activity into the chloroform and pH 1 ether extracts between neomycin preparation-treated and untreated rats would seem to indicate that the other metabolites which are extracted under these conditions are unaffected by the neomycin treatment.

While it cannot be said that the absence of the dihydroxy metabolite from the urine following neomycin treatment is unequivocal proof that intestinal microbes are involved in the transformation of parent compound to α-(3,5-dihydroxycinnamido)isobutyric acid, there does not appear to be an alternative explanation for this observation if we bear in mind the fact that neomycin is not absorbed when orally administered. If we also take into account the earlier observations of Griffiths[2,3] and Smith and Griffiths,[4] and the similar structures which are involved in the transformations, it appears that intestinal microbes are involved in the conversion of *N*-(3,3-dimethylpropynyl)-3,4,5-trimethoxycinnamide to α-(3,5-dihydroxycinnamido)isobutyric acid.

These experiments again show that due care must be exercised in the interpretation of drug metabolism data when *in vivo* systems are used; it would be important that the investigator distinguish between mammalian and microbial metabolites of a given drug, especially since intestinal flora differ in various species and even among individual human beings.

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REFERENCES

1. R. R. SCHELINE, *J. pharm. Sci.* **57,** 2021 (1968).
2. L. A. GRIFFITHS, *Biochem. J.* **113,** 603 (1969).
3. L. A. GRIFFITHS, *Experientia* **26,** 723 (1970).
4. G. E. SMITH and L. A. GRIFFITHS, *Biochem. J.* **118,** 53P (1970).
5. G. J. IKEDA and C. B. ESTEP, *Fedn Proc.* **30,** 391 (1971).
6. J. T. MATSCHINER, T. A. MAHOWALD, W. H. ELLIOTT, E. A. DOISY, JR., S. L. HSIA and E. A. DOISY, *J. biol. Chem.* **225,** 771 (1957).

Biochemical Pharmacology, Vol. 21, pp. 2153–2155. Pergamon Press, 1972. Printed in Great Britain.

Effect of morphine on esterified fatty acids in plasma and brain of the nontolerant, tolerant and abstinent rat

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IN A CONTINUING effort to ascertain the effect of morphine on lipid metabolism,[1,2] an attempt was made to determine the relationship, if any, between the action of morphine and the levels of esterified fatty acids in the plasma and brain of nontolerant, tolerant and abstinent rats. Such an effect might directly implicate fatty acid metabolism mechanistically in the agonistic as well as drug-dependent action of morphine either independently or possibly coupled with the phospholipid effects of this drug.[3–6] The results of this study are reported in this communication.

Nontolerant male Wistar rats weighing between 150 and 260 g were housed in cages in air-conditioned animal quarters and fed a Rockland mouse/rat diet and water *ad libitum*. Ten rats each were sacrificed at 1 and 2 hr by decapitation after the administration of 10 mg/kg (free base) of morphine subcutaneously. The blood was collected in heparinized beakers and the brain rapidly removed and immediately frozen.

Male Wistar rats were made tolerant to morphine by initially administering 20 mg/kg of morphine (free base) subcutaneously at 8 a.m. and 5 p.m. each day. The dose was increased by 20 mg/kg every 3–5 days until a stabilized level of 100 mg/kg twice each day was achieved. The rats were stabilized at this dose for a period of 8–26 days. Five rats, weighing 240–270 g, were each sacrificed at 1 and 2 hr after the 8 a.m. injection of morphine by decapitation.

Five male Wistar tolerant-dependent rats weighing 245–300 g were abruptly withdrawn from morphine at 24, 48 and 120 hr prior to sacrifice.

Ten male nontolerant rats weighing 150–270 g were sacrificed at 1 and 2 hr after a subcutaneous injection of 0·9% saline in a volume equivalent to 10 mg/kg based on a 40 mg/ml solution of morphine.

All food was removed 24 hr before decapitation from all the animals in these experiments with collection of the blood and brains as described for the nontolerant acutely injected morphine rats.

The blood collected (about 5 ml) was centrifuged at 3500 rpm for 20 min. One ml of plasma was extracted with 15 ml of chloroform–methanol (2:1, v/v). The extract was filtered through no. 591 S & S paper. Three-ml aliquots of the filtrate were used for the analysis. The brain after thawing was weighed and homogenized in 25 vol. of chloroform–methanol (2:1, v/v), filtered as described, and 0·5-ml aliquots of the filtrate were used for analysis.

The determination of the esterified fatty acids in the plasma and brain extracts were performed by modifications of the methods of Rapport and Alonzo[7] and Antonis.[8] In order to avoid the problem of maintaining diethyl ether and isopropyl ether free from peroxides and aldehydes, methylene chloride was substituted as the initial solvent for the reaction. The 0·2% ferric perchlorate solution was prepared from the commercially available compound (K & K Labs., Plainview, N.Y.). The procedure consisted of drying under a stream of N_2 at 35° the extracts of plasma and brain. Each residue was dissolved in 1 ml of methylene chloride and 0·5 ml of the hydroxylamine hydrochloride, sodium hydroxide mixture added. After mixing by gently swirling, the tubes were allowed to stand for 30 min and 6 ml of ferric perchlorate solution was added to each tube, stoppered, mixed and allowed to remain in the dark for 30 min. The optical density was determined with a Zeiss PMQ II spectrophotometer at 530 mμ. Internal standards consisting of 0·5 to 1 mg of methyl stearate in chloroform–methanol (2:1, v/v) were treated identically to the unknown samples to provide a continuous check on the efficiency of the assay.

Concentrations of methyl stearate from 0·05 mg to 1 mg in triplicate were carried through the procedure as described. A straight line relationship was obtained when the optical density was plotted against the concentration. All calculations were made from this relationship. The minimal reliable sensitivity for detection was 50 μg.

TABLE 1. EFFECT OF MORPHINE ON ESTERIFIED FATTY ACIDS (EFA) IN PLASMA AND BRAIN OF NONTOLERANT, TOLERANT AND ABSTINENT RATS*

	EFA (mg/g or ml) ± S.E.M.					
	Plasma			Brain		
Physiologic state	No.†	1 hr	2 hr	No.†	1 hr	2 hr
Saline control	10	1·14 ± 0·08	1·19 ± 0·06	10	33·35 ± 0·48	33·08 ± 0·37
Nontolerant	10	1·08 ± 0·04	1·12 ± 0·06	10	33·75 ± 0·27	33·66 ± 0·75
Tolerant	5	1·31 ± 0·11	0·92 ± 0·09‡	5	34·53 ± 0·41	33·95 ± 1·05
Abstinent§	5	1·15 ± 0·08		5	31·36 ± 0·28‖	

* Nontolerant rats received a 10 mg/kg (free base) s.c. injection of morphine. The tolerant rats were stabilized on 100 mg/kg of morphine s.c. twice each day. The abstinent rats were sacrificed at 24, 48 and 120 hr after the last injection of 100 mg/kg s.c. of morphine. At 48 and 120 hr the abstinent values for EFA in the brain were 30·86 ± 0·48‖ and 32·43 ± 1·06 mg/g respectively.

† Number of rats sacrificed at each time interval for each experimental condition.

‡ $P < 0·05$ in comparison to the saline control data at 2 hr in the plasma.

§ The EFA data for plasma and brain was obtained from rats sacrificed 24 hr after the last 100 mg/kg s.c. injection of morphine.

‖ $P < 0·01$ in comparison to all experimental data except the 48 and 120 hr abstinent values in the brain.

Tolerance to morphine in the rat was evaluated by measuring analgesia with a modification of the hot plate method described by Eddy and Leimbach.[9] The data indicated that complete tolerance to 100 mg/kg of morphine (as free base) twice each day was achieved following stabilization at this dosage level for at least 7 days.

The abstinence syndrome in abruptly withdrawn and naloxone antagonized morphine-dependent rats was evaluated by measuring the following: (a) activity; (b) "wet dog shakes"; (c) weight; (d) water consumption; (e) temperature; and (f) respiration in a manner similar to that described by Martin *et al.*[10] A maximal abstinence syndrome was observed at 24 hr and was still quite evident at 72 hr.*

Data were statistically analyzed by the Wilcoxin rank sums test,[11] a two-sample test of significance for unmatched groups.

In Table 1 appears the data concerning the effect of morphine on esterified fatty acids (EFA) in plasma and brain of the nontolerant, tolerant and abstinent rats. In plasma, the primary statistically significant effect was the depressed level of EFA in the tolerant rats (0·92 $\pm$ 0·09 mg/ml) at 2 hr in comparison to the saline control (1·19 $\pm$ 0·06 mg/ml) rats at the same time interval. The values obtained at 1 hr in the tolerant rat were also significantly higher ($P < 0·05$) than the corresponding 2-hr levels of EFA in the plasma of the tolerant rats. In brain, there was practically no effect on the EFA after either acute or chronic administration of the drug. However, during abstinence, a highly significant ($P < 0·01$) depression of the EFA was observed at 24 (31·36 $\pm$ 0·28 mg/g) and 48 (30·86 $\pm$ 0·48 mg/g) hr after abrupt withdrawal of morphine. After 5 days of abstinence, the EFA levels (32·43 $\pm$ 1·06 mg/g) were not significantly different from the control values. The levels of EFA in the brain of the abstinent rats (24 and 48 hr abstinent) were significantly lower than all other physiologic states (saline control, nontolerant and tolerant rats) whether 1 or 2 hr after drug. No effect on EFA was observed in the plasma at 24, 48 or 120 hr after withdrawal of morphine.

The results of this study with regards to the data obtained in brain suggest an inhibitory effect induced by the state of abstinence on acyltransferase.[12] Alternate possibilities, i.e. increased catabolism of EFA and direct effects on the synthesis of fatty acids, appear unlikely because of the absence of an effect of morphine in the brain of the nontolerant and the tolerant-dependent rats. Therefore, it appears that morphine has no significant direct effect on EFA, but induces a secondary effect related to the severe physiological[10]* and biochemical[13] changes that accompany withdrawal from morphine in highly tolerant-dependent animals.

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REFERENCES

1. S. J. Mulé, in *Narcotic Drugs: Biochemical Pharmacology* (Ed. D. H. Clouet), ch. 8, p. 190. Plenum Press, New York (1971).
2. S. J. Mulé, in *N.Y., The Addictive States* (Ed. A. Wikler), Vol. 46, pp. 32–52. Assoc. Res. Nerv. Ment. Dis. (1968).
3. S. J. Mulé, *J. Pharmac. exp. Ther.* **154,** 370 (1966).
4. S. J. Mulé, *J. Pharmac. exp. Ther.* **156,** 92 (1967).
5. S. J. Mulé, *Biochem. Pharmac.* **19,** 581 (1970).
6. E. J. Simon, in *Chemical and Biological Aspects of Drug Dependence* (Ed. S. J. Mulé and H. Brill), Chemical Rubber Company, Cleveland, in press (1972).
7. M. M. Rapport and A. Alonzo, *J. biol. Chem.* **217,** 193 (1955).
8. A. Antonis, *J. Lipid Res.* **1,** 485 (1960).
9. N. B. Eddy and D. Leimbach, *J. Pharmac. exp. Ther.* **107,** 385 (1953).
10. W. R. Martin, A. Wikler, C. G. Eades and F. T. Pescor, *Psychopharmacologia* **4,** 247 (1963).
11. J. V. Bradley, *Distribution Free Statistical Tests*, pp. 105–111. Prentice Hall, Englewood Cliffs, N.J. (1968).
12. W. E. M. Lands, *A. Rev. Biochem.* **34,** 313 (1965).
13. D. H. Clouet (ed.), *Narcotic Drugs: Biochemical Pharmacology*, Plenum Press, New York (1971).