In conclusion, aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity accumulates in five liver-derived cell lines treated with polycyclic hydrocarbons, biogenic amines or phenobarbital in the growth medium. The established lines are: H-4-II-E, derived from rat Reuber hepatoma H-35; MH_1C_1, from the rat transplantable Morris hepatoma 7795; Hepa-1, from the transplantable hepatoma BW 7756 originally developed in the C57/JL mouse; and TRL-2-Cl-2 and ERL-2-Cl-3, derived, respectively, from normal liver of 10-day-old and 8-week-old rats of the BD-6 strain. We feel that there are advantages in using such established cell lines instead of fetal rat hepatocyte primary cultures.

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Effect of chemical sympathectomy on morphine antinociception and tolerance development in the rat

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IN A PREVIOUS paper,[1] it was reported from this laboratory that 6-hydroxydopamine (6-OHDA) which has been reported to cause a selective degeneration of the adrenergic neurons in the brain,[2–5] reduced the analgetic response to morphine in the mouse but did not affect materially the development of tolerance to morphine. Evidence was also provided indicating that the signs of morphine withdrawal were exacerbated by 6-OHDA. The present communication presents findings of a similar study performed in the rat.

Male Sprague–Dawley rats weighing 150–180 g from Horton Laboratories, Calif., were used. Chemical sympathectomy was effected by intraventricular injection[6] of 6-OHDA HBr (Regis Chemical Co., Chicago, Ill.), dissolved in nitrogen-saturated physiologic saline containing 0·1% ascorbic acid; control rats received the vehicle. Two dosage schedules of 6-OHDA were used.

In expt. 1, a dose of 0·5 mg/kg of 6-OHDA was injected for 2 consecutive days, and 5 days after the second dose, the analgetic response to morphine was determined. Four other animals in this series were utilized for estimation of brain norepinephrine (NE) and dopamine (DA) according to

Chang[7] and Brodie *et al.*[8] In expt. 2, 1·3 mg/kg was injected daily for 2 days and the same estimations were made 1 day after the second dose of 6-OHDA. Three hr after the analgetic determination in both series, the animals were rendered tolerant to morphine as reported previously[9] by implanting two pellets of morphine containing 75 mg of morphine base. After implanting one pellet, a second one was implanted 36 hr later and after an additional 36 hr, both pellets were removed. The analgetic response to morphine was determined 6 hr later. Four other animals in each experimental series were utilized for NE and DA estimation.

The analgetic response to morphine was assessed by the classic tail flick procedure. A quantal assay was performed using latency in response to thermal stimulus after morphine as the index. After establishing the baseline tail flick response (1·5 ± 0·5 sec), varying doses of morphine sulfate were administered s.c. using eight animals per dose. After 30 min, the tail flick response was redetermined and a reaction time of 3·5 sec above the baseline was considered to be a positive response. The morphine AD_{50} and its 95 per cent confidence limits were estimated by the method of Litchfield and Wilcoxon.[10] The degree of tolerance developed after pellet implantation was indicated by the increase in the morphine AD_{50}, that is the ratio of the AD_{50} after implantation over the AD_{50} before implantation.

In both non-tolerant and morphine-tolerant rats, brain NE and DA were reduced by 6-OHDA pretreatment. The findings are compatible with those previously reported by others.[2–5] As can be seen in Table 1, with the 0·5 mg/kg dose in non-tolerant animals (expt. 1), NE was decreased 57 per cent and DA 38 per cent. With development of tolerance after pellet implantation, NE was reduced 45 per cent and DA 32 per cent by 6-OHDA. With the 1·3 mg/kg dose (expt. 2), NE was lowered 75 per cent and DA 51 per cent in non-tolerant animals; in tolerant animals, NE was reduced 70 per cent and DA 53 per cent. Thus, the lowering effect of 6-OHDA on brain catecholamine levels was not affected materially by the development of tolerance to morphine.

The lowering of brain catecholamine levels effected by 6-OHDA in both non-tolerant and tolerant rats was accompanied by a decrease in sensitivity to morphine, but development of tolerance to morphine was not affected. As can be noted in Table 1, a decreased analgetic response to morphine with 6-OHDA pretreatment was evidenced by an increase in the morphine AD_{50}. With either the 0·5 mg/kg or 1·3 mg/kg dose of 6-OHDA, the morphine AD_{50} before pellet implantation was higher than its respective saline control by nearly 3-fold. After pellet implantation, all groups in both

TABLE 1. EFFECT OF INTRAVENTRICULAR 6-HYDROXYDOPAMINE (6-OHDA) ON BRAIN LEVELS OF NOREPINEPHRINE (NE) AND DOPAMINE (DA) AND ON THE ANALGESIC RESPONSE TO MORPHINE (AD_{50}) IN NON-TOLERANT AND TOLERANT RATS*

Experiments	NE (μg/g ± S.E.) (N = 5)	DA (μg/g ± S.E.) (N = 5)	Morphine AD_{50} (mg/kg)
1. Before pellet implantation			
Saline	0·46 ± 0·03	1·03 ± 0·06	2·00 (1·8–2·3)
6-OHDA	0·18 ± 0·02	0·66 ± 0·06	5·40 (4·4–6·7)
After pellet implantation			
Saline	0·51 ± 0·04	1·19 ± 0·10	5·6 (4·8–6·5)
6-OHDA	0·28 ± 0·04	0·71 ± 0·04	10·2 (7·8–13·4)
2. Before pellet implantation			
Saline	0·52 ± 0·03	1·14 ± 0·05	1·5 (1·3–1·8)
6-OHDA	0·13 ± 0·01	0·56 ± 0·03	4·2 (3·4–5·2)
After pellet implantation			
Saline	0·50 ± 0·04	0·90 ± 0·05	4·4 (3·9–5·0)
6-OHDA	0·15 ± 0·01	0·42 ± 0·02	11·0 (9·4–12·9)

*Animals were rendered tolerant to morphine by the implantation of two pellets of morphine (75 mg). The first pellet was implanted 3–4 hr after the first AD_{50} determination and the second pellet 36 hr after the first one. Both pellets were removed 36 hr after implantation of the second pellet, and the morphine AD_{50} was estimated 6 hr later. The figures in parentheses denote the 95 per cent confidence limits. NE and DA were estimated for the same interval in four animals selected randomly from the same group. In experiment 1, the daily dose of 6-OHDA for 2 days was 0·5 mg/kg. Catecholamines and AD_{50} estimations were made 5 days after the second injection of 6-OHDA. In experiment 2, the daily dose of 6-OHDA for 2 days was 1·3 mg/kg. Catecholamines and AD_{50} estimations were made in separate groups of animals 1 day after the second injection.

experiments developed about a 2-fold tolerance to morphine as evidenced by the ratio of the morphine AD_{50} of tolerant animals over that of non-tolerant animals. Despite the increase in morphine AD_{50} as a result of tolerance development, the antagonism of morphine analgesia by 6-OHDA was not affected as evidenced by a further increase in the morphine AD_{50}. With the 0·5 mg/kg dose, 6-OHDA produced a 1·9-fold increase in the morphine AD_{50} and with 1·3 mg/kg, a 2·6-fold increase. Thus, the increase in morphine in AD_{50} effected by 6-OHDA in tolerant animals was almost the same as that obtained in non-tolerant ones. These findings are consistent with the report[11] that the analgetic effects of morphine are antagonized by 6-OHDA.

The dependent state was greatly altered by 6-OHDA. The development of tolerance which occurred after pellet implantation was accompanied by the development of physical dependence. In saline-treated animals, the latter state was evidenced by the fact that 12–24 hr after pellet removal, typical abstinent signs were noted (sneezing, wet shakes, ear blanching and increased locomotor activity and vocalization upon touching). In comparison to the 6-OHDA-treated group, however, these signs were relatively mild. At the time of pellet removal, the 6-OHDA groups appeared to be a little more difficult to handle than the control group. One hr later, the animals began to exhibit hyperactivity and within another 2 hr extreme aggressive behavior. Fierce and frequent fighting developed among the animals which were housed two per cage. They would rear on their hind legs and bite each other on the mouth and paws until bleeding was profuse. The animals were extremely difficult to handle; they squealed and attempted to bite the experimenter when touched. This aggressive behavior persisted over a 4-week period of observation.

The results in the rat confirm our previous findings in the mouse that 6-OHDA alters the acute effect of morphine but does not affect materially the development of tolerance.[1] In agreement with the data obtained in mice, 6-OHDA antagonized the acute analgetic effects of morphine in both non-tolerant and tolerant rats to about the same degree. Since the 6-OHDA was injected prior to rendering the animal tolerant to morphine by pellet implantation, we interpret the results to mean that 6-OHDA did not affect materially the development of tolerance to morphine.

The extreme and protracted withdrawal response observed in the 6-OHDA-treated rats is of considerable interest. It should be noted that 6-OHDA alone has been reported to produce some degree of hyperirritability.[1,5,12] Also, aggressive behavior was reported to occur in the rat during chronic morphine administration after 6-OHDA treatment.[5,13] However, our data suggest that the latter response does not reflect a direct drug effect but rather a morphine withdrawal response, since the irritability became evident in our study only after removal of the morphine pellet at a time when animals were maximally dependent. The exaggerated response in 6-OHDA-treated animals reflects a profound interaction between morphine and 6-OHDA. It has been pointed out by Jaffee and Sharpless[14] that morphine abstinence might reflect a state of rebound hyperexcitability resulting from central denervation supersensitivity. Also, Ungerstedt[15] has shown that 6-OHDA produces supersensitivity of central dopaminergic receptors to L-dopa and apomorphine. However, neither drug alone produced responses that were comparable in onset, intensity and duration to those observed when given in combination. We previously[1] noted also that the abstinence-like state precipitated by the antagonist naloxone in morphine-dependent mice was enhanced by 6-OHDA.

It might be expected that 6-OHDA should exacerbate the withdrawal state, since the prime manifestations of withdrawal in the rat and mouse appear to involve dopaminergic pathways. We have found, for example, that the spontaneous jumping which occurs after abrupt or naloxone-precipitated withdrawal can be related to a sudden elevation of brain dopamine[16] and, moreover, the phenomenon can be induced in the morphine-dependent mouse by administering the monoamine oxidase inhibitor pargyline. Consistent with these results is the report that the activity of 6-OHDA on dopaminergic neurons in the rat is inhibited by chronic morphine treatment as a consequence of altered changes in the uptake process of the nigrostriatal system.[12]

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Inhibition of dopamine-β-hydroxylase by spinochrome A and echinochrome A, naphthoquinone pigments of echinoids

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THE NATURAL naphthoquinone pigments in echinoids (sea urchins), spinochrome A (2-acetyl-3,6-dihydroxynaphthazarin) and echinochrome A (2-ethyl-3,6,7-trihydroxynaphthazarin), were found to be potent inhibitors of tyrosine hydroxylase.[1] Other naphthoquinone derivatives such as aquayamycin[2,3] and deoxyfrenolicin[4] were also potent inhibitors of tyrosine hydroxylase. Among these naphthoquinones, aquayamycin was also found to be an inhibitor of dopamine-β-hydroxylase,[5] tryptophan hydroxylase and tryptophan 2,3-dioxygenase.[6] Dopamine-β-hydroxylase, a copper-containing enzyme, and tryptophan 2,3-dioxygenase, a heme-containing enzyme, both require ascorbic acid as a cofactor, whereas tyrosine hydroxylase and tryptophan hydroxylase require a tetrahydropterin as a cofactor.

Considering these previous results, we have examined the possibility of inhibition of dopamine-β-hydroxylase by spinochrome A and echinochrome A, and the mechanism of the inhibition. A preliminary report has appeared.[7]

Crystalline spinochrome A and echinochrome A were kindly supplied by Drs. Asashima and Kinoshita. The pigments were dissolved in ethanol in desired concentrations. The concentration of echinochrome A was estimated based on the extinction coefficient of $18{\cdot}9 \times 10^3$ M^{-1}, cm^{-1} at 263 nm in ethanol. Dopamine-β-hydroxylase was purified from fresh bovine adrenal medulla by the method of Friedman and Kaufman.[8] The enzymic activity was measured by two methods as described before.[9] The standard reaction mixture for the enzymic assay contained (μmoles): potassium phosphate buffer (pH 6·5) 200; tyramine or dopamine, 10; fumarate, 10; ascorbic acid, 10; an appropriate amount of the enzyme (5–30 μg of protein), enough catalase to give maximum stimulation and water to 1·0 ml. The reaction mixture was incubated for 30 min at 37°. When tyramine was substrate, the conversion of tyramine to norsynephrine was followed according to the spectrophotometric procedure of Creveling *et al.*[10] When dopamine was used as substrate, norepinephrine formed from dopamine was assayed according to the fluorometric procedure of von Euler and Floding[11] by an Aminco–Bowman spectrophotofluorometer.

When tyramine was used as substrate, both spinochrome A and echinochrome A inhibited dopamine-β-hydroxylase in the presence of ascorbic acid (Table 1). Spinochrome A and echinochrome A inhibited the enzyme by 50 per cent at 5×10^{-6} M and 3×10^{-5} M, respectively.

Nozaki *et al.*[6] have recently reported that aquayamycin at $1{\cdot}5 \times 10^{-6}$ M strongly inhibited the ferric form of tryptophan 2,3-dioxygenase in the presence of ascorbic acid, probably due to a rapid