Virus Growth and Cellular Energy Production: Effect of Substances Chemically Related to Thyroxin on Influenza Virus.* (20540)

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Growth of influenza virus in tissue cultures has been partially inhibited with 2,4-dinitrophenol (DNP) under conditions which indicate that high energy bonds derived from oxidative phosphorylation play a part in growth of this virus(1-3). An antagonist of thyroxin, butyl 3,5 diiodo-4-hydroxybenzoate (4), has an even more powerful action than DNP in uncoupling phosphorylation from oxidation(5,6). This suggested studies of the effect of this compound on virus growth and a comparison with the action of DNP. Thyroxin itself and other related substances have also been investigated.

Materials and methods. The PR8 allantoic passage strain of influenza virus was used in all experiments. Methods of tissue culture in flasks, and the preparation of deembryonated eggs have been described previously(3). In addition, a modification of the roller tube technic was adopted which permitted direct observation of fragments of chick embryo chorioallantoic membrane sticking to the glass walls of the tubes. The medium used was Hanks' balanced salt solution with certain combinations of sodium pyruvate and glucose. The pH was adjusted to 7.6 and maintained as close to this level as possible. No sodium bicarbonate, serum, plasma or chick embryo juice was added because the duration of the experiments was short and it was desired to employ as simple a medium as possible. The roller tubes were closed with plastic screw caps which were kept loose to permit escape of CO_2. Because of their limited solubility in aqueous media the butyl† and methyl esters of the substituted benzoic acid were sterilized by heating concentrated alcoholic solutions and then diluted 1:1000 or more in alkaline balanced salt solution. Alcohol alone at concentrations as high as 1% had no effect on the growth of virus or on proliferation of the chorioallantoic tissue. Diiodotyrosine and 3,5 diiodo-4-hydroxybenzoic acid (Eastman) were sterilized by heating the aqueous suspensions to 80°C for 20 minutes and dissolved by adding NaOH to bring the pH slightly above neutrality. At the conclusion of the experiments pooled samples of the tissue were suspended in fresh balanced salt solution with 0.1% glucose and measurements of oxygen consumption were made by the usual Warburg method.

Results. Warburg experiments were done with fresh minced chorioallantoic membrane to test for stimulation or depression of respiration by butyl 3,5-diiodo-4-hydroxybenzoate. The tissue was rinsed 3 times in balanced salt solution and suspended in this same solution containing the test substance. One set of experiments was done with 0.1% pyruvate in the balanced salt solution and a second set with 0.1% glucose. In the presence of pyruvate both 20 γ/ml and 5 γ/ml of the butyl ester produced an approximately 50% increase in the respiration within a few minutes, and some stimulation was evident, particularly with the lower concentration, after 7 to 8 hours. At 24 hours there was no significant difference in O_2 consumption between the treated and control tissue. The medium remained alkaline. In the presence of glucose, on the other hand, the butyl ester produced a rapid drop in pH, and there was almost complete inhibition of oxygen consumption within 3 to 4 hours. No initial stimulation was detected. These results are essentially the same as those previously detailed for dinitrophenol and chorioallantoic tissue(3). As with DNP a drop in the pH of the medium causes a relatively higher toxicity of the drug.

The results of experiments with influenza virus in flask and roller tube cultures are presented in Table I. The flask cultures with

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† A supply of the butyl ester was furnished by Dr. A. E. Heming of Smith, Kline and French, Inc. The methyl ester was prepared according to the method of Wilkinson and his associates(4).

TABLE I. Effect of Butyl and Methyl Esters of 3,5-diiodo-4-hydroxybenzoic Acid on Growth of Influenza Virus in Tissue Culture.

Tissue culture	Ester, γ/ml		Day tested	Hemagg. titer	Final pH	Epithelial† growth	Fibroblast growth	O_2, μl/g/hr
Flask*	Butyl	8	6	<2	7.7	±	±	45
	"	4	6	4	7.9	+, ±	+	145
	"	2	6	175	7.8	+, ±	+	150
	—	0	6	265	7.7	+	+	160
Roller tube*	Butyl	5	3	<2	6.9	±	0	0
	"	2.5	3	2	7.5	+	+	140
	"	1	3	40	7.6	+	++	212
	"	0	3	215	7.5	++	++	202
	Methyl	10	3	<2	6.8	0	±	0
	"	5	3	25	7.0	+	+	73
	—	0	3	112	7.9	+	+	218
	Free acid	200	3	65	7.2	+, ±	+	N.D.
	Thyroxin	50	3	80	7.1	±	++	80

Virus inoculum 0.1 cc of 10^{-4} or approximately 10 tissue culture infectious doses. Results represent averages of duplicate experiments.

* Medium in flasks was Hanks' balanced salt solution with 0.02% glucose and 0.1% sodium pyruvate, in roller tubes 0.05% glucose and 0.1% pyruvate.

† For flask cultures growth was observed in fragments of tissue explanted to fibrin clot medium; in roller tubes growth was observed directly by microscopic examination of tissue sticking to wall of tube.

a medium containing 0.1% pyruvate and 0.02% glucose were gently shaken in a special apparatus during the period of incubation(3). The hemagglutination titer remained low for periods as long as 6 days in the presence of 4 γ/ml‡ of the butyl 3,5 diiodo-4-hydroxybenzoate. There was only slight retardation of tissue growth in explants and little depression of oxygen consumption. Concentrations of 8 γ/ml were found to be toxic in this medium. With 0.1% glucose in place of pyruvate, concentrations as low as 2 γ/ml were toxic even when $CaCO_3$ or $NaHCO_3$ were added to control the drop in pH.

In roller tube cultures 2.5 γ/ml of the butyl ester produced a hundredfold reduction in the hemagglutination titer at 3 days without noticeable effects on fibroblastic outgrowth but with slight depression of epithelial proliferation and some inhibition of oxygen consumption. The methyl ester at concentrations of 5 γ/ml produced only a slight depression of hemagglutinin formation despite rather marked inhibition of oxygen uptake. Other experiments were done with the sodium salt of 3,5 diiodo-4-hydroxybenzoic acid. This substance produced noticeable effects on virus growth only at concentrations of 200 γ/ml or higher but it caused development of acidity, probably attributable to increased glycolysis, at concentrations between 100 and 1000 γ/ml.

Wilkinson(4) has reported that the antithyroxin effect of the butyl ester is about twice that of the methyl ester and that unesterified 3,5 diiodo-4-hydroxybenzoic acid is inactive. Attempts to reverse the virus inhibitory effect of the butyl ester by adding thyroxin at concentrations of 5 to 50 γ/ml to the roller tube tissue cultures at the beginning of the experiment have so far been unsuccessful. Thyroxin by itself had little effect on the growth of influenza virus under those conditions but at concentrations of 50 γ/ml it tended to inhibit tissue proliferation and oxygen consumption after 3 days' incubation (Table I). Further studies with this hormone are in progress.

The more rapid growth of virus in deembryonated eggs made it possible to study the early effects of the butyl ester both on infectivity and hemagglutination. Little inhibition of virus was obtained below 10 γ/ml but at this concentration the ratio of the hemagglutination titers, control/treated, after 72 hours' incubation was 8 in one experiment and 70 in another. At concentrations of 20 γ/ml hemagglutinins were detectable in only

‡ This is approximately 8.5 x 10^{-6} M.

TABLE II. Effect of Butyl Ester on Influenza Virus in Deembryonated Eggs.

		Time in hr 18	24	48	72
Hemagg.*	Treated	<2	<2, <2, <2, 512	<2, <2, <2, 256	<2, <2, <2, 100
	Control	2, 8, 160, 160	1900	7700	2600
Infect.† (log)	Treated	4.0, 5.0	5.5	5.0	—
	Control	6.5, 7.0	8.5	7.5	—
O_2 uptake, μl/g/hr	Treated	169	200	108	75
	Control	240	240	122	180

Concentration of butyl ester 20 γ/ml; alcohol 1% by volume in this set of experiments.

* Where there were marked differences, titers of individual deembryonated eggs are presented. Elsewhere the single figure represents the avg.

† Infectivity titrations were done on pools of 4 individual fluid samples. Initial titer approximately 10^2 E.I.D. 50.

about 25% of the treated cultures. Several experiments were done at this level of the butyl ester and minced samples of the membranes from 3 to 4 preparations were pooled and tested for O_2 uptake at various periods from 18 to 72 hours. In addition the fluids were titrated for infectivity by allantoic inoculation of chick embryos. The results are summarized in Table II. In the first 24 hours an increase in infectivity titer occurred in both treated and control preparations but both hemagglutination and infectivity titers of the treated cultures remained below the controls for a period of 48 hours. During the period of maximum inhibition of virus growth the butyl ester reduced the oxygen consumption only by 15 to 30% but after 72 hours the concentration of 20 γ/ml began to produce deleterious effects as indicated by more definite depression of respiration.

Diiodotyrosine and sodium 3,5 diiodo-4-hydroxybenzoate at a concentration of 2000 γ/ml produced little or no inhibition of virus growth in deembryonated eggs but oxygen consumption after 48 hours' incubation was depressed by about 30%.

Experiments in chick embryos inoculated by the allantoic route indicated that the LD50 of the butyl ester was 2 to 4 mg per egg when given as a suspension. Because of the low solubility of the compound it is likely that only a small part of the amount injected was actually in solution in the allantoic fluid. With the maximum possible dosage no inhibition of virus growth was found at periods of 18 to 48 hours after inoculation.

Discussion. In most respects the butyl ester of 3,5 diiodo-4-hydroxybenzoic acid closely resembles 2,4-dinitrophenol(3) in its action on tissue cultures and deembryonated eggs infected with influenza virus but on a molar basis it is about 5 to 10 times as active as the latter compound in its inhibitory action both on virus and tissue. The reported low toxicity of the butyl ester for mice(4) by subcutaneous injection, 3500 mg/kg is of interest in view of the considerably higher toxicity, about 80 mg/kg, in chick embryos inoculated by the allantoic route, and the still higher toxicity in tissue cultures. Although dinitrophenol at concentrations which were effective in tissue cultures also produced temporary inhibition of influenza virus in the allantoic sac of chick embryos, this effect was not observed with the butyl ester possibly because of its insolubility or rapid destruction.

Thyroxin itself produced very little inhibition of the growth of influenza virus in tissue culture even at concentrations of 50 γ/ml which tended to depress epithelial growth and respiration. Although thyroxin has been reported to have an effect like dinitrophenol on oxidative phosphorylation(5,7), in certain mitochondrial preparations, unknown factors must prevent this uncoupling action from inhibiting the growth of influenza virus in intact cells. Delayed absorption or a latent period before activation may be concerned. The failure of thyroxin to reverse the virus inhibition suggests that the observed effects may be separate from the mechanism of antithyroxin action of the butyl ester in thyroidectomized rats(4).

Conclusion. Butyl 3,5-diiodo-4-hydroxy-

benzoate, a substance which uncouples phosphorylation from oxidation, has effects very similar to dinitrophenol in inhibiting the growth of influenza virus in tissue cultures and deembryonated eggs.

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In vivo Inhibition of Monoamine Oxidase Studied with Radioactive Tyramine. (20541)

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In an earlier publication evidence was presented for the participation of monoamine oxidase in epinephrine metabolism and for inhibition of this enzyme *in vivo*(1). Epinephrine, because of the complexity of its metabolism(2) is not an adequate substrate for extended studies of monoamine oxidase *in vivo*. A more nearly ideal substrate, tyramine, a compound widely used for *in vitro* studies on monoamine oxidase, has therefore been synthesized in radioactive form. It is shown that with the exception of a small amount of conjugation, tyramine is metabolized by rats and mice only through monoamine oxidase action.

A simple method for testing *in vivo* inhibition of monoamine oxidase has been devised and applied to a number of compounds which not only have *in vitro* inhibitory power but also have pronounced pharmacological activity. Because of the relationship of some of these compounds to the sympathetic nervous system, a clarification of their effect on monoamine oxidase in intact animals is of considerable importance. It has been proposed by Gaddum and Kwiatkowski(3) that the sympathomimetic activity of ephedrine may be due to inhibition of monoamine oxidase and consequent protection of epinephrine from destruction. However, in the present study, no relationship is found between sympathomimetic activity and *in vivo* inhibitory power for monoamine oxidase.

Isotopic compounds. Tyramine, labeled with C^{14} in the α-position of the side chain was prepared by a small scale modification of the method of Johnson and Daschavsky (4). It involves thermal decarboxylation of DL-tyrosine, the latter being purchased in radioactive form from Tracerlab, Inc. The crude tyramine was purified by sublimation and converted to the picrate. The melting point of the radioactive tyramine picrate was 201°; Barger(5) reported 200°. For a non-isotopic sample synthesized by the same procedure, calculated C 45.9, H 3.85, N 15.3; found C 46.2, H 4.12, N 15.6. The compound showed only one radioactive spot on paper chromatograms. The specific activity was 3.3×10^6 c.p.m. per mg measured in a flow counter. Tyramine was converted to the hydrochloride before injection into animals.

Metabolism of tyramine. In order that radioactive tyramine be of maximum value in these experiments it was necessary to establish the extent to which monoamine oxidase is responsible for its metabolism. Previous workers had shown that in dogs, cats and

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