DISCUSSION

We have demonstrated that the amphetamine derivative, CMPEP, has an *in vitro* effect on adipose tissue metabolism, which is dose dependent. A dose of 0·5 mg/ml produced an increase in the rate of lipolysis, affecting very slightly the reutilization of the glycerol by the tissue, since the reduction of (^{14}C) CO_2 and ^{14}C-total lipids formation from (1-^{14}C) glycerol could simply be explained by a decrease in the specific activity of the tracer and not by an action on the net synthesis of these compounds. The effect is biphasic as higher doses of the drug produced an intense inhibition of the lipolysis and of the reutilization of glycerol by the tissue, specially for the synthesis of glyceride glycerol. This biphasic effect of CMPEP on adipose tissue lipolysis could be related to the biphasic effect of this drug on the release of catecholamines by the perfused adrenals.[8] It is possible that the drug acts on adipose tissue metabolism by affecting the release of catecholamines from the sympathetic terminals, still present in the fat pads incubated *in vitro*, and not by a direct action. Further investigation is required to support or refute this possibility.

A second explanation of the biphasic effect of the drug could be that, at high concentrations, it inhibits lipolysis by an enhanced accumulation of free fatty acids into the adipocyte as a result of a previous activation of lipolysis.[9] This possibility seems unlikely as data not shown demonstrate that these doses of CMPEP produce an inhibition in the release and reutilization of glycerol by adipose tissue after shorter periods of incubation (30 and 60 min) than those used here.

In the present study we have demonstrated once more that adipose tissue reutilizes glycerol for the synthesis of glyceride glycerol and for its complete oxidation to CO_2 and it seems therefore that glycerokinase activity might be considerably higher than previously thought. The rate of reutilization of glycerol by adipose tissue does not necessarily parallel the rate of lipolysis, it must therefore be taken into account when the true rates of lipolysis and esterification are calculated.

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Inhibition of brain adenylate cyclase by ethacrynic acid and dithiobisnitrobenzoic acid

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THE ESSENTIAL role of cyclic AMP as a second messenger in biological systems is well established.[1] The biochemical and physiological effects of cyclic AMP are frequently studied by utilizing inhibitors of phosphodiesterase which delay degradation of cyclic AMP. A more direct evaluation of the role of cyclic AMP in biological responses might be possible if potent inhibitors of adenylate cyclase, the

enzyme responsible for cyclic AMP formation, were available. In fact, very few inhibitors of this enzyme have been described. Calcium, copper, zinc and cobalt ions have been observed to have an inhibitory effect on the enzyme.[2] Cohen and Bitensky[3] found that alloxan also inhibits activity of adenylate cyclase from several mammalian tissues.

The purpose of this report is to describe two inhibitors of brain adenylate cyclase which apparently inactivate the enzyme by interaction with critical sulfhydryl groups.

Adult, female Swiss–Webster mice (NLW strain) were killed by decapitation. The brain was quickly exposed and a portion of the cerebral cortex was removed, weighed and homogenized with a ground glass homogenizer in 125 vol. of cold 50 mM Tris buffer, pH 7·5. This homogenate was assayed for adenylate cyclase activity using a modification of the method described by Steiner *et al.*[4] Approximately 80 μg of brain (10 μl) was added to 90 μl of a reaction mixture (enzyme assay reagent) containing 50 mM Tris, pH 7·6; 1·25 mM ATP; 12·5 mM theophylline; 5 mM $MgCl_2$; 0·05% bovine serum albumin; 60 mM creatine phosphate; and 700 μg/ml of creatine kinase. The latter two constituents were included to provide an ATP regenerating system. The mixture which had been maintained at 0° was then incubated for 10 min at 30° and the reaction was stopped by placing the reaction tube in boiling water for 3 min. Cyclic AMP in the supernatant fluid was then measured with the radioimmunoassay method of Steiner *et al.*[5] Under these conditions cyclic AMP formation was linear with respect to time for up to 15 min of incubation, and with respect to tissue concentration at least up to 150 μg of brain, wet weight.

The effects of several compounds on adenylate cyclase activity were tested. These agents were added to the brain homogenate in small volumes, either singly or in combination, and the mixtures were incubated for various intervals. Cyclic AMP formation in these brain homogenates was then measured as described above. Levels of cyclic AMP in blanks (i.e. samples which were not incubated at 30°, but were transferred directly from an ice bath to boiling water) were the same in the absence or presence of the various compounds tested. This indicates that none of the substances used had any effect on the radioimmunoassay for cyclic AMP.

Adenylate cyclase activity in homogenates of mouse cerebral cortex varied between 35 and 70 pmoles cyclic AMP formed/mg wet weight/min at 30° in the absence of NaF. Addition of NaF (10 mM) to the enzyme assay reagent or freezing the brain prior to homogenization increased the activity 2- to 3-fold. These findings are similar to those of others.[6]

The effects of ethacrynic acid and dithiobisnitrobenzoic acid (DTNB) on cerebral cortical adenylate cyclase activity are shown in Fig. 1. Both compounds inhibited formation of cyclic AMP in a concentration-related fashion. Fifty per cent inhibition was observed at an ethacrynic acid concentration of 3×10^{-4} M and at a DTNB concentration of 2×10^{-4} M. To obtain maximum inhibition of adenylate cyclase activity with ethacrynic acid it was necessary to incubate this compound with the brain homogenate for at least 30 min. Little or no inhibition of cyclic AMP formation was observed when ethacrynic acid and brain homogenate were added simultaneously to the enzyme assay reagent. Ethacrynic acid forms a covalent linkage with tissue sulfhydryl groups and this reaction would be expected to require some definite period of time.[7] In contrast, no incubation period was required for DTNB to produce maximum inhibition of the enzyme. DTNB causes an almost instantaneous addition of thionitrobenzoic acid to sulfhydryl groups forming a disulfide linkage.[8]

As noted above, ethacrynic acid did not inhibit the rate of formation of cyclic AMP when it was added to the enzyme assay reagent simultaneously with brain homogenate. Furthermore, the inhibitory effects of both ethacrynic acid and DTNB on cyclic AMP formation were the same regardless of whether the two inhibitors were only added to brain homogenates or whether they were also added, at an equivalent concentration, to the enzyme assay reagent. These observations indicate that the decreased formation of cyclic AMP observed in the presence of ethacrynic acid or DTNB were not due to some alteration of the ATP regenerating system in the enzyme assay reagent.

The effects of several ethacrynic acid analogs kindly supplied by Merck, Sharp & Dohme, including 4-(α-ethylacrylyl)-phenoxyacetic acid; [2,3-dichloro-4-(2-ethylidene-butyryl) phenoxy]-acetic acid; (2,3-dichloro-4-butyryl-phenoxy)-acetic acid and [2,3-dichloro-4-(2 methyl butyryl) phenoxy]-acetic acid on adenylate cyclase activity were tested. These compounds do not alkylate or alkylate only very slowly with sulfhydryl groups and none of them inhibited formation of cyclic AMP at a concentration (0·1 mM) at which ethacrynic acid produced 75 per cent inhibition of activity. Furosemide, which is not a structural analog of ethacrynic acid but has similar pharmacological properties,[9] also had no effect on adenylate cyclase activity at concentrations up to 1 mM.

Addition of cysteine or dithiothreitol to the brain homogenate had no effect in themselves on adenylate cyclase activity, but prevented inhibition of enzyme activity by both ethacrynic acid and DTNB (Fig. 2). However, this prevention was observed only when the concentration of cysteine or dithiothreitol exceeded the level of ethacrynic acid or DTNB and only when they were added to the brain homogenate prior to or simultaneously with ethacrynic acid or DTNB. Under similar circumstances, dithiothreitol also prevented inhibition of adenylate cyclase activity by alloxan.

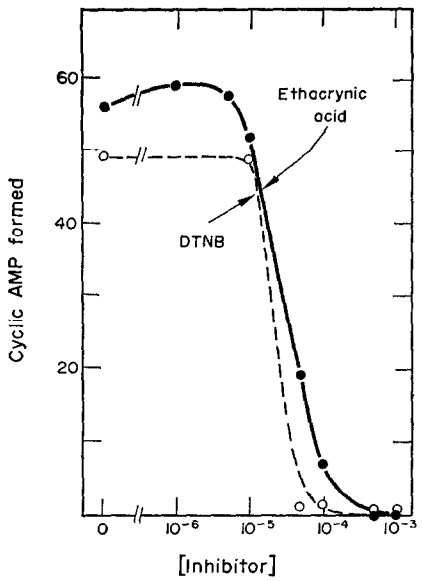

FIG. 1. Effect of ethacrynic acid and dithiobisnitrobenzoic acid (DTNB) on brain adenylate cyclase activity. Mouse cerebral cortex was homogenized in 125 vol. of cold 50 mM Tris-HCl buffer, pH 7·5, and incubated for 30 min at 30° in the absence or presence of various concentrations of ethacrynic acid or DTNB. The homogenate was then assayed for adenylate cyclase activity. The results are expressed as pmoles cyclic AMP formed/mg brain (wet weight)/min at 30° vs concentration of inhibitor (ethacrynic acid or DTNB). Each point represents the mean of three to four experiments.

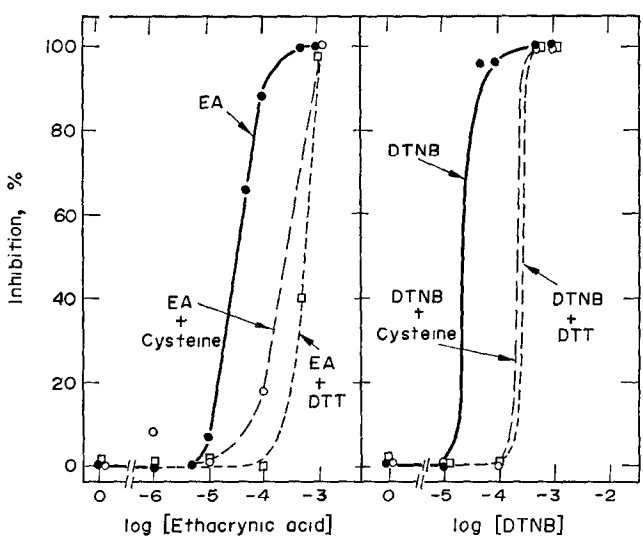

FIG. 2. Protective effects of cysteine and dithiothreitol (DTT) on inhibition of brain adenylate cyclase by ethacrynic acid (EA) and dithiobisnitrobenzoic acid (DTNB). Mouse cerebral cortex was homogenized in 125 vol. of 50 mM Tris–HCl buffer. To aliquots of the homogenate cysteine, 1 mM, DTT, 1 mM, or nothing was added. The mixtures were then incubated for 30 min at 30° in the absence or presence of various concentrations of ethacrynic acid or DTNB. After this, the homogenate was assayed for adenylate cyclase activity. The results are expressed as per cent inhibition of adenylate cyclase activity as a function of ethacrynic acid (left) or DTNB (right) concentration. Each point represents the mean of two to four samples.

Sulfhydryl reagents have been reported to be necessary in maintaining activity of adenylate cyclase prepared from avian and amphibian erythrocytes.[10,11] The apparent importance of sulfhydryl groups for activity of this enzyme in mammalian brain has not been previously noted.

The results of this study indicate that free sulfhydryl groups are necessary for full expression of adenylate cyclase activity in brain homogenates. Presumably these sulfhydryl groups are located in the enzyme itself. However, an alternate possibility is that ethacrynic acid and DTNB may interact with sulfhydryl groups of structural proteins in membranes. This, in turn, could alter the configuration of the membrane bound adenylate cyclase and inhibit its enzymatic activity.

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SHORT COMMUNICATION

Effects of methylcholanthrene on 2-acetamidofluorene association with liver cell components

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2-ACETAMIDOFLUORENE (AAF) can be converted to a highly reactive, ultimate carcinogen which is capable of forming conjugates with cell components under physiological conditions.[1] The reactions involved are *N*-hydroxylation followed by esterification to a molecule which can ionize and react with cell nucleophiles.[2] Resistance to the hepatocarcinogenic effects of AAF is observed in male rats treated simultaneously with AAF and small quantities of methylcholanthrene (MC).[3,4] One metabolic effect of MC in AAF-dosed rats is to reduce the proportion of AAF excreted as the glucuronide of *N*-hydroxy-AAF.[5] This result is due to a reduction in *N*-hydroxylating activity, an increase in *N*-hydroxy-AAF reduction or to an increase in the reactions producing phenolic metabolites. Because *N*-hydroxy-AAF is more carcinogenic than the parent compound,[6] it seemed that, through diminishing the concentration of *N*-hydroxy-AAF for further reaction, MC blocked an essential step in the activation of AAF. Consequently, interaction of the carcinogen with key components in target cells should be reduced below a threshold carcinogenic level in MC-treated animals and provide a basis for the observed inhibition of AAF induced hepatocarcinogenesis.[3,4] These key components have not been identified.