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DIETHYL CHELIDONATE, A SPECIFIC INHIBITOR OF HORMONE-STIMULATED LIPOLYSIS*

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Abstract—Diethyl chelidonate (DEC), diethyl ester of 4-oxo-4H-pyran-2,6-dicarboxylic acid, was synthesized and found to depress lipolysis stimulated by ACTH, norepinephrine, isoproterenol, theophylline, and N^6-2′-*O*-dibutyryl cyclic 3′,5′-adenosine monophosphate. In some respects, the type of blockade resembled that of the alpha-adrenergic blocking agents. DEC did not inhibit the stimulation of adipose tissue phosphorylase by norepinephrine or ACTH, nor did it affect the action of catecholamines on smooth muscle, indicating a specificity for the blockade of lipolysis. The compound exhibited a slight inhibitory effect on norepinephrine-stimulated adenyl cyclase activity and some stimulatory effect on cyclic 3′,5′-AMP phosphodiesterase. It produced no inhibition of lipases such as porcine pancreatic lipase, an adipose tissue monoglyceride lipase, and an adipose tissue long-chain triglyceride lipase, nor did it prevent the spontaneous release of free fatty acids from isolated adipocytes.

THE IMPLICATION of the involvement of plasma lipids in various cardiovascular diseases has aroused great interest in the metabolism of adipose tissue. The mechanism by which many agents increase the release of free fatty acids from adipose tissue has received much attention and the adipokinetic action of catecholamines, polypeptide hormones and methyl xanthines has been well established.[1–7] These agents have been shown to cause an increase in the intracellular level of cyclic 3′,5′-adenosine monophosphate (3′,5′-AMP),[8] which is postulated to act as a second messenger in the activation of a triglyceride lipase.[9]

Along with the interest in adipose tissue lipolysis and lipolytic agents, inhibitors of this process have received much attention. Antagonists, in fact, represent not only useful tools for the investigation of the molecular mechanisms by which the agonists exert their activity, but constitute as well, a series of compounds with potential for development into clinically useful agents. Included among the large number of compounds with lipolytic blocking activity are the classical alpha- and beta-adrenergic blocking agents,[10–14] insulin,[15–18] nicotinic acid[19] and the prostaglandins.[20–21] Despite their wide range of chemical and physical properties, none of these compounds is specific for lipolysis.

In the search for blocking agents with specificity for lipolysis, it was hypothesized that compounds bearing some structural relationship to triglycerides may selectively inhibit the triglyceride lipase in adipose tissue. Such a hypothesis led to the synthesis

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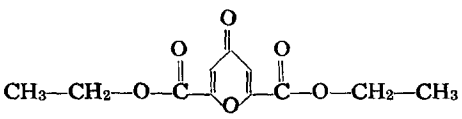
of diethyl chelidonate (DEC), which was found to depress significantly the activity of various lipolytic agents. This paper reports on the investigation of the mode and site of action of the new antilipolytic agent, DEC. A preliminary report has been published.[22]

METHODS AND MATERIALS

Chemicals. *l*-Norepinephrine was obtained from Winthrop Laboratories, New York, N.Y.; Oxycel purified ACTH (103 USP units/mg) was obtained from Wilson Laboratories, Chicago, Ill.; N^6-2′-*O*-dibutyryl cyclic 3′,5′-AMP and cyclic 3′,5′-AMP-^{3}H was supplied by Schwartz Bioresearch Company; ATP, 5′-AMP, cyclic 3′,5′-AMP and glucose 1-phosphate were furnished by PL Biochemicals, Milwaukee, Wis.; adenosine-5′-triphosphate-α-^{32}P was obtained from ICN, City of Industry, Calif.; *Crotalus atrox* venom was obtained from the Ross Allen Reptile Institute, Silver Springs, Fla. Chelidonic acid was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. All other chemicals were of highest purity commercially available.

Preparation of DEC. DEC was synthesized from chelidonic acid by esterification with ethanol. Chelidonic acid (10 g, 54·3 m-moles) was refluxed with 50 ml of alcoholic hydrogen chloride (20%) for 8 hr. The solvent was evaporated under reduced pressure at 20° and the dry residue was extracted with anhydrous ether in a Soxhlet extractor. The ether was removed *in vacuo* and the extract dissolved in water and passed through an anion-exchange resin column (Dowex-1-Cl × 8, 100–200 mesh, 20 × 1 cm). Upon drying *in vacuo* at 20°, the effluent yielded 2·60 g (10·86 m-moles, yield 20%) of DEC, m.p. 60–61 (reported 60–62). The structure of DEC is depicted below.



Adipose tissue. Epididymal fat pads were obtained from male albino rats of the Holtzman strain, starved 24 hr prior to sacrifice. At the time of use, the animals weighed 180–200 g.

Isolation of fat cells. Adipocytes were isolated from epididymal adipose tissue by Rodbell's procedure modified as described previously.[23]

Ghost cells. Fat cell "ghosts" were prepared by the method of Rodbell,[24] with the exception that the hypotonic lysing medium contained Na-ATP instead of Tris-ATP.

Partially purified cyclic 3′,5′-AMP phosphodiesterase. Epididymal fat was homogenized with 1·5 vol. of 1 mM Tris HCl–1 mM $MgCl_2$ buffer, pH 7·5, at 0° for 1 min in a Waring blender. The homogenate was centrifuged at 27,000 *g* for 20 min. The supernatant was passed through gauze and adjusted to 0·5 saturation with solid ammonium sulfate, keeping the pH at 7·5 with 0·01 N NaOH. After 10 min of stirring, the suspension was centrifuged at 15,000 *g* for 10 min. The pellet was suspended in Tris-Mg buffer and recentrifuged at 20,000 *g* for 15 min. The supernatant was dialyzed for 24 hr against Tris-Mg buffer. The dialyzed enzyme was stored at −20°.

Protein determination. Protein content in the various preparations was determined by the method of Lowry *et al.*[25]

Analytical procedures. To measure lipolysis, aliquots of the fat cell suspension were added to polyethylene incubation flasks containing warm (37°) Krebs–Ringer phosphate–5% bovine serum albumin buffer, pH 7·4. The lipolytic agent was added and the mixture was incubated at 37° for 1 hr. When DEC was used, a preincubation of the cells with the blocking agent for 3 min at 37° was performed. The free fatty acids produced were determined in aliquots taken from the incubation vessels prior to and after the incubation according to the method of Dole and Heinertz.[26] Free fatty acid (FFA) production is reported as the net amount (i.e. 60 min incubation minus 0 time flask).

Phosphorylase activity was assayed by the method of Sutherland and Wosilait, as described by Jungas.[16]

The adenyl cyclase activity was measured by the assay developed by Krishna *et al.*[27] Ghost cells were employed as the source of enzyme.

Cyclic 3′,5′-AMP phosphodiesterase was assayed by the method described by De Lange *et al.*[28]

RESULTS

Figure 1 shows the lipolytic activity of various doses of ACTH and the depression of this activity when $1{\cdot}35 \times 10^{-4}$ M and $2{\cdot}7 \times 10^{-4}$ M DEC were present in the incubation mixture. The curves clearly indicate a noncompetitive type of inhibition. When the concentration of DEC was raised to $5{\cdot}4 \times 10^{-4}$ M, the activity of the whole range of concentrations of the lipolytic agent used was virtually eliminated.

The norepinephrine (NE)-stimulated release of FFA is also significantly inhibited by DEC, as is shown in Fig. 2. Similar to ACTH, the inhibition of NE by DEC appears to be noncompetitive. DEC was equally effective in depressing isoproterenol-induced lipolysis.

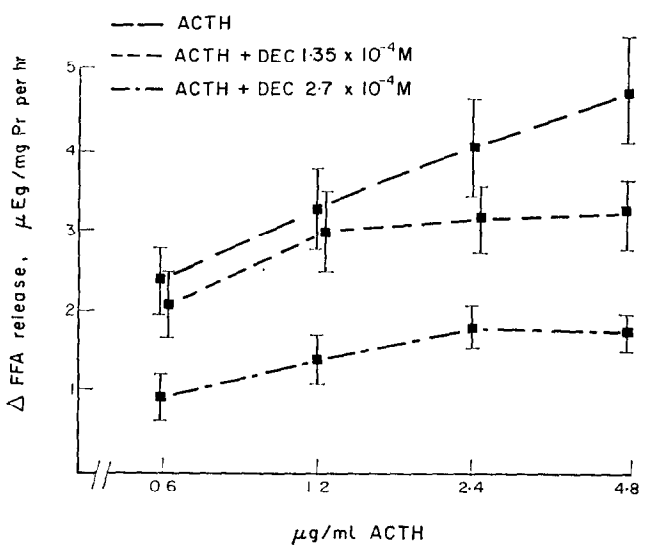


FIG. 1. Effect of DEC on ACTH-stimulated lipolysis. Each point represents the mean of four or more experiments. The bars represents ± 1 S.E. Experimental conditions are described under analytical procedures in Methods.

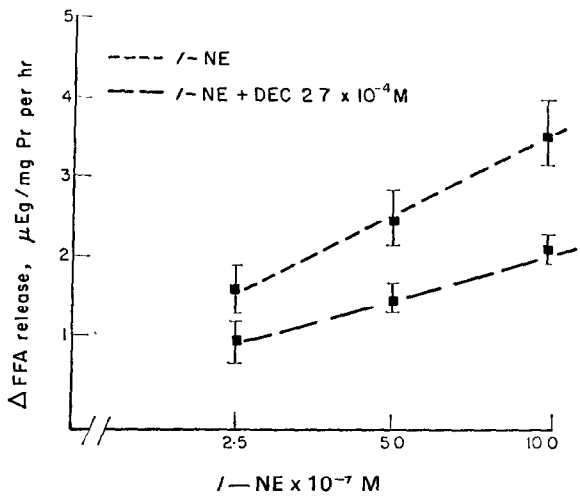


FIG. 2. Effect of DEC on NE-stimulated lipolysis. Each point represents the mean of four or more experiments. The bars represent ± 1 S.E. Experimental conditions are described under analytical procedures in Methods.

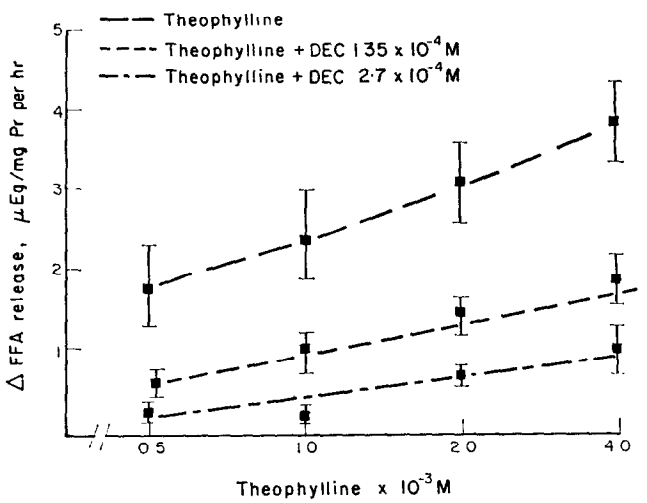


FIG. 3. Effect of DEC on theophylline-stimulated lipolysis. Each point represents the mean of four or more experiments. The bars represent ± 1 S.E. Experimental conditions are described under analytical procedures in Methods.

To circumvent the adenyl cyclase system, which has been implicated as the site of action of the above agents, experiments were carried out in which theophylline or N^6-2′-*O*-dibutyryl cyclic 3′,5′-AMP was employed as the lipolytic substance. Figure 3 shows the effect of DEC on the theophylline-induced lipolysis. From the data, it appears that the lipolytic activity of the methyl xanthine is inhibited more strongly than that of any other agent. Moreover, the lipolytic activity of N^6-2′-*O*-dibutyryl

cyclic 3′,5′-AMP was also strongly inhibited by the addition of DEC to the incubation system (Fig. 4).

It is of interest that, although DEC effectively blocks the lipolytic activity of NE and ACTH, the stimulation of phosphorylase produced by either of the two hormones is not affected (Table 1).

Table 2 reports the effect of DEC and NE on adenyl cyclase activity. Although the blocking agent seemed to possess a stimulant activity of its own, it was found to depress, to some extent, the stimulation of adenyl cyclase by NE.

DEC was also found to possess some stimulating effect on the phosphodiesterase, which effect is not statistically significant.

The possibility that DEC, being an ester, might act as a substrate for the lipolytic

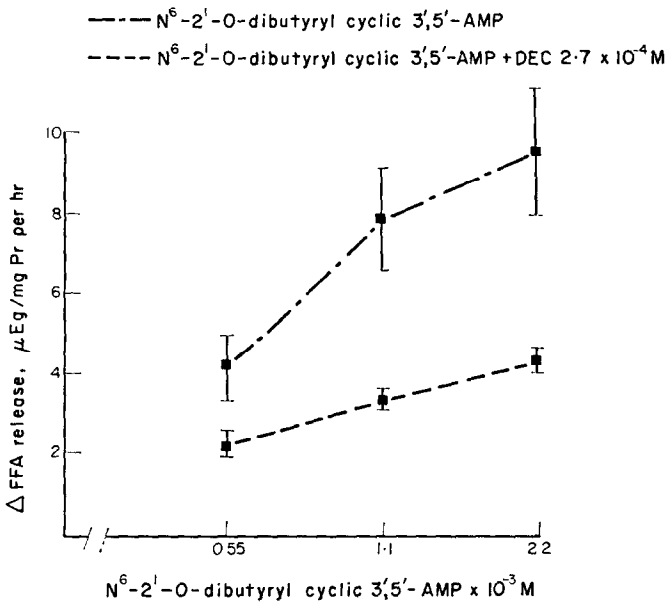


FIG. 4. Effect of DEC on dibutyryl cyclic AMP-stimulated lipolysis. Each point represents the mean of four or more experiments. The bars represent ± 1 S.E. Experimental conditions are described under analytical procedures in Methods.

TABLE 1. EFFECTS OF NE, ACTH AND DEC ON PHOSPHORYLASE ACTIVITY

Addition to assay medium	N	Phosphorylase activity*
None	5	1·22 ± 0·17
DEC ($2·7 \times 10^{-4}$ M)	5	1·31 ± 0·25 ($P > 0·7$)
NE (1×10^{-6} M)	5	1·81 ± 0·13 ($P < 0·05$)
DEC + NE	5	1·75 ± 0·18
None	4	1·15 ± 0·17
DEC ($2·7 \times 10^{-4}$ M)	4	1·24 ± 0·14 ($P > 0·6$)
ACTH (0·24 U)	4	1·92 ± 0·26 ($P < 0·05$)
DEC + ACTH	4	2·06 ± 0·26

* Micromoles of P_i produced/100 mg of adipose tissue/15 min ± S.E.

TABLE 2. EFFECTS OF NE AND DEC ON ADENYL CYCLASE ACTIVITY

Addition to assay medium	Adenyl cyclase activity*
None	0·37 ± 0·06
DEC ($5·5 \times 10^{-3}$ M)	0·99 ± 0·42
NE (1×10^{-4} M)	1·17 ± 0·29
DEC + NE	0·60 ± 0·11

* Nanomoles of cyclic 3′,5′-AMP formed/mg protein/10 min ± S.E. (N = 6).

TABLE 3. EFFECT OF DEC ON VARIOUS LIPASE ACTIVITIES

Lipase system	Reference for experimental conditions	Substrate	Activity (μ equiv. FFA hydrolyzed/mg protein/min)	
			Control	+ DEC (66 μg/ml)
1. Partially purified monoglyceride lipase	29	1-Monolaurin	0·33	0·35
2. Crude triglyceride lipase	*	Natural endogenous	0·011	0·013
3. Purified pancreatic lipase (porcine)	30	Triolein	123	127

* Incubation conditions for this enzyme preparation are as follows: whole adipose cells are divided into two portions and to one portion is added 10^{-5} M norepinephrine for 5 min; to the other portion nothing is added. Both portions are sonicated and incubated for 60 min as a homogenate in an incubation system containing 10 μmoles PO_4 buffer, pH 7·4, and albumin (4%) in 1 ml. One of the incubation vessels contained DEC and the other did not. The numbers reported represent the increment of FFA released in the presence of NE over that in the absence of NE.

enzymes was also investigated. However, no evidence of ethanol could be found in the incubation mixture. The magnitude of blockade of lipolysis was not significantly altered by preincubation of the fat cells with DEC for 1 hr and the compound did not inhibit the spontaneous release of FFA by isolated adipocytes; it depressed only hormone-stimulated lipolysis.

The activities of three different lipolytic enzyme systems were tested in the presence of ten times the concentration of DEC needed to inhibit hormone-induced lipolysis in intact cells. None of the enzymes was inhibited by DEC (Table 3).

Since the alpha- and beta-adrenergic blocking agents have been shown to inhibit hormone-stimulated lipolysis, the question arose as to whether DEC possessed any such sympathetic blocking activity. However, no hemodynamic changes could be observed when various doses of DEC were injected directly into the heart of anesthetized cats and dogs, and experiments employing isolated intestinal strips of rabbits or isolated rat vas deferens disclosed neither sympathomimetric nor sympathetic blocking activity.

DISCUSSION

DEC has been shown to depress the lipolytic activity of various agents on epididymal fat pads and isolated adipocytes. Its inability to depress the stimulation of phosphorylase or to affect the action of beta- and alpha-adrenergic agents on smooth muscle

indicates a specificity of action for hormone-stimulated lipolysis, although the exact site of inhibition has not been established.

According to the hypothesis advanced by Butcher *et al.*,[31] catecholamines and polypeptide hormones act on the adenyl cyclase system, stimulating the formation of cyclic 3′,5′-AMP, which, through a single or multiple step operation, activates a triglyceride lipase resulting in an increased release of FFA and glycerol from adipocytes. Compounds such as the methyl xanthines, capable of preserving cyclic AMP by inhibition of cyclic 3′,5′-AMP phosphodiesterase, produce similar lipolytic effects.[7, 32] N^6-2′-*O*-dibutyryl cyclic 3′,5′-AMP mimics the cyclic nucleotide in its lipolytic effect. An antilipolytic agent may exert its activity at any place in such a lipolytic pathway. Depending on the site and type of inhibition, the blocking agent may be selective for a specific lipolytic agent, or possess no specificity at all. Accordingly, the beta-adrenergic blockers in low concentrations are effective in preventing the stimulation of adenyl cyclase by catecholamines, whereas nicotinic acid inhibits the action of a variety of lipolytic agents such as the catecholamines, various polypeptides and the methyl xanthines.

Although DEC possesses some features in common with the above agents, it differs from them in the lack of effect on NE- and ACTH-stimulated phosphorylase activity and the lack of any significant effect of vascular and smooth muscle responses to catecholamines. The effects of DEC both as a stimulant of and as a partial blocker of adenyl cyclase are somewhat difficult to interpret. One would expect on the basis of the current hypothesis[17] that the stimulant effect would result in lipolysis, while the blocking action on NE-stimulated adenyl cyclase would block lipolysis. However, one must be aware that the concentration of NE required to demonstrate adenyl cyclase activation is at least 100 times that needed to demonstrate lipolysis or a cyclic AMP accumulation in intact cells[17] and that the cyclase assay system contained 1 mM theophylline. Such vastly different assay conditions must temper an attempt to attribute the blocking effects of DEC on lipolysis to the blockade of adenyl cyclase activation. Others have also reported the necessity to employ such large concentrations of hormones on ghost or broken cell preparations[33, 34] in order to demonstrate activation of adenyl cyclase.

In terms of the effective concentration for inhibition and the lack of specificity for different lipolytic agents, DEC resembles the alpha-adrenergic blocking agents.

Possibly, in accordance with the hypothesis which led to its synthesis, DEC competes with triglyceride for the lipase. The reversed direction of the ester bonds possibly makes DEC resistant to hydrolysis, resulting in a relatively stable enzyme–substrate complex with a decreased amount of enzyme available for the triglycerides. The lack of effect of DEC on the isolated lipases may be explained by the possibility that none of the lipases tested is the hormone-sensitive lipase. The resistance to hydrolysis would also explain the inability to detect ethanol in the incubation mixture and the long-lasting blocking effect.

It should be emphasized that these are mere speculations and that the mode of action of DEC can be truly elucidated only when the lipolytic pathway is fully understood and the hormone-sensitive lipase(s) characterized.

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