Biochemical Pharmacology, 1964, Vol. 13, pp. 1655–1661. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECT OF MORPHINE ON ^{14}C-LEUCINE INCORPORATION BY RAT LIVER MICROSOMES *IN VITRO*

MILTON RATNER and DORIS H. CLOUET

New York State Research Institute for Neurochemistry and Drug Addiction,
and Columbia University College of Physicians and Surgeons,
New York, N.Y., U.S.A.

(*Received* 3 *June* 1964; *accepted* 6 *July* 1964)

Abstract—The addition of morphine *in vitro* had no effect on the incorporation of ^{14}C-leucine into protein by rat liver microsomal preparations. When morphine was administered in a single dose (30 mg/kg) the response was biphasic: a decreased incorporation *in vitro* at 2 hr after the injection, followed by an increased amino acid incorporation at 5 hr; both effects were due to the changes in activity of the microsomal fraction. During chronic morphine administration at a low dose, an increased amino acid incorporating activity was found at 11 and 15 days, which returned to control level after 30 days of treatment. Similarities have been found between these responses and those produced by the so-called "inducer" drugs.

THE administration of certain "inducer" drugs into animals results in an increase in the activity of some drug-metabolizing enzymes localized in the liver microsomal fraction.[1,2] A concomitant increase in the protein synthetic activity of liver microsomes has been found after the administration of the inducer drugs: 3-methylcholanthrene,[3] phenobarbital,[4] and aminofluorene.[5] On the other hand, the administration of narcotic drugs such as morphine produces a decrease in the activity of some of the same drug-metabolizing enzymes in liver microsomes and a decrease in liver wet weight and the level of protein in the microsomal–supernatant fraction of liver. After a single injection of morphine the protein level recovers only after 60 h, as does the activity of meperidine N-demethylase and the hexobarbital-metabolizing enzymes.[6] During the chronic administration of morphine, liver size, microsomal–supernatant protein (calculated per gram liver wet weight), and demethylase activity all remain depressed.[7] In this paper we describe the response to the administration of morphine of the protein-synthesizing activity in the rat liver microsomal fraction as measured by amino acid incorporation into protein *in vitro*.

EXPERIMENTAL

Materials

DL-Leucine-1-^{14}C (4 mc/mmoles), creatine phosphokinase, and disodium creatine phosphate were obtained from the California Corp. for Biochemical Research, and guanosine-5′-triphosphate (GTP) from the Sigma Chemical Co. The experimental animals were adult male rats of the Sherman strain maintained on a stock diet.

Assay of amino acid incorporation

The rats were killed by decapitation and the liver homogenized in two volumes of 0·25 M sucrose containing 10^{-2} M $MgCl_2$, 10^{-3} M EDTA, and $3{\cdot}5 \times 10^{-2}$ M Tris·HCl buffer, pH 7·6, in a conical glass homogenizer for 1 min. The microsomal–supernatant fraction was obtained by discarding the sediment from the centrifugation of the homogenate at 15,000 *g* for 10 min. The microsomal fraction was sedimented at 105,000 *g* for 1 hr, removed from the soluble fraction, and homogenized in a small volume of fortified sucrose solution. The supernatant soluble fractions were combined for each group of rats similarly treated. The microsomal fractions were not pooled but were assayed separately in duplicate for each rat, except in the experiments testing the effect of morphine added *in vitro*, in which the microsomal fractions from the livers of untreated rats were pooled.

The incubation mixture contained 3 μmoles of reduced glutathione, 0·01 mg creatine phosphokinase (0·16 E.U.), 40 μmoles creatine phosphate, 10 μmoles $MgCl_2$, 2 μmoles ATP, 120 μmoles KCl, 0·4 μmoles GTP, 60 μmoles Tris·HCl buffer (pH 7·6), 40 μmoles potassium phosphate buffer (pH 7·6), 0·03 ml DL-leucine-^{14}C (0·075 μmoles), 0·4 ml of the microsomal preparation (about 10 mg protein), and 0·4 ml of the soluble fraction (about 8 mg protein) in a total volume of 2 ml. The stimulatory effect of high potassium has been described by Sachs.[8]

Incubation was carried out at 37° with shaking for 20 min, when the reaction was stopped by the addition of 2 ml of 10% trichloroacetic acid (TCA). The protein precipitate was washed according to the procedure of Siekevitz[9] and transferred to planchets with the aid of formic acid. The TCA-soluble fraction was also transferred to planchets, and both fractions were counted in a low-background counter. The amount of radioactivity incorporated into the protein fraction was expressed as counts per minute/10 mg microsomal protein/100,000 counts per minute in the TCA-soluble fraction (the average number of counts in this fraction was about 100,000 cpm).

Protein was determined by the method of Lowry *et al.*[10]

RESULTS

Effect of morphine added in vitro

Pooled microsomes from the livers of untreated rats were incubated in the presence or absence of morphine. There was no difference in the amount of ^{14}C-leucine incorporated when morphine was absent or added in a final concentration of 10^{-4} M or 10^{-3} M (Table 1).

TABLE 1. THE EFFECT OF MORPHINE *IN VITRO*

Final concentration of morphine	Incorporation (cpm/10 mg protein/100,000 counts in TCA-soluble fraction) (1)	(2)
None	166 ± 16*	142 ± 19*
10^{-4} M		147 ± 11
10^{-3} M	157 ± 16	137 ± 9

Morphine sulfate was added at zero time in the described concentrations. Pooled microsomal fractions from three rat livers were assayed in triplicate for each experiment.

* Standard deviation.

Effect of a single dose of morphine

In each experiment two rats were injected subcutaneously with 30 mg morphine base/kg at the appropriate time and two served as controls. The microsomal fraction from the liver of each rat was incubated in duplicate assays with pooled soluble fraction from the pair of rats similarly treated and also with the pooled soluble fraction from the other group of rats. The leucine incorporation for each liver microsomal preparation from the morphine-treated rats was compared to that from the control rats of the same experiment. At time points of maximal change two or three experiments were performed.

In Fig. 1 is shown the ratio ($\times 100$) of the incorporation of ^{14}C-leucine by liver microsomes from morphine-treated rats assayed with the soluble fraction from

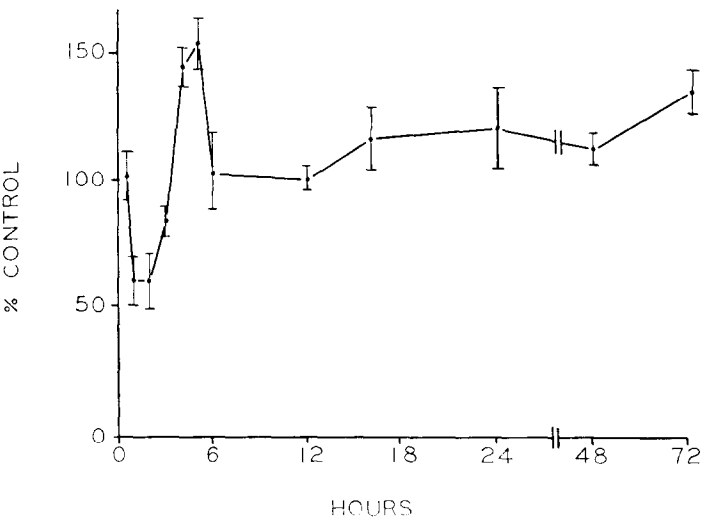

FIG. 1. Effect of a single dose of morphine (30 mg/kg) on the change in ^{14}C-leucine incorporation into rat liver microsomes in the presence of soluble fraction from the same animals. The control values in each case were obtained from animals killed at the same time as the morphine-treated animals. Each point is the average of duplicate determinations on tissue from at least two morphine-treated rats, measured separately, compared to a like number of assays from control rats. At 5, 6, 16, and 48 hr after the injection, the number of assays was 8, and at 2 and 4 hr, the number was 12 (derived from 6 rats killed at the appropriate time after the injection of morphine). The bar indicates the standard deviation at each point.

morphine-treated rats to that for microsomes and soluble fractions from control rats. There was a multiphasic response. In the first 2 hr there was a depression of incorporating activity by almost 50%, followed by an increase in activity of the same magnitude, reaching a peak at 5 hr after the single injection. After a return to the control level at 6 to 12 hr, a slightly increased activity was found from 16 to 72 hr.

The rectal temperature of the morphine-treated rats fell from 39° to a low of 32°, 90 min after the injection. This fall could be prevented by keeping the morphinized rats in a 30° incubator for the first 2 hr after the injection of morphine. When the incorporation by the liver microsomes from injected rats maintained at 30° was compared to that by microsomes from morphine-treated rats whose temperature was

allowed to drop, there was no difference: a 60% decrease with temperature control vs. a 58% decrease without temperature control, 2 hr after the injection.

The same liver microsomal fractions from morphinized rats were assayed in the presence of control soluble fraction, and the liver control microsomal fractions were incubated with the soluble fractions from the morphinized animals (Fig. 2). The

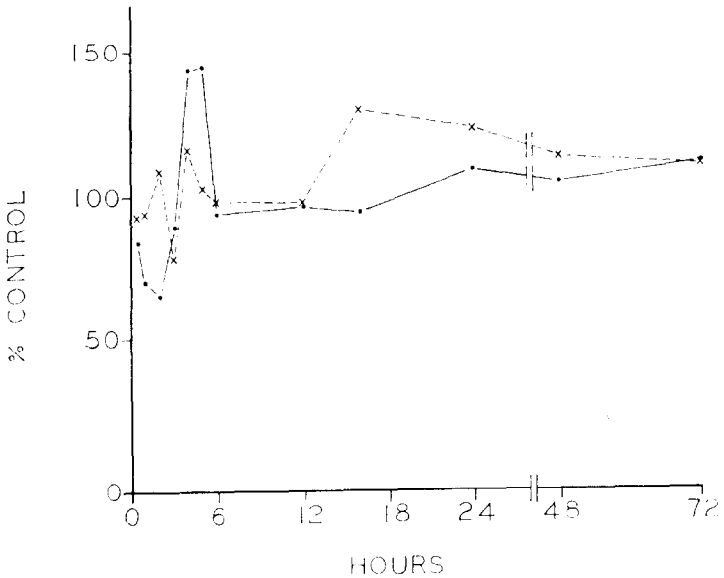

FIG. 2. Effect of morphine on amino acid-incorporating activity of microsomal and supernatant fractions of rat liver. The animals and number of assays are the same as in Fig. 1. The microsomes from the morphinized animals assayed in the presence of control soluble (·————·) and control microsomes assayed in the presence of soluble fraction of livers from morphinized rats (X- - - - -X) are compared to the complete system from untreated rats.

change in amino acid incorporating activity in the first 6 hr after a single injection of morphine was due completely to the lower activity in the microsomal fraction. However, the soluble fraction from rats treated with morphine 16 hr previously had significantly higher activity when combined with the microsomal fraction from control rats than when the soluble fraction from control animals was incubated with the microsomal fractions from the same animals. This increased activity of the soluble fraction probably contributed to the rise at 16 hr in incorporating activity.

In the livers from morphine-treated rats the amount of protein in the fraction, defined as microsomes by centrifugation characteristics, varied significantly from the average control level in an upward direction at 0·5 hr after the injection of morphine, and in a downward direction at 6 and 12 hr after the injection (Fig. 3). The changes in protein were absolute (calculated per gram weight of individual liver) as well as relative, since the liver weights did not vary after a single injection of morphine in the time periods examined. The injection of a larger dose of morphine (60 mg/kg) produced a decrease in the amount of protein in the liver microsomal–supernatant fraction in a very similar fashion (dashed line in Fig. 3 from Ref. 7), except that the recovery to control level of the microsomal protein 16 hr after the injection was not reflected in the entire microsomal–supernatant fraction.

Effect of chronic morphine administration

Morphine was administered in drinking water to a group of 40 rats kept in individual cages so that the liquid consumption could be measured. During an initial 3-day period, both water and a morphine solution were available to the rats. On the third day the water bottles were removed, and the experimental period began. For the first 15 days the concentration of morphine (in solution as morphine sulfate) was 0·1 mg base/ml water. The concentration was doubled in the 16–30 day period. On this regime the rats were completely tolerant to the analgesic effect of an injected dose of 30 mg morphine/kg by the 11th day.

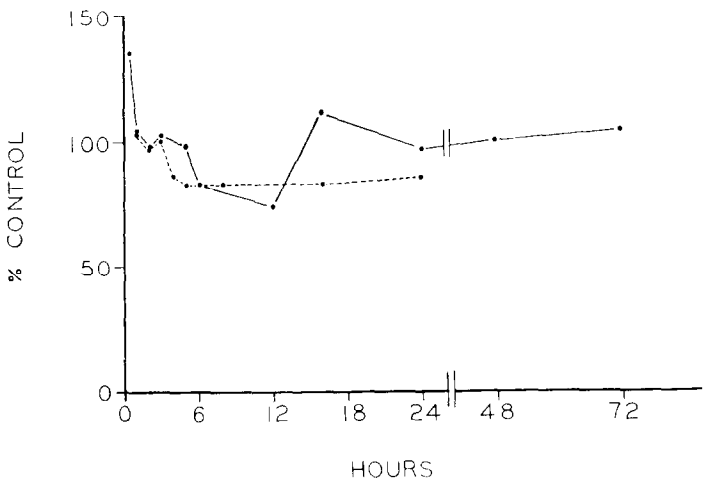

FIG. 3. Effect of morphine on the amount of protein sedimenting as microsomal fraction in the liver of rats after the injection of 30 mg morphine/kg (·————·). The total microsomal protein from each liver is compared to the average value of the microsomal protein from the livers of untreated animals. For comparison, a curve is included (from Ref. 7) of the protein in the combined microsomal–supernatant fraction after an injection of 60 mg morphine/kg (·– – – – –·). The total protein of the combined fractions is compared to control levels found in the same fractions of livers from untreated animals.

The *in-vitro* incorporation of ^{14}C-leucine by liver microsomes from two of these rats after 11 days of drinking morphine solution was double from that a control group killed at the same time and only slightly lower in the livers of two rats killed after 15 days of treatment (Table 2). By 30 days there was no difference in amino acid incorporation between control and morphinized rats. The response to a single injection of 60 mg phenobarbital/kg administered 48 hr before killing the animals was measured in four of the rats treated for 30 days, and also in four of the appropriate unmorphinized rats. The response of the liver microsomal fraction to phenobarbital was greater in the unmorphinized than in the morphinized rats. However, the response to phenobarbital in the livers from morphinized rats was a significant ($P < 0{\cdot}01$) increase in the amino acid-incorporating activity by liver microsomes.

DISCUSSION

The increase in activity of drug-metabolizing enzymes in liver microsomal fractions by "inducer" drugs and the depression in activity of some of the same enzymes by

morphine and other narcotic drugs may be more closely related than is at first apparent. Both phenomena involve drug-metabolizing enzymes localized in the smooth endoplasmic reticulum[6,11] and include a multiphasic effect: an initial depression of enzyme activity followed by an increase above the normal level. After a single injection of phenobarbital, the decrease in hexobarbital oxidation *in vitro* by microsomal enzymes

TABLE 2. AMINO ACID INCORPORATION AFTER CHRONIC MORPHINE ADMINISTRATION

Morphine-treated			Control
Days of treatment	(mg/kg/day)	Incorporation (cpm/10 mg protein/100,000 cpm in TCA-soluble fraction)	
11	11·5	266 ± 21* (2)	132 ± 8* (2)
15	12·4	254 ± 17 (2)	158 ± 16 (2)
30	23·5	146 ± 6 (4)	138 ± 11 (2)
		Plus 60 mg phenobarbital/kg	
30	22·3	196 ± 18 (4)	243 ± 13 (2)

The microsomal fraction from each rat was examined individually in duplicate. The number of rats is given in parentheses. Morphine was administered in drinking water at a level of 0·1 mg/ml for 15 days and 0·2 mg/ml from 16 to 30 days. Phenobarbital was injected subcutaneously 48 hr before the rats were killed.

* Standard deviation.

is maximal 2 to 4 hr after the injection, and the induction is maximal at 48 hr.[12] After a single injection of morphine, the maximal depression of meperidine N-demethylase activity occurred 8 hr after the injection and the increase above normal at 60 hr.[6] The action of the drug-metabolizing enzyme inhibitor, SKF 525A, is similarly biphasic, which led Remmer[12] to suggest that the biphasic response is peculiar to all such "activating" drugs.

Evidence is accumulating which suggests that the drug-induction phenomenon in the liver microsomal fraction includes the synthesis of new protein. In addition to the evidence of increased synthetic activity after the administration of 3-methylcholanthrene or phenobarbital, an inhibition of the effect has been found with ethionine[13] and puromycin,[14] and the incorporation of labeled amino acid into protein *in vitro* was increased when RNA isolated from the livers of induced rats was included in the incubation medium.[15] The response of the amino acid-incorporating system in liver to the administration of morphine described in the present paper is another point of similarity of the inducer drug and narcotic drug effects, since the same biphasic response of the amino acid-incorporation system has been described after aminofluorene.[5]

The initial biochemical lesion produced by the administration of the drug is most likely to involve steps prior to the final synthesis of the protein molecule on the ribosome, in view of the stimulatory effect of isolated messenger RNA from induced rats.[15]

The parallelism between the effects of inducer drugs and narcotic drugs after a single injection of the drug does not continue on chronic administration of the drug since,

with inducer drugs, the response to chronic treatment is an increase of drug-metabolizing enzyme activity and, with narcotic drugs, a decrease in the activity of the enzymes. This, too, may be a matter of timing, since a new dose of narcotic drug may be injected during the period when the enzyme levels are depressed, whereas the new dose of an inducer drug may be introduced after the induction has begun. This question must be explored further. Pertinent is the finding reported here that the stimulation of amino acid-incorporating activity of the liver microsomes produced by a low dose of morphine in drinking water is diminished after 30 days of treatment, even though the administration of phenobarbital still produces an increase in protein synthesis.

REFERENCES

1. H. Remmer, *Naturwissenschaften* **45**, 189 (1958).
2. A. H. Conney and J. J. Burns, *Nature (Lond.)* **184**, 363 (1959).
3. A. von der Decken and T. Hultin, *Arch. Biochem.* **90**, 201 (1960).
4. H. V. Gelboin and L. Sokoloff, *Science* **134**, 611 (1961).
5. E. Arrhenius and T. Hultin, *Cancer Res.* **22**, 823 (1962).
6. D. H. Clouet, *J. Pharmacol. exp. Ther.* **144**, 354 (1964).
7. D. H. Clouet and M. Ratner, *J. Pharmacol. exp. Ther.* **144**, 362 (1964).
8. H. Sachs, *J. biol. Chem.* **228**, 23 (1957).
9. P. Siekevitz, *J. biol. Chem.* **195**, 549 (1952).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
11. J. R. Fouts, *Biochem. biophys. Res. Commun.* **6**, 373 (1961).
12. H. Remmer in *Proceedings*, First Int. Pharmacol. Meetings, B. B. Brodie and E. G. Erdös, Eds., **6**, 235 (1962).
13. A. H. Conney and J. J. Burns, *Ann. N.Y. Acad. Sci.* **86**, 167 (1960).
14. A. H. Conney and A. G. Gilman, *J. biol. Chem.* **238**, 3682 (1963).
15. L. A. Loeb and H. V. Gelboin, *Nature (Lond.)* **199**, 809 (1963).