

EFFECT OF BLOOD SERUM FROM PATIENTS WITH MYASTHENIA GRAVIS ON THE SYNTHESIS OF ACETYLCHOLINE *IN VITRO*¹

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INTRODUCTION

Walker's observation (1) that neostigmine,² a choline esterase inhibitor, benefited patients with myasthenia gravis, suggested that the acetylcholine metabolism was disturbed in such patients. Assuming that this conception has validity, the dominant possibilities concerning myasthenia gravis are: (a) excessive destruction of acetylcholine, due to unusually large amounts of choline esterase; (b) defects in the ability of muscle to respond to acetylcholine; and (c) defects in the synthesis or release of acetylcholine.

The choline esterase level of the serum is not unusual in patients with myasthenia gravis, as was shown by Milhorat (2). Lanari (3), and Harvey and collaborators (4 to 6) demonstrated that the skeletal muscle of patients with myasthenia gravis is capable of reacting to acetylcholine and, indeed, is especially sensitive. Harvey and Lilienthal (5) suggested that this increased sensitivity represented a change in muscle threshold because of its exposure to diminishing amounts of acetylcholine.

The most plausible hypothesis is that there exists a fundamental defect in the synthesis of acetylcholine in patients with myasthenia gravis. Dr. Otto Loewi suggested that such synthesis be investigated.³

MATERIAL

The sera of 5 healthy subjects and 59 patients with diseases other than myasthenia gravis were used as controls. Summaries of the clinical state of 9 patients with myasthenia gravis are presented in Table I. In the ninth patient (N), the diagnosis of myasthenia gravis, although probable, was difficult to establish.

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² Prostigmine (Hoffmann-La Roche).

³ We are immeasurably indebted to Dr. Loewi for his enthusiastic interest in the development of the problem, and for his valuable advice on technic.

EFFECT OF HUMAN SERUM ON THE SYNTHESIS OF ACETYLCHOLINE

Principles and outline of method. The method of Quastel, Tennenbaum, and Wheatley (7) was modified for use in this demonstration. The bases of the method utilized are: (a) uniform samples of nerve tissue under standard environmental conditions will synthesize acetylcholine at a fixed rate; (b) the amount of acetylcholine thus synthesized may be biologically assayed by measuring its effect in inducing contraction in a properly sensitized muscle. The relative ability of 2 media to influence the synthesis of acetylcholine can thus be measured. In this series of experiments, the nerve tissue was frog brain. The indicator of acetylcholine content was the rectus abdominis muscle of the frog. The effect on acetylcholine synthesis of sera from patients with myasthenia gravis was compared with the effect on acetylcholine synthesis of control sera.

Collection and preparation of samples. The frog brain⁴ medium was prepared by mincing with scissors the freshly dissected whole brains of 10 to 25 frogs. Hundred mgm. samples were rapidly weighed from this homogeneous stock.

Blood samples were usually collected before breakfast. All blood samples were immediately defibrinated by shaking with glass perles for 5 minutes, and then promptly centrifuged. The supernatant serum was used for analysis. Only clear yellow sera were used and specimens with hemolysis were discarded. From each individual serum, specimens in triplicate were prepared as follows: 1 cc. of serum was mixed with 100 mgm. of frog brain, 1 cc. of Ringer's solution at pH 7.4, 1 cc. of Ringer's solution containing 3 mgm. of physostigmine salicylate, and 0.3 cc. of Ringer's solution containing 4.8 mgm. of glucose.

The Ringer's solution used in preparing mixtures for incubation was of the mammalian type and each 100 cc. contained 0.9 gram of NaCl, 0.4 gram of KCl, 0.011 gram of CaCl₂, and phosphate buffer (M/100) to give a pH value of 7.4. (The final concentration of the phosphorus was 0.03 gram per 100 cc. of Ringer's solution.) The pH of all mixtures was corrected to 7.4, the pH of blood, before incubation because the amount of acetylcholine synthesized depends on the pH.

Incubation. The mixtures were placed in vessels mounted on a shaking machine and shaken and incubated for 4 hours in a water bath. At short intervals through-

⁴ The results were more uniform and small differences in the synthesis in the presence of serum were more readily demonstrable with frog brain than with mammalian brain.

TABLE I
Short summary of the clinical state of 8 patients with myasthenia gravis

Name	Sex	Age	Severity of disease	Duration	Thymectomy	X-ray treatment	Symptomatology	Neostigmine (Prostigmine Bromide Hoffmann-La Roche)		
								Dose before thymectomy	Dose	Achievement after medication
H	F	43	5+	15	Yes	Yes	Bedridden, severe lid ptosis, diplopia, constant difficulty in chewing and swallowing, very severe muscular fatiguability	225	120 to 180	Chews food, turns in bed, sits on chair
G	F	39	4+	3	No	Yes	Almost bedridden, severe lid ptosis, diplopia, difficulty in chewing and swallowing, severe muscular fatiguability		135	Sits in chair for a greater number of hours, can walk a few steps
R	F	23	3+	9	No	No	Moderate lid ptosis, occasional diplopia, occasional difficulty in chewing, moderate muscular fatiguability		90	Walks 1 to 2 blocks
Sa	F	32	3+	7	No	No	Moderate lid ptosis, occasional diplopia, occasional difficulty in chewing, moderate muscular fatiguability		90 to 150	Housework
S	M	22	2+	2	Yes	No	Slight lid ptosis, rare diplopia, moderate muscular fatiguability	120	15 to 45	Works as an inspector of machinery
V	F	20	2+	5½	No	No	Slight lid ptosis, rare diplopia, occasional difficulty in chewing, mild muscular fatiguability		45 to 75	Walks, goes to parties
P	M	36	2+	2	No	No	Difficulty in chewing, moderate muscular fatiguability		45 to 75	Walks, but unable to work
M	F	27	2+	6	No	No	Slight lid ptosis, rare diplopia, occasional difficulty in chewing, mild muscular fatiguability		15 to 45	Works as a cashier (against advice takes as high as 135 mgm. for almost normal function)
N*	F	47	1+	12	No	No	Mild muscular fatiguability			Housework

* Doubtful.

out the incubation, the gas above the mixtures was replaced by oxygen. Incubations were made at 23° C. and at 38° C.

After 4 hours of incubation, 1.2 cc. of distilled water were added to the samples to convert the osmotic pressure to that of frog Ringer's solution and the volume was made up to 10 cc. with frog Ringer's solution. (The Ringer's solution of the frog type contained 0.67 gram of NaCl, 0.02 gram of KCl, and 0.015 gram of CaCl₂ in 100 cc.) The pH was corrected to 6.8, and kept constant throughout all following steps of the analysis. (Both the magnitude of the contraction of the rectus abdominis muscle and the stability of the acetylcholine before and during boiling are modified by variations in the pH.)

The mixtures were then centrifuged. After centrifuging, the acetylcholine content of the supernatant fluid was ascertained by measuring the magnitude of contractions of the sensitized rectus abdominis muscle of the frog.

Preparation of the rectus abdominis muscle. The rectus abdominis muscle of the frog was excised, immersed in

Ringer's solution, and kept in an ice-box (2° C.) for 2 hours to promote uniform responses of the muscle. The muscle was then suspended in a muscle chamber of 10 cc. capacity (method of Riesser (8) and Chang and Gaddum (9)), and sensitized by immersion for half an hour in Ringer's solution (at room temperature) containing 2 mgm. physostigmine salicylate per 100 cc. Thereupon, the physostigmine solution was replaced for 2 minutes by a solution containing 10 µgm. acetylcholine per 100 cc. The magnitude of contraction was registered by an isotonic lever on a kymograph. The muscle was then immersed for 5 minutes in Ringer's solution, followed by 5 minutes in a solution of physostigmine, and then again by immersion in the above acetylcholine solution. The procedure was repeated until 3 successive exposures to the solution of acetylcholine gave similar responses. (Between all subsequent immersions in any contraction inducing solution, the muscle was washed for 5 minutes with Ringer's solution and for 5 minutes with the Ringer's solution containing physostigmine.) The writing lever of the kymograph was so

weighted as to bring the contraction within the submaximal range where the muscle is sensitive to small changes in the concentration of acetylcholine.

These preliminary steps completed, the muscle was immersed for 2 minutes in one of the unknown solutions. Then, after measuring the contraction induced by the unknown solution, the muscle was immersed in sequence in a series of standard acetylcholine solutions, of concentrations covering the range of the unknown solutions. The acetylcholine content was thus ascertained for each unknown solution.

Assay of acetylcholine synthesis. The acetylcholine content of the mixtures, enumerated in Table II, was assayed.

It has been suggested (10, 11) that boiling brain tissue makes available the intracellular bound acetylcholine. Therefore these assay procedures were repeated after boiling. The residue of brain previously removed by centrifugation was suspended in the diluted supernatant fluid used in the above described procedure, and the whole boiled for 2 minutes at pH 6.8. It was then re-centrifuged and the supernatant fluid assayed for acetylcholine content by measuring the magnitude of contraction of the rectus abdominis muscle of frog as described above.

The stability of the muscle was tested before and after the assay of each unknown solution by immersion in standard acetylcholine solutions.

The acetylcholine content of each mixture was assayed on 2 to 12 different rectus abdominis muscle preparations. Thus, calculations on acetylcholine synthesis in each individual serum specimen were based on results obtained from at least 5 muscles.

Demonstration that the contractions induced by the mixtures are due to acetylcholine. To be certain that the contraction was induced by acetylcholine contained in the unknown solution and not by other substances, the muscles were immersed in the same unknown solution before and after being sensitized by immersion in physostigmine. Since only acetylcholine effects are significantly modified by physostigmine, it was inferred that any contraction that was augmented by physostigmine was due to acetylcholine.

Test for the occurrence of a substance that interferes with the action of acetylcholine on the rectus abdominis muscle. There is a possibility that during the 4 hours of incubation, other substances were produced in unequal quantities in

the mixtures containing frog brain-control serum and frog brain-serum from patients with myasthenia gravis. Some of these substances might prevent acetylcholine from inducing contraction of the rectus abdominis muscle. If so, it is conceivable that the magnitude of the contraction of the rectus abdominis muscle on immersion in the incubated mixtures is the result of 2 effects: (a) the contraction inducing action of acetylcholine, and (b) the effect of an agent which prevents the acetylcholine from acting on the muscle. To investigate this possibility, we incubated for 4 hours mixtures containing frog brain, glucose, and control serum, or serum from a patient (H) with myasthenia gravis, but not containing physostigmine. In these mixtures, the acetylcholine synthesized was destroyed by the choline esterase present. Therefore, the incubated mixtures did not induce contraction of the rectus abdominis muscle. After incubation, the mixtures were diluted to 10 cc., as described above, and rectus abdominis muscles were immersed in these solutions for 30 minutes and afterwards immersed for 2 minutes in solutions of acetylcholine of known concentrations. It was found that neither mixture ((a) frog brain-control serum and (b) frog brain-serum from patient H with myasthenia gravis) modified the expected effect of acetylcholine. Therefore, it may be assumed that in such incubated mixtures, no substance was developed in sufficient concentration to prevent acetylcholine from acting on the rectus abdominis muscle of the frog.

Calculation of synthesis of acetylcholine. The acetylcholine synthesis within the incubated mixtures was calculated by subtracting from the acetylcholine content of the incubated mixtures the acetylcholine content of identical non-incubated mixtures. Comparisons were made between the acetylcholine synthesis resulting from the mixtures containing control serum, and those containing the serum from patients with myasthenia gravis.⁵

⁵ The method employed for the assay of acetylcholine, as with all bioassays of this type, gave considerable variation in the results obtained in individual experiments. However, a sufficient number of experiments were performed in each group to assure the significance of the difference reported between the effect of control serum and serum from patients with myasthenia gravis.

TABLE II
Assay of acetylcholine synthesis

State	Frog brain	Ringer's solution	Serum	Physostigmine salicylate		Glucose	
					Final concentration		Final concentration
	mgm.	cc.	cc.	mgm.	grams per 100 cc.	mgm.	grams per 100 cc.
IA. non-incubated	100	3.3	0	3	0.09	4.8	0.15
IB. non-incubated	100	2.3	1	3	0.09	4.8	0.15
IIA. incubated	100	3.3	0	3	0.09	4.8	0.15
IIB. incubated	100	2.3	1 (control serum)	3	0.09	4.8	0.15
IIC. incubated	100	2.3	1 (myasthenia gravis)	3	0.09	4.8	0.15

RESULTS

Series 1. A comparison of the effect of control serum and serum from patients with myasthenia gravis on acetylcholine synthesis

I. Fresh non-incubated mixtures

A. Frog brain—Ringer's solution. The acetylcholine content of mixtures of frog brain-Ringer's solution, as assayed before boiling, averaged 0.39 $\mu\text{gm.}$ and, as assayed after boiling, averaged 0.80 $\mu\text{gm.}$ per 100 mgm. frog brain (Table III).

B. Frog brain-serum. Mixtures containing frog brain-control serum and frog brain-serum from patients with myasthenia gravis gave less constant results but, when averaged, the acetylcholine content was approximately the same as that of frog brain-Ringer's solution mixtures (Table III).

II. Incubated mixtures

A. Frog brain-Ringer's solution. The amount of acetylcholine synthesized at 23° C. by a mixture of frog brain-Ringer's solution, as assayed before boiling, averaged 0.70 $\mu\text{gm.}$ and, as assayed after boiling, averaged 1.50 $\mu\text{gm.}$ acetylcholine per 100 mgm. of frog brain. The amount of acetylcholine synthesized at 38° C., as assayed before boiling, averaged 0.41 $\mu\text{gm.}$ acetylcholine,

and, as assayed after boiling, averaged 1.02 $\mu\text{gm.}$ acetylcholine per 100 mgm. of frog brain (Tables IV and V).

B. Frog brain-control serum. Five healthy persons and 59 patients with diseases other than myasthenia gravis served as controls. The patients had the following diseases: multiple sclerosis, brain tumor, cerebral accident, paresis, encephalitis, peripheral neuritis, ruptured disk, arterial hypertension, subacute bacterial endocarditis, secondary anemia, uremia, asthma, diabetes, lead poisoning, "gastro-enteritis," hyperthyroidism, myalgia, and undiagnosed muscular weakness. One hundred and two separate blood samples were collected and incubated in triplicate. The amount of acetylcholine synthesized at 23° C. by a mixture of frog brain-control serum, as assayed before boiling, averaged 1.16 $\mu\text{gm.}$ acetylcholine, and as assayed after boiling, averaged 1.73 $\mu\text{gm.}$ acetylcholine per 100 mgm. of tissue. The amount of acetylcholine synthesized at 38° C., as assayed before boiling, averaged 1.45 $\mu\text{gm.}$ acetylcholine, and as assayed after boiling, averaged 2.08 $\mu\text{gm.}$ acetylcholine per 100 mgm. of tissue. The amount of acetylcholine synthesized in the presence of serum of healthy subjects and of the patients with diseases

TABLE III

Acetylcholine content of freshly prepared non-incubated mixtures expressed in $\mu\text{gm.}$ per 100 mgm. of tissue

Mixtures containing frog brain and	Assays of non-boiled mixtures				Assays of boiled mixtures			
	No. of separate mixtures	No. of rectus abdominis muscles	Acetylcholine content		No. of separate mixtures	No. of rectus abdominis muscles	Acetylcholine content	
			Mean	S. E.			Mean	S. E.
Ringer's solution Serum	40	157	0.39	± 0.011	20	59	0.80	± 0.022
	40	157	0.38	± 0.029	20	59	0.82	± 0.035

TABLE IV

Acetylcholine synthesis during 4 hours' incubation in mixtures containing frog brain and Ringer's solution expressed in $\mu\text{gm.}$ per 100 mgm. of frog brain

Assays of non-boiled mixtures (38° C.)				Assays of boiled mixtures (38° C.)				Assays of non-boiled mixtures (23° C.)				Assays of boiled mixtures (23° C.)			
No. of incubated mixtures	No. of rectus abdominis muscles	Acetylcholine synthesis		No. of incubated mixtures	No. of rectus abdominis muscles	Acetylcholine synthesis		No. of incubated mixtures	No. of rectus abdominis muscles	Acetylcholine synthesis		No. of incubated mixtures	No. of rectus abdominis muscles	Acetylcholine synthesis	
		Mean	S.E.			Mean	S.E.			Mean	S.E.			Mean	S.E.
22	60	$\mu\text{gm.}$ 0.41	± 0.013	28	55	$\mu\text{gm.}$ 1.02	± 0.033	9	14	$\mu\text{gm.}$ 0.70	± 0.023	9	14	$\mu\text{gm.}$ 1.50	± 0.044

TABLE V

Comparison of the amounts of acetylcholine synthesized in the mixtures containing: 1. Frog brain—Ringer's solution; 2. Frog brain—control serum; and 3. Frog brain—serum from patients with myasthenia gravis

Average of the acetylcholine synthesized during 4 hours of incubation expressed in $\mu\text{gm.}$ per 100 mgm. of frog brain

Mixtures containing frog brain, glucose, physostigmine, and	Assays of mixtures			
	Non-boiled 23° C.	Boiled 23° C.	Non-boiled 38° C.	Boiled 38° C.
Ringer's solution	0.70	1.50	0.41	1.02
Control serum	1.16	1.73	1.45	2.08
Myasthenia gravis serum				
H +++++*	0.47	0.75	0.53	0.90
G +++++			0.65	
R +++++	0.59	0.92	0.79	1.11
Sa +++++			0.82	1.16
S ++			0.88	
M ++			0.91	
P ++			0.91	1.29
V ++	0.75	1.10	0.92	1.30
N +?	0.93	1.38	1.17	1.69

* Most seriously ill patient: +++++; least seriously ill patient: +.

other than myasthenia gravis deviated from the average not more than ± 15 per cent and usually less than ± 10 per cent.

C. Frog brain-serum from patients with myasthenia gravis. The synthesis of acetylcholine in

the mixtures of frog brain and serum from seriously incapacitated patients with myasthenia gravis was less than half that found in mixtures of frog brain-control serum. The amount of acetylcholine synthesized at 23° C. by mixtures of frog brain and serum from patient H with severe myasthenia gravis, as assayed before boiling, averaged 0.47 $\mu\text{gm.}$ acetylcholine, and, as assayed after boiling, averaged 0.75 $\mu\text{gm.}$ acetylcholine per 100 mgm. of tissue. The amount of acetylcholine synthesized at 38° C., as assayed before boiling, averaged 0.53 $\mu\text{gm.}$ acetylcholine, and, as assayed after boiling, averaged 0.90 $\mu\text{gm.}$ acetylcholine per 100 mgm. of tissue (Tables V and VI).

The most serious decrease in the synthesis of acetylcholine was noted in the mixtures containing serum from the patients most seriously ill with myasthenia gravis. The samples from the patient with the least impairment (N), constituting a borderline myasthenic state, presented a defect in synthesis barely below the level of some of the controls (Tables V and VI).⁶

⁶ While this paper was in press, results similar to those of case S were obtained using serum of 3 additional and comparable patients with myasthenia gravis.

TABLE VI

Acetylcholine synthesized during 4 hours' incubation in mixtures containing frog brain and serum from patients with myasthenia gravis expressed in $\mu\text{gm.}$ per 100 mgm. of tissue

Subject	Assays of non-boiled mixtures (38° C.)								Assays of boiled mixtures (38° C.)							
	No. of separate blood samples	No. of incubated mixtures	No. of rectus abdominis muscles	Acetylcholine synthesis					No. of separate blood samples	No. of incubated mixtures	No. of rectus abdominis muscles	Acetylcholine synthesis				
				Minimum-maximum	Mean	S.E.	Deviation in percentage of average	Percentage of control †				Minimum-maximum	Mean	S.E.	Deviation in percentage of average	Percentage of control
H+++++	11	32	85	0.42 to 0.81	0.53	± 0.024	-20/+15	36.6*	1	2	6	0.86 to 0.95	0.90	± 0.017	-5/+5	43.2**
G+++++	4	12	27	0.60 to 0.75	0.65	± 0.010	-3/+15	44.9								
R++++	4	12	46	0.70 to 0.90	0.79	± 0.012	-11/+14	54.6	4	12	24	1.02 to 1.19	1.11	± 0.014	-8/+7	53.4
Sa++++	3	9	20	0.76 to 0.88	0.82	± 0.013	-7/+7	56.6	3	9	20	1.08 to 1.23	1.16	± 0.016	-7/+6	55.7
S++	4	12	40	0.82 to 0.94	0.88	± 0.010	-7/+7	60.8								
M++	1	3	6	0.87 to 0.95	0.91	± 0.014	-5/+4	62.9								
P++	1	3	6	0.86 to 0.94	0.91	± 0.013	-6/+3	62.9	1	3	6	1.20 to 1.36	1.29	± 0.028	-7/+5	62.1
V++	1	3	6	0.86 to 0.94	0.91	± 0.013	-6/+3	62.9	1	2	6	1.22 to 1.34	1.30	± 0.026	-6/+3	62.5
N+?	4	12	21	1.07 to 1.23	1.17	± 0.015	-9/+5	80.8	4	12	24	1.49 to 1.80	1.69	± 0.031	-12/+6	81.3
	(23° C.)								(23° C.)							
H+++++	1	2	6	0.42 to 0.52	0.47	± 0.022	-11/+11	40.5***	1	2	6	0.70 to 0.81	0.75	± 0.022	-7/+8	43.3****
R++++	4	12	30	0.54 to 0.64	0.59	± 0.010	-8/+8	50.9	4	12	30	0.86 to 1.02	0.92	± 0.014	-7/+11	53.0
V++	1	2	6	0.69 to 0.80	0.75	± 0.024	-8/+7	64.6	1	2	6	1.05 to 1.16	1.10	± 0.024	-4/+5	63.5
N+?	2	8	20	0.87 to 0.97	0.93	± 0.011	-6/+4	80.1	2	8	20	1.30 to 1.43	1.38	± 0.015	-6/+4	79.8

† The percentage of control is calculated by taking the average amount of acetylcholine synthesized in the serum of controls as 100 per cent: * 1.45; ** 2.08; *** 1.16; **** 1.73.

‡ Most seriously ill patient: +++++; least seriously ill patient: +.

Incubated mixtures containing serum collected before and after the administration of 15 mgm. neostigmine bromide by mouth to 2 healthy adults, yielded similar acetylcholine content. All patients with myasthenia gravis were taking neostigmine bromide during these experiments. Blood samples were collected both before and after the morning medication. No significant difference between the 2 sets of blood samples was noted as regards acetylcholine synthesis.

Series 2. The effect on acetylcholine synthesis of the mixture of the control serum and the serum from patients with myasthenia gravis

A mixture of the serum of controls and the serum from patients with myasthenia gravis synthesized at 38° C. an amount of acetylcholine approximately equivalent to the sum of the synthesis of the 2 sera.

Series 3. Effect of the dialysate of serum on the synthesis of acetylcholine

To ascertain whether the factors which modify the synthesis of acetylcholine in the frog brain *in vitro* are dialyzable, serum was dialyzed through a semi-permeable cellophane membrane. Three cc. of serum were placed in a flat glass container (4 cc. volume), separated from another similar glass container by a cellophane membrane. This second vessel contained 3 cc. of Ringer's solution (mammalian type) and the apparatus was shaken for 4 hours at 23° C. The effect of the dialysate on the acetylcholine synthesis was then compared with the effect of Ringer's solution: 2 cc. of dialysate and 2 cc. of Ringer's solution were added to mixtures of frog brain, glucose, and physostigmine, incubated, and assayed as described above. The results are summarized in Table VII.

Frog brain mixed with 2 cc. of dialysate obtained from control sera, synthesized 0.68 μ gm. acetylcholine at 38° C. The amount of acetylcholine synthesized at 38° C. in mixtures containing frog brain-Ringer's solution averaged 0.41 μ gm. acetylcholine per 100 mgm. of frog brain. An increase of acetylcholine synthesis can best be demonstrated at 38° C., a temperature at which the difference between synthesis in mixtures of frog brain-Ringer's solution and frog

TABLE VII

Acetylcholine synthesized during 4 hours' incubation in mixtures containing 1. Frog brain and Ringer's solution; 2. Frog brain and dialysate of control serum; and 3. Frog brain and dialysate of serum from patients with myasthenia gravis expressed in μ gm. per 100 mgm. of frog brain (assays of non-boiled mixtures)

Temperature	Mixtures containing frog brain and	No. of expts.	Amount of acetylcholine synthesis	
			Mean	S.E.
38° C.	Ringer's solution	22	0.41	± 0.013
38° C.	Dialysate from controls	5	0.68	± 0.049
23° C.	Ringer's solution	9	0.70	± 0.023
23° C.	Dialysate from R (myasthenia gravis)	5	0.55	± 0.038

brain-control sera is greater than at 23° C. The results indicate that at least some of the potentiator substances in the serum are dialyzable.

To ascertain whether sera from patients with myasthenia gravis contain dialyzable substances that decrease the acetylcholine synthesis, mixtures of frog brain-dialysate of sera from patients with myasthenia gravis and mixtures of frog brain-Ringer's solution were incubated at 23° C., a temperature favorable for acetylcholine synthesis in mixtures containing frog brain and Ringer's solution and therefore suitable for demonstrating a depression. The amount of acetylcholine synthesized in mixtures containing frog brain-Ringer's solution averaged 0.70 μ gm. acetylcholine. Mixtures containing frog brain and 2 cc. dialysate obtained from the serum of patient R with myasthenia gravis synthesized, on the average, 0.55 μ gm. acetylcholine. The results suggest that at least some of the substances responsible for the decrease of the acetylcholine synthesis in patients with myasthenia gravis are dialyzable. Although it appears that there is contained in the serum of patients with myasthenia gravis an agent that inhibits acetylcholine synthesis, it may not be inferred that such agent is absent in the control sera.

DISCUSSION

These data reveal that the serum from patients with myasthenia gravis is an unfavorable medium for the synthesis of acetylcholine. The acetylcholine synthesis in mixtures of frog brain-Ringer's solution-control serum is almost 3 times as great as in mixtures of frog brain-Ringer's

solution-serum from a patient seriously incapacitated by myasthenia gravis (12).

This difference is significant, since variation from subject to subject, both among the healthy and among those with diseases other than myasthenia gravis, was less than ± 15 per cent, and it was usually under ± 10 per cent. The difference in acetylcholine synthesis between samples of serum of different patients with myasthenia gravis was considerable, but the variation in the sample of serum of any one myasthenic patient was less than ± 15 per cent. The serum from patient H with severe myasthenia gravis was examined over a long period during which the clinical state changed slightly. This may have a bearing on the wider variation (-20 , $+15$ per cent from the average) observed in this patient. Hence, the usually more than 100 per cent greater synthesis of acetylcholine with control serum, as compared with the serum from patients seriously incapacitated by myasthenia gravis, represents a major difference in the nature of the serum samples.⁷

Moreover, this reduction of acetylcholine synthesis is not an expression of debility, cachexia, immobility, or prostration.

It is of interest that the magnitude of the decrease in acetylcholine synthesis is related to the severity of the myasthenia gravis in this small group of patients. The serum from patients with the more serious clinical disability supported acetylcholine synthesis less well than did serum of patients in a better clinical state. With the method now employed, the determinable reduction in the less seriously ill is so small that obviously this technic is not of value for diagnostic purposes.

⁷ The spinal fluid from patients with myasthenia gravis exerts a similar effect on the synthesis of acetylcholine to that of the serum (13).

While this paper was in press, Stoerk and Morpeth (14), using rat brain as a source of enzyme, reported that they could detect no difference in the amounts of acetylcholine synthesized in the presence of serum from control subjects and serum from patients with myasthenia gravis. Since they were also unable to demonstrate any difference in the amounts of acetylcholine synthesized in the presence of control serum as compared to Locke's solution, it is inferred that their adaptation of the method of Quastel, Tennenbaum, and Wheatley was not adequate for the demonstration of such differences in the synthesis of acetylcholine as are relevant to this discussion.

Since the dialysate of serum affects acetylcholine synthesis, it may be inferred that some of the modifying agents, both potentiators and depressors, are of a relatively small molecular size and not colloidal in nature.

Since acetylcholine synthesis is greater in frog brain-Ringer's solution-control serum mixtures than in frog brain-Ringer's solution mixtures, it is likely that the serum furnishes additional substrate to the enzyme responsible for acetylcholine synthesis. Also, a temperature of 38°C ., that interferes with acetylcholine synthesis in frog brain-Ringer's solution mixtures, permits, in frog brain-Ringer's solution-serum mixtures, more synthesis than is found at 23°C . Apparently at 38°C ., this particular enzyme is more active than at 23°C ., and though the substrate contributed by frog brain is converted at 38°C . into a state less suitable for acetylcholine synthesis, the substrate added by the serum compensates for the loss.

The decrease in acetylcholine synthesis demonstrated in these *in vitro* studies suggests that there may be similar defects in acetylcholine synthesis in patients with myasthenia gravis. There is reason to assume that the serum of patients with myasthenia gravis which poorly supports acetylcholine synthesis *in vitro* acts similarly *in vivo*.

It seems probable that in patients with myasthenia gravis, the acetylcholine available at the synapses eventually becomes insufficient for effective and repeated contraction of the muscle. Neostigmine aids these patients by impeding the breakdown of the diminished quantities of acetylcholine.

SUMMARY

1. Using frog brain-serum and frog brain-Ringer's solution mixtures as preparations for studying rate of acetylcholine synthesis, and the sensitized rectus abdominis muscle of frog for bioassay of acetylcholine content of the mixtures, the effect of control sera and sera from patients with myasthenia gravis on acetylcholine synthesis was investigated.

2. In frog brain-Ringer's solution mixtures, acetylcholine synthesis is greater at 23°C . than at 38°C ., while the reverse is true of frog brain-serum mixtures.

3. Acetylcholine synthesis in frog brain-control serum mixtures or in frog brain-dialysate of control serum mixtures exceeded that from frog brain-Ringer's solution mixtures.

4. Acetylcholine synthesis in mixtures of frog brain and serum from patients with myasthenia gravis was significantly less than that in frog brain-control serum mixtures. The more severe the myasthenia gravis, the less the acetylcholine synthesis. In mixtures of frog brain and dialysate of serum from patients with myasthenia gravis, acetylcholine synthesis was less than in mixtures of frog brain-Ringer's solution.

5. These results suggest that there is a defect in acetylcholine synthesis in patients with myasthenia gravis which can explain the fatiguability and weakness of these patients.

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