Biochemical Pharmacology, Vol. 18, pp. 2711–2721. Pergamon Press, 1969. Printed in Great Britain

INHIBITION OF MORPHINE TOLERANCE AND PHYSICAL DEPENDENCE DEVELOPMENT AND BRAIN SEROTONIN SYNTHESIS BY CYCLOHEXIMIDE

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(*Received* 7 *February* 1969; *accepted* 9 *May* 1969)

Abstract—Mice were rendered tolerant to and physically dependent on morphine by daily subcutaneous (s.c.) injections of increasing doses of morphine for 3 weeks. The degree of tolerance was determined by measuring the increase in the median analgetic dose of morphine in relation to response to thermal stimulus, and dependence was determined by the precipitation of withdrawal jumping with the antagonist, naloxone. The concomitant daily administration of cycloheximide with morphine prevented the development of tolerance and physical dependence. The increase in brain serotonin turnover, which was noted to accompany development of tolerance and physical dependence, was also blocked. The findings suggest that the three responses to morphine may be closely related.

THE CONCURRENT development of tolerance to and physical dependence on morphine administered repeatedly has led many investigators to believe that a common underlying mechanism operates within the matrix of the central nervous system. It has been proposed that development of tolerance to and physical dependence on a pharmacologic agent are the consequences of drug alterations of the steady state level of the receptor enzyme resulting from repression or derepression of its synthesis.[1,2] Cohen *et al.*[3] reported that treatment of rats and mice with the protein synthesis inhibitor, dactinomycin (actinomycin D), blocks the development of tolerance to morphine. The possibility that the protein involved may be concerned with brain serotonin (5HT) synthesis was suggested by our recent findings indicating that an accelerated rate of 5HT synthesis accompanied the development of tolerance to and physical dependence on morphine.[4] These results have been extended, and the present communication describes studies with cycloheximide (Acti-dione) showing that all three responses to chronic morphinization can be inhibited by cycloheximide.

METHODS AND MATERIALS

Swiss albino mice of both sexes weighing 20–25 g were employed throughout. In the tolerance studies, the mice were randomly divided into four groups, which included at least twenty-four animals per group, for two series of experiments. In each series, two of the four groups were injected with increasing s.c. doses of morphine sulfate three times daily for 3 weeks; the starting dose of morphine was 10 mg/kg and the final dose over the last 2 days was 200 mg/kg every 8 hr. One of the morphine-treated

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groups received, in addition, cycloheximide daily at a dose of 20 mg/kg, i.p. As controls, the third group received only the cycloheximide each day and the fourth group received injections of physiologic saline. The median "analgetic" dose (AD_{50}) of morphine sulfate was determined for each group before and after drug treatment. In the latter instance, the estimation was performed 8 hr after the last dose of each treatment. This interval appeared adequate for elimination of any residual morphine in morphinized animals, which may interfere with AD_{50} estimations, as evidenced by the return of the tail-flick reaction time to thermal stimulus to pre-drug control levels of $1 \pm 0{\cdot}2$ sec (S.E.) In the first series, the morphine AD_{50} for the s.c. route was determined; in the second series, the i.v. AD_{50} was determined. Analgesia was determined by the D'Amour-Smith[5] procedure, using a 1·5-sec prolongation in tail-flick reaction time to thermal stimulus as a quantal response to varying doses of morphine. The Litchfield–Wilcoxon[6] procedure was used to estimate the AD_{50} and the 95 per cent confidence limits. The degree of tolerance to morphine was measured by the increase in the AD_{50} of morphine at the end of the 3-week treatment period.

The presence of physical dependence on morphine in animals receiving repeated morphine injections was established by inducing an abstinence-like syndrome with the antagonist, naloxone.[7] Precipitated abstinence is characterized by defecation, urination, increased motor activity and tremors, occasionally by convulsions, and most distinctly by stereotyped jumping.[7, 8] The jumping response can be used as a criterion for determining the degree of dependence by simply counting the proportion of animals that jump from a circular platform after injecting naloxone.

The effect of cycloheximide on dependence was assessed by comparing the incidence of withdrawal jumping in morphinized mice with and without cycloheximide treatment. Two groups of twenty mice were rendered dependent on morphine by injection with 40 mg/kg of morphine every 8 hr. One of the two groups received, in addition, 20 mg/kg of cycloheximide, i.p. once daily. After five, ten and fourteen injections of morphine, the animals were challenged with naloxone (4 mg/kg, i.p.) 6 hr after giving morphine. The animals were placed on a circular platform in groups of five and the proportion of animals which jumped off within 15 min was recorded.

The brain 5HT turnover was determined according to the method of Tozer *et al.*[9] With this procedure, the conversion of 5HT to 5-hydroxyindole acetic acid (5HIAA) is blocked by the monoamine oxidase inhibitor, pargyline (75 mg/kg, i.p.), and on the assumption that brain 5HT is converted solely to 5HIAA, the rate of 5HT synthesis may be calculated from the initial rise in 5HT level. The brain 5HT was estimated according to the procedure of Bogdanski *et al.*[10] at 30, 60, 90 and 120 min, using four animals per interval. The animals were from the same four groups used in the s.c. AD_{50} determination. An additional set of experiments was performed in mice rendered tolerant to and dependent on morphine by implantation of a pellet of morphine in the manner previously described.[7] A second group received, in addition to the implanted pellet, cycloheximide 40 mg/kg, i.p. twice daily. Estimations for 5HT turnover after pargyline were performed 4 days after implantation.

The rate of decay of morphine was determined in additional animals from the four groups in the s.c. series. Eight hr after the last morphine dose on day 21, each animal was given an i.v. injection of morphine sulfate, 2 mg/kg, containing $0{\cdot}7$ μg of the N-^{14}C-H_3-labeled compound with a specific activity of $65{\cdot}3$ μc/mg. Various organs and tissues were removed at $\frac{1}{2}$, 1, 2 and 4 hr for estimation of morphine content.

A suitable aliquot of 10% homogenate, equivalent to 300–400 mg tissue, was transferred to 50-ml centrifuge tubes containing 0·5 mg of unlabeled morphine carrier, and the morphine was extracted by the procedure of Fujimoto *et al.*[11] Twenty ml *n*-butanol and 1 ml of 16 N KOH were added and the mixture was shaken for 15 min in a mechanical shaker. After centrifuging for 10 min, a 16–ml aliquot of the organic phase was transferred to a 20-ml scintillation counting vial and evaporated overnight on a Fisher slide-warmer at 55°. The residue was dissolved in 1·0 ml of *n*-amyl alcohol. Ten ml of a scintillation solution, containing 6 g of 2, 5-diphenyloxazole and 200 mg of 1, 4-*bis*-2-(5-phenyloxozolyl)-benzene in 2000 ml of analytical grade toluene, was added and the radioactivity was determined in a Packard model 3375 series liquid scintillation counter. The samples were counted for 3 × 10 min. Background and control determinations with known amounts of labeled morphine were run concurrently to serve as a check on the technique and performance of the counter. Thin-layer chromatograms of the solvent extract of tissue revealed only one radioactive spot, indicating that the extraction procedure limits the removal of any radioactive material other than labeled morphine.

RESULTS

Effect of cycloheximide on body weight and mortality. The effects on body weight of one series of experiments are summarized in Fig. 1. A slight gain in weight was recorded in mice given repeated injections of either saline or cycloheximide over a 2-week observation period, while those animals receiving morphine or morphine plus cycloheximide showed a significant loss in body weight. Of the latter two groups, the one treated with the combined drugs exhibited a greater rate of decline for the initial 7 days, after which the mean body weight of both groups was about the same.

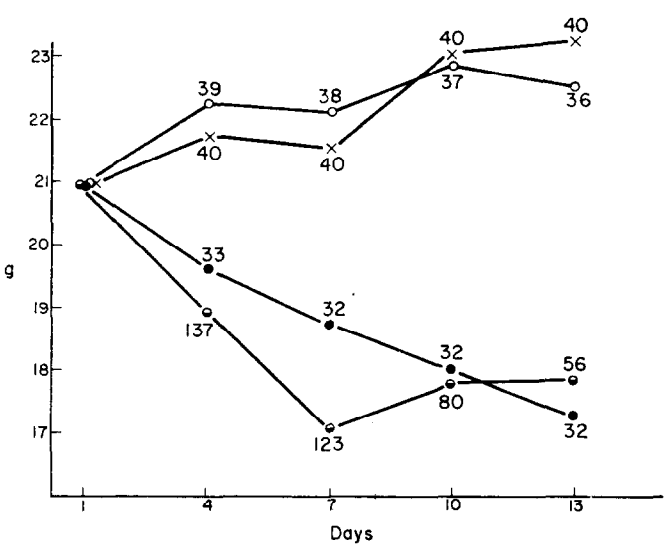

FIG. 1. Mean body weight of mice treated daily with: saline (X); cycloheximide (open circles); morphine (black circles); and morphine and cycloheximide (semi-black circles). The numerals at each observation point denote the number of animals.

However, there were a considerable number of deaths in the group receiving the combined medication and the animals with the lowest weights generally were the ones that failed to survive.

The cumulative mortality rate of the same series of animals is shown in Fig. 2. At the end of 2 weeks, the mortality was less than 10 per cent in the group of mice which received cycloheximide or morphine alone, but in the cycloheximide plus morphine group a synergistic toxic effect was noted. After 1 week of combined treatment,

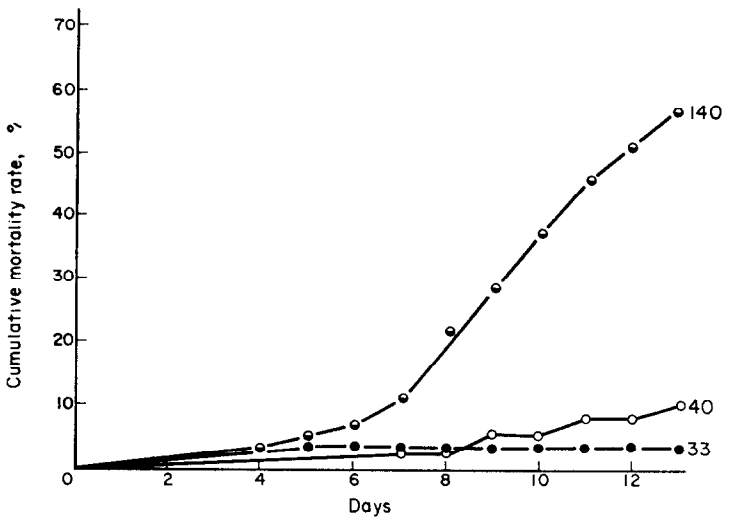

FIG. 2. Cumulative mortality of mice treated daily with: cycloheximide (open circles); morphine (black circles); and morphine and cycloheximide (hemi-black circles). The number of animals used in each group at the beginning of the experiment is identified with each curve.

the animals gradually developed increasing cachexia and the mortality rate of this group started to climb sharply. By day 13, the cumulative mortality was 60 per cent and after 21 days, it was 85 per cent. The last value is not shown in the figure, since the records were lost on a few animals in the other groups. However, the mortality for the saline group at the end of 3 weeks was less than 10 per cent and was between 15 and 30 per cent for the groups of animals on either morphine or cycloheximide. These findings are in essential agreement with two other series of experiments.

Cycloheximide and tolerance development. After 3 weeks of chronic morphine administration in mice there was nearly an 8-fold increase in tolerance development to morphine as judged by the elevation in the tail-flick i.v. AD_{50} of morphine from 0·23 mg/kg before treatment to 1·60 mg/kg after treatment. The concurrent administration of cycloheximide with morphine largely inhibited tolerance development; the change in AD_{50} of morphine before (0·20 mg/kg) and after (0·17 mg/kg) chronic morphinization was not significantly different. While these animals had lost considerable body weight, their tail-flick response to thermal stimulus was not impaired. The control reaction times of all four experimental groups determined prior to administering morphine did

not differ significantly from one another and were the same as those noted when the experiments were initiated (i.e. 1·0 ± 0·2 sec). No significant change in the AD_{50} of morphine was noted in animals treated with saline or cycloheximide for 3 weeks. While the AD_{50} of the cycloheximide group appeared to be lower than that of the saline group, their confidence limits overlapped. The AD_{50} values for the various treatments are shown in Fig. 3 and their slope functions, 95 per cent confidence limits, and potency ratios appear in Table 1.

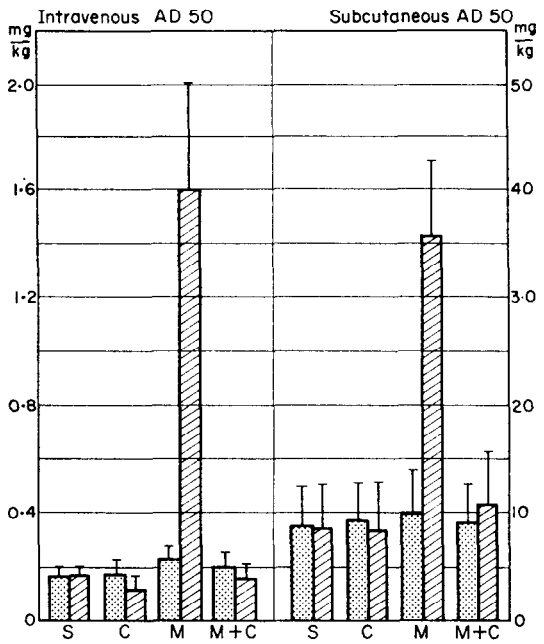

FIG. 3. The analgetic response to morphine before and after repeated daily treatment for 21 days with: saline (S); cycloheximide (C); morphine (M); and morphine and cycloheximide (M + C). The brackets denote the S.E.

The results with the s.c. AD_{50} experiments were in essential agreement with those of the i.v. experiments. The s.c. AD_{50} for morphine sulfate, before any drug was given, was found to be about 10 mg per kg for all groups. After 3 weeks of morphine, the morphine, the AD_{50} in the morphinized group was found to increase 4-fold (37 mg/kg), but no significant change in the AD_{50} was noted in the morphinized group which received cycloheximide concomitantly (8·4 mg/kg). Likewise, the AD_{50} of morphine in the saline and the cycloheximide control groups was also not affected significantly.

In a separate experiment carried out over a shorter period, cycloheximide effected a blockade of tolerance development to morphine without producing overt toxic effects. Over a 5-day interval, daily injections of morphine (40 mg/kg, s.c.) resulted in an increase in the s.c. AD_{50} of morphine to 19 mg/kg but with concurrent cycloheximide treatment the AD_{50} of morphine was 12 mg/kg, which was virtually identical with the value (10 ± 2 mg/kg) noted repeatedly in normal animals. The mean body weight of

TABLE 1. EFFECT OF FOUR MODES OF CHRONIC MEDICATION ON THE ANALGETIC POTENCY OF MORPHINE*

Treatment	Day 1		Day 21			
	Slope function	AD_{50} (mg/kg)	Slope function	AD_{50} (mg/kg)	Slope ratio	Potency ratio
Intravenous saline	1·91 (1·18–3·09)	0·17 (0·12–0·25)	1·85 (1·08–3·16)	0·17 (0·12–0·24)	1·03 (0·50–2·12)	1·00 (0·58–1·70)
Morphine	1·93 (1·27–2·91)	0·23 (0·16–0·32)	2·11 (0·88–5·06)	1·60 (0·95–2·67)	1·09 (0·40–2·96)	6·95 (3·75–12·8)
Morphine and cycloheximide	2·26 (1·19–4·29)	0·20 (0·13–0·30)	1·58 (1·23–2·02)	0·17 (0·12–0·23)	1·43 (0·71–2·86)	0·85 (0·51–1·42)
Cycloheximide	2·26 (1·19–4·29)	0·18 (0·12–0·27)	2·11 (1·16–3·84)	0·12 (0·08–0·18)	1·07 (0·44–2·58)	0·65 (0·36–1·16)
Subcutaneous saline	2·52 (1·10–5·74)	8·4 (5·31–13·3)	2·06 (1·03–13·7)	8·3 (5·03–13·7)	1·22 (0·45–3·27)	0·98 (0·50–1·93)
Morphine	1·93 (1·16–3·20)	9·7 (6·42–14·7)	1·81 (1·13–2·89)	36·5 (26·1–51·1)	1·06 (0·53–2·10)	3·76 (2·27–6·39)
Morphine and cycloheximide	1·92 (1·17–3·15)	9·0 (6·04–13·4)	2·13 (1·16–3·89)	11·0 (7·19–16·8)	1·10 (0·50–2·38)	1·22 (0·67–2·19)
Cycloheximide	1·97 (1·18–3·09)	9·2 (6·21–13·6)	2·07 (1·05–4·06)	8·4 (5·09–13·9)	1·08 (0·47–2·45)	0·91 (0·48–1·72)

*The potency ratio is expressed by the median analgetic dose (AD_{50}) of morphine after and before repeated medication for 21 days. The figures in parentheses denote the 95 per cent confidence limits.

the group receiving the combined treatment on the first and last experimental day showed a decrease from 23·1 ± 0·16 (S.E.) to 20·3 ± 0·30 g but this loss was not significantly different from the mean body weight of animals on morphine alone, which also decreased from 23·4 ± 0·16 g to 21·0 ± 0·38 g. At the end of 5 days, both groups appeared relatively healthy and it was not possible to make any distinctions in their physical condition.

Cycloheximide and development of physical dependence. In twenty animals receiving 40 mg/kg of morphine every 8 hr, the degree of physical dependence was found to be directly dependent on the number of morphine injections. As early as 40 hr (that is, after five injections of morphine), physical dependence on morphine was detectable as evidenced by the fact that 30 per cent of the mice exhibited withdrawal jumping in response to naloxone challenge (Table 2). After ten injections of morphine, the response

TABLE 2. EFFECT OF CYCLOHEXIMIDE ON THE INCIDENCE OF NALOXONE-PRECIPITATED WITHDRAWAL JUMPING IN MICE RENDERED DEPENDENT BY INJECTIONS OF 40 mg/ MORPHINE SULFATE EVERY 8 hr

No. of morphine injections	No. jumping/No. tested	
	Morphine only	Morphine and cycloheximide
5	6/20	0/20
10	11/20	0/20
14	16/20	0/20

to naloxone was 55 per cent and after fourteen injections (112 hr), 80 per cent. On the other hand, in twenty morphine-injected animals receiving, in addition, 20 mg/kg of cycloheximide once daily, none jumped when injected with naloxone at any of the three time intervals tested. Before challenge with naloxone, animals appeared healthy and there were no deaths. The body weight of these animals was not significantly different from that of the group receiving morphine alone.

Cycloheximide and brain 5HT synthesis. The brain 5HT levels at fixed time intervals in mice treated with saline, morphine, or morphine plus cycloheximide by injection, before and after pargyline, are shown in Fig. 4. The levels of all three groups at zero time, i.e. before administering pargyline, were nearly the same, at about 0·7 μg/g. However, at 30 and 60 min after pargyline, brain 5HT levels in morphine tolerant mice were roughly double those of animals in which development of tolerance to morphine was blocked by cycloheximide administration. After 60 min, the mean 5HT level in all animals tended to level off and in tolerant animals an actual decrease in brain 5HT was noted. Levels of 5HT in cycloheximide-treated animals at 30 and 60 min were identical with those of saline controls.

Similar results were noted in morphine-implanted animals. After four days of implantation, brain 5HT levels after pargyline increased from 0·67 μg/g at zero time to 3·1 μg/g at 30 min, whereas in implanted animals receiving cycloheximide in addition, the brain 5HT rose from 0·80 μg/g to 1·70 μg/g. As was the case with the injected mice, the tolerant implanted animals showed a decrease in 5HT levels after 60 min.

Rate of tissue uptake and decay of morphine. Brain concentrations of morphine at

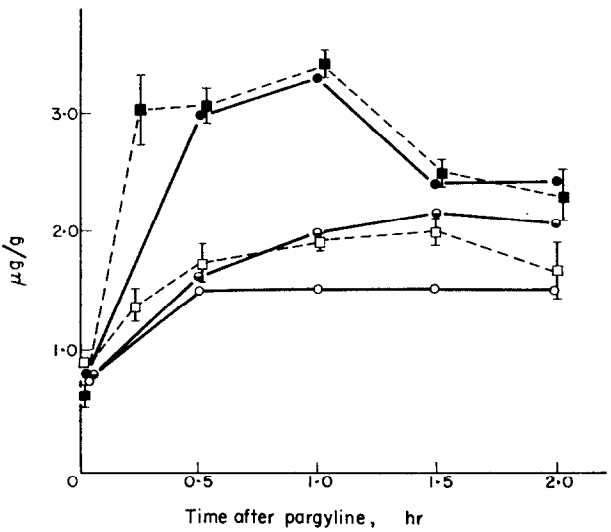

FIG. 4. Brain 5HT levels in mice at various times after pargyline; four animals were used per interval. The solid lines represent the results in mice treated daily by injection for 21 days with: saline (open circles); morphine (black circles); and morphine + cycloheximide (semi-black circles). The broken lines with black squares denote data obtained in mice implanted with a morphine pellet for 4 days; the open squares represent data on animals which received two daily injections of cycloheximide in addition to the pellet. Levels of 5HT at 30 and 60 min in animals receiving cycloheximide only (not shown in figure) were similar to those of saline controls.

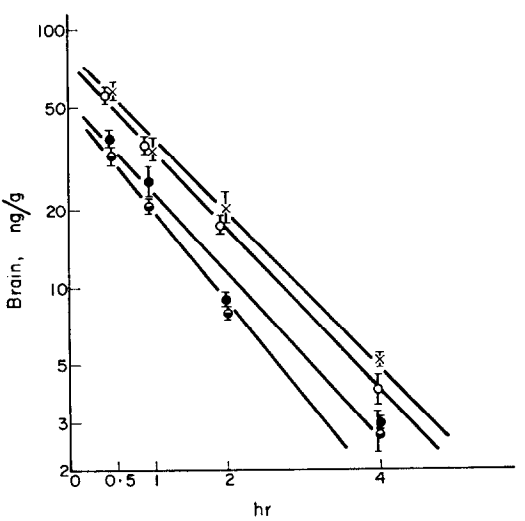

FIG. 5. Rate of decline of morphine in the brain of mice treated daily for 21 days with: saline (X); (open circles); morphine (black circles); and morphine and cycloheximide (semi-black circles). N-$C^{14}H_3$-labeled morphine was given on day 21, 8 hr after the last dose of each treatment. The bars indicate the S.E. of three or four determinations at each time interval.

varying times after administration of the labeled drug for the four treatment procedures are shown in Fig. 5. The saline-treated animals showed the highest levels of morphine at all time intervals. Cycloheximide-treated animals appeared to have slightly lower levels, but the difference was not significant. However, both chronically morphinized groups consistently exhibited lower levels than did two control groups; the concomitant administration of cycloheximide did not seem to alter significantly the brain morphine uptake. The rate of disappearance of the morphine from the brain in all groups appeared to be similar, the mean half-life being approximately 50 min.

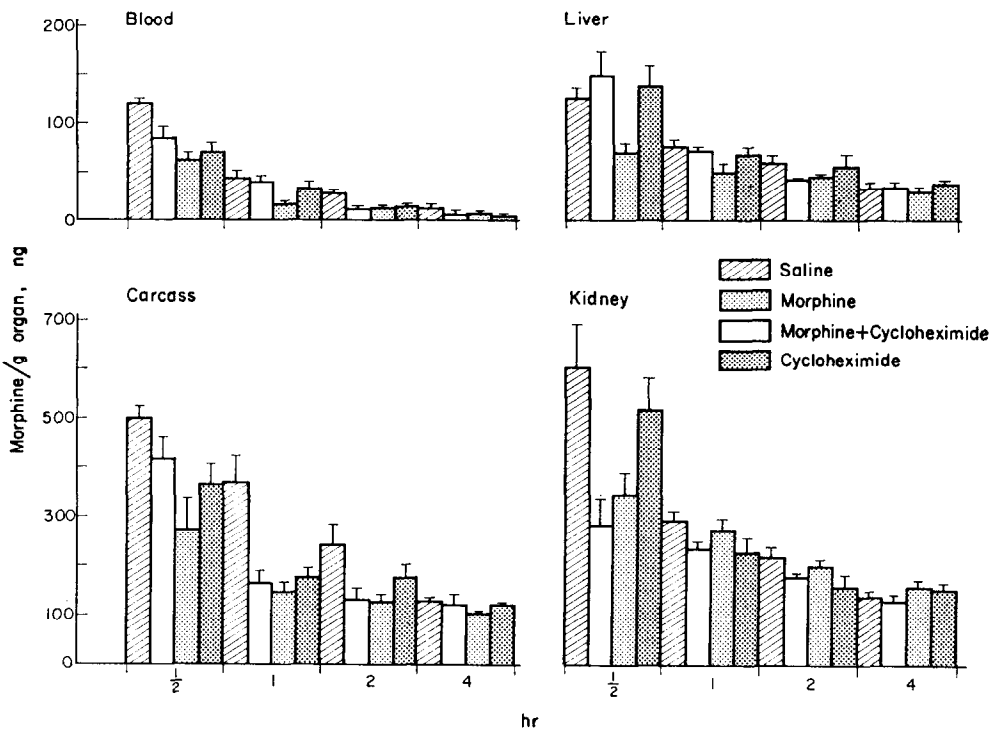

FIG. 6. Mean tissue and organ levels of morphine in mice subjected to repeated daily treatment with: saline; cycloheximide; morphine; and morphine plus cycloheximide. Radioactive morphine was given on day 21, 8 hr after the last dose of each treatment. The bars, indicate the S.E. of three or four determinations at each time interval.

The decay of morphine in the blood and carcass (total body excluding brain, blood, liver and kidneys) of the four experimental groups generally paralleled the levels found in the brain. Again, nonmorphinized animals had higher concentrations than chronically morphinized ones and cycloheximide did not appear to affect the rate of disappearance of morphine from the carcass in morphinized animals (Fig. 6). Kidney levels of morphine were appreciably higher in nonmorphinized than in morphinized animals at 30 min, but thereafter there was little difference among the four experimental groups. Liver levels of morphine were lower in the morphine plus cycloheximide group at ½ hr and perhaps at 1 hr, but not at 2 and 4 hr.

DISCUSSION

The present studies confirm our previous findings that tolerance to and physical dependence on morphine can be produced in the mouse,[7] and indicate clearly that cycloheximide can inhibit the onset and intensity of the two syndromes and the increased synthesis of 5HT in the brain as well. These effects of cycloheximide were elicited at a dose that was $\frac{1}{2}$ its reported LD_{50}.[12] This dose produced no acute overt pharmacologic effects and, when repeated, no toxic manifestations were apparent for 1 week. However, the cumulative mortality of chronically morphinized animals was enhanced considerably by cycloheximide treatment, particularly after the seventh day of injection. Since the mortality in the two groups receiving either drug alone was much less, the increased cumulative toxicity of combining the two agents could only have resulted from drug interaction. It would be difficult to state at this time whether these effects on morphine sensitivity were direct or indirect. Despite the poor condition of these animals, their tail-flick reaction time to thermal stimulus appeared normal. Moreover, in shorter term experiments, when toxic effects had not become apparent, development of tolerance to and physical dependence on morphine was inhibited by cycloheximide.

In view of the known inhibitory effects of cycloheximide on the synthesis of protein, it is only reasonable to infer that such a mechanism might be operating in tolerance or physical dependence development or in both. However, agents which inhibit the synthesis of proteins are likely to have many other effects, and the selectivity of such responses needs to be defined. Dactinomycin has been reported to inhibit tolerance development to repeated morphine administration and the response has been interpreted to occur by protein synthesis inhibition resulting from suppression of new RNA synthesis,[3] but our unpublished studies suggest that other mechanisms might also be involved. We have found that dactinomycin may alter brain permeability to morphine and facilitate its access into the CNS. The mechanism of inhibition of tolerance development to morphine by dactinomycin, therefore, might also be attributed in part to enhancement of sensitivity to morphine and this might have greater consequence. It has also recently been reported that dactinomycin inhibits the development of acute tolerance to morphine and, since the action was manifested within 2 hr after dactinomycin administration without affecting urinary nitrogen excretion, it appears unlikely that inhibition of protein synthesis was the prime mechanism.[13]

The prevention of tolerance and dependence development to morphine by cycloheximide, however, appears not to be primarily related to factors of physiologic disposition governing the organ distribution and biotransformation of the drug. The brain uptake of morphine in morphinized animals was the same in tolerant animals and in those rendered nontolerant by cycloheximide administration. Moreover, the decay of morphine in other body organs and tissues of the two groups was also quite similar. The cause for the lower levels of labeled morphine in morphinized animals was not established. If more extensive biotransformation of the drug is involved, the metabolic routes must be other than glucuronide conjugation or *N*-demethylation, since there is abundant evidence indicating that both processes are generally depressed as tolerance develops after chronic morphinization.[14, 15]

The reduction of tolerance and dependence development to morphine by cycloheximide suggests that changes in the pattern of protein synthesis have occurred, but these alterations do not appear to be requisite for analgetic effects, since the AD_{50} of

both the morphine–cycloheximide-treated group and the cycloheximide controls appeared to be nearly the same as that of the saline controls. This suggests that the protein concerned with tolerance and dependence development is not the morphine-receptor protein itself, but some other protein or macromolecule that has a more rapid turnover rate. Such reasoning would be analogous to that invoked to explain the selective antitumor effect of certain antibiotic agents on rapidly dividing malignant tissue.

If selective inhibition of a protein is involved, the task of selecting the particular protein is formidable. Alteration in the activity of demethylating enzyme has been proposed,[14] but numerous arguments have been advanced to challenge this concept.[15] Cycloheximide has been reported to inhibit protein synthesis by preventing the transfer of activated amino acids from s-RNA to the nascent polypeptide chain,[16] blocking the read-out process and preventing polysome breakdown during protein synthesis.[17] At the current state of our knowledge, it would be difficult to relate these effects to morphine tolerance and dependence mechanisms, but our present findings on cycloheximide inhibition of increased synthesis of brain 5HT may have greater relevance. Structurally, the chemical models of morphine and 5HT appear to be highly complementary and the fact that *p*-chlorophenylalanine, an agent which has been reported to inhibit specifically brain 5HT synthesis,[18] prevents to a large measure the development of tolerance and dependence to morphine suggests that a protein involved with 5HT synthesis should be considered.[4] Since the rate-limiting step in 5HT synthesis is hydroxylation,[19] the role of tryptophane hydroxylase should be assessed. Our current studies are designed to explore in greater depth the possible role of increased brain synthesis of 5HT in tolerance and physical dependence mechanisms.

Acknowledgements—This study was supported by grants from the National Institutes of Health, GMS-01839, the NAS-NRC Committee on Problems of Drug Dependence, and the Breon Fund of the School of Medicine.

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