4. The effects of these various treatments on the appearance of the rods has been followed microscopically.

5. Very pure cattle rhodopsin solutions have been obtained showing maxima at 498, 345 and 275 mμ. The last band can be accounted for by the presence of tyrosine and tryptophan only. Making some plausible assumptions, rhodopsin appears to contain 6% tyrosine and 3% tryptophan on the dry weight of the protein.

We would like to thank the Medical Research Council for grants. One of us (F.D.C) participated in this work as a holder of an Imperial Chemical Industries Research Fellowship.

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Estimation of Protamine and Insulin in Protamine Zinc Insulin

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(*Received* 5 *September* 1951)

In a recent publication, Franklin & Quastel (1950) reported that mixtures of proteins could be separated into their individual components by paper chromatography using solutions of sucrose or sodium potassium tartrate for development. The difficulty of locating the position of the protein zones was overcome by adding haemin to the protein mixture and then streaking the paper with a solution of benzidine and hydrogen peroxide. Jones & Michael (1950) separated proteins on columns of cellulose by development with buffer solutions containing ammonium sulphate and located the position of the individual components by treatment with suitable dyestuffs which stained the zones occupied by the proteins, but did not give coloured spots with amino-acids or peptides. More recently, Papastamatis & Wilkinson (1951) used bromothymol blue and tetrabromophenolphthalein ethyl ester to indicate the position occupied by proteins in paper chromatograms. The publication of this last paper prompts us to report our own work on the chromatographic separation and estimation of protamine and insulin.

The initial experiments on the separation of insulin and protamine by paper chromatography were carried out with strips of Whatman no. 1 filter paper 2 cm. wide, using 10 μl. spots of a solution containing 1% protamine sulphate and 1% insulin (w/v). The strips were developed in a downwards direction in the usual way according to the method of Consden, Gordon & Martin (1944) with the phases of a mixture (by vol.) of *n*-butanol (40%), glacial acetic acid (10%) and water (50%), a mixture generally used in the paper chromatography of amino-acids. Solway purple, as recommended by Jones & Michael, was used for indicating the position of the protein bands. Two bands were formed, one due to protamine at the point of application and the other due to insulin, some 6 cm. below the point of application; the solvent front travelled about 25 cm. The separation was not entirely complete, however, and a number of other solvent mixtures were tried. Better results were eventually obtained with the upper phase obtained by equilibrating a mixture of *n*-butanol and glacial acetic acid (3:1 by vol.) with an equal volume of water. This resulted in the formation of two well-defined bands with R_F values of 0 and 0·43 respectively, and this mixture was used in all subsequent work.

The estimation of substances separated by paper chromatography often presents considerable difficulty, although fairly satisfactory methods of estimating individual amino-acids on paper chromatograms have recently been described. It occurred to us that a protein might be estimated with a fair degree of accuracy by the method of retention analysis described by Wieland & Fischer (1948). These workers showed that if a spot of an amino-acid solution was placed near the edge of a

sheet of filter paper and the paper then dipped into a solution of a cupric salt, the latter was drawn up by the paper and cupric ions became uniformly distributed throughout the paper except in the area immediately above the amino-acid spot. In this region no copper could be detected on subsequently spraying with a suitable indicator, because the amino-acid retained the copper ions in the form of a complex, thus preventing them from passing further up the paper. The area of the white copper-free wedge formed above the spot was proportional to the amount of amino-acid present. It was thought possible that the same principle could be applied to the estimation of a protein using instead of a copper salt a solution of a dyestuff with an affinity for the particular protein concerned.

To test this hypothesis spots of different sizes of solutions of insulin, protamine sulphate, blood albumin and edestin were applied to rectangular sheets of filter paper, and the filter papers dried and dipped into solutions of Solway purple or erythrosine. The dyes were allowed to rise about 5 cm. above the protein spots. In every instance a wedge-shaped area above the protein spot remained white and the areas of these wedges were approximately proportional to the amount of protein present.

Attempts were then made to apply retention analysis to mixtures of protamine sulphate and insulin after separation by paper chromatography. A spot of the solution to be tested was applied at one corner of a rectangular sheet of filter paper. The paper was developed along the longer axis with the aqueous butanol-acetic acid mixture and dried. It was then cut in such a way as to separate the protamine which remained at the point of application from the insulin spot which had moved about 10 cm. away from it. Both halves of the paper were then dipped in a dye solution until the dye had risen about 5 cm. above the protein spot. Estimates of the amount of protamine calculated from the area of the unstained wedge above the protamine spot were in fair agreement with the amount of protamine known to be present, but it was impossible to estimate the amount of insulin in a similar manner because the insulin spot, in contrast to the protamine spot, was very diffuse, marked 'tailing' generally having occurred. All attempts to sharpen the insulin zone so as to make it amenable to retention analysis have been unsuccessful.

At this stage we learnt that Dr W. Dickinson had used bromocresol green for indicating the position of different protein bands in paper chromatograms of crude insulin solutions and had succeeded in eluting the insulin bands from such paper chromatograms by means of a borate buffer solution of pH 9·2; the colour of the eluate was proportional to the amount of insulin present. We therefore investigated this method and found that we obtained satisfactory results for the estimation of insulin after chromatographic separation from protamine. A number of other dyes were examined, but none gave better results than bromocresol green.

EXPERIMENTAL

Separation of insulin from protamine by chromatography

In preliminary experiments 10 μl. drops of a 1% (w/v) protein solution, as well as a mixture of protamine sulphate and insulin solutions, were placed on Whatman no. 1 filter-paper strips (2 cm. broad) and developed with a large number of solvent mixtures. Each of the protein solutions when examined separately gave single spots. The protamine sulphate was derived from salmon milt and the insulin from ox pancreas.

The mixture found to be most satisfactory for the separation of protamine from insulin was the upper phase obtained by equilibrating *n*-butanol, glacial acetic acid and water (3:1:4 by vol.). This solvent mixture gave a clean separation of protamine and insulin with R_F values of 0·00 and 0·43–0·45 respectively.

Retention analysis of proteins

Preliminary experiments were carried out with solutions of insulin, protamine sulphate, blood albumin and edestin, varying the concentrations and volumes of the solutions used. A drop of each solution was placed 2 cm. from the lower edge of a rectangular piece of Whatman no. 1 filter paper, which was suspended vertically in a covered beaker so that the lower edge of the paper dipped about 1 cm. into the dye solution. The dye was allowed to rise about 5 cm. above the protein spots, and the paper was then dried. The white portion above the spot was traced on to graph paper and its area measured.

Using a 0·1% (w/v) solution of erythrosine, the results recorded in Table 1 were obtained. Fig. 1 illustrates the

Table 1. *Effect of concentration and drop size on the retention analysis of protein solutions with erythrosine*

(For experimental procedure see text.)

Protein	Concentration of solution (%, w/v)	Volume of drop (μl.)	Retention area (sq.mm.)	Relative retention areas
Insulin	1	5	61	1·0
	1	10	102	1·7
	1	20	202	3·3
Protamine sulphate	1	5	99	1·0
	1	10	198	2·0
	1	20	356	3·6
Protamine sulphate	0·2	5	81	1·0
	0·4	5	180	2·2
	0·6	5	285	3·5
	0·8	5	365	4·5
Blood albumin	0·125	10	55	1·0
	0·25	10	114	2·1
	0·5	10	167	3·0
Edestin	1	10	18	1·0
	2	10	38	2·1

appearance after retention analysis of filter papers to which 5 μl. spots of 0·2, 0·4, 0·6 and 0·8% (w/v) protamine sulphate solutions had been applied.

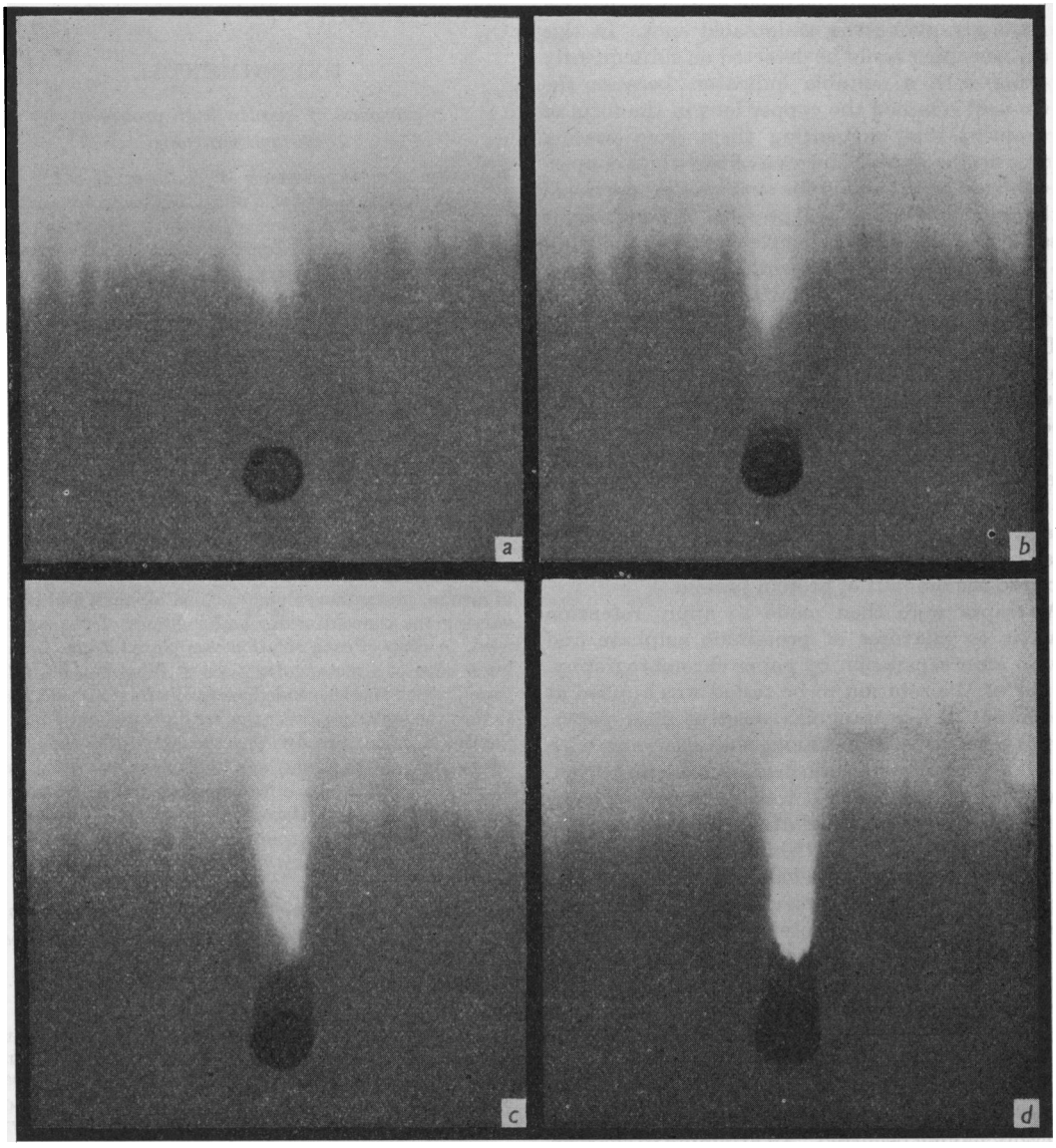

Fig. 1. Paper chromatograms obtained by retention analysis of 5 μl. spots of (*a*) 0·2, (*b*) 0·4, (*c*) 0·6 and (*d*) 0·8% (w/v) protamine sulphate solution with 0·1% erythrosine solution.

Attempted estimation of insulin and protamine by retention analysis

Portions (20 μl.) of 1% insulin solution and of 1% protamine sulphate solution were mixed and half of the mixture applied to each of two 10 cm. strips of Whatman no. 1 filter paper, which were then developed with the aqueous butanol-acetic acid mixture. The strips were dried and one was dipped into a 0·1% (w/v) Solway purple solution; from the positions of the two coloured zones thus formed, the positions of the insulin and protamine on the undyed strip were noted.

This assay strip was cut into four portions each about 8 cm. wide, one portion containing the protamine zone, another the insulin zone and the third and fourth portions containing no protein. Measured amounts (10 μl.) of 1% insulin solution and of 1% protamine sulphate solution were applied to the third and fourth portions respectively to serve as standards.

These four portions of the chromatogram were retention-analysed as described above with a 0·1% solution of

erythrosine, this having been found to work better with protamine than did Solway purple.

The following retention areas were obtained: standard (10 μl. of 1% insulin solution) = 123 sq.mm.; test solution (insulin band) = 179 sq.mm.; standard (10 μl. of 1% protamine sulphate solution) = 312 sq.mm.; test solution (protamine band) = 320 sq.mm.

From these results the insulin zone is apparently equivalent to 14·5 μl. of a 1% insulin solution, whereas only 10 μl. of 1% insulin solution had been chromatographed. The protamine zone, on the other hand, gave an area equivalent to 10·2 μl. of 1% protamine sulphate solution, a result in excellent agreement with the theoretical value of 10 μl.

Presumably, therefore, before insulin can be estimated by retention analysis it is essential that a more compact zone should be obtained than is formed when insulin is chromatographed with the solvent mixture described above. In an attempt to obtain a more suitable insulin zone, another chromatogram was prepared as before. The portion containing the protamine zone was cut off and the insulin zones were developed further with dilute acetic acid (1 part glacial acetic acid to 4 parts of water), which had been shown in preliminary experiments to form a compact zone at the solvent front. The resulting zone was retention-analysed as before, with the following results: standard (10 μl. of 1% insulin solution) = 117 sq.mm.; test solution (insulin band) = 151 sq.mm. Again the result was higher than anticipated, the area being equivalent to 12·9 μl. instead of 10 μl. of 1% insulin solution.

Estimation of protamine in protamine zinc insulin

As the retention analysis of insulin after separation from protamine by chromatography gave unsatisfactory results, attention was concentrated on the estimation of protamine, since this remained in the form of a compact spot readily amenable to retention analysis. Various samples of protamine zinc insulin were freed from insulin by paper chromatography and the residual protamine spot was assayed by retention analysis. Portions of the precipitates and supernatant liquors from these samples were also examined in the same way in order to obtain an estimate of the distribution of the protamine between the precipitates and the supernatant solutions. This was done by centrifuging portions of the protamine zinc insulin sample under investigation, separating the supernatant liquor from the precipitate, dissolving the latter in a few drops of dilute HCl and diluting the solution thus obtained to the original volume with distilled water. Portions (50 μl.) of the original protamine zinc insulin suspension, the supernatant liquor, and the dissolved precipitate were chromatographed and the protamine-containing portions of the filter papers were examined by retention analysis as described above. The protamine content was calculated from the retention areas by reference to a standard curve prepared by treating in a similar manner standard solutions of protamine sulphate. The results are shown in Table 2.

Table 2. *Protamine and insulin contents of various samples of protamine zinc insulin*

(For analytical procedures see text. Figures in table are mg./100 ml. Values given are mean and range. Figures in brackets represent number of assays done.)

Protamine zinc insulin sample no.	Insulin			Protamine		
	Total	Precipitate	Supernatant	Total	Precipitate	Supernatant
AB836 (40 units)	162± 6 (5)	162±30 (4)	Too low to measure	30± 9 (6)	34± 7 (2)	Too low to measure (2)
C208165 (40 units)	204± 3 (2)	250 (1)	Too low to measure	50±10 (6)	51± 6 (3)	5±5 (3)
S919 (40 units)	170± 7 (3)	133± 3 (2)	Too low to measure	36±10 (6)	39± 6 (3)	4±4 (4)
SD342442 (40 units)	160±13 (2)	151 (1)	Too low to measure	41±11 (5)	31±10 (2)	Too low to measure (1)
L4134 (40 units)	158± 8 (2)	178± 1 (2)	Too low to measure	42± 8 (5)	46± 3 (2)	3±3 (2)
O7510 (40 units)	—	—	—	8± 2 (5)	4± 4 (3)	7±6 (3)
B119 (40 units)	150±34 (5)	134±10 (3)	Too low to measure	43± 4 (6)	44± 0 (2)	Too low to measure (1)
B120 (40 units)	155±18 (7)	156± 1 (2)	Too low to measure	48± 9 (5)	38± 7 (4)	Too low to measure (1)
B121 (40 units)	187±35 (5)	150±19 (3)	Too low to measure	51± 6 (6)	42± 1 (2)	Too low to measure (1)
B122 (80 units)	335±31 (3)	338±25 (3)	Too low to measure	96± 8 (6)	86± 7 (2)	Too low to measure (2)

Estimation of insulin in protamine zinc insulin

The amount of insulin present in the volume of protamine zinc insulin suspension adequate for the estimation of protamine was found to be insufficient for the estimation of insulin by Dickinson's method. Accordingly, a second chromatographic separation was carried out on a rectangular sheet of Whatman no. 1 filter paper. Up to 400 μl. of the solution were streaked in a broad band across one end of the paper and about 6 cm. from the edge. The chromatogram was then developed with the upper phase obtained by equilibrating a mixture of *n*-butanol (75%) and acetic acid (25%) with water. After drying, the paper was soaked

in a 0·02% (w/v) aqueous solution of bromocresol green for 5 min. This resulted in adsorption of the dye by both the protamine band and the insulin band. The background colour was removed by washing the strips for 20 min. in three changes of 2% (w/v) acetic acid and the strips were then dried. Fig. 2 illustrates the appearance of a typical chromatogram after exposure to ammonia vapour to intensify the colour. A section 6 cm. wide containing the insulin band was cut out and supported vertically with the upper end bent over and dipping into a borate buffer solution of pH 9·2. This was prepared by mixing 50 ml. of 0·1M-boric acid in 0·2M-KCl solution with 26·7 ml. of 0·2N-NaOH solution and diluting to 200 ml. This eluted the insulin-dye complex, and the eluate, which was collected at the lower end of the strip, was made up to 10 ml.

A 50 µl. spot of the solution was applied to a rectangular sheet of filter paper (10 × 30 cm.) 2 cm. from the longer and 8 cm. from the shorter edge. The latter was bent over so that it dipped 3 cm. into the trough of the chromatographic apparatus containing the upper phase of the *n*-butanol-glacial acetic acid-water mixture described above. After development in a closed vessel for about 8 hr., during which time the solvent front travelled the whole length of the paper and the insulin spot moved about 15 cm., the filter paper was dried in a current of warm air. A 3 cm. strip was then cut from the end of the paper that had been immersed in the solvent, and discarded. The remaining sheet was cut into two portions 10 cm. from the new edge thus formed, and the smaller of the two pieces, containing the protamine spot 2 cm. from one edge, was supported with this edge

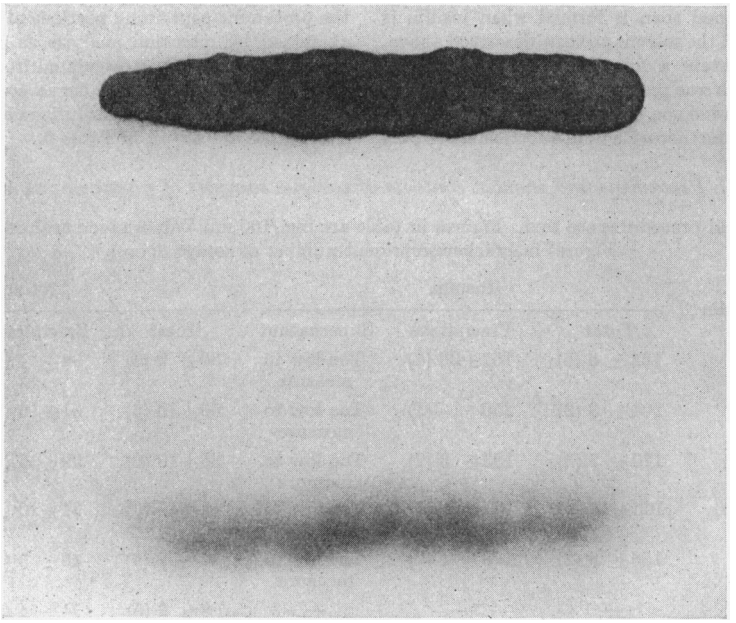

Fig. 2. Paper chromatogram showing bands of protamine (above) and insulin (below) obtained by developing protamine zinc insulin solution with aqueous butanol-acetic acid and staining with bromocresol green solution.

A 6 cm. portion containing no protein band was cut out from the paper and eluted in the same way for use as a blank. The optical density of the eluate was measured against the blank solution in a Spekker absorptiometer using Ilford filter no. 607.

Portions of insulin solutions of various concentrations were chromatographed and the insulin bands treated as described above. A graph was plotted connecting the insulin concentration with the absorptiometer reading. The relation was found to be linear between 0·25 and 1·5% of insulin. The insulin content of the unknown solution was calculated from this standard curve. The results are shown in Table 2.

Recommended method for the estimation of protamine and insulin in protamine zinc insulin

The procedure finally adopted for the estimation of protamine and insulin in protamine zinc insulin suspension was as follows:

dipping into a 0·1% solution of erythrosine and the dye allowed to rise about 5 cm. above the spot. The area of the white V-shaped wedge was then measured by tracing on to graph paper and the results calculated from a standard curve prepared by treating known amounts of protamine in the same manner.

To estimate the insulin content a fresh chromatogram was prepared by streaking 200–400 µl. of protamine zinc insulin solution about 6 cm. from and parallel to the shorter edge of a rectangular sheet of filter paper similar to that used in the protamine estimation. The chromatogram was developed and dried as before. The paper was dipped into a 0·02% solution of bromocresol green for 5 min. and then washed for 20 min. in three changes of 2% acetic acid to remove the background colour. The paper was again dried, the portion containing the insulin zone was cut out and the insulin-dye complex eluted with a borate buffer solution (pH 9·2) and subjected to absorptiometry exactly as described above.

SUMMARY

1. Insulin can be separated from protamine by paper chromatography developing with the upper phase of a mixture of *n*-butanol, glacial acetic acid and water (3:1:4 by vol.). A dye such as bromocresol green is used for locating the zones.

2. The concentration of protamine and of various proteins in a solution can be estimated on filter paper by retention analysis with a suitable dye, e.g. erythrosine. The area of the unstained wedge formed above the protein spot is proportional to the amount of protein present. The method can be used to estimate the amount of protamine in protamine zinc insulin after removal of insulin by paper chromatography.

3. The concentration of insulin in protamine zinc insulin can be estimated, after separating the insulin from protamine by paper chromatography, by staining with bromocresol green solution, eluting the insulin-dye complex and comparing the colour of the eluate with that given by a standard solution of insulin treated in the same way.

4. Data are recorded on the composition of a number of suspensions of protamine zinc insulin and on the distribution of the components between the phases of the suspension.

We thank the Directors of Allen and Hanburys Ltd. and The British Drug Houses Ltd. for permission to publish this paper.

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Studies on Cholinesterase

7. DETERMINATION OF THE MOLAR CONCENTRATION OF PSEUDO-CHOLINESTERASE IN SERUM

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(*Received* 17 *October* 1951)

The classical theory of reversible enzyme-substrate and enzyme-inhibitor reactions postulates that enzyme and substrate, or enzyme and inhibitor, form a reversible combination according to mass law principles (Michaelis & Menten, 1913). The mechanism of enzyme-inhibitor reactions has been analysed from this theoretical standpoint by Straus & Goldstein (1943) and Goldstein (1944), with special consideration being given to the concentration of free enzyme and free inhibitor. They pointed out that the degree of inhibition of enzyme activity effected by a certain concentration of inhibitor will be dependent in part upon the enzyme concentration when the so-called 'dissociation' or Michaelis constant of the enzyme-inhibitor system is of the same order of magnitude or much smaller than the molar enzyme concentration employed.

The theoretical treatments given by Goldstein (1944) were illustrated with experimental data obtained, using a pseudo-cholinesterase preparation as the source of enzyme and eserine as the enzyme inhibitor. In this system it is possible to obtain appreciable inhibition of the cholinesterase activity with eserine concentrations as low as 10^{-8} M; nevertheless, the degree of inhibition is practically independent of enzyme concentration under the experimental conditions. Thus it was not possible to confirm the theoretical formulations completely at that time, or to arrive at any definite value for enzyme concentration in the cholinesterase system.

Since that time, other investigators, notably Bain (1949), have attempted to determine cholinesterase concentration by the use of irreversible alkylphosphate inhibitors such as di*iso*propyl fluorophosphonate (DFP) and tetraethyl pyrophosphate (TEPP). The lack of theoretical criteria which could be applied to the results with these inhibitors makes this a rather unreliable method for determining cholinesterase concentration. Moreover, the results obtained do not correspond with those expected of