Incorporation of Protein and Nucleic Acid Precursors into Frog Nervous Tissue *in vitro*. (31213)

JACOB SHAPIRA, PETER C. F. CASTELLANI, AND JOHNNIE O. COLEMAN
NASA, Ames Research Center, Moffett Field, Calif.

In higher animals, 1,1,3-tricyano-2-amino-propene (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). Jacob and Sirlin reported(3) 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. It has been shown(4) that TCAP can accelerate limb regeneration in the newt, a process limited by nerve growth, and also that it can induce the growth of fibers from chick ganglia maintained *in vitro*. These effects of TCAP on nucleic acid metabolism and nervous tissue are of particular interest because of the role played by RNA in learning and memory(5,6) and the observation that when 15 mg/kg of TCAP was given to rats, there was an enhanced retention of learning in an avoidance conditioning situation(7).

We were interested in determining whether TCAP would produce changes in frog nervous tissue nucleic acids similar to those observed in higher vertebrates. It is not known how general a phenomenon the effects of TCAP on nervous tissue are and further, it was hoped that these studies might shed some light on its mechanism of action. Our approach was to devise an *in vitro* system which would maintain frog nervous 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 bullfrog), weighing approximately 350 g, were obtained from the Dahl Co., Berkeley, Calif., 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 3 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 × 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(8) for maintaining dissociated *Rana catesbeiana* kidney and heart cells, contained 10% Hanks' 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 gas phase, frog brain tissue could be maintained for up to 3 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 described for brain tissue.

The isotopic precursors used were obtained from the New England Nuclear Corp. and had the indicated specific activities: H^3-uridine (15.3 mc/mg), H^3-cytidine (25 mc/mg),

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

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 the material was separated into low molecular weight (LMW), RNA, DNA, and protein fractions by a modification of the Schmidt-Thanhauser method(9). 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 0.35% terphenyl, 0.025% 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 formation 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 then 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. The quite similar degrees of inhibition of both the RNA and protein precursor suggest a general toxicity in this tissue rather than a specific effect on RNA metabolism. 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.

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

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

* Data are summarized from 2 different experiments. Incorporation is mean total cpm ± S.E. for 3 replicate tubes each containing 0.5 ml of medium and 10 mg of tissue.

The results of an experiment performed to compare the effects of TCAP with those of DNP and azide are shown in Table II. The concentrations of 75 μg/ml of TCAP, 2 μg/ml DNP, and 15 μg/ml azide were selected since they have been shown(10,11) to inhibit by 50% the oxidative phosphorylation of mitochondria. The lower concentrations of TCAP studied are in the range of concentrations known to produce *in vivo* and *in vitro* stimulation of growth of nervous tissue. The incorporation of precursors into the DNA and protein fractions was negligible and is not shown.

It can be seen that TCAP, DNP and azide all inhibited the incorporation of cytidine and guanine into the LMW fraction whereas the incorporation of uridine and adenine was unaffected. However, TCAP alone of these compounds reduced the incorporation of adenine into RNA whereas all 3 inhibited cytidine incorporation to about the same degree. Again, there was no evidence of a stimulation of nucleic acid metabolism at 5 μg/ml, a concentration known(4) to stimulate outgrowth of fibers from chick ganglia *in vitro*. It is of interest to note that Eghhazi and Hyden observed(2) a decrease in content of cytidine in rabbit nerve cells after administration of TCAP *in vivo*.

Discussion. The biochemical processes involved whereby TCAP can produce an alteration in the RNA base composition of nervous

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

Additions	Uridine	Adenine	Cytidine	Guanine
	Incorporation (cpm/unit O.D.$_{260\,m\mu}$)*			
Low molecular weight fraction				
None	5,250 ± 400	11,600 ± 550	10,000 ± 1,900	5,100 ± 70
5 μg/ml TCAP	5,590 ± 600	9,900 ± 700		
20 " "	4,700 ± 600	9,700 ±1,300		
75 " "	5,650 ±1,200	10,600 ±1,800	7,350 ± 1,700	3,200 ± 400
2 " DNP	4,400 ± 500	11,100 ±1,100	5,400 ± 1,250	2,000 ± 600
15 " azide	6,100 ± 400	13,300 ± 950	5,600 ± 1,300	3,100 ± 300
2 mg/ml azide	1,800 ± 200	1,800 ± 400		
RNA fraction				
None	1,380 ± 210	1,380 ± 140	2,760 ± 500	300 ± 50
5 μg/ml TCAP	1,330 ± 110	930 ± 100		
20 " "	1,010 ± 170	980 ± 80		
75 " "	1,320 ± 230	900 ± 130	1,740 ± 230	270 ± 90
2 " DNP	1,280 ± 215	1,450 ± 260	1,530 ± 500	300 ± 50
15 " azide	1,450 ± 400	1,720 ± 310	1,980 ± 360	320 ± 130
2 mg/ml azide	120 ± 20	230 ± 50		

* Values given are the mean ± S.E.M. for 6 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}. See text for details.

tissue are not understood. A variety of metabolic effects of TCAP has 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 moniodotyrosine to diiodotyrosine(12) 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 formation of ATP, since Eberts(10) 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 uridine into frog central nervous tissue, but did not inhibit its incorporation into ganglia. Valine incorporated into ganglionic tissue was inhibited 20% by 5 μg/ml TCAP and 36% by 20 μg/ml TCAP, which was about the same as that observed in brain tissue. When the effect of TCAP was compared with that of 2 inhibitors of oxidative phosphorylation using ganglia, the only situation where a difference was observed was the incorporation of adenine into RNA. These data may be compared to the observation of Eberts(13) that TCAP had no effect on 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(14). Again, TCAP at low concentrations appreciably inhibited the reduction of crotonyl-CoA by rat liver microsomes(15). Most analogous to the results obtained here is the observation by Mendelson and Warmouth(16) 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 in general quite similar to those produced by DNP and azide. However, TCAP did inhibit adenine incorporation into RNA whereas DNP and azide did not.

1. Grenell, R. G., P.N.Y.A.S., 1961, v96, 345.
2. Eghhazi, E., Hyden, H. J., J. Biophys. Biochem. Cytol., 1961, v10, 403.
3. Jacob, J., Sirlin, J. H., Science, 1964, v144, 1011.
4. Houlihan, R. T., DaVanzo, J. P., Exp. Neurol., 1964, v10, 183.
5. Barondes, S. H., Jarvik, M. E., J. Neurochem., 1964, v11, 187.
6. Barondes, S. H., Nature, 1965, v205, 18.
7. Chamberlin, T. J., Rothchild, G. H., Gerard, R. W., P.N.Y.A.S., 1963, v49, 918.
8. Wolf, K., Quimby, M. C., Pyle, E. A., Dexter, R. P., Science, 1960, v132, 1890.
9. Scott, J. F., Fraccastoro, A. P., Taft, E. P., J. Histochem. Cytochem., 1956, v4, 1.
10. Eberts, F. S., Biochem. Pharmacol., 1961, v8, 367.
11. Hunter, F. E., Methods in Enzymology, 1955, v2, 615.
12. Ingbar, S. H., J. Chem. Endocrin. Met., 1961, v21, 128.
13. Eberts, F. S., Upjohn Co. Report, 1961.
14. Miller, W. L., Krake, J. J., Proc. Soc. Exp. Biol. and Med., 1961, v107, 585.
15. O'Connell, P. W., Eberts, F. S., Biochem. Pharmacol., 1961, v8, 337.
16. Mendelson, J. H., Warmouth, M., Nature, 1963, v198, 1202.

Received December 9, 1964. P.S.E.B.M., 1966, v122.

Studies on Homologous Disease Using Germfree Mice.* (31214)

J. C. SALOMON AND J. C. LECOURT (Introduced by Morris Pollard)
Institut de Recherches Scientifiques sur le Cancer, Centre National de la Recherche Scientifique, B.P. 8 Villejuif (Val de Marne), France.

Intravenous injection of newborn mice with spleen cells from an adult mouse of a different strain can induce very regularly a complex syndrome called homologous or runt disease (1,2,3,4). The disease is fatal whenever the cell inoculum is large.

Does this graft *versus* host (G.V.H.) reaction develop if the animals are maintained free of infectious agents, as provided by germfree methodology? In other words, is the disease in conventional animals the effect of the G.V.H. reaction alone, or is it the result of a combination of the G.V.H. reaction plus some enhancing effect of infection, the G.V.H. reaction alone being relatively benign? In the first hypothesis, axenic mice should react like conventional mice; in the second case, axenic mice should show only the basic G.V.H. reaction.

The second hypothesis seemed plausible because certain microorganisms can induce runting but not complete homologous disease in newborn mice: strain Copenhagen of *Salmonella typhimurium*(5), polyoma virus(6,7, 8), lymphocytic choriomeningitis virus(9), reovirus(10), thymic agent(11). We also know that neonatal injection of a large dose of hydrocortisone(12,13) or neonatal thymectomy(14,15,16) will induce symptoms of runting in conventional but not in germ free mice. However, contrary to the infectious hypothesis of the homologous disease, it is known that runting can be induced by spleen cells which are free of infectious agents injected by intraperitoneal(16) or intravenous (17) routes.

Axenic animals have an immunologic status different from that of the conventional counterpart. It is underdeveloped or dormant: the lymph nodes have few germinal zones and the serum globulin levels are far below that of the conventional animal. The immunogenic mechanism of the axenic animal is functional-

* Supported by a grant from l'Association pour le Dévelopment de la Recherche sur le Cancer à Villejuif.