibited LPS synthesis 50 per cent and 0·5 mM 150C47 inhibited LPS synthesis 30 per cent.

The effects of the phenanthridinium drugs on L5178Y cells are given in Fig. 2. At 0·5 mM, ethidium, 150C47, and carbidium were very inhibitory to RNA, DNA, protein and glycoprotein synthesis. Prothidium inhibited RNA, DNA and protein synthesis at 0·5 mM to a lesser degree but was the most potent inhibitor of glycoprotein synthesis. Ethidium bromide was by far the most potent inhibitor tested; even at 75 μM, RNA synthesis was inhibited 33 per cent (Fig. 2). At equimolar concentrations, the phenanthridinium compounds were somewhat more inhibitory to macromolecular synthesis in the mouse leukemic cells (Fig. 2) than in *E. coli* (Fig. 1), except for uridine incorporation into RNA.

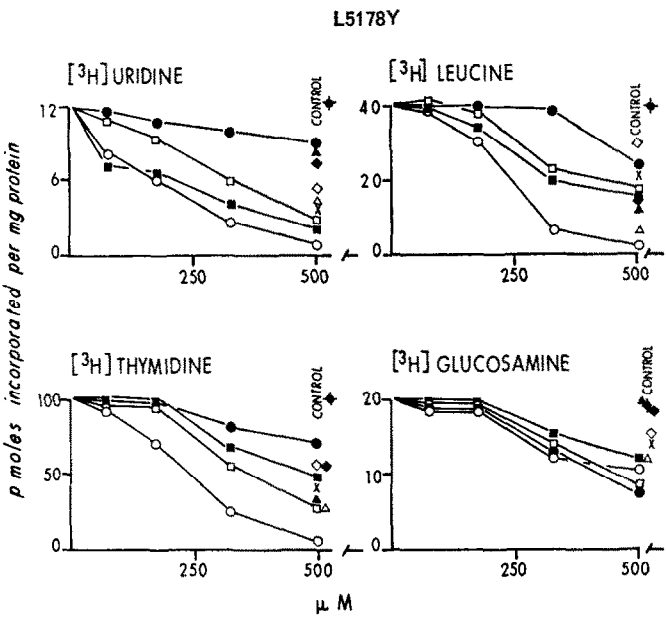

FIG. 2. Effects of phenanthridinium compounds and acetylated derivatives on the synthesis of macromolecules in L5178Y cells. Experiments were performed as described. Each point is the mean of five or more independent experiments. Variability between experiments was less than 15 per cent. Activities of acetylated compounds were determined at 0·5 mM even though space on the graph may have necessitated offsetting the points.

Key as for Fig. 1.

The data on the effects of the phenanthridium compounds on macromolecular synthesis in rat cerebral cortex mitochondria are given in Fig. 3. None of the compounds had any significant effect on DNA synthesis. At 0·5 mM, RNA synthesis was inhibited 50 per cent by 150C47, 33 per cent by prothidium, 30 per cent by ethidium, and 28 per cent by carbidium. All the phenanthridiniums inhibited protein and glycoprotein synthesis in the rat cerebral cortex mitochondria severely; ethidium was the most potent inhibitor (about 90 per cent inhibition at 0·5 mM) of protein and glycoprotein synthesis (Fig. 3).

The compounds had essentially no effect on RNA synthesis in the rat liver mitochondria as shown in Fig. 4. DNA synthesis was inhibited at 0·5 mM, with the order of greatest inhibition being carbidium > prothidium > 150C47 > ethidium. Prothidium was the most potent inhibitor of both protein and glycoprotein synthesis in the rat liver mitochondria. 150C47 did not affect glycoprotein synthesis and carbidium had little significant effect on protein synthesis (Fig. 4).

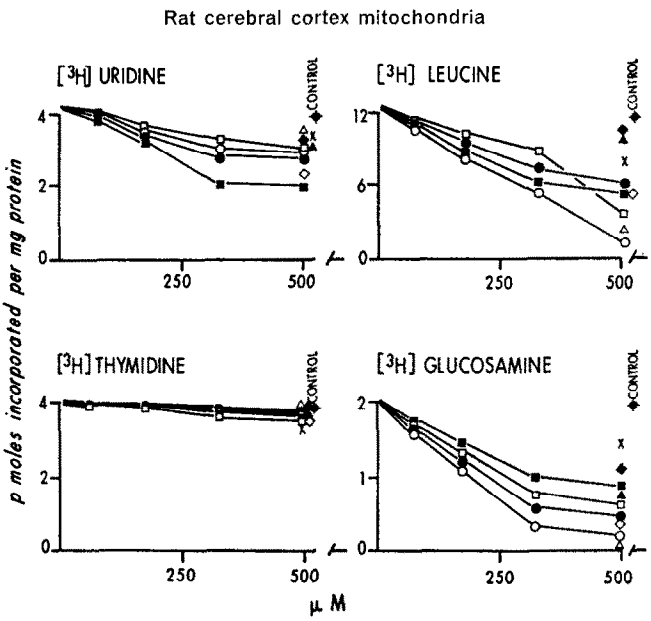

FIG. 3. Effects of phenanthridinium compounds and acetylated derivatives on the synthesis of macromolecules in rat cerebral cortex mitochondria. Experiments were performed as described. Each point is the mean of five or more independent experiments. Variability between experiments was less than 15 per cent. Activities of acetylated compounds were determined at 0·5 mM even though space on the graph may have necessitated offsetting the points.

Key as for Fig. 1.

The data given in Fig. 1 show that in general acetylation of the phenanthridiniums decreased their toxicity to nucleic acid synthesis in *E. coli*; e.g. fully acetylated carbidium did not inhibit DNA synthesis whereas carbidium itself inhibited DNA synthesis almost 40 per cent. This was not true for inhibition of protein and LPS synthesis. In general, acetylation did not greatly decrease the inhibition of protein or LPS synthesis in *E. coli* seen with the parent compound. Indeed, monoacetylated 150C47 inhibited LPS synthesis to a much greater extent than 150C47 (Fig. 1).

In Fig. 2 are data indicating that the acetylated derivatives of the phenanthridinium compounds were in every instance less inhibitory to macromolecular synthesis in L5178Y cells than their parent compounds. It is of interest that in most instances the acetylated compounds were themselves still toxic.

The data in Fig. 3 show that in most cases acetylation of the parent compound produced a compound which was less inhibitory to macromolecular synthesis than the parent compound in rat brain mitochondria. The monoacetyl derivative of ethidium, which appears in rat bile,[13,14] however, appears to be more toxic, or at least as toxic as ethidium itself to glycoprotein synthesis. The monoacetyl derivative of 150C47 was also more toxic to glycoprotein synthesis than was 150C47 itself.

The data in Fig. 4 show that the acetylated derivatives, like the parent compounds, do not affect RNA synthesis in rat liver mitochondria. The monoacetylated derivatives of ethidium and 150C47, however, inhibit DNA synthesis to a greater extent than the respective parent compounds, while the completely acetylated derivatives show about the same degree of inhibition as the parent compounds. The acetylated derivatives inhibited protein and glycoprotein synthesis slightly less than their parent compounds.

The effect of ethidium on DNA, RNA and protein synthesis in *E. coli* has previously been investigated by Tomchick and Mandel.[2] The major effect of ethidium reported by these workers was a decrease in the DNA content of the bacteria. The most pronounced effect of ethidium in the *E. coli* system of the present work was an inhibition of ^{3}H-uridine incorporation into RNA. The incorporation of uridine was only about 15 and 33 per cent of control at 500 μM and 175 μM, respectively, while the incorporation of thymidine-^{3}H was still 60 and 95 per cent at these same concentrations respectively.

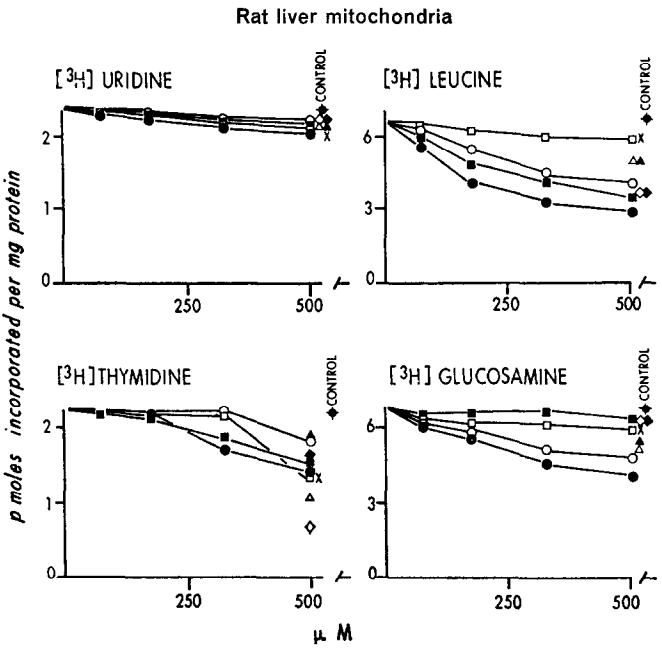

FIG. 4. Effects of phenanthridinium compounds and acetylated derivatives on the synthesis of macromolecules in rat liver mitochondria. Experiments were performed as described. Each point is the mean of five or more independent experiments. Variability between experiments was less than 15 per cent. Activities of acetylated compounds were determined at 0·5 mM even though space on the graph may have necessitated offsetting the points.

Key as for Fig. 1.

The most striking observation in the present work, however, is that the phenanthridinium compounds investigated exhibit specificity for different synthetic systems. For example, 500 μM carbidium reduced RNA synthesis to about 10 per cent of control but did not affect LPS synthesis at all; 150C47 had very little effect on DNA or protein synthesis but decreased RNA synthesis markedly and LPS synthesis moderately; and ethidium inhibited RNA, protein, and LPS synthesis markedly.

Henderson[3] investigated the effects of ten phenanthridinium compounds on the incorporation of glycine into nucleic acids and protein and of adenine into nucleic acids in Ehrlich ascites carcinoma cells. These results are qualitatively similar to the present results with L5178Y cells, although prothidium seems to be a somewhat more potent inhibitor of nucleic acid synthesis in Ehrlich ascites cells than in L5178Y cells.

Ethidium reduced protein and glycoprotein synthesis in the cerebral cortex mitochondria to about 10 per cent of control at 500 μM, while DNA synthesis was not affected at all and RNA synthesis remained at 75 per cent of its control value at this same concentration. In the rat liver mitochondria prothidium was the most potent inhibitor of protein and glycoprotein synthesis. Furthermore, none of the agents affected RNA synthesis in the rat liver mitochondria, while in rat cerebral cortex mitochondria DNA synthesis was unaltered. In both types of mitochondria these agents produced much more pronounced effects on protein and glycoprotein synthesis than on DNA and RNA synthesis. The lack of effect of ethidium on rat liver mitochondrial RNA synthesis is surprising since Zylber *et al.*[4] report a 60 per cent inhibition of HeLa cell mitochondrial RNA synthesis by a 1 μM concentration of ethidium. This same concentration caused almost complete inhibition of synthesis of the 21s and 12s mitochondrial RNA fractions[4] and also inhibited the synthesis of 4s mitochondrial RNA in HeLa cell mitochondria.[22,23] DeVries and Kroon[24] have recently reported that ethidium caused a decreased synthesis of cytochrome *c* oxidase in regenerating rat liver and suggested that the site of inhibition was probably the mitochondrial translation system. It is now known that ethidium is extensively metabolized to a monoacetylated form by the rat,[13,14] and the present results indicate that both ethidium and its monoacetylated metabolite affect RNA synthesis much less than protein, glycoprotein, and DNA synthesis in rat liver mitochondria. It is, of course, possible that a small part of total

RNA synthesis which is responsible for cytochrome *c* oxidase production is specifically inhibited, while the major part of RNA synthesis is not affected.

The fact that 150C47 dramatically inhibits glycoprotein synthesis in rat cerebral cortex mitochondria but not at all in rat liver mitochondria is extremely interesting and may indicate a basic biochemical difference in these mitochondria. The fact that 150C47 inhibits protein synthesis to about the same degree in both types of mitochondria indicates that permeability to the compound is probably not the determining factor. Acetylation of the amino groups of these compounds generally reduced their toxicity but usually did not abolish it entirely. The interesting exception to this generality is that the monoacetyl forms of both 150C47 and ethidium which are formed by the rat[13,14] are in some cases even more toxic than the parent compounds.

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