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THE INEFFECTIVENESS OF (±)-CARNITINE PREVENTING THE TWITCHINGS OF STRIATED FROG MUSCLE IN 0.7% SODIUM CHLORIDE SOLUTION

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The spontaneous twitchings of isolated frog sartorius muscles in 0.7% NaCl solution have been studied. Addition of 1 mg./ml. of (±)-carnitine hydrochloride, or of (±)-carnitine base, to the bath fluid had no influence on the spontaneous activity of the muscles, their excitability or their ability to liberate potassium. This indicates that carnitine is not a natural inhibitor of striated frog muscle. Fluids enriched with potassium either from twitching muscle or by addition of KCl inhibited the activity of muscles reversibly.

The wide distribution of carnitine in biological materials, especially in skeletal muscle, has been known for over 50 years (Gulewitsch and Krimberg, 1905). Despite many investigations of the physiological properties of this substance (Weger, 1936; Strack and Försterling, 1953; Fraenkel, 1954) its function in muscle is unknown. Hayashi (1956), however, allocated to carnitine an important rôle in his hypothesis concerning the chemical physiology of excitation in muscle and excitable tissues.

Hayashi (1956) studied the rhythmic contractions of frog sartorius muscle immersed in a solution of isotonic NaCl (Mines, 1908; Adrian and Gelfan, 1933). He observed that, when a freshly excised muscle is put into the bath fluid in which another muscle had previously been immersed, it failed to exhibit rhythmic contractions. He concluded that the bath fluid contained a factor which inhibited movements, and having detected carnitine in the solution suggested that this was the inhibiting factor; he called it "inhibitine."

The theory of Hayashi (1956) was mentioned in several recent reviews (for example, Purpura, Girado, Smith, Callan, and Grundfest, 1959), and it was considered of interest to confirm two of his basic experiments, namely the appearance of an inhibiting factor in the bath fluid of a twitching muscle and the inhibiting effect of carnitine, prepared synthetically, on the twitching muscle. In addition the influence of carnitine on muscular excitability and on the liberation of potassium during immersion of the muscle in saline was studied.

METHODS

Two samples of carnitine hydrochloride were used; one was received from Dr. Fraenkel and the other from Dr. Deltour. They contained crystals of different sizes but of the same melting point. Carnitine base (synthesized by Dr. Deltour; m.p. 201 to 203°, free of chloride) had not more than 2% of impurities. A modified Ringer solution of the following composition was used: NaCl, 111 mM.; KCl, 2.7 mM.; $CaCl_2$, 1.1 mM.; NaH_2PO_4, 0.08 mM.; $NaHCO_3$, 2.4 mM.; glucose, 11 mM. The *p*H of this solution was 7.2.

Rana temporaria (male and female) were obtained from Rheinland water meadows. Experiments were done from January to April in two successive years. In all experiments the temperature was kept at 20° (±1°). When twitching was studied by visual observation, both sartorii were dissected from tendon to tendon. For mechanical recording of the twitches the pubic symphysis was cut under a binocular microscope and each muscle was left attached to a splinter of bone. After dissection, the muscles were immediately suspended in calcium-free Ringer solution for 45 sec. by a thread tied to the tendon. Finally, the muscles were placed in test-tubes containing 5 ml. of bath fluid. One muscle of each pair was put into the test fluid, the other was used as a control.

The action of various solutions on the twitching induced by exposure to NaCl was studied visually to see if twitching was suppressed in the treated muscle. The observation periods varied from 30 min. to 3 hr. A rough quantitative idea of the effectiveness of the

suppression was obtained by attaching the muscle to the moving arm of a condenser microphone (Brecht and Boucke, 1953) by means of a thread tied to the tendon, the pelvic end being fixed. The output from the microphone was fed into a conventional electroencephalogram amplifier feeding into a pen recorder. Excitability was tested directly by stimulating the muscles with rectangular current pulses of 2 msec. duration at 1 sec. intervals, and with current strengths of up to 100 mA. The electrodes were two silver wires of 0.5 mm. diameter, set 2 mm. apart in plastic ground down to expose the wires for a distance corresponding to the width of a muscle. They were placed under the muscle at its pelvic end. Excitability measurements were only used to give a general idea of the onset of conduction block.

Potassium was analysed with a Beckman flame photometer, Model D.U.

Results

The left-hand muscles of a pair were placed in the fluid in which other muscles (100 to 500 mg. wet weight) had previously been immersed for several hours until they stopped twitching. The corresponding right-hand muscles were put into fresh 0.7% saline. Left-hand muscles, immersed in a bath which had contained previously a muscle of approximately 100 mg., had activities similar to that of the corresponding control muscle. In nine experiments, muscles were immersed in fluid in which several muscles (about 500 mg.) had previously twitched (referred to as "used" fluid). In these experiments twitching was inhibited, though not completely suppressed. The potassium contents of "used" fluids which had contained 530, 580, and 630 mg. of fresh muscle for 6 hr. were found to be 2.4, 2.3, and 2.6 mM. KCl respectively. If these muscles were removed from this potassium enriched saline they commenced twitching once again.

Starting from the above observation that enrichment of 0.7% saline by KCl is accompanied by depression of muscular activity, 64 left-hand muscles were immersed in 0.7% saline and their corresponding right-hand muscles in 0.7% saline containing additional KCl (0.67–2.7 mM.). Some depression of activity was observed in 21 of 25 experiments with a concentration of 1.3 mM.-KCl. With 2 and 2.7 mM.-KCl, twitching was strongly depressed in 30 of 34 experiments immediately after transferring the muscles into the solution containing KCl, but some activity remained during the observation period of 3 hr. The behaviour of these muscles was similar to that of muscles placed in "used" saline.

The action of carnitine itself was studied on 27 muscle pairs using two samples of (±)-carnitine hydrochloride or carnitine base at bath concentrations of 125, 250, 500, or 1,000 μg./ml. in 0.7% saline. Carnitine hydrochloride solutions are strongly acid. Therefore the *p*H was usually brought to 7.5, but in some experiments it was varied from 4 to 9 by the addition of 0.1 M NaOH. Changes of *p*H in this range were found to have no influence on the twitching. Pairs of muscles were first suspended for 45 to 60 min. in 0.7% saline to assess their normal response. One of each pair was then transferred to the bath containing carnitine and the other to fresh 0.7% saline and both were observed for a further 30 min. or more. Neither carnitine hydrochloride nor carnitine base inhibited twitching.

The excitability of muscle suspended in Ringer solution was tested at various stimulus strengths, but no change occurred when 1 mg./ml. carnitine hydrochloride or carnitine base was added. Likewise there was no appreciable effect of carnitine on the rate of liberation of potassium from muscles in 0.7% saline (Table I).

TABLE I

ACTION OF CARNITINE ON THE RATE OF LIBERATION OF POTASSIUM

Frog sartorii muscles (right-hand) in 0.7% saline for 3 hr.; the corresponding left-hand muscles in the same solution with carnitine (1 mg./ml.).

	Potassium in Bath (μg./100 mg. of Muscle)			
With carnitine ..	53	56	62	71
Without ,, ..	55	58	73	67

Discussion

The highest concentrations of carnitine found by Fraenkel (1954) and other workers in mammalian tissue were 150 to 450 μg./g. of fresh muscle. The carnitine content of frog muscle, therefore, would not be likely to exceed 100 to 500 μg./g. Hayashi (1956) found that 500 mg. of frog muscle immersed in 5 ml. of 0.7% saline for 6 to 7 hr. produced enough "inhibitine" to suppress the twitching. Such inhibition corresponded in his experiments to the effect of 24 to 48 μg. of extracted "inhibitine"/ml. or 240–480 μg. of carnitine/ml., as synthesized by himself. The carnitine content which he found necessary to produce an inhibition, therefore, exceeds the quantity which a frog muscle might be expected to release. In the present experiments a concentration of 1,000 μg./ml. of synthetic carnitine had no effect on the twitch activity of the muscles, on their excitability or on their ability to liberate potassium.

Hayashi (1956) prepared his carnitine by a method ascribed to Tomita (Hayashi, 1956).

Bhattaharyya (quoted by Friedman, Galun, and Fraenkel, 1957) tried to use this method, but his attempts to duplicate the results of Tomita, or achieve the same results by other chemical means, consistently failed. It seems possible, therefore, that the attempts of Hayashi (1956) to produce carnitine led either to a substance different from carnitine or that his carnitine contained an impurity which itself had a high inhibiting effect.

The inhibiting factor appearing in the fluid of a twitching muscle may be the potassium which has diffused out of the muscle fibres (Dulière and Horton, 1929). Ringer (1886) added KCl (100 μg./ml. in 0.7% saline) to the bath and observed that the contractions all but ceased. Our results show that additions of 1.4–2.7 mM.-KCl to the saline reduced the activity reversibly. Therefore, the potassium content of a "used" fluid, found by Hayashi (1956) to be equivalent to 1.4 mM.-KCl, could be sufficient to produce a depression.

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