ON ATEBRIN ACTION ON SUCCINOXIDASE SYSTEM I. COMPETITION BETWEEN ATEBRIN AND THE PROSTHETIC GROUP OF THE FLAVIN ENZYME IN THE SUCCINOXIDASE SYSTEM

by

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Thunberg[14]) conducted the oxidation of succinic acid to the fumaric acid under the influence of the succinodehydrogenase as a biological oxidation. Szent-Györgyi[13]) and his co-workers postulated its place in the chain of enzymatical respiratory processes, as an intermediate in carrying over the hydrogen from the dihydrocodehydrogenase to the oxygen activated by the Warburg-Keilin-system. Succinodehydrogenase belongs to enzymes which reduce cytochrome c. Flavin-enzymes especially cytochrome reductase were known as the only suitable enzymes for this reducing function. Potter[10, 11]) has shown that succinodehydrogenase is an analogous enzyme.

All animal tissues contain succinodehydrogenase and it is one of the most active dehydrogenases. A common opinion is that it belongs, along with α-glycerophosphatedehydrogenase, to the enzymes which act without coenzyme. It contains sulfhydril groups, known to be blocable by specific compounds and thus being able to diminish or completely to destroy the action of the enzyme. Glutathione (GSH), cysteine and 2,3-dimercaptopropanol renew its activity.

Its function in the chain of respiratory enzymes is apparent only in the oxidation of triosephosphoric acid. Succinodehydrogenase has not yet been isolated and therefore its chemical properties are not yet quite known. Its place in the chain shows it to be similar to the flavin-enzymes.

Axelrod and co-workers[1, 4]) have found that avitaminosis B_2 in rats diminishes the concentration of the flavin-enzyme, d-amino-acid-oxidase and xanthinoxidase in the tissues. In the same way they showed by experiments with succinodehydrogenase and succinoxidase system[2, 3]), that the action of the latter is diminished. They have concluded that succinodehydrogenase or a component of the succinoxidase system which acts between itself and Warburg-Keilin system is a flavin-enzyme.

We have tried to prove this fact using atebrin, which has been shown by Haas[5]) to be a competitor of riboflavine in the prosthetic group of flavin-enzyme, and to diminish respiration. Haas worked on an isolated system of cytochrome oxidase,

cytochrome c, cytochrome reductase, triphosphopyridinnucleotide, Zwischenferment and glucose-6-phosphate, and he based his investigations upon the discovery of Wright and Sabine[15]), who have observed the inhibitory action of atebrin in a rat's liver, brain and kidney. It was found that the oxidation of glucose, lactate, pyruvate, malate, citrate and fumarate is inhibited by atebrin in the latter tissues, whereas it does not inhibite the oxidation of succinate. The action of d-amino-acid-oxidase is also inhibited, though its prosthetic group (flavin-adenin-dinucleotide) protects it from atebrin. We have investigated the action of succinodehydrogenase i. e. the succinoxidase system isolated by Hoff-Jörgensen and Lehmann[9]) and by Keilin and Hartree[7]), though Wright and Sabine[15]) have shown that the ability for the oxidation of succinate in the tissues was unimpaired.

The method

The enzyme preparation by Hoff-Jörgensen and Lehmann does not contain fumarase. A pig's heart of some 250 g is put through the grinder five times, then it is rinsed twice for ten minutes with distilled water, and finally ground half an hour in a mortar with glass grit and a successive addition od 2×10^{-2} M Na_2HPO_4. That hash is cooled for an hour to 0°C. It is centrifuged, the sediment discarded and water added to the liquid to treble its volum. 0,5 M sodium hydroxyde is added until the pH is 9,5, the liquid left standing for half an hour at room temperature and is then precipitated with 0,5 M sulphuric acid until the pH is 4,3. After being centrifuged the sediment is suspended in 50 ccm of M/15 Na_2HPO_4. The coenzyme and parts of cytochrome c are rinsed aut. As the preparation lacks fumarase, the fumaric acid can not induce the formation of oxalacetic acid which, owing to its better adsorption of the succinodehydrogenase, inhibits further oxidation of the succinic acid.

Investigations on the activity of succinodehydrogenase were carried out according a test by Lehmann in Thunberg-vacuum tubes at 40°C.

enzyme solution	0,2 ml
sodium succinate 0,2 M	0,2 ml
methylene-blue 1 : 5000	0,5 ml
phosphate buffer M/15 pH 7,4	1,0 ml
distilled water	0,6 ml

The decolourising times were 9—10 minutes, according to the preparation. A similar test for proving the presence of

fumarase was carried out after Quastel[12]) with a polarimeter. The test consists of:

enzyme solution	2,0 ml
sodium fumarate 0,4 M	1,0 ml
phosphate buffer M/15 pH 7,3	1,0 ml

This mixture remains for one hour in a water bath at 37°C, after which 1 ml of glacial acetic acid and 10 ml of a 14,2 p. c. solution of ammonium molybdate are added and filtered. The clear filtrate is put into polarimeter tubes of 2 dm. As a source of light served a sodium-lamp. If the enzyme solution does not contain fumarase, the rotation is $0{,}00 \pm 0{,}05^{0}$.

The preparation by Keilin and Hartree[7]) differs from the above mentioned by the circumstance that the hash from ground muscle is not washed and the preparation is carried out with an acetate buffer of pH 4,5 . The enzyme solution contains along with the succinodehydrogenase also cytochrome oxidase, fumarase, cytochromes, α-glycerophosphatedehydrogenase, malicodehydrogenase and some lacticodehydrogenase. At 0°C it keeps its activity for several weeks. The activity was also tested by Lehmann as described earlier.

The experiments were carried out in Thunberg-vacuum tubes at 40°C, and by Warburg's manometric method. The measurements gave identical values with and without sodium hydroxide for the absorption of carbon dioxide, and in later investigations sodium hydroxide was omitted. The values for atebrin are given as final concentrations for the entire contents of the Warburg flasks.

The results

Succinodehydrogenase. The oxidation ability of sodium succinate was measured after Lehmann's test in Thunberg-vacuum tubes. As the yellow colour of atebrin added to methylene blue gives a green colour, the time needed by the green colour to become yellow was measured and compared with controls without the addition of methylene blue. The enzyme solution contained 2 ml of the preparation and 1 ml of 0,5 M sodium succinate solution. As seen from Table 1. atebrin did not influence any of the preparations within the time needed for decolourising of the methylene blue.

From results given in Table 1 we see, that the sucinodehydrogenase does not contain the flavin component with which atebrin needs to compete and so it does not prolong the reduction time of methylene blue.

Table 1

Preparation by			
Hoff-Jörgensen and Lehmann		Keilin and Hartree	
without atebrin	with atebrin	without atebrin	with atebrin
min.	min.	min.	min.
9	10	8	8
11	10	7	7
11	11	8	7
8	9	8	8
10	9	9	11
12	11	11	12
11	10	9	8
10	11	9	8
11	11	7	7
9	9	8	8
10,2	10,1	8,2	8,4

Succinoxidase system. The activity of this system was investigated using Warburg's manometric method. As it is shown in Table 2. the consumption of oxygen has considerably diminished by the addition of atebrin in a final concentration of 1×10^{-3} M.

The table shows that the oxygen consumption of succinoxidase system is inhibited by atebrin. The preparations of the enzyme were made according Hoff-Jörgensen and Lehmann[6]) and according Keilin and Hartree[7]). Each flask contained 2 ml of the enzyme preparation, 1 ml M/15 of the phosphate buffer pH 7,4, 1 ml of atebrin in the final concentration of 1×10^{-3} M dissolved in the phosphate buffer of the same concentration and pH 7,4, and in the side arm of the flask 1 ml 0,5 M sodium succinate. Readings are made every 15 minutes. Gaseous phase: air. Temperature 40°C.

The results show the average values of 18 equal experiments with preparations of the enzyme prepared in the same way. Haas[5]) has shown that atebrin in concentration of 1×10^{-4} M inhibits cytochrome oxidase for about 60 p. c. using p-phenylendiamine as a substrate at 25°C. If ascorbic acid is added to the enzyme preparation as a substrate, the inhibition of the oxygen consumption using atebrin could not be observed in concentrations mentioned above. In Haas[5]) the system

Table 2

Preparation by				
	Hoff-Jörgensen and Lehmann		Keilin and Hartree	
	without atebrin	with atebrin	without atebrin	with atebrin
	cmm of oxygen consumption			
after minutes				
15	42,3	21,0	38,7	13,5
30	98,6	45,1	81,0	20,7
45	153,5	87,1	128,6	50,4
60	198,9	108,1	178,8	91,9
Per cent of final inhibition	0,0	45,6	0,0	49,1

of cytochrome oxidase — cytochrome c is not inhibited with atebrin and the consumption of oxygen is proportional to the concentration of cytochrome c. This has been proved and it was found that atebrin was not able to inhibit the oxidation of ascorbic acid with enzyme preparations because the cytochrome concentration remained constant whereas cytochrome itself cannot be inhibited. This would indicate that atebrin enters into a component of the succinoxidase system which is placed between the dehydrogenase and the Warburg-Keilin system.

It was endeavoured to find out, whether this component forms a flavin enzyme, by adding riboflavin. According to Haas[5]) atebrin concurs with riboflavin, replaces it and thus disrupts the chain of transfer of hydrogen upon oxygen activated by cytochrome oxidase. By adding chemically pure riboflavin the inhibitory action could not develop, i. e. the inhibitory action was diminished as shown on Table 3. We see that flasks with atebrin plus riboflavin demonstrate an inhibition about half smaller than in flasks with atebrin alone.

Table 3 shows the protective action of riboflavin on the atebrin inhibition of succinoxidase system. The preparations were made according to Hoff-Jörgensen and Lehmann and according to Keilin and Hartree. Each flask contained 2 ml of the enzyme preparation, 1 ml M/15 of the phosphate buffer of pH 7,4 and the contents indicated in the final concentration. The side arm of the flask contained 1 ml M/2 sodium

succinate which after incubation of fifteen minutes was poured into the flask. Gaseous phase: air. Temperature 40°C. Readings every 15 minutes.

Table 3

	Preparation after					
	Keilin and Hartree			Hoff-Jörgensen and Lehmann		
	1 Without atebrin	2 With 1 ml of atebrin 1×10^{-3} M	3 With 1 ml of atebrin 1×10^{-3} M and 1 ml of riboflavin $0,5\times10^{-3}$ M	1a Without atebrin	2a With 1 ml of atebrin 1×10^{-3} M	3a With 1 ml of atebrin 1×10^{-3} M and 1 ml of riboflavin $0,5\times10^{-3}$ M
	cmm of oxygen consumption					
after minutes						
15	38,7	12,6	12,8	39,2	14,1	13,2
30	80,6	21,0	37,8	90,2	24,0	21,3
45	129,5	52,2	69,3	148,1	60,0	78,1
60	181,2	93,0	134,5	194,0	101,3	148,8
Per cent of final inhibition	0,0	48,7	25,8	0,0	47,8	23,3

Figure 1. shows clearly the increase of oxygen consumption through addition of riboflavin. We see besides that restitution of oxygen consumption during the first 15 minutes is either diminutive or nonexisting but that it increases rapidly during the last fifteen minutes.

Discussion

The experimental results shown in this paper point out that atebrin in its final concentration 1×10^{-3} M is able to inhibit preparations of succinodehydrogenase respective a succinoxidase system prepared after Keilin and Hartree[7]) and after Hoff-Jörgensen and Lehmann[6]). These results collide with the statements of Wright and Sabine[15]) who have experimented with bits of tissues and have obtained results which show that atebrin could not inhibit the oxidation of succinate. The oxidation of ascorbic acid as well as the reduction

time of methylene blue remains unchanged by atebrin, showing thus that the succinodehydrogenase and cytochrome oxidase system remain unimpeded in their function in spite of the action of atebrin. As the inhibiton in these cases was 40—50 p. c. it would correspond according to H a a s to the values found for

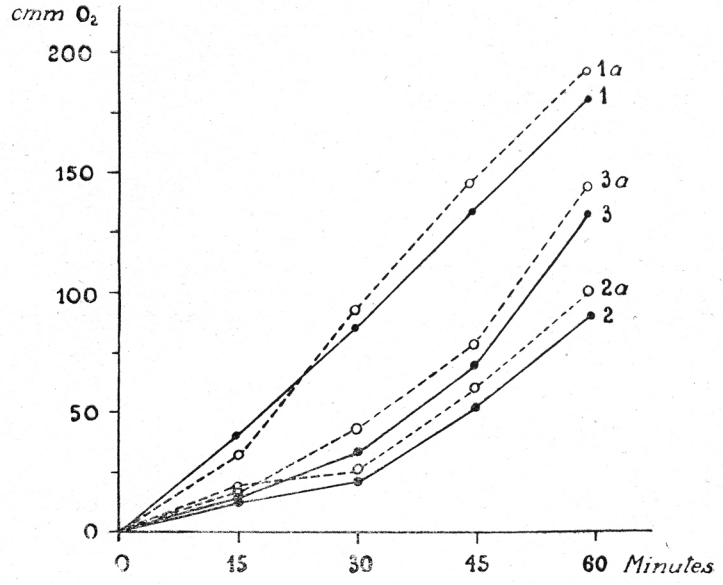

Figure 1.

Consumption of oxygen acording to Table 3. The full line marks the preparation according to H o f f - J ö r g e n s e n and L e h m a n n whereas the dotted line gives us the preparation after K e i l i n and H a r t r e e. The numbers correspond to the indicated columns in the above mentioned table.

the atebrin inhibition of flavin enzymes. The place of action of atebrin being in a link between succinodehydrogenase and the W a r b u r g - K e i l i n system, it would follow that this link should be the flavin enzyme. H a a s[5]) has demonstrated on isolated enzyme system that atebrin concurs with the prosthetic group of the flavin enzyme, and as the experiments quoted above show, there exists a similar condition in the succinoxidase system also, as chemically pure riboflavin was able to diminish for about 50 p. c. the atebrin inhibition. K u h n and R u d y[9]) have already shown that riboflavin alone possesses a certain catalytic activity, as has been shown at the beginning of the experiment, but at the end of the first hour of the duration of the experiment there is an obvious increase of the catalytic

activity of riboflavin, probably because the resting in the tissue developes phosphorylation of riboflavin thus forming the original prosthetic group.

Summary

The atebrin inhibition of succinoxidase system at the level of a link placed between the cytochrome oxidase system and succinodehydrogenase is shown. This inhibition can be restituted by adding riboflavin. From these observations it follows that within the succinoxidase system atebrin also competes with the riboflavin-phosphoric acid, i. e. with the prostetic group of the flavin enzyme. It is concluded that the succinoxidase system contains the flavin enzyme as a component in the oxidation of the succinic acid.

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I Z V O D

Djelovanje atebrina na sistem sukcinoksidaze
I. Konkurencija između atebrina i prostetske skupine flavin-enzima u sistemu sukcinoksidaze

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Prema A x e l r o d - u i suradnicima[1, 2, 3, 4]) smanjuje se u tkivu štakora koncentracija ksantinoksidaze i oksidaze d-amino kiselina, ako su stavljeni na dijetu bez riboflavina. Oni su našli i to, da se smanjuje

aktivnost sistema sukcinoksidaze, te su zaključili, da je ili jedna karika u gornjem sistemu ili pak, da je sama sukcinodehidraza flavin-enzim.

Gornju pretpostavku preispitali smo služeći se nalazom Wright i Sabine[15]) te Haas-a[5]), da atebrin konkurira sa prostetskom skupinom flavin-enzima, ulazi na njezino mjesto i prekida kontinuitet karika u lancu oksidativnih enzima. I ako su Wright i Sabine našli, da je oksidacija jantarne kiseline u tkivu ostala neoštećena, ipak smo proveli ispitivanja u ovom smislu služeći se pripravcima enzima prema Hoff-Jörgensen i Lehmann-u[6]), te prema Keilin i Hartree-u[7]). Opisano je dobivanje pripravaka i određivanje njihovih aktivnosti po Lehmann-u. Sukcinodehidraza je ispitana Lehmann-ovom metodom u Thunberg-ovim vakuum-cjevčicama, te je nađeno da atebrin ne produljuje vrijeme odbojadisanja metilenskog plavila, što se vidi iz tabele 1.

Sistem sukcinoksidaze je ispitan Warburg-ovom manometričkom metodom, pa se je vidjelo, da je potrošnja kisika kod obih preparacija inhibirana za 45—50% kod konačne koncentracije atebrina od 1×10^{-3} m. Da se ne radi o inhibiciji djelovanja citokrom-oksidaze ni citokroma c pokazano je time, što je oksidacija askorbinske kiseline ostala neoštećena djelovanjem atebrina (Tabela 2). Dodavanjem riboflavina (kemijski čistog) u koncentraciji $0{,}5 \times 10^{-3}$ m uz atebrin, povećana je ponovno potrošnja kisika za oko 25% prema onoj kod djelovanja atebrina. (Tabela 3). Iz gornjeg se može zaključiti, da se u sistemu sukcinoksidaze nalazi jedna karika, sa kojom atebrin konkurira i koja se nalazi između sukcinodehidraze i Warburg-Keilin-ovog sistema, nadalje, da je u analogiji sa pronalaskom Haas-a[5]) gornja karika flavin-enzim, jer se njegova akcija dade obnoviti sa riboflavinom (Sl. 1).

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