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Classical Conditioning of a Morphine Abstinence Phenomenon, Reinforcement of Opioid-Drinking Behavior and "Relapse" in Morphine-Addicted Rats

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Introduction

Anamnestic and experimental data obtained in studies on opioid addicts (HIMMELSBACH, 1942; WIKLER, 1948, 1952, 1953, 1955) suggest that physical dependence on morphine (or other drugs with morphine-like properties) may contribute to the disposition of the "cured" addict[1] to relapse through: 1. long-term persistence of low-grade physiological (autonomic) deviations from normal; 2. classical conditioning of abstinence phenomena to environmental situations frequently associated with acute withdrawal distress; and 3. operant conditioning of opioid-seeking behavior through repeated reduction of acute withdrawal distress by self-administration of such drugs. In terms of a "two-factor" learning theory analogous to that of MOWRER (1950), classical conditioning of abstinence phenomena could, in the presence of the adequate conditioned stimulus, result either in augmenting an existing unconditional "drive" state due to persistence of low-grade physiological abnormalities after withdrawal of opioids, or in the recurrence of a physical dependence-like "drive" state long after recovery from the last previous withdrawal syndrome; and operant conditioning during previous episodes of physical dependence could provide the organism with a "problem solving" technique (opioid-acquisitive behavior) for reducing the unconditioned and/or conditioned "drive", thereby increasing the probability of relapse (WIKLER, 1958, 1961, 1965).

[1] In this paper, the terms "addict", or "addicted" mean subjects (human or rat) that are physically dependent on morphine or any other opioid. The term "postaddict" refers to subjects previously made physically dependent on morphine or any other opioid, but who have been withdrawn from the drug ("cured" in that sense only, but not necessarily in any other sense). "Nonaddict" refers to subjects that have never been "addicted" as defined above.

The present report deals with an attempt to analyze the relative importance of previous physical dependence *per se*, as well as of classical and operant conditioning in generating "relapse" in the rat. Basic to the methodology employed are a number of findings reported earlier (WIKLER *et al.*, 1960; MARTIN *et al.*, 1963; WIKLER *et al.*, 1963): 1. Increased frequency of "wet dog" shakes (sudden, brief body twitches resembling those of a dog shaking water off its back) is a reliable indicator of the early or "primary" morphine abstinence syndrome in the rat (at least in males of the Wistar strain), roughly paralleling other signs such as increased activity, hypothermia, loss of body weight, decreased water intake, and increased defecation, urination and hostility (all compared with measures or observations made concurrently on normal control rats). This "primary" abstinence syndrome becomes manifest 8—16 hours after the last dose of morphine, is fully developed by 24 hours and lasts about 72 hours, though increased activity and frequency of "wet dog" shakes persist for about a week. 2. As the "primary" abstinence syndrome subsides, a "secondary" abstinence syndrome emerges, consisting of a rapid gain in body weight, elevated body temperature and metabolic rate and an increase in water consumption, together with renewed (though non-significant) increases in activity and frequency of "wet dog" shakes. In contrast to the "primary" abstinence syndrome, the "secondary" abstinence syndrome is protracted and small differences between postaddict and normal control rats may be detected for as long as four to six months after withdrawal of morphine. 3. Whereas most rats (normal, morphine-tolerant or morphine-abstinent) will not drink a very dilute aqueous solution of morphine (e.g., 0.5 mg/ml) without severe water-deprivation, they will drink a 5 or 10 mcg/ml aqueous solution of etonitazene[2] (a benzimidazole derivative with morphine-like properties which is 1,000 times as potent as morphine for analgesia in the rat) without any prior water deprivation. 4. The presence of etonitazene (5 or 10 mcg/ml) in the drinking water reinforces drinking behavior in "primarily" morphine-abstinent rats (which consume significantly greater volumes of the drug solution than they do of water), and after allowing such rats to drink a 10 mcg/ml aqueous solution of etonitazene for 17 hours, no significant differences from normal control animals drinking water during the same period can be detected in respect of frequency of "wet dog" shakes, activity, body temperature, metabolic rate or general behavior. In contrast, both normal control rats and morphine-tolerant (but not abstinent) rats drink a 5 mcg/ml aqueous solution of etonitazene in volumes not significantly different from water.

[2] The authors wish to express their appreciation to the Ciba Pharmaceutical Company, Basle, Switzerland, for furnishing generous supplies of etonitazene for use in the studies described in this report.

These data were utilized in the two studies described in the present report, which were designed to test the hypotheses stated in the Introduction. As these studies were rather complicated because of the large number of variables involved, a synopsis of the main procedures employed and of the theoretically anticipated findings is given here for the purpose of orientation to the details of methods and results that follow.

Rats were made physically dependent on morphine by intraperitoneal injection in gradually increasing doses over several weeks and were then maintained on a single dose (200 mg/kg) of morphine given intraperitoneally every morning. On this schedule, such rats exhibited signs of morphine-intoxication every morning after injection and throughout most of the afternoon, whereas in the evening and throughout the night (until the next morning injection of morphine) they exhibited the signs of "primary" morphine-abstinence. In both studies, conditions favorable to classical conditioning of morphine-abstinence phenomena were created by confining each rat to one end-compartment of a three-compartment linear maze[3] several nights weekly over a six-week "training" period with food and water, thereby establishing temporal contiguity between a specific environment and the unrelieved morphine abstinence syndrome. Also in both studies, reinforcement of etonitazene-consumption was accomplished during the six-week "training" period with food and a 10 mcg/ml aqueous solution of etonitazene for drinking in the other end-compartment of the linear maze. Discriminative cues in the first study were gustatory-olfactory (anise oil flavor added to the etonitazene solution), while in the second study, they were visual-tactile. In both studies, controls included normal rats receiving intraperitoneal injections of 0.9 % aqueous sodium chloride solution ("saline") each morning, which were subjected to the same "training" procedures as the morphine-injected animals, except that the etonitazene solution for drinking was 5 mcg/ml (to avoid fatalities and minimize the possible development of tolerance to and physical dependence on etonitazene). In addition, the first study included both morphine-injected and normal rats "pseudo-reinforced" with anise-flavored water in the linear mazes, while the second study included morphine-injected and normal (saline-injected) rats that resided in home cages without any "training" at all during the six-week period.

After completion of the six-week period in each study, all injections were terminated and rats heretofore residing in linear mazes were returned to individual home cages. Thereafter, at intervals a week or more apart for several months, all rats were tested on a given morning for evidence

[3] The valuable services of Mr. WESLEY PROCOP in constructing the linear mazes are acknowledged with thanks.

of "conditioned abstinence" by comparing frequencies of "wet dog" shakes in home cage and in linear maze. In the evening of the same day, each rat was placed in the middle compartment of a linear maze with portals open to both end-compartments and allowed to drink a 5 mcg/ml aqueous solution of etonitazene (anise-flavored in the first study; cued by tactile-visual stimuli in the second study) at one end, or water at the other end *ad libitum* until the next morning. "Relapse" was evaluated by comparisons of the mean percent of total fluids consumed in the form of etonitazene solution among the various groups of rats.

By theory, it was predicted that: 1. The mean frequency of "wet dog" shakes would be higher in the linear maze than in the home cage in the case of postaddict rats that had been morphine-abstinent repeatedly in the linear maze, while the reverse would be true for the postaddict rats that had been abstinent each night in the home cage prior to permanent withdrawal of morphine; in contrast, the mean frequency of "wet dog" shakes in the linear maze would not be significantly different from that in the home cage for normal control rats, regardless of place of residence prior to termination of saline injections. And 2., the highest mean "free choice" consumption of etonitazene solution would be found in the postaddict rats that had been reinforced repeatedly (by drinking etonitazene solution when acutely morphine-abstinent) in the linear maze prior to abrupt withdrawal of morphine; next highest would be postaddict rats that had been "pseudo-reinforced" in the linear maze, or had resided in the home cage drinking water only prior to abrupt withdrawal of morphine; while the lowest consumption of etonitazene in "free choice" tests would be found in normal control rats regardless of their treatments prior to termination of saline injections.

Methods and Results

Study 1

Methods

1. Preparatory. Twenty-eight male Wistar rats, 4 months of age, were randomly assigned in equal numbers to experimental (M) and control (S) groups. Over a six-week period, M rats received intraperitoneal injections of morphine sulfate, beginning with 10 mg/kg twice daily and increasing by 10 mg/kg steps for each dose about every third day until the daily dose level reached 200 mg/kg. Thereafter, they were maintained on a single intraperitoneal injection of morphine (200 mg/kg) given between 0730 and 0800 each day. Concomitantly, S rats were treated in an identical manner except that the intraperitoneal injections consisted of "saline" in volumes equivalent on a body-weight basis to those of morphine in the M group.

Nine weeks after beginning injections, all rats were transferred from individual home cages to individual "linear mazes" (73.7 × 17.8 × 17.8 cm) constructed of solid metal except for hardware cloth floors and tops which were permanently divided into three equal compartments connected with each other by portals (7.6 × 7.6 cm) in the otherwise solid metal partitions, either of which could be closed completely by insertion of solid metal sliding panels. The laboratory was air-conditioned (22.2° to 23.3° C) and illuminated only by ceiling lights which were turned on at 0730 and off at 1600 every day. Over a two-week period following transfer of the animals to the linear mazes, measures were made of total daily consumption of distilled H_2O from two 100 ml graduated glass drinking tubes placed respectively in the end-compartments (free access to both, with ample food rations, in the form of Purina Chow bars always available). These measurements served to establish the "preferred" end-compartment for each rat.

2. *Training.* After linear maze end-preferences were determined, a six-week "training" period was begun. M and S rats were randomly assigned to subgroups of seven animals each, *defined in accordance with the contents of the drinking fluids made available to them in the "non-preferred" end-compartment from 2000 to 0730 on certain nights of each week* (see below). For all subgroups, this drinking fluid was "tagged" by passing it through filter paper on which a few drops of anise oil had been placed, thus imparting to the fluid an odor and taste which the observer, and presumably the rats could readily detect. The four subgroups were:

MTF: morphine-injected, "trained" (offered anise flavor-tagged etonitazene, 10 mcg/ml, designated ETZ^{10}-F).

MPF: morphine-injected, "pseudo-trained" (offered anise flavor-tagged distilled water, designated H_2O-F).

STF: saline-injected, "trained" (offered anise flavor-tagged etonitazene, 5 mcg/ml, designated ETZ^{5}-F).

SPF: saline-injected, "pseudo-trained" (offered anise flavor-tagged distilled water, H_2O-F).

In the linear mazes, access to the end-compartments (each with one drinking tube) was so arranged that on certain afternoons and evenings of each week, all rats could drink only from the tube in the "*preferred*" *end, which contained unflavored distilled water,* while on the other evenings of each week, the animals could drink only from the tube in the "*non-preferred*" *end which contained the anise-flavored fluid* assigned to each subgroup as indicated above. Details of these arrangements are shown in Table 1. This design provided opportunities on repeated occasions for the occurrence of the following spatial, gustatory-olfactory and

Table 1. *Study 1. Weekly "training" schedule. End-compartment access, time periods and drinking fluids (all rats)*

	Both compartments	"Preferred" only	"Non-preferred" only
Monday	0800—1400 (H_2O)	1400—0730 (H_2O)	(excluded 1400—0730)
Tuesday	0800—2000 (H_2O)	(excluded 2000—0730)	2000—0730 (flavored fluids)
Wednesday	0800—1400 (H_2O)	1400—0730 (H_2O)	(excluded 1400—0730)
Thursday	0800—2000 (H_2O)	(excluded 2000—0730)	2000—0730 (flavored fluids)
Friday	0800—1400 (H_2O)	1400—0730 (H_2O)	(excluded 1400—0730)
Saturday	—	0800—0730 (H_2O)	(excluded 0800—0730)
Sunday	—	0800—2000 (H_2O)	(excluded 0800—2000)
		(excluded 2000—0730)	2000—0730 (flavored fluids)

physiological-pharmacological contiguities in the cases of the various subgroups:

MTF: unrelieved "primary" morphine abstinence in the "preferred" end-compartment; suppression of "primary" abstinence in the "non-preferred" end-compartment (contingent on consumption of the etonitazene solution); and possibly, other effects of etonitazene and/or anise-flavor *per se*.

MPF: unrelieved "primary" morphine-abstinence in both end-compartments; possible effects of anise-flavor *per se*.

STF: "preferred" end-compartment "neutral"; possible effects of etonitazene and/or anise-flavor *per se* in the "non-preferred" end-compartment.

SPF: both end-compartments "neutral" except for possible effects of anise-flavor *per se* in the "non-preferred" end-compartment.

On alternate Sunday evenings, the "training" sessions described above were replaced by "free choice" sessions, in which the portals to both end-compartments were open from 0800 to 0730 the next morning, making both the tube of distilled water in the "preferred" and the anise-flavored fluid in the "non-preferred" end-compartment available to each rat. In all, each rat had 14 "training" and three "free choice" sessions during this six-week period (a 15th "training" session was added later, as explained below).

At the end of the six-week period, all rats were treated as on Mondays, Wednesday, and Fridays for five continuous days for the purpose of equalizing the degree of physical dependence on morphine for the two M subgroups (this was deemed advisable because the etonitazene solution consumed by MTF in the "training" sessions could have resulted in stronger

physical dependence in this subgroup as compared with MPF). Then all injections (morphine and saline) were discontinued permanently and all rats were excluded from the "non-preferred" end-compartments. 24 hours after termination of injections, each rat was removed from the linear maze, weighed, replaced in the linear maze (excluded from the "non-preferred" end-compartment) and immediately thereafter, "wet dog" shakes were counted for 15 consecutive min. At the end of the 36th hour after termination of injections, a final (15th) "training" session was made in the "non-preferred" end-compartments from 0800 to 0730 (each subgroup offered its anise-flavored fluid), after which all rats were removed from the linear mazes, weighed and placed in individual home cages where counts of "wet dog" shakes in 15 min were made immediately after the transfer.

3. Testing (Pre-Extinction). In the home cages, all rats were provided with food and tap water (in bottles fitted with glass drinking spouts) *ad libitum*. Except on week-ends, they were weighed daily throughout the remaining period of the study. To follow the course of "primary" abstinence (in the two M subgroups), frequency counts of "wet dog" shakes were also made on all rats in the home cages immediately after weighing at 0800 on the 3rd, 6th and 8th days after termination of injections. Thereafter, measurements of "wet dog" shake frequencies were made according to the procedure described below on "relapse"-test days (9th, 23rd, 44th, 58th, 72nd, 87th, and 94th post-injection days), when "free choice" consumption of distilled water and of anise-flavored fluid was also measured.

a) "Wet-dog" Shake Frequencies. 24 hours before each "relapse" test, counts of "wet dog" shakes in 15 min were made in the home cages and total tap water consumption there from then till 0800 the next morning was measured for each rat. At 0800 on the morning of the "relapse" test day, "wet dog" frequency counts were made in two ways for each rat: After removing from the home cage, weighing and replacing in the home cage; and after removing from the home cage, weighing and placing in the linear maze, with access to the "non-preferred" end-compartment excluded. Within each subgroup, the order of home cage and linear maze "wet dog" counts was reversed for successive rats. After completion of "wet dog" counting, those rats remaining in the home cages were also transferred to the linear mazes ("non-preferred" end-compartment excluded) and all rats stayed there (with food and H_2O in the "preferred" end-compartment *ad libitum*) until 2000.

b) "Free Choice" Fluid Consumption. At 2000, a drinking tube filled with anise-flavored etonitazene in a 5 mcg/ml concentration was placed in the "non-preferred" end-compartment of the linear maze for *each rat (all subgroups)* and the portal to that compartment was also opened,

permitting each rat to "choose" between H_2O and ETZ[5]-F until 0800 the next morning, after which all rats were transferred to the home cages.

4. Extinction. After the seventh "relapse" test (94th post-injection day), two successive "extinction" procedures were carried out on all rats, the first of which may be designated, "etonitazene extinction", and the second, "etonitazene and anise-flavor extinction." The "etonitazene extinction" procedure consisted of three separate trial days (99, 101 and 104 postinjection) conducted in exactly the same manner as the "relapse" tests, except that H_2O-F was substituted for ETZ[5]-F in the "non-preferred" tube. From the 105th through the 111th post-injection day, all rats resided continuously in the linear mazes, and they were permitted to drink *ad libitum* throughout the 24 hours of each day from the "preferred" tube containing H_2O, or the "non-preferred" tube containing H_2O-F. On the 112th post-injection day, all rats were transferred to their home cages at 0730. On this day and also on the 139th post-injection day "etonitazene extinction" trials were again conducted exactly as in the "relapse" tests except, of course, that the "non-preferred" tube contained H_2O-F instead of ETZ[5]-F. The etonitazene and anise-flavor extinction" procedure was conducted between the two last mentioned "etonitazene extinction" trial days in the following manner. From the 113th through the 134th post-injection day, all rats resided in the linear mazes continuously and were permitted to drink *ad libitum* throughout the 24 hours of each day from either the "preferred" or the "non-preferred" tube, both containing only unflavored H_2O. On the 135th post-injection day, the rats were transferred to the home cages at 0730 and on this day an "etonitazene and anise-flavor extinction" trial was conducted exactly as in the "relapse" tests except that both the "preferred" and the "non-preferred" tubes contained only unflavored H_2O. These two "extinction" procedures were designed to provide data on the "symbolic significance" that remained attached to the flavor-tag after the seventh "relapse" test in each subgroup, and to furnish a new "baseline" from which to assess the results on the eighth (final) "relapse" test.

5. Testing (Post-Extinction). On the last "relapse" test day (142nd post-injection day), "wet dog" frequencies and "free choice" consumption of H_2O in the originally "preferred", and of ETZ[5]-F in the originally "non-preferred" tubes were measured exactly as in the pre-extinction period. In addition, "wet dog" frequencies in the home cages and in the linear mazes were measured on the 145th, 148 and 155th post-injection days, as in the "relapse" tests.

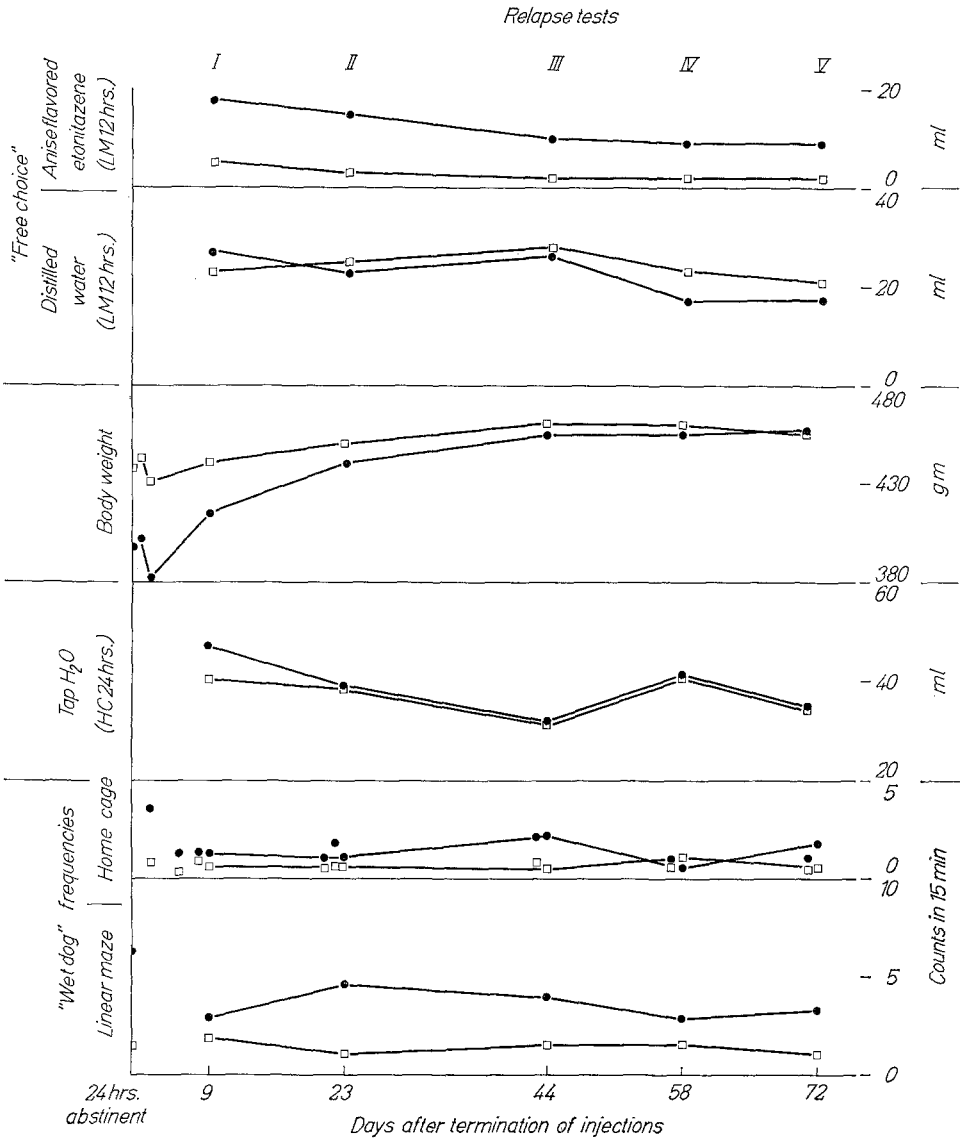
6. Interpolated "Forced Drinking" Tests. Measures were also obtained on the volumes of ETZ[5]-F each rat would consume when only this solution was offered from 2000 to 0730 in the "non-preferred" end-

compartments of the linear mazes on the 10th, 85th, 92nd and 155th post-injection days, and on the volumes of H_2O-F and H_2O respectively that each rat would drink under otherwise identical conditions, on the 146th and 149th post-injection days. These measures were made to obtain data on drinking of the respective fluids "by contraint" (though without any prior water-deprivation) for comparison with the amounts drunk "by choice" in the various "relapse" tests, to assist in assessment of the role, if any, played by "residual tolerance" in the consumption of ETZ[5]-F and H_2O-F by the two M subgroups in the "relapse" and "extinction" trials.

7. *Supplementary Study.* In addition, a supplementary "Anise-Flavor Control" study was conducted on eight other male Wistar rats (10 months old) as follows. Four of these rats (M) received intraperitoneal injections of morphine on the same schedule (final maintenance level, 200 mg/kg at 0730 each morning) as the M rats in the main study, while the other four (S) received intraperitoneal injections of "saline" in volumetrically equivalent amounts on a body-weight basis in an otherwise identical manner. Up to the time of this supplementary study, these eight rats had resided in individual home cages and had received no other treatments. After transfer to individual linear mazes and assignment of "preferences" on the basis of continuous, 24-hour *ad libitum* consumption of H_2O from either tube in the linear maze (both tubes containing only H_2O) over a period of 3 days, the M and S groups were "pseudo-trained" on a schedule exactly as that employed for the MPF and SPF subgroups on the main study except that only three "pseudo-training" trials (forced drinking of H_2O-F from the "non-preferred" tube, from 1430 to 0730 without prior water-deprivation) were given over a period of 7 days, and no "free choice" trials were conducted as long as daily morning injections were continued. As in the main study, a final "pseudo-training" session took place in the linear mazes between the 36th and 48th hours after termination of injections, after which all rats were returned to their home cages on food and tap water *ad libitum.* On the 9th, and again on the 23rd day after termination of injections, exactly the same procedures were carried out as in the "relapse" tests in the main study (at the corresponding times for the first and second "relapse" tests), except that the "free choice" offered was between H_2O ("preferred" tube) and H_2O-F ("non-preferred" tube).

Results

In the MTF subgroup, one died after the tenth "training" trial. In the MPF subgroup, one died after the eighth "training" trial and another after the first "free choice" trial during training. Two deaths occurred in the STF subgroup, one after the fourth "relapse" test and



a

Fig. 1a and b. Study 1. Behavior of previously morphine-injected (MTF + MPF) rats (dots) and previously saline-injected (STF + SPF) rats (squares) after termination of injections. In Fig. 1a, "free choice" was between H_2O in the "preferred", and ETZ^5-F in the "non-preferred" tube in all five "relapse" tests. In Fig. 1b, the "preferred" tube always contained H_2O but the contents of the "non-preferred" tube varied because of interpolation of extinction procedures (A, etonitazene extinction; B, etonitazene and anise-flavor extinction). "Free choices" on the various tests were: a, H_2O vs ETZ^5-F; b, H_2O vs H_2O-F; c, H_2O vs H_2O. Circled points on "wet dog" frequency graphs refer to counts made 24 hours after forced dringking of ETZ^5-F for 12 hours on two occasions between the 85th and 95th days after termination of injections. See text for explanation of symbols for animal subgroups and drinking fluids

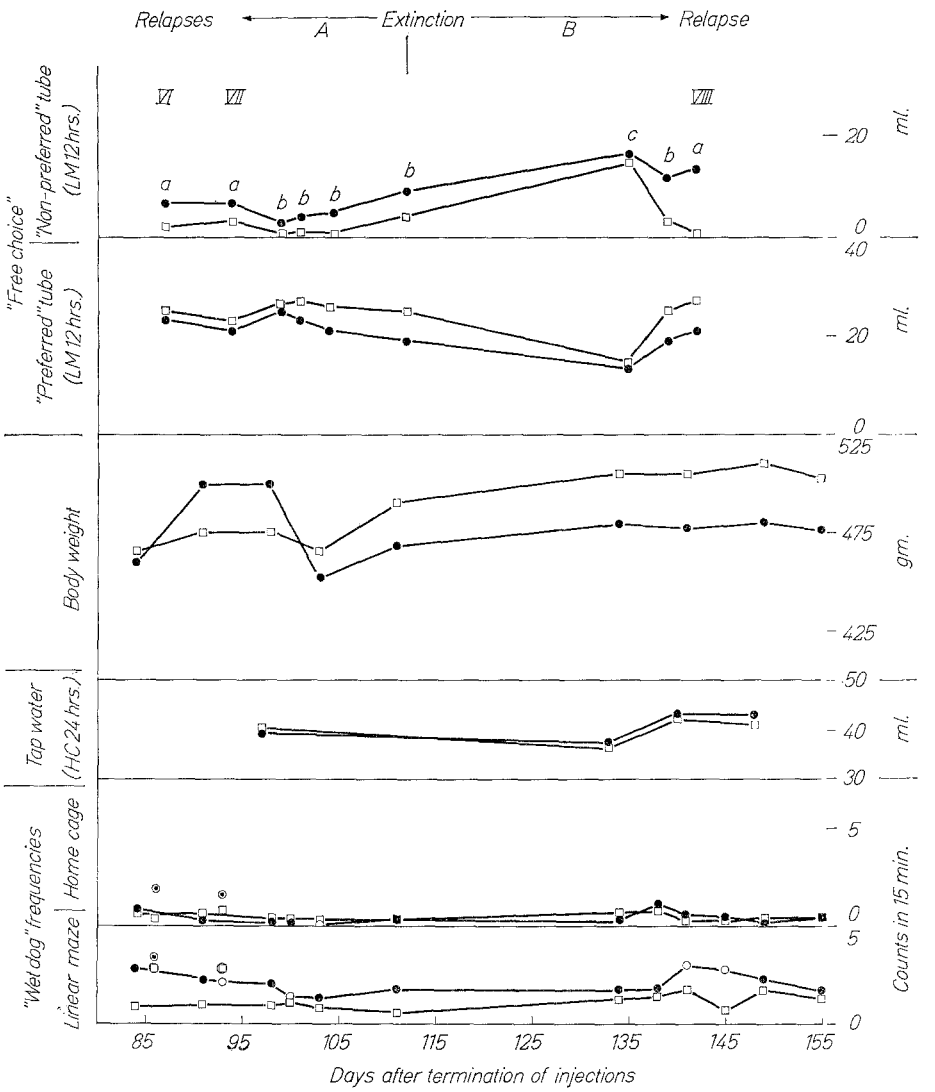


Fig. 1 b

the other during the continuous "etonitazene and anise-flavor extinction" period. In the SPF subgroup, one rat died after the fifth "relapse" test. At the end of the main study, therefore, the Ns for each subgroup were: MTF, 6; MPF, 5; STF, 5; SPF, 6.

For convenience, the results of the main study may be considered in relation to 1. classical conditioning of "wet dog" shakes; and 2. reinforcement of opioid (etonitazene) drinking behavior and "relapse".

1. Morphine abstinence syndrome and conditioning of "wet dog" shakes. 24 hours after cessation of all injections (designated as "1st post-injection day"), the mean "wet dog" response frequency for the MTF and MPF subgroups was 7.1 and for the STF and SPF subgroups, 1.4, both measured in the linear mazes (Fig. 1 a, "Linear Maze Wet Dogs"). By the t-test for independent groups, the significance of this difference was $p < .001$. As between the two subgroups of each major group, there were no significant differences in mean "wet dog" frequencies (MTF, 7.3 and MPF, 7.2; STF, 1.1 and SPF, 1.7). On the 2nd post-injection day, immediately following the last "training" session, the mean "wet dog" frequency for the MPF group was 5.8, and for the SPF group, 2.0, both measured in the home cage (significance of the difference, $p < .01$). Confirming earlier findings (WIKLER *et al.*, 1963), no "wet dog" responses were observed in the MTF group, which had drunk ETZ^{10}-F during the preceding 12 hours; the significance of the difference from the mean "wet dog" frequency of the MPF group, which had drunk H_2O-F during that same period, was $p < .001$. At the same time, the mean "wet dog" frequency for the SPF group (H_2O-F preceding 12 hours) was 2.0, and for the STF group (ETZ^5-F preceding 12 hours), 0.1, the significance of the difference being $p < .05$. In the home cages, mean "wet dog" frequencies of all groups declined progressively during the first week of abstinence, appearing to "level off" at mean frequencies of about 1.4 for the MTF and MPF subgroups, and 0.7 for the STF and SPF subgroups. That of the latter remained fairly constant at this low level throughout the 155-day period after termination of injections. During this time, "wet dog" frequencies of MTF and MPF subgroups were also very low in the home cages, but generally somewhat higher and more variable than those of the STF and SPF subgroups through the fifth "relapse" test (72nd post-injection day); thereafter, no differences in "wet dog" frequencies could be discerned between the experimental and control groups when these were measured in the home cages (Figs. 1 a and 1 b, "Wet Dog" Frequencies, Home Cage). Measures of 24-hour tap water consumption (home cages) were equal for the two groups by the 23rd day of abstinence. After a relatively larger loss of body weight on the 3 rd day of abstinence, the weights of the experimental groups were very close to those of the control group by the 23rd day of abstinence and remained so through the 72nd day of abstinence (Fig. 1 a); thereafter, both groups continued to gain weight steadily, that of the control group "levelling off" at a mean of about 30 gm higher than the experimental group during the last three weeks of observation (Fig. 1 b).

In the linear mazes, however, the differences in "wet dog" frequencies between the experimental and control groups were numerically greater than in the home cages on every "relapse" test and with rare exceptions,

Table 2. *Study 1. Analysis of variance (mixed type) for "wet dog" frequencies after termination of injections*

Relapse test No.	I		II		III		IV		V		$\bar{x}$ of 12 tests	
Days after end of injections	9		23		44		58		72		84—155	
Source	df	MS	df	MS	df	MS	df	MS	df	MS	df	MS
M *vs* S	1	11.69	1	48.17*	1	51.40**	1	2.47	1	37.50**	1	3.58*
Error, between subjects	23	4.93	23	6.22	23	6.47	23	5.36	22	4.21	22	0.65
Between subjects	24	5.21	24	7.97	24	8.34	24	5.24	23	5.66	23	0.78
Within subjects	25	2.70	25	5.30	25	2.32	25	2.70	24	2.00	24	0.99
LM *vs* HC	1	19.22**	1	40.50**	1	18.02**	1	16.82**	1	10.29*	1	14.46**
Interaction (M *vs* S×LM vs HC)	1	0.15	1	26.88**	1	1.15	1	8.51*	1	3.82	1	2.86**
Error (LM *vs* HC×subjects)	23	2.09	23	2.83	23	1.69	23	1.83	22	1.54	22	0.30
Total	49	3.93	49	6.61	49	5.27	49	3.94	47	3.79	47	0.89

M = rats previously injected with morphine ("trained," MTF + "pseudotrained," MPF). S = rats previously injected with saline ("trained," STF + "pseudotrained," SPF). LM = Linear maze. HC = Home cage.

* $p < .05$. — ** $p < 01$.

on every other test throughout the study (Fig. 1 a and 1 b, "Wet Dog" Frequencies, Linear Mazes). The results of a mixed type of analysis of variance (EDWARDS, 1957)[4] of "wet dog" frequencies on each of the first five "relapse" tests (through the 72nd post-injection day) and on mean frequencies over 12 tests from the 84th through the 155th post-injection day (including those obtained on the sixth, seventh and eighth "relapse" tests as well as on the "extinction" trials) are shown in Table 2. The strongest evidence in support of the predictions made from theory is that obtained on the second "relapse" test (23rd post-injection day) and of the means of 12 tests from the 84th through the 155th post-injection days, where the F ratios due to previous treatment (experimental, or morphine-tolerant *versus* control, or non-tolerant), test conditions (linear maze, *versus* home cages) and interaction (between previous treatment and test conditions) were all significant. Less strong, but still substantial supportive evidence was found in Relapse III and V, where F ratios for variances due to previous treatment and those due to test conditions were significant, even though that for previous treatment alone was not (i.e., total frequencies of "wet dog" responses, regardless of whether they occurred in the linear maze or the home cage, were not significantly different in the experimental and control groups). On relapse I only the F ratio for test conditions was significant; failure to obtain significant F ratios for previous treatment or interactions was apparently due to the fact that on this first "relapse" testing occasion, the relative increase in mean "wet dog" frequency of the control group exceeded that of the experimental group on testing in the linear mazes, as compared with testing in the home cages (Fig. 1 a). As would be expected from theory, no significant differences in overall "wet dog" frequencies (all tests) were found between the two subgroups of each major group (MTF *vs* MPT, or STF *vs* SPF), either in the home cages or in the linear mazes.

A curious phenomenon was the transient increase in "wet dog" frequencies, both in the home cages and in the linear mazes, observed in both the experimental and the control groups on two occasions, each 24 hours after a test on forced drinking of ETZ[5]-F (Fig. 1 b, "Wet Dog" Frequencies, circled points). It is tempting to ascribe this to "acute physical dependence" since not only the experimental but also the control animals exhibited this phenomenon, but further studies are needed to elucidate the nature of this transient reaction.

2. Reinforcement of opioid drinking behavior and "relapse". a) Drinking patterns during training period. The means (with standard errors) of the

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[4] The authors are grateful to Dr. CHARLES A. HAERTZEN, Research Psychologist, NIMH Addiction Research Center for advice and assistance with regard to the statistical analyses of the data.

Table 3. *Study 1. Drinking patterns during "training" period*

Subgroups	Reinforcement (forced drinking from "non-preferred" tube only. 2000-0730). Mean volumes ingested on 15 trials		Free choice (H_2O) in "preferred" or flavored fluid in "non-preferred" tube (0800-0730)					
			Mean on 3 trials			Last (3rd) trial		
			Total volumes of fluids ingested	Percent ingested from "non-preferred" tube		Total volumes of fluids ingested	Percent ingested from "non-preferred" tube	
	ETZ-F (ml) $\bar{x} \pm$ S.E. $\bar{x}$	H_2O-F (ml) $\bar{x} \pm$ S.E. $\bar{x}$	ETZ-F + H_2O-F (ml) $\bar{x} \pm$ S.E. $\bar{x}$	ETZ-F (%) $\bar{x} \pm$ S.E. $\bar{x}$	H_2O-F (%) $\bar{x} \pm$ S.E. $\bar{x}$	ETZ-F + H_2O-F (ml) $\bar{x} \pm$ S.E. $\bar{x}$	ETZ-F (%) $\bar{x} \pm$ S.E. $\bar{x}$	H_2O-F (%) $\bar{x} \pm$ S.E. $\bar{x}$
MTF ($N = 6$)	38.5 ± 4.0[a]		26.2 ± 5.0	30.4 ± 9.8[b]		30.3 ± 7.3	21.1 ± 9.8[c]	
STF ($N = 7$)	27.3 ± 2.9[d]		32.6 ± 3.8	15.3 ± 9.3[e]		39.1 ± 5.8	9.7 ± 7.8[f]	
MPF ($N = 5$)		8.0 ± 2.5	8.1 ± 1.1		33.6 ± 11.8	9.0 ± 2.0		28.6 ± 19.7
SPF ($N = 7$)		23.5 ± 2.6	25.8 ± 2.6		40.5 ± 10.4	28.0 ± 2.2		23.6 ± 9.8

[a] ETZ, 10 mcg/ml, est. $\bar{x}$ "total dose": 974.7 mcg/kg. — [b] ETZ, 10 mcg/ml, est. $\bar{x}$ "total dose": 268.4 mcg/kg. — [c] ETZ, 10 mcg/ml, est. $\bar{x}$ "total dose": 186.3 mcg/kg. — [d] ETZ, 5 mcg/ml, est. $\bar{x}$ "total dose": 320.2 mcg/kg. — [e] ETZ, 5 mcg/ml, est. $\bar{x}$ "total dose": 106.7 mcg/kg. — [f] ETZ, 5 mcg/ml, est. $\bar{x}$ "total dose": 67.6 mcg/kg (see Table 2 for designations of subgroups).

Table 4. *Study 1. Drinking behavior in "free choice" relapse tests*

		Mean percent total fluids (H_2O + ETZ[a]-F) ingested in form of ETZ[a]-F							
"Relapse" test No.		I	II	III	IV	V	VI	VII	VIII[a]
Days after termination of injections		9	23	44	58	72	87	94	142
Groups	MTF + MPF[b]	40.5	38.4	24.1	31.5	28.2	17.1	16.9	29.2
	STF + SPF[c]	16.2	12.9	7.3	5.2	8.7	6.7	10.9	1.0
MANN-WHITNEY "U"		34.5*	28.5**	38.5*	33.5**	48.0	35.0	45.0	18.0**

* $p < .05$. — ** $p < .02$.

[a] After successive etonitazene and anise-flavor extinction periods. — [b] $N = 11$ on all "relapse" tests. — [c] $N = 14$ on "relapse" tests I—IV, 13 on V, 12 on VI and VII, 11 on VIII (see Table 2 for designations of subgroups).

volumes of fluids consumed under all conditions and of percentage of fluids consumed in the form of anise-flavored "reinforcing fluid" in "free choice" trials by all four subgroups during the "training" period are shown in Table 3. In examination of this table, cognizance should be taken of the differences in diurnal drinking patterns between the M rats and the S rats while both groups are still receiving morning intraperitoneal injections of morphine (MTF and MPF) or saline (STF and SPF). The data in the two columns under "Reinforcement" show that addition of anise-flavor to the fluids does not alter the patterns described earlier (WIKLER *et al.*, 1963) for the M and S groups when only a single tube containing etonitazene solution or water is available for drinking during the evening and nocturnal hours. It may be noted again that under such conditions of "constraint" (but without prior water-deprivation), S rats drink at least as much (here, slightly more) ETZ^5-F than H_2O-F, whereas M rats (morphine-abstinent) drink far greater volumes of ETZ^{10}-F than H_2O-F. The data in the two columns under "free choice" show that under this condition (computing absolute volumes of "reinforcing fluid" consumed on mean of three trials) S rats drink less of ETZ^5-F (5.0 ml) than H_2O-F (10.5 ml) whereas M rats drink more ETZ^{10}-F (8.0 ml) than H_2O-F (2.7 ml). It would appear, therefore, that by the time the "free choice" trials were made, some discriminative "learning" had taken place in the MTF and STF subgroups (appetitive for the former and aversive for the latter) on the basis of temporal contiguity between the flavor (and/or "non-preferred" tube position) and the effects of etonitazene.

b) Drinking patterns after termination of injections. (i) Behavior of combined experimental (MTF+MPF) and combined control (STF+SPF) subgroups. Mean "free choice" consumptions of ETZ^5-F and of H_2O on the first seven "relapse" tests (through the 94th post-injection day) and again on the eighth "relapse" test (142nd post-injection day, after the "extinction" procedure) are shown in Figs. 1a and 1b ("Free Choice"). It will be noted that on the first seven "relapse" tests, the volumes of H_2O-F consumed by the experimental and control animals were almost identical, or slightly smaller for the former. In contrast, the volumes of ETZ^5-F consumed by the experimental animals were greater than those consumed by the control rats on each of the seven "relapse" tests, though the difference was maximal on the first "relapse" test and declined progressively thereafter. In terms of percentage of total fluids (H_2O + ETZ^5-F) consumed in the form of ETZ^5-F, the differences (MTF + MPF > STF + SPF) were significant by the Mann-Whitney "U" test (SIEGEL, 1956) on the first four "relapse" tests (through the 58th post-injection day) but non-significant on the fifth, sixth and seventh tests (72—94 post-injection day), as shown in Table 4.

Drinking behaviors during the "etonitazene extinction" procedures (Fig. 1 b, H_2O vs. H_2O-F) were rather different from those anticipated. As expected, M rats drank less H_2O-F when this fluid was substituted for ETZ[5]-F in the first "drug extinction" trial than they drank of the latter fluid on the last previous (seventh) "relapse" test, but S rats did likewise. Over the next three "drug extinction" trials, the volumes of H_2O-F consumed by M rats increased, while the volumes of H_2O they consumed decreased. A similar, but less marked trend was also exhibited by S rats. However, the difference between M and S rats as regards relative "free choice" consumption of H_2O-F and H_2O became very striking on retesting after "etonitazene and anise-flavor extinction" (H_2O, "preferred" tube *vs* H_2O, "non-preferred" tube for 21 days continuously). It will be noted (Fig. 1 b, 135th post-injection day) that when offered a "choice" between H_2O only in either tube, neither group exhibited any "preference". In other words, this test revealed that somewhere in the long course of the study, the rats in both groups had lost their initial "position-preference". Viewed against this new "neutral" baseline, the results of the "etonitazene-extinction" trial conducted on the 139th post-injection day are especially revealing. With H_2O-F now in the originally "non-preferred" tube (H_2O in the originally "preferred" tube), the decrease in "free choice" intake of fluid from the former (H_2O-F) and increase from the latter (H_2O) is far more marked for S than for M rats. Furthermore, this difference is retained, in even more pronounced form on the eighth "relapse" test (142nd post-injection day), when all rats were offered a choice between H_2O (originally "preferred" tube) and ETZ[5]-F (originally "non-preferred" tube), the difference being significant at the $< .02$ level by the Mann-Whitney "U" test (Table 4).

Comparison of the volumes of H_2O-F consumed in "free choice" tests by the SPF subgroup during "training" (Table 3) with the volumes of H_2O-F consumed in "free choice" tests by S rats (STF + SPF) during and after the "extinction" procedures (Fig. 1 b) suggests that for this group, the anise-flavor had acquired aversive properties as a result of association of the flavor with the pharmacological effects of etonitazene (for the SPF subgroup in "relapse" tests, and for the STF subgroup both during "training" and in "relapse" tests), which resisted the "extinction" procedures employed. Furthermore, such "aversion" to etonitazene far outweighed any "attraction" the drug may have had for S rats, since the amounts of ETZ[5]-F consumed by them by "choice" in any "relapse" test was very small (Figs. 1 a and 1 b). Similar comparison of data for the MPF subgroup during "training" and for M rats (MTF + MPF) during and after the "extinction" procedures in respect to "free choice" consumption of H_2O-F reveals that for M rats also, the anise-flavor had acquired aversive properties which, however, were

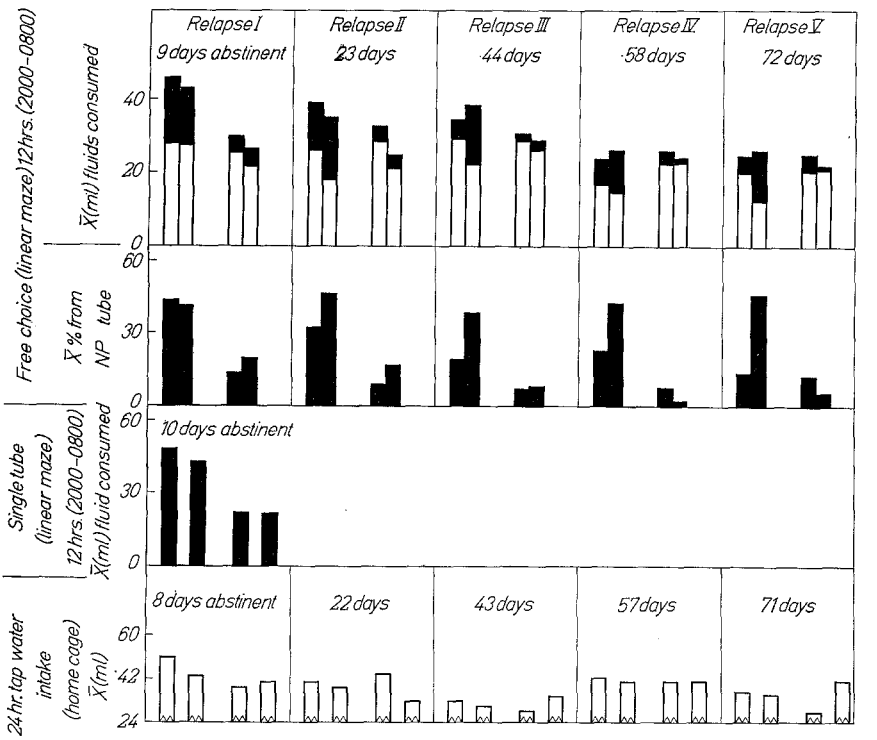

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Fig. 2a and b. Study 1. Drinking behavior of individual subgroups after termination of injections. In all quartets of bargraphs, data are shown for subgroups in the following order (left to right); MTF, MPF, STF, SPF. Fluids available in "free choice" tests were as specified in legend for Figs. 1a and 1b on corresponding "days of abstinence" (days after termination of injections). ■ Anise-flavored etonitazene, 5 μg/ml; ▨ anise-flavored water; □ water

susceptible to the "extinction" procedures employed. Furthermore, in contrast with S rats, such "aversion" was either not displayed by M rats at all in the first four, and in the eighth "relapse" tests (Table 4), or if present, it was outweighed by the "attraction" etonitazene had for the experimental animals.

(ii) Behavior of subgroups. Examination of the data acquired on the first five "relapse" tests for each subgroup (Fig. 2a) reveals the surprising finding that the "aversive" factor referred to above became manifest earlier in the MTF than in the MPF subgroup. Thus, in terms of percentage of total fluids (H_2O and ETZ[5]-F) consumed in the form of ETZ[5]-F "by choice" the high values for MPF are well-sustained throughout the five "relapse" tests, while that for MTF, equally high on the first "relapse" test, falls progressively. However, on the sixth and seventh "relapse" tests, the values for MPF drop to those of MTF (Fig. 2b).

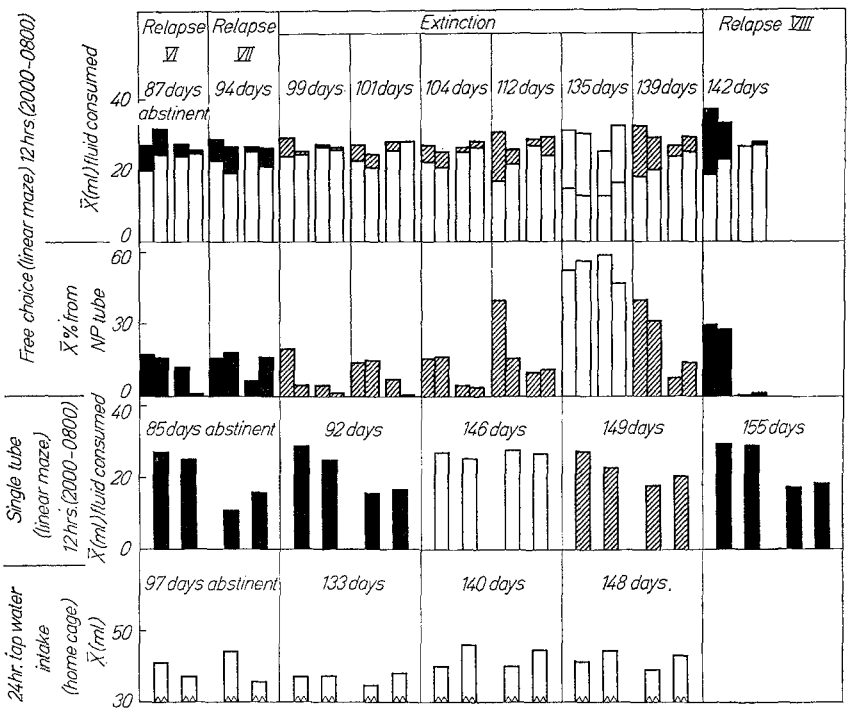

Fig. 2 b

The subgroup data acquired during the "extinction" procedures and on the eighth "relapse" test show the same general trends already discussed for the combined experimental and combined control groups, and also show that the acquired "aversion" to the anise-flavor was extinguished earlier in the MTF than in the MPF subgroup. As between the STF and SPF subgroups, no noticeable differences were observed in any of the tests.

(iii) Forced drinking tests (without prior water-deprivation). These tests (Figs. 2 a and 2 b) reveal how much of each fluid used in the various phases of the study the four subgroups were capable of drinking (from the originally "non-preferred" tube only) when they had no "choice". On the first forced ETZ[5]-F drinking test (10th post-injection day), the MTF and MPF subgroups drank comparable amounts, which were twice those of the STF and SPF subgroups (likewise comparable with each other). On the three additional tests of forced drinking of ETZ[5]-F (85th, 92nd and 155th post-injection day), each subgroup drank approximately the same as its paired subgroup, but the volumes consumed by MTF and MPF were about one-third again as large as those consumed by STF and SPF. Comparison of the last forced ETZ[5]-F drinking test with the

immediately preceding tests of forced drinking of H_2O and H_2O-F (Fig. 2b) reveals that while the consumption of ETZ[5]-F by the MTF or MPF subgroup was not less great than its consumption of either H_2O or H_2O-F, the consumption of ETZ[5]-F or H_2O-F by the STF or SPF subgroup was about one-third less than its consumption of H_2O. These differences are especially noteworthy since 24-hour *ad libitum* drinking of tap water (home cage) was virtually the same for all subgroups. Noteworthy also is the vastly greater amount of ETZ[5]-F consumed by the STF and SPF subgroups in the forced drinking tests than in the "free choice" tests for "relapse" in which H_2O was also available (in the "preferred" tube), contrasting with the relative intake of ETZ[5]-F by the MTF and MPF subgroups under these conditions. Thus, the ratio of ETZ[5]-F consumed by "constraint" (10th post-injection day) to that consumed "by choice" (9th post-injection day) was about 5:1 for STF or SPF, and 8:3 for MTF or MPF (Fig. 2a), and for the 155th ("constraint") and 142nd ("choice") post-injection days, the corresponding constraint-to-choice ratios for the control animals approached infinity, whereas for the experimental animals they were only about 2:1 (Fig. 2b).

Table 5. *Study 1. Supplementary anise-flavor control study. Drinking patterns after "pseudo-training" (forced drinking of H_2O-F from "non-preferred" tube) and termination of injections (morphine, M; saline, S)*

		"Free choice" (H_2O in "preferred" or H_2O-F in "non-preferred" tube) 2000-0800			
		9 days after end of injections		23 days after end of injections	
		Total volumes of fluids ingested $\bar{x}$ (ml)	Percent ingested in form of H_2O-F $\bar{x}$ (%)	Total volumes of fluids ingested $\bar{x}$ (ml)	Percent ingested in form of H_2O-F $\bar{x}$ (%)
Groups	M ($N = 4$)	37.0	33.8	29.7	34.8
	S ($N = 4$)	31.8	55.7	31.9	50.6
Diff. (M − S)		5.2	−21.9*	−2.2	−15.8

* $p < .05$.

(iv) Supplementary Study. The results obtained in the two "free choice" tests (9th and 23rd post-injection days) in the supplementary "Anise-Flavor Control" study are shown in Table 5. In both tests, M rats consumed about one-third, and S rats about one-half of total fluids in the form of H_2O-F. These data indicate that *per se*, anise-flavor holds no particular attractiveness to M rats (as compared with S), and that in the main study, S rats (STF and SPF) developed an absolute aversion to H_2O-F through its association with etonitazene, whereas in M rats (MTF + MPF) such aversion, if present, was not sufficient to inhibit

consumption of ETZ[5]-F in relatively large amounts (compared to S) on the first, second and eighth "relapse" tests (Table 4).

Study 2

The purposes of this second study were to test the possibilities that 1., the failure of the MTF subgroup to exceed the MPF subgroup in "free choice" consumption of ETZ[5]-F on "relapse" tests in the first study was due to a "ceiling" set on consumption of anise-flavored drinking fluid by the mildly aversive properties of anise-flavor for post-addict rats which was revealed in the supplementary "Anise-Flavor Control" study (see above); and 2. that the higher "wet dog" shake frequencies exhibited by post-addict rats in the linear mazes (compared with frequencies in the home cages) in the first study was due, not to the presumed classical conditioning process, but to some unknown irrelevant property of the linear maze.

Methods

To these ends, the first study was replicated with the following modifications:

1. Anise-flavor was eliminated entirely.

2. Provision of cues for discriminating the drinking tube containing etonitazene from that containing distilled water in the linear mazes was accomplished by painting the inner walls of one end-compartment black and fitting it with a graduated glass drinking tube outside, the "well" of which protruded inside; the inner walls of the other end-compartment were painted white, as was a strip of corrugated aluminium fixed to the floor extending from the portal to a smooth metal ramp leading to a metal drinking "spout" inside, which was connected with a graduated glass drinking tube outside.

3. Throughout the six-week "training' period and in all "relapse" tests, the drinking fluid containing etonitazene was placed in a given end-compartment (and distilled water in the other) for a given rat, although initially (prior to "training"), all rats were assigned randomly to one or the other end-compartment drug conditions (etonitazene solution or distilled water).

4. The middle compartment was fitted with a large custard bowl containing tap water for drinking purposes, and all "trained" rats were confined therein (portals to both end-compartments closed) at all times except from 2000 to 0800 on "training" nights, when they were confined to one or the other end-compartment, and on "relapse" test nights, when both portals were open (tap water bowl removed from middle compartment). Otherwise, "training" and "relapse"-testing were conducted on two subgroups exactly as in the first study (except for omission

of "extinction" and forced drinking trials after termination of injections): MT (morphine-injected, trained) and ST (saline-injected, trained), corresponding to MTF and STF in the first study.

5. In addition, two other subgroups remained in home cages throughout the six-week period (tap water and food available *ad libitum* without any "training": MU (morphine-injected, untrained) and SU (saline-injected, untrained). For "relapse"-testing (in the linear mazes) assignment of the end-compartment drug condition (etonitazene solution or distilled water) for a given rat was made according to a table of random numbers prior to the first "relapse" test and remained the same for each rat throughout the remainder of the study. Like MT and ST, these rats resided in their individual home cages between "relapse" tests, as in the first study.

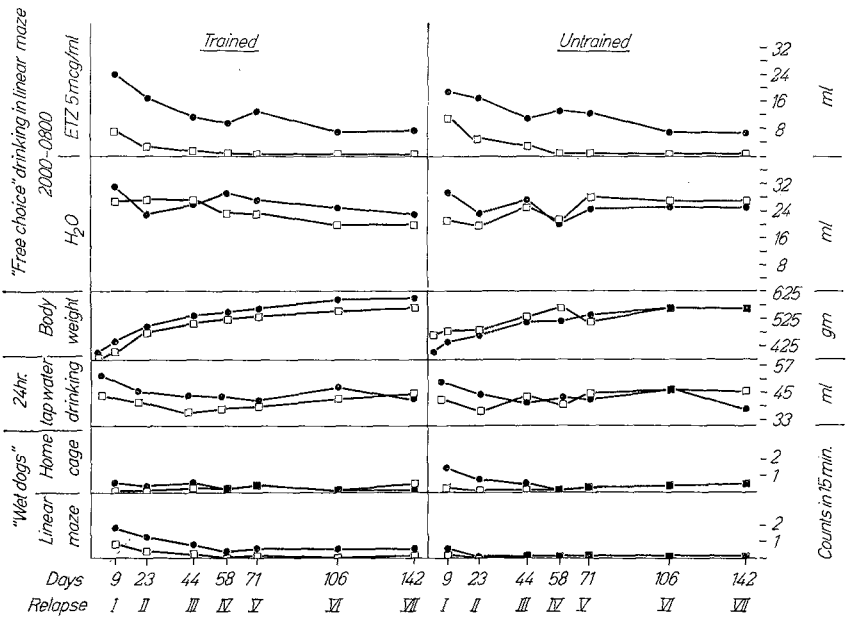

Fig. 3. Study 2. Behavior of individual subgroups after termination of injections. MT and MU, dots; ST and SU, squares. See text for explanation of symbols for subgroups

Results

Although this second study was begun with 14 animals in each subgroup (56 in all), three successive replications with new animals (male Wistar rats, 4 months old) had to be made before the cumulated Ns were sufficient for unequivocal statistical evaluation of near-significant trends, because of the deaths of numerous animals during the prolonged addicting, "training" and "relapse"-testing procedures.

Table 6. *Study 2. Analysis of variance (mixed type) for "wet dog" frequencies after termination of injections*

Relapse test No	I		II		III		IV—VII	
Days after end of injections	9		23		44		58—142 days, combined	
Source	df	MS	df	MS	df	MS	df	MS
Training (MT + ST *vs* MU + SU)	1	11.10**	1	4.15**	1	3.56	1	0.09
Previous addiction (MT + MU *vs* ST + SU)	1	25.76**	1	10.35**	1	2.96*	1	0.13
Error, between subjects	101	1.02	100	0.52	68	0.60	44	0.11
Between subjects	104	1.32	103	0.63	71	0.62	47	0.11
Within subjects	105	0.73	104	0.30	72	0.29	48	0.10
Place of abstinence L.M. (MT and ST) + H.C. (MU and SU) *vs* H.C. (MT and ST) + L.M. (MU and SU)	1	15.47**	1	4.92**	1	3.06**	1	1.05**
Training × abstinence place	1	3.61**	1	0.77+	1	0.00	1	0.01
Previous addiction × abstinence place	1	6.70**	1	4.12**	1	1.01*	1	0.15
Error, within	101	0.50	100	0.22	68	0.24	44	0.08
Total	209	1.02	207	0.46	143	0.45	95	0.11

MT = previously injected with morphine, "trained". MU = previously injected with morphine, "untrained". ST = previously injected with saline, "trained". SU = previously injected with saline, "untrained". L.M. = Linear maze. H.C. = Home cage.

* $p < .05$. — ** $p < .01$. — + $p < .05$ only by one-tailed test.

Table 7. *Study 2. Drinking behavior in "free choice" relapse tests*

Relapse test No.	I			II			III			IV—VII		
Days after termination of injections	9			23			44			58—142		
Subgroups	Mean percent total fluids (H_2O + ETZ[a]) ingested in form of ETZ[a]											
	N	$\bar{x}$ % S.D.		*N*	$\bar{x}$ % S.D.		*N*	$\bar{x}$ % S.D.		*N*	$\bar{x}$ % S.D.	
MT	21	43.7 ± 25.54		20	41.9 ± 31.43		17	27.3 ± 33.03		11	19.4 ± 25.56	
ST	31	21.0 ± 19.38		31	8.1 ± 10.18		17	4.8 ± 5.77		10	1.7 ± 1.98	
MU	16	43.8 ± 37.41		16	42.4 ± 33.97		14	29.1 ± 26.50		10	27.8 ± 32.71	
SU	37	34.0 ± 31.92		37	21.1 ± 29.60		24	9.8 ± 21.50		17	7.2 ± 23.20	
Comparisons	Differences											
	N	*D* %	*t*	*N*	*D* %	*t*	*N*	*D* %	*t*	*N*	*D* %	*t*
MT *vs* ST	52	22.7	3.45***	51	33.8	4.65***	34	22.5	2.77***	21	17.7	2.29*
MU *vs* SU	53	9.8	0.91	53	21.3	2.18*	38	19.3	2.32*	27	20.6	1.75+
MT *vs* MU	37	−0.1	−0.01	36	−0.5	−0.05	31	−1.8	−0.17	21	−8.4	−0.65
ST *vs* SU	63	−13.0	−2.06*	68	−13.0	−2.50**	41	−5.0	−1.09	27	−5.5	−0.97

* $p < .05$. — ** $p < .02$. — *** $p < .01$. — + $p < .05$ by one-tailed test only (see Table 6 for designations of Subgroups).

The final results (initial and all three replications combined) of this second study are shown in Fig. 3 and Tables 6 and 7. Consistent with the results of the first study (see above) is the finding that at least on the first and second "relapse" tests, "wet dog" shake frequencies of postaddict rats were higher in the "place of abstinence"—i.e., the linear maze for MT, and the home cage for MU (Fig. 3, "wet dogs"). While ST (but not SU) showed an analogous trend, a mixed type of analysis of variance (EDWARDS, 1957) revealed significant "previous addiction × abstinence place" interactions through the third "relapse" test on the 44th post-injection day (Table 6). Also, postaddict rats (MT + MU) displayed higher total "wet dog" shake frequencies than control rats (ST + SU) through the 44th post-injection day (Table 6, previous addiction).

Likewise consistent with the results of Study 1 are the "free choice" drinking patterns of the various subgroups in the "relapse" tests (Fig. 3). Although in these tests MT and MU consumed about the same volumes of distilled water as ST and SU, each of the postaddict groups drank much more of the etonitazene solution than their respective nonaddict controls, and for MT *vs* ST this difference was significant (by Student's "*t*" test for independent groups) on every "relapse" test through the 142nd post-injection day (Table 7). In the case of MU *vs* SU, the differences were also significant on every "relapse" test (on Relapse IV—VII by a one-tailed test) except the first when, apparently, the "untrained", naive control rats had not had an opportunity to develop aversion to the etonitazene solution (contrast with lower etonitazene consumption by ST on Relapse I, Fig. 3). However, such aversive "learning" was quickly demonstrated by SU in subsequent "relapse" tests in which their "free choice" consumption of etonitazene, like that of ST, fell to very low levels. Again consistent with the results of Study 1 is the absence of evidence for positive effects of previous etonitazene-reinforcement during "training" upon subsequent "free choice" consumption of etonitazene solution in "relapse" tests, as revealed by comparison of postaddict groups MT and MU, the differences between which were non-significant throughout (Table 7).

Discussion

The results obtained in both studies lend strong support to the hypothesis (WIKLER, 1948) that at least some morphine-abstinence phenomena are conditionable according to the classical (Pavlovian) paradigm. In terms of the murine "wet dog" phenomenon, such conditioning may be viewed as a process of conditioned facilitation—i.e., "association" (through neural mechanisms still not understood) of "place of abstinence" (conditioned stimulus) with the unconditioned facilitatory effect of "primary" morphine-abstinence upon the unconditioned "wet dog" response

to the standardized handling procedure (unconditioned stimulus). As a result, facilitation of the "wet dog" response occurs as a conditioned phenomenon on return to the earlier "place of abstinence" even long after the "primary" morphine-abstinence syndrome has subsided.

If it is assumed (this remains to be demonstrated) that conditionable opioid abstinence phenomena are mediated by neural systems that are also involved in the generation of that "drive" state experienced by opioid addicts as "craving" for opioids then, as noted in the Introduction, the process of conditioned facilitation of opioid-abstinence phenomena could play an important role in generating relapse of "cured" opioid addicts *provided* that 1. such persons had been acutely abstinent on repeated occasions under more or less constant environmental conditions during previous episodes of physical dependence and are returned to those environmental conditions after "cure"; and 2. they had previously learned the "problem-solving" technique (active acquisition and self-administration of opioids when acutely abstinent) for reducing unconditioned and/or conditioned "craving".

The experimental designs employed in the studies reported here met both provisos but surprisingly, the results indicate that regardless of "training" conditions, previous morphine-addiction alone is sufficient to dispose rats to "relapse", as judged by comparison of their "free choice" consumptions of etonitazene solution with those of non-addicted control rats over substantial period of time following termination of morphine or saline injections respectively. Presumably, this factor was sufficiently potent to obscure whatever differential effects environment-specific conditioning of "wet dog" shakes and/or prior etonitazene reinforcement may have had on the performance of postaddict rats in the "relapse" tests. In retrospect, the "relapse" testing method employed may not have been appropriate for detection of such differential effects inasmuch as the nocturnal 12-hour drinking period was sufficiently long to allow adaptation of the "wet dog" response (cf. MARTIN *et al.*, 1963) and even "self-education" of "pseudo-trained" or "untrained" postaddict rats in regard to the reinforcing properties of etonitazene (see below).

Incidentally, it should be noted that for nonaddict control rats, repeated exposure to the etonitazene solution had aversive effects, as indicated by the rapid decline in their consumption of the drug solution "by constraint" and by "free choice" (see Results, above). To a lesser degree, such an aversive trend was also manifested by the postaddict rats; indeed, their behavior vis-à-vis etonitazene solution is better described as a "lesser aversion" than as a "preference", for in no "relapse" test did they consume larger volumes of the etonitazene solution than of water "by choice".

The basis of such "lesser aversion" to etonitazene on the part of postaddict rats is to be sought in their histories of previous addiction to morphine. Inasmuch as morphine-tolerant rats are cross-tolerant to etonitazene (WIKLER *et al.*, 1963), the "free choice" drinking behavior of postaddict rats might be explained on the basis of residual cross-tolerance. However, such a seemingly simple explanation actually begs the question, since in the case of opioid drugs, tolerance and physical dependence develop *pari passu* and the underlying mechanisms of the two phenomena may very well be the same; hence, it would be equally justifiable to invoke residual physical dependence as an explanation, and indeed, the "lesser aversion" of postaddict rats to etonitazene may plausibly be ascribed to relatively long persistence of disturbed homeostasis in consequence of previous tolerance to *and* physical dependence on morphine. In the case of man, evidence for long-lasting disturbances of homeostasis, appearing phenomenologically as persistence of low-grade morphine-abstinence signs, has been adduced by HIMMELSBACH (1942). In the case of the rat, the post-withdrawal disturbances in homeostasis appear to be of two qualitatively different sorts: those manifested by the signs of the early or "primary" abstinence syndrome, which is of short (3—5 days) duration, and those manifested by the signs of the later, or "secondary" abstinence syndrome, which may persist up to 6 months following abrupt withdrawal of morphine from a daily addiction dose level of 320 mg/kg (MARTIN *et al.*, 1963). In the present studies, a lower daily addiction dose level of morphine was used (200 mg/kg) and no measurements were made of several of the signs of "secondary abstinence" (colonic temperature, metabolic rate, "activity") but nevertheless suggestive evidence of "secondary" abstinence syndromes was obtained in both studies, namely, long-lasting elevation of home-cage "wet dog" frequencies in the first study and of home-cage tap water consumption in the second study (Figs. 1 a,1 b and 3). Unfortunately, no studies have been made on the effects of morphine or etonitazene upon the signs of "secondary abstinence", and therefore it cannot be assumed that the etonitazene-drinking of the postaddict rats was governed by a homeostatic drive, but this hypothesis does fit the data that are available. Persistence of an unconditioned homeostatic drive long after withdrawal of morphine could provide a source of reinforcement for postaddict rats even in the absence of the theoretically postulated classically conditioned drive, and would explain the "relapsing" behavior of the "pseudo-trained" and "untrained" postaddict rats under the particular experimental conditions employed, which provided 12 continuous hours for such "learning" to take place in each "relapse" test.

Insofar as patterns of "free choice" opioid-drinking behaviors are concerned, the results of the studies reported here are in agreement

with those of NICHOLS *et al.*, (1956) and DAVIS and NICHOLS (1962), to the extent that rats without previous physical dependence on morphine showed no disposition to "relapse" even if they had been forced to drink substantial quantities of an opioid solution repeatedly (etonitazene, 5 mcg/ml in our experiments or morphine, 0.5 mg/ml in those of NICHOLS and associates) prior to the "free choice" tests. However, in contrast to our findings, NICHOLS and associates (*loc cit.*) reported that postaddict rats that had not been forced to drink morphine solution repeatedly while presumably still physically dependent on morphine, consumed significantly less of the morphine solution in "free choice" tests (morphine, 0.5 mg/ml or water) than postaddict rats that had been so forced (in "training cycles"), over periods of up to 6 or 7 weeks following withdrawal of morphine. Though our methods differed in other ways as well (e.g., use of severe water-deprivation by NICHOLS and associates to force rats to drink morphine solution during "training"), the differences in our results with regard to "free choice" drinking behavior of "untrained" postaddict rats may be due to the far greater aversive properties of the morphine solution used in their experiments than those of the etonitazene solutions used in ours. Thus, without prior water-deprivation, normal rats drink volumetrically as much as, and acutely morphine abstinent rats much more etonitazene solution (5 or 10 mcg/ml) than water when offered a single tube containing one or the other fluid for 17 hour periods (WIKLER *et al.*, 1963) but they reject morphine (0.5 mg/ml) completely under comparable conditions (WIKLER and PESCOR, unpublished data). Presented with this concentration of morphine for the first time and having a "choice" between it and water (as in the "choice" tests of NICHOLS *et al.*, 1963), postaddict rats would not be likely to sample enough of the morphine solution to permit reinforcement through reduction of a long-persisting homeostatic drive. The experiments of NICHOLS and associates, however, do demonstrate more clearly than our own that such reinforcement is powerful, as it is sufficient to overcome the strongly aversive properties of the morphine solution, as in the case of the postaddict rats that were forced to drink the morphine solution (by prior water-deprivation) before the "choice" tests were initiated (NICHOLS *et al.*, 1956; DAVIS and NICHOLS, 1962). In the human situation, too, morphine has aversive properties, not only of pharmacological (e.g., emetic effect) but also of social origin, and therefore the experimental design of NICHOLS and associates, while tending to conceal the importance of long-persisting homeostatic disturbances following withdrawal of morphine, does make manifest the importance of reinforcement in the genesis of relapse.

As for evaluation of the importance of classically conditioned abstinence phenomena (and the inferred "conditioned drive") in the genesis

of relapse, a method permitting a more time-locked stimulus-response criterion than drinking behavior is needed. Perhaps the intravenous self-injection technique developed by WEEKS (1962) may lend itself to this purpose.

Summary

1. For 6-week periods in two studies, rats made tolerant to and maintained on intraperitoneal injection of morphine (200 mg/kg) once daily in the morning resided on alternate nights in one end of a 3-compartment linear maze with water for drinking and on the intervening nights in the other end-compartment with etonitazene (10 mcg/ml) for drinking. On this schedule, temporal contiguity was provided between the unrelieved nocturnal "primary" morphine abstinence syndrome (including elevated frequency of "wet dog" shakes) and the specific environment of the water-end of the linear maze, while in the other end, opportunity was provided for reinforcement of etonitazene-drinking through reduction of the nocturnal "primary" morphine abstinence syndrome. Saline-injected normal rats were trained identically except that the concentration of etonitazene was 5 mcg/ml. In one study, the etonitazene solution was tagged with anise-flavor while in the other study, tactile-visual cues were used. Also, other morphine-tolerant and normal rats were maintained on intraperitoneal injections of morphine (200 mg/kg) or saline respectively once each morning for 6 weeks in home cages without any training.

2. At the end of the 6-week periods, all injections were terminated and all rats in linear mazes were transferred to home cages. On test days at intervals of one or more weeks thereafter, the previously morphine-injected rats exhibited higher "wet dog" shake frequencies in their former "abstinence places" (linear maze or home cage) over periods of 155 and 44 days after termination of injections in the two studies, while conditions of previous housing were not systematically related to "wet dog" shake frequencies in the previously saline-injected normal rats.

3. In "free choice" tests (etonitazene, 5 mcg/kg, *versus* water) conducted on the nights of the same test days, the previously morphine-injected rats (both studies) drank more of the etonitazene solution than the previously saline-injected normal rats up to 58 days after termination of injections in one study and 44 days in the other, but "trained" and "untrained" previously morphine-injected rats did not differ significantly from each other in this regard.

4. It is concluded that although classical conditioning of morphine-abstinence phenomena (and by inference, "craving" for the drug) is demonstrable, the pre-potent factor in disposing to relapse, at least in the rat under the experimental conditions described, is the long-term

persistence of unconditioned disturbances in homeostasis following withdrawal of morphine which can provide a source of reinforcement for operant conditioning of opioid-seeking behavior during "relapse-testing" sessions even without benefit of previous "training".

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