Biochemical Pharmacology, 1966, Vol. 15, pp. 1309–1316. Pergamon Press Ltd,. Printed in Great Britain.

# THE ROLE OF MICROSOMAL ENZYMES IN THE ACTIVATION AND INACTIVATION OF MODALINE SULFATE*

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(*Received* 6 *December* 1965; *accepted* 21 *March* 1966)

**Abstract**—The incubation of modaline sulfate ($5 \times 10^{-5}$ M) with NADPH and rat liver microsomes results in the rapid formation of a highly active monoamine oxidase inhibitor. Prolonged incubation under these conditions leads to a subsequent decrease in inhibitor concentration. The use of various manipulations and microsomal enzyme antagonists indicates that both the bioactivation and inactivation of the inhibitor are carried out by the drug-metabolizing enzymes in liver microsomes. When modaline is exposed to microsomes from phenobarbital-pretreated rat, the rate of activation to the inhibitor intermediate increases markedly. However, the rate of inactivation of the inhibitor by liver microsomes from phenobarbital-pretreated rats is only slightly increased above controls. Assay of MAO activity of rat livers after administration of modaline in controls and in rats pretreated with drugs that alter microsomal activity yielded information essentially the same as that in the experiments *in vitro*.

THE discovery of the monoamine oxidase (MAO)-inhibiting property of iproniazid (1-isonicotinyl-2-isopropyl hydrazine, Marsilid) by Zeller *et al.*,[1] and the subsequent correlation of this action with its antidepressant property, have resulted in the search for more effective and specific anti-MAO agents. The hydrazine derivatives were the first class of such drugs, but because of numerous side effects, other inhibitors of MAO have become of primary interest. These include compounds such as tranylcypromine, the harmala alkaloids, and pargyline. The most recent of the nonhydrazine inhibitors is the compound 2-methyl-3-piperidinopyrazine (modaline sulfate, W3207B) which was first described by Dubnick *et al.*[2] and Gylys *et al.*[3]

Modaline sulfate is an interesting compound not only because of its unusual chemical structure but also because of its requirement of initial activation by liver microsomes to yield the active enzyme inhibitor. This compound, therefore, requires an initial biotransformation to an active intermediate before the antienzyme effect can be demonstrated. The nature of the biotransformation product of modaline is at present unknown, but Dubnick *et al.*[2] have reported that the inhibition of MAO in liver homogenates occurs provided that the coenzyme NADPH is present in adequate concentrations. Under such conditions, modaline exhibits considerable anti-MAO activity at concentrations as low as $4 \times 10^{-6}$ M.

Although the structure of the active intermediate is not at present available, we have characterized some of the properties of modaline as a MAO inhibitor. The

* This investigation was supported in part by Grant MH-02435-07, 08 from the National Institute of Mental Health.

present paper describes some of the properties of the biotransformation of modaline to the active intermediate and also its subsequent inactivation.

## METHODS

*Bioactivation and inactivation of modaline* in vitro

In order to demonstrate the formation and inactivation of the anti-MAO activity of modaline sulfate *in vitro*, it was necessary to expose it to liver microsomes in the presence of NADPH. As the microsomal source, we employed the 9000-*g* supernatant of 20 per cent rat liver homogenate (prepared in 1·15% KCl) which had been centrifuged for 15 min at 4° in the Spinco model L ultracentrifuge. One ml of the supernatant was incubated with $10^{-4}$ M NADPH, 0·067 M phosphate buffer (pH 7·4), and $5 \times 10^{-5}$ M modaline sulfate in a final volume of 3·0 ml. Inhibitors (e.g. SKF-525A) were also added at this time in certain experiments. The mixtures were incubated for varying intervals of time, after which aliquots of 1·5 ml (unless otherwise indicated) were withdrawn and transferred to flasks containing a buffered suspension of rat liver mitochondria which acted as the source of MAO. After preincubating this mixture for 15 min, 6 μmoles serotonin creatinine sulfate was added (final volume 3·0 ml) as the substrate and incubated further for 50 min. An aliquot was then removed for assay of residual serotonin and MAO activity by the method described by Udenfriend *et al.*[4] The mitochondrial suspension in these instances served as a bioassay system for the amount of active intermediate present in the first preincubation of modaline with the microsomal fraction.

When the experiments on the mode and rate of inactivation of the active intermediate were undertaken, several additional procedures were included. In the instance of determining whether the inactivation was a function of the microsomal fraction, the following steps were taken. Modaline sulfate was converted maximally to the active intermediate by incubation with microsomes for 10–15 min according to the method described above. At this time, part of the incubation mixture was centrifuged at 100,000 *g* for 60 min to separate the soluble fraction containing the activated inhibitor (A) from the microsomal fraction. The other part of the mixture (B) was placed under ice for 60 min. After this time, both the soluble fraction (A) and the drug plus the 9000 *g* supernatant mixture (B) were reincubated for varying times at 37° and their aliquots tested for changes in anti-MAO activity.

A similar procedure was employed when the effect of phenobarbital pretreatment on the inactivation of the inhibitor was examined. Modaline sulfate was first activated as described above. The mixture was then centrifuged at 100,000 *g* for 60 min to separate the microsomal from the soluble fraction (containing the active intermediate). An aliquot of this supernatant fluid was then added to liver microsome which had been prepared just previously from control rats and rats which had been pretreated with phenobarbital (100 mg/kg, 18–20 hr previously). The mixtures of the soluble fractions containing the inhibitor and the microsomes were thoroughly mixed and incubated at 37° for varying periods. Samples of the incubation mixtures were removed at these times and assayed for anti-MAO activity as described earlier.

*Bioactivation and inactivation* in vivo

In the intact rat, the demonstration of anti-MAO activity upon administration of modaline did not require the use of an exogenous source of the coenzyme NADPH.

The influence of inhibitors or enzyme inducers (such as phenobarbital) on the activation process was readily demonstrated by the appropriate pretreatment of the animals with the drugs, which was then followed by modaline. These animals were sacrificed 2–4 hr later and their livers (as homogenates) examined for MAO activity, which indicated the extent of conversion of modaline to the active intermediate.

An analysis of the rate of inactivation of the active inhibitor required a procedure similar to that described for the methods *in vitro*. Rats were pretreated with rather large doses of modaline (20–40 mg/kg, i.p.), and after various intervals of time they were sacrificed and their livers removed, homogenized, and centrifuged at 100,000 *g* for 60 min. Aliquots of the supernatant fraction were then assayed for anti-MAO activity on the mitochondrial MAO assay system.

## RESULTS

The incubation of modaline sulfate with NADPH and the 9000-*g* supernatant (containing the liver microsomes) results in the formation of an anti-MAO intermediate within the first 5 min. Maximal formation of the active substance appears to occur under these conditions at about 10–15 min of incubation, and further exposure of the compound to the microsomal fraction results in a decrease in the anti-MAO activity in the supernatant fraction. As indicated in Fig. 1, prolonging the incubation

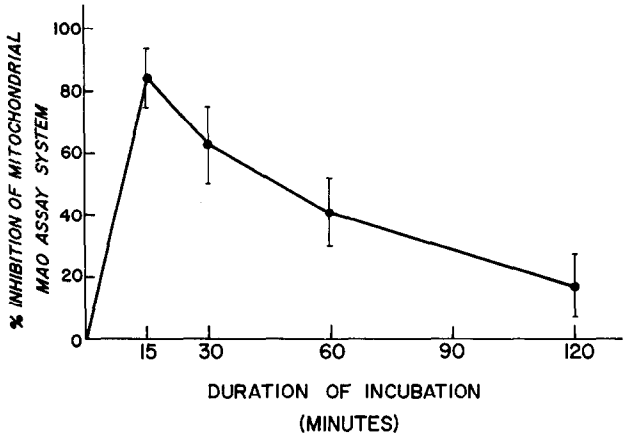


FIG. 1. The rate of formation and inactivation of the active anti-MAO intermediate upon incubation of rat liver microsomes, NADPH, and modaline sulfate. Aliquots of this mixture were added to rat liver mitochondria which acted as the source of the MAO assay system. Each point represents the mean value of per cent MAO inhibition from 8 experiments. Vertical lines indicate the standard deviations of the means in all figures.

period to 120 min decreases the amount of active inhibitor to approximately 20 per cent or less of the peak concentration. The fall in peak anti-MAO activity beyond the first 15-min incubation period was clearly evident, but the mechanism by which this phenomenon occurred was not known. In order to determine whether the fall in anti-MAO activity was a spontaneous destruction of the active intermediate or whether it was exerted by enzymatic processes, experiments were undertaken to study the nature of this inactivation process. Since the microsomal enzymes of liver represented

a possible mechanism for the inactivation, several of the subsequent procedures involved separation of the microsomal from the soluble fractions. This was done in order to avoid possible errors due to microsomal activity that might occur when this fraction was added to the mitochondrial MAO assay system.

One such experiment consisted of first preincubating the modaline sulfate with the 9000-*g* supernatant of liver homogenate and NADPH to produce an optimal yield of the active intermediate. A part of this preparation was then centrifuged at 100,000 *g* to separate the soluble fraction from the microsomal phase. The supernatant containing the active intermediate was then further incubated at 37° and at the various intervals aliquots were assayed on mitochondrial MAO to determine anti-MAO activity. A second part of the activated intermediate fraction (containing the activated inhibitor, NADPH, and the 9000-*g* supernatant) was also incubated for the same time period and also assayed for anti-MAO activity. As indicated in Table 1, it was quite apparent

TABLE 1. THE RATE OF INACTIVATION OF THE ACTIVE INTERMEDIATE OF MODALINE SULFATE IN THE 9000-*g* SUPERNATANT OR THE SOLUBLE FRACTION (100,000 *g*) OF RAT LIVER HOMOGENATES*

| Time of incubation (min) | Per cent inhibition ± S.D. of MAO system after incubation of active intermediate in: | |
|---|---|---|
| | Soluble fraction | 9000-*g* supernatant |
| 0 | 92 ± 4 (6) | 80 ± 5 (5) |
| 30 | 86 ± 4 (5) | 72 (2) |
| 60 | 75 ± 6 (7) | 22 ± 3 (4) |
| 90 | 65 (2) | 18 ± 3 (4) |
| 120 | 68 ± 3 (4) | |

* Modaline sulfate was converted to the active anti-MAO intermediate by incubating it with the 9000-*g* supernatant of rat liver homogenate with NADPH ($10^{-4}$ M) for 15 min. Half of this mixture (A) was centrifuged at 100,000 *g* for 60 min to separate the soluble from the microsomal fraction. The other half (B) was placed in an ice bath for 60 min. At the end of this time, both mixtures were again incubated at 37° for the designated times and their aliquots assayed for residual anti-MAO activity. Figures in parentheses indicate number of experiments.

that the decrease in anti-MAO activity with prolonged incubation was greatly enhanced in the fraction containing the 9000-*g* supernatant, although the fraction containing only the soluble phase also demonstrated a progressive loss in enzyme inhibitory activity. This would suggest that the active intermediate of modaline sulfate is susceptible to degradation by the microsomal fraction of liver and also that it is somewhat unstable even in the absence of the microsomes. Subsequent studies indicate that the deterioriation of anti-MAO activity in the soluble fraction is a spontaneous but oxygen-dependent phenomenon, since it occurred even after boiling the incubation mixture, but not under a nitrogen atmosphere.

Having found the indications that the liver microsomes were involved not only in the activation but also in the inactivation of the active intermediate of modaline sulfate, we proceeded with several other experiments to confirm these findings and to characterize

the nature of this inactivation process. Figure 2 shows the effectiveness of the compound SKF-525A (3-diethylaminoethyl-diphenylpropylacetate)($10^{-4}$ M) in antagonizing the rate of loss of anti-MAO activity when it is added to the incubation mixture after the peak concentration of the activated intermediate had been formed. Since SKF-525A is a well-known inhibitor of the microsomal enzymes, this would be

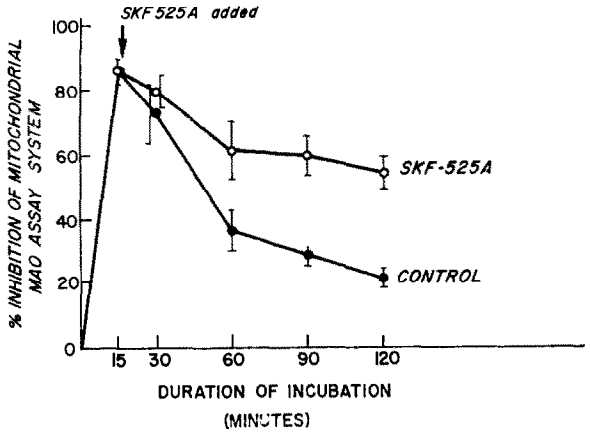


FIG. 2. The effects of SKF-525A ($10^{-4}$ M) on the rate of inactivation of the anti-MAO property of modaline sulfate *in vitro*. SKF-525A was added at the arrow in the experimental mixtures; in the control mixtures it was added just prior to transference of an aliquot to the mitochondrial MAO assay system at the times designated. The latter procedure was employed to assure that the SKF-525A per se was not interfering with the assay system. Each point represents the mean per cent inhibition of the mitochondrial MAO assay system from 3 to 5 experiments.

expected if the microsomes were involved in the degradation of the active intermediates. However, even with higher concentrations of SKF-525A ($5 \times 10^{-4}$ M), complete inhibition of the inactivation process was never accomplished, further supporting the presence of a nonmicrosomal pathway of degradation as described above.

An experiment was also carried out to determine whether the rate of activation and inactivation of the active intermediate of modaline was influenced by liver microsomes which had been removed from animals previously treated with phenobarbital. Incubation of such preparations with modaline resulted in a much higher rate of formation of activated inhibitor, as indicated by the smaller volumes of mixture necessary to block the mitochondrial MAO assay system (Fig. 3). Under these conditions, the phenobarbital-pretreated liver microsomes were capable of producing from three to six times as much of the activated inhibitor.

The rate of inactivation of activated modaline was also enhanced by liver microsomes of phenobarbital-pretreated rats. This process, however, was much less affected than the activation step in that the rate of loss of anti-MAO activity was only slighter greater than control preparations (Table 2).

A characteristic of the rat is the sexual dimorphism in the activity of the microsomal enzymes to inactivate various drugs. We attempted to determine the extent of difference in the activation and inactivation of modaline in both male and female rats, but because of the great variation, especially in the female rat, it was not possible to conclude

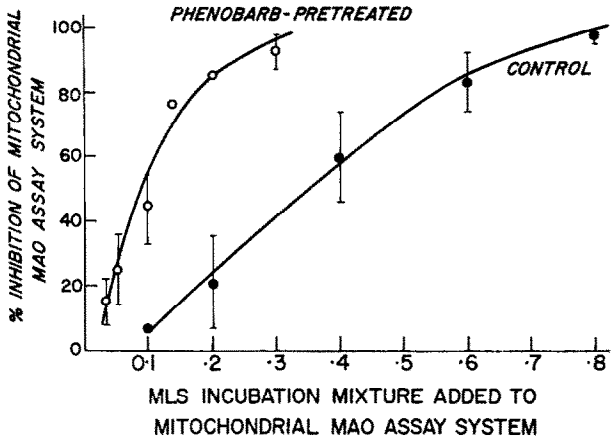


FIG. 3. The effect of phenobarbital pretreatment on the ability of rat liver microsomes to transform modaline to its active anti-MAO intermediate *in vitro*. The graph represents the degree of inhibition of the mitochondrial assay system upon incubation with varying volumes of modaline sulfate activated by the 9000-*g* supernatant of liver homogenates from control and phenobarbital-pretreated rats. Each point represents the mean value from 2 to 5 experiments.

TABLE 2. INACTIVATION *IN VITRO* OF THE ANTI-MAO INTERMEDIATE OF MODALINE UPON EXPOSURE TO MICROSOMES FROM NORMAL AND PHENOBARBITAL-PRETREATED RATS*

| Time of incubation (min) | Per cent inhibition ± S.D. of MAO system after incubation of activated intermediate in liver microsomes obtained from: | |
|---|---|---|
| | Controls | Phenobarbital-pretreated |
| 0 | 92 ± 2 (5) | 91 ± 5 (5) |
| 30 | 79 ± 3 (5) | 63 ± 8 (4) |
| 60 | 64 ± 3 (5) | 50 ± 10 (4) |
| 120 | 37 ± 4 (5) | 21 ± 7 (5) |

* Modaline sulfate was converted to the active anti-MAO intermediate by incubating it with the 9000-*g* supernatant of rat liver homogenate with NADPH ($10^{-4}$ M) for 15 min. From this mixture the soluble fraction containing the active intermediate was obtained and added to tubes containing liver microsomes prepared from control and phenobarbital-pretreated rats. These mixtures were incubated for the times designated and their aliquots assayed for residual anti-MAO activity. The figures in parentheses indicate the number of experiments.

whether a definite difference existed. However, the trend indicated that the rate of activation of modaline to an active inhibitor of MAO was lower in the female, whereas the rate of inactivation of the anti-MAO activity by female liver microsomes did not seem to differ significantly from that of the male.

Several experiments were also made *in vivo* in order to correlate the observations *in vitro* with those seen in the intact animal. The activation of modaline sulfate to an inhibitor of MAO and the antagonism of this activation by SKF-525A have been described previously.[5] However, no observations have been reported of the influence

of phenobarbital pretreatment on the formation of the active substance. In order to establish this phenomenon *in vivo*, we pretreated animals with phenobarbital 18 hr prior to the administration of varying doses of modaline, and the resulting inhibition of MAO in the liver was determined. As in the experiments *in vitro*, the phenobarbital-pretreated animals demonstrated the presence of a far greater amount of the active intermediate (Fig. 4). The difference between the phenobarbital-pretreated and control animals ranged from two to four times as much, depending upon the points on the two curves.

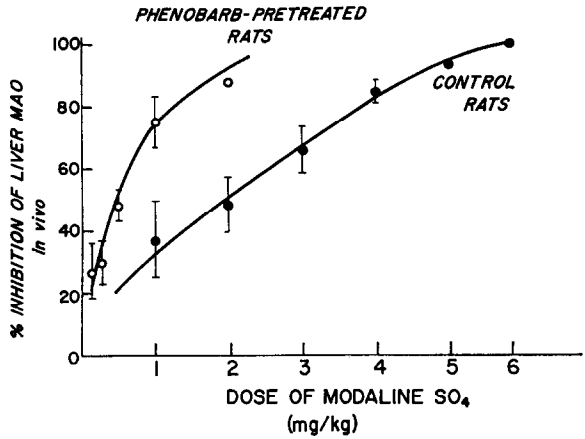


FIG. 4. The effect of phenobarbital pretreatment on the inhibitor activity of modaline sulfate on liver MAO of intact rats. The designated doses of modaline sulfate were injected i.p.; 2 hr later the animals were sacrificed and their livers assayed for the extent of MAO inhibition. Each point represents the mean value of 2 to 5 experiments.

Attempts were also made to determine the extent of degradation or inactivation of the active intermediate *in vivo*. If a large dose of modaline was injected into the male rat, the appearance of a peak concentration of inhibitor activity in the supernatant fraction of liver occurred within a period of minutes; this rapidly decreased within a period of 30 min, and within 2 hr, the active inhibitor in the livers was essentially absent. We attempted to determine the influence of SKF-525A and phenobarbital pretreatment *in vivo* on the degradation of activated modaline, but because of the inability to isolate the active product, this was not feasible.

## DISCUSSION

The present studies have provided evidence that the compound modaline sulfate (W3207B) is an active inhibitor of monoamine oxidase by virtue of its biotransformation to an active intermediate. Furthermore, after activation, this substance undergoes inactivation, most probably by way of the microsomal enzymes. The two processes may not involve identical enzymes, since their sensitivities appear to be quite different with respect to treatment with SKF-525A or the pretreatment with phenobarbital. With the SKF-525A *in vitro* and *in vivo*, it appears that the bioactivation process is much more sensitive than the inactivation process. According to Dubnick *et al.*,[2] a dose of 50 mg SKF-525A/kg prevented completely the inhibitory activity of modaline sulfate. Moreover, whereas the bioactivation process was markedly enhanced by

phenobarbital pretreatment, the degradation of the active intermediate in microsomes prepared from both normal and phenobarbital-pretreated rats showed only slight differences in rate.

The chemical structure of the active intermediate of modaline sulfate is at present unknown. Dubnick *et al.*[2] have synthesized a series of compounds related to modaline but none of these represented the active structure. Although the active intermediate is not available in pure form, it has been possible to determine some of its characteristics as an inhibitor by the method described in this paper. By employing microsomes to biosynthesize *in vitro* the active compound, it may be possible to produce sufficient amounts of the inhibitor substance to permit various physicochemical procedures that might aid in its identification. If such a procedure is to be employed, the use of phenobarbital-pretreated microsomes is recommended, since the bioactivation by this system is much greater than in normal rat microsomes. Furthermore, since it appears that the degradation of the active metabolite is enhanced only slightly by phenobarbital pretreatment, it would represent an ideal method by which to obtain a sufficient amount of intermediate.

The nature of MAO inhibition as exerted by modaline is somewhat reminiscent of the action of pheniprazine. It was reported earlier that pheniprazine produced its anti-MAO activity quite rapidly after exposure to the enzyme, and that the inhibition was irreversible and similar to that produced by iproniazid.[5] However, the presence of the active inhibitor in tissues was short-lived in that it disappeared from tissue fractions within a period of 30–60 min, although MAO inhibition persisted for several days. Modaline sulfate exerts its anti-MAO activity in a rapid fashion, although it requires initial biotransformation. This step appears to be extremely rapid, since minutes after its administration or exposure to liver homogenates containing NADPH, inhibition of the enzyme is observed, and within 5–10 min, it is complete. Upon reaching a peak concentration, the active intermediate gradually decreases in activity. This last step appears to be primarily a function of the liver microsomes *in vitro*. In the intact animal, the rate of removal of inhibitor activity appears to be even more rapid, and preliminary evidence indicates that extra-microsomal mechanisms are also involved in the removal or termination of modaline action *in vivo*.

Modaline sulfate represents the newest of the nonhydrazine types of inhibitor of MAO. It appears to have the anti-MAO property of the hydrazine-type inhibitors but, because of the absence of a hydrazine structure, it possesses fewer of the reactions that have been attributed to hydrazine-type agents. Upon identification of the active intermediate, we should have a new addition to the family of MAO inhibitors, many of which have contributed significantly to an understanding of the enzyme and its possible functions in biological systems.

*Acknowledgements*—The author wishes to acknowledge Mrs. M. Carino for her valuable assistance in these studies. Modaline sulfate (W-3207B) was generously provided by the Warner Lambert Research Institute, Morris Plains, N.J., research affiliate of Warner Chilcott Laboratories.

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