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Investigation of the Kinetic Characteristics of Red Blood Cell Acetylcholinesterase in Abo, Rh Hemolytic Disease of the Newborn and Thalassemia Major Cases

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Recent interest has been focused on erythrocyte acetylcholinesterase activity in clinical disorders with various erythrocyte abnormalities.[1 2 3 4] Except in cases with paroxysmal nocturnal hemoglobinuria (PNH)[5 6] and in one family with definitely reduced erythrocyte acetylcholinesterase,[7] in all the cases studied the values reported showed a great deal of overlap with normal controls, but the results give no clue as to the nature of the defect.[3 6] The activity was investigated only at one substrate concentration, and as this stromal enzyme shows a complex kinetic behavior towards its substrates and inhibitors,[8 9] such results are not truly relevant to the state of the enzyme in the erythrocyte. Hence we have investigated the detailed kinetic behavior of this enzyme with its various substrates and inhibitors in the hope of obtaining more information on the nature of the defect. We found no significant difference in

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the kinetic behavior of the enzyme in newborns with ABO or Rh hemolytic disease and thalassemia major. The results indicate that there may be a diminished amount of enzyme rather than a variant in newborns, and that this decrease is more pronounced in ABO hemolytic disease.

Methods and Subjects

The normal adults studied were selected from healthy blood bank donors, and the normal newborn group from the babies in our Department of Obstetrics, none of whom were older than three days of age. In each patient the diagnosis was confirmed by proper laboratory tests. The presence of ABO hemolytic disease was certain in all cases, by the serologic findings in the infants'blood (1. e.. Coombs test and the usual blood group incompatibility), microspherocytosis and increased reticulocytosis. The results of suspected cases of ABO hemolytic disease are not included. The usual diagnostic criteria were adopted for Rh hemolytic disease. Thalassemia major was confirmed with the peripheral blood picture, osmotic fragility and hemoglobin electrophoresis findings in both the siblings and parents.

The blood samples were withdrawn prior to transfusion in all patients and were washed with saline three times to remove any trace of plasma. A hematocrite value was obtained from the last packed cell sediment, and it was assumed that 10^{10} erythrocytes (1 ml) were present at 100 per cent hematocrite value. The erythrocytes were then hemolyzed in $5x10^{-3}M$, pH 7 potassium phosphate; in some control experiments the hemolysis was achieved with sodium deoxycholate at a concentration of 2 mg. per ml. The hemolysate was immediately frozen and kept at–20 C° until the time of analysis, however most of the samples were assayed right away.

Acetylthiocholine (ATC) was used for the kinetic studies as substrate. The assay system, as described previously,[10] was composed of potassium phosphate $2x10^{-2}M$, 5.5[1] dithiobisnitrobenzoic acid $8x10^{-5}M$, varying concentrations of inhibitor, hemolysed erythrocyte finally diluted 1/10000-20000 at pH 8; the hydrolysis was followed at 412 mμ with a Becmann DU spectrophotometer equipped with a tempering cell holder at 24-25 C°. The assays were run at least three and sometimes four times; the average of these results was used for kinetic calculations. In each instance initial velocities obtained before one percent of the hydrolysis obtained were used for the calculations. The enzyme manifests substrate inhibition with ATC similar to the finding of Nachmansohn described for acetylcholine.[9] At least eight substrate inhibited and non-inhibited

concentrations were selected. The inhibition of acetylthiocholine hydrolysis caused by substrates, substrate analogues and most of the inhibitors can be successfully investigated. However, among the group of quarternary nitrogen carrying inhibitors, only mytelase could be used because most compounds of this group manifest an unsteady degree of inhibition with time, and hence are not suitable.[8] This stromal enzyme can be completely solubilized by sodium deoxycholate treatment.[8] This treatment does not change the kinetic characteristics of the enzyme as was found in most of the normal blood samples; in fact a twohundredfold purified enzyme behaved similarly.[8]

The kinetic characteristics of the enzyme was established by using Lineweaver-Burke plots drawn for each sample with and without inhibitors; K_m, K_i, V_m values and inhibition percents were obtained from these graphics.[10] K_m and K_i were expressed in M, V_m as μ Moles of ATC hydrolyzed per 10^{10} (1 ml.) erythrocyte per hour at 24-25 C°. Standard biostatistical methods were used for the evaluation of results.

Results

Various kinetic findings of the erythrocyte acetylcholinesterase in normal adults, newborns, ABO, Rh hemolytic diseases of the newborn and cases of thalassemia major are shown in Table 1. The statistical distribution of the values is as would be expected from the presence of a single population.[8] There was no evidence of the existence of a variant enzyme in the total 137 cases studied. Statistically we found no significant difference in K_m, K_i or in any one of the inhibition values, which suggests the existence of similar enzyme (enzymes) in all these groups. The maximal velocity of the enzyme, however, differed in newborns and ABO hemolytic disease; this was significant despite overlapping values; it was less in normal newborns, and more so in ABO hemolytic disease. The maximal velocity reflects the amount of enzyme protein, and in view of the lack of any other kinetic parameter change such as K_m, K_i and inhibition percentages, this finding may be interpreted as the existence of a diminished amount of enzyme protein in these erythrocytes.

Discussion

Interest in erythrocyte acetylcholinesterase has been aroused by the findings of Kaplan and his co-workers who reported a decreased enzyme activity in ABO hemolytic disease of the newborn.[1] Burman reported values within the normal range in two cases afflicted with the same disorder.[2] Choremis et al reported the enzyme activity as lower than nor-

mal in cases of thalassemia major.[3] Erythrocte acetylcholinesterase has been investigated in Rh hemolytic disease of the newborn, in other types of hemolytic anemias, as well as in anemias due to malnutrition and erythrocyte enzyme defects;[1 2 3 4] in none of these cases was there any difference. In the hope of eliciting the nature of the decrease, Herz and his co-workers investigated the distribution of acetylcholinesterase activity among erythrocytes of various ages in normals, ABO hemolytic disease and PNH.[6] They observed that whereas the distribution of activity was similar to normal in ABO hemolytic disease, it was different in PNH erythrocytes. They reported thet thay had found no difference in heat sensitivity and inhibitory properties although they give no experimental values.

All these experiments were carried out with substrate at one concentration. No special consideration was given to obtain initial velocities, and in some experiments the concentration of the substrate had changed as much as ten per cent by the end of the experiment. The velocity of the enzyme changes significantly at the concentrations of the substrate generally used in such experiments. Both this critical change and the usual splay of results observed render the interpretation of overlapping results difficult and conclusions as to the nature of the defect hazardous.

The results shown in Table 1 indicate that the only difference in the kinetic behavior of acetylcholinesterase was the maximal velocity. The distribution of values for all the kinetic parameters yielded a normal distributional pattern denying the existence of a variant enzyme. A two-hundredfold purified enzyme behaved similarly[8] as did the sodium deoxycholate solubulization. Thus we may presume that the results presented are reasonably representative of the state of the enzyme in the erythrocyte.

The decreased maximal velocity in newborn and ABO hemolytic disease erythrocytes may be interpreted as the existence of a lesser amount of enzyme in these groups. Should it have been that the enzyme was either a variant or under the influence of an **in vivo** acting inhibitor, significant differences would also have been observed in K_m, K_i and inhibition percentages with inhibitors, substrate and substrate analogues. This of course cannot be definitely ruled out unless quantitative extraction of the enzyme from the erythrocyte is achieved, which is at present impossible. Hence we may speculate that the decreased enzyme amount observed in normal newborn erythrocytes is physiological and is reminiscent of the delayed appearance of bilirubin conjugating activity normally absorbed in the newborn liver. In fact, the results obtained by Burman support such an interpretation.[2] The reasons leading to a more pronounced

TABLE I

COMPARISON OF VARIOUS KINETIC PARAMETERS OF ERYTHROCYTES ACETYLCHOLINESTERASE BETWEEN NORMALS AND VARIOUS HEMOLYTIC ANEMIAS

Parameter investigated	Normal subjects (84) *	Normal newborns (25) **	Rh incompatibility (10)	ABO incompatibility (10)	Thalassemia major (8)
K_m ($\times 10^{-5}$M)					
Range	1.3 – 14.0	1.1 – 9.3	2.9 – 9.8	3.3 – 6.3	4.7 – 7.9
Mean ± S. E.	4.8 ± 0.3	5.0 ± 0.6	5.5 ± 0.8	5.1 ± 0.3	6.1 ± 0.5
P value		n.s.*	n.s.	n.s.	n.s.
K_1 ($\times 10^{-3}$M)					
Range	1.0 – 26.2	1.3 – 12.0	3.3 – 19.2	4.8 – 12.5	0.3 – 7.8
Mean ± S. E.	6.4 ± 0.3	6.6 ± 0.8	7.6 ± 1.7	7.3 ± 0.8	5.2 ± 0.9
P value		n.s.	n.s.	n.s.	n.s.
V_m					
Range	625 – 1389	313 – 909	402 – 741	312 – 943	862 – 1299
Mean ± S. E.	1032 ± 34	649 ± 23	596 ± 38	460 ± 60	1053 ± 46
P value		<0.001	n.s.	$0.01 < P < 0.001$ n.s.	
Inhibition experiments :					
Caffeine inhibition					
Caffeine 3.3×10^{-4}M, ATC $+10^{-4}$M					
Range		20 – 44	30 – 43	28 – 49	27 – 44
Mean ± S. E.		35 ± 1	37 ± 1	38 ± 2	40 ± 2
P value			n.s.	n.s.	n.s.
Caffeine 6.6×10^{-4}M, ATC 5×10^{-4}M					
Range		19 – 48	24 – 48	27 – 49	33 – 45
Mean ± S. E.		35 ± 1	37 ± 2	38 ± 2	37 ± 2
P value			n.s.	n.s.	n.s.
2) **Acetylcholine inhibition**					
A) Acetylcholine 3.3×10^{-4}M, ATC 10^{-4}M					
Range		40 – 81	55 – 96	50 – 73	44 – 69
Mean ± S. E.		62 ± 1	67 ± 4	62 ± 2	61 ± 3
P value			n.s.	n.s.	n.s.

* The number in parenthesis denotes the number of samples analysed.

** The newborn group gave essentially the same inhibition percentages.

* S. E.: Standard error; n. s.: Not significant which is $P<0.05$.

ql Inhibitions are expressed as percent.

Parameter investigated	Normal subjects	Rh incompatibility	ABO incompatibility	Thalassemia major
B) Acetylcholine 10^{-3}M, ATC 5×10^{-4}M				
Range	46 –74	52 –65	47 –70	60 –83
Mean ± S. E.	59 ± 1	58 ± 1	59 ± 2	66 ± 3
P value		n.s.	n.s.	n.s.
3) Mecholyl inhibition				
A) Mecholyl 1.33×10^{-3}M ATC 10^{-4}M				
Range	41 –65	50 –64	50 –60	50 –75
Mean ± S. E.	51 ± 2	58 ± 2	54 ± 2	56 ± 2
P value		n.s.	n.s.	n.s.
B) Mecholyl 5×10^{-3}M, ATC 5×10^{-4}M				
Range	40 –70	32 –64	50 –73	50 –75
Mean ±	64 ± 2	57 ± 3	63 ± 2	61 ± 3
P value		n.s.	n.s.	n.s.
4) Mytelase inhibition				
A) Mytelase 5×10^{-9}M ATC 5×10^{-5}M				
Range	30 –78	36 –90	30 –79	44 –86
Mean ± S. E.	62 ± 6	58 ± 6	58 ± 5	70 ± 6
P value		n.s.	n.s.	n.s.
B) Mytelase 5×10^{-9}M ATC 5×10^{-4}M				
Range	38 –64	25 –78	31 –72	39 –71
Mean ± S. E.	50 ± 7	52 ± 6	54 ± 4	59 ± 6
P value		n.s.	n.s.	n.s.

delay in the appearance of enzyme protein in the erythrocyte of ABO hemolytic disease, but not in Rh disease, were not investigated and should be studied.

We failed to detect any difference in our cases of thalassemia major. This may be due either to the splay of results usually observed at one fixed substrate concentration, or to the difference of other physiological factors such as the age of the patient, the severity of the disease or its duration existing between our group and that of Choremis et al.[3]

Summary

Various kinetic parameters of erythrocyte acetylcholinesterase in normal cases and patients afflicted with ABO or Rh hemolytic disease or thalassemia major have been investigated. No evidence for the presence of a variant enzyme was found. However, the maximal velocity was lower in normal newborns and was more pronounced in ABO hemolytic disease. A delayed appearance of enzyme protien is speculated to be the explanation for this.

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