

## Effects of morphine on choline acetyltransferase levels in the caudate nucleus of the rat

K. DATTA, L. THAL\* AND I. J. WAJDA

*New York State Research Institute for Neurochemistry and Drug Addiction, Ward's Island, New York, NY 10035*

### Summary

1. Choline acetyltransferase (choline-o-acetyltransferase 2.3.1.6.) concentrations were determined in the caudate nucleus, thalamus, and cortex of control and morphine treated rats. The enzyme was assayed using a modified radiochemical method on a number of selected days, one hour after the last injection of 30 mg/kg of morphine and also during the subsequent phase of abstinence from morphine.
2. Significant lowering of choline acetyltransferase activity in the caudate nucleus area was found in two cases, one hour after the first dose of morphine and upon subsequent abstinence from morphine.
3. The enzyme activity in the two other parts of the brain remained at the normal levels.
4. The presence of endogenous inhibitors formed during morphine administration was excluded.
5. The relationship of a possible effect of morphine on the tissue binding of the enzyme and the subsequent lowering of its activity was tested by homogenization of the caudate nucleus area in different media. The decrease in enzyme activity occurred in all extraction media one hour after morphine administration.
6. Inhibitory effects of *in vitro* addition of morphine to caudate nucleus homogenate, obtained from normal and morphine treated rats, were found to occur only at very high concentrations of the drug, negating the possibility of direct inhibitory effects of morphine.
7. These experiments suggest two possible causes of the observed effects, which can be responsible for the lowering of enzyme activity, and can be operative simultaneously: (1) a negative feedback mechanism of accumulated acetylcholine, occurring after the first dose of morphine, and (2) the possible changes in enzyme configuration produced by morphine treatment.

### Introduction

Numerous theories have been proposed in recent years to explain morphine addiction on a biochemical basis. It is generally accepted that biochemical alterations in the central nervous system play an important role. Interference with or

\* Present address: Kings County Medical Center, Department of Medicine, 451 Clarkson Avenue, Brooklyn, N.Y. 11203.

alteration of neurotransmitter systems by morphine has been considered a promising approach.

Because of autonomic nervous system hyperactivity during withdrawal and symptoms suggestive of parasympathetic stimulation in the intact animal, early work concentrated on changes in acetylcholine levels during morphine administration. Subcutaneous administration to rats of a single dose of morphine caused an increase in total cerebral acetylcholine, which reached a peak in about 30 min and then gradually returned to normal (Hano, Kaneto, Kakunaga & Moribayashi, 1964; Maynert, 1967; Giarmen & Pepeu, 1962). This increase has generally been assumed to occur as a result of decrease in the release of acetylcholine, first demonstrated in the intestine (Schaumann, 1957; Paton, 1957) rather than as a result of increased production or decreased destruction (Maynert, 1967). However, the absence of increased production has not been experimentally disproved. Chronic administration did not cause significant changes in acetylcholine levels and, *in vivo*, morphine had no effect on cholinesterase activity (Hano *et al.*, 1964). Recently, Large & Milton (1970) reported changes in the acetylcholine content in the brain of rats recorded (1) after acute administration of morphine, which caused an increase in brain concentration of acetylcholine, (2) during chronic administration, when no increase in ACh levels was recorded, and (3) during subsequent abstinence from morphine (39–46 h), when again an increase in the concentration of acetylcholine was found. In the central nervous system depression of acetylcholine release by morphine was demonstrated in cat brain by Beleslin & Polak (1965).

Cholinergic mechanisms have been demonstrated in the central nervous system, most notably in the striatum. Iontophoretic application of acetylcholine to the caudate nucleus has resulted in a demonstration of two distinct regions: one excited and the other depressed by acetylcholine (McLennan & York, 1966). Choline acetyltransferase, the enzyme responsible for the production of acetylcholine, occurs in highest concentrations in the striatum (Wajda, 1951; Hebb, 1963; Fahn & Cote, 1968). The acetylcholine concentration is also exceptionally high in this region of the brain, and the striatal content in rats was found by Sattin (1966) to be  $47 \pm 3$  nmol/g of initial wet weight.

After receiving a large dose of morphine, rats become rather immobile, indeed catatonic. In the rat, the caudate nucleus is believed to coordinate the vast bulk of motor activity (Elliot, 1963). The convergence of biochemical and electrophysiological evidence of cholinergic caudate activity, plus the behavioural evidence in the form of morphine induced catatonia mediated by the caudate nucleus, strongly implies a cholinergic role of morphine action in the central nervous system. To approach the relationship between morphine and the cholinergic system in the brain a study of the three different parts of the effect of morphine on choline acetyltransferase activity in the central nervous system was undertaken. A preliminary report of the work has been presented (Thal & Wajda, 1969).

## Methods

### *Schedule of injections*

Wistar female rats, 175–225 g, were subcutaneously injected with 30 mg/kg of morphine. Animals were injected daily, for 1–12 days, in order to obtain a series

of animals on morphine for varying periods of time. Animals were generally killed one hour after receiving their last injection of morphine unless otherwise stated. One group of animals received injections for 12 consecutive days, then were killed 24, 48, 72 and 96 h after withdrawal. Normal values were determined both on saline injected and uninjected rats.

Another group of rats was treated daily for 6–7 weeks with increasing doses of morphine. Starting with 30 mg/kg during the first 2 weeks, followed by 50 mg/kg during the next 2 weeks, the rats received in the last 2–3 weeks 80 mg/kg of morphine administered subcutaneously. Saline treated animals were used as controls.

Nearly all the values of choline acetyltransferase activity were obtained using commercial  $^{14}\text{C}$  acetyl coenzyme A (New England Nuclear Corp.), a purer preparation than the one on which the experiments presented in Fig. 3 are based. The shape of the curve presented in Fig. 3 has since been confirmed with a purer preparation of acetyl coenzyme A, but those results are nevertheless presented in the original values because of the large number of experiments included in them. Lowering of choline acetyltransferase activity depending on the purity of commercial preparations is known to other authors (Potter, Glover & Saelens, 1968).

#### *Tissue sample preparation*

All animals were killed by rapid decapitation. The brains were quickly removed, placed on ice, and divided along the sagittal plane. The thalamic structures from three rat brains were pooled, as were representative samples of motor cortex, thereby resulting in two tissue samples. The two caudate nuclei from each rat were pooled and assayed as one unit. Three rats then ultimately produced five tissue specimens: three of caudate nuclei and one of each from cortex and thalamus. The tissue specimen was weighed, then diluted to 1 to 9 (w/v) in 0.1 mM  $\text{Na}_4\text{EDTA}$  plus 1% butanol. Homogenization was carried out for 2 min in a motor driven ground glass tissue homogenizer. Each tissue sample was ultimately assayed in triplicate.

#### *Enzyme assay*

Enzyme assay was carried out by a modification of the method described by Potter *et al.* (1968). Activity was estimated by determining the rate of acetylation of choline with  $^{14}\text{C}$ -acetyl CoA using homogenized brain as source of enzyme. The reaction mixture contained: choline chloride 2.0 mM;  $^{14}\text{C}$ -acetyl CoA 0.2 mM, specific activity 10  $\mu\text{Ci}/\mu\text{mol}$ ; physostigmine sulphate 0.2 mM; neutralized EDTA 0.2 mM; potassium phosphate buffer (equimolar mono- and di-basic salt, pH 7.4), 20 mM; KCl 600 mM; 1-butanol 2% by volume. To carry out the assay, 20  $\mu\text{l}$  of this reaction mixture was pipetted into a 0.2 ml microcentrifuge tube and warmed to 37° C. Crude enzyme (10  $\mu\text{l}$ ) was then added and the reaction was allowed to proceed for 10 min. The reaction was stopped by the addition of 10  $\mu\text{l}$  of 1 N acetic acid containing unlabelled carrier acetylcholine chloride (0.02 M) and by returning the test tube to an ice bath. Ten microlitres of this final mixture were then spotted onto strips of Whatman No. 1 filter paper and electrophoresed for 1 h at 200 V in a Gelman tank. The buffer used for electrophoresis was 0.125 M acetic acid and 0.125 M sodium acetate, pH 4.7. After electrophoresis, the paper was air dried in a hood. The bands of acetylcholine were located in iodine vapour, cut out and counted by a liquid scintillation technique, in a toluene base phosphor con-

taining 10% ethanol. Enzyme blanks, using 10  $\mu$ l of water in place of homogenate, gave values less than 0.5% of the substrate value.

## Results

Initial experiments showed that the enzyme values are similar to those reported by other workers. For the whole brain of normal rats, the mean value for choline acetyltransferase activity was 3.21  $\mu$ mol acetylcholine formed/g fresh tissue each hour. For caudate nucleus the value was 8.9  $\mu$ mol acetylcholine formed/g fresh tissue each hour. Obtained values are compared in Table 1 with those reported by other workers (Fahn & Cote, 1968; Potter *et al.*, 1968; McCaman & Hunt, 1965; Schrier & Shuster, 1967).

To establish the dependence of the assay on the quantity of enzyme present, an enzyme concentration curve was determined. A linear dependence between the amount of enzyme present and the quantity of  $^{14}$ C-acetylcholine produced was noted over the range of 0–15  $\mu$ l of crude enzyme (see Fig. 1).

The effect of hypodermic injection of saline on choline acetyltransferase concentrations was tested. Enzyme concentrations in the caudate nucleus of saline injected and non-injected normal animals were compared. No significant difference was found between the two groups.

TABLE 1. *Choline acetyltransferase activity in CNS homogenates*

Species	Tissue	Units	Reference
Rat	Brain	3.21	Present study
Rat	Brain	5.00	Potter <i>et al.</i> (1968)
Rabbit	Brain	3.50	McCaman & Hunt (1965)
Mouse	Brain	3.44	Schrier & Shuster (1967)
Rat	Caudate nucleus	8.9	Present study
Rabbit	Caudate nucleus	12.5	McCaman & Hunt (1965)
Monkey	Caudate nucleus	12.1	Fahn & Cote (1968)

Units represent  $\mu$ mol of acetylcholine (synthesized/h)/g of fresh tissue.

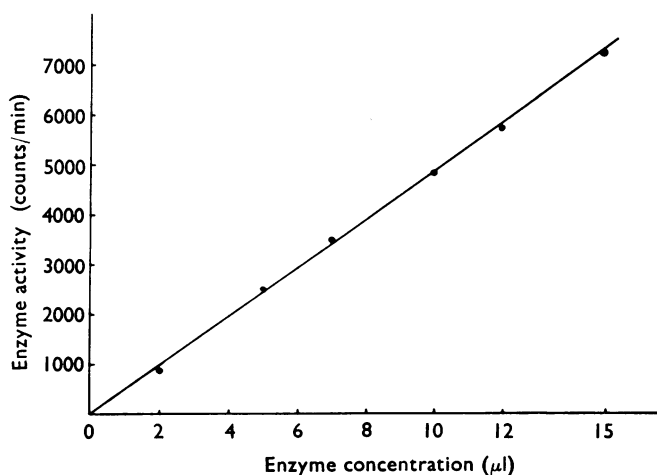


FIG. 1. Effect of enzyme concentration on activity. Counts/min represent the amount of  $^{14}$ C-acetylcholine produced.

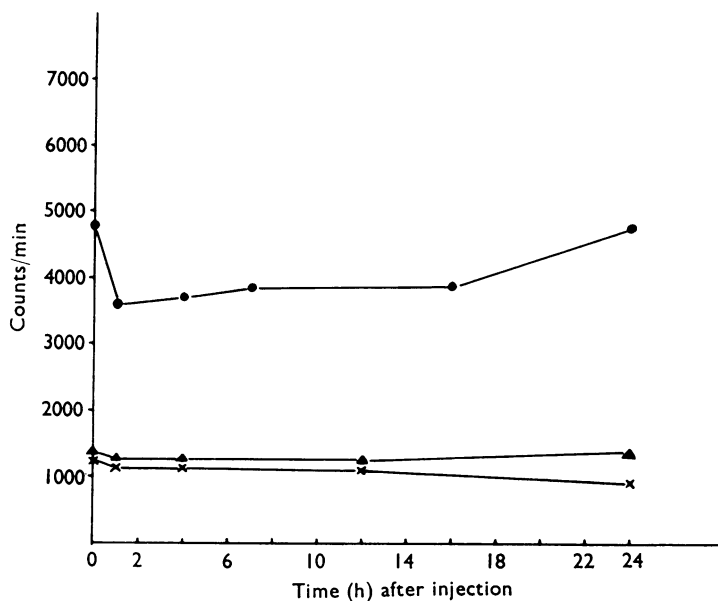


FIG. 2. Effect of a single dose of morphine on choline acetylase activity. One single dose of morphine sulphate, 30 mg/kg, was given subcutaneously. Choline acetyltransferase activity is in counts/minute. ●—●, Caudate nucleus; ▲—▲, thalamus; ×—×, cortex.

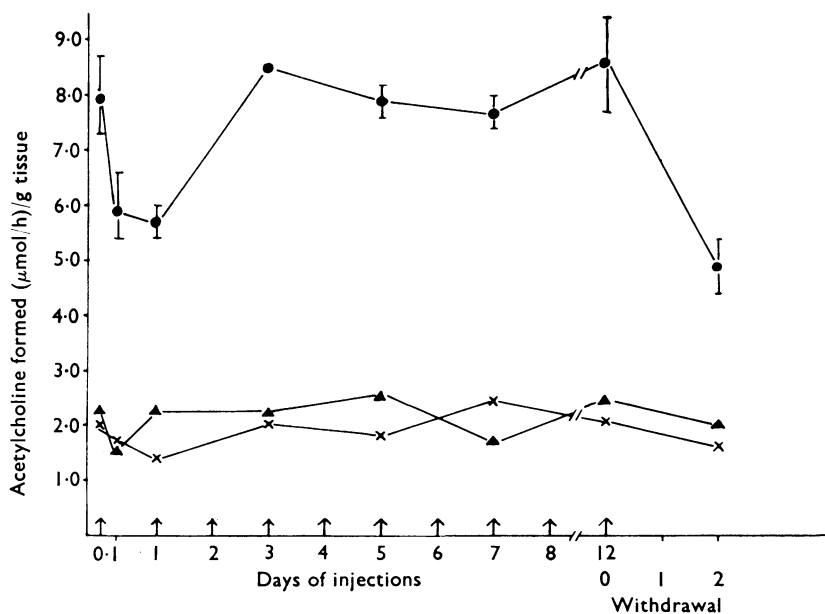


FIG. 3. Choline acetyltransferase activity during morphine administration and withdrawal. Rats received 30 mg/kg of morphine sulphate. The arrows indicate daily injections of drug. Up to the twelfth day the animals were killed always one hour after the last injection. The last value on the chart represents 48 h after the injections (treatment for 12 days). Mean values were obtained from six to fourteen animals. Standard deviations are indicated by vertical lines. ●—●, Caudate nucleus; ▲—▲, thalamus; ×—×, cortex.

Early experiments involved investigation of events following a single subcutaneous dose of 30 mg/kg of morphine. Choline acetyltransferase activity of the caudate nucleus decreased 26% after one hour, then slowly climbed back up to normal after 24 hours. No such comparable change took place in the thalamus or motor cortex (see Fig. 2).

Chronic administration of morphine was then studied. Animals were injected with morphine daily for 1–12 days. Choline acetyltransferase activity was determined in the caudate nucleus, thalamus, and cortex. In this experiment the animals were killed one hour after the last injection of morphine (see Fig. 3). After one single injection and after two injections during 2 consecutive days, choline acetyltransferase activity was 26% below basal values in the caudate nucleus. After 3 days, the choline acetyltransferase activity had almost returned to normal despite the continued daily injection of morphine. On days 5, 7, 10 and 12, the choline acetyltransferase activity in the caudate nucleus was either slightly above or essentially the same as the basal levels. No significant changes in the enzyme activity took place in either the thalamus or motor cortex of these animals. Slight lowering of the enzyme activity was noticed in the thalamus after one single dose of 30 mg/kg morphine. However, the difference from normal was not significant (see Fig. 3). Choline acetyltransferase activity was determined 48 h after the final dose of morphine. Enzyme activity in the caudate nucleus dropped precipitously, reaching a low point of over 30% below basal values. Lowered choline acetyltransferase activity was not found in either the thalamus or cortex after withdrawal from morphine (Fig. 3).

The next experiment was designed to find out if longer treatment with increasingly higher doses of the narcotic would have also an increased depressant effect on the enzyme upon withdrawal. Subcutaneous injections of 30 mg/kg of morphine given daily during 2 weeks were followed by 2 weeks of 50 mg/kg, and finally 2–3 weeks of 80 mg/kg. One group of rats thus treated was analysed (group II in Table 2) one hour after the last morphine dose. In this experiment the controls were treated for the same period with daily injections of saline. When compared with saline treated rats the activity of choline acetyltransferase of the caudate nucleus was slightly lower than the controls, but the difference was insignificant. On withdrawal, lowering of enzyme activity was again observed (group III), the difference with controls was statistically significant, but only 19.5% of activity was lost.

The presence of endogenous inhibitors in crude homogenates obtained from morphine treated animals was excluded by experiments on an additive basis. As can be seen from Table 3 the quantity of acetylcholine formed is practically identical in all experiments presented, disproving the formation of possible inhibitors in the

TABLE 2. *Effect of prolonged morphine treatment on choline acetyltransferase in the caudate nucleus of rats*

Group	No. of rats	Treatment of animals	( $\mu\text{mol ACh/h}$ )/g of wet weight	P value
I.	9	Saline	$8.78 \pm 0.47^*$	—
II.	6	Morphine tolerant	$8.01 \pm 0.48$	0.01
III.	7	Withdrawn from morphine for 48 h	$7.07 \pm 0.56$	0.001

\* Standard deviation of the mean. Estimations of choline acetyltransferase activity for each individual animal were performed in triplicates. Treatment of rats lasted 6–7 weeks, with increasing doses as described in **Methods**. Observed changes were calculated using Student's method.

material obtained from morphine treated rats. Homogenates were inactivated by treatment for 5 min at 50° C; we can, therefore, exclude inhibitors with one exception: an enzyme-like activity dependent on mild heat treatment.

It is interesting to correlate the biochemical events and the animals' behaviour during this period of time. After the first injection of morphine, often the rats become quite immobile—almost catatonic. Spontaneous movement disappeared although the animals continued to respond to strong physical and auditory stimuli. After 1, 2 and 3 days of daily morphine injections, the animals became progressively less immobile after morphine administration. On the fourth day, no signs of abnormal behaviour could be observed after injection of morphine. After 12 days the administration of morphine was stopped. Forty-eight hours later the animals were more excited than usual, but the typical signs of loss of weight and 'wet dog' appearance were not really present. Therefore, we cannot call this a typical withdrawal syndrome, although the animals were withdrawn from the administration of the drug.

During the prolonged treatment with morphine, when the dose was increased every 2 weeks, the animals were losing weight during the third week, when the dose was changed to 50 mg/kg. However, during the last week, even when given 80 mg/kg, they were again gaining weight and their appearance improved. Upon withdrawal again, we were unable to observe the 'wet dog' behaviour, but the animals were obviously uneasy and looked different from either normal rats or the animals which received their daily doses of narcotic.

The rapid lowering of choline acetyltransferase activity (one hour after morphine administration) seemed to point in the direction of altering either the properties or the binding of the enzyme, rather than changing its rate of synthesis or degradation. To investigate the possible role of binding, tissue homogenates from both morphine and control animals were prepared in a variety of media. Homogenization in

TABLE 3. *Test for endogenous inhibitors in the homogenates obtained from morphine treated rats*

Addition to normal enzyme (10 $\mu$ l)	Acetylcholine synthesized ( $\mu$ mol/g fresh tissue)/h)
Normal homogenate	9.01
After morphine treatment for one hour	8.94
After morphine treatment for 4 days, abstinence from morphine for 24h	9.24

All additions to normal enzyme were inactivated by keeping the homogenates for 5 min at 50° C. Incubation conditions were the same as described in **Methods**. The dose of morphine was 30 mg/kg given subcutaneously.

TABLE 4. *Effect of different extraction media on choline acetyltransferase activity in the caudate nucleus*

Homogenizing medium	$\mu$ mol of (ACh formed/h)/g of C.N.		Percentage decrease after morphine
	Normal rats	1 h after morphine	
0.25M sucrose	3.15 $\pm$ 0.34*(10)	2.9 $\pm$ 0.32*(11)	— 8.0
300 mM NaCl	3.29 $\pm$ 0.45 (9)	2.52 $\pm$ 0.29 (10)	— 24.0
EDTA-butanol	9.20 $\pm$ 0.55 (9)	6.7 $\pm$ 0.64 (11)	— 27.0
0.5% Triton X-100	10.9 $\pm$ 0.55 (9)	8.4 $\pm$ 0.40 (9)	— 23.0
NaCl+ether	9.84 $\pm$ 0.45 (9)	8.7 $\pm$ 0.48 (9)	— 11.0
Distilled water	7.99 $\pm$ 0.30 (10)	6.56 $\pm$ 0.57 (8)	— 19.0
Water+ether	10.04 $\pm$ 0.50 (9)	8.14 $\pm$ 0.47 (10)	— 24.0

The dose of morphine was 30 mg/kg given subcutaneously. \* Standard deviation of the mean. The figures in parentheses represent the number of estimation.

0.25 M sucrose was chosen as the gentlest, and ether treatment as the most vigorous. EDTA-butanol, 300 mM NaCl, distilled water, and 0.5% Triton X-100 were also investigated (see Table 4).

In normal animals, Triton X-100 treatment produced the greatest amount of enzyme activity while isotonic sucrose produced the least. However, the decrease in caudate nucleus enzyme activity in rats which had been treated with morphine for one hour occurred in all extraction media, negating the possibility that the binding alone of the enzyme to particulates was responsible for the decreased activity.

To test the inhibitory effects of morphine on choline acetyltransferase activity *in vitro*, several concentrations of morphine were used. As enzyme source, crude homogenates of caudate nuclei from normal and differently treated rats were obtained. The treatment consisted of 30 mg/kg of morphine given subcutaneously, and the animals were always killed one hour after the last dose of morphine. Homogenates were obtained from normal animals and animals that had been treated for one hour, 4 days and 12 days and also from rats which had been withdrawn from the drug for 48 hours. All these samples were inhibited equally by morphine. A final concentration of morphine of  $10^{-3}\text{M}$  inhibited choline acetyltransferase activity by 4–6% ; 20% inhibition was obtained with  $5 \times 10^{-3}\text{M}$  ;  $10^{-2}\text{M}$  inhibited the enzyme activity up to 30%, and  $2.5 \times 10^{-2}\text{M}$  morphine inhibited the activity up to 50%. We conclude from these results that our inhibitory effects *in vivo* could not be produced by a direct action of the drug on the enzyme activity as such concentrations are not obtained in the CNS after injections of morphine.

## Discussion

It is interesting to note that choline acetyltransferase activity follows a pattern similar to tolerance. On administration of morphine, enzyme activity falls sharply and remains low for a few days, in spite of daily administration of the drug. After 3 days, the enzyme activity returns almost to normal. It is also after 3–4 days that the injection of a constant daily dose of morphine is no longer able to produce catatonia. From day 5 on, enzyme activity remains slightly above normal until the drug is withdrawn. Withdrawal of morphine after 12 days produces a marked decrease in enzyme activity, but longer treatment and higher doses of morphine do not increase this effect on choline acetyltransferase activity.

The lowering of choline acetyltransferase levels follows the initial morphine administration ; however, after continuous administration the opposite procedure, namely withdrawal from the drug, produces a decrease in enzyme activity. At some point during the first 4 days a crossover phenomenon occurs so that instead of merely inhibiting the enzyme activity, the maintenance of normal choline acetyltransferase activity slowly becomes dependent on the presence of morphine. In spite of this similarity, we have no direct proof that the effect of morphine on the enzyme activity has any relation to morphine tolerance.

It is not easy at this point to explain fully the observed facts. To postulate actual enzyme drops of 25% in an hour requires the turnover rate of the enzyme to be 4 hours. While this is possible, a decrease in enzyme activity rather than a decrease in the quantity of the enzyme present seems more probable.

A tentative hypothesis can be constructed to explain the experimental data. Theoretically, morphine induced changes in enzyme activity can be brought about

in three ways: (1) possible formation of endogenous inhibitor; (2) indirect effect of acetylcholine production by a negative feedback mechanism that can regulate enzyme activity; (3) changes in enzyme configuration, or formation of a new enzyme. The first possibility is excluded as no endogenous inhibitor was formed (Table 3). The other two possibilities can be operative, and they might not be mutually exclusive. Cerebral increase of acetylcholine 30–60 min after morphine administration has been reported (Hano *et al.*, 1964; Maynert, 1967; Giarman & Pepeu, 1962; Large & Milton, 1970). This involves an accumulation of the reaction product and a feedback inhibition such as postulated by Saelens & Potter (1966) and by Kaita & Goldberg (1968, 1969). The enzyme returns to normal after a few days, but this induced enzyme is morphine dependent. When morphine is ultimately withdrawn, the morphine dependent, induced enzyme loses its activity as morphine is cleared from the system, and the enzyme activity drops sharply. Once morphine has disappeared, the animal once again begins to produce normal choline acetyltransferase, and enzyme levels slowly climb towards normal values. On the other hand, the lowering of enzyme activity after the drug administration has been stopped following prolonged treatment (2 weeks and 6–7 weeks) can also be explained by a mechanism similar to that operating after the first dose of narcotic. The concentrations of acetylcholine rose on withdrawal from morphine (Large & Milton, 1970), and although this was reported for the whole brain, a similar negative feedback of the product of the reaction may take place.

The concept that drugs might affect the enzyme levels is not without previous experimental support. Depression of choline acetyltransferase activity by very high doses of morphine has been reported (Torda & Wolfe, 1947). Also, administration of scopolamine has been reported significantly to increase choline acetyltransferase in guinea-pig parietal cortex (Beani, Bianchi & Megazzini, 1964).

The possibility that morphine merely increases the percentage of inactive membrane bound choline acetyltransferase seems remote. The fact that after one dose of morphine, brain tissue extracted with Triton showed a 23% decrease in choline acetyltransferase activity does not support simple membrane binding since Triton is generally considered a potent disruptor of membranes, similar to lipid solvents (Hebb & Smallman, 1956).

The effect of morphine on the overall metabolism of the animal cannot be invoked to explain the data. The failure of morphine to depress choline acetyltransferase activity in the thalamus or cortex militates against the concept of a shotgun effect on the animal's metabolism. In an attempt to elucidate these problems further, experiments involving kinetic studies of choline acetyltransferase, acetylcholine levels in striatum, and the inhibitory effects of acetylcholine and morphine on the enzyme are in progress. A possibility of conformational changes in the enzyme molecule under the influence of morphine has to be considered. A preliminary communication on some of the results (Wajda & Datta, 1970) has been presented, and the increase in the  $K_m$  value of a partially purified enzyme preparation obtained from the caudate nucleus of morphine treated rats has been reported.

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