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EFFECTS OF CHRONIC CONSUMPTION OF METANIL YELLOW BY DEVELOPING AND ADULT RATS ON BRAIN REGIONAL LEVELS OF NORADRENALINE, DOPAMINE AND SEROTONIN, ON ACETYLCHOLINE ESTERASE ACTIVITY AND ON OPERANT CONDITIONING

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Abstract—Metanil yellow is the principal non-permitted food colour used extensively in India. The effects of long-term consumption of metanil yellow on the developing and adult brain were studied using Wistar rats. Regional levels of noradrenaline, dopamine and serotonin, activity of acetylcholine esterase (AChE), and operant conditioning with food reward were assessed in rats fed, metanil yellow and in controls. In the treated rats the amine levels in the hypothalamus, striatum and brain stem were significantly affected, and the changes were not generally reversible even after withdrawal of metanil yellow in developing rats. The striatum showed an early reduction of AChE activity, whereas the hippocampus showed a delayed but persistent effect of reduced AChE activity. Treated rats also took more sessions to learn the operant conditioning behaviour. These effects on these major neurotransmitter systems and on learning, indicate that chronic consumption of metanil yellow can predispose both the developing and the adult central nervous system (CNS) of the rat to neurotoxicity.

INTRODUCTION

With the introduction of coal-tar dyes the use of synthetic colourings in foodstuffs has increased. Their easy availability and long shelf-life have prompted substitution of synthetic colourings for natural colourings. Although the common food colouring, flavouring and preservatives were provisionally regarded as safe because they lacked overt toxicity or carcinogenicity (Lipton *et al.*, 1979), evaluation of the physiological and behavioural effects of sub-toxic doses was felt to be of paramount importance (Augustine and Levitan, 1979). Feingold (1975) first suggested that food additives, particularly colouring agents, can cause hyperactivity and learning disabilities, based on his observations on adults and hyperactive children sensitive to food colourings.

Metanil yellow is one of the non-permitted dyes used for food colouring in India, and is present in nearly 25% of all coloured foodstuffs (Khanna *et al.*, 1985). Metanil yellow is a monoazo dye, the sodium salt of *m*-[(*p*-anilinophenyl)azo]benzenesulphonic acid. Its toxicity and mutagenicity have been assessed (Giri *et al.*, 1986; Khanna *et al.*, 1980; Vaidya and Godbole, 1982); however, there have been no studies on brain neurotransmitters. The study reported here was therefore conducted on rats to assess the following: (a) the consequences of chronic consumption of metanil yellow, particularly by animals in which the brain is developing, on regional levels of monoamine neurotransmitters and AChE activity in the developing brain, as well as in adult rats; (b) the changes in performance in operant conditioning for food reward, and (c) the reversibility or otherwise of such changes on withdrawal of metanil yellow of the diet of developing rats.

MATERIALS AND METHODS

Animals and exposure. The study was conducted on Wistar rats of either sex of roughly equal numbers in each group. They received a freshly constituted diet (Mascarenhas *et al.*, 1984) provided *ad lib.* with water. Weaned animals were housed singly in polypropylene cages (37.5 × 22 × 16 cm) having husk bedding, in approximately 12 hr/12 hr light/dark cycles at 27 ± 3°C at 60–62% relative humidity. Metanil yellow (90% purity) was obtained from Eastman Kodak Laboratory and Research Products Division (Rochester, NY, USA). For studies on the developing brain, 20 mg metanil yellow/kg body weight as a 0.2% solution in distilled water was administered daily by gastric intubation from the second postnatal day to 60 days of age. Control animals were given equivalent

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✠Deceased.
Abbreviations: AChE = acetylcholine esterase; CNS = central nervous system; CRF = continuous reinforcement; DA = dopamine; FR5 = fixed ratio 5 schedule of reinforcement; 5-HT = serotonin; NA = noradrenaline.

amounts of distilled water by gastric intubation. Very few (< 2%) pups were lost in handling or misdosing. At 60 days the exposure was stopped and the animals were allowed to rehabilitate up to 160 days of age. In studies on adults, rats consumed, through their daily food intake of about 20.7 ± 1.5 g containing 400 ppm, approximately 20 mg metanil yellow/kg body weight/day, from 90 to 180 days of age. Food intake and body weights of all the rats were monitored regularly.

At 20 and 60 days of age, and at 160 days of age (at the end of rehabilitation) in the case of developing animals, and at 180 days of age for adult animals, the age-matched control and treated rats were decapitated and the brains were quickly removed in the cold. The following seven regions were dissected according to the methods described by Glowinsky and Iversen (1966) and Lindgren *et al.* (1982): motor cortex, striatum, nucleus accumbens, hippocampus, hypothalamus, cerebellum and ventral brain stem. The wet weight of the whole brain and of each region was noted.

Estimation of noradrenaline (NA), dopamine (DA) and serotonin (5-HT). NA, DA and 5-HT were simultaneously estimated in each brain region by the method of Cox and Perhach (1973) with minor modifications, and the concentrations were expressed as ng/g wet tissue. The amines were extracted from the tissue by homogenization in acidified butanol (10 vol). The catecholamines (NA and DA) were adsorbed on to alumina. 5-HT was extracted into an acidic, aqueous phase and reacted with *o*-phthalaldehyde to form a fluoroescent complex. NA and DA were eluted with perchloric acid and fluoroescent derivatives were formed by reacting with iodine, alkaline sulphite and acetic acid. 5-HT was measured (against reagent blank) at excitation 360 nm and emission 470 nm, NA at 380 nm and 495 nm and DA at 325 nm and 380 nm using a Hitachi 650-40 fluorescence spectrophotometer.

Estimation of acetylcholine esterase (AChE) activity. The activity of AChE was estimated by the method of Ellman *et al.* (1961). Tissue was homogenized (10 mg/ml) in 0.1 M-phosphate buffer at pH 8. 5 min after adding 2.6 ml phosphate buffer (0.1 M, pH 8) to 0.4 ml homogenate in a cuvette, the AChE activity was determined at room temperature (25°C) for 10 min in the presence of 0.1 ml 0.01 M-dithiobisnitrobenzoic acid and 0.02 ml 0.075 M-acetylthiocholine iodide in an LKB-4050 spectrophotometer at 412 nm.

Operant conditioning. (According to the procedure of Shailesh Kumar and Desiraju, 1992). The rats were tested in a Skinner box at 60 and 160 days of age in developing animals and at 180 days of age in adult animals. In the experimental set-up, the manipulation of a horizontal lever with 20 g force activated a solid food dispenser. Rats were trained to press the lever in 30-min sessions after 24 hr food deprivation. After learning, each rat was tested in 15-min sessions to record the number of reinforcements earned in continuous reinforcement (CRF) and then in fixed ratio 5 (FR5) schedules. The time taken for extinction of the learned response was also recorded. Learning behaviour was quantified in terms of number of

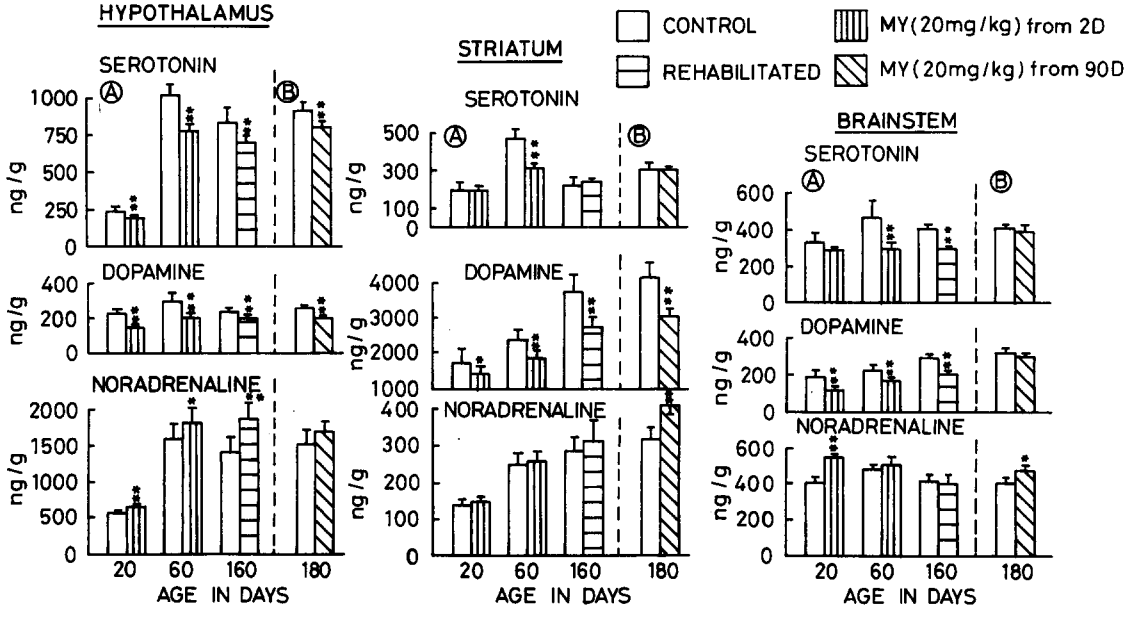

Fig. 1. Effects of chronic consumption of metanil yellow on brain regional monoamine levels. Panel A in each block shows changes in developing animals that were exposed from postnatal day 2 to 60 days and then rehabilitated up to 160 days; panel B in each block shows changes in adult animals that were exposed from 90 to 180 days of age. Each value (bar) is mean $\pm$ SD for six rats (significances compared using Student's *t*-test): $^{*}P < 0.05$; $^{**}P < 0.01$.

sessions taken for acquisition, rate of performance in CRF and FR5 schedules and time taken for extinction.

Statistical analysis. The results were analysed by Student's t-test (two tailed) and differences were considered to be significant at $P < 0.05$.

RESULTS

Animals exposed to metanil yellow did not display any behavioural anomalies at any stage of exposure. Food intake, body and brain weights were also unaffected.

Of the brain regions studied, the striatum, hypothalamus and brain stem were greatly affected by the consumption of metanil yellow and showed altered amine levels (Fig. 1). In the developing animals, the hypothalamus contained increased NA levels and decreased DA and 5-HT levels during the period of exposure, and these changes were not reversible even after the rehabilitation period. In adult animals, too, the hypothalamus contained lower levels of DA and 5-HT. The striatum showed decreased DA levels during exposure, which persisted even after rehabilitation. At late stages of exposure the striatum also showed decreased 5-HT levels, which recovered after rehabilitation. In adult animals the striatum contained increased NA levels and lowered DA levels. The brain stem showed a reduction in DA and 5-HT levels during exposure and also after rehabilitation; it also contained elevated NA levels in adult animals at 180 days of age.

Effects of AChE activity also showed regional variations (Fig. 2). The striatum and brain stem showed decreased activity at 20 days of age, but normal levels returned spontaneously by 60 days even while the consumption of metanil yellow was continuing. On the other hand, the hippocampus showed a delayed response, in that AChE activity decreased by 60 days of age, which reduction persisted even after rehabilitation. In the adult animals only the striatum was affected, showing decreased AChE activity.

Developing animals exposed to metanil yellow took more time to acquire learning behaviour at 60 days.

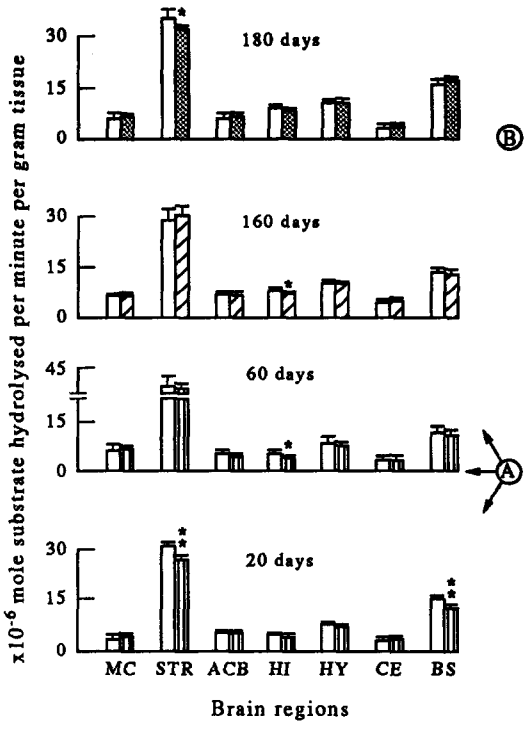

Fig. 2. Effects of chronic consumption of metanil yellow on acetylcholine esterase activity in brain regions. Each value is mean ± SD for five rats. MC = motor cortex; STR = striatum; ACB = nucleus accumbens; HI = hippocampus; HY = hypothalamus; CE = cerebellum; BS = brain stem. Other details as in Fig. 1.

This deviation was not present in the group tested after rehabilitation (Table 1). In the adult group also, treated rats took longer to learn the necessary behaviour. However, once it had been learned, no subsequent differences from controls were observed either in performance in CRF and FR5 schedules, or in extinction.

DISCUSSION

Although metanil yellow is known to be toxic and mutagenic (Giri *et al.*, 1986; Khanna *et al.*, 1980), the

Table 1. Effects of chronic consumption of metanil yellow on acquisition, performance and extinction of operant conditioning with food reward

Age† (days)	Group‡	No. of training sessions needed for acquisition	Performance§ (pedal press rate/15 min) CRF	Performance§ (pedal press rate/15 min) FR5	Time taken for extinction (min)
60	Control	1.75 ± 0.5 (3)	66.0 ± 14.9 (3 × 2)	35.6 ± 8.7 (3 × 2)	18.1 ± 2.6 (3)
	MY	2.87 ± 0.3 (3)*	60.6 ± 9.5 (3 × 2)	31.7 ± 6.8 (3 × 2)	15.4 ± 1.3 (3)
160	Control	2.3 ± 0.5 (4)	130.3 ± 19.1 (4 × 3)	57.4 ± 9.4 (4 × 2)	15.4 ± 3.4 (4)
	MY	2.2 ± 0.7 (4)	136.4 ± 15.7 (4 × 3)	61.6 ± 8.5 (4 × 2)	18.7 ± 2.2 (4)
180	Control	1.66 ± 0.6 (3)	85.0 ± 14.8 (3 × 4)	44.5 ± 12.2 (3 × 2)	13.0 ± 1.9 (3)
	MY	4.0 ± 0.1** (3)	84.3 ± 16.1 (3 × 4)	43.7 ± 6.8 (3 × 2)	16.8 ± 1.8 (3)

†Age 60 and 160 days represent the developing animals exposed during development up to 60 days of age, and rehabilitated from 60 to 160 days. Age 180 days represents the adult animals exposed from 90 to 180 days of age.
‡MY = exposed to metanil yellow.
§CRF = continuous reinforcement; FR5 = fixed ratio 5 schedule of reinforcement. Values are means ± SD of number of rats × performance sessions (in parentheses).
Significant from controls ($P < 0.05$; **$P < 0.01$).

effects of its chronic consumption on brain and behaviour have not been studied; indeed, such studies are not available even for many permitted food colourings.

The present study revealed alterations of levels of biogenic amines and AChE activity in certain regions of the brain, as a result of metanil yellow consumption. The hypothalamus, striatum and brain stem seemed particularly vulnerable. Of the neurotransmitters studied, dopaminergic and serotonergic systems were observed to be more vulnerable than the noradrenergic system. Although these monoaminergic neurotransmitter systems have their cell bodies in the brain stem, it is interesting to note that their terminals (containing the amines) seem to be affected differently in the different regions. Changes in the brain stem were not always accompanied by similar changes in the regions affected. Similarly, only the hippocampus showed reduced AChE activity in the developing animals. It is possible that differences in local factors in different regions of the brain may have a role in determining the effects of metanil yellow on neurotransmitter metabolism in axon terminals in the different regions of brain and that different neurotransmitter systems are affected in different regions.

There are as yet no data on differences in distribution of metanil yellow in the brain. Metanil yellow is metabolized by liver into non-toxic metanilic acid and toxic *p*-aminodiphenylamine (Khanna *et al.*, 1980). *p*-Aminodiphenylamine is known to be lipophilic and hence may be able to cross the blood–brain barrier and exert its toxic effects. The xanthine dye erythrosine-B (a permitted food dye) has been shown to inhibit DA uptake and binding in caudate synaptosomes in experiments involving acute exposure (Lafferman and Silbergeld, 1979). Hence, metanil yellow metabolites over a long period of exposure may cause altered amine levels, as observed in the present study.

Metanil yellow delayed operant learning. Previous reports suggest that even permitted food colours can bring about behavioural abnormalities in laboratory animals and in humans. Goldenring *et al.* (1980) exposed developing rat pups to a mixture of eight permitted food dyes for 26 days and observed increased activity and impaired performance in avoidance learning; however, they observed no changes in whole brain NA and DA levels. As whole brain estimations can mask regional alterations, these authors may have failed to observe regional changes. The study reported here revealed regional brain transmitter changes and also slowing in appetitive learning; however, in our gross observations, hyperactivity was not apparent in the metanil yellow group of rats. It is tempting to speculate that this may be due to a reduction in striatal DA level and also a tendency for cholinergic synaptic function to increase because of the reduction in AChE activity observed in this region.

Swanson and Kinesbourne (1980) found that hyperactive children fed with a mixture of approved food dyes showed impaired performance in paired-associated learning tests. Although the altered neurotransmitter levels may have led to changes in learning, the complete neural mechanisms involved in inducing changes in learning ability in children or in laboratory animals need to be explored further with regard to different types of food dyes.

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