THE USE OF MITOGENETIC SPECTRAL ANALYSIS FOR DETECTION OF ACETYLCHOLINE IN FROG'S MUSCLE DURING VARIOUS TYPES OF STIMULATION

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The application of mitogenetic spectral analysis to the problem of nerve stimulation has shown that the radiation is a sensitive indicator of the functional condition of the neuromuscular system. This may be judged by the change in its spectral composition attending the most insignificant functional changes [1-4, 6, 7]. These changes in the spectrum, resulting from definite molecular conversions of the substrate of excitation, enable the character of several processes to be elucidated (for example, the degree of dispersion and polymerization of peptides) and their connection with the observed functional changes to be established. For this reason the use of mitogenetic spectral analysis in the detection of acetylcholine in vivo was held to be important, although its part in the stimulation process is the subject of lively discussion in the literature, since it made possible the further study of the link between the metabolism of the mediator and the state of the molecular substrate.

The work, which was performed during the autumn and winter of 1956-1957, consisted of two parts: preliminary experiments to determine the standard spectrum of acetylcholine and experiments to detect acetylcholine in vivo in the muscle of the frog (Rana temporaria, male) by the spectral method. We used a mitogenetic method that has been developed with an enzyme biodetector [4].

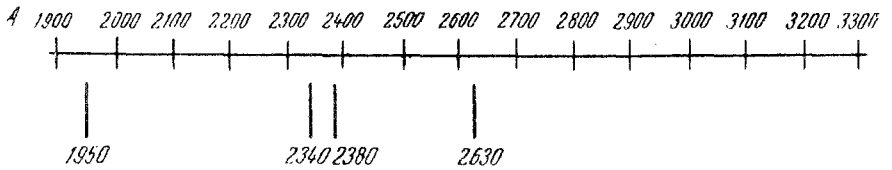
The instability of acetylcholine in solution prompted the suggestion that by analogy with other compounds already studied, it is a source of spontaneous mitogenetic radiation. The preliminary experiments showed that in fact a solution of acetylcholine chloride, prepared freshly, is quite a powerful source of radiation, the maximum effect of which appears in a concentration of $0.5 \cdot 10^{-2}$ with an exposure of 25-30 sec. The radiation from the solution falls off from 5-6 min after preparation.

The spectrum was obtained by means of a spectrograph* A quartz dish containing a freshly prepared solution of acetylcholine was placed in front of the slit of the collimator. The whole field of mitogenetic activity in the ultraviolet (1900-3200 A) was subdivided by means of standard stencils with apertures of 100 A into corresponding areas. The 100 A areas in which the mitogenetic effect was found were subdivided into areas of 20 A by means of monochromatic apertures. Mitogenetically active areas of 20 A were further divided into areas of 10 A and in the same way these were divided into lines 5 A in width. Acetylcholine chloride was used in the experiments, and it was therefore necessary to exclude from the general spectrum the spectral lines of the Cl ion, whose mitogenetic radiation takes place in the form of a sensitized fluorescence [4]. The spectrum of sensitized fluorescence of the Cl ion was studied in 1939 between limits of 1900-2500 A [5], and hence in this range the chloride lines were excluded by simple comparison. However, on determining the spectrum of acetylcholine, mitogenetic effect was also found in the range 2600-2700 A; for this reason in order to exclude the possibility that this was due to chloride we performed an additional series of experiments to study the sensitized fluorescence of a solution of NaCl in the range 2500-3200 A.

The results of this series of experiments showed no effect on the lines in the range 2600-2700 A from sensitized fluorescence of Cl and Na ions. Consequently the effect on this area, which was obtained in the experiments to study the radiation from acetylcholine chloride solution, occurs as the result of the acetylcholine molecule. The spectrum of acetylcholine thus obtained included three lines 5 A wide (1945-1950 A, 2335-2340 A, 2375-2380 A) and one line 10 A wide (2620-2630 A) (see figure).**

* Fiuess spectrograph. Transliterated.

**This refers, of course, to maxima of intensity of radiation in given areas of the spectrum, i.e., to the so-called half-breadth of the lines.



Mitogenetic spectrum of acetylcholine.

The spectral lines obtained were checked by the method of sensitized fluorescence of an acetylcholine solution of a concentration $0.5 \cdot 10^{-2}$. As a control, in addition to the spectral lines of acetylcholine, adjacent areas of the spectrum, 5 A wide, situated in the long and short wave sides of the main line were checked. The results of the experiments showed the presence of mitogenetic effect in the region of the main lines and its absence from the adjacent areas at the sides. This coincidence of the spectra of spontaneous radiation and of sensitized fluorescence of acetylcholine was confirmation of the correctness and accuracy of the spectrum obtained.

TABLE 1

Test of the Acetylcholine Lines in the Resting Muscle*

Wavelength in A	Intensity of acetylcholine line in % exposure in sec			
	20	30	40	60
1940—1945	—	—13	9	—9
1945—1950	—5	0	5	9
1950—1955	—	—6	6	—4
2330—2335	—	0	—	0
2335—2340	1	—4	—6	4
2340—2345	—	—15	3	10
2370—2375	—	4	11	20
2375—2380	10	8	2	3
2380—2385	—	—2	6	6
2615—2620	—	—7	8	—3
2620—2630	—11	—4	—2	5
2630—2635	—	10	—7	—6

* In this and subsequent tables the figures show the mean values of several experiments. The variation of the figures between limits of ±15% gives the margins of error of the statistical method of biodetection.

The second part of the work consisted of the detection of acetylcholine in stimulated muscle in vivo by means of the standard spectrum obtained. The experiments were performed on the upper third of the gastrocnemius muscle (an area 4 × 3 mm) from which the skin had been removed. The frog was pinned sparingly to a board and wrapped over with a damp cloth. In all the experiments adjacent spectral areas to the main lines, 5 A in width, on both the long and short wave sides were checked.

Experiments on resting muscles with exposures of 20, 30, 40 and 60 sec showed absence of radiation both at the main lines and in the areas at the sides. In experiments on resting muscles their spontaneous radiation was tested at the end, giving a maximum which was concentrated in the region of 2200-2300 A, at an optimal exposure of the order of 8-10 sec. The spontaneous radiation acted as a criterion of the satisfactory physiological condition of the muscle, for only in such a case did we consider the results of the main experiment to be valid (Table 1).

In the experiments to detect acetylcholine in the stimulated muscle two forms of stimulation were used. In the first experiments, stimulation of the sciatic nerve was effected by electric impulses from a stimulator of the GRAKh-1 system. The physical characteristics of stimulation were: form of impulse — square, duration of impulse — 5 msec, frequency — 25 hertz. At threshold amplitude (varying in different specimens from 0.7 to 1.7 v) weak, rhythmical contractions of the gastrocnemius muscle were obtained. Rest periods of 1-2 min were given between exposures, and after six stimulations the frogs were replaced. Exposures of 5 and 8 sec were effective.

In the second series of experiments a more physiological tactile stimulus was used on the contralateral limb, by means of a revolving disc operated by an electric motor. The edge of the disc which performed the stimulation was bent at right angles to its surface; in consequence of the graded reduction in the radius of the disc, which gave the edge of the disc a graded spiral shape, this device caused continuous and successive stimulation of the whole of the skin of the limb from the foot to the upper third of the thigh. With correct selection of the threshold of stimulation, regulated by altering the speed of revolution of the disc, a weak interrupted tetany of the whole musculature of the contralateral limb was obtained. Duration of exposures — 8 and 12 sec.

TABLE 2

Test of the Acetylcholine Lines in the Muscle During Electrical Stimulation of the Nerve*

Wavelength in A	Intensity of the acetylcholine lines in %	
	exposure in sec	
	5	8
1940—1945	4	—9
1945—1950	36	62
1950—1955	—2	0
2330—2335	—10	—2
2335—2340	29	33
2340—2345	—2	14
2370—2375	8	8
2375—2380	60	75
2380—2385	4	—5
2615—2620	0	7
2620—2630	8	26
2630—2635	0	—

* The difference in the intensity of individual acetylcholine lines is not surprising in view of the different spectral data.

TABLE 3

Test of the Acetylcholine Lines in the Muscle During Tactical Stimulation of the Contralateral Limb

Wavelength in A	Intensity of the acetylcholine lines in %	
	exposure in sec	
	8	12
1940—1945	—4	—8
1945—1950	54	10
1950—1955	9	9
2330—2335	—11	0
2335—2340	10	29
2340—2345	7	7
2370—2375	4	4
2375—2380	40	—14
2380—2385	2	—2
2615—2620	8	—8
2620—2630	—2	20
2630—2635	—	—2

The results of both series of experiments showed clearly a marked mitogenetic effect in all 4 acetylcholine lines and no effect in series of lateral areas of the spectrum 5 A in width (Tables 2 and 3).

The results obtained suggest that during stimulation acetylcholine appears in the muscle in the free state. The immediate purpose of the investigation — the possibility of detection of acetylcholine in vivo by the method of mitogenetic spectral analysis — was thus fulfilled.

The view that the acetylcholine detected spectrally was identical with that detected during perfusion of a stimulated muscle was most convincing. This view does not touch the question whether this acetylcholine is the mediator secreted by the nerve endings, according to the classic neurohumoral theory (O. Levi, Dale, A. V. Kibiakov) or whether it appears in the muscle as a result of leakage according to the concept of Nachmansohn [9]; the latter attributes to acetylcholine the leading role in the generation of action currents and considers that there is no intrinsic difference in carrying out stimulation through the axon or the synapse. The possibility cannot be entirely excluded also that the acetylcholine found in large quantity in nerve plexi and endings during stimulation may also be picked up spectrally, which can be judged indirectly by the high concentration of cholinesterase in these structures.*

SUMMARY

Experimental data demonstrate the possibility of detection of acetylcholine with the aid of mitogenetic spectral analysis in the frog's muscle in vivo.

The spectrum of acetylcholine may be easily revealed during the stimulation of the gastrocnemius. There are no characteristic spectral lines when the muscle is at rest.

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* The mitogenetic radiation from the deeper layers of the exposed area of muscle can be detected as a result of the phenomenon of "secondary radiation" [9].

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