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SOME BIOCHEMICAL ACTIONS OF CARNITYLCHOLINE, A NEUROMUSCULAR BLOCKING AGENT*

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Abstract—Incubation of carnitylcholine with either of the cholinester hydrolases failed to destroy the molecule. Carnitylcholine has weak anti-cholinesterase activity, and it competitively inhibited the hydrolysis of acetylthiocholine by either cholinester hydrolases. The acetylation of carnitylcholine by brain preparations containing both choline and carnitine acetyl transferases was not achieved.

The intramuscular injection of carnitylcholine promotes efflux of several enzymes from muscle into plasma. Of the enzymes investigated, creatine kinase likely appears in the plasma in greatest amounts after treatment with the drug, followed by aldolase. In the muscle itself, phosphorylase activity showed the greatest decrease. On the other hand, citric acid cycle enzymes such as succinic dehydrogenase do not migrate from muscle cell to plasma.

BRAIN, heart and skeletal muscle extracts contain both carnitine and choline.[1–3] As biochemicals they are important substrates for the formation of acetylcarnitine, acetylcarnityl CoA, acetyl-1-carnitylcholine, acetylcholine and choline containing phospholipids respectively. Acetylcarnitine has been isolated from brain and heart tissue by Hosein *et al.*,[2,3] and Friedman and Fraenkel[4] have suggested that this substance possesses a high group potential. Fritz and Marquis[5,6] and Bremner[7] have shown that acetylcarnitine transfers its acetyl group through the mitochondrial membrane to form acetyl CoA. An identical reverse reaction involving palmitylcarnitine and palmityl CoA has also been described.[8] Thus carnitine serves as a carrier of acyl groups across the mitochondrial membrane. Acetyl-1-carnitylcholine and acetylcholine are both present in brain extracts;[9] acetyl-1-carnitylcholine is, however, on a molar basis, one half as powerful as acetylcholine in its cholinomimetic activity.[9,10] These substances, however, are both destroyed by acetylcholine acetylhydrolase (EC 3.1.1.7) (true cholinesterase) into acetylcarnitine and choline on the one hand and acetate and choline on the other; all without cholinergic activity.† Acetyl-1-carnitylcholine is, however, not destroyed by cholinesterase (EC 3.1.1.8) (pseudo cholinesterase). The biosynthesis of acetyl-1-carnitylcholine by enzymes present in brain acetone powder has been described.[11]

The deacetylation of acetyl-1-carnitylcholine leads to the formation of carnitylcholine. There is no evidence that carnitylcholine is present in tissues although, as mentioned above, both carnitine and choline have been found in brain and muscle extracts. However, carnitylcholine could well be synthesized *in vivo* either by (a) the deacetylation of acetyl-1-carnitylcholine or (b) the condensation of carnitine (as carnityl CoA) with choline.

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† E. A. Hosein and S. J. Booth, in preparation.

On intravenous injection into animals, carnitylcholine was found to be an extremely powerful neuromuscular blocking agent with low cholinergic activity.[10] Because carnitylcholine is a powerful depolarizing substance, its effect on enzymes concerned with the synthesis and destruction of acetylcholine was investigated. Again, because carnitylcholine is a neuro-muscular blocking agent of the depolarizing type, its action in permeability, or rather the efflux of enzymes from cells treated with this substance, was also studied.

EXPERIMENTAL

Section A

Hydrolysis of choline and carnitinesters by cholinester hydrolases. The action of the cholinester hydrolyses on various choline and carnitinesters was measured by determining the disappearance of carboxylesters with the Hestrin reaction[12] as modified by Friedman and Fraenkel.[4]

Depending on the experiment, various concentrations of the appropriate substrate were incubated either with acetylcholine acetylhydrolase (bovine erythrocyte cholinesterase, Sigma) or with cholinesterase (human plasma cholinesterase, Sigma) in a mixture containing 0·8 ml phosphate buffer (pH 7·4; 0·1 M). The reaction was terminated on addition of 0·1 ml NH_2OH-HCl. Appropriate blanks contained heat-inactivated enzyme.

Anti-cholinesterase activity. Compounds to be tested for anti-cholinesterase activity were incubated with acetylthiocholine and the cholinester hydrolases. Enzyme activity was followed by the method of McOsker and Daniel.[13] The sulfhydryl (SH) group was measured by the method of Grunert and Phillips.[14]

Acetylation. Acetylation of choline, carnitine and the carnitylcholine was performed following the method of Feldberg.[15] Rabbit brain acetone powder, prepared according to Kaplan and Lipmann,[16] was the source of the enzymes. Product formation was followed colorimetrically by determining the appearance of ester linkage with the method of Friedman and Fraenkel.[4]

Section B

Hooded rats, of either sex and weighing 150–200 g, were used throughout this investigation. In the experiments described in Section B, each animal served as its control. All experimental animals were prepared in the following manner before any enzymic analyses were performed on the particular tissues.

Rats were anaesthetized by intraperitoneal injection of 10 mg of nembutal, dissolved in 0·3 ml distilled water. Ten mg of carnitylcholine in 0·3 ml physiological saline (0·9% NaCl) was injected into the right hind leg of the rat and simultaneously 0·3 ml of saline alone was injected into the left hind leg which served as the control when analysis of enzymes in muscle were to be performed. For the analysis of enzymes in blood, control animals were injected intramuscularly solely with 0·3 ml saline. Fifteen min after the injection, the animal was killed by decapitation. Samples of muscle or blood were obtained for enzyme assays as described below.

Solutions of carnitylcholine (10 mg/0·3 ml) were freshly prepared daily. A brilliant cresyl blue dye (0·1%) was added to the carnitylcholine and saline solutions to mark the site of intramuscular injection.

Source of enzymes

Enzymic assays were done on muscle and plasma samples obtained from rats as described above.

Muscle. One g of muscle was removed from the site of injection (as marked by the dye) from each hind leg of the rat. The muscle was blotted with tissue paper. Fascia and other extraneous material were cleared from the sample. The muscle (0·1–0·5 g) was weighed and prepared for enzyme assay. The muscle was usually suspended in an appropriate medium and homogenized in a Virtis homogenizer in the cold (0·5°). Unless otherwise stated, 0·2 ml of the homogenate was used as the source of enzyme from muscle.

Plasma. Before injection of carnitylcholine or physiological saline solution (0·9% NaCl) into the rat, a control sample of blood (1 ml) was obtained from the tip of the tail of the anaesthetized animal. The blood sample was suspended in 0·5 ml 3·2% solution of sodium citrate. Fifteen min after injection of carnitylcholine or saline, the animal was decapitated and another blood sample (2 ml) was collected dropwise in 1 ml of 3·2% sodium citrate, mainly from the carotid arteries. The blood samples were centrifuged for 10 min at 2000 rev/min in a clinical centrifuge. The plasma was used as the source of enzyme in blood. Unless otherwise stated, 0·2 ml of this plasma was used in all analyses.

Methods for enzymatic assays

Aldolase. Aldolase activity was determined both in muscle and plasma. Muscle (0·5 g) was homogenized in 25 ml of distilled water at 0–5° as described above. The homogenate was well mixed and diluted 1 : 4 with distilled water. Aldolase activity was determined with a commercial kit purchased from Sigma Chemical Co., St. Louis, Mo. The procedure is based upon a simplification of the complicated calibration technique of Sibley and Lehninger.[17] Trizma buffer at pH 8·6 was used instead of collidine buffer at pH 7·2. Enzymes from muscle and plasma were both assayed and optical density was read at 540 mμ in a Coleman spectrophotometer.

Phosphorylase. The activity of this enzyme in muscle was determined by the method of Sutherland and Wosilait.[18]

Muscle (0·5 g) was homogenized in 10 ml of 0·1 M sodium fluoride at 0–5°. The homogenate was centrifuged at 2000 rev/min for 10 min (0–5°). The supernatant (0·2 ml) was mixed with 0·2 ml of freshly prepared 0·05 M cysteine (pH 6·0) and 0·2 ml of 0·1 M sodium fluoride. The mixture was preincubated for 2 min at 37°. The incubated solution was immediately used as the enzyme source.

Glutamic-oxaloacetic transaminase. The plasma level of glutamic-oxaloacetic transaminase was determined with the kit developed by Sigma Chemical Co. The optical density was measured at 505 mμ in a Coleman spectrophotometer.

Creatine phosphokinase. The method of Nada *et al.*[19] was used to determine the enzyme activity in both muscle and plasma. Muscle (0·1 g) was homogenized in 25 ml of 0·001 M glycine (pH 9·0). One ml of homogenate was then diluted to 25 ml with glycine solution before being used as an enzyme source.

Succinic dehydrogenase. The enzyme activity in muscle alone was investigated. Muscle (0·5 g) was homogenized in 10 ml 0·25 M sucrose. One ml of the homogenate was added to a tube containing 0·9 ml 0·02 M sodium succinate (pH 7·5), 0·5 ml; 0·5 M phosphate buffer (pH 7·5); 0·5 ml freshly prepared 0·2% triphenyltetrazolium chloride;

and 0·1 ml distilled water. A control without substrate was processed simultaneously. The tubes were incubated for 30 min in a shaking water bath at 37°. To stop the reaction 7·0 ml acetone was added. A small amount of ammonium sulphate (0·5 g) was added and the tubes were centrifuged at 2000 rev/min for 10 min. The optical density was measured at 540 mμ with a Coleman spectrophotometer using 70% acetone as blank.[20]

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The activities of these two enzymes were measured in the muscle only. Muscle (0·1 g) was homogenized in 25 ml 0·15 M potassium chloride at 0–5°. Homogenate (0·25 ml) was introduced into the reaction mixture.[21] After incubation and precipitation of the protein, the tubes were centrifuged for 5 min and the clear supernatant used for spectrophotometric assay of reduced NADP. The increase in optical density at 340 mμ during the first 5 min was taken as a measure of dehydrogenase activity.

The glucose 6-phosphate dehydrogenase activity was taken as the difference between the activities measured separately at pH 7·6 in the presence of 0·5 ml 0·005 M 6-phosphogluconate and 0·5 ml of a solution of glucose 6-phosphate and 6-phosphogluconate (0·005 M with respect to each other).

RESULTS

Section A

Susceptibility of carnitylcholine and other carnitine and choline esters to hydrolysis by cholinester hydrolases. Before studying the influence of carnitylcholine on muscle enzymes described below in Section B, it was decided to determine the stability of this substance to tissue cholinesterases. Instability would have meant either: (a) that it would have been impossible to carry out the experiments described, or (b) that conclusions drawn from such experiments attributable to the drug might well be invalid

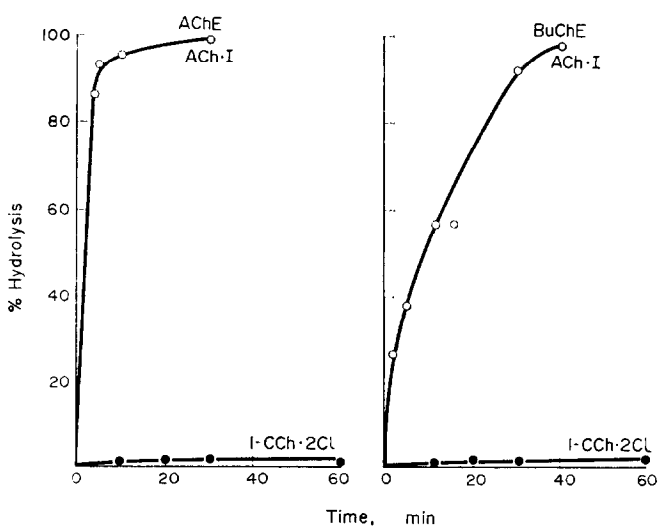

FIG. 1. Susceptibility of acetylcholine iodide and carnitylcholine dichloride to cholinester hydrolases. Bovine erythrocyte cholinesterase (AChE, 1 mg/ml) or human plasma cholinesterase (BuChE, 1 mg/ml) is incubated with 3 μmoles acetylcholine (ACh). One or 3 μmoles 1-carnitylcholine dichloride (1-CCh.2Cl) at 37°.

if the material is unstable *in vivo*. Accordingly, the influence of the cholinester hydrolases on the hydrolysis of carnitylcholine was studied and comparisons made with other choline and carnitine esters.

The incubation of 3 μmoles acetylcholine with either acetylcholine acetylhydrolase (AChE) (1 mg/ml) or cholinesterase (BuChE) (1 mg/ml) resulted in complete hydrolysis of the ester. Under identical reaction conditions 2, 3 and 4 μmoles carnitylcholine were not hydrolysed by either enzyme as shown in Fig. 1. Carnitylethylchloride was equally resistant to the action of both enzymes.

The inhibitory action of choline, carnitylethylchloride, succinyldicholine and carnitylcholine on the hydrolysis of acetylthiocholine iodide (AcTCh) by the cholinester

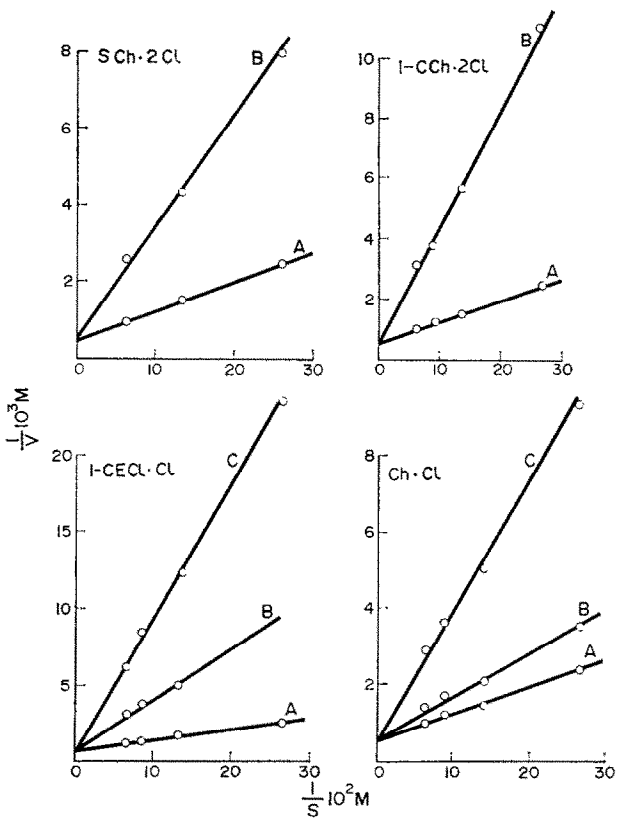

FIG. 2. Competitive inhibition by choline and carnitine esters (Lineweaver–Burk plots). Bovine erythrocyte cholinesterase (AChE, 1 mg/ml) is incubated with acetylthiocholine iodide $1{\cdot}5 \times 10^{-3}$ μmoles in the presence and absence of inhibitors (indicated below) for 10 min at 37°.

Inhibitor	Concentration (M)		
	A	B	C
Succinyldicholine dichloride (SCh.2Cl)		$7{\cdot}1 \times 10^{-3}$	
Carnitylcholine dichloride (1-CCh.2Cl)		$1{\cdot}4 \times 10^{-2}$	
Carnitylethylchloride chloride (1-CECl.Cl)		$1{\cdot}4 \times 10^{-2}$	$7{\cdot}1 \times 10^{-2}$
Choline chloride (Ch.Cl)		$1{\cdot}4 \times 10^{-2}$	$7{\cdot}1 \times 10^{-2}$

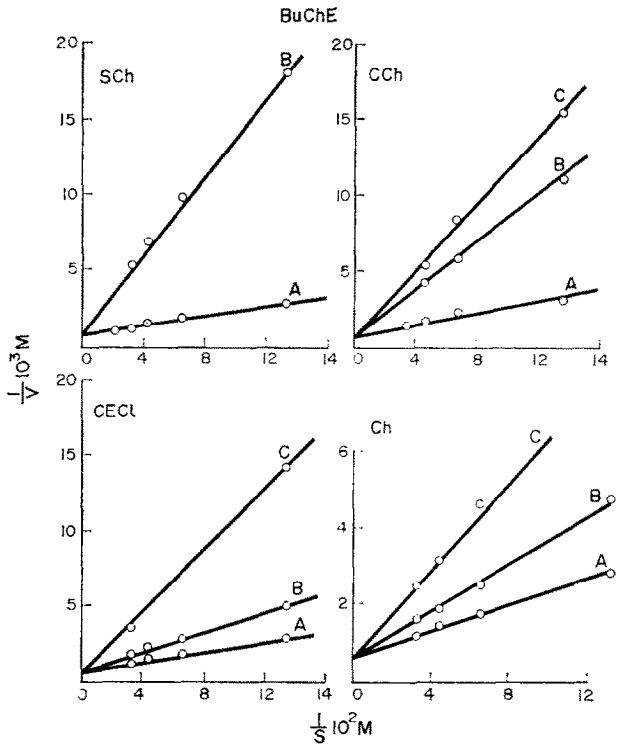

FIG. 3. Competitive inhibition by choline and carnitine esters (Lineweaver–Burk plots). Human plasma cholinesterase (BuChE, 0·5 mg/ml) is incubated with acetylcholine iodide 3×10^{-3} μmoles in the presence and absence of inhibitors (indicated below) for 10 min at 37°.

Inhibitor	Concentration (M) A	B	C
Succinyldicholine dichloride (SCh)		$7{\cdot}1 \times 10^{-2}$	
Carnitylcholine dichloride (CCh)		$2{\cdot}7 \times 10^{-3}$	$4{\cdot}3 \times 10^{-3}$
Carnitylethylchloride chloride (CECl)		$7{\cdot}1 \times 10^{-3}$	$7{\cdot}1 \times 10^{-2}$
Choline chloride (Ch)		$7{\cdot}1 \times 10^{-2}$	$2{\cdot}1 \times 10^{-1}$

hydrolases was next investigated. Acetylthiocholine ($1{\cdot}5 \times 10^{-3}$ M) was the optimal substrate concentration used for maximal activity with 1 mg/ml acetylcholine acetylhydrolase. The optimal substrate concentration was not so well defined for cholinesterase; 3×10^{-3} M acetylthiocholine was the concentration used for maximal activity with 0·5 mg/ml cholinesterase. The data on the inhibitory action of choline, carnitylethylchloride, succinyldicholine and carnitylcholine are presented as Lineweaver–Burk plots in Figs. 2 and 3. It is apparent from these results that the mono-quaternary ammonium compounds, choline and carnitylethylchloride, and the bis-quaternary ammonium compounds, succinyldicholine and carnitylcholine, inhibit the hydrolysis of acetylthiocholine by cholinester hydrolases in a competitive manner.

The I_{50} values for succinyldicholine, carnitylcholine, carnitylethylchloride, and choline are given in Table 1.

TABLE 1. ANTI-CHOLINESTERASE ACTIVITY OF NEUROMUSCULAR BLOCKING COMPOUNDS

Inhibitor	I_{50}(M)*	
	Acetylcholinesterase (Bovine erythrocyte)	Butyrylcholinesterase (Human serum)
	Acetylthiocholine	
	($1{\cdot}5 \times 10^{-3}$M)	(3×10^{-3} M)
Succinyldicholine dichloride	$3{\cdot}3 \times 10^{-3}$	$6{\cdot}3 \times 10^{-3}$
Carnitylcholine dichloride	10^{-2}	$2{\cdot}5 \times 10^{-2}$
Carnitylethylchloride chloride	$1{\cdot}1 \times 10^{-2}$	$4{\cdot}8 \times 10^{-3}$
Choline chloride	$3{\cdot}5 \times 10^{-2}$	$1{\cdot}7 \times 10^{-1}$

*I_{50} is that concentration of the drug needed to produce 50 per cent enzyme inhibition.

Enzymic acetylation of carnitylcholine. Results from the incubation of choline and 1-carnitine chloride (1-C.Cl) with rabbit brain acetone powder extract, known to contain the enzymes choline acetyl transferase and carnitine acetyl transferase leading to the formation of acetylcholine and acetylcarnitine, are shown in Fig. 4. Under identical reaction conditions carnitylcholine was not acetylated by this enzyme preparation.

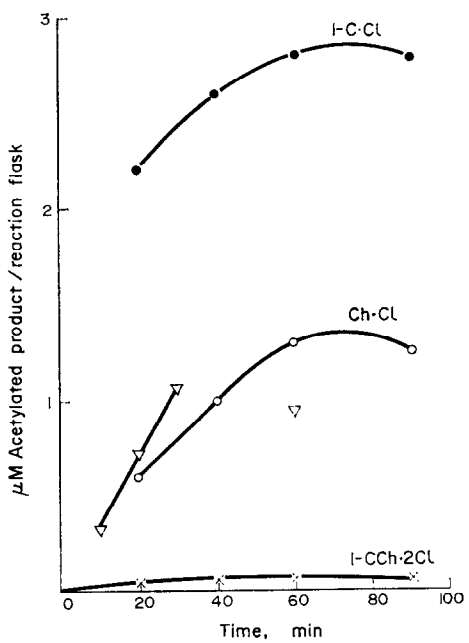

FIG. 4. Susceptibility of choline chloride, carnitine chloride and carnitylcholine dichloride to acetylating enzymes. Rabbit brain acetone powder extract is incubated with carnitylcholine dichloride (1-CCh.2Cl) (×), choline chloride (Ch.Cl) (△, ○) and 1-carnitine chloride (1-C.Cl) (●) at 37°

Since it was shown above that carnitylcholine is neither destroyed by cholinester hydrolases nor can it be acetylated to form acetyl-l-carnitine choline, it was concluded that this substance was stable enough to be used in experiments *in vivo*. In the experiments to be described below, carnitylcholine was injected intramuscularly into rats to study its effect on the enzyme concentration in the muscle and blood of these animals.

In these experiments, the "control" value represents the enzyme activity in muscle of one hind leg of the rat after saline injection and the "experimental" value is from the muscle in the other hind leg injected with an equal volume of carnitylcholine.

Efflux of enzyme from muscle to plasma. The effect of intramuscular injection of carnitylcholine on the activity of several enzymes in rat muscle and plasma is shown in Table 2. There was a loss of 40 per cent of the muscle aldolase and an increase of 150 per cent of the plasma aldolase after a single injection of the drug. Muscle phosphorylase diminished by 65 per cent.

From these data on the efflux of muscle aldolase and phosphorylase after carnitylcholine treatment, it is likely that metabolism of both glycogen and glucose 6-phosphate would be altered in the carnitylcholine-treated muscle. What then might be the metabolic fate of glucose 6-phosphate in such muscles?

As shown in Table 2, an increase of 110 per cent muscle glucose 6-phosphate dehydrogenase activity and 105 per cent of muscle-6-phosphogluconate dehydrogenase activity was observed after intramuscular injections of carnitylcholine into rats.

Intramuscular injection of carnitylcholine into rats caused an increase of 45 per cent in plasma glutamic-oxaloacetic transaminase activity.

So far it was shown that after intramuscular injection of carnitylcholine, there was movement of three enzymes from muscle to plasma, but the measurements were not made simultaneously. Table 3 contains data which show the effect of a single intramuscular injection of this drug on the simultaneous movement of these three enzymes in each of several rats. It can be seen that, on the basis of units of enzyme activity and under the conditions of the experiment, there was greater efflux of aldolase than glutamic-oxaloacetic transaminase from muscle to plasma.

The effect of intramuscular injection of carnitylcholine on creatine phosphokinase activities in rat muscle and plasma showed that there was a 30 per cent loss of enzyme in the muscle and an increase of 400 per cent in the plasma activity of the enzyme.

From the results described above, it is apparent that the intramuscular injection of carnitylcholine into rats caused efflux of several enzymes from the injected muscle into the plasma. It was, therefore, of importance to determine whether this property of carnitylcholine was restricted to the efflux of the so-called soluble enzymes or whether, in fact, insoluble enzymes might similarly be affected.

Intramuscular injections of carnitylcholine did not cause any change in the succinic dehydrogenase activity in muscle (Table 2).

DISCUSSION

The permeability of the cell membrane appears to be a necessary condition for the release of enzymes from tissues into plasma and the rate of diffusion is limited by the permeability of the membrane.[22] A variety of findings indicate that the permeability of the membrane can be varied physiologically and thus it might be under metabolic

TABLE 2. EFFECT OF INTRAMUSCULAR INJECTION OF CARNITYLCHOLINE ON ENZYME ACTIVITIES IN RAT TISSUES

Enzymes	Enzyme activities*					
	Muscle			Plasma		
	Control	Experimental	% Change	Control	Experimental	% Change
Aldolase	41,436 ± 2920	27,117 ± 2339	−38 ± 5	39 ± 3	91 ± 10	+152 ± 39
Phosphorylase	186 ± 31	74 ± 73	−65 ± 31			
Serum GOT				78 ± 10	113 ± 15	+46 ± 10
Creatine kinase	12·43 ± 2·33	9·05 ± 3·08	−29 ± 19	1·50 ± 0·23	7·69 ± 2·41	+406 ± 111
Succ. dehydrogenase	1·44 ± 0·22	1·46 ± 0·27	+1 ± 7			
G 6-p dehydrogenase	23·2 ± 1·7	47·9 ± 1·32	+108 ± 17			
6-Phosphogluconate dehydrogenase	58·0 ± 2·0	119·6 ± 14·2	+106 ± 17			

* The unit of measure for each enzyme is described under "Methods".

TABLE 3. EFFECT OF A SINGLE INTRAMUSCULAR INJECTION OF CARNITYLCHOLINE ON THE MOVEMENT OF THREE ENZYMES IN RATS

Rat no.	% Change in enzyme activities*			
	Glutamic-oxaloacetic transaminase (plasma)	Phosphorylase (muscle)	Aldolase (muscle)	Aldolase (plasma)
1	+41	−70	−60	+109
2	+16	−59	−67	+74
3	+19	−77	−18	+75
4	+48	−80	−79	+176
5	+65	−10	−21	+180
6	+39	−55	−33	+123

*The unit of measure for each enzyme is described under "Methods".

control. On the other hand, increase of cellular permeability to aldolase in muscle by insulin,[23] as well as the increase of plasma aldolase activity in rats and rabbits after ACTH or cortisone treatment,[24] strongly point to hormonal control of membrane function. Disturbance of permeability may also be found when there is an insufficient energy supply to the membrane. Loss of enzymes from the cell might well be the direct consequence of biochemical lesions[17,25] involving either oxidative phosphorylation, glycolysis, ATP synthesis, carbon monoxide poisoning, thyrotoxicosis or localized anoxia.[26,27]

Impairment of cellular respiration causing a fall in the concentration of ATP or the ratio ATP : ADP × P_i leads to changes in the water content of the cells, which manifests itself in cellular swelling.[28] Swelling leads to a disturbance of permeability which results in the loss of soluble cytoplasmic components including enzymes.[23]

The intramuscular injection of carnitylcholine caused the rapid efflux of several enzymes from the site of injection into the blood. It was shown that on the basis of per cent changes in enzyme activity more creatine phosphokinase than any other enzyme appeared in the plasma following the injection of the drug. Loss of muscle aldolase and phosphorylase from such tissues would impair glycolysis on the one hand and lead to accumulation of several hexosephosphate esters on the other.

In previous work[29] it was shown that in the rat intramuscular injection of the ethyl ester of gamma-butyrobetaine, a substance whose pharmacologic properties are identical with those of carnitylcholine, caused loss of muscle aldolase, phosphorylase, transaminase,[29] and creatine phosphokinase,[30] with concomitant rise of these enzymes in the plasma. In addition, intramuscular injection of the ethyl ester of gamma-butyrobetaine to rats caused a decrease in the rate of muscle anerobic glycolysis and a fall in the tissue concentration of citrate, α-keto-glutarate,[30] creatinine, ATP, creatine phosphate, and inorganic phosphate, the effect being related to the dosage of the drug used. Amino aciduria was produced in these animals[31] as well as an elevation in the excretion of creatine and inorganic phosphate with diminished creatinine.[29] It is therefore apparent that, subsequent to the intramuscular injection of substances such as ethyl ester gamma-butyrobetaine and carnitylcholine, which have long-lasting cholinomimetic activity, profound alterations take place in the tissue concentration

of certain enzymes which metabolize carbohydrate and protein in the muscle. The effects observed in the present and previous studies indicate that the presence of these cholinomimetic substances in muscle affects a variety of unrelated biochemical processes which, because of the eventual failure to synthesize ATP, may lead to further degenerative changes in the afflicted cells.

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