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Incorporation of Protein and Nucleic Acid Precursors into Frog Nervous Tissue *in vitro*

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In higher animals, 1,1,3-tricyano-2-aminopropene (TCAP) has a variety of effects on central nervous tissue. Grenell (1) reported that it caused a specific increase in the content of ultraviolet absorbing material (presumably nucleic acids) in rabbit spinal ganglion, cord anterior horn, cerebellar Purkinje and cortical pyramidal cells of the rabbit. When administered at 20 mg/kg body weight, it caused an increase in the RNA and protein content of Deiter's nerve cells while reducing their content in associated glia, and also accentuated differences in composition of the RNA of the two cell types (2). It was shown recently that when 15 μg/gm of TCAP was given to rats, there was an enhanced retention of learning in an avoidance conditioning situation (3). Most recently, Jacob and Sirlin reported (4) that TCAP greatly stimulated the *in vitro* incorporation of uridine into the nucleolar RNA of dipteran salivary glands. Further, it reversed the inhibition of uridine incorporation into nucleolar RNA by actinomycin D, which suggests that TCAP stimulated DNA-dependent RNA synthesis.

We were interested in determining whether TCAP would produce changes in frog nervous tissue nucleic acids similar to those observed in higher vertebrates. If TCAP affects structures of the nervous system associated with learning, it might be expected to

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have a lesser effect on lower animals. Our approach was to devise an in vitro system which would maintain these tissues in a viable condition for a sufficient period to permit the determination of their ability to incorporate various radioactive precursors of the nucleic acids and proteins.

Materials and Methods. The frogs, Rana catesbeiana (American bull frog), weighing approximately 350 gm, were obtained from the Dahl Company, Berkeley, California and used immediately or kept in storage at 10° C. for a short time. After anesthesia with ether, the brain was removed under sterile conditions. The tissue, minus the olfactory bulbs, was washed with cold medium, and cut into three major portions: the medulla, optic tract and bulb, and cerebral hemispheres. These were in turn separately cut into pieces approximately 1 mm^3, which were then placed in stoppered 11 x 75 mm glass tubes in such a manner that each tube had the same distribution of material. Ganglia were obtained by sterile dissection, rejecting the large second ganglion, and incubated individually.

The medium, a modification of one described by Wolf (5) for maintaining dissociated Rana catesbeiana kidney and heart cells, contained 10% Hank's Balanced Salt Solution,* 45% Earle's Balanced Salt Solution, 30% Medium 199, 10% fetal bovine serum, 400 units/ml each of penicillin and streptomycin, and was adjusted to pH 7.35 with 7.5% $NaHCO_3$. With tri-weekly changes of fluid and air as the

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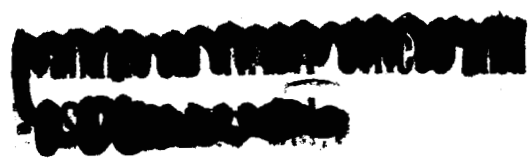
* All tissue culture components were obtained as prepared solutions from Microbiological Associates, Albany, California.

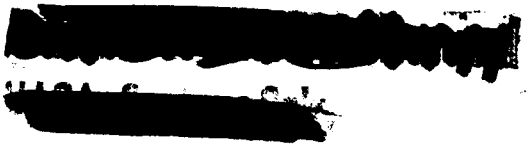


gas phase, frog brain tissue could be maintained for up to three weeks. However, the tissue was used immediately in the experiments to be reported here and the incubation time was 18 hours at 16-18° C. Ganglia were incubated in 90% Medium 199, 10% fetal calf serum, and 0.2% glucose with antibiotics, pH, and conditions as before.

The isotopic precursors used were obtained from the New England Nuclear Corporation and had the indicated specific activities: H^3-uridine (15.3 mc/mg), H^3-cytidine (25 mc/mg), H^3-thymidine (31 mc/mg), adenine-2-C^{14} (0.059 mc/mg), guanine-2-C^{14} (0.022 mc/mg), and valine-1-C^{14} (0.053 mc/mg).

Incubations were terminated by washing the tissue twice with cold phosphate-buffered saline, and then separating the material into a low molecular weight (LMW), RNA, DNA, and protein fractions by a modification of the Schmidt-Thanhauser method (6). The absorbancy of the LMW, RNA, and DNA fractions was determined using a Beckman Model DB spectrophotometer. A Packard liquid scintillation counter was used to determine the content of radioactivity of an aliquot of the individual fractions dispersed in a toluene counting solution containing .35% terphenyl, .025% POPOP, 4% hyamine, and 1% formic acid.

Results. Initial experiments were conducted to determine whether the tissues were able to incorporate significant amounts of radioactivity during an 18-hour incubation. Easily determinable amounts of all the precursors except H^3-thymidine were incorporated. Approximately 0.4% of the added radioactivity was found in the LMW fraction and 0.2% in the RNA fraction. The relatively slight incorporation of H^3-thymidine was expected since the tissues were not engaged in for-



mation of new DNA. It was further observed that only a negligible amount of radioactivity from the other precursors was incorporated into the DNA. These early experiments were conducted with only a single isotopic precursor in the medium. Dual labeling experiments with paired H^3- and C^{14}-precursors were initiated. Comparison of results with earlier experiments showed this to be a quite satisfactory procedure.

A study was performed to determine the effect of increasing amounts of TCAP on the incorporation of H^3-uridine into the RNA fraction and C^{14}-valine into the protein fraction of central nervous tissue. It can be seen from Table I that there is a progressively greater inhibition of incorporation with no indication of stimulation at 5 μg/ml. When the data is plotted on "probit" paper, straight lines with differing slopes are obtained for the different precursors. The greater slope with H^3-uridine suggests a more direct effect on newly synthesized nucleic acids than on proteins. The residual incorporation of radioactivity into tissues poisoned with a high concentration of azide may be due to either non-specific adsorption or to metabolic activities using energy stores present in the cells prior to inhibition of respiration by the azide.

The results of a representative experiment performed to compare the effects of TCAP with those of DNP and azide are shown in Table II. The concentrations of TCAP, DNP, and azide selected were those which have been shown (7, 8) to inhibit by 50% the oxidative phosphorylation of mitochondria. The incorporation of precursors into the DNA and protein fractions was negligible and is not shown. In general,

the compounds were somewhat inhibitory. In a separate experiment, 20 μg/ml of TCAP inhibited C^{14}-valine incorporation into the protein fraction by 40%. The apparently greater incorporation of H^3-uridine into the LMW fraction in the presence of TCAP in this experiment was not a reproducible result, nor was the apparent greater inhibition of H^3-cytidine incorporation. In general, the variable nature of the experimental material precluded exact replication of results as is evident from the large standard errors shown in the table for some conditions.

Discussion. The biochemical processes involved whereby TCAP can produce an alteration in the RNA base composition of nervous tissue are not understood. A variety of metabolic effects of TCAP have been described, but it is probable that most of these are of secondary nature. Thus, the observation that it inhibits organic binding of iodine by the thyroid, suppresses the formation of thyroxine, and inhibits the conversion of monoiodotyrosine to diiodotyrosine (9) is difficult to relate to nucleic acid metabolism.

In the experiments reported here, the radioactive precursors must first be phosphorylated before they can be incorporated into RNA. Incorporation into the LMW pool only partially reflects this activity. There is some evidence that TCAP has an effect on oxidative phosphorylation and the formation of ATP, since Eberts (7) demonstrated that it uncoupled phosphorylation associated with oxidation of various tricarboxylic acid cycle intermediates in a manner similar to 2,4-DNP.

The results were not the same in the two tissues investigated. TCAP inhibited the in vitro incorporation of valine and uridine into frog central nervous tissue, but only valine incorporation was inhibited in ganglia. When the effect of TCAP was compared with that of two inhibitors of oxidative phosphorylation using ganglia, no major effect was observed. These data may be compared to the observation of Eberts (10) that TCAP had no effect on the incorporation of U-C^{14}-glucose or C^{14}-lysine into protein, RNA, or DNA of the cerebrum, cerebellum, midbrain, cord, or whole brain of rabbits.

It is possible that some other metabolic activity of TCAP is more closely related to its in vivo effect on nucleic acid metabolism. It has a direct effect on respiration since it increased oxidation of acetate, tripalmitin, leucine, and glycine in rats with increased CO_2 output (11). Again, TCAP at low concentrations appreciably inhibited the reduction of crotonyl-CoA by rat liver microsomes (12). Most analogous to the results obtained here is the observation by Mendelson and Warmouth (13) that TCAP, in rat brain cortex slices, produced a marked stimulation of O_2 uptake, whereas slices removed from rats administered TCAP in vivo respired at the same rate as controls. In contrast, 2,4-DNP, which uncoupled oxidative phosphorylation, did not stimulate O_2 uptake.

Summary. The effects of TCAP on the incorporation of C^{14}-adenine, C^{14}-guanine, H^3-uridine, H^3-cytidine, and C^{14}-valine into frog central and peripheral nervous tissue maintained in vitro was observed. TCAP inhibited C^{14}-valine incorporation into the protein fraction of both tissues. However, H^3-uridine incorporation into RNA was inhibited

only in brain tissue. TCAP caused changes in the nucleic acid metabolism of frog ganglia quite similar to those produced by DNP and azide.

TABLE I. Effect of Varying Concentrations of TCAP on the *in vitro* Incorporation of H^3-Uridine and C^{14}-Valine by Frog Central Nervous Tissue*

Addenda	Incorporation	
	Uridine-H^3 (2μc/ml)	Valine-C^{14} (0.5μc/ml)
None	7,300 ± 600	160 ± 60
5 μg/ml TCAP	6,600 ± 600	150 ± 40
20 μg/ml TCAP	2,900 ± 200	50 ± 5
50 μg/ml TCAP	1,900 ± 200	35 ± 5
100 μg/ml TCAP	1,100 ± 100	40 ± 5
2 mg/ml azide —	170 ± 90	20 ± 5

* Data is summarized from two different experiments. Incorporation is mean total cpm ± S.E. for three replicate tubes each containing 0.5 ml of medium and 10 mg of tissue.

TABLE II. Effect of Oxidative Phosphorylation Inhibitors on the *in vitro* Nucleic Acid Metabolism of Frog Ganglia

Additions	Incorporation (cpm/unit O.D. $_{260\ m\mu}$)*			
	Uridine	Adenine	Cytidine	Guanine
Low Molecular Weight Fraction				
None	5,600 ± 390	12,210 ± 860	8,500 ± 2,690	5,060 ± 1,460
75 µg/ml TCAP	7,140 ± 1,990	13,570 ± 2,260	4,530 ± 630	3,770 ± 620
2 µg/ml DNP	3,680 ± 460	10,020 ± 1,930	6,500 ± 2,550	2,920 ± 890
15 µg/ml azide	5,660 ± 220	13,550 ± 1,090	5,470 ± 470	3,600 ± 1,490
RNA Fraction				
None	1,200 ± 280	1,260 ± 130	1,970 ± 730	300 ± 30
75 µg/ml TCAP	1,300 ± 370	1,110 ± 190	1,030 ± 50	220 ± 70
2 µg/ml DNP	840 ± 60	1,160 ± 220	1,070 ± 70	260 ± 90
15 µg/ml azide	560 ± 150	1,180 ± 40	1,810 ± 500	470 ± 180

* Values given are the mean ± S.E. for three replicate incubations. The medium (0.5 ml) contained either 1 µc each of uridine-H^3/adenine-C^{14} or cytidine-H^3/guanine-C^{14}.

BIBLIOGRAPHY

(1) Grenell, R. G. P.N.Y.A.S. 96:345 (1961).

(2) Eghhazi, E. and Hyden, H. J. J. Biophys. Biochem. Cytol. 10:403 (1961).

(3) Chamberlin, T. J., Rothchild, G. H., and Gerard, R. W. P.N.A.S. 49:918 (1963).

(4) Jacob, J. and Sirlin, J. H. Science 144:1011 (1964).

(5) Wolf, K., Quimby, M.C., Pyle, E. A., and Dexter, R. P. Science 132:1890 (1960).

(6) Scott, J. F., Fraccastoro, A. P., and Taft, E. P. J. Histochem. Chtochem. 4:1 (1956).

(7) Eberts, F. S. Biochem. Pharmacol. 8:367 (1961).

(8) Hunter, F. E. Methods in Enzymology 2:615 (1955).

(9) Ingbar, S. H. J. Chem. Endocrin. Met. 21:128 (1961).

(10) Eberts, F. S. Upjohn Co. Report (1961).

(11) Miller, W. L. and Krake, J. J. P.S.E.M.B. 107:585 (1961)

(12) O'Connell, P. W. and Eberts, F. S. Biochem. Pharmacol. 8:337 (1961).

(13) Mendelson, J. H. and Warmouth, M. Nature 198:1202 (1963).