ON ATEBRIN ACTION ON SUCCINOXIDASE SYSTEM
II. PROTECTION FROM ATEBRIN INHIBITION IN TISSUES

by

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In a previous paper[2]) the atebrin inhibition on the succinoxidase system, which occurs in the component of the system situated between the dehydrogenase and the Warburg-Keilin system has been described. Wright and Sabine[8]) have noted the atebrin inhibition, while investigating the influence of bits of tissues on the oxidation of glucose lactate, pyruvate, citrate and fumarate but have not observed such an inhibition on the oxidation of succinate. Meanwhile it has been shown that the atebrin inhibition occurs in a link of the chain, which is situated between the dehydrogenase and the Warburg-Keilin system in those cases where the preparations of succinodehydrogenase i. e. of the succinoxidase system are made after Hoff-Jörgensen and Lehmann[3]) and after Keilin and Hartree[5]). Reexamining the experiments made by Wright and Sabine[8]) we found again that a distinct difference exists between the behaviour of strips of tissues in their reaction to sucinate, as far as the action of atebrin in concerned. This difference shows that the action of these preparations of succinoxidase does not imitate completely the oxidation of succinate which occurs in vivo. We were interested to find out which factor is lacking in the preparations, thus making the atebrin inhibition possible.

The method

The muscle hash was prepared from fresh muscle tissues. The muscle tissue was put five times through the grinder, washed two times with distilled icewater and suspended in an equal volume of M/15 phosphate buffer pH 7,4.

The preparation of the succinoxidase system after Hoff-Jörgensen and Lehmann[3]) and after Keilin and Hartree[5]) has been described in the paper mentioned above[2]).

Cytochrome c is prepared by using the Keilin and Hartree method[4]) but the dialysis was not made against 1 p. c. sodium chloride but against redistilled water.

Kochsaft (boiled muscle exstract) was prepared after Ahlgren[1]) from fresh rabbit muscle. The fresh muscle tissue was hashed and boiled for several minutes with a sufficient quantity of redistilled water, filtered and neutralised to pH 7,30.

The preparation was kept at 0°C and reboiled before each experiment.

The activity of the enzyme was tested after Lehmann. The decolourising time of methylene blue was 9—11 minutes.

When precipitating the enzyme with acid, the supernant liquid was recovered and used in the experiments under the name of the »supernatant liquid« after being neutralised to pH 7,4.

The experiments were made according to Warburg's manometric method.

The results

The difference between the behaviour of the muscle hash and the succinoxidase system preparations against the inhibitory action of atebrin is clearly shown in Table 1, which shows the atebrin inhibition of the oxidation of succinate in muscle hash, and in the sucinoxidase system preparation by Keilin and Hartree[5]) and by Hoff-Jörgensen and Lehmann[3]). The final concentration of atebrin was 1×10^{-3} M. Each flask contained 1 ml of muscle hash or the enzyme preparation, 1 ml of the above mentioned concentration of atebrin, and 1 ml of M/15 phosphate buffer pH 7,4. After the temperature in the flask became constant we added 1 ml of 0,5 M sodium succinate to the muscle or the enzyme preparation. Gaseous phase: air. Temperature 40°C. The numbers show in cmm the oxygen consumption for every fifteen minutes.

Table 1

	Muscle hash		Preparation by Keilin and Hartree		Preparation by Hoff-Jörgensen and Lehmann	
	no atebrin	atebrin	no atebrin	atebrin	no atebrin	atebrin
	cmm of oxygen consumption					
after minutes						
15	48,2	46,2	40,2	16,2	43,1	23,0
30	50,4	50,2	88,6	24,8	100,4	51,2
45	81,3	78,1	139,0	60,3	166,8	96,3
60	112,2	110,4	191,0	98,9	202,5	112,6
per cent of final inhibition	0,0	1,6	0,0	50,1	0,0	44,5

As we see the amount of inhibition in muscle hash is quite unimportant compared to the effect in enzyme preparations. This fact confirms the findings of Wright and Sabine[8]) about the impossibility of inhibition of oxidation of the succinate by atebrin. It is of course clear that we cannot imitate the biological process in the preparations and that there must be another factor present in vivo which guards the system from atebrin action. Ahlgren[1]) has reported a thermostabile factor X in boiled muscle extract (Kochsaft) which greatly activates the oxidation of succinate. Potter and Schneider[7]) have demonstrated that calcium and aluminum ions can replace the action of Kochsaft, and they concluded therefrom, that this action is due to the Kochsaft having the same ions. Potter[6]) has further coined the term »diluting effect« which results from the dissolving of the cytochrome c in water and from its too weak concentration in the enzyme preparations. The addition of cytochrome c to the intact preparation does in fact raise the consumption of oxygen. All these substances have been used during the tests in such a way as to reactivate the oxygen consumption, which was weakened by the atebrin action. Table 2 reviews twenty eight experiments where different preparations have been used.

The results shown in table 2, point out that none of the substances quoted have been able to renew the atebrin inhibition, with the exception of »supernatant liquid« which is the only one able to replace almost completely the physiological conditions. Riboflavin has only partially restituted the inhibitory effect of atebrin, and in two experiments also cytochrome c. That cytochrome c, however is not identical with the activating factor in this liquid, is evidenced by its thermostability. The activity of the activating factor has disappeared after boiling.

The »supernant liquid« shows an intensive yellow-green fluorescence which after standing for several days disappears and at the same time loses its activity, though after boiling the fluorescence does not disappear. Thus the activity of the »supernatant liquid« cannot depend on the fluorescent matter in it. Cytochrome c is thermostable which excludes its identity with the active substance mentioned above. The activity of calcium and aluminum ions ought to remain unimpaired by boiling, and they are instrumental in giving a specific value to the active matter in the »supernatant liquid«. Though the »supernatant liquid« gives a positive luminol reaction, it is probably only owing to some remains of blood or even of cytochrome. Experiments, however, in succinate oxidation with the »supernatant liquid« alone remained negative. The »supernatant liquid« be-

Table 2

	Preparations by Keilin and Hartree ml									Preparations by Hoff-Jörgensen and Lehman ml								
Enzyme preparation	2	2	2	2	2	2	2	2		2	2	2	2	2	2	2	2	
Phosphate buffer pH 7,4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Na-Succinate (0,5 m)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Atebrin 1×10^{-3} M		1	1	1	1	1	1	1			1	1	1	1	1	1	1	
Ca^{++} ($CaCl_2$) $1,2 \times ^{-6}$ M			1		1							1		1				
Al^{+++} ($Al\ Cl_3$) $1,2 \times 10^{-6}$ M				1	1								1	1				
Cytochrome c						1									1			
Supernatant liquid							1		1							1		1
Riboflavine 1×10^{-3} M								1									1	
	O_2 consumption in cmm									O_2 consumption in cmm								
	203,8	98,2	96,1	96,0	101,6	95,1	202,4	168,8	22,6	166,2	72,2	76,2	76,3	73,2	78,9	166,3	128,4	8,2
Inhibition in per cent	0	51,8	52,9	52,9	50,1	53,3	0,7	17.2		0	56,6	54,1	56,0	55,9	52,5	0	22,7	

haved in a similar manner in the oxidation of ascorbic acid and that shows again, that an increase of oxidation in sodium succinate is not caused by addition of cytochrome oxidase. It only remains to accept succinic acid or some other substrate as a probable cause of this increased oxidation. This cause must of course be contained in the »supernatant liquid«. In order to solve this problem, experiments were made by adding larger quantities of succinate, fumarate, α-glycerophosphate, malate or lactate. All these were unable to diminish the atebrin inhibition. We found, however, an increase of oxygen consumption when adding a surplus of α-glycerophosphate or malate, but this could in no way be compared with the increase of oxygen consumption accomplished by an addition of the »supernatant liquid«. We did not succeed in isolating or identifying the substance contained in »supernatant liquid« responsible for its function. Further experiments will give a better survey of its composition and function.

Summary

The atebrin inhibition of succinoxidase system preparations by Keilin and Hartree and by. Hoff-Jörgensen and Lehmann cannot be induced in succinate oxidation with the tissue itself i. e. with muscle hash. It was possible to accomplish a renewal of oxygen consumption in succinate oxidation with the supernatant liquid after the precipitation of enzyme with acid. This liquid called »supernatant liquid«, its function and its comparison with other substances which activate the oxygen consumption in succinate oxidation with the above mentioned preparations, is discussed on the base of experimental findings.

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IZVOD

Djelovanje atebrina na sistem sukcinoksidaze
II. Zaštita od atebrinske inhibicije u tkivu

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U prvom saopćenju bilo je prikazano inhibitorno djelovanje atebrina na potrošnju kisika kod oksidacije jantarne kiseline sa pripravcima sistema sukcinoksidaze po Hoff-Jörgensen-u i Lehmann-u. Ovo se ne slaže sa rezultatima Wright i Sabine, koji su našli ispitujući djelovanje atebrina na potrošnju kisika kod oksidacije jantarne kiseline pomoću komadića mišićnog tkiva, da nema inhibicije potroška kisika kod ove oksidacije. Ispitano je djelovanje raznih tvari, za koje se predmnijevalo da bi zbog toga, jer se normalno nalaze u mišiću, mogle utjecati na ovu inhibiciju. »Kochsaft« po Ahlgren-u, kalcijev i aluminijev jon, i citokrom c nisu obnavljali sistem nakon atebrinske inhibicije, a riboflavin je samo neznatno utjecao. Dodavanje većih količina sukcinata, zatim fumarata, α-glicerofosfata, malata i laktata nije također imalo utjecaja na obnavljanje smanjene potrošnje kisika. Ako smo u istu svrhu upotrijebili »gornju tekućinu«, koja preostaje kod obaranja enzima kiselinom, vidjeli smo, da je ova u stanju sasvim obnoviti potrošak kisika. Ova »gornja tekućina« pokazuje intenzivnu žuto-zelenu fluorescenciju, koja stajanjem iščezava. Kuhanjem gubi svoju aktivnost, dok fluorescencija ostaje sačuvana. Prema ovome tvar, koja je nosilac ove aktivnosti gornje tekućine, nema sličnosti niti sa citokromom c, koji je termostabilan, a niti se riboflavinom, koji fluorescira i nakon kuhanja.

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