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Received October 4, 1957. P.S.E.B.M., 1957, v96.

Acetylcholine-Cholinesterase Relationships in Embryonic Chick Lung Cultivated *in vitro*.* (23597)

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Acetylcholine has been shown to influence the level of cholinesterase activity of embryonic chick intestine cultured *in vitro*(1,2). Jones *et al.*(1) suggested that acetylcholine induced the formation of cholinesterase in intestinal cells, but since the added ester served only to prevent a consistent fall in cholinesterase activity during the culture period, no definite statement as to whether acetylcholine actually induced the formation of new cholinesterase could be made. The present report deals with the influence of acetylcholine and similar esters on the cholinesterase activity of embryonic chick lung, a tissue chosen because the authors hoped its normally relatively low level of cholinesterase activity would provide a setting wherein the formation of new cholinesterase following a substrate stimulus could be demonstrated. Lung cells served well for this purpose, and the system was used to study the relationship between enzyme induction and time of exposure to substrate, as well as the type of cholinesterase induced.

Methods. Primary explants of 15-day chick embryo lung were incubated at 37°C for 8 days in roller tubes in 25% embryo extract in a highly buffered salt solution according to methods described by Jones *et al.* (1). Acetylcholine and other compounds were added to the medium in amount sufficient to provide an initial concentration of 0.02 Molar. The *cholinesterase* activity and protein contents of the cells in individual roller tubes were measured by the microchemical methods described by Bonting and Featherstone(3). In experiments comparing the hydrolysis of several concentrations of various substrates, the cells of many roller tubes were pooled and homogenized, and enzyme activity was determined manometrically. Acetylcholine chloride and acetyl-β-methylcholine chloride were purchased from Merck and Company; propionylcholine iodide and butyrylcholine iodide were purchased from Dajac Laboratories at Leominster, Mass., and tributyrin was purchased from Fisher Scientific Co. Benzoylcholine was a gift from Hoffmann-LaRoche.

Results. The effects of adding 0.02 M acetylcholine or the products of its hydrolysis to the medium of embryonic chick lung cultivated *in vitro* for 8 days are shown in Table I for 2 experiments—one in which there was good growth (protein increase) of the lung cells during incubation, the other where there was no such growth. A 2 to 6-fold increase in the cholinesterase activity over the non-incubated controls occurred. If the assumption is made that cholinesterase concentration is proportional to cholinesterase activity, then these results strongly support the hypothesis that acetylcholine induced the formation of the cholinesterase. That acetyl-

* This research has been supported by grants from Natl. Inst. of Health.

† This material is from dissertation submitted in partial fulfillment of degree Doctor of Philosophy, in the Dept. of Pharmacology, Graduate College, State University of Iowa.

TABLE I. Effects of Adding Acetylcholine (ACH) and Products of Its Hydrolysis to Medium of Embryo Chick Lung Incubated *In Vitro*.

Exp.	Treatment	No. tubes	mg protein per tube (mean)	μMoles ACH hydrolyzed/hr/mg protein (mean ± S.D.)
1 a	Not incubated	10	2.65	.58 ± .04
b	Incubated 8 days with .02 M ACH	10	4.39	3.70 ± .05*
2 a	Not incubated	6	2.10	.64 ± .09
b	Incubated 8 days with .02 M ACH	6	2.82	1.47 ± .12*
c	Incubated 8 days with .02 M choline	6	2.52	.57 ± .06
d	Incubated 8 days with .02 M Na acetate	6	2.69	.70 ± .06
e	Incubated 8 days with .02 M choline + .02 M Na acetate	5	2.07	.85 ± .04

* Significantly different from group not incubated ($p < .05$). Student's t test according to Mather(6) was used to test the significance of differences.

choline itself, and not a product of its hydrolysis, was the inducing agent was supported by the fact that sodium chloride, choline, acetate or choline plus acetate was not able to cause an increase in cholinesterase activity comparable to that produced by acetylcholine. The small increases in cholinesterase activity caused by adding choline, acetate, or both to the medium might have been due to the creation of a favorable chemical environment for the synthesis of acetylcholine by the cultured lung cells, with the acetylcholine thus synthesized acting as the inducing agent. These results are similar to those obtained with intestine(2).

The time course of the acetylcholine effect during the 8 day incubation period was studied and the results are summarized in Fig. 1, where it can be seen that the maximal increase in cholinesterase activity was reached 4 days after adding acetylcholine and was maintained only by the continued addition of acetylcholine to the medium. Enzyme activity expressed as μmoles acetylcholine hydrolyzed per hour per mg protein is the ordinate, and the abscissa is time of incubation in days. Note that incubation for 2, 4, 6 or 8 days did not change the cholinesterase level of the lung tissue, as shown by curve H. However, the addition of acetylcholine to the medium caused an increase in cholinesterase activity, as shown by curve A. There were no significant differences in the protein content of tissue incubated with or without ACH. In this particular experiment the protein content of the incubated tissue was only slightly higher than the non-incubated tissue indicating poor growth; however, the cells appeared to be multiplying well during the incubation period. Curves B, C, and D represent the fate of cholinesterase content as the result of leaving acetylcholine out of the medium from the 6th, 4th or 2nd day, respectively. Curves E, F and G show what happens if the addition of substrate to the medium is delayed until the 2nd, 4th or 6th day, respectively. When acetylcholine was added for the first 2 (Curve D) or 4 (C) days only, there remained no significant increase in enzyme activity at 8 days. When acetylcholine was added for the first 6 days only (B), the cholinesterase activity at 8 days was significantly higher than the activity of tissue incubated without acetylcholine (H),

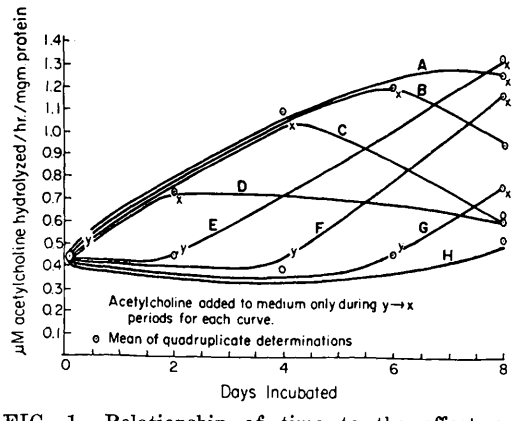

FIG. 1. Relationship of time to the effect on cholinesterase of acetylcholine added to the medium of embryonic chick lung incubated *in vitro*. Each point represents mean value of determinations from 4 roller tubes.

TABLE II. Substrate Specificity of Not-Incubated and Incubated Lung Tissue Cholinesterase.

Substrate	Substrate conc., molar	μl CO_2/hr/100 mg wet wt tissue (mean of duplicates) Not incubated	Incubated 8 days	Incubated 8 days with .02 M ACH
Acetylcholine	.1	45	50	195
	.01	55	138	428
	.001	43	83	350*
Acetyl-β-methylcholine	.1	13	73	163
	.01	13	48	118
	.001	0	13	40
Propionylcholine	.1	73	113	358
	.01	70	183	495
	.001	50	108	313*
Butyrylcholine	.1	43	1	10
	.01	41	3	6
	.001	28	0	7
Tributyrin	.1	714	126	100
	.01	352	142	94
	.001	144	92	72
Benzoylcholine†				

* Extrapolated values from 30 min. readings.
† Not hydrolyzed in any of the concentrations used.

but was also significantly lower than in the tissue incubated with acetylcholine (A) for 8 days. When acetylcholine was added for the last 4 (curve F) or last 6 (E) days only, the increase in activity produced was equivalent to that caused by adding acetylcholine for 8 days. Addition of acetylcholine for the last 2 days only (G) produced a small but statistically significant increase in cholinesterase activity.

The type of cholinesterase present in both incubated and non-incubated embryo chick lung tissue and the qualitative effect of acetylcholine were also studied. Table II shows the enzymatic hydrolysis of 6 substrates by non-incubated lung tissue. Both relative rates of hydrolysis and shape of the curves for the choline esters do not fit those of either of the 2 classical types of cholinesterase described by Augustinsson(5). Benzoylcholine was not hydrolyzed to an appreciable extent by the lung enzyme. The high enzymatic hydrolysis of tributyrin suggested the presence of a non-specific type of esterase since this substrate is reportedly not hydrolyzed by the specific acetylcholinesterase(4). On the other hand acetyl-β-methylcholine is reportedly not hydrolyzed by the non-specific cholinesterase(5); therefore, the presence of more than one type enzyme was suggested by these results.

Similar experiments were carried out with incubated tissue and the results are also summarized in Table II. After incubation for 8 days with no acetylcholine in the medium, the enzymatic hydrolysis of acetylcholine, acetyl-β-methylcholine and propionylcholine was increased while the opposite was true for tributyrin and butyrylcholine. Also after incubation an optimal substrate concentration was observed for acetylcholine and propionylcholine, which suggested the presence of an acetylcholinesterase, or "true" cholinesterase. The addition of acetylcholine to the medium increased the hydrolysis of acetylcholine, acetyl-β-methylcholine and propionylcholine and did not affect the hydrolysis of tributyrin and butyrylcholine.

Since the data on the substrate specificity of the enzyme in the incubated tissue indicated that there were at least 2 enzymes present, a suggested explanation of the results would be the following: The original non-incubated lung tissue contained both an acetylcholinesterase and a non-specific esterase capable of hydrolyzing certain choline esters. This non-specific esterase was more specific for aliphatic esters, as evidenced by

the high hydrolysis rates of tributyrin and butyrylcholine compared to the low rate for benzoylcholine, and exhibited maximal hydrolyzing powers at high substrate concentrations. When the lung was incubated *in vitro* for 8 days, the acetylcholinesterase activity increased with the growth of the cells and the non-specific esterase activity decreased. Thus, the acetylcholinesterase activity would tend to become "unmasked," explaining the optimal substrate concentrations observed for acetylcholine and propionylcholine. Addition of acetylcholine to the medium increased the activity (concentration) of acetylcholinesterase only, explaining why the enzymatic hydrolysis of tributyrin and butyrylcholine was not affected. This suggested explanation is compatable with the data and indicates that, at least under the experimental conditions of the reported study, a physiological substrate of an enzyme has a role in the formation of that enzyme.

Summary. Addition of 0.02 Molar acetylcholine to the medium of 15-day embryo chick lung cultivated *in vitro* for 8 days caused a 2- to 6-fold increase in the cholinesterase activity of that tissue. The increase in enzyme activity was maximal 4 days after addition of acetylcholine to the medium, and was maintained only by the continued addition of acetylcholine to the medium. Neither choline nor acetate alone nor choline and acetate together produced an increase in enzyme activity comparable to that produced by acetylcholine. Results of substrate specificity studies on the cholinesterases of chick embryo lung suggested that there was both an acetylcholinesterase and a non-specific esterase in the lung cells. Only the acetylcholinesterase was affected by the added acetylcholine. The results indicated that acetylcholine was an inducing agent for the formation of acetylcholinesterase in the embryo chick lung cells cultivated *in vitro*.

Thanks are due to Miss Patricia Sandy and William Coffey for their technical assistance.

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Received October 7, 1957. P.S.E.B.M., 1957, v96.

Relationship Between Penicillin Susceptibility and Virulence of *Staphylococcus aureus*. (23598)

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Penicillin susceptibility and virulence as measured by intraperitoneal mouse pathogenicity with mucin(1) of 80 recently isolated strains of staphylococci were determined and compared to ascertain whether or not any relationship exists between both characteristics. A good degree of reproducibility for a biological procedure has been observed with the animal virulence test for staphylococcal pathogenicity provided young Swiss mice and a constant, moderate-sized inoculum of microorganisms are employed. To further elucidate this problem, 4 penicillin-sensitive staphylococcal strains were assessed for virulence before and after induction of penicillin resistance *in vitro*. Since repeated artificial passage on media containing graded concentrations of penicillin might conceivably result in loss of virulence by itself, each strain was also transferred on antibiotic-free media an equal number of times as that required to induce resistance and also tested for virulence.