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Morphine-induced kinetic alterations of choline acetyltransferase of the rat caudate nucleus

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Summary

1. In order to explain the decrease of choline acetyltransferase (2.3.1.6.) activity observed in the caudate nucleus of morphine-treated rats, partially purified preparations of the enzyme were used in kinetic studies, with choline as substrate.

2. The apparent Michaelis constant for the enzyme obtained from normal rats was found to be 0·9 mM choline; this value doubled when the animals were killed one hour after a single injection of morphine (30 mg/kg). When the rats were injected daily for 4 or 15 days, and killed one hour after the last injection, the apparent *Km* value was 2·1 mM in each case. Prolonged daily treatment with morphine, followed by 48 h withdrawal, or by administration of 4 mg/kg of naloxone (given half an hour after the last injection of morphine) resulted in apparent *Km* values of 1·3–1·5 mM of choline, suggesting a gradual return to the lower, normal substrate requirement. *Vmax* changes were insignificant.

3. The effect of morphine added *in vitro* to different enzyme preparations was also studied. The *Km* values of 0·9 mM, in the enzyme isolated from normal rats, increased to 2·0 after incubation *in vitro* with 12·5 mM morphine. Similar increases were found in enzymes obtained from rats 48 h after the withdrawal of morphine or from rats injected with naloxone after prolonged morphine treatment. The high apparent *Km* values, found in enzyme obtained from animals killed one hour after the last dose of morphine, did not change upon incubation with 12·5 mM morphine. A similar pattern of *Km* changes was noticed after incubation with 25 mM acetylcholine.

4. An increase of 32% in acetylcholine (ACh) level was found in the caudate nucleus one hour after subcutaneous injection of 30 mg/kg of morphine. Return to normal values was observed when morphine was administered daily. After two to three weeks of daily treatment and subsequent withdrawal from morphine for 48 h, the levels of ACh were normal. If the daily treated rats were given naloxone within half an hour of the last injection of morphine, and killed 30 min later, the levels of ACh remained normal.

5. Fifty per cent inhibition of enzyme activity was observed upon *in vitro* incubation with 75 mM acetylcholine, or with 25 mM morphine. The same degree of inhibition was noticed when the enzyme was obtained from normal or from morphine-treated rats.

Introduction

An increase in total acetylcholine (ACh) content of the rat brain, after a single morphine administration, has been reported by Giarman & Pepeu (1962). More recently, Large & Milton (1970) published a similar observation of increased ACh levels in the whole brain of rats, recorded after a single injection of morphine. They also report a return to normal levels in animals treated daily with the drug.

However, the activity of the enzyme involved in the synthesis of ACh was found to be considerably lower after a single injection of morphine, when tested in the caudate nucleus area (Thal & Wajda, 1969). Sharkawi (1970) also reported a decreased ability to form ACh in the cortical slices obtained from morphine-treated rats. When choline acetyltransferase activity was assayed in the caudate nucleus, thalamus and cortex, after acute or prolonged treatment with morphine (Datta, Thal & Wajda, 1971), it was found that the enzyme activity decreased significantly in the caudate nucleus after a single dose of morphine and returned to normal within four days in chronically treated rats. Such a decrease in the ability to synthesize neurohormone could result from an accumulation of ACh, since the inhibition of ACh release by morphine in the intestinal wall (Shaumann, 1957 ; Paton, 1957) or in the brain (Beleslin & Polak, 1965), has been described. An accumulation of the product of the reaction could result in feedback inhibition of the enzyme. In the present study, total ACh content in the striatal area, and *in vitro* effects of ACh on the purified enzyme were taken into account (Datta & Wajda, 1970). Since the decrease in enzyme activity after morphine administration could also be produced by a direct inhibitory effect of the narcotic on choline acetyltransferase, this possibility, although less probable, was tested by studying the direct inhibitory action of morphine on the enzyme. Assuming that morphine could also directly influence the enzymatic properties of choline acetyltransferase, the enzyme kinetics were examined by determining the values of apparent *Km* and *Vmax* of choline acetyltransferase with choline as the substrate. The enzyme was obtained from caudate nuclei of normal or of morphine-treated rats (Wajda & Datta, 1970).

Methods

Animals used

Female rats of the Wistar strain, weighing 190–210 g, were injected subcutaneously with 30 mg/kg of morphine base. In prolonged experiments, the first dose was lowered to 20 mg/kg to reduce mortality ; the subsequent injections of 30 mg/kg were given daily, during morning hours. Unless otherwise stated, the rats were killed one hour after the last injection of morphine. Daily injections were repeated for 4 days and for 15 days. In each particular experiment groups of 8–10 rats were used. One group treated daily for 4 days was withdrawn from the drug for 24 hours. Another, treated daily for 15 days was withdrawn from morphine for 48 hours. Still another group treated for 15 days with morphine, was given naloxone hydrochloride (4 mg/kg, subcutaneously) 30 min after the last injection of morphine, and the rats were killed 30 min later. Each such experiment was repeated three and sometimes four times. The animals were killed by decapitation, and the caudate nucleus area was dissected on ice.

Preparation of the enzyme

Choline acetyltransferase was partially purified according to the method of Potter, Glover & Saelens (1968), up to step 3, and was finally suspended in 200 mM KCl. This preparation is stable for one week when kept at 4° C. Further purification was not attempted, owing to the scarcity of the original material.

Enzyme assay

The activity of choline acetyltransferase was determined by the modified method of Potter *et al.* (1968), as described previously by Datta, Thal & Wajda (1971). When the inhibition by morphine or acetylcholine was studied, the solutions of the inhibitors were added to the incubation mixture prior to the addition of the enzyme, and equivalent amounts of water were used in the control tubes. Kinetic studies were performed with varying concentrations of choline chloride; the concentration of acetyl coenzyme A was constant; the incubation was carried out at 37° C for 10 minutes. The *Km* and *Vmax* values were calculated from Lineweaver-Burk plots; the results were confirmed by the Dowd & Riggs (1965) method of calculation.

Acetylcholine assay

Total acetylcholine content of the rat striatum was estimated by biological assay. The method used was similar to that described by Edge (1970).

Rana pipiens (males of medium size—4 inches long) were kept at 4° C until used. The rectus abdominis muscle was dissected and placed in a 3 ml bath filled with frog-Ringer solution through which oxygen was bubbled. The composition (g/l) of the frog-Ringer was as follows: NaCl, 6·50; KCl, 0·14; $CaCl_2$, 0·12; $NaHCO_3$, 0·20; NaH^2PPO^4, 0·01 and glucose 2·0; shortly before use, physostigmine salicylate was added (0·01 mM final concentration). The pH of the Ringer was 7·8. The assays were conducted at room temperature, and the muscle movements were recorded with a lightly weighted frontal lever with about 5 times magnification. A 5 min interval between individual contractions was used, and the test solutions were in contact with the muscle for 1 min; after this time, with the kymograph switched off, the test solutions were replaced twice with fresh frog-Ringer. The 50% response of the maximal effect obtainable, bracketed between two doses of standard, was taken for final estimation of the potency of the unknown.

Total acetylcholine content was estimated in the striatum of normal and morphine-treated rats, using two animals for each estimation. The caudate nuclei (about 150 mg wet weight) were dissected immediately after decapitation, with all the material kept on ice. They were homogenized in glucose-free frog-Ringer. The pH was 3·0 and the Ringer contained 0·1 mM of eserine. Total acetylcholine content was liberated by placing the tubes in a boiling water bath for 5 minutes. After centrifuging and washing the precipitate twice, the supernatants were combined and kept at 4° C till used. On the day of the assay, they were neutralized and made up to the desired volume.

Results

Inhibition of choline acetyltransferase by morphine in vitro

Partially purified preparations of choline acetyltransferase were incubated with different concentrations of morphine (Table 1). In all experiments, the enzyme

was obtained from the striatal areas of the brain of normal and morphine-treated rats. The concentrations of morphine used were high, but it is evident that the degree of inhibition of enzyme activity increased gradually with increasing concentrations of morphine. This effect, however, was identical in all preparations used, regardless of the treatment of animals. It also became clear that the concentration of morphine in brain necessary to inhibit the enzyme activity was too high to be attained by the injection of animals with 30 mg/kg of morphine.

Inhibitory effect of ACh on choline acetyltransferase

Taking into account that the enzyme might be inhibited directly by ACh, different concentrations of ACh were tested. It is known that total ACh content is increased up to 47% in the brain of rats injected with 50 mg/kg of morphine (Giarman & Pepeu, 1962). More recently Kaita & Goldberg (1969) reported inhibitory effects of ACh on choline acetyltransferase. Table 2 shows the results of *in vitro* addition of ACh in four consecutive concentrations to the partially purified preparations of the enzyme. As in the experiments with morphine, the increasing concentrations of acetylcholine resulted in a graded response and the degree of inhibition was the same in all differently treated groups.

TABLE 1. In vitro *effect of morphine on choline acetyltransferase of caudate nucleus of normal, morphine and naloxone treated rats*

	Percentage inhibition of enzyme activity						
		Morphine treated for:			Withdrawn from morphine for:		
Final concentration of morphine (mM)	Normal	1 h	4 days	15 days	24 h (4 days)	48 h (15 days)	Naloxone* treated
1·0	6	6	4	5	4	7	—
5·0	13	19	19	19	12	20	24
10·0	26	27	31	28	28	29	31
25·0	50	48	55	50	41	48	69

Partially purified preparations of choline acetyltransferase, as described in **Methods**, were used. All estimations were done in triplicate, and the values are a mean of 3 to 4 different experiments. Unless otherwise stated, all rats were killed 1 h after the last morphine injection (30 mg/kg of morphine base). * Naloxone hydrochloride (4 mg/kg) was given 30 min after the last injection of morphine and the rats were killed 30 min later. The columns indicating withdrawal from morphine give the days of morphine treatment in parentheses, and the time elapsed after the last injection in hours (24 h and 48 h).

TABLE 2. In vitro *effect of acetylcholine on choline acetyltransferase obtained from normal, morphine and naloxone treated rats*

	Percentage inhibition of enzyme activity						
		Morphine treated for:			Withdrawn from morphine for:		
Final concentration of ACh (mM)	Normal	1 h	4 days	15 days	24 h (4 days)	48 h (15 days)	Naloxone treated
10·0	12	6	7	8	7	9	11
25·0	20	16	16	14	15	20	17
50·0	27	25	24	32	27	31	35
75·0	53	58	55	48	55	56	—

The treatment of animals was as described in the Legend to Table 1. The values represent the average of 3 to 4 different experiments, and each time the enzyme was assayed in triplicate.

Total ACh content in the striatum of morphine-treated rats

Since ACh was found to exert an inhibitory effect on choline acetyltransferase, it was of interest to establish the levels of the neurohormone in the caudate nucleus of morphine-treated rats. It was found (Table 3) that only one hour after a single injection of morphine the levels of acetylcholine in the caudate nucleus were significantly increased in comparison with the normal; the increase was 32% of normal values. After prolonged morphine treatment, the acetylcholine content returned to normal; 48 h after withdrawal from daily morphine treatment, or after naloxone administration, normal, or even slightly lower acetylcholine levels were observed respectively.

Since it has been found (Datta *et al.*, 1971) that the enzyme shows lower activity after a single injection of morphine when acetylcholine content is high, and also after withdrawal from morphine treatment, when the acetylcholine content was found to be normal, another reason for the decrease of choline acetyltransferase activity, in animals withdrawn from morphine, was sought.

TABLE 3. *Effect of morphine and naloxone on total acetylcholine content of the caudate nucleus of the rat*

Exp.	Drug	Treatment	ACh in nM/g* of wet weight	No. of exps.	*P*
1	—	—	28·5±0·88	12	$0{\cdot}01>P<0{\cdot}001$
	Morphine	Single injection	36·2±1·29	12	
2	—	—	30·6±1·10	6	$<0{\cdot}4$
	Morphine	3-4 weeks of daily treatment	30·9±1·08	6	
3	—	—	29·7±0·49	13	$<0{\cdot}9$
	Morphine	48 h withdrawal after 2-3 weeks of daily treatment	31·4±0·99	14	
4	—	—	28·9±0·63	12	$<0{\cdot}2$
	Morphine plus Naloxone	3-4 weeks daily One single dose	27·4±1·30	16	
5	—	—	30·6±1·11	6	$<0{\cdot}4$
	Morphine	48 h withdrawal after high doses	30·8±1·08	6	

* Mean±S.E.M. The experimental conditions were the same as those described in Table 1, except for experiment No. 5, where 10 daily injections of morphine were followed for an additional 10-15 days, with doses of morphine (30 mg/kg at 9.00 a.m. and 50 mg/kg at 5.00 p.m.) resulting in a daily intake of 80 mg/kg. Saline treated animals were used as controls with every estimation. Each experiment represents the combined material from two rats and the estimations of ACh were in duplicate.

TABLE 4. *Kinetic constants of choline acetyltransferase obtained from caudate nucleus of normal, morphine and naloxone treated rats*

Treatment of animal	*Km* (mM)	*Vmax* (μmol of ACh/mg protein)/h
Normal	0·883±0·05	1·32±0·03
1 h morphine treated	1·86 ±0·16	1·90±0·07
4 days morphine treated	2·10 ±0·07	2·07±0·09
15 days morphine treated	2·12 ±0·11	1·79±0·08
4 days morphine treated—24 h withdrawn	1·53	1·55
15 days morphine treated—48 h withdrawn	1·49 ±0·18	1·30±0·07
15 days morphine treated—naloxone (4 mg/kg) injected	1·33	1·41

Groups of 1 h, 4 days and 15 days were killed 1 h after the last subcutaneous injection of 30 mg/kg of morphine. The last group was given naloxone subcutaneously 30 min after the injection of morphine and the rats were killed 1 h after the last dose of morphine. Each figure is the average of four different enzyme preparations, each of them tested twice. The kinetic constants were determined using choline as substrate.

Kinetic studies on choline acetyltransferase

The *in vitro* effect of morphine on the kinetic parameters of the enzyme were studied by determining the values for apparent *Km* and *Vmax* of partially purified choline acetyltransferase obtained from the caudate nuclei of rats subjected to the different treatments. It was of interest to note that the *Km* values for the enzyme obtained from normal rats agreed with those published by Kaita & Goldberg (1969). As can be seen from Table 4, *Vmax* or molecular activity did not differ significantly from normal; however, the apparent Michaelis constant, or *Km app* of the enzyme, increased significantly in all treated animals. Acute (1 h) and prolonged treatment with morphine (4 days, 15 days), in which the rats were killed one hour after the last injection of morphine, resulted in two- to three-fold increase in *Km* values. It was found, after 15 days of injections, followed by 24 or 48 h withdrawal of the drug, and also after naloxone administration, that the increase of *Km* values was not as great as in the previous groups, but the levels were still higher than normal.

The influence of *in vitro* addition of morphine and acetylcholine on the enzyme kinetics is represented in Table 5. The addition of morphine to the enzyme obtained from normal rats resulted in an increase of *Km* value, which was nearly as high as that noted after morphine administration. When the same procedure was applied to the enzyme obtained from acutely or daily treated rats, the already increased *Km app* did not alter. Fifteen days of treatment with morphine followed by 48 h withdrawal of the drug or naloxone administration, combined with *in vitro* addition of morphine, resulted in a further increase of *Km app* values.

A similar pattern of *Km app* alterations was observed when acetylcholine was used instead of morphine; the changes were, however, on a smaller scale.

Graphic representations of the determination of *Km* and *Vmax* values using a double reciprocal plot are given in Fig. 1, Fig. 2, and Fig. 3. The inhibitory effect of *in vitro* addition of morphine is observed in each case. The values of *Km* obtained by double reciprocal plot were confirmed by the method of Dowd & Riggs (1965), in which the two parameters were calculated from observed data by programming a computer. Since the results obtained by the two methods were nearly identical, we retained the values obtained from the double reciprocal plot.

Discussion

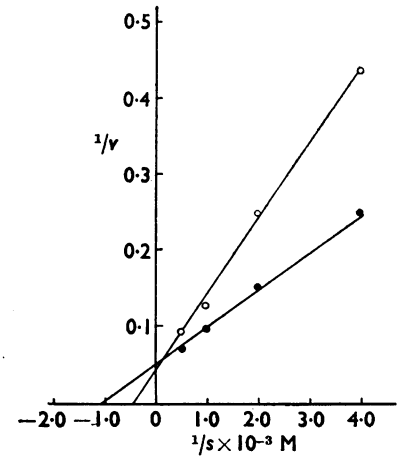
The lower choline acetyltransferase activity in the caudate nucleus of rats treated with morphine was reported previously (Datta *et al.*, 1971). The mechanism by

TABLE 5. In vitro *effect of morphine* (12·5 mM) *and acetylcholine* (25 mM) *on the kinetic constant of choline acetyltransferase of normal, morphine and naloxone treated rats*

Treatment of animals	*Km* (mM)		
	In absence of ACh or morphine	In presence of morphine (12·5 mM)	In presence of ACh or (25 mM)
Normal	0·86	2·0	1·68
1 h morphine treated	1·83	1·80	1·34
15 days morphine treated	1·82	2·0	1·54
15 days morphine treated 48 h withdrawn	1·33	2·22	1·73
15 days morphine treated naloxone (4 mg/kg) injected	1·38	2·36	2·11

The treatment of animals was the same as described in the legends of Table 1 and Table 4. The results are the average of two different experiments performed in duplicate.

FIG. 1. Normal



1 h after morphine treatment

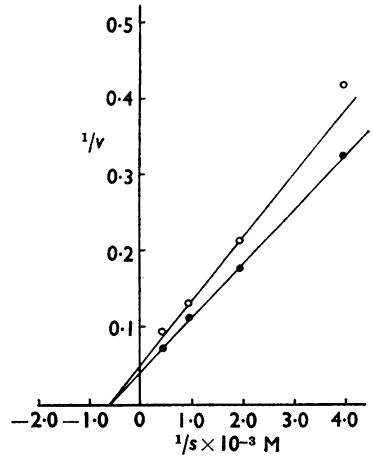
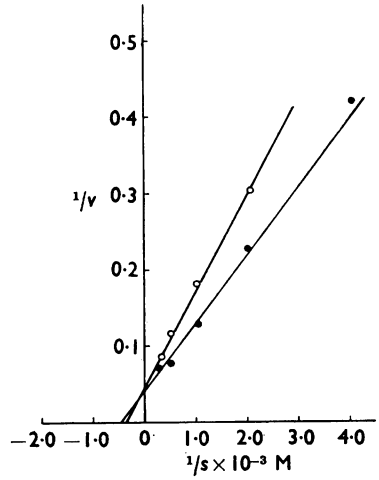


FIG. 2. 4 Days morphine treatment (withdrawn 1 h after last dose)



4 Days morphine treatment (withdrawn 24 h after last dose)

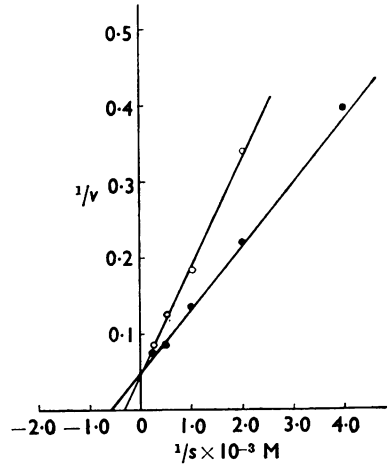
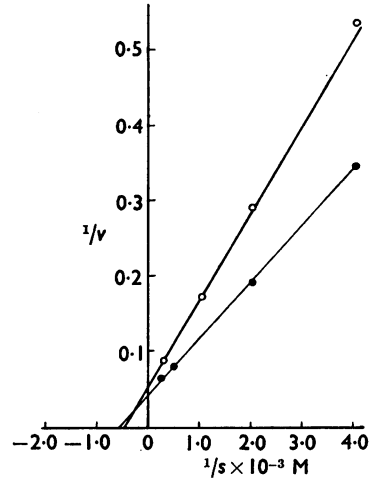
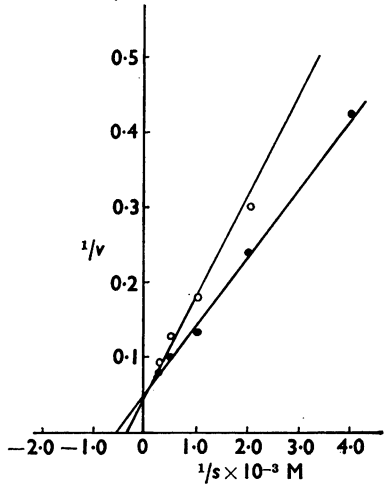


FIG. 3. 15 Days morphine treatment (withdrawn 1 h after last dose)



15 Days morphine treatment (withdrawn 48 h after last dose)



FIGS. 1, 2 & 3. Results given in Table 4 are represented in graphic form, to illustrate the inhibitory effects of additions of morphine (12·5 mM) to partially purified enzyme preparations obtained from normal and morphine-treated rats (as indicated in the headings). Full circles represent in each case the values of control enzyme preparation. The open circles represent experiments where *in vitro* addition of morphine was tested. The *Km* values were determined, varying the concentrations of choline ; the incubation time was 10 minutes.

which morphine changes the enzyme activity is still an open question. The simplest explanation could be provided by proof of a direct inhibitory effect of the narcotic on the enzyme activity. However, only high concentrations of morphine produced measurable inhibitory action when tested *in vitro*. Johannesson & Milthers (1962), found anything from a trace to 4 μg of morphine per g wet weight in the brains of rats injected with 500 mg/kg of morphine, a dose exceeding the one used in this study by about twenty times. Seven mg/kg (base) given subcutaneously to rats results in about 0·3 μg/g of brain content of ^{3}H-dihydromorphine measured 30 min after the injection (Yeh & Woods, 1970). Of course, even such low concentrations as can be produced by 30 mg/kg of morphine, if present in the vicinity of the enzyme *in vivo*, might affect the enzyme molecule.

Another possible direct inhibition could be produced by the neurohormone itself. Like morphine, acetylcholine was found to inhibit equally all choline acetyltransferase preparations irrespective of the previous treatment of rats. The concentration of ACh (75 mM) effective in producing 50% inhibition *in vitro* can exist in the intact synaptosomes, according to Whittaker & Sheridan (1965). Moreover, morphine has been shown to increase the acetylcholine content of the CNS of rats (Giarman & Pepeu, 1962 ; Maynert, 1967 ; Large & Milton, 1970). The inhibitory effect of acetylcholine on choline acetyltransferase, reported by Kaita & Goldberg (1969), could provide a feedback inhibition of the enzyme after morphine injection. This mechanism, postulated in the previous work (Datta *et al.*, 1971) and by Sharkawi (1970), could also contribute to the lowering of normal activity of the enzyme.

It was of interest to establish the levels of acetylcholine in the caudate nucleus of rats treated in various ways with morphine. The total acetylcholine content of the rat striatum 4·89 μg/g wet weight, was found to agree very closely with the values published by Fischer, Westermann & Oelssner (1969) (4·68 μg/g). A 32% increase in acetylcholine content was found after one dose of morphine, and the levels came back to normal in animals treated daily. In chronically treated animals, either withdrawal for 48 h, or naloxone administration, resulted in a lowering of acetylcholine levels in the striatum. This finding differs from the one published by Large & Milton (1970), who reported an increase in ACh content measured 39–46 h after withdrawal. This difference could be due to the fact that they used the whole brain, or that their schedule of injections and dosage of morphine were different from those used here.

It became obvious that the changes in acetylcholine levels alone could not explain the pattern of variations in the enzyme activity. Kinetic studies provided an additional important clue to this question. From those experiments it became evident that even a single morphine administration changes the substrate affinity for choline, without greatly affecting the maximal velocity. This fact alone suggests that a conformational alteration had occurred in the enzyme molecule after a single administration of morphine. This drug-induced alteration at a cellular level in the central nervous system, observed also after prolonged morphine treatment, seems to represent a very persistent characteristic, since even 48 h after withdrawal from the drug (fifteen days treatment) the *Km* values were still at a high level. If any conclusions can be drawn from the *Vmax* values, which did not change considerably in our experiments, it may be assumed that there is no increase in the synthesis of the enzyme protein.

Of interest, however, is the fact that the incubation of the normal enzyme with morphine brings the normal *Km* values up to that obtained by morphine injections. This implies that a minute amount of morphine *in vivo* is able to induce changes in the enzyme kinetics similar to those induced *in vitro* by a very much larger concentration. Since the brain levels of acetyl CoA and of choline are much below those chosen arbitrarily for the reaction mixture, a proportionately lower concentration of morphine could be expected to inhibit the enzyme activity *in vivo*.

Choline acetyltransferase, already changed by either a single dose or prolonged administration of morphine does not show any further increase in *Km* values when morphine is added *in vitro*. But upon withdrawal from the drug for 24 or 48 h the enzyme seems to regain the ability to respond to morphine, as can be seen from the values of *Km* in the presence and in the absence of the drug.

The experiments presented suggest that the changes in choline acetyltransferase activity are produced by a combination of several factors. Initial decrease in enzyme activity may be due to the negative feedback mechanism of increased accumulation of acetylcholine, produced by morphine. Simultaneously, the substrate requirement of the enzyme decreases under morphine action, suggesting an almost immediate change in molecular conformation. Upon daily administration of the narcotic, the enzyme adapts itself to the presence of morphine, and is able to produce the usual amount of acetylcholine.

It may be concluded that both morphine and acetylcholine inhibit the enzyme *in vivo*. Changes in *Km* values, recorded after a single dose of morphine, suggest that the concentration at the site of enzyme is adequate, in those *in vivo* conditions, to exert some inhibitory effect. In the case of prolonged administration the changed enzyme, when tested one hour after the last injection of the drug, is able to produce the usual amounts of acetylcholine; further changes in the *Km* value of the enzyme, once exposed to the narcotic, are not obtained after *in vitro* addition of either morphine or acetylcholine.

We would like to recall that Goldstein & Goldstein (1961) presented a hypothesis proposing that an addicting drug may inhibit an enzyme whose synthesis is repressed by its product. Although we cannot claim to have proved this hypothesis, nevertheless, our results show that in acute morphine treatment such negative feedback action exists for choline acetyltransferase.

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