Biochimica et Biophysica Acta, 448 (1976) 314–324

BBA 77453

A NEW ASSAY FOR ENDOCYTOSIS IN ERYTHROCYTE GHOSTS BASED ON LOSS OF ACETYLCHOLINESTERASE ACTIVITY

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(Received April 2nd, 1976)

SUMMARY

A new method for assaying endocytosis in erythrocyte ghosts is presented. The method involves measuring the percentage loss of acetylcholinesterase activity which occurs when vacuoles form, making the acetylcholinesterase on the vacuole surface inaccessible. This method is compared to other methods of measuring endocytosis in this system, including phase contrast microscope estimation of vesiculation, stereological analysis of electron micrographs to determine vesiculation and loss of sialic acid accessible to neuraminidase due to endocytosis. Comparison of the percentage loss of acetylcholinesterase activity with the electron micrographic and sialic acid methods showed that all three methods gave a quantitative measure of the percentage of total membrane area taken in as vesicles. Since the acetylcholinesterase method was fast, easy, inexpensive, and quantitative, it was the preferred method for assay of endocytosis. The inhibition of endocytosis by Ca^{2+} was observed with this method: the success of this experiment demonstrated the applicability of the method to the study of inhibitors of endocytosis.

INTRODUCTION

Endocytosis, encompassing the processes of phagocytosis and pinocytosis, refers to vacuole formation by invagination of the outer membrane [1]. These processes occur in essentially all cells, but the red cell ghost provides an unusually simple system for the study of endocytosis.

Non-energy-dependent endocytosis in erythrocyte ghosts can be induced by the combination of low ionic strength and drugs such as primaquine [2, 3] or by detergents [4]. At higher ionic strengths, endocytosis requires an input of energy generated by Mg^{2+}-ATP hydrolysis [5]. Further study of endocytosis has been hampered by lack of an easy, inexpensive and quantitative assay.

Abbreviations: TES, *N*-Tris-(hydroxymethyl)methyl-2-aminoethane-sulfonic acid; Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid).

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Many assays are available for endocytosis in red cell ghosts. Observation of ghosts under a phase contrast microscope allows one to estimate the percentage of ghosts which have vesicles inside [6]. A stereological method, which requires measurements on a large number of electron micrographs, is available for quantitating the percentage of total membrane as vesicles [7]. In resealed ghosts, a method involving radio-labeled cobalamine has been used [8].

However, these assays all have drawbacks which make them inadequate for systematic studies of endocytosis. The phase contrast microscope estimates are accurate to within only ± 10 % [6]. The stereological method involves lengthy analysis of large numbers of electron micrographs and, like other electron microscope techniques, is expensive, time consuming, and cumbersome. The cobalamine method is expensive, difficult to carry out and not applicable to unresealed ghosts.

In order to obtain a more useful assay for endocytosis, we have utilized the acetylcholinesterase (Acetylcholine hydrolase; EC 3.1.1.7) of the red cell membrane. Acetylcholinesterase activity can be measured rapidly and reproducibly by the method of Ellmann et al. [9]. This enzyme is located on the outside of erythrocytes and has been used as a surface marker [10]. Invagination of the outer membrane to form vesicles would make these vesicles inside out and acetylcholinesterase would then have its active center on the inner surface of the vesicle membrane. If either the outer or vesicle membrane acts as a barrier between acetylcholinesterase and its substrate, a loss of acetylcholinesterase activity would be observed due to endocytosis.

To demonstrate that such a loss of activity can be used as an assay for endocytosis, three questions must be answered affirmatively. (1) Does this assay agree with the other applicable methods which are based on different principles? (2) Does this loss of accessibility of the enzyme to its substrates agree with the loss of accessibility of other surface markers? (3) Does the percentage decrease of enzymic activity quantitatively measure the percentage of total membrane taken in as vesicles?

METHODS

Human or porcine blood was used throughout. Human blood was collected in heparin by venipuncture of healthy, fasted volunteers. Porcine blood was collected from a slaughterhouse in U.S.P. Acid Citrate Dextrose (0.15 ml/ml of blood).

Erythrocyte ghosts were prepared essentially by the method of Dodge et al. [11] with the following modifications: EDTA was present at every step from lysis onward at a concentration of 1 mM, except where otherwise reported. For the last three washes of the ghosts, the hypotonic phosphate was replaced by 28 mM Tris · HCl, pH 7.4. EDTA was left out of the final wash and the ghosts were stored at 4 °C in this final wash buffer at a concentration of 5 mg membrane protein/ml. Porcine ghosts were used in the experiment presented in Table I. All other experiments were performed on human ghosts.

Preparation of primaquine hydrochloride. Primaquine was prepared from the diphosphate by titration to pH $\simeq$ 10 with NaOH and extracted repeatedly into methylene chloride. The extract was dried by filtration over anhydrous sodium carbonate into a weighed flask. Dry HCl gas was bubbled through the extract and, after the appearance of crystals, the solvent was removed by vacuum distillation. The orange, hygroscopic crystals of primaquine HCl were stored in a vacuum desic-

cator in the dark until a constant weight was obtained. A 10 mM solution was prepared and stored at 4 °C in the dark.

Preparation of TES-triethanolamine buffer. To remove interfering divalent cations, 500 ml of 1 M *N*-Tris-(hydroxymethyl) methyl-2-aminoethane-sulfonic acid (TES) was passed over a protonated chelex column of known void volume. To ensure no dilution, TES was not collected until 25 ml had passed through the column after the pH of the effluent had become acid. Then 450 ml of 1 M TES were collected. This TES was adjusted to pH 7.4 at 37 °C with triethanolamine, diluted to 500 mM and stored at 4 °C until needed.

Preparation of Tris-ATP. All operations were performed at 0 °C. Na_2ATP (4 g/100 ml) was passed over a column of Tris-Dowex 50, and the pH of the effluent adjusted when necessary to pH 7.0 at 0 °C with Tris or hydrochloric acid. The concentration was adjusted to 30 mM Tris-ATP using $\varepsilon = 15.4\ l \cdot cm^{-1} \cdot mmol^{-1}$ at 259 nm [12]. Inorganic phosphate was determined [13] and in all cases was less that 0.02 % the concentration of ATP. The Tris-ATP was stored at −30 °C.

Determination of membrane protein. Membrane protein was determined, except where otherwise reported, by the Biuret method as described previously [6]. Bovine serum albumin (Fraction V), was the standard in all cases.

Endocytosis. Endocytosis in porcine ghosts was induced in a test tube by incubation at 37 °C for 30 min in a medium which was 1 mg membrane protein/ml, 25 mM TES-triethanolamine, 3 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 3 mM Tris-ATP, and 1 mM primaquine-HCl. Endocytosis in human ghosts was carried out in a test tube by incubation at 37 °C for 30 min except where otherwise reported, in a medium which was 1 mg membrane protein/ml, 50 mM TES-triethanolamine, 3 mM $MgCl_2$ and 3 mM Tris-ATP. Endocytosis was started in all cases by the addition of ghosts to the otherwise complete medium which had been prewarmed for 5 min in a 37 °C water bath. Endocytosis was stopped in all cases by transferring the test tubes to an ice bath.

Acetylcholinesterase assay. Acetylcholinesterase activity was assayed by a modification of the method of Ellman et al. [9]. A 5,5′-dithiobis-(2-nitrobenzoic acid) (Nbs_2) phosphate buffer that was 0.4 mM in Nbs_2 and 0.1 M sodium phosphate (pH 8.0 at 20 °C) was made up at 5 times this concentration and stored at −30 °C. This concentrated stock solution remained stable for several months and the dilute solution was stable at room temperature for several weeks.

The modified assay was as follows. To a cuvette was added 3 ml of Nbs_2 phosphate buffer, 25 μl of 75 mM acetylthiocholine chloride, and 50 μg of porcine ghost protein or 25 μg of human ghost protein. The cuvette was inverted to mix and the change in absorbance at 412 nm recorded for 1 min at room temperature (about 20 °C) between the first and fourth minute of the reaction. The activity was calculated as $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$ using $\varepsilon = 13.6\ l \cdot cm^{-1} \cdot mmol^{-1}$.

For the purpose of this paper, "zero time acetylcholinesterase activity" refers to the activity of ghosts that were put in the endocytosis incubation medium and immediately placed in an ice bath. In all cases, this refers to the acetylcholinesterase activity of ghosts which had not undergone endocytosis as determined by phase contrast microscopy.

Determination of sialic acid accessible to neuraminidase. To prevent ATP from interfering with the assay, the ghosts were centrifuged from the endocytosis medium

at 0 °C at 25 000 × *g* for 20 min, washed once with 50 mM sodium acetate buffer (pH 6.0 at 0 °C) and then diluted to half the initial volume with the same buffer. Each sample was divided into two equal aliquots. One aliquot was used for the acetylcholinesterase and light microscope determination of endocytosis and for protein determination by the Folin-Ciocalteau method [15]. To the other aliquot was added 0.033 units of neuraminidase (Sigma type V from *Cl. perfringens*) per mg membrane protein and the mixture was incubated at 37 °C for 60 min. The reaction was stopped by transfer to an ice bath and the sialic acid released was determined by the 2-thiobarbituric acid method of Warren [14] with *N*-acetylneuraminic acid as the standard.

Phase contrast microscope assay of endocytosis. Slides were made of the standard endocytosis suspension using glass slides and coverslips. The percentage of ghosts with vesicles inside was estimated by observation with a 100 X oil immersion objective of 5 or more fields. These estimates vary ±10 % between individual observers.

Stereological determinations of endocytosis. The percentage of total membrane taken inside human ghosts as vesicle membrane was determined by the multipurpose grid method of Weibel [7], with a coarse test grid of total line length 63.8 cm and a fine test grid of total line length 270 cm. The linear magnification of the electron micrographs was 13 000 times. Human erythrocyte ghosts were either allowed to undergo endocytosis for 15 min at 37 °C or were immediately transferred to an ice bath. Acetylcholinesterase was determined and the membranes observed under the phase contrast microscope. The membranes were then fixed, embedded and sectioned as described previously [16] and mounted on carbon coated specimen grids.

Areas of the sections to be photographed were selected for randomness as follows. All areas which were framed by a corner of a specimen grid square and which contained 3 or more outer membranes were photographed regardless of the quality of the resulting micrograph. More than 20 micrographs were analyzed for each determination. Membranes that were incubated at 37 °C and those which were not were observed by both light microscopy and electron microscopy. In analysis of the electron micrographs, two definitions were used. (1) An outer membrane is the outermost closed figure. (2) A vesicle must lie within an outer membrane.

Observation of the electron micrographs demonstrated considerable infolding of the membranes at both the 0 and 15 min incubation times. This infolding was obvious from the irregular shape of the outer membranes and resulted in invaginations which looked like very large vesicles when sectioned and examined under the electron microscope. These invaginations could be distinguished from vesicles by their large size and elliptical shape, which contrasted with the small size and circular shape of the vesicles. Ghosts observed by the light microscope never contained vesicles larger than 1.2 μm and this finding was used to formulate a third definition. (3) Any closed figure inside an outer membrane that had any dimension greater than 1.6 μm was the result of infolding.

If only definitions 1 and 2 were used, 36.3 % of the total membrane area was calculated to be contained as vesicles while the application of definition 3 reduced this to 27.7 %. Definition 3 still did not exclude a few cases of obvious infolding but no further correction was attempted. For this reason, the data presented should be considered as an upper limit of the percentage of total membrane area contained as vesicles.

Using the following symbols: L_f, total length of the lines on the fine grid (270 cm); L_c, total length of the lines on the coarse grid (63.8 cm); I_f, total number of intersections of vesicle membranes with the fine grid; I_c, total number of intersections of outer membranes with the coarse grid; the percentage of total membrane area contained as vesicle membrane is equal to:

$$\frac{I_f \cdot L_c}{I_f \cdot L_c + I_c \cdot L_f}$$

The standard error of the mean was calculated from the results of applying this equation to each micrograph of the 15 min incubation time membranes.

Na_2 ATP (from equine muscle), primaquine diphosphate neuraminidase, *N*-acetyl neuraminic acid (Type IV), acetylthiocholine chloride and Nbs_2 were all obtained through Sigma. All common chemicals used were of reagent grade.

RESULTS

In the porcine ghost system, omission of $MgCl_2$, ATP, primaquine-HCl or the 37 °C incubation resulted in no endocytosis as observed under the phase contrast microscope, while the complete system resulted in 95 % of the membranes containing vesicles.

Table I shows that ATP, $MgCl_2$, primaquine-HCl, and 37 °C incubation were all required to obtain the decrease in acetylcholinesterase activity; about 36 % of this activity disappeared upon endocytosis. These data suggested that omission of any of these factors could have been utilized to obtain a control in which endocytosis had not occurred. However other experiments showed that the requirement for primaquine was often not absolute even when porcine ghosts were prepared as described. Furthermore, primaquine has an inhibitory effect on acetylcholinesterase activity which varies from preparation to preparation. In order to have the components of the control and experimental systems identical, so that the effect of inhibitors or activators of endocytosis could be easily observed, omission of the 37 °C incubation was chosen as the control for all subsequent experiments.

Fig. 1 shows that the decrease of acetylcholinesterase activity followed the time

TABLE I

ACETYLCHOLINESTERASE ACTIVITY

The same conditions required to obtain endocytosis were required to observe a loss of acetylcholinesterase activity. Endocytosis was carried out as described in methods for porcine erythrocyte ghosts. The data are presented as the mean ± the standard deviation ($n = 5$). The activity was calculated as nmol · min^{-1} · mg membrane $protein^{-1}$.

Component	Without component	Complete medium	% Acetylcholinesterase activity loss
$MgCl_2$	251 ± 7.4	160 ± 5.1	36.4 ± 2.8
ATP	257 ± 4.3	167 ± 3.3	35.1 ± 1.7
Primaquine	261 ± 6.1	170 ± 6.3	35.0 ± 2.8
37 °C incubation	264 ± 7.6	170 ± 6.3	35.6 ± 3.0

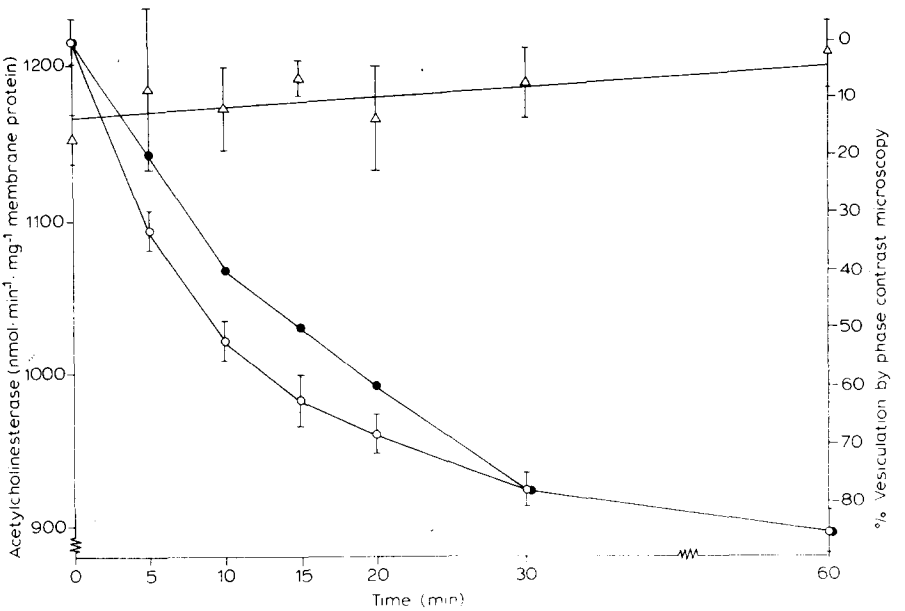

Fig. 1. The agreement between the acetylcholinesterase method and light microscope estimation of endocytosis is demonstrated. Endocytosis was carried out as described in Methods on human ghosts for the incubation times shown. Each sample was then divided into two aliquots. One aliquot was used for phase contrast microscope observation (●) and for acetylcholinesterase activity determination (○). The other aliquot was made 0.5 % Triton X-100 and the acetylcholinesterase activity was determined (△). The points shown represent the mean ($n = 6$) and the bars the standard deviations. The line shown for the Triton data (△) is the result of linear least squares analysis of the data shown.

course of endocytosis as measured by light microscopy. This loss of activity was due to membrane barrier effects rather than inactivation as was shown by recovery of acetylcholinesterase activity in the presence of 0.5 % Triton X-100. Under the phase contrast microscope it was observed that at this concentration of Triton the solution was clear, with no intact membranes and only a few particles.

In human or porcine ghosts it has been observed that $CaCl_2$ inhibits endocytosis [17]. In Fig. 2 is plotted acetylcholinesterase and light microscopy results versus the $CaCl_2$ concentration of the endocytosis media. The microscopic and enzymic assays were performed on the same tubes. The data demonstrate the agreement of the acetylcholinesterase and microscope assays in this inhibitor study.

In Table II is presented a comparison between the loss of acetylcholinesterase activity and sialic acid accessible to neuraminidase in endocytosis of human ghosts. Phase contrast microscope estimates are also provided. Notice the agreement in the amount of these two outer membrane markers lost upon endocytosis.

The stereological method as described determined 27.7 ± 2.1 % (standard error of the mean, $n = 23$) of the total membrane was taken inside as vesicles. The percentage of acetylcholinesterase activity lost upon endocytosis was 25.1 ± 1.2 % ($n = 2$); 80 % of the membranes contained vesicles when observed under the light microscope.

Fig. 3 is an electron micrograph of ghosts from the experiment just described, fixed and embedded after the 15 min incubation. The irregular shape of the outer membranes and elliptical "vesicle" shapes due to infolding of the membranes are obvious.

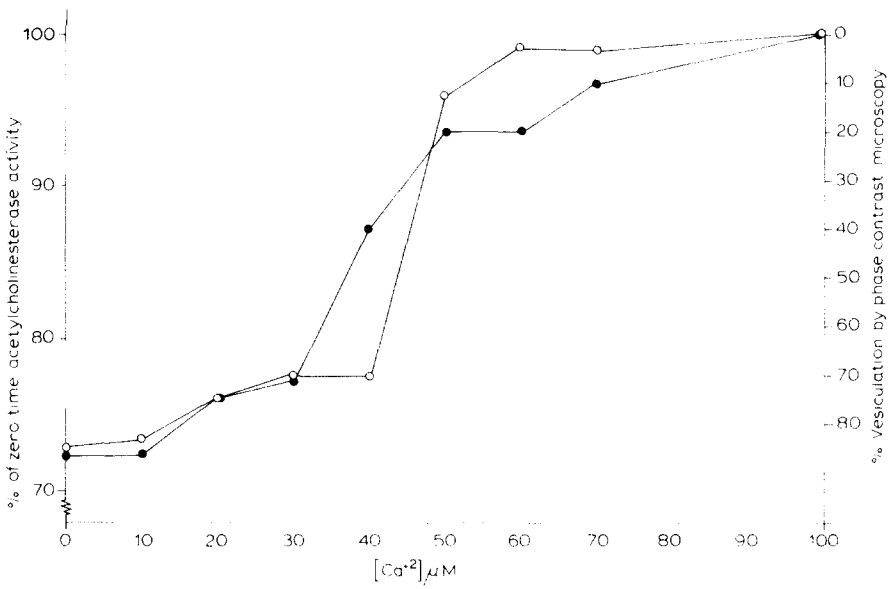

Fig. 2. The acetylcholinesterase method is shown to agree with light microscope estimates of endocytosis in this study of Ca^{2+} inhibition of endocytosis. Endocytosis was carried out as described in Methods on human ghosts at the calcium concentrations shown. A zero incubation time control was included for each calcium concentration. The data shown represent the percent of the control acetylcholinesterase activity (●). The results of phase contrast microscope estimations (○) performed on the same samples used for the acetylcholinesterase determinations are also shown. Each point represents the mean of duplicate samples.

TABLE II

After endocytosis, a similar percentage loss of acetylcholinesterase activity and of sialic acid accessible to neuraminidase was observed. Endocytosis was carried out as described in Methods on human erythrocyte ghosts for the incubation times shown. Sialic acid refers to the sialic acid accessible to neuraminidase as described in Methods. The data is presented as the mean ± the standard deviation with $n = 6$ for the zero incubation time and $n = 5$ for the 30 min incubation time.

Incubation time (min)	Light microscope estimate of % vesiculation	Sialic acid (nmol · mg protein^{-1})	Acetylcholinesterase (nmol · min^{-1} · mg protein^{-1})
0	0	51.3±2.6	1012±27
30	85	31.0±1.5	625±15
% Loss		39.6±4.2	38.2±2.2

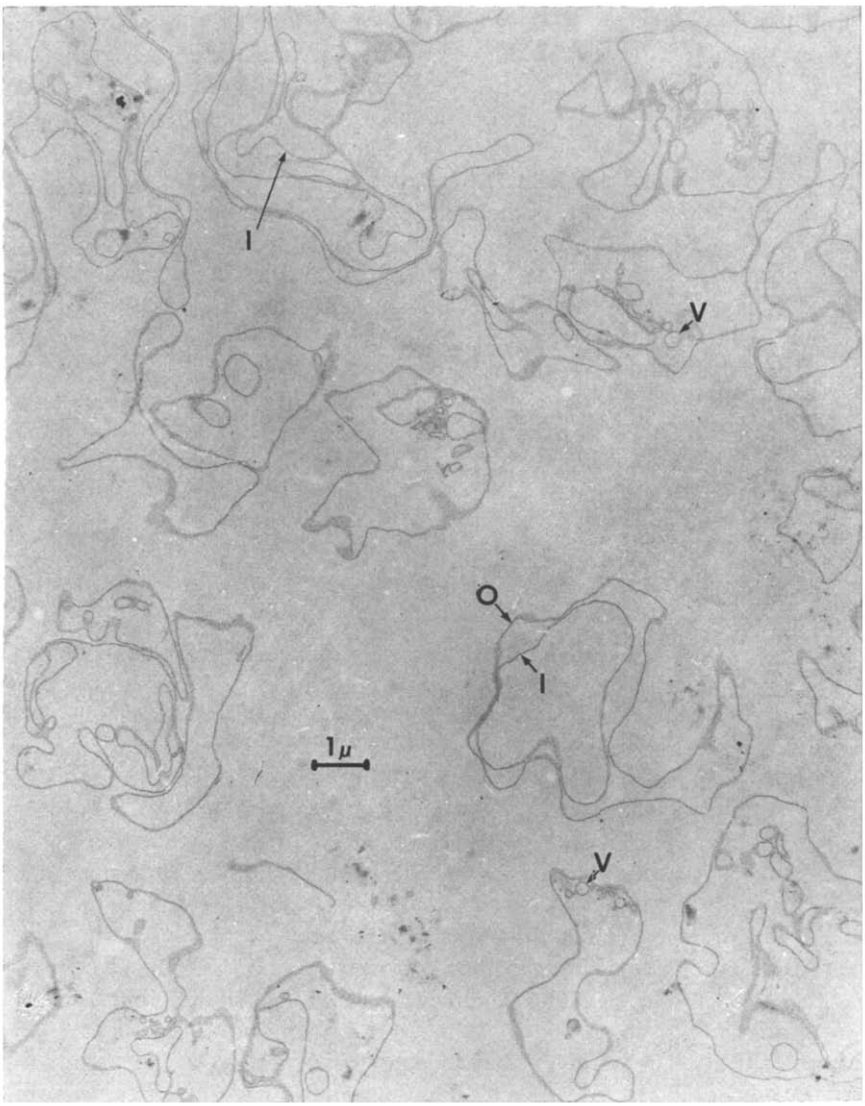

Fig. 3. A representative electron micrograph demonstrating the definitions used. An example of an infolding (I), a true vesicle (V), and an outer membrane (○) are shown. Notice the irregular outline of the outer membrane.

DISCUSSION

Conclusions

The data presented allow us to draw several conclusions.

1. The assay depended on membrane integrity, as demonstrated by the Triton results (Fig. 1), but was not necessarily dependent on the membranes being resealed. The observed loss of acetylcholinesterase activity may be due to one or a combination of the following factors. (a) Upon endocytosis, the vesicles taken in may be resealed.

Vesicles formed by invagination of the outer membrane would be inside out and, if resealed, would sequester the acetylcholinesterase from its substrate. Steck et al. [18] have observed such a loss of acetylcholinesterase activity due to resealing of inside out vesicles. (b) The outer and vesicle membranes may both still be partially permeable to acetylthiocholine but the combination of two partial permeability barriers may effectively sequester acetylcholinesterase from its substrate.

Since both factors involve a permeability barrier, either partial or total, we were unable to test which of these was responsible for the observed loss of activity.

2. The acetylcholinesterase method agreed quantitatively with all other methods tested. The percentage loss of acetylcholinesterase activity corresponded to the percentage of the total membrane taken in as vesicles. This would suggest that the enzyme is randomly distributed over the outer surface of the ghosts, or that if patches of the enzyme exist on the membrane, they are not taken into vesicles preferentially. The agreement in percentage loss of acetylcholinesterase activity and of sialic acid accessible to neuraminidase shows that the enzyme and sialic acid have a similar distribution over the surface of the membrane.

Advantages of the acetylcholinesterase method

The acetylcholinesterase assay of endocytosis has several advantages over the other methods tested.

Time requirements. The acetylcholinesterase assay of endocytosis can be performed more rapidly than any other method tested, with the possible exception of estimation under the light microscope. An accurate determination of acetylcholinesterase activity requires recording the change in absorbance at 412 nm for only a minute. With experience, an investigator can perform the assay in an average of 1.5 min per experimental determination.

The method measures the percent membrane taken in as vesicles directly. This is a decided advantage over the phase contrast microscope method, which measures the percentage of ghosts containing vesicles. We have found this difference to be important. For example, a comparison of Fig. 1 and Table II shows that the light microscope method observed 85 % of the ghosts as containing vesicles in both experiments while the acetylcholinesterase method demonstrated a substantial difference between the two, with 28.4 % and 35.6 % of the membrane taken in as vesicles in Fig. 1 and Table II respectively. This ability of the acetylcholinesterase method to distinguish levels of endocytosis not distinguished by light microscopy allows a better comparison between ghost preparations and between methods for obtaining endocytosis.

Reproducibility. The acetylcholinesterase method is more reproducible than any of the other methods tested, with the possible exception of the stereological determination. The standard deviation of the stereological determination is determined by the number of intersections counted. The standard error reported was the result of counting over 1000 intersections of the fine grid with vesicle membranes and over 900 intersections of the coarse grid with outer membranes in 23 electron micrographs. The acetylcholinesterase results presented were the result of determination of the activity of the enzyme on two zero min and two 15 min incubation time samples of human ghosts. The standard errors of the mean reported for the two methods are similar, but the acetylcholinesterase results were obtained in less than 10 min, while the stereological results required over 100 man-hours. Having accurate results from an

experiment almost immediately instead of as much as 1 month or more after the experiment was performed makes the acetylcholinesterase method preferable.

Applicability. We have presented results using the acetylcholinesterase method on ghosts from two species (porcine and human) which have very different levels of activity of this enzyme. We have used this method in other studies on bovine ghosts and found it applicable there as well.

Since the method described here depends on the percentage change in acetylcholinesterase activity due to endocytosis, it is applicable to ghosts from species with widely varying acetylcholinesterase activities. The use of inhibitors of endocytosis such as Ca^{2+} (Fig. 2) raises the question of whether such inhibitors may affect the acetylcholinesterase assay. In the Ca^{2+} experiment, the Ca^{2+} had minimal effect on acetylcholinesterase activity and this effect was corrected by using a zero incubation time control for each calcium concentration.

Other reagents can strongly inhibit acetylcholinesterase, and the effect of such reagents has recently been reviewed [19]. Other than by the classic nerve gases and insecticides, this enzyme is also inhibited by various proteases, sulfhydryl reagents, and antibiotics [19]; tetra-substituted ammonium compounds [20]; cross-linking reagents [19, 21]; and fatty acid esters and phenothiazines [22]. It is interesting to note that the fatty acid ester inhibition of acetylcholinesterase was found to be dependent on resealing of the membrane. Sodium chloride tonicity also effects acetylcholinesterase activity [19], presumably due to solubilization of the acetylcholinesterase [23].

Since the assay discussed here depends only on the ratio of final to initial acetylcholinesterase activity, it should be applicable to studies involving reagents which activate or inhibit this enzyme, if suitable control experiments are carried out. If the reagent which affects acetylcholinesterase is present throughout the experiment, and has already had its full effect at zero time, the zero time control will be adequate, with a separate control for each concentration of the reagent. If the effect of the reagent on acetylcholinesterase activity changes during the 37 °C incubation, then the assay must be carried out with the no Mg^{2+} or no ATP control. Such a situation may occur if the reaction of the reagent with acetylcholinesterase is slow.

In all such studies, the resulting membrane suspensions should be observed by light microscopy to verify that the ghosts have not been fragmented or solubilized and to check the amount of endocytosis.

Since the acetylcholinesterase method is applicable to membranes which are not resealed, it heightens the advantages of studying endocytosis in unresealed ghosts. In these ghosts, substrates and effectors of endocytosis can be added directly and allowed to diffuse across the membrane. In resealed ghosts, substrates and effectors must be resealed inside the membrane. Resealed ghosts also contain a high concentration of hemoglobin, which is known to bind calcium and other substances which influence membrane shape.

We have not compared this method with the cobalamine method in resealed ghosts. We have not as of this writing studied its applicability to resealed ghosts or whole cells, but the method should be applicable to them also.

Thus the acetylcholinesterase method reported here has the qualities set forth as desirable for an assay of endocytosis in erythrocyte ghosts. It is fast, inexpensive and quantitative, and has proved useful in this and other studies carried out on endocytosis in erythrocyte ghosts.

ACKNOWLEDGMENTS

The excellent technical assistance of Mr. James M. McKinney and the helpful advice and encouragements of Dr. Hideo Hayashi, Ms. Lucy Vaughan, Dr. Gordon Plishker, and Mr. James M. McKinney are greatly appreciated. The encouragements of Ms. Karen Mears were invaluable. This work was supported by the NIH(AM17588, DE02668 and RR05333).

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