synchronous population. The rate of inactivation at high doses is not particularly unusual and compares favorably with many other organisms not exhibiting such an extensive shoulder at low dose.

Growth and plating of the *D. discoideum* cells on *E. coli* B_{s-1} (more radiation-sensitive than *E. coli* B/r) did not alter the survival curve as it might have if repair enzymes of *E. coli* B/r had been ingested intact into the slime mold cells.

Hemocytometer counts of aerated liquid suspensions after irradiation indicated an absolute cessation of cell division followed by a sharp return to an almost normal rate of division. The duration of the division lag was linear with dose up to at least 120 krad where it was 6.3 hours. This division delay did not depend on whether the slime mold cells were grown on unirradiated or irradiated bacteria (up to 800 krad). Growth of control *D. discoideum* cells occurred normally in liquid suspensions of bacteria given doses of 800 krad, with only a small effect for doses as high as 3.2 Mrads.

Irradiation in the presence of nitrogen rather than air increased the resistance of *D. discoideum* even further, giving a 10 percent survival dose of 500 krad (*14*).

The presence of an extensive low dose shoulder and the well-defined division lag followed by resumed growth are strong indications of a mechanism for repairing sublethal damage (*6, 8, 9, 12*). Dose fractionation experiments further elaborate such mechanisms. During an incubation period of 2.5 or 5 hours between doses (Fig. 1) the cells recover the ability to cope with additional radiation and the shoulder of the survival curve reappears, in the sense that the 5-hour curve superimposes on the 0-hour curve if translated to 100 percent survival and zero dose. This response is typical of dose fractionation experiments with organisms capable of repairing sublethal damage (*6, 8, 9, 12*). The recovery is virtually complete by 5 hours for this dose combination (Fig. 1, inset). No change in survival after a single dose of 200 krad is observed up to 10 hours of incubation. For the fractionated doses, once recovery has reached its maximum at 5 hours, it does not change further up to at least 10 hours. An effect from sensitivity changes due to progression through the cell-division cycle is not observed as has been shown for some other systems (*6, 8*). Other fractionation survival curves done after initial doses of 120 krad and 275 krad were analogous.

The capacity to repair ionizing radiation damage is an important feature of the radiation response of vegetative cells of *D. discoideum*, and a knowledge of the repair processes will be essential in order to understand their metabolic capacities.

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Morphine Tolerance, Physical Dependence, and Synthesis of Brain 5-Hydroxytryptamine

Abstract. *Tolerance and physical dependence development to morphine in mice can be prevented by concomitant administration of cycloheximide. The fact that the rate of synthesis of brain 5-hydroxytryptamine (5HT) increases with tolerance to morphine suggests that the protein involved may be associated with 5HT synthesis. Inhibition of this synthesis with* p*-chlorophenylalanine markedly decreases tolerance and physical dependence development to morphine.*

The development of tolerance with chronic administration of morphine is generally accompanied by the development of physical dependence. Indeed, the intimate relation between physical dependence and tolerance has led many investigators to believe that a common underlying or closely related mechanism operates within the matrix of the central nervous system (*1*). It is important to distinguish between central receptor tolerance, which might be linked to physical dependence and nonrelated mechanisms that prevent access of morphine to the locus of action (*2*).

It has been proposed that tolerance and physical dependence to a pharmacologic agent are the consequences of drug alterations of the steady-state level of the receptor enzyme, and that tolerance and dependence may be the consequence of repression and derepression of enzyme synthesis (*3*). If tolerance to morphine at the central receptor is linked to enzyme synthesis, it should be possible to prevent tolerance development with inhibitors of protein synthesis, and such studies have been reported (*4*). Since the effects of protein inhibitors are widespread, the task remains to select the possible reactions or enzymes that might be involved in interaction with morphine. Alterations in demethylating enzyme activity have been proposed (*5*), but numerous arguments have been invoked to challenge this concept (*2*). As an alternative, it appears logical to consider those reactions or enzymes associated with the biogenic amines in the central nervous system.

The relation of catecholamines to morphine has been extensively studied (*6*) and, to a lesser degree, that of acetylcholine (*7*). While changes in the concentrations of these substances in the brain occur with development of morphine tolerance, the changes have not been uniform or dramatic and do not occur consistently from species to species. The change in amount of 5-hydroxytryptamine (serotonin or 5HT) in the brain after repeated morphinization has been only cursorily examined. Several laboratories have reported that levels of 5HT in the brain remain unchanged after long-term morphinization (*8*). Since such measurements reflect essentially the steady-state level of brain 5HT resulting from equal

rates of synthesis and efflux, a comparison of the rate of brain turnover of 5HT might provide more meaningful data.

Initially, mice were rendered tolerant and dependent to morphine by subcutaneous injection of increasing doses of morphine three times daily for 3 consecutive weeks. Subsequently, by modifying the Huidobro and Maggiolo implantation technique (*9*), we produced a comparable degree of tolerance to morphine within 2 or 3 days. The procedure involves the subcutaneous implantation in the back of a conscious mouse of a specially formulated pellet or tablet of morphine (75 mg) base (*10*). The rate and extent of tolerance developed to morphine was followed from the increase in the median "analgetic" dose (AD_{50}) of morphine at various days after morphine implantation, with the use of a 2.5-second delay in the reaction time of the mouse tail to thermal stimulus as a quantal response to varying doses of morphine.

In repeated control experiments with at least 24 animals, the subcutaneous AD_{50} of morphine sulfate was consistently close to 10 mg/kg. In animals injected with morphine daily for 3 weeks, the AD_{50} generally increased about three- or fourfold. In implanted mice, development of tolerance was discernible within 24 hours and was maximum on day 3 or 4. The increase in the AD_{50} was generally about four- or fivefold. The morphine pellet was removed 8 hours before each AD_{50} determination. This interval appeared adequate for elimination of any residual morphine which may interfere with AD_{50} estimations, as evidenced by the fact that the tail reaction time to thermal stimulus had returned to predrug control levels of approximately 1.0 ± 0.2 second.

Physical dependence to morphine was observed by precipitating an abstinence syndrome with the morphine antagonist naloxone. The precipitated abstinence in mice is characterized by defecation, urination, increased motor activity, tremors, convulsions, and, most characteristically, by an uncontrollable urge to jump (*9*). The jumping response can be measured by determining the percentage of animals that jump off a circular platform, 70 cm high and 35 cm in diameter, within 15 minutes after injection of naloxone. In animals receiving morphine (40 mg per kilogram of body weight, subcutaneously every 8 hours) the naloxone-precipitated jumping could be detected in some animals in less than 2 days. In animals implanted for 3 days, naloxone precipitated jumping within 5 minutes, even in the presence of the morphine pellet, and less naloxone was required to evoke the response as the animal became more tolerant and dependent. A withdrawal response could be elicited with naloxone as early as 3 hours after implantation with morphine, although the dose required of naloxone was 70 times that needed after 72 hours of implantation. Thus, the degree of physical dependence at any interval after implantation can be measured by estimating the amount of naloxone needed to precipitate the withdrawal jumping syndrome. Abrupt withdrawal can also be induced by removal of the pellet but the signs are relatively mild. The jumping response is maximum 6 to 8 hours after removal of the morphine tablet and disappears at about 30 hours. The withdrawal response is selective for morphine since it can be suppressed by morphine or one of its surrogates, which exhibits cross-tolerance to morphine. On the other hand, other central nervous depressants such as chlorpromazine and pentobarbital failed to affect materially the jumping response at doses high enough to produce pronounced sedation.

Table 1. Effect of cycloheximide on the development of tolerance to morphine in mice as measured by the median effective dose (AD_{50}) of morphine to inhibit the tail response to thermal stimulus. Mice were rendered tolerant by increasing doses of morphine injected three times daily for 21 days. The figures enclosed by parentheses denote the 95 percent confidence limits calculated according to Litchfield and Wilcoxon (*14*).

Substance	AD_{50} morphine (mg/kg)	
	Before injection	After 21 days
Saline	8.4 (5.3–13.3)	8.3 (5.0–13.7)
Morphine	9.7 (6.4–14.7)	36.5 (26.1–51.1)
Morphine + cycloheximide	9.0 (6.0–13.4)	11.0 (7.2–16.8)
Cycloheximide	9.2 (6.2–13.6)	8.4 (5.1–13.9)

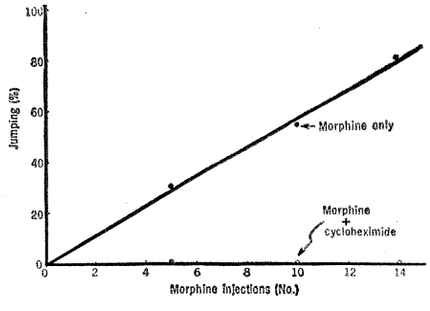

Fig. 1. Inhibition of development of physical dependence to morphine by cycloheximide. Effect of repeated morphine injections and concomitant cycloheximide (20 mg/kg) administration on the incidence of withdrawal jumping in mice receiving morphine (40 mg/kg) every 8 hours. The withdrawal tests were performed 6 hours after the last morphine dose, by challenging with naloxone (4 mg/kg).

An association of tolerance and physical dependence to morphine with increased protein synthesis is suggested by our experiments showing that tolerance and physical dependence to morphine could be prevented with cycloheximide (Actidione) at a dose which elicited no overt pharmacologic effects.

Inhibition of morphine tolerance development by cycloheximide was demonstrated as follows. The AD_{50} to morphine was determined in four groups of mice in the usual manner. Two of the four groups were then injected with morphine three times daily for 3 weeks but one of two groups was also injected once daily with cycloheximide (20 mg/kg) intraperitoneally. For controls, a third group received saline and a fourth, cycloheximide daily. At the end of 3 weeks, the AD_{50} was again determined.

Cycloheximide almost completely inhibited tolerance development to morphine (Table 1). In the group receiving morphine, the AD_{50} increased almost fourfold, whereas in the morphinized group which received cycloheximide concomitantly, the change in AD_{50} to morphine was not statistically significant. No significant change in the AD_{50} was noted in the saline and cycloheximide control animals.

It was also determined that in animals with an implant of morphine and receiving cycloheximide (40 mg/kg) twice daily for 4 days, no significant change in the AD_{50} to morphine occurred, whereas in implanted mice receiving saline alone the AD_{50} increased nearly fivefold. Similar results on tolerance development to morphine have been reported with toxic doses of actinomycin (*4*), but our unpublished observations suggest that at least part of the actions mediated by actinomycin may not be related to protein synthesis.

Inhibition of physical dependence development to morphine by cycloheximide is shown in Fig. 1. In animals receiving 40 mg of morphine per kilogram of body weight every 8 hours, development of physical dependence was shown to be a function of the num-

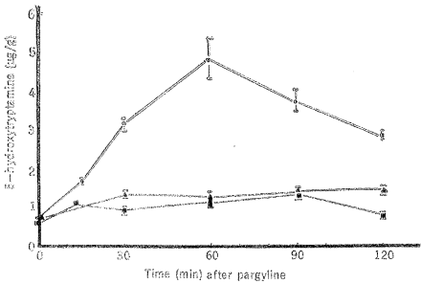

Fig. 2. Concentration of 5-hydroxytryptamine in the brain after pargyline (75 mg/kg) intraperitoneally in mice rendered tolerant to morphine by pellet implantation (circles), nontolerant controls (triangles), and in implanted mice 2 weeks after pellet removal (squares). Four animals were used for each time interval. The brackets indicate the S. E.

ber of morphine injections. Precipitated withdrawal jumping after challenge with naloxone was observed after five injections of morphine and was maximum after 14 injections; in animals receiving cycloheximide concomitantly none jumped when injected with naloxone, indicating that cycloheximide had prevented development of physical dependence as well as tolerance to morphine.

The possibility that one of the proteins whose synthesis is blocked by cycloheximide may be involved with 5HT metabolism is suggested by our finding that an increase in 5HT synthesis in the brain occurred with tolerance development to morphine. The turnover in 5HT in the brain in morphine tolerant and nontolerant mice was determined by blocking the rate of conversion of 5HT to 5-hydroxyindole acetic acid (5-HIAA) with the monoamine oxidase inhibitor (pargyline) at a dose of 75 mg/kg intraperitoneally. If we assume that brain 5HT is converted solely to 5-HIAA, the rate of 5HT synthesis may be calculated from the initial increase in 5HT (*12*).

In repeated experiments on mice made tolerant to morphine by morphine implantation or injection, a significant increase in brain 5HT was consistently obtained 30 and 60 minutes after pargyline. The 5HT levels of both groups before pargyline (zero time) were nearly the same, but after pargyline the mean increase in brain 5HT in the morphine tolerant group was twice that of nontolerant controls; in one series of experiments, the difference between the two groups was as much as fivefold. In implanted mice, estimations of brain 5HT were made without prior removal of the pellet. The brain 5HT levels at fixed time intervals before and after pargyline for one set of experiments are shown in Fig. 2. The zero-time level of both groups were comparable, but after pargyline the morphine tolerant animals exhibited higher 5HT levels than the nontolerant controls at all time intervals. In another group of implanted mice whose pellets had been removed for 2 weeks, the brain 5HT levels at various intervals were similar to those of the nontolerant controls. No change in 5HT turnover was noted in acute studies following a single 100 mg/kg dose of morphine subcutaneously.

The selectivity of the morphine effect on synthesis of 5HT rather than on other biogenic amines was demonstrated in the studies with *p*-chlorophenylalanine (PCPA). This compound inhibits the synthesis of 5HT, but has little or no effect on the catecholamine amines. After three consecutive injections of PCPA daily, the concentrations of 5HT in the mouse brain were reduced to 25 percent of the normal within 24 hours; in the rat, maximum effects were obtained at 3 days and persisted for up to 6 to 8 days (*11*). In mice treated with PCPA (320 mg/kg) intraperitoneally 1 day before the morphine pellets were implanted, the AD_{50} of morphine was three times that of untreated controls; however, in the implanted mice receiving no PCPA, the AD_{50} had increased seven times. Thus, PCPA reduced markedly but did not completely block the development of tolerance to morphine. *p*-Chlorophenylalanine also inhibited the development of physical dependence to morphine, as evidenced by a decreased response to withdrawal jumping precipitated by naloxone. In eight animals receiving morphine subcutaneously (40 mg/kg) at 8-hour intervals for 6 days, all eight animals exhibited precipitated withdrawal jumping when challenged 6 hours after the last injection with naloxone (4 mg/kg) subcutaneously. On the other hand, in a group of eight animals that received PCPA (320 mg/kg) intraperitoneally at 1 and 2 days before and 5 days after initiating the repeated morphine injections, only one out of eight animals exhibited precipitated withdrawal jumping to naloxone. These findings suggest that the tolerance, physical dependence, and increased 5HT synthesis which occur after chronic morphinization may be part of closely related phenomena.

The manner in which increased 5HT synthesis is induced needs to be clarified. Presumably, tryptophan hydroxylase would be involved since hydroxylation is the rate-limiting step in 5HT synthesis (*13*). Apart from the tolerance and physical dependence mechanisms, the confirmation of these actions of morphine in other species is mandatory because the compound might be helpful in delineating the functional role of 5HT.

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