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INFLUENCE OF VARIOUS AGONISTS AND ANTAGONISTS ON THE RELEASE OF FREE FATTY ACIDS FROM ADIPOSE TISSUE *IN VITRO**

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Abstract—Various agonists and antagonists of free fatty acid (FFA) release from rat epididymal fat tissue have been investigated. It has been established that all catecholamines tested have equal intrinsic activities but vary in their affinity constants (pD_2 values) in this system. Thus the order established for affinity is isopropyl arterenol > epinephrine > norepinephrine >> dopamine. Tyramine and amphetamine were evaluated and found to be inactive in releasing FFA. Desmethylimipramine, imipramine, and chlorpromazine were found to be antagonists of the norepinephrine-induced release of FFA *in vitro*. Noncompetitive inhibition was evident for desmethylimipramine and imipramine, whereas chlorpromazine appeared to be a competitive inhibitor of the system.

EVIDENCE that the sympathetic nervous system may have an important role in the mobilization of free fatty acids (FFA) from adipose tissue has been accumulating at a rapid pace during the past few years. For example, it has been shown that stimulation of the sympathetic nerve supply to the epididymal fat pad results in the release of FFA[1]; it has been similarly shown that injection of either epinephrine, or norepinephrine results in an increase in plasma FFA[2–4] and that, when catecholamines are added to a medium containing adipose tissue slices, an increase in the hormone-sensitive lipase results,[5] which in turn leads to an increased mobilization of FFA. These and other studies have provided the basis for the current interest in the effects of the catecholamines on fat mobilization. Other studies [6–9] primarily concerned with the investigation of compounds capable of inhibiting the catecholamine-induced release of FFA have given impetus to experimentation directed toward elucidating the role of the fat mobilization phenomena in certain cardiovascular diseases. Perusal of the literature in this field suggests that much remains to be learned about the nature of the catecholamine–adipose tissue interactions and the influence of various drugs, both inhibitors and potentiators, on this interaction and the metabolic processes concerned with the release of FFA.

This report presents data concerning the structural specificity shown by the adipose tissue system for several phenethylamine agonists and the effect of certain antagonists on the catecholamine-induced mobilization of FFA from rat epididymal fat pads *in vitro*.

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MATERIALS AND METHODS

The chemicals employed in this study and their sources are as follows: desmethylimipramine and imipramine, Geigy Research Labs.; chlorpromazine, Smith, Kline and French Labs.; l-epinephrine bitartrate, Winthrop Labs.; l-norepinephrine bitartrate, Nutritional Biochemicals Corp.; *d,l*-isopropyl arterenol HCl, tyramine HCl, and dopamine HCl, Mann Research Labs.; *d*-amphetamine, K and K Labs.; and bovine albumin, fraction V, Nutritional Biochemicals Corp. All concentrations expressed in this paper refer to free base.

FAA *Release, assay procedures.* Male Holtzman rats, weighing between 200 and 250 g, were maintained in our animal quarters for at least one week prior to being employed in an experiment. The animals were sacrificed by stunning and decapitation, and the anterior third of the epididymal fat pads was rapidly removed, placed in freshly prepared Krebs-Ringer bicarbonate buffer (pH 7·4), washed with buffer, and minced with a small scissors to yield pieces weighing approximately 5–10 mg. Fat pads from six rats were pooled for each experiment.

Into each 30-ml narrow-neck incubation flask containing 9·0 ml of a 4% bovine albumin solution was added 600 mg adipose tissue slices. Each flask was fitted with a rubber-stopper assembly through which were placed no 20 hypodermic needles fitted with polyethylene tubing to serve as vents for the gassing procedure and also for sampling the media in the assay for FFA.

All inhibitors were preincubated with the adipose tissue slices for 15 min prior to gassing. The catecholamines or other agonists were added in 0·2 ml volume, and all flasks were then gassed with the 95% O_2–5% CO_2 mixture for 10 min

The incubations were carried out in a Dubnoff metabolic shaker maintained at 37° and oscillating at 120 cycles per minute. At 0, 20, 40, and 60 min 0·5 ml samples were withdrawn from the flasks and assayed for FFA by a modification[10] of the method of Dole[11] (Nile blue A as the indicator for titration).

The FFA concentrations determined at the times given above were then plotted vs. time and the rates of FFA release, expressed in micromoles per gram per hour, were calculated from the slope of the line obtained. Controls (no agonist present) were run. and the amount of FFA released in the absence of agonist was subtracted from the amount released in the presence of agonist.

RESULTS

Standard dose–response relationships. Preliminary experimentation indicated that the release of FFA induced by the catecholamines was linear with respect to time for approximately 2 hr. However, it was observed that deviations from linearity were most frequent in the 90- to 120-min period, and the initial rate period of 0–60 min was therefore chosen for all assays The shorter time interval also minimized errors due to degradation of the catecholamines, as has been reported previously.[12]

In Fig. 1, the response of the adipose tissue system to various concentrations of norepinephrine is shown. It can be seen that the response rate is linear with respect to time over this period and that as the concentration of norepinephrine is increased from $0{\cdot}65 \times 10^{-7}$ M to $5{\cdot}2 \times 10^{-7}$ M, there occurs an increase in the rate of FFA release. Higher concentrations of norepinephrine caused no further increase in the FFA release rate over that obtained at $5{\cdot}2 \times 10^{-7}$ M. In the system employed in this study, the maximal release rate was found to be 18 μmoles/g/hr, and was constant irrespective

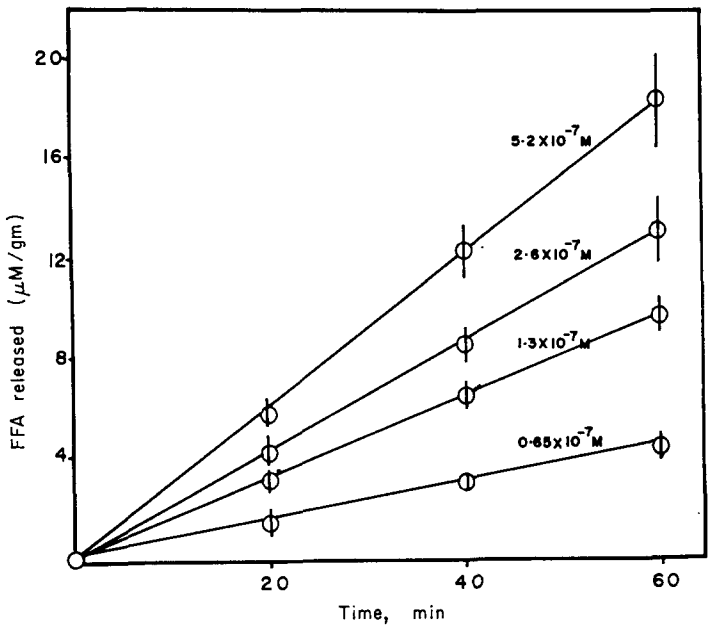

FIG. 1. Effect of various concentrations of norepinephrine on release of FFA from rat epididymal fat tissue, *in vitro*, as a function of time. Norepinephrine concentrations are expressed in moles per liter. The values plotted represent the mean ± S.E.

of the agonist employed This figure was utilized to calculate the per cent response of the tissue system in all studies described below.

Dose–response relationships similar to that shown in Fig. 1 for norepinephrine were determined for each agonist and the rate of FFA release calculated as described above.

The relative effectiveness of epinephrine, norepinephrine, and isopropyl arterenol in releasing FFA from adipose tissue *in vitro* was determined, and the results are illustrated in Fig. 2, where the reciprocal of the FFA release rate is plotted against the reciprocal of the agonist concentration. It can be seen in Fig. 2 that the data for both epinephrine and norepinephrine reside about a common line, indicating their similarity in intrinsic activity and affinity in the adipose tissue system. A plot of the data obtained with isopropyl arterenol lies on a slope that is much less steep, indicating that this substance has a much greater affinity for the adipose tissue system (see Table 1).

Dose–response relationships for other agonists. In an attempt to determine the degree of chemical specificity shown by the adipose tissue system, the activity of certain other substituted phenethylamines in stimulating the release of FFA was determined. Dopamine, another catecholamine, was selected for testing because it differed from noreprinphrine in lacking the β-hydroxyl function in the ethyl amino side chain and thus provided an opportunity to assess the role played by the β-hydroxyl group in determining the relative affinity and intrinsic activity in this system. Tyramine and amphetamine were selected for their structural characteristics and because one of their

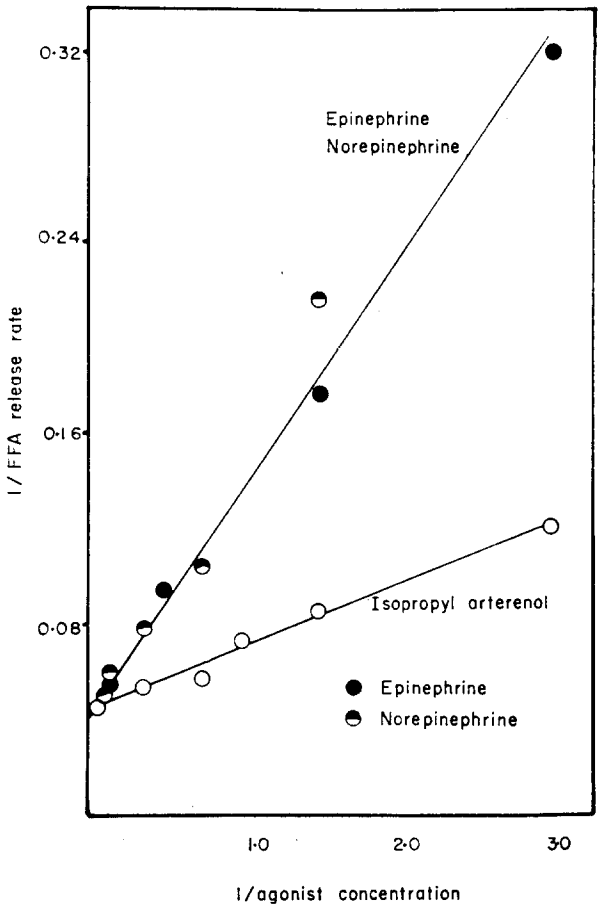

FIG. 2. Double reciprocal plot illustrating the effects of epinephrine, norepinephrine, and isopropyl arterenol on the release of FFA from rat epididymal fat tissue *in vitro*. The rate of FFA release was calculated from FFA release data as explained in text and expressed in micromoles per gram per hour. The concentrations of agonists employed were in moles per liter $\times 10^{-7}$.

TABLE 1. THE INTRINSIC ACTIVITY AND AFFINITY (pD_2) CONSTANTS FOR SEVERAL PHENETHYLAMINES AS DETERMINED FROM THEIR FFA-RELEASING ACTIVITY

Phenethylamine	Intrinsic activity	pD_2*
Epinephrine	1·0	6·6
Isopropyl arterenol	1·0	7·6
Norepinephrine	1·0	6·6
Dopamine	1·0	4·4
Amphetamine	0	—
Tyramine	0	—

* pD_2 = Negative logarithm of the concentration of agonist required to produce 50 per cent of the maximal response obtained in the system.

proposed mechanisms of action is an indirect action mediated by the release of endogenous catecholamine. The results of these experiments are shown in Fig. 3, and the calculated intrinsic activity and affinity (pD_2) constants[13] for the compounds tested are presented in Table 1.

It can be seen that dopamine stimulated the release of FFA from rat epididymal fat tissue but was much weaker in this regard than any of the other catecholamines tested. The lower affinity possessed by dopamine is apparent from the marked shift of the dose-response curve to the right in Fig. 3 and from the much lower affinity

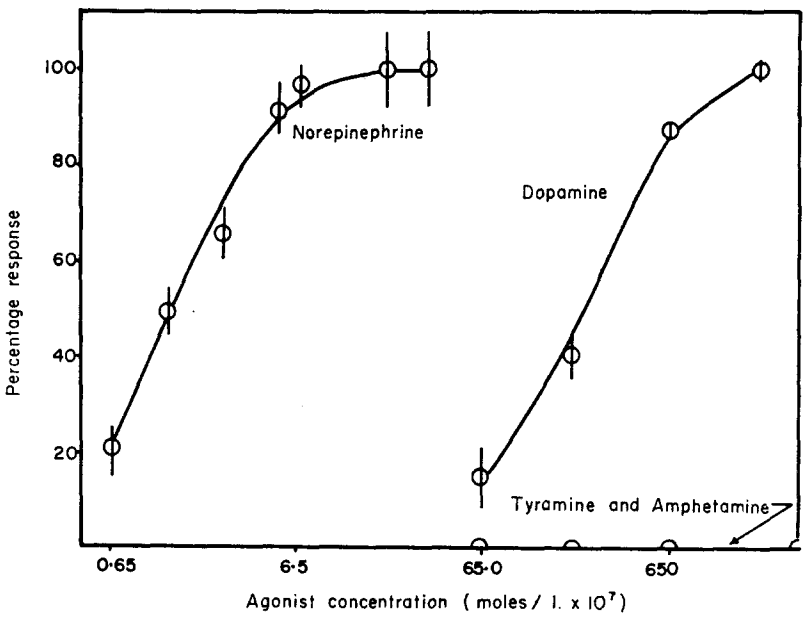

FIG. 3. The effect of norepinephrine, dopamine, tyramine, and *d*-amphetamine on the release of FFA from rat epididymal fat tissue, *in vitro*, expressed as the per cent of the maximal response obtainable in the system. (see text). Tyramine and amphetamine did not stimulate FFA release: i.e. zero per cent response. The values plotted represent the mean ± S.E.

constant presented in Table 1. The intrinsic activity found for dopamine is equal to that observed for the other catecholamines.

Tyramine and amphetamine, on the other hand, failed of produce a significant increase in FFA release above that of the controls. Thus, these compounds possess no intrinsic activity in this system. Preliminary experiments, not reported here, also indicated that these two compounds did not inhibit norepinephrine induced FFA release at the relatively high concentrations employed.

Inhibition of FFA *release by desmethylimipramine* (DMI). Various concentrations of DMI were preincubated with the adipose tissue slices prior to the addition of norepinephrine to the media and the effect of the added DMI on FFA release determined. It is apparent from the data shown in Fig. 4 that DMI is a relatively potent inhibitor of norepinephrine-induced FFA release. The inhibition appears to be noncompetitive. Indeed, very high concentrations of norepinephrine failed to reverse the inhibition

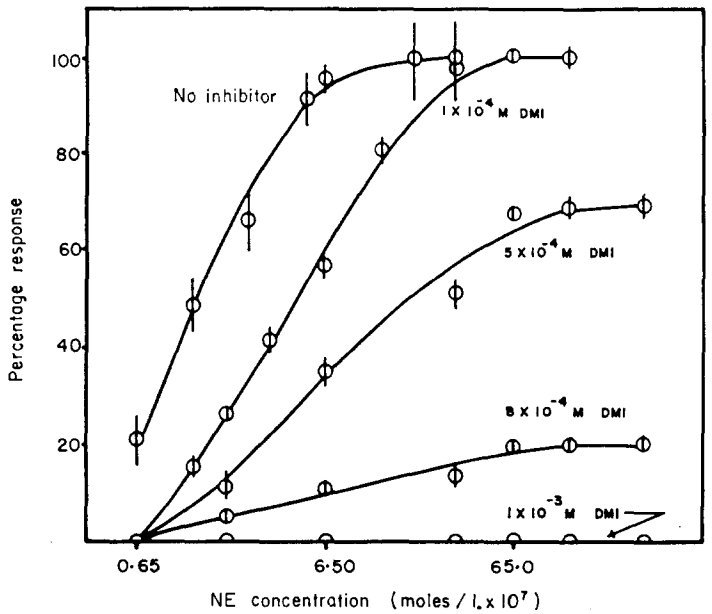

FIG. 4. Inhibitory effect of DMI on norepinephrine induced FFA release from rat epididymal fat tissue, *in vitro*, expressed as a per cent of the maximal response obtainable in the system. The values plotted represent the mean ± S.E.

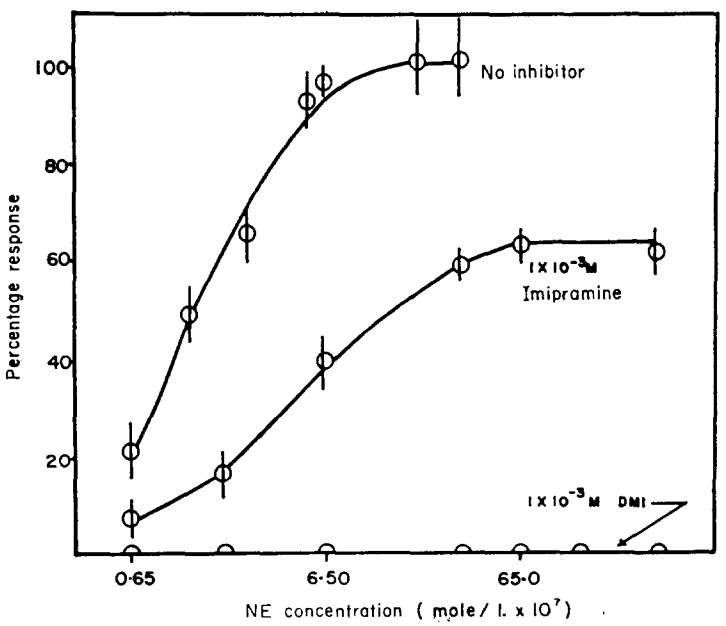

FIG. 5. Comparison of the inhibitory effects of imipramine and DMI on the norepinephrine-induced FFA release from rat epididymal fat tissue, *in vitro*, at equimolar concentrations of inhibitor (1×10^{-3} M). The values plotted represent the mean ± S.E.

by DMI at DMI concentrations of 5×10^{-4} M and above. These data do not suggest the site of inhibitory activity, and additional studies are under way to elucidate the possible mechanisms by which DMI may be acting.

Inhibition of FFA release by imipramine. Imipramine, the N-dimethyl analogue, was also evaluated as an inhibitor of norepinephrine-induced FFA release. The comparison of the inhibitory activities of imipramine and DMI are shown in Fig. 5. It can be seen

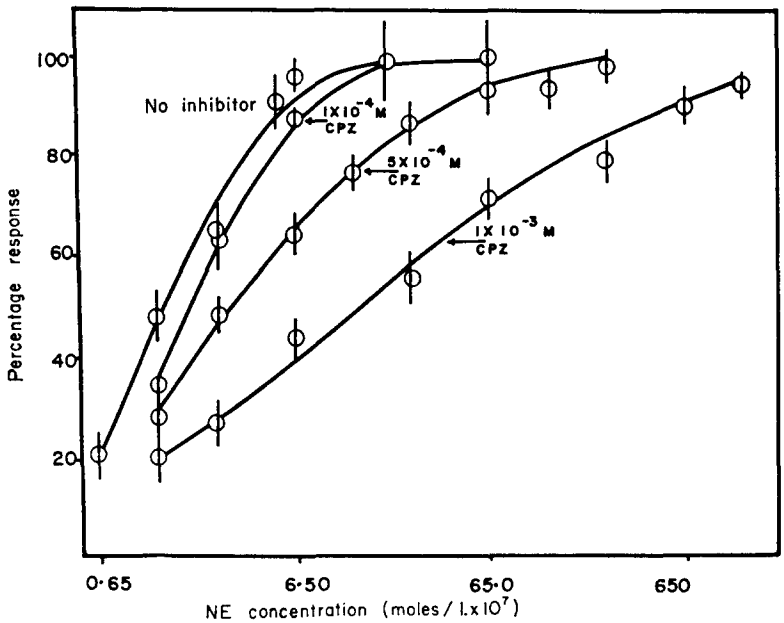

FIG. 6. Inhibitory effect of chlorpromazine (CPZ) on norepinephrine-induced FFA release from rat epididymal fat tissue, *in vitro*, expressed as a per cent of the maximal response obtainable in the system. The values plotted represent the mean ± S.E.

that imipramine is a much weaker inhibitor of the system than is DMI; however, like DMI, it appears to inhibit in a noncompetitive manner.

Inhibition of FFA *release by chlorpromazine.* Chlorpromazine has been shown to be an inhibitor of adrenergic systems, and for this reason it was of interest to determine the effect of chlorpromazine in the adipose tissue assay. When tested at concentrations comparable to those employed for the DMI studies, chlorpromazine was found to inhibit norepinephrine-induced FFA release but was much weaker in this regard than DMI. These data are illustrated in Fig. 6.

DISCUSSION

Recent studies have shown that catecholamine-induced release of FFA from adipose tissue appears to be mediated by the action of the catecholamines on adenyl cyclase.[14,15] That is, acting through adenyl cyclase, the catecholamines stimulate the production of 3′,5′cyclic AMP which in turn appears to be responsible for the activation of lipolytic activity in adipose tissue. Thus, the maximal and rate-limiting step observed in this and other studies probably is due to the limiting concentation of 3′,5′AMP in the adipose tissue.

The present study has shown that all catecholamines studied were capable of inducing the same maximal rate of FFA release from adipose tissue *in vitro*, thus implying that in this system, all catecholamines studied possess equal intrinsic activities. However, modifications of the basic catecholamine structure resulted in rather pronounced alterations in the apparent affinity of the molecules for the adipose tissue receptor system. Epinephrine and norepinephrine possessed identical affinity constants (pD_2 values of 6·6), while N-substitution of an isopropyl group greatly enhanced the affinity of the catecholamine for the adipose tissue system, as evidenced by the pD_2 value for isopropyl arterenol of 7·6.

The present studies have also shown the importance of the β-hydroxyl function on the ethyl amino side chain of the catecholamines in determining the affinity of the compounds for the adipose tissue system. Evidence for this may be derived from the comparison of the dose–response curve obtained for dopamine with that obtained for norepinephrine. The dose–response curve for dopamine is shifted far to the right, indicating a much lower affinity. Thus, dopamine possessed a pD_2 value of 4·4 (Table 1), which represents an approximately 100-fold decrease in affinity when compared to norepinephrine (pD_2 6·6). These results point up the importance of the β-hydroxyl function in determining the relative affinity of the catecholamine in this system and suggest that any model system designed to illustrate the interaction of catecholamine with a receptor moiety must take into account the orientation and presence or absence of this chemical group. A similar admonition was expressed by Ariens, based on data obtained in other adrenergic systems.[16]

The negative results obtained with tyramine and amphetamine suggest that the catechol moiety may be essential for effective FFA mobilization by a phenethylamine agonist. Certainly, more complete structure–activity relationship studies must be done to establish the absolute requirement for the catechol nucleus in this regard; however, these data suggest such a requirement. Paoletti *et al.*[17] and others[18] have shown that adipose tissue contains appreciable quantities of norepinephrine, and inasmuch as tyramine acts, at least in part, by an indirect action mediated by the release of endogeneous norepinephrine,[19, 20] we fully expected to observe some FFA-releasing activity with tyramine in our system. The fact that we did not observe an effect with either tyramine or amphetamine in this system may be interpreted in at least two ways: (1) the inability of these compounds to stimulate the release of FFA *in vitro* may be due to the inability of these compounds to reach the site of norepinephrine storage; or (2) if they did reach the site of norepinephrine storage, they failed to cause a release of the endogenous catecholamine. The former possibility would suggest that either tyramine and amphetamine failed to diffuse through the fat tissue to the storage sites or they were accumulated preferentially by the adipose tissue, thus being effectively removed from the aqueous phase within the cells. The latter alternative, involving the failure of tyramine and amphetamine to cause the release of endogenous catecholamine, is in direct opposition to the currently offered explanation of the mechanism of action of these compounds. Uptake studies with tyramine are in progress in an attempt to explain these findings.

The finding that imipramine, desmethylimipramine, and chlorpromazine were capable of inhibiting the catecholamine-induced mobilization of FFA is important from several points of view. Certainly, more potent inhibitors of this system have been reported, but relatively little has been reported concerning the mechanisms by

which the inhibitors act or the competitive or noncompetitive nature of their inhibition. The three compounds investigated as part of this study possess certain chemical similarities, yet they appear to differ in their inhibitory characteristics. Imipramine and desmethylimipramine do not follow the requirements for competitive inhibitors, whereas chlorpromazine, in the concentrations studied, appeared to be a competitive inhibitor of the adipose tissue system. In such a biochemically complex system as adipose tissue, there exist several points of attack for an inhibitor. Thus an inhibitor might block the catecholamine stimulation of the cyclic AMP formation, the action of the cyclic AMP in activating lipase, or inhibit the lipase enzyme directly. Studies currently proceeding in our laboratory are attempting to establish the site of inhibition for these compounds. In this regard, Hulsmann *et al.*[21] have reported that phenothiazines inhibit lipase activity in lung and adipose tissue homogenates and have suggested that this inhibitory activity may be responsible for the gain in weight frequently observed in patients receiving phenothiazines. These authors did not suggest, however, a mechanism for the inhibition.

Blei[22] has reported a finding which may have relevance to our present findings. He studied the physicochemical interactions existing between various phenothiazines and ATP in aqueous solutions. Such a complex, if it were sufficiently stable, could represent the mechanism by which the chlorpromazine inhibits catecholamine-induced FFA release and perhaps explain some of its other antiadrenergic effects.

Imipramine and desmethylimipramine, on the other hand, have been shown by Sigg *et al.*[23] and by Ursullo and Jacobson[24] to potentiate the effects of catecholamines on certain α-adrenergic receptor systems both *in vitro* and *in vivo*. In the adipose tissue system employed in the present study, both imipramine and desmethylimipramine were shown to be inhibitors of the system. Indeed, desmethylimipramine possessed appreciable inhibitory activity at a concentration of 1×10^{-4} M. Although the mechanism of inhibitory activity of these compounds is still under study, it is possible that the site of inhibition is remote from the adrenergic receptor *per se;* i.e. the compounds may have an effect on lipase itself. Recently, Santi and Fassina[25] have reported that both imipramine and desmethylimipramine, *in vivo*, were capable of causing a rise in plasma FFA, presumably mediated through their stimulating activity on the central nervous system. It is interesting to note that the time of maximal elevation reported by these authors for imipramine and desmethylimipramine was 150 min as compared to the 30 min required for amphetamine. It is possible, but not proven, that metabolic transformetion of imipramine and desmethylimipramine occurred in these studies to yield moieties retaining CNS-stimulating properties but lacking adipose tissue inhibitory activities. It is also possible that imipramine and desmethylimipramine are inhibitors of adipose tissue FFA release *in vitro* but do not possess this activity *in vivo*. These possibilities are also under investigation.

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