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RED CELL CHOLINESTERASE IN MENTAL PATIENTS

by
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A.B., University of California, Berkeley, 1963

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

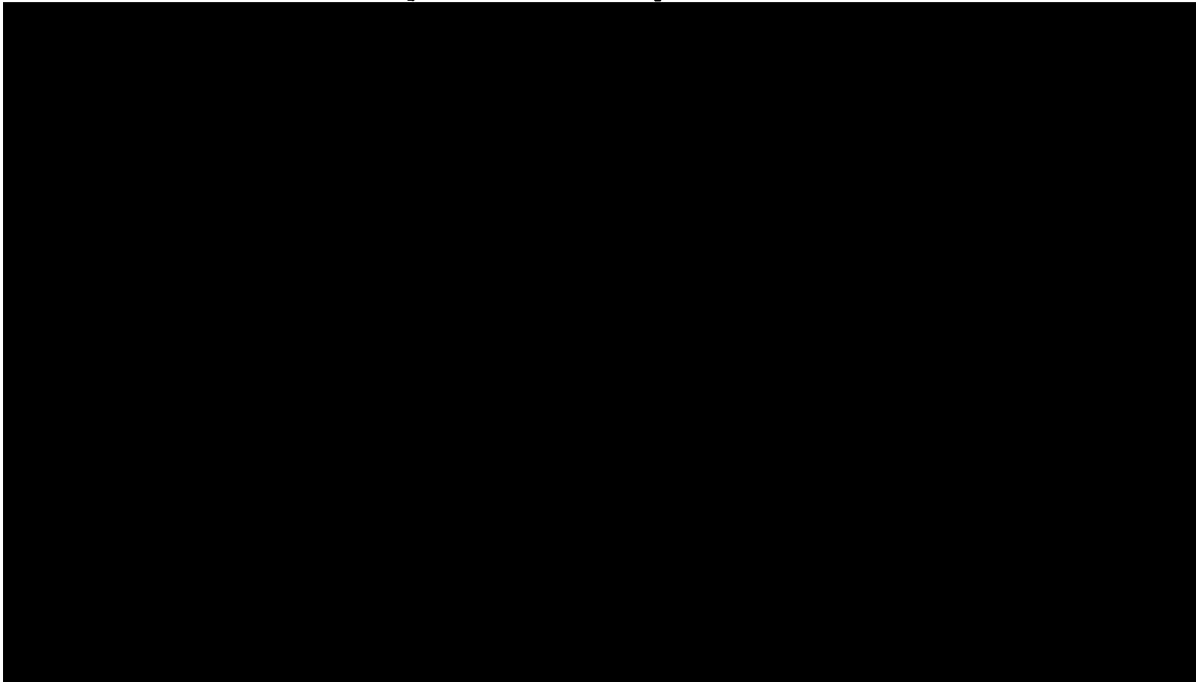
in

BIOCHEMISTRY

in the

GRADUATE DIVISION

[San Francisco]



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Introduction

Red blood cells are rich in acetylcholinesterase[1], the enzyme being bound to the cell membrane. The abbreviation RBC AChE will be used to denote the activity of this enzyme and when the enzyme itself is referred to, acetylcholinesterase will be used. According to Sabine, the exact role of this enzyme is still unknown. One would expect that if the enzyme were to have a function, a substrate for it would be found in the blood stream. Holland and Greig have found that red cells contain choline acetylase[2], the enzyme required for acetylcholine formation. After inactivating acetylcholinesterase with physostigmine (.002M) they were unable to detect any measurable amount of acetylcholine. They used the bioassay method of Chang and Gaddum, which is capable of detecting the production of 1 microgram of acetylcholine per hour (1).

Red cell acetylcholinesterase exhibits a much greater substrate specificity than the plasma cholinesterase and, in regard to its sensitivity to inhibitors and kinetic behavior, is similar to the cholinesterase of the central nervous system. The latter two enzymes appear to have activities which are linked in some indirect fashion. Oberst and Christensen exposed rats to an atmosphere of Sarin[3] (an anti-cholinesterase drug) and found, in the animals displaying toxic symptoms, a consistent reduction of AChE below 10% of normal for erythrocytes and 5% of normal for brain. However it was found that brain AChE was not appreciably

[1] Acylcholine acyl-hydrolase (3.1.1.7)
[2] Acetyl-CoA:Choline O-acetyl-transferase (2.3.1.6)
[3] Isopropyl methylphosphonofluoridate

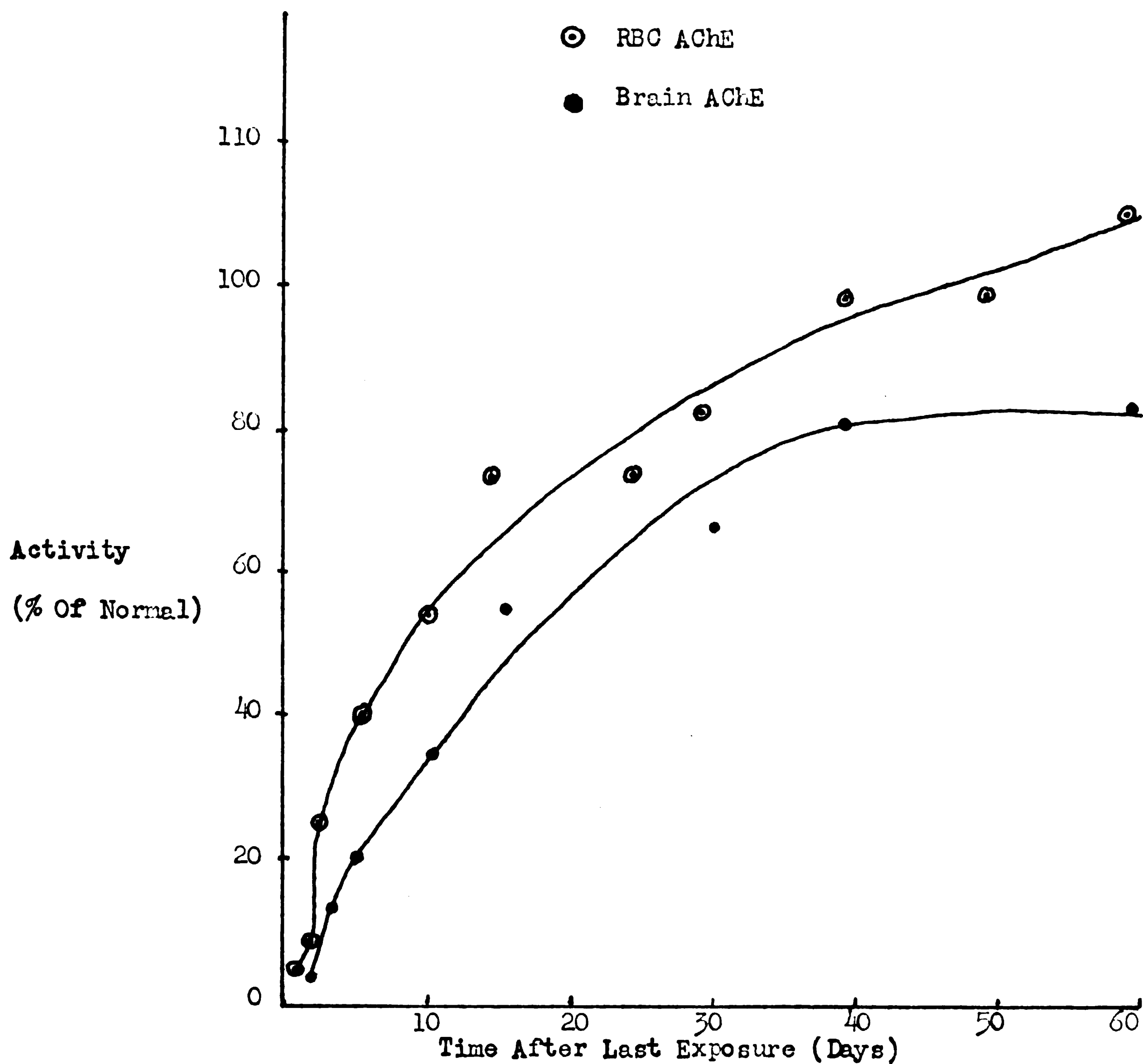


Figure (1) Regeneration Of Erythrocyte And Brain Cholinesterase In Animals Showing Severe Toxic Signs After Exposure To Sarin Vapor

Adapted from a table of Oberst and Christensen (3)

inhibited until the RBC AChE fell below 28% of its normal level (3). The data from Oberst and Christensen's experiment concerning recovery of activity following Sarin inhibition is plotted in figure (1).

From figure (1) it would seem that there is a strong correlation between brain and RBC AChE. Using di-isopropylfluorophosphate (DFP) as an inhibitor, several other workers have obtained slightly different results. This could easily be accounted for on the basis of the difference in the ability of the two drugs to penetrate the blood-brain barrier. Koelle and Gilman have concluded that the RBC AChE level following DFP inhibition does not yield an accurate measure of brain AChE except possibly during the period of erythrocyte regeneration during which the brain AChE recovery was very rapid (figure 2). This was followed by a rapid recovery in the red cells, accompanying a marked slowing of AChE production in the brain (5). Similarly Freedman, Willis and Himwich have reported that after giving DFP, the residual activity of brain AChE tripled during the first 48 hours while the RBC AChE showed no significant recovery. Subsequently the brain AChE regeneration rate decreased markedly while that of the red cell increased more rapidly (6). With both DFP and Sarin the inhibition is irreversible and caused by phosphorylation of the enzyme. Restoration of enzyme activity is not due to formation of new erythrocytes because the life cycle of red cells in the rat is 8-9 days (4) and one can see from figure (3) that restoration is not complete at the end of this time. Thus it seems that a considerable amount of the activity renewal may be due to formation of new enzyme by mature, enucleate erythrocytes. Another possibility is an increased rate of formation and/or release of erythrocytes from the bone marrow. DFP^{32} labeling experiments have shown that in humans the effect of DFP is not due to a shortening of the red

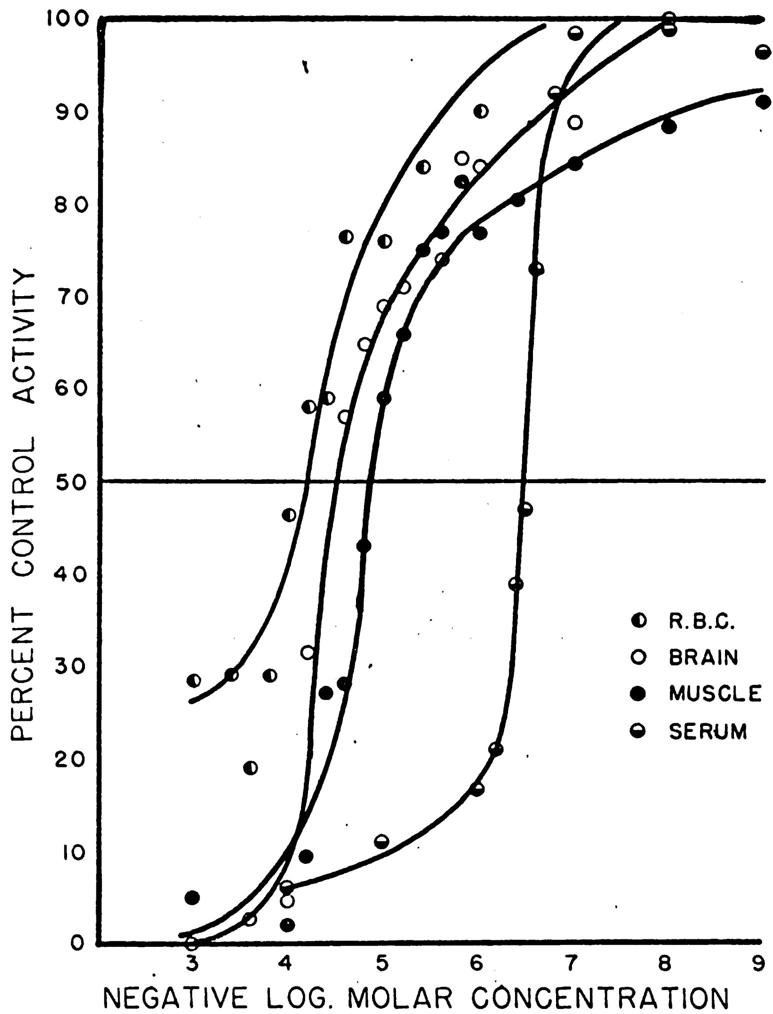


FIG. 1. THE INHIBITION OF RAT CHOLINESTERASE BY DI-ISOPROPYL FLUOROPHOSHATE IN VITRO

The "pK values" (negative logarithm of the molar concentration of DFP required to produce 50% inactivation) were found to be as follows:

	Rat	*Dog*
Serum	6.5	6.8
Red cells	4.2	4.3
Brain	4.5	3.8
Muscle	4.8	

Figure (2) Koelle and Gilman (5)

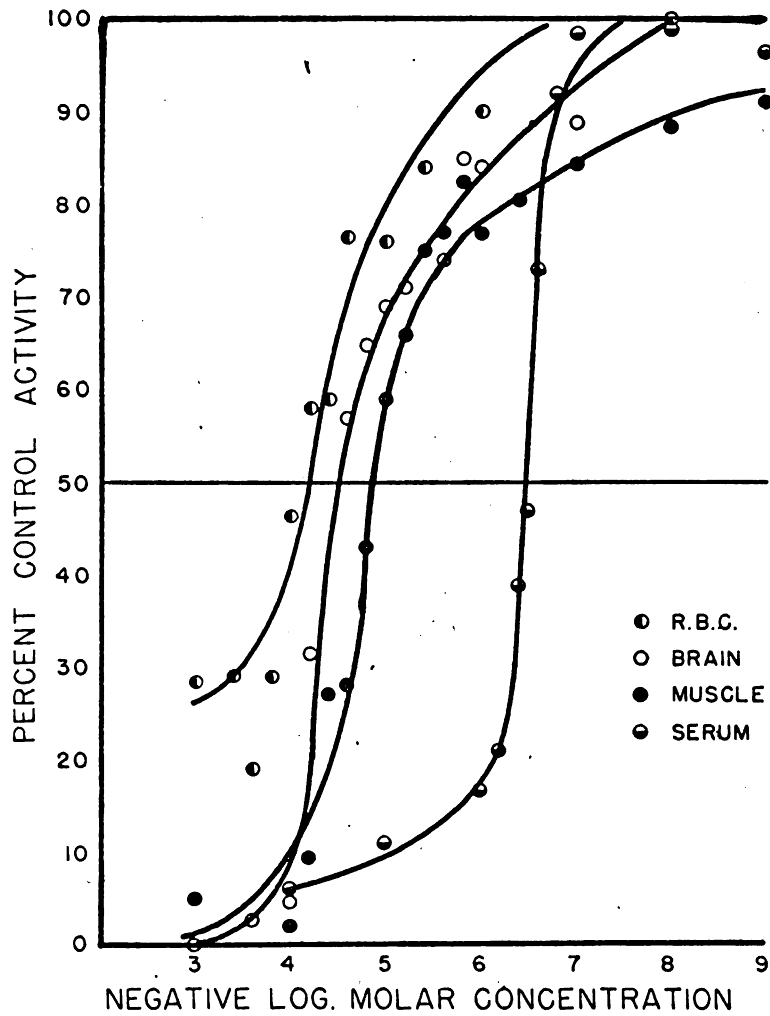


Fig. 1. The Inhibition of Rat Cholinesterase by Di-isopropyl Fluorophoshate in Vitro

Figure (2) Koelle, G.B., Gilman, A. (5)

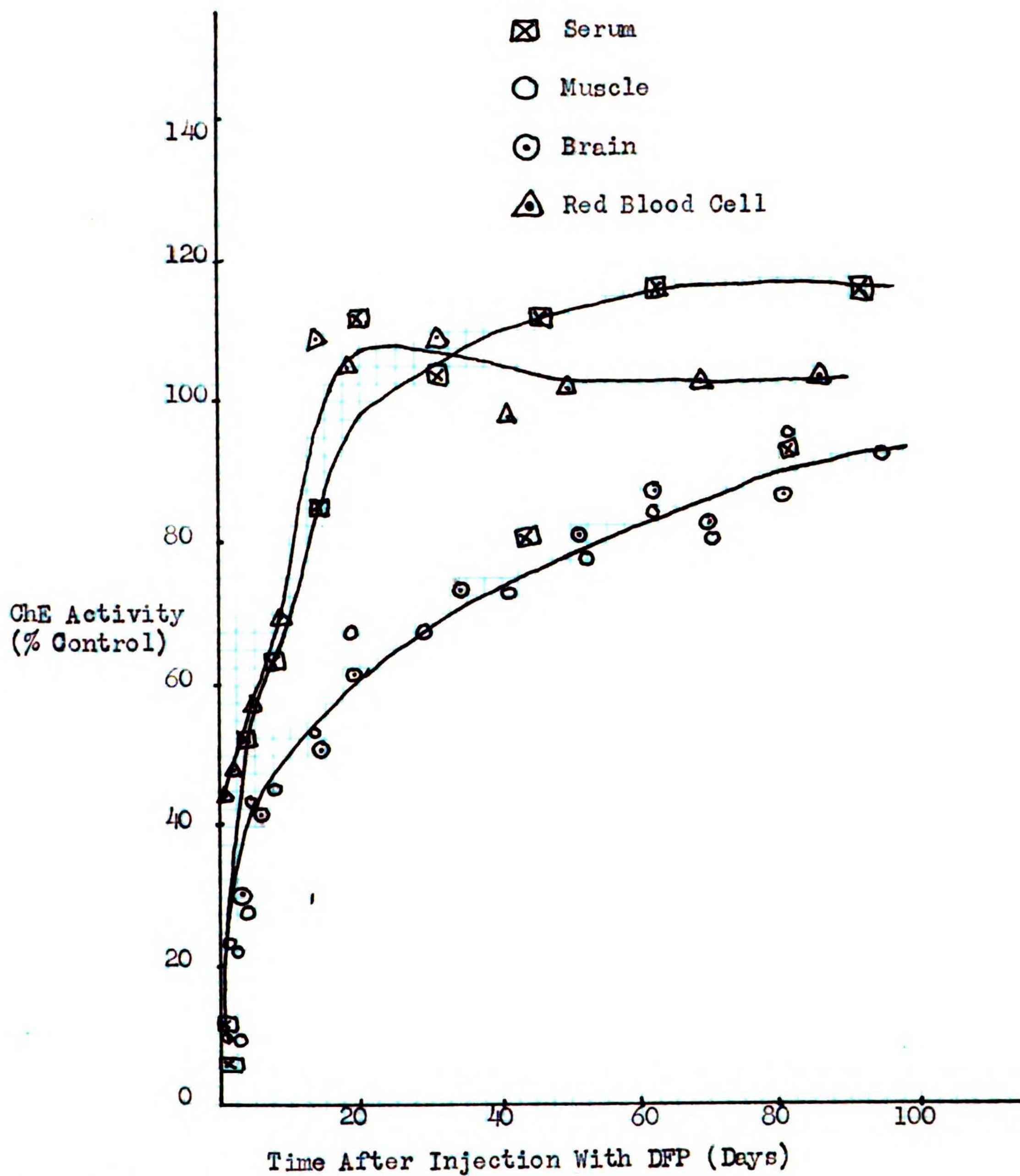


Figure (3) Recovery Of Cholinesterase Activity After DFP Inhibition In Rats

Adapted from a table of Koelle and Gilman (5)

cell's life span.

Krech, Rosenzweig and Bennet feel that it is rash to assume a relationship between the red cell and brain enzyme because even in different parts of the central nervous system they have found correlations of AChE activity which varied from -0.08 to + 0.52 depending on the strain of animal used (7). Thus the relationship, if any, between the two enzymes appears to be unresolved at present.

Evidence has been brought forward which suggests that there may be abnormal levels of either AChE or acetylcholine in abnormal mental states such as schizophrenia. Plotting the log of the threshold luminance versus the time the subject has spent in the dark, Granger has found that schizophrenics are able to adapt to the dark more rapidly than normals (7). In relation to this, Rubin and Goldberg have found that inhalation of the anti-cholinesterase drug Sarin produced an increased dark adaptation which decreased following treatment with atropine [a drug which competes with acetylcholine for binding at the receptor site on acetylcholinesterase (9).] It was then postulated by Rubin that the mechanism relating dark adaptation changes and psychiatric disorders may be an abnormality in the ACh-AChE system. On the basis of atropine's ability to decrease dark adaptation one would suppose that adaptation would be improved by increasing the concentration of acetylcholine or decreasing the activity of acetylcholinesterase. As a test of this hypothesis Rubin measured RBC AChE levels in mental patients and normals and obtained the results shown in table (1).

Subjects	Hydrolysis Rate	RBC AChE $\Delta[H^+]$ Moles/L/Min.
Mental Patients	Slow	-0.0169
Normals	Normal	-0.0218
Mental Patients	Fast	-0.0256

Table (1) A modification of the table from Rubin (9a)

In the study above Rubin examined only 10 normals and 23 mental patients, 16 of whom were schizophrenics. Rubin's results appear to be questionable because he examined so few patients and because he chose to separate the results of his mental patients into two categories. If one combines the results for all 23 patients one obtains an average hydrogen ion change of -.0206 Moles/l/min., which is not significantly different from the activity of normals.

Nevertheless there are findings which support Rubin's hypothesis. Sherwood, Ridley and McCulloch found, in cats partially recovered from a lesion which produced catatonic stupor, that intraventricular injection of a few micrograms of acetylcholine produced a recurrence of the stuporous state (10). An intraventricular injection of DFP or physostigmine was also found to produce a state of catatonic stupor (11). In a seemingly hopeless case, Sherwood succeeded in interrupting catatonic stupor by intraventricular injections of human red cells with a very high acetylcholinesterase activity, producing a remission that lasted several weeks. Similar results were obtained using pentamethonium, a drug which could counteract the effects of an excess of acetylcholine. He was always

successful in terminating catatonic stupor and in some cases no further treatment was necessary (12).

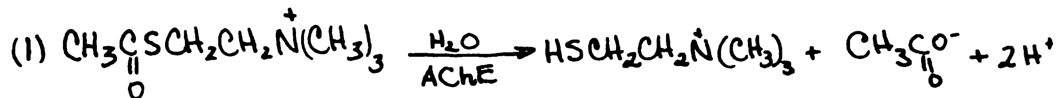
After obtaining experimental evidence suggesting the possibility of abnormal AChE levels in mental illness and a possible connection between AChE of the blood and brain, an effort was made to verify these points. The present paper is based on work done under the direction of Dr. George Ellman, Chief Research Biochemist at the Langley Porter Neuropsychiatric Institute. In a previous paper Ellman and Callaway have published data concerning RBC AChE in mental patients (13). They found that the mean enzyme activity was the same in both hospitalized patients and normal controls. However it was noted that in certain groups of hospitalized patients the standard deviation was considerably larger than in the normals. Since that time, Gal has published data indicating that a group of schizophrenic patients in a veteran's hospital had higher AChE levels than normal controls (14). In an attempt to resolve this difference we have repeated our previous work, this time using a larger number of patients.

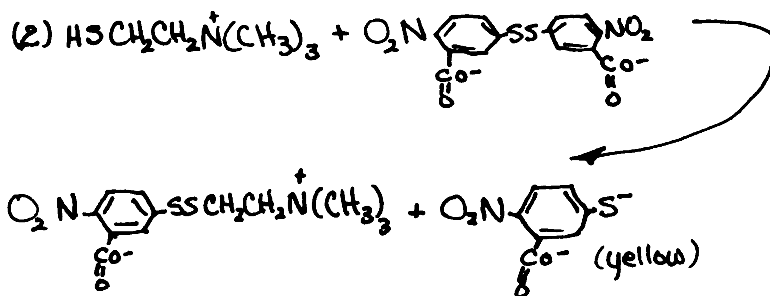
Materials And Methods

Over a 4 month period blood samples were obtained from patients who had been recently admitted to the Langley Porter Neuropsychiatric Institute. A sample was taken at the time of admission and if the RBC AChE was abnormal further samples were taken at approximately weekly intervals. The blood samples were obtained by the clinical laboratory staff and were assayed without knowledge on our part of the specific psychiatric classification of the patients.

The assay of RBC AChE was an adaptation of a method published earlier and can be performed on whole blood without washing the erythro-

cytes (15). It involves the enzymatic cleavage of acetylthiocholine; the thiocholine then reacts with dithiobisnitrobenzoate (DTNB) to produce nitrothiobenzoate, an intensely-yellow anion. The development of this yellow color is recorded as a function of time. The method consists of a coupling of reactions (1) and (2) below and using quinidine sulfate to selectively inhibit the plasma cholinesterase (16).





Technique

20 microliters of blood were hemolyzed in 5.0 ml of water and 5.0 ml of 0.1M pH 8.0 phosphate buffer was added. To this were added 0.2 ml DTNB, made by dissolving 40 mg dithiobisnitrobenzoic acid[1] and 10 mg $NaHCO_3$ in 10.0 ml 0.1M pH 7.0 phosphate buffer, and .05 ml of 8.43×10^{-4} M quinidine sulfate[2]. The tubes were brought to 37 degrees C. in a water bath prior to assay.

[1] Aldrich Chemical Co.
[2] Nutritional Biochemicals Corp.

3.0 ml of this mixture was pipetted into each of two cuvettes. To the sample cell, 20.0 microliters of acetylthiocholine iodide[1] (.074M) was mixed in by blowing through the micro-pipette to agitate the solution. The absorbance at 412 millimicrons was recorded for 4-6 minutes, using a Beckman DK-1 Recording Spectrophotometer. In calculating the activity from the recording of optical density vs. time, a six inch line was drawn from the point where the curve became smooth to another point. (See Figure 4).

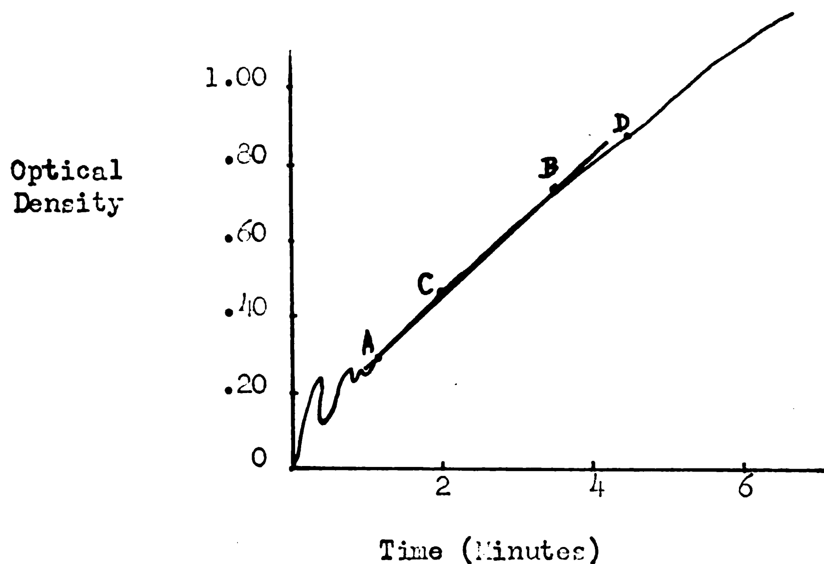


Figure (4) Typical Curve Obtained From An Assay Of RBC AChE According To The Method Described Above

Assays were performed using a chart speed of $2\frac{1}{2}$ inches/minute. Since one might want to assay a sample using a different chart speed and because the curve may assume a regular form at different times from sample to sample, it might have been better to make this line starting

[1] California Biochemical Co.

at a certain time after the addition of substrate and connect it to another definite time. This is important because the optical density plot is not perfectly straight (it is concave downward) and the longer one waits before starting the six inch line, the less the slope will be. Thus a 6 inch line from A to B (figure 4) would have a greater slope than a line drawn from C to D. Once the slope is calculated, the enzyme activity can be determined by using the following formula:

$$\text{Activity} = (0.038)\left(\frac{\Delta A}{H}\right)$$

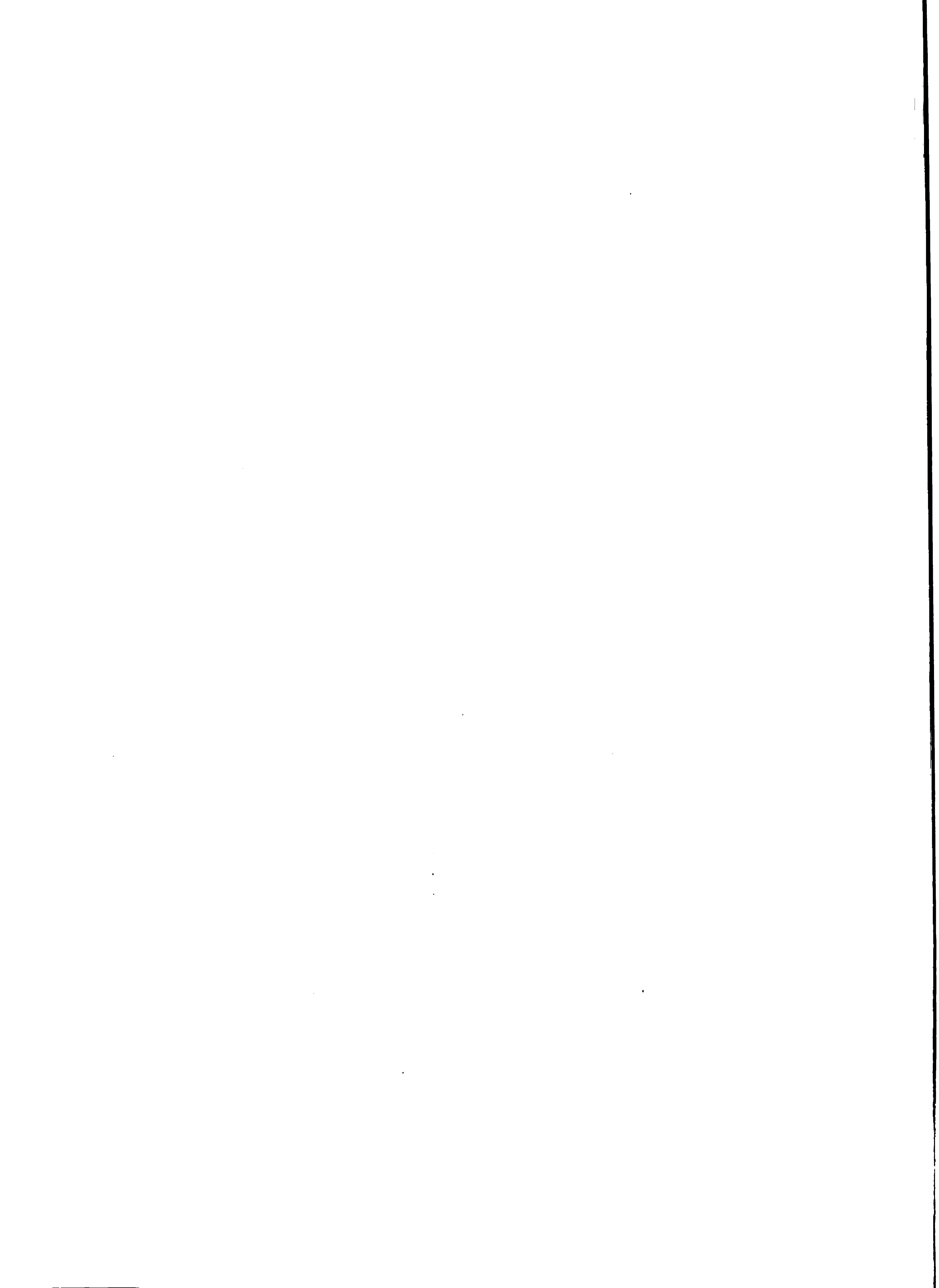
A is the slope of the line expressed as change in optical density per minute and H is the hematocrit of the blood used. In its most complex form the activity is expressed as:

$$\text{Activity} = \frac{\text{dilution factor}}{\text{extinction coefficient}}\left(\frac{\Delta A}{H}\right)$$

The extinction coefficient is 13,600. In order to calculate the dilution factor one must take into account the volumes of all the reagents added.

buffer and water	10.0 ml
blood	0.02 ml
quinidine sulfate	0.05 ml
DTNB	0.20 ml
	10.27 ml

The next step is to take 3.0 ml of the above and add 0.02 ml of acetylthiocholine iodide. This is equivalent to adding 0.02 (10.27/3.0) ml of substrate to 10.27 ml. Therefore the total volume for the dilution calculations would be 10.27+ 0.02 (10.27/3.0) or 10.34. Thus for 0.02 ml of substrate the dilution factor would be 10.34/0.02 or 517 and dividing



517 by the extinction coefficient (13,600) one obtains the factor 0.038.

In an effort to see whether or not the downward curvature as seen in figure (6) was due to oxidation, assays were performed under a reducing atmosphere (nitrogen) and in the presence of reducing agents. Neither was successful in eliminating the activity drop. Using a preparation of whole red cells, ferric ion (0.035M) did not cause any inhibition. Because it was felt that hemoglobin from the hemolyzed cells was the cause of the reduced activity, crystalline hemoglobin was added to the reaction mixture (20.0 microliters of a solution containing 15 gm/100 cc). The results showed clearly that hemoglobin had no demonstrable effect on the activity. (Assays were done on both whole red cells and red cell ghosts). The drop in activity as the reaction proceeds was not due to an insufficient amount of substrate as the same drop was observed when the concentration of substrate was raised to twice the optimal amount.

If one does not hemolyze the red cells there are fluctuations in the optical density record which make it difficult to get similar results on duplicate samples. This is due to the motion of erythrocytes in the cuvette after the substrate has been blown in. One of the first things attempted, in an effort to stabliize the curve, was varying the slit width, thereby changing the amount of solution exposed to light. This was done to average out any local fluctuations in optical density. However the slit width setting seemed to make no significant difference in the results obtained. See Table (2).

Slit Width	Activity (mMoles/L RBCs/Min.)	Patient
0.23 0.23 0.30 0.40	12.9 13.5 12.5 12.3	#1
0.24 0.30 0.40 0.58	23.6 24.7 25.0 24.3	#2

Table (2) Effect Of Slit Width On RBC AChE

RBC AChE values for the mental patients studied covered a range of 13.9± 2.1 (mMoles/l RBCs/min.). If the results from duplicate samples varied by more than one of these activity units the assay was repeated. Thus the percentage variability for the data included in the study was between 6.5 and 8.5%.

The effect of hemolysis on the replicability of runs is shown in Table (3) below:

Patient	Non-hemolyzed Blood Activity (mMoles/L RBCs/Min.)	Patient	Hemolyzed Blood Activity
1	27.8 20.7 24.4 28.3	6	15.1 15.9 15.0 15.7
2	14.0 11.5 11.6 9.0	7	14.6 15.0
		8	14.3 14.8
3	13.6 18.0 20.3 17.5 17.3 13.3	9	20.0 20.6
4	16.5 14.7 10.6	10	18.0 18.3
5	19.1 14.9		

Table (3) The Effect Of Hemolysis On Replicability In RBC AChE Assays

In an effort to improve the consistency of non-hemolyzed blood samples various agents were added to the assay medium to increase the viscosity and thereby reduce the movement of the erythrocytes. In Table (4) one can see the effects of glycerol and methyl cellulose.

% Glycerol	RBC AChE (mMoles/L RBCs/Min.)	Average Activity	Activity Unit Variation
0	22.9 26.1 21.3 22.0	23.1	4.8
10	20.4 25.0	22.7	4.6
20	19.6 18.4	19.0	1.2
30	16.2 16.7	16.5	0.5
% Methyl Cellulose			
0	16.8 14.2 16.5 14.7 10.6	14.6	6.2
10	14.4 15.2	14.8	0.8
20	16.6 17.7	17.1	1.1
30	15.1 16.0	15.5	0.9

Table (4) Effect Of Increasing Viscosity On Non-hemolyzed Blood Samples

One can see from the above table that there is perhaps a tendency for replicate values to become similar at the higher concentrations of glycerol, but there is an inhibitory effect. It can also be seen that, using methylcellulose, one can obtain quite consistent results without any inhibition. However, due to the difficulties encountered in

pipetting these "thickening agents", it was felt that it would be better to work with hemolyzed erythrocytes.

Since the non-specific cholinesterase of plasma will also hydrolyze acetylthiocholine it is necessary to inhibit it with quinidine sulfate. The concentration of quinidine sulfate used causes nearly complete inhibition of the plasma cholinesterase while having a minimal effect on the red cell enzyme (figure 5). Quinidine sulfate is a reversible inhibitor and the concentration required for 50% inhibition is comparable to that for physostigmine. To determine whether or not the amount of quinidine sulfate used was sufficient to effectively inhibit the plasma cholinesterase the amount of quinidine sulfate was varied and the resulting cholinesterase activity was plotted as shown in figure (6). Although apparently enough quinidine sulfate was used to inhibit the plasma cholinesterase, it might have been better to have used a slightly higher concentration to allow a greater margin of safety.

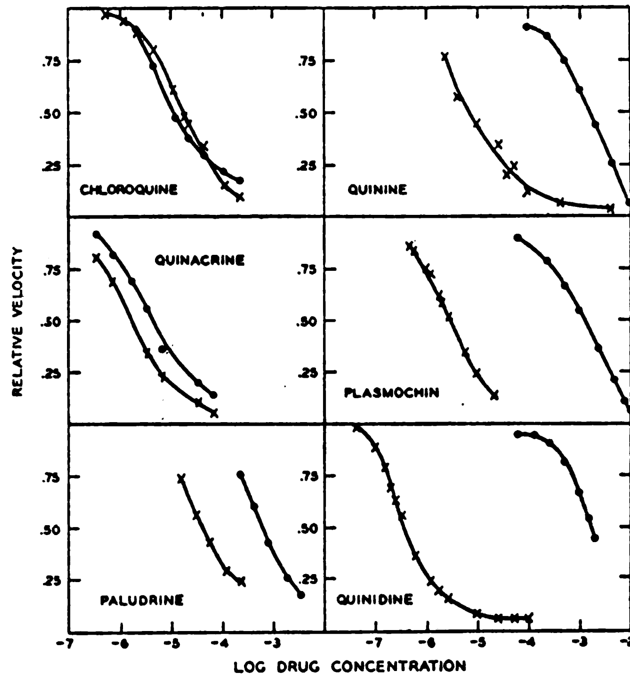
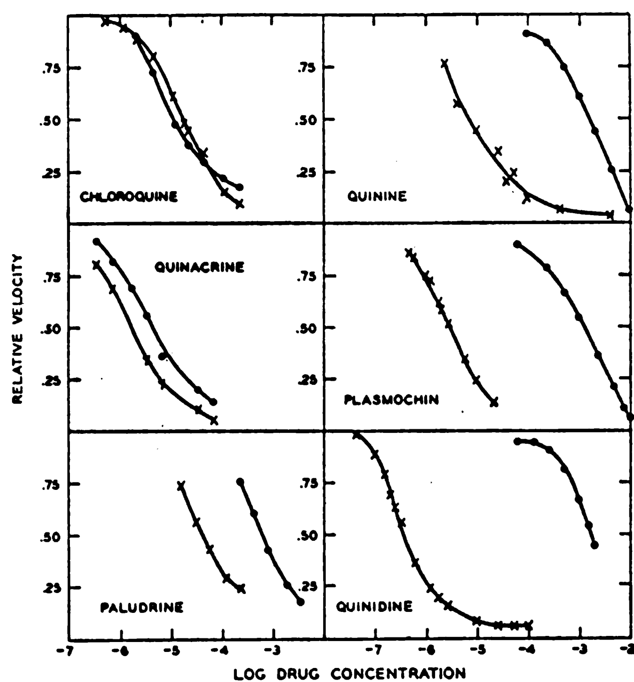


FIG. 5. Relative velocity of hydrolysis of 0.003 M *ACh* by plasma (x) and erythrocytes (●) in the presence of varying drug concentrations.

Figure (5) Wright, C.I., Sabine, J.C. (16)



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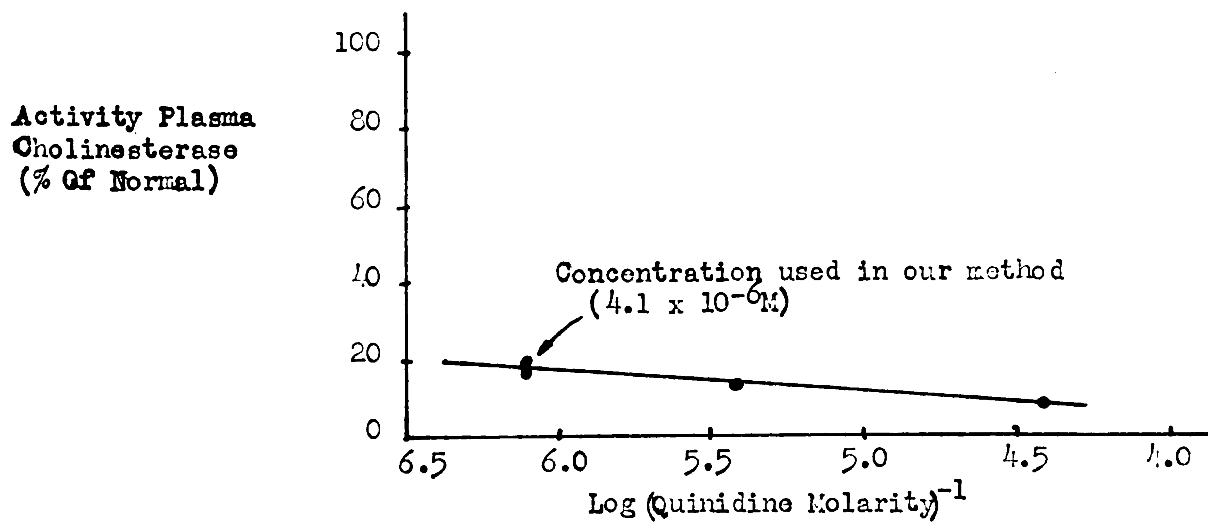

Figure (6) Inhibition Of Plasma Cholinesterase With Quinidine Sulfate

Results

In our present study we observed that no significant difference exists between the mean RBC AChE activities of mental patients and normal controls or between the standard deviations of the two groups. (See table 5.)

	Number	Mean RBC AChE (mM/L RBCs/Min.)	Standard Deviation
Controls	40	13.6	±0.23
Patients	144	13.9	±0.21

Table (5) RBC AChE In Mental Patients And Controls

In our present study we measured RBC AChE activity in 144 different mental patients. Of these 94.4% fell within the normal range. One would expect 95% of the values in a given population to fall within two standard deviations of the mean value. We can conclude that we would have found the same number of high cholinesterase levels in a normal population of similar size. In figure (7) one can see the similarity in RBC AChE levels between normals and mental patients.

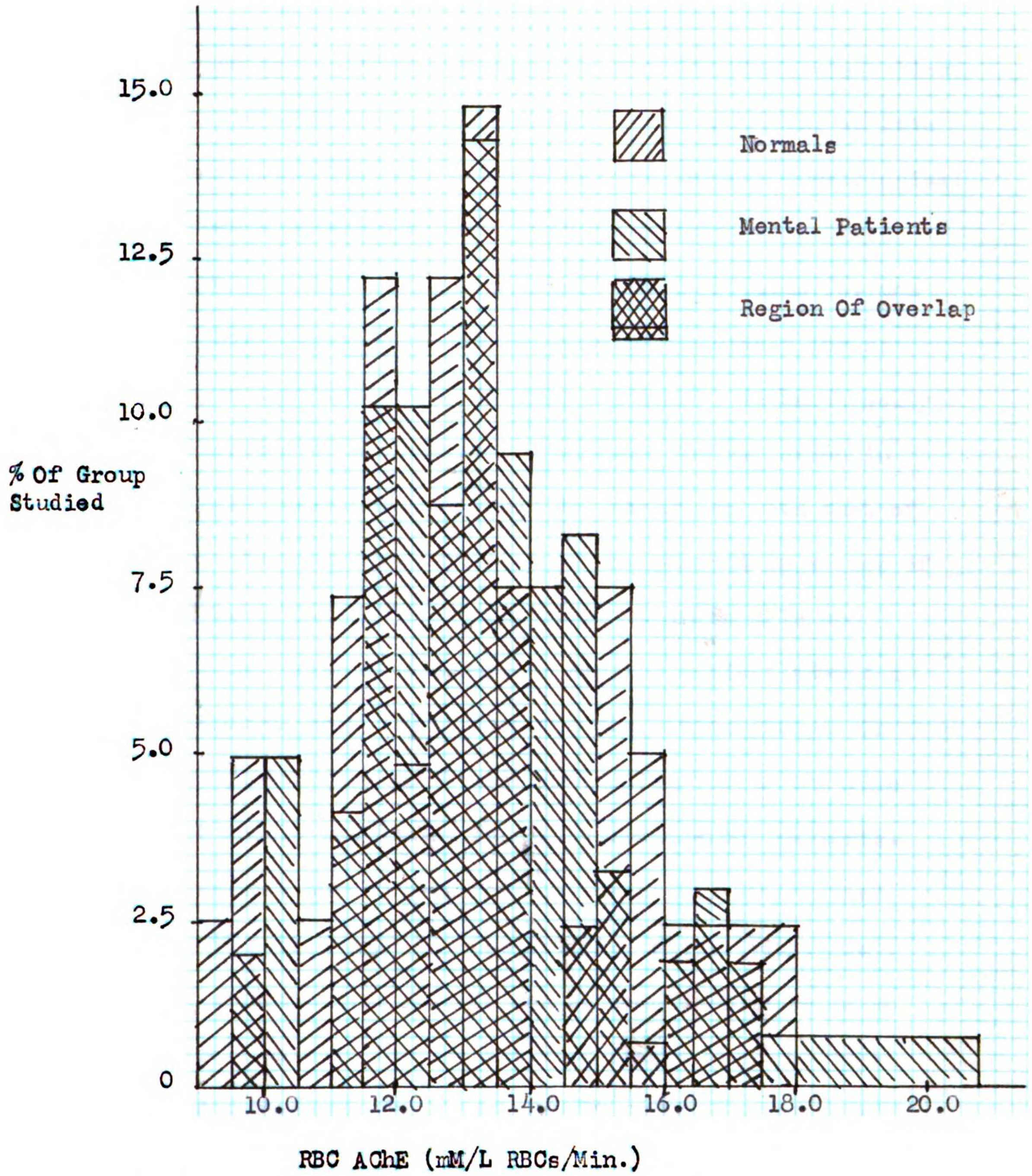

Figure (7) Distribution Of RBC AChE In Mental Patients And Normals

We have examined in greater detail those individuals whose blood samples had an elevated cholinesterase level. In addition, we have attempted to correlate the AChE activities with various hematological parameters. When a person was found with a cholinesterase level greater than two standard deviations beyond the mean value for the control group, his enzyme level was followed at intervals for as long as he was in the Institute. In some instances, we have weekly values for patients over a three-month period. The variation in levels of a single individual was small in comparison with that of the group; e.g., the standard deviation of one patient (a series of 9 values) was ± 0.13, i.e., 55% of that observed in normals and other patients. A series of 6 assays on another patient showed a standard deviation of only 31% of that for the entire group. In figure (8) are plotted cholinesterase activities versus the time after the first sample was drawn, for individual mental patients. We felt if there were any correlation between a patient's mental state and his RBC AChE level that the activity should either fall or rise as his condition varied. It can be seen that there is no consistent overall pattern for the activity change and that most patients showed activities which were fairly constant over a 2-4 week interval. An attempt to correlate the shape of the curve with the patient's diagnosis gave negative results.

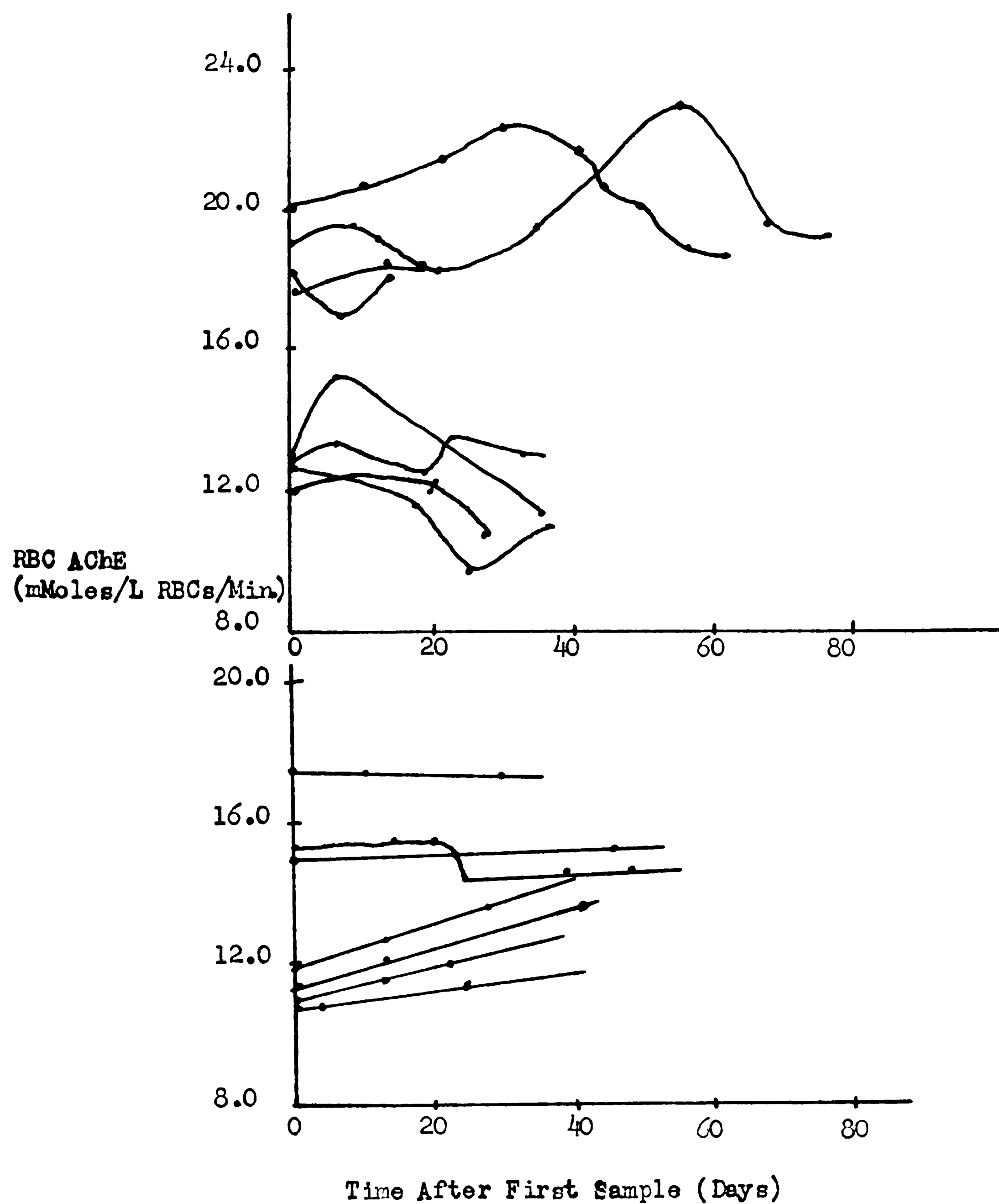

Figure (8)

RBC AChE Variation In Individual Mental Patients

We have divided our data into two patient groups: schizophrenic and non-schizophrenic. The former were further sub-divided into paranoid and non-paranoid, and whether of the process or reactive type. Of the schizophrenic patients, only in the paranoid-process and non-paranoid process groups were there sufficient individuals to warrant further examination of the data. Here, the mean activities were, respectively, 13.3 and 13.2 mMoles/l RBCs/min. For both groups the standard deviations were ±2.2. This is not significantly different from the means or standard deviations of the normals or of the patient group as a whole.

When the patient population which had high cholinesterase levels was examined we found that, with one exception, they had other blood abnormalities. For example, 4 of the 8 patients with high enzyme activities showed low hemoglobin levels (table 6), whereas in a group of 25 mental patients, with RBC AChE activities very close to the mean of the control group, no abnormal hemoglobin levels were found. No significant differences in the incidence of elevated sedimentation rates, white blood cell or reticulocyte counts were observed. A patient's hematological state was regarded as abnormal if it fell outside the ranges listed in table (7).

		RBC AChE			
		Activity Above Normal Range (8)		Normal Activity (25)	
		Number	%	Number	%
Hemoglobin	Below Normal	4	50	0	0
	Above Normal	0	0	1	4
Hematocrit	Below Normal	2	25	0	0
	Above Normal	0	0	0	0
Sedimentation Rate	Above Normal	5	63	13	52
White Blood Cell Count	Below Normal	0	0	0	0
	Above Normal	3	37	7	28

Table (6) Relation Between Hematological Findings And Red Cell Cholinesterase Activity In Mental Patients
(Clinical work was done by Langley Porter Neuropsychiatric Institute Clinical Laboratory)

	Normal Range	
	Male	Female
Hemoglobin	14-18 grams/100cc	12-16 grams/100cc
Hematocrit	40-54 volume %	37-47 volume %
Sedimentation Rate	0-6.5 mm/hr.	0-15 mm/hr.
White Blood Cell Count	5-10,000/mm^3	same
Reticulocyte Count	0.5-1.5%	same

Table (7) Normal Ranges For Certain Hematological Parameters
Pfizer Laboratories (19)

Discussion

Rubin (9) and Gal (4) have brought forth evidence which they feel may indicate a relationship between mental illness and RBC AChE activity. In carrying out a similar study we have assayed blood samples from over 200 patients and over 150 non-patients. In both series of studies, no statistically-valid differences were obtained.

A possible explanation for the difference between our data and that of Gal (14) may depend on the type of patient population found in our respective institutions. The principal difference might be in the relative number of chronic schizophrenic individuals involved. Sabine has found that when a hemolytic tendency appeared to be the primary disorder of a patient his RBC AChE was high when the disease was active and normal when it was in remission (17). Thus, one might find indication of erythrocyte abnormalities in individuals with high cholinesterase levels. On the basis of the limited data available it seems that low hemoglobin levels and low hematocrits might be associated with elevated enzyme activity. The incidence of patients with high cholinesterase levels in this Institute is so low, however, that a direct test of this hypothesis would have to extend over a rather long period of time. Thus, if there is any significant correlation between a particular type of mental illness and the RBC AChE level it may be at the level of the synthesis and/or release of newly-formed cells. Nevertheless there may be other factors involved because following irreversible inhibition the restoration of activity can not be accounted for solely on the basis of formation of new erythrocytes. For example, Allison and Burn calculated that the RBC AChE of reticulocytes was three times the activity in the erythrocyte population of circulating

normal blood (18). During a reticulocytosis they found a rise in the RBC AChE and that the higher the proportion of reticulocytes, the higher the rise. They felt that the results of their experiments were consistent with the interpretation that there is little or no synthesis of enzymes in the mature erythrocyte and that there is a continuous decrease in the enzyme activity as the cell grows older. However we did not find any elevation of reticulocyte count in the patients who had the highest AChE activity. Elevated RBC AChE accompanying a low reticulocyte count may be explained by the persistence of a high concentration of enzyme on the erythrocyte after the stainable reticulum has disappeared. Consequently, one m ght expect that the RBC AChE activity might be a more sensitive index of erythropoiesis than the reticulocyte count, as proposed by Sabine (17).

Summary

Blood samples were obtained from mental patients and assayed for erythrocyte acetylcholinesterase activity (RBC AChE), using a modification of the Ellman method (15). This assay consists of measuring the yellow anion produced when thiocholine reacts with dithiobisnitrobenzoate. The time course of the development of the yellow color is followed on a recording spectrophotometer. The thiocholine is produced by enzymatic cleavage of acetylthiocholine. In order to differentiate between red cell and plasma cholinesterase, the latter is selectively inhibited with quinidine sulfate (16).

It is hard to obtain reliable activity measurements using unhemolyzed erythrocytes because of fluctuations in the optical density record. The fluctuations were not reduced by variation of the slit width or by the

addition of glycerol as a "thickening agent", the latter inhibiting the reaction. Increasing the viscosity of the reaction mixture with methyl cellulose seemed to reduce the fluctuations but it was felt that it was easier to use hemolyzed red cells as a means of improving the reliability of the assay.

Evidence from the literature was presented which suggested that the activity of RBC AChE was in some way related to the activity of the AChE of the central nervous system. Other workers have also shown that the two enzymes are related in exhibiting a similar sensitivity to inhibitors, substrate specificity and kinetic behavior. Circumstantial evidence was also brought forth by a number of investigators which implicated the acetylcholine-AChE system as being involved in certain abnormal mental states.

The present study was an effort to determine whether or not there is a relationship between RBC AChE and mental illness. Previously Gal had reported that such a relationship existed (14). Using a much larger sample of patients we have confirmed our previous work by showing that there is no significant difference in RBC AChE levels between mental patients and normal controls. It was found that both the mean activity and the standard deviation was the same in both groups. When the schizophrenic group was divided into paranoid-process and non-paranoid process groups, again no difference between the enzyme levels was found. In following the RBC AChE of individual mental patients no consistent change in the pattern of activity with time was observed and the activities of most patients were fairly constant, or changed in a linear fashion with time.

An interesting finding was the fact that there is a rather high incidence of hematological disorders in the mental patients showing elevated AChE levels. Of disorders in this category, low hemoglobin levels and hematocrits were the most common, suggesting that the increased rate of formation of young erythrocytes in such patients may be the cause of their high enzyme activity. If this is the case, the formation of young erythrocytes might be followed more sensitively by measurement of RBC AChE rather than by the traditional reticulocyte count (16).

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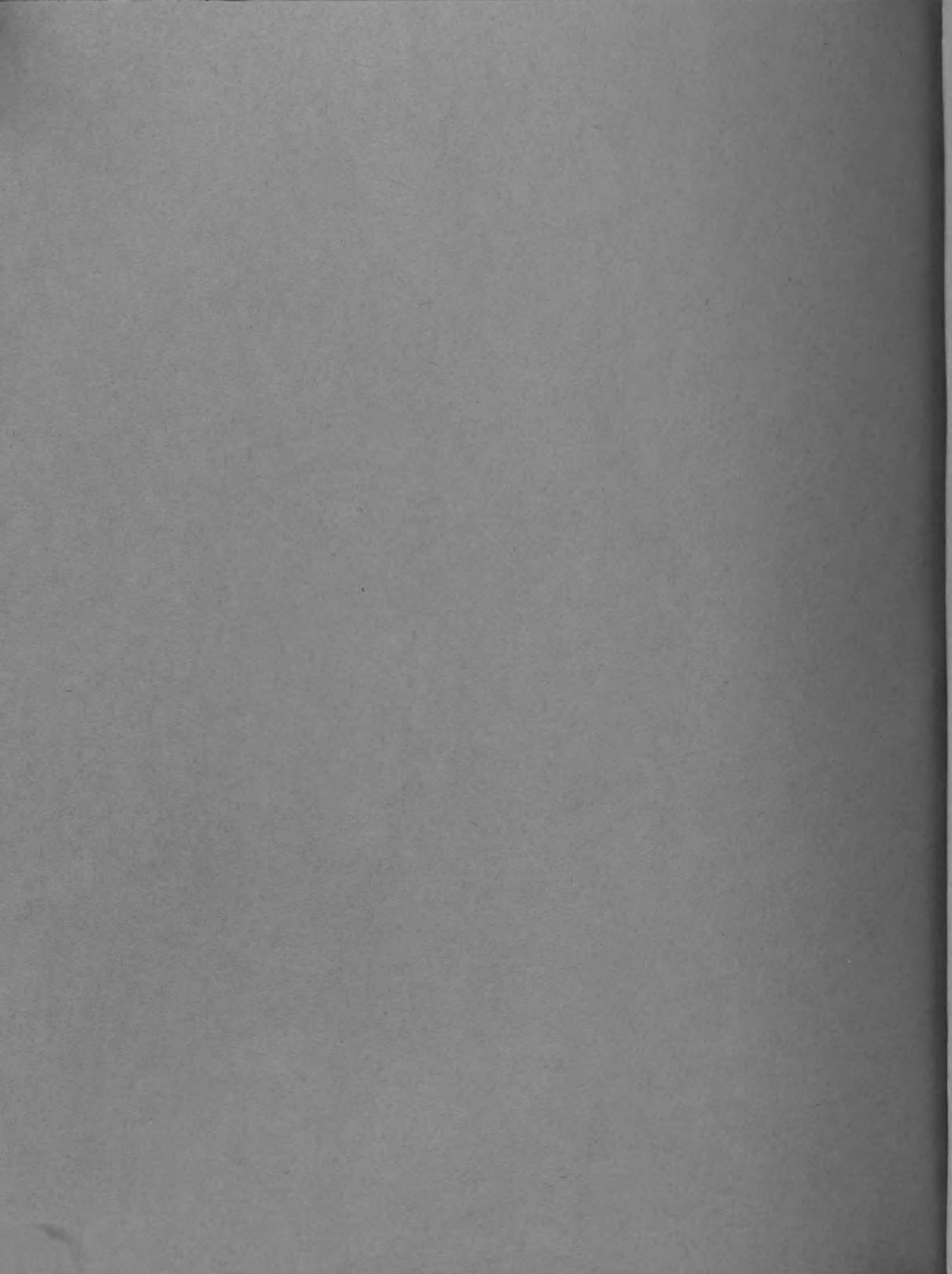

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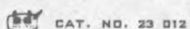
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