STUDIES ON THE STORAGE STABILITY OF HUMAN BLOOD CHOLINESTERASES: I

Charles R. Crane, Ph.D.
Donald C. Sanders, M.S.
John K. Abbott, B.A.

Approved by

J. Robert Dille, M.D.
Chief, Civil Aeromedical Institute

Released by

P. V. Siegel, M.D.
Federal Air Surgeon

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I. Introduction.

The widespread use of organophosphate pesticides by aerial and surface applicators has necessitated the development of methods for assessing the degree of exposure to these toxic chemicals. The principal biochemical action of this class of compounds is the inhibition of hydrolytic enzymes, such as the cholinesterases; therefore, the measurement of blood cholinesterase activity has become the standard clinical method for evaluating exposure. Although many techniques are used for assaying blood cholinesterase, the method of titration at constant pH (pH-Stat) is one of the most precise[1 2 3].

It has been recommended that aerial applicators have periodic cholinesterase determinations during the spraying season, and particularly if a known spill occurs, but the scarcity of clinical laboratories equipped to perform this test often dictates shipping blood samples elsewhere for this analysis. Changes in cholinesterase activity which occur during such shipments, or during storage, are of some concern but have received little consideration. The interpretation of cholinesterase values in blood stored or shipped under unknown conditions is questionable because subnormal values may be due to either pesticide poisoning or enzyme denaturation.

The present study resulted from two problems which had confronted our laboratory periodically: (1) Receipt of blood samples shipped by a physician who was unable to obtain cholinesterase assays in his locale, and (2) the receipt of more samples (freshly-drawn or not) than could be assayed in one day, requiring their storage overnight, over a weekend, or occasionally for longer periods. Most storage studies have dealt with changes in activity over periods of a few hours to a few days[4 5 6 7 8], and many of the earlier studies used less precise methodology than is currently available[9 10 11]. We have, therefore, examined the changes in cholinesterase activity over a longer period of time and under various storage conditions.

II. Materials and Methods.

Reagents:

1. Acetylcholine perchlorate (K and K Laboratories, Plainview, N.Y.) was dried overnight at 110° C. and stored in a desiccator over Drierite. Aqueous stock solution was 0.102M (25 mg./ml.) prepared fresh weekly and stored at 4° C.

2. Butyrylcholine iodide (Mann Research Laboratories, New York, N.Y.) was dried overnight at 110° C. and stored in a desiccator over Drierite. Aqueous stock solution was 0.163M (49 mg./ml.), prepared fresh weekly and stored at 4° C.

3. Triton X–100 (Rohm and Haas, Philadelphia, Penna.); a 20% (v/v) aqueous solution was prepared and stored at room temperature.

4. Sodium hydroxide, standard solution, 0.005N. Aqueous stock solution of approximate normality was prepared using CO_2-free distilled water. Solution was stored in titrant reservoir of pH-Stat and protected from CO_2 absorption by a drying tube filled with Mallcosorb, 30–50 mesh, indicating, CO_2 absorbent (Mallinckrodt). This solution was standardized daily by titration with potassium hydrogen phthalate (primary standard grade).

5. Ringers salts solution: 0.9 g. NaCl, 0.03 g. KCl, 0.025 g. $CaCl_2$, 0.02 g. $NaHCO_3$ per 100 ml. distilled water.

Titration equipment:

Radiometer pH-Stat (Radiometer A/S; Copenhagen, N.V., Denmark) was used for all

constant-pH titrations, and consisted of the following units:

PHM26—expanded scale pH meter.

TTT11b—electronic titration control unit.

ABU1b—semi-micro automatic buret for titrant delivery, 2.5 ml. capacity.

TTA3—glass-jacketed reaction vessel (3 ml.) with plastic-coated magnetic stirring disc.

VTS13c—constant-temperature water bath and circulator, set to maintain 25° C. in reaction vessel.

SBR2c—recorder for indicating volume of titrant delivered per unit time.

Blood collection:

Blood for studies lasting more than 7 days was secured by advance arrangement with a local blood bank. Processing began within 2 hours after collection in heparinized, 500 ml. vacuum bottles. Blood for short-term studies was drawn from laboratory personnel into heparinized *Vacutainers* (Becton, Dickinson & Co., Rutherford, N.J.) and processed immediately.

Preparation of storage samples:

Fresh, whole blood, with sufficient solid heparin added to prevent clotting over the planned storage period, was added to a volumetric flask five times the size of the blood aliquot. To each flask was added 20% Triton X–100 in the ratio of 0.05 ml. per ml. whole blood. The contents were mixed thoroughly, allowed to stand for 3 to 5 minutes and diluted to volume with Ringers salts solution. Duplicate 1–ml. samples of this whole blood hemolysate were assayed immediately to determine the "zero-day" activity. The remainder of the hemolysate was divided into three parts and each stored at a different temperature, namely, room temperature (23° – 24° C.), refrigerator temperature (4° C.) and freezer temperature (−20° C.).

Plasma samples were collected from fresh, heparinized, whole blood after centrifugation at 800 X *g* for 10 minutes and were diluted 1:10 with Ringers salts solution. This diluted plasma was divided for immediate assay and for storage at the three temperatures designated for whole blood.

Red blood cells were collected from fresh, heparinized whole blood by centrifuging at 800 X *g* for 10 minutes, carefully removing the plasma with a Pasteur pipet, resuspending the cells in Ringers salts solution and recentrifuging. This washing was repeated three times. Triton X–100, 20%, (0.05 ml. per ml. whole blood) was added to the washed cells and mixed by gently touching to a rotary mixer (*SUPER-MIXER*, Matheson Sci.). The resulting hemolysate was quantitatively transferred to a volumetric flask five times the volume of the whole blood sample and diluted to volume with Ringers salts solution. Aliquots of the diluted hemolysate were prepared for immediate assay and for storage at the three temperatures designated for whole blood.*

Assay Procedure:

One ml. of the enzyme solution (whole blood hemolysate, diluted plasma or RBC hemolysate) was added to the reaction vessel, the magnetic stirrer was turned on and the solution was allowed to come to temperature equilibrium (25° C.). The pH was adjusted to 8.10 by addition of dilute aqueous NaOH from a Pasteur pipet for whole blood or RBC, for plasma the adjustment was made with the automatic titrator. The appropriate type and volume of substrate (see below) was added and the titrator and recorder chart drive turned on.

The reaction was allowed to continue until the recorder had plotted a straight line (titrant volume *vs* time) for a minimum of 3 minutes or until a titrant volume of 0.5 ml. had been added.**

Substrates:

For whole blood and RBC assays, 100 microliters of acetylcholine perchlorate solution (0.102M) was added with a 250 microliter Ham-

* The activity of 1 ml. of this hemolysate multiplied by a dilution factor of five gives the activity of the volume of red blood cells contained in 2 ml. of whole blood. Multiplication of this value by 1/hematocrit yields the activity of exactly 1 ml. of packed red blood cells. This technique was used in order to avoid the direct measurement and quantitative transfer of packed red blood cells.

** Since full scale on the recorder (100 scale divisions) represents 2.5 ml. of titrant, 0.5 ml. represents 20 scale divisions. The recorder trace can be read to ±0.2 scale division; therefore, the precision in reading titrant volume is ±1%. With a chart speed of 2 cm. per min., the precision in reading elapsed time of 3-min. trace is also ±1%.

ilton syringe. For plasma assays, 0.50 ml. of butyrylcholine iodide solution (0.163M) was added with a long-tipped glass pipet. These quantities of substrate were chosen to give a concentration in the optimum activity range and still furnish adequate substrate to yield zero-order kinetics for at least 3 minutes.

Calculations:

All activities were expressed as micromoles of substrate hydrolyzed/minute/ml. of sample. Calculations were made as follows:

Activity = (S.D./min.) (0.025) (N) (1,000) (D.F.)

where

S.D. = recorder scale divisions (titrant volume axis).

min. = minutes corresponding to S.D. on "time" axis.

0.025 = ml. of titrant per S.D.

N = normality of titrant.

1,000 = μmoles/mmole

D.F. = dilution factor.

D.F. for whole blood is 5.

D.F. for plasma is 10.

D.F. for RBC assay is 5/Hct

where

Hct is the fractional hematocrit value of the whole blood.

III. Results and Discussion.

The results of the storage studies are presented in Figures 1–3. Each point on the graphs represents the mean of 1 to 10 samples assayed in duplicate, with the vertical bars representing ±one standard deviation.

Whole blood:

Whole blood hemolysates maintained 90% of their original cholinesterase activity for about 6 days when stored at room temperature. When stored either refrigerated or frozen, they maintained at least 90% activity for 50 days.

Those whole blood hemolysates which were stored frozen occasionally appeared to form a stringy, gelatinous precipitate on subsequent thawing. The presence of this precipitate made it difficult to pipet homogeneous samples.

An interesting and unexplained aspect of the storage of whole blood was the apparent increase in activity during the first four days, regardless of storage temperature, a phenomenon which has been noted previously[5 6 12]. Although the average increase in activity was only 6–8%, with some individual samples it was as high as 14%. This increased activity could be the result of any of several processes.

The erythrocyte enzyme is known to be bound to the cell membrane. To our knowledge, no determination has been made of the relative specific activity of the enzyme in the bound and solubilized forms, nor of the rate of enzyme solubilization under different conditions. If the soluble form of the enzyme has a faster reaction rate (a larger V_{max}) than the bound form, which would not be illogical, then the observed increase in activity could be the result of enzyme solubilization.

Other possibilities include an activation of the enzyme due to conformational changes produced by some aspect of the treatment. Also, it has been established that both the erythrocyte and plasma cholinesterase molecules are composed of dissociable sub-units[13 14] which are capable of reaggregating into multiple molecular forms (isozymes). The exact sub-unit composition of these reaggregates has been shown to be influenced by pH, ionic strength, temperature, and enzyme concentration[13 15]. It is not inconceivable that the treatment and storage conditions in this study might have favored formation of an isozyme population having an increased reaction rate.

Plasma:

Cholinesterase activity in the plasma samples exhibited a remarkable stability when either refrigerated or frozen; activity remained above 90% for the entire 50-day storage period. At room temperature, the activity appeared to be stable for 6 days and then decreased steadily to about 40% after 3 weeks. There was no difference in the activity of plasma samples stored undiluted or diluted 1 : 10 with Ringers; therefore, both values are included in the data.

No difficulty was experienced with the formation of fibrin strands or any other precipitates in the stored samples which had been adequately heparinized.

Red blood cells:

These hemolysates exhibited a very rapid loss of cholinesterase activity when stored at room temperature; the activity decreased to about 80% in the first 24 hours. Refrigeration of the

hemolysates allowed storage for 6 days before the 80% level was reached, and freezing maintained the activity above 90% for 50 days. As was observed with whole blood preparations, freezing of the RBC hemolysates produced a gelatinous substance which sometimes interfered with sampling.

Only in the case of frozen RBC hemolysates was there evidence for an increased activity in the first few days of storage. This is interesting when compared with the behavior of whole blood samples, since most of the measured activity in whole blood is due to the erythrocyte enzyme when acetylcholine is the substrate. Another related anomaly is the observed difference in stability of the RBC and whole blood activities when storage is at room temperature. Once again, since the same enzyme is being measured in both cases, the obvious difference in stability is hard to explain. A possible factor is the presence of stabilizing elements in the plasma portion of whole blood which decrease the rate of denaturation of solubilized erythrocyte enzyme.

Assay of samples prepared from stored whole blood:

We have obtained preliminary information on the storage stability of untreated whole blood. Two blood specimens were stored without treatment at each of the experimental temperatures. At intervals during the storage period, RBC and whole blood hemolysates were prepared from these stored samples and assayed in duplicate. After the first few days of storage, it became difficult to obtain homogeneous whole blood hemolysates due to red cell agglutination and formation of a stringy sediment. It was also difficult to prepare a red cell hemolysate as an accurate aliquot of the whole blood because of this agglutination and because of the unknown relationship between enzyme activity and hematocrit in an appreciably hemolyzed sample. However, the data indicate that values obtained during the first week of storage were at least as high as for samples which were stored as hemolysates. We, therefore, believe that useful clinical values can be expected if the assays are performed within the time limits discussed for stored hemolysates, namely: for RBC's, 12 hours at room temperature or 7 days refrigerated or frozen; for whole blood, 7 days at any of the three temperatures.

IV. CONCLUSIONS

From a clinical viewpoint, we consider assay values to be useful as long as we can expect them to represent at least 90% of the original activity. Therefore, we have selected this value of 90% as a lower limit and store samples under conditions which will insure at least this level of activity at the time of assay.

These conditions allow storage of red cell hemolysates for less than 12 hours at room temperature, up to three days at 4° C., and up to 6 weeks at −20° C. Whole blood hemolysates and plasma may be stored for 6 days at room temperature and in excess of 6 weeks if refrigerated or frozen.

We recommend that samples for shipment be drawn into heparinized tubes, adequately mixed, and refrigerated until shipment. Shipment should be made in an insulated container containing sufficient ice to last during transit. If the samples are to be shipped frozen, one-half of each whole blood sample should first be separated into plasma and red cells, and these three preparations placed in separate containers; otherwise the hemolysis resulting from freezing whole blood prevents a separate assay on plasma and renders the accurate measurement and assay of red cells more difficult.

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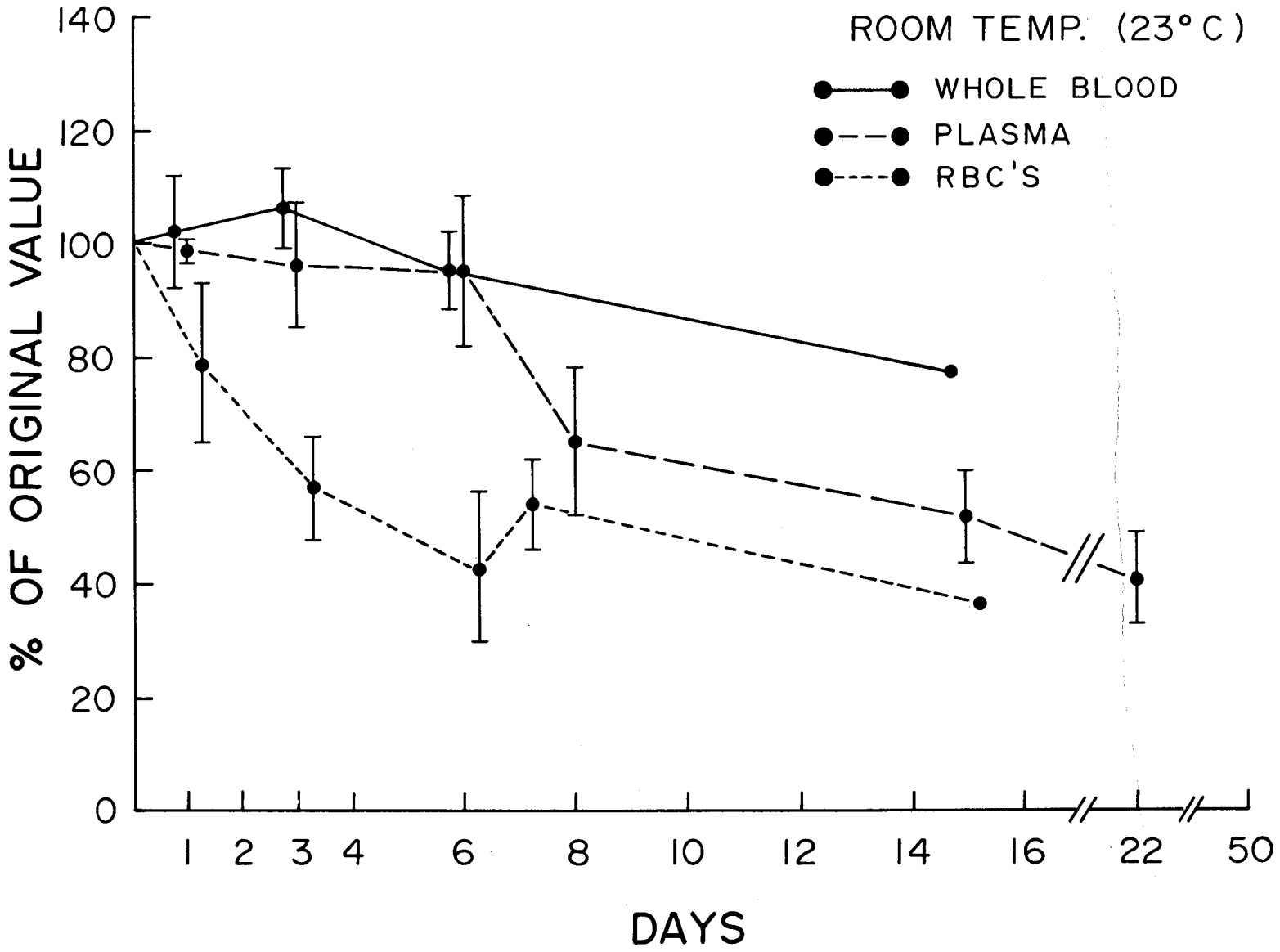

FIGURE 1. Cholinesterase activity as a function of storage time (days) at +23° C.; activity is expressed as percentage of original (day zero) activity.

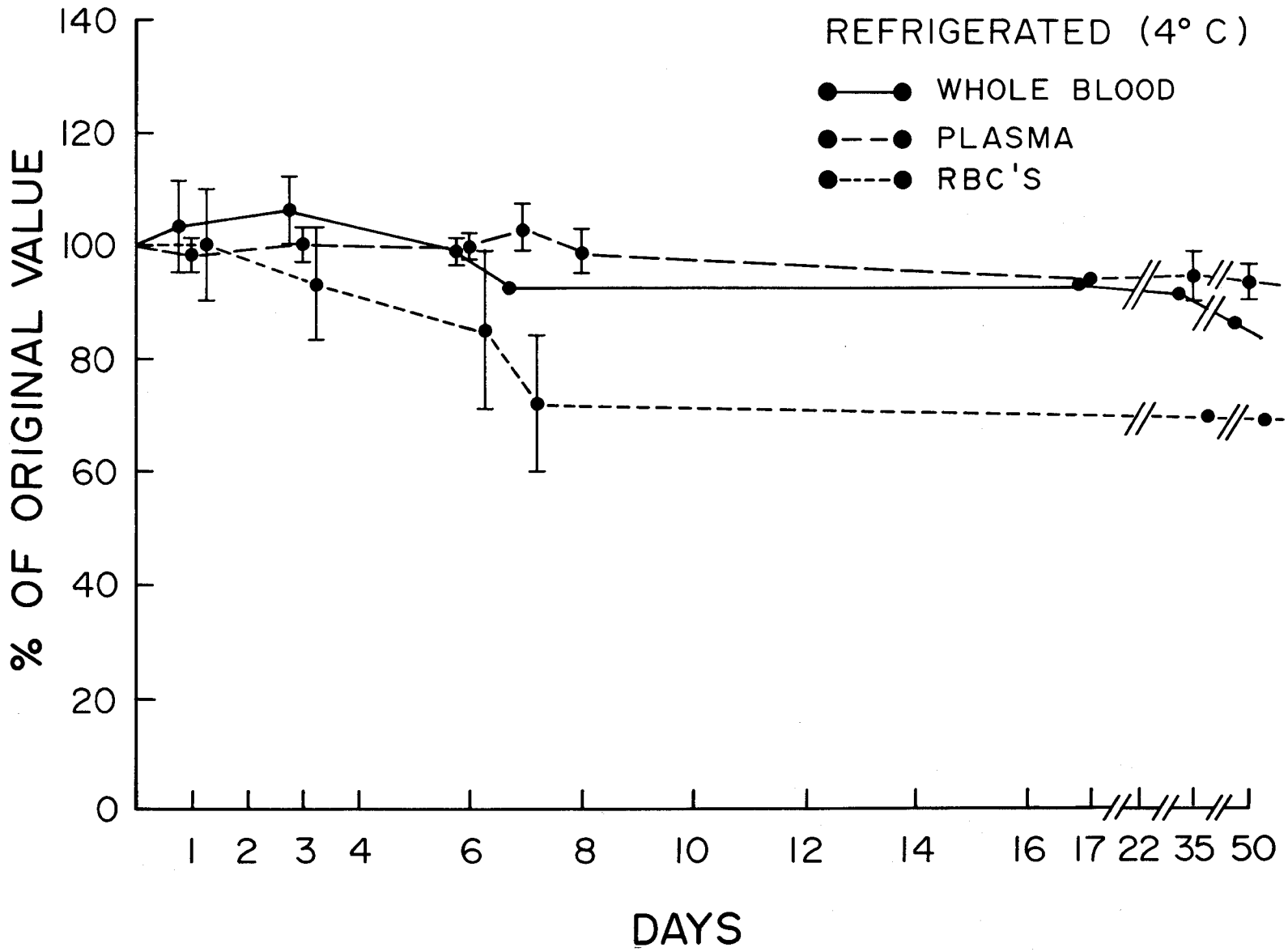

FIGURE 2. Cholinesterase activity as a function of storage time (days) at +4° C.; activity is expressed as percentage of original (day zero) activity.

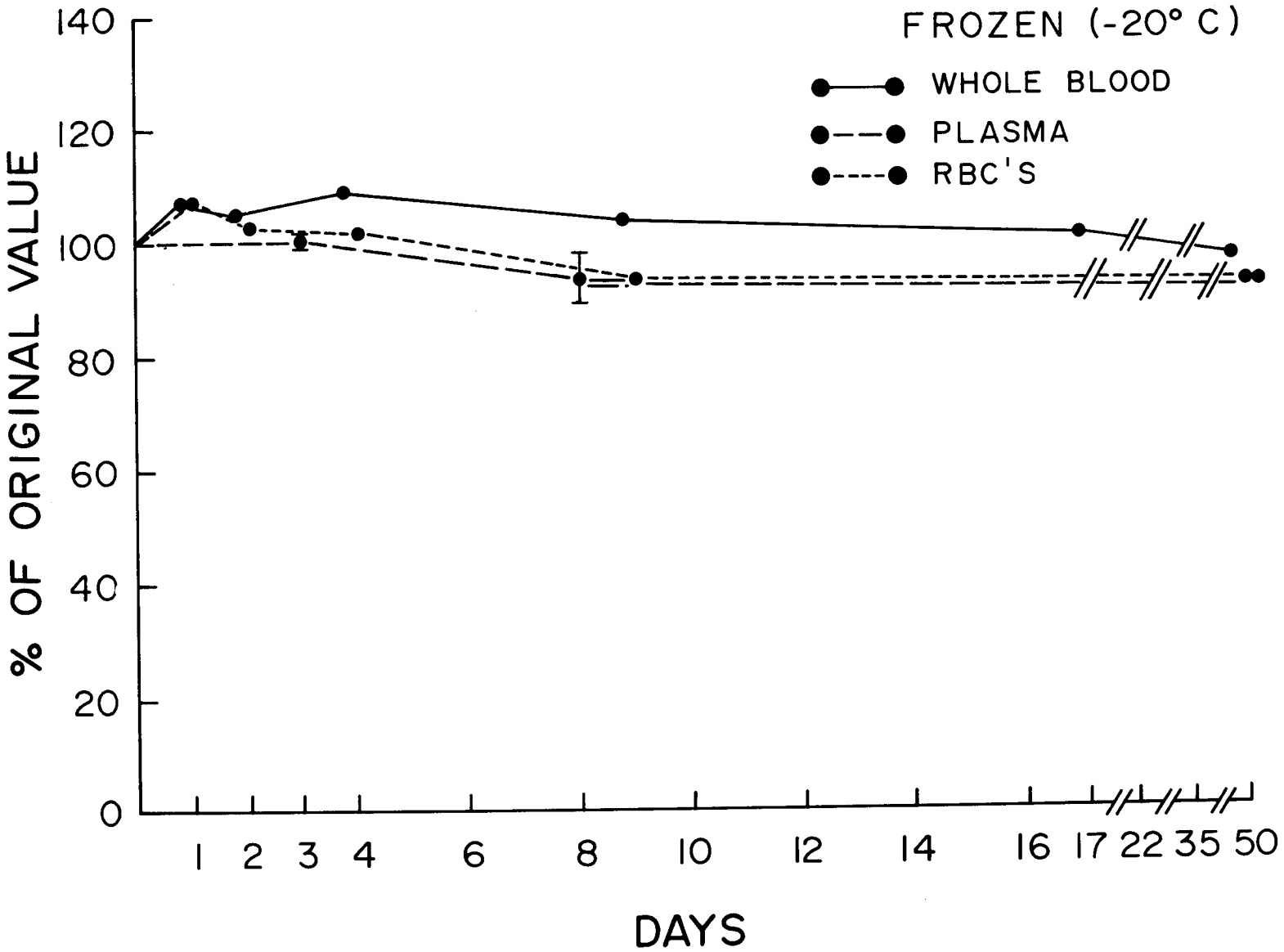

FIGURE 3. Cholinesterase activity as a function of storage time (days) at −20° C.; activity is expressed as percentage of original (day zero) activity.