that some amount of tris-BP can be readily released from fabric and may be absorbed through the skin, but further studies are needed to confirm this.

While positive results in mutagenicity tests in bacteria are usually reliable indicators of carcinogenic potential in mammals, some mutagens may not be carcinogens (*3, 10*). Thus, our results do not conclusively demonstrate that tris-BP is a cancer-causing agent. Another concern raised by the results is that tris-BP may cause heritable mutations in humans. Mutagenicity in bacteria does not, in itself, imply that a chemical poses a genetic risk to humans because the chemical might not reach the germ cells of exposed humans in an active form or might not be mutagenic to eukaryotic cells. The latter possibility is excluded for tris-BP by observations indicating that this chemical induces heritable mutations (sex-linked recessive lethals) in *Drosophila melanogaster* (*11*) as well as unscheduled DNA synthesis (*12*) and repairable breaks in DNA (*13*) in human cells in culture. A complete assessment of the mutagenic hazard associated with the use of tris-BP in children's sleepwear requires additional data on the possible oral and dermal absorption of the chemical from fabric as well as data on whether or not tris-BP or its active metabolites, once absorbed, can reach germinal tissue. More definitive evaluation of the carcinogenic potential of tris-BP must await the completion of tumor-induction studies in mammals.

MICHAEL J. PRIVAL*
Office of Toxic Substances WH-557, Environmental Protection Agency, Washington, D.C. 20460
ELENA C. MCCOY
BEZALEL GUTTER
HERBERT S. ROSENKRANZ
Department of Microbiology, New York Medical College, Valhalla 10595

References and Notes

1. T. J. McGeehan and J. T. Maddock, *A Study of Flame Retardants for Textiles* (Report No. PB251-441/AS, National Technical Information Service, Springfield, Va., 1976).
2. A. F. Kerst, *JFF/Fire Retardant Chem.* **1**, 205 (1974).
3. J. McCann, E. Choi, E. Yamasaki, B. N. Ames, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 5135 (1975); J. McCann and B. N. Ames, *ibid.* **73**, 950 (1976).
4. B. N. Ames, J. McCann, E. Yamasaki, *Mutat. Res.* **31**, 347 (1975).
5. J. McCann, N. E. Springarn, J. Kobori, B. N. Ames, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 979 (1975).
6. L. E. St. John, Jr., M. E. Eldefrawi, D. J. Lisk, *Bull. Environ. Contamin. Toxicol.* **15**, 192 (1976).
7. H. S. Rosenkranz, *ibid.* **14**, 8 (1975).
8. This compound was purchased as "tris (dichloropropyl) phosphate." Analysis by nuclear magnetic resonance (NMR), performed by the Stauffer Chemical Company, demonstrated that it was tris(1-chloromethyl-2-chloroethyl) phosphate.
9. R. W. Morrow, C. S. Hornberger, A. M. Kligman, H. E. Maibach, *Am. Indus. Hyg. Assoc. J.* **37**, 192 (1976).
10. H. S. Rosenkranz and W. T. Speck, *Biochem. Pharmacol.* **25**, 1555 (1976).
11. R. Valencia, personal communication.
12. H. F. Stich, cited by A. Blum and B. N. Ames, *Science* **195**, 17 (1977).
13. B. Gutter and H. S. Rosenkranz, unpublished data.
14. W. T. Speck and H. S. Rosenkranz, *Photochem. Photobiol.* **21**, 369 (1975).
15. We thank the Michigan Chemical Company for the highly purified tris-BP, the Stauffer Chemical Company for NMR analysis of the tris(1-chloromethyl-2-chloroethyl) phosphate, Dr. B. N. Ames for the *Salmonella* strains, and Sears, Roebuck, and Company and others who provided the samples of tris-BP–treated and untreated fabrics. Supported by contract 68-01-3246 from the Environmental Protection Agency Office of Toxic Substances.

* Present address, Genetic Toxicology Branch, HFF-156, U.S. Food and Drug Administration, Washington, D.C. 20204.

23 September 1976; revised 12 November 1976

Activation of the Nigrostriatal Dopaminergic Pathway by Injection of Cholera Enterotoxin into the Substantia Nigra

Abstract. *Twenty-four hours after unilateral injection of cholera enterotoxin into the rat substantia nigra there is an increase, in the striatum on the injected side, of basal adenylate cyclase activity, 3,4-dihydroxyphenylacetic acid, and 3-methoxy-4-hydroxyphenylacetic acid. Moreover, there is an increase of motor activity, and rats tend to circle contralateral to the side of the injection. Injection of cholera enterotoxin into brain nuclei may be a useful procedure for pharmacologically activating selected neuronal systems of brain and for studying the pharmacology of drugs that are suspected of interacting with these systems.*

Cholera enterotoxin (choleragen) binds to cell surface receptor glycolipids and stimulates membrane-bound adenylate cylase (*1*). The stimulation by choleragen is long lasting. We found that selected neuronal systems of rat brain were biochemically activated for prolonged periods if choleragen was injected into specific nuclei of brain. Activation may be the consequence of stimulating adenylate cyclase systems.

The dopaminergic neurons of the striatum originate in the substantia nigra (*2*), and destruction of the substantia nigra leads to a loss of dopaminergic neurons in the striatum (*3*). In contrast, we found that injection of choleragen into the substantia nigra produced biochemical changes in the striatum that are consistent with the hypothesis that dopaminergic neurons are activated. Biochemical activation of the striatum was manifested by an increase of basal adenylate cyclase activity, an elevation of 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-methoxy-4-hydroxyphenylacetic acid [homovanillic acid (HVA)] concentrations, and an increase of motor activity. These changes lasted for more than 24 hours. Local injection of choleragen into nuclei of brain may be a useful procedure for pharmacologically activating specific neuronal pathways and for evaluating the actions of drugs that are thought to alter these pathways.

Male Sprague-Dawley rats (150 to 180 g), anesthetized with chloral hydrate, were stereotaxically injected with 1 μg of choleragen (Schwarz/Mann, Orangeburg, N.Y.) in 1 μl or with vehicle unilaterally into the substantia nigra. The stereotaxic coordinates, according to Konig

Table 1. Basal and dopamine-stimulated adenylate cyclase activity of the rat striatum after injection of choleragen into the substantia nigra. Choleragen (1 μg) was injected unilaterally into the substantia nigra 24 hours before adenylate cyclase was assayed in the striata from the injected and contralateral sides of the brain. Enzyme activity (nanomoles of cyclic AMP per milligram of protein per minute) was measured in the absence (basal activity) and presence (dopamine-stimulated activity) of 0.1 m*M* dopamine. Values are means ± standard error of the mean (S.E.M.). Incubation was for 3 minutes at 30°C. A paired *t*-test was used to compare basal and dopamine-stimulated adenylate cyclase activity from the same side of the brain and activity in the striatum from the injected and contralateral sides of the brain.

Treatment	Enzyme activity			
	Injected side		Contralateral side	
	Basal	Dopamine-stimulated	Basal	Dopamine-stimulated
Toxin ($N = 10$)	0.13 ± 0.02*	0.18 ± 0.02*†	0.071 ± 0.005	0.11 ± 0.01‡
Vehicle ($N = 5$)	0.065 ± 0.006	0.11 ± 0.01§	0.068 ± 0.003	0.11 ± 0.01§

*$P < .05$ compared to contralateral. †$P < .05$ compared to basal. ‡$P < .001$ compared to basal. §$P < .01$ compared to basal.

and Klippel (*4*), were anteroposterior 1.9 mm, lateral 2.0 mm, and dorsoventral 2.5 mm. The injection site was verified by visual inspection of the needle track at the time of killing. Twenty-four hours after the intranigral injection, the animals were decapitated and the striata from the injected and contralateral side of the brain were rapidly removed and assayed for adenylate cyclase activity by a modification of the method of Kebabian *et al.* (*5*). Tissue was homogenized in 25 volumes of cold 4 m*M* tris-maleate buffer (*p*H 7.4) containing 0.15 m*M* EGTA. The reaction mixture contained, in a final volume of 100 μl, 55 m*M* tris-maleate, 1.5 m*M* $MgSO_4$, 6.5 m*M* theophylline, 0.17 m*M* EGTA, 0.5 m*M* [^{14}C]adenosine triphosphate (0.5 μc per sample), and 25 μl of homogenate. Incubation was for 3 minutes at 30°C. The reaction was terminated by adding 1*N* perchloric acid and the radioactive adenosine 3′,5′-monophosphate (cyclic AMP) formed was isolated on an alumina column (*6*). Enzyme activity was measured in the presence and absence of dopamine (0.1 m*M*). Locomotor activity was measured in a photocell activity cage for 15 hours before the time of killing. Dopamine, HVA, and DOPAC were assayed by gas chromatography–mass fragmentography (*7*) and protein was assayed by the method of Lowry *et al.* (*8*) with bovine serum albumin as standard.

Twenty-four hours after the injection of choleragen, basal activity of adenylate cyclase in the striatum from the treated side of the brain was about double the activity in the striatum on the contralateral side of the brain (Table 1). The finding that basal enzyme activity increased in the striatum on the injected side of the brain and not in the contralateral side is evidence that the choleragen did not diffuse to the striatum but acted at the site of injection. Choleragen is a nonspecific activator of adenylate cyclase (*1*) and could activate the enzyme in many cell types at the site of injection in addition to dopaminergic neurons. The addition of dopamine (0.1 m*M*) to homogenates increased adenylate cyclase activity by about the same extent in striata from both sides of the brain (Table 1). The vehicle had no apparent effect on basal or dopamine-sensitive adenylate cyclase activity, and in another series of studies (not presented here) treatment with the choleragen vehicle did not change basal or dopamine-stimulated activity compared with that in normal untreated animals.

Results consistent with those described above were found when the striatal dopaminergic system was evaluated (Table 2). Dopamine metabolism was apparently enhanced on the choleragen-treated side of the brain. Concentrations of DOPAC and HVA were increased, whereas dopamine levels remained unchanged. Similar changes occur in the striatum when dopaminergic neurons are activated with neuroleptic drugs or by electrical stimulation of the substantia nigra (*9*). Stable catecholamine levels accompanied by accelerated metabolism of the catecholamines during periods of increased neuronal activity are characteristic of peripheral sympathetic neurons as well (*10*). Catecholamine levels probably remain constant because of their ability to modulate the activity of tyrosine hydroxylase (*11*).

Table 2. Concentration of dopamine, HVA, and DOPAC in the rat striatum after injection of choleragen into the substantia nigra. Choleragen (1 μg) was injected unilaterally into the substantia nigra 24 hours before assaying dopamine and its metabolites in the striata from the injected and contralateral sides of the brain. Dopamine, DOPAC, and HVA were assayed by mass fragmentography. Normal, uninjected rats had substrate values that were similar to the values reported for vehicle-treated animals. A paired *t*-test was used to compare concentrations on the injected and conralateral sides. Values are means ± S.E.M.; $N = 6$.

Treatment	Concentration (nmole/g)	
	Injected side	Contralateral side
	Dopamine	
Toxin	26 ± 3	26 ± 2
Vehicle	28 + 2	28 + 1
	DOPAC	
Toxin	9.9 ± 0.7*	7.3 ± 0.6
Vehicle	5.6 + 0.6	6.2 + 0.3
	HVA	
Toxin	4.5 ± 0.6†	3.5 ± 0.3
Vehicle	2.8 + 0.2	2.7 + 0.2

*$P < .01$ compared to contralateral. †$P < .05$ compared to contralateral.

In support of the idea that dopaminergic neurons are activated by choleragen, we found that toxin-treated rats were approximately four times more active than vehicle-treated rats. Moreover, when the rats approached a cage wall or were prodded with a pencil they circled contralateral to the side of choleragen treatment. This finding is consistent with the hypothesis that turning is in the direction of lesser dopaminergic activity in nigrostriatal neurons (*3*). Our findings are in agreement with other reports of increased motor activity following an intracerebral injection of choleragen (*12*).

Our results are consistent with the hypothesis that choleragen injected into the substantia nigra activates biochemical systems associated with dopaminergic neurons that innervate the striatum. Activation appears to be specific. We found no changes of choline acetyltransferase or glutamate decarboxylase activities or of gamma aminobutyric acid concentrations in the striatum after treatment with choleragen (*13*). Activation of the system is long lasting; in fact, we found that basal adenylate cyclase activity and motor activity were enhanced for more than 7 days. Whether choleragen activates dopaminergic cell bodies directly, activates neurons that impinge on the cell bodies, or enters neurons and migrates to the nerve endings in the striatum where activation takes place remains to be studied.

Treatment with choleragen is a novel procedure for prolonged pharmacological activation of selected neuronal systems, and thus offers the possibility for biochemically tracing neuronal pathways in brain. Moreover, schizophrenia may be a consequence of enhanced sensitivity to dopamine (*14*), and pharmacological activation of dopaminergic systems of brain with choleragen may be a useful model for studying the pathophysiology and treatment of this disease. In addition, it may offer new avenues of research for the treatment of parkinsonism, a disease associated with a reduction of nigrostriatal dopaminergic neurons (*2*).

L. F. QUENZER
C. L. GALLI, N. H. NEFF
Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeths Hospital, Washington, D.C. 20032

References and Notes

1. P. Cuatrecasas, *Biochemistry* **12**, 3546 (1973); J. Holmgren, I. Lonnroth, L. Svennerholm, *Infect. Immun.* **8**, 208 (1973); J. Holmgren, I. Lonnroth, L. Svennerholm, *Scand. J. Infect. Dis.* **5**, 77 (1973); C. A. King and W. E. van Heyningen, *Infect. Dis. Rev.* **127**, 639 (1973); N. F. Pierce, *J. Exp. Med.* **137**, 1009 (1973); S. van Heyningen, *Science* **183**, 656 (1974).
2. O. Hornykiewicz, *Pharmacol. Rev.* **18**, 925 (1966).
3. U. Ungerstedt and T. Ljungberg, in *Frontiers in Catecholamine Research*, E. Usdin and S. H. Snyder, Eds. (Pergamon, Oxford, 1973), p. 689.
4. J. F. R. Konig and R. A. Klippel, *The Rat Brain: A Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem* (Williams & Wilkins, Baltimore, 1967).
5. J. W. Kebabian, G. L. Petzold, P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2145 (1972).
6. K. H. Jakobs, E. Bohme, G. Schultz, *Acta Endocrinol. Copenhagen Suppl.* **199**, 431 (1975). The perchloric acid extract was placed on a column (inner diameter, 0.4 cm) of 1 g of alumina. The column was washed with 9 ml of 1*N* perchloric acid, 10 ml of water, and 1.5 ml of 0.2*M* sodium acetate. Cyclic AMP was eluted from the column by the addition of 1.5 ml of sodium acetate solution. Recovery was 84 percent for cyclic AMP, and no interfering radioactive nucleotides were detected in the final eluate.
7. F. Karoum, J. C. Gillin, R. J. Wyatt, *J. Neurochem.* **25**, 653 (1975); S. H. Koslow, F. Cattabeni, E. Costa, *Science* **176**, 177 (1972).

8. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
9. R. H. Roth, L. C. Murrin, J. R. Walters, *Eur. J. Pharmacol.* **36**, 163 (1976).
10. J. V. Luco and F. Goni, *J. Neurophysiol.* **11**, 497 (1948); M. Vogt, *J. Physiol. (London)* **123**, 451 (1954).
11. T. Nagatsu, M. Levitt, S. Udenfriend, *J. Biol. Chem.* **239**, 2910 (1964).
12. R. J. Miller and P. H. Kelly, *Nature (London)* **255**, 163 (1975); G. Nistico, J. D. Stephenson, P. Preziosi, *Eur. J. Pharmacol.* **36**, 459 (1976).
13. B. K. Schrier and L. Shuster, *J. Neurochem.* **14**, 977 (1967); J. L. Meek, *Anal. Chem.* **48**, 375 (1976).
14. S. Matthysse, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 200 (1972).

15 September 1976

Participation of a Nonenzymatic Transformation in the Biosynthesis of Estrogens from Androgens

Abstract. *The biosynthesis of estrogens from androgens proceeds via three enzymatic hydroxylations, of which the first two take place on the C-19 methyl group and convert it to aldehyde. The final and rate-determining hydroxylation occurs at the 2β position, and the product rapidly and nonenzymatically collapses to an estrogen.*

All the estrogens in man are formed from androgen precursors by a sequence of enzymatic reactions referred to as aromatization (*1*). Location of this transformation, originally thought to be limited to the gonads and the placenta, has now been extended also to specific brain sites (*2*), as well as to adipose tissue (*3*). The implications of estrogen biosynthesis in the brain for behavior regulation (*4*) and in fat for the induction of postmenopausal carcinoma (*5*) emphasizes further the need for an understanding of the precise nature of the androgen to estrogen conversion as a prerequisite to any rational attempts at its modification or control. Previous studies have shown that aromatization involves the loss of the angular C-19 methyl group and the stereospecific elimination of the 1β and 2β hydrogens (*6*). The process includes the participation of three enzymatic hydroxylations requiring 3 moles of O_2 and NADPH (nicotinamide adenine dinucleotide phosphate reduced) per mole of estrogen formed (*7*). The first two of the hydroxylations take place on the C-19 methyl group giving rise successively to the 19-hydroxy (*8*) and 19-aldehyde structures (*9*). The site of the third hydroxylation and the nature of the final stage of the androgen to estrogen transformation remain unknown. The synthesis of 2β-hydroxy-19-oxoandrost-4-ene-3,17-dione (**1d**) (*10*) and its collapse to estrone in aqueous solution have been described. The extremely rapid aromatization of **1d** under physiological conditions and the coincidence of its structural features with the known requirements of biological aromatization suggested that 2β may be the site of the third and rate-determining hydroxylation in the aromatization process and that **1d** may be the immediate precursor of the female sex hormone. We now present experimental evidence in support of the above hypothesis and describe the complete mechanism of estrogen biosynthesis from androgen precursors including the unusual nonenzymatic transformation as a final step.

Because of the extremely rapid conversion of **1d** to estrone in neutral or basic aqueous solutions, we were unable to isolate labeled **1d** during the aromatization of labeled androstenedione with human placental aromatase under the usual conditions of *p*H 7.2 (*11*). Since **1d** is distinctly more stable under acidic conditions we repeated the incubation with ^3H- or ^{14}C-labeled androstenedione at *p*H 6, the lowest *p*H still consistent with adequate aromatase activity (*12*), and also added carrier **1d** to the labeled substrate to assist in the trapping of the labeled intermediate. To permit isolation and purification of **1d** without its further decay to estrone, we found it necessary to work up the incubation under acidic conditions and to convert the product to a stable derivative early in the isolation procedure. Reduction with sodium cyanoborohydride [$NaB(CN)H_3$], which is rapid and can be carried out in acid solutions, was the method we selected. This reagent converts the labile aldehyde **1d** to the stable tetrol **2c**, and also reduces both of the other aromatization intermediates **1b** and **1c** to the triol **2b**. The recovered substrate **1a** and the end product estrone are at the same time transformed to the 3β, 17β-diol **2a** and 17β-estradiol, respectively. Reduction of the 3-ketone function with $NaB(CN)H_3$ generated some 3α epimers in addition to the predominant 3β alcohols, but we isolated and analyzed only the 3β alcohol. The 3β stereochemistry in tetrol **2c** was established by means of NMR spectroscopy and conforms to the known orientation of the other main reduction products, **2a** and **2b** (*13*).

Table 1 shows the results obtained from the incubation of [1,2-^3H, 4-^{14}C]androstenedione without addition of carrier **1d** (A), with carrier **1d** added (B), and from the nonenzymatic control (C). The yields of the recovered substrate, intermediates, and product, isolated as their reduced forms, are given as percent of ^{14}C substrate content. Labeled **1d** as the tetrol **2c** derivative could be isolated only in experiment B, in which the car-

Table 1. Products isolated from the incubation of doubly labeled androst-4-ene-3,17-dione ($^3H/^{14}C$ = 5.8) and subsequent reduction. [1,2-^3H,4-^{14}C]Androst-4-ene-3,17-dione (0.1 μ*M*) (with a tritium content of 5.8×10^7 count/min and a ^{14}C content of 1.0×10^7 count/min) was incubated with placental microsomes in the presence of NADPH. The incubation was carried out in 3 ml of phosphate-citrate buffer (*p*H 6) for 30 minutes at 37°C. Simultaneously, another incubation with the same placental preparation was run exactly as above except that 950 μg (3 μ*M*) of the 2β-hydroxy derivative **1d** was added to the substrate before the start of incubation. A third incubation in which the placental microsomes were heated at 100°C for 3 minutes served as the nonenzymatic control. At termination, the incubation mixtures were adjusted to *p*H 3 with 0.1*N* HCl, and 6 ml of ethanol was added. The precipitated protein was removed and washed with ethanol. The combined ethanolic extracts (22 ml) were reacted with 200 mg of $NaB(CN)H_3$. After 3 hours at room temperature, excess reagent was decomposed with acetone, the reaction mixture was neutralized with 0.1*N* NaOH, and the volume was reduced. The remaining aqueous solution was extracted with ethyl acetate–(*n*)-butanol (10:1) and the organic layer was taken to dryness. The residue was purified by successive paper chromatography as follows: (system a) isooctane, toluene, methanol, water (75 : 125 : 160 : 40); (system b) isooctane, toluene, methanol, water (3:1:3:1); (system c) benzene, methanol, ethyl acetate, water (1:1:0.1:1); (system d) benzene, methanol, water (1 : 1 : 1). Radioactive regions corresponding to androst-4-ene-3β,17β-diol (**2a**), androst-4-ene-3β,17β,19-triol (**2b**), androst-4-ene-2β,3β,17β,19-tetrol (**2c**), and estradiol were eluted, diluted with the appropriate carrier steroid, and recrystallized until constant specific activity and isotope ratio were achieved. The tetrol **2c** was acetylated to the tetraacetate before crystallization. Abbreviations: A, substrate only; B, substrate + 950 μg of **1d**; C, inactivated enzyme control.

Product	Yield (percent ^{14}C)			$^3H/^{14}C$			Percent ^3H lost		
	A	B	C	A	B	C	A	B	C
Diol **2a**	31	38	65	5.0	5.0	5.0	13.8	13.8	13.8
Triol **2b**	1.7	6.1		5.0	5.0		13.8	13.8	
Tetrol **2c**		0.08			3.9			32.8	
Estradiol	12.0	5.9		1.5	1.5		74.1	74.1	