Biochem. J. (1974) **139**, 123–127
Printed in Great Britain

The Effects of Two Disulphides on Cholinesterase Activity in the Spectrophotometric Assay

By KLAS-BERTIL AUGUSTINSSON and HÅKAN ERIKSSON
Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-10405 Stockholm, Sweden

(*Received* 10 *September* 1973)

1. 5,5′-Dithiobis-(2-nitrobenzoate) did not influence serum cholinesterase activity, whereas 2,2′-dithiodipyridine had an inhibitory effect. 2. The lowering of the molar extinction coefficients observed in the presence of physostigmine may be a result of a reaction between thiolate ions with carbamate moieties. 3. The use of 5,5′-dithiobis-(2-nitrobenzoate) is still recommended in investigations, especially where the quantitative aspects are significant.

The use of the spectrophotometric method first described by Ellman *et al.* (1961) for the determination of cholinesterase activity and recently applied in a similar manner for measuring arylesterase (Augustinsson *et al.*, 1972) is very attractive because of its simplicity and adaptability for micro determinations. The method is based on the use of thiocholine esters as substrates and the reaction of the thiocholine formed with 5,5′-dithiobis-(2-nitrobenzoate) to give 5-thio-2-nitrobenzoate, which has an extinction maximum at 405–420nm.

In a recent report (Brownson & Watts, 1973), another disulphide, 2,2′-dithiodipyridine (aldrithiol-2), is suggested to be a better choice than 5,5′-dithiobis-(2-nitrobenzoate) because the latter was found to activate both acetylcholinesterase (EC 3.1.1.7) and serum cholinesterase (EC 3.1.1.8). However, in the work reported here this effect of 5,5′-dithiobis-(2-nitrobenzoate) could not be confirmed. Instead, the 'activation' was found to have another explanation.

Materials and Methods

Enzymes

Acetylcholinesterase was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Butyrylcholinesterase was a partly purified preparation from horse serum and was obtained from Organon, Oss, Holland.

Other reagents

The choline and thiocholine esters (used in the form of iodide salts), 5,5′-dithiobis-(2-nitrobenzoate acid) and physostigmine sulphate were obtained from Sigma, and aldrithiol-2 was from Aldrich–Europe, Beerse, Belgium. The properties of aldrithiol-2 were described by Grassetti & Murray (1967). All other chemicals used were standard commercial products, and all solutions were made up in double-deionized water.

Assay of esterase activities

All spectrophotometric measurements were made at 30°C with a Beckman DB-G spectrophotometer with a Contron 3012 recorder. Unless otherwise stated, the reaction mixture was composed of 3.0ml of 50mM-sodium phosphate buffer, pH7.4, containing 0.35mM-disulphide (5,5′-dithiobis-(2-nitrobenzoate) or aldrithiol-2), 0.02ml of the substrate solution and 0.05ml of the enzyme solution, added in that order.

In a number of determinations of the esterase activity in the absence of disulphide, a slight modification of a method described by Brownson & Watts (1973) was used. The composition of the reaction mixtures was as above minus the disulphide, and the reaction tubes were equilibrated at 30°C before addition of the enzyme. After suitable time-intervals, samples (0.4ml) were transferred to a stopping mixture, composed of 0.50mM-disulphide and 1.5mM-physostigmine sulphate, giving a total volume of 1.0ml. Then the extinction of this 'stopped' reaction mixture was measured at the usual wavelength (5-mercapto-2-nitrobenzoate): $\lambda_{max.} = 412$nm, $\varepsilon = 13\,600\,M^{-1}\cdot cm^{-1}$; 2-thiopyridine: $\lambda_{max.} = 343$nm, $\varepsilon = 7060\,M^{-1}\cdot cm^{-1}$). The sampling times were 0.50, 1.0, 1.5 and 2.0min. As controls, parallel series of measurements by this same method were done in the presence of 0.35mM-disulphide (the disulphide concentration in the stopping mixture was in this case 0.35mM).

The esterase activity was also determined by the Warburg technique at 25°C in a $NaHCO_3$–CO_2 buffer of pH7.4 with the choline esters as substrates (Augustinsson, 1957).

Esterase activity was expressed in mkatals/kg, i.e. mmol of substrate hydrolysed/s per kg of protein (according to the *Recommendations* (1972) *of the IUPAC and the IUB* on enzyme units).

Results

Effect of two disulphides on cholinesterase activity

The effects of 5,5′-dithiobis-(2-nitrobenzoate) and aldrithiol-2 on cholinesterase activity, when the concentration of the two disulphides was varied and the spectrophotometric assay was used, are shown in Fig. 1(*a*). The activity in the presence of 5,5′-dithiobis-(2-nitrobenzoate) was independent of the concentration used. On the other hand, the activity in the presence of aldrithiol-2 increased as the concentration of the disulphide decreased and reached the value obtained with 5,5′-dithiobis-(2-nitrobenzoate) at a concentration of about 0.05 mM when acetylcholinesterase was used as enzyme. The inhibiting effect of aldrithiol-2 was even stronger for butyrylcholinesterase.

Fig. 1(*b*) illustrates an experiment with butyrylcholinesterase by the Warburg technique. The results were the same as those obtained with the spectrophotometric assay; consequently, all the assays presented below were performed spectrophotometrically. Fig. 1(*b*) also shows that the activity in the absence of disulphides was identical with that obtained in the presence of 5,5′-dithiobis-(2-nitrobenzoate). Fig. 2 shows that the results are valid over a wide substrate-concentration range.

The kinetic behaviour of butyrylcholinesterase catalysis did not follow the Michaelis–Menten equation, as shown by the plot (Fig. 2).

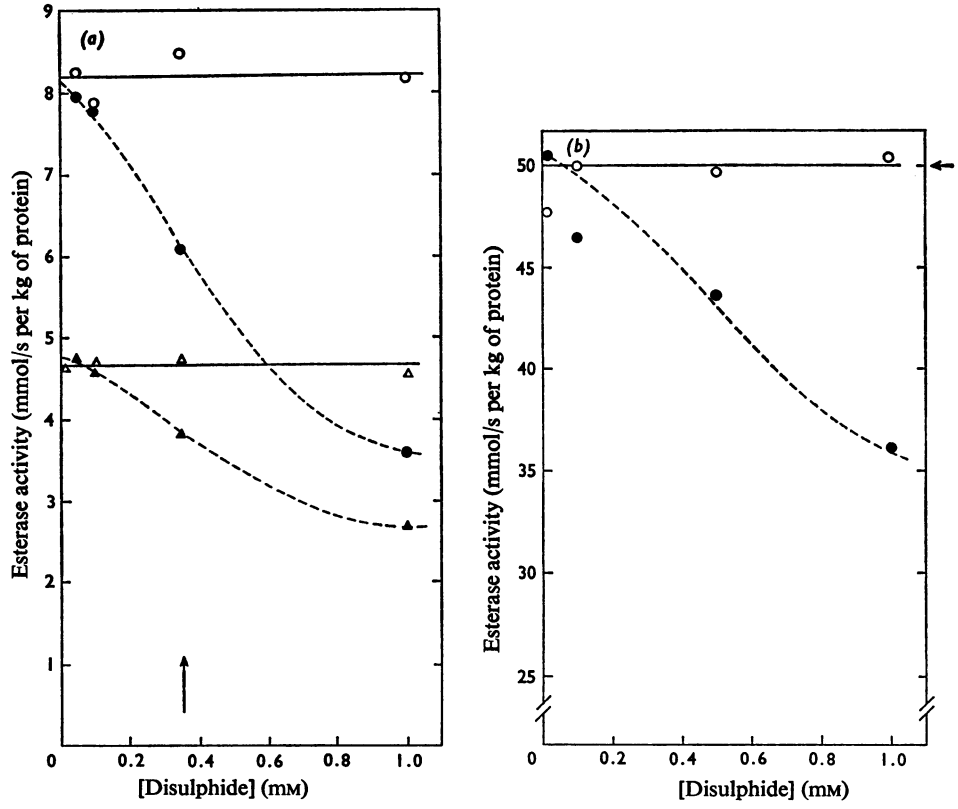

Fig. 1. *Effects of 5,5′dithiobis-(2-nitrobenzoate) and aldrithiol-2 at various concentrations on the activity of bovine erythrocyte acetylcholinesterase and horse serum butyrylcholinesterase*

The substrates used in (*a*) were acetylthiocholine iodide (0.1 mM) and butyrylthiocholine iodide (0.1 mM). The activities were measured in the spectrophotometer at 30°C. In (*b*) the Warburg technique was used (at 25°C) with butyrylcholine (10 mM) as substrate. Open symbols represent the presence of 5,5′-dithiobis-(2-nitrobenzoate), and closed symbols the presence of aldrithiol-2. △, Acetylcholinesterase; ○, butyrylcholinesterase. The arrow in (*a*) indicates the disulphide concentration normally used, and that in (*b*) the activity obtained when no disulphide was present.

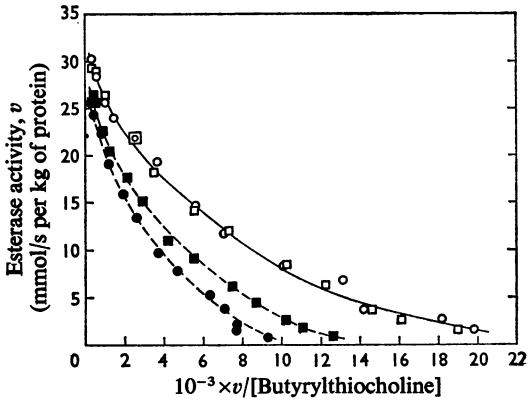

Fig. 2. *Effects of 5,5'-dithiobis-(2-nitrobenzoate) and aldrithiol-2 on the activity of butyrylcholinesterase over a wide range of substrate concentrations*

The results are shown as Augustinsson–Hofstee plots, and the butyrylthiocholine concentration was varied over the range 0.01–10mM in the presence of: ■, 0.35mM-aldrithiol-2; ●, 0.10mM-aldrithiol-2; □, 0.35mM-5,5'-dithiobis-(2-nitrobenzoate); ○, 0.10mM-5,5'-dithiobis-(2-nitrobenzoate).

Comparison of cholinesterase activity in the presence and absence of disulphides by a spectrophotometric technique

To be sure that 5,5'-dithiobis-(2-nitrobenzoate) does not influence the cholinesterase activity even at low substrate concentrations, another method (described in the Materials and Methods section) was used. Fig. 3 shows no significant difference between the activity with 5,5'-dithiobis-(2-nitrobenzoate) in the reaction mixture and that obtained in the absence of disulphide.

The difference in extinction at zero time was due to the fact that the disulphide concentration was somewhat higher (0.35mM rather than 0.30mM) in the final stopped mixture when the disulphide was also present in the reaction mixture.

Fig. 4 demonstrates that aldrithiol-2 caused a significant inhibition of the enzyme activity. Several other substrate concentrations were also used in this experiment and the results were essentially the same as those shown in Figs. 3 and 4.

Similar results were obtained when the same assay procedure was tested with acetylcholinesterase and acetylthiocholine.

Effect of physostigmine on the extinction measured in the spectrophotometric assay

The reference and sample cuvettes were filled with the same solution (at pH7.4) without cysteine for

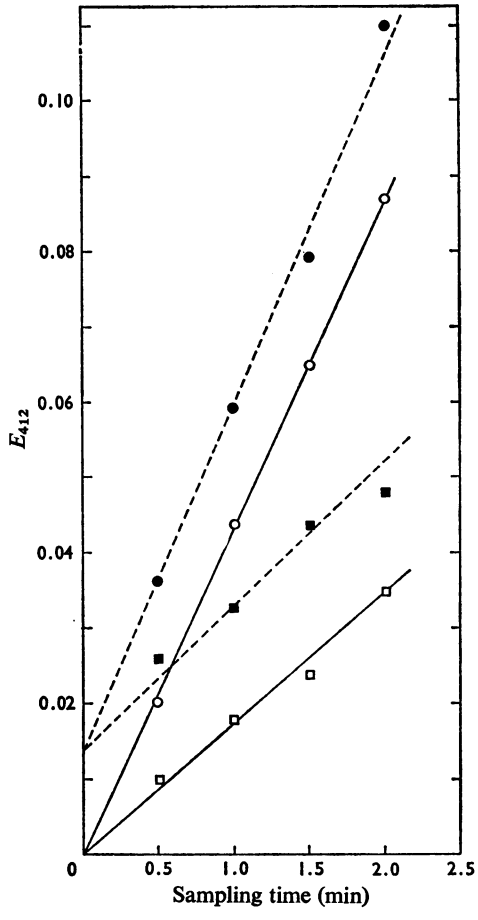

Fig. 3. *Effect of 5,5'-dithiobis-(2-nitrobenzoate) on the activity of butyrylcholinesterase*

The results are presented as increases in extinction at 412 nm as a function of time. Open symbols indicate that 5,5'-dithiobis-(2-nitrobenzoate) was present in the reaction mixture and closed symbols that 5,5'-dithiobis-(2-nitrobenzoate) was added with the stopping mixture. ○, 0.1mM-Butyrylthiocholine; □, 0.05mM-butyrylthiocholine. For experimental details see the Materials and Methods section.

determination of the molar extinction coefficients. The determinations were made over a wide range of cysteine concentrations (0.001–0.1mM) and within this range the extinction seemed to be a linear function of the cysteine concentration. Fig. 5 shows part of the ranges and demonstrates that in the presence of physostigmine the extinction decreased and continued to fall with time when 5,5'-dithiobis-(2-nitrobenzoate) was used as the disulphide. This decrease was shown to be not due to autoxidation of cysteine.

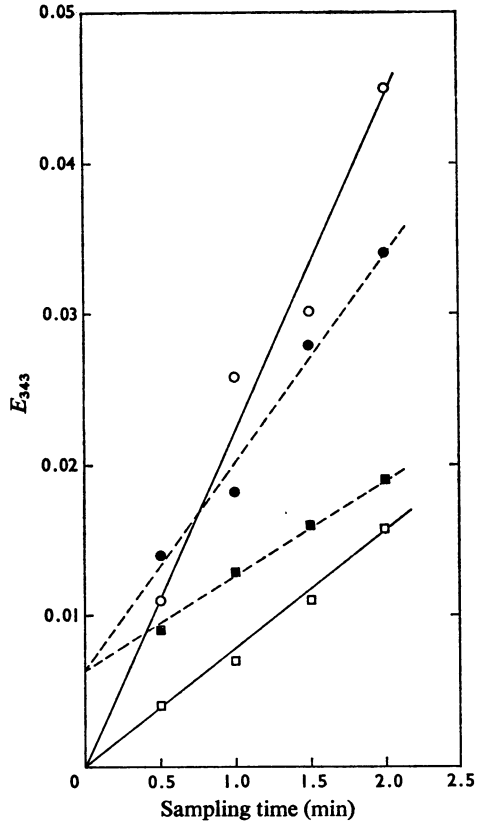

Fig. 4. *Effect of aldrithiol-2 on the activity of butyrylcholinesterase*

Methods and symbols are as indicated in Fig. 3, except that aldrithiol-2 was used instead of 5,5'-dithiobis-(2-nitrobenzoate).

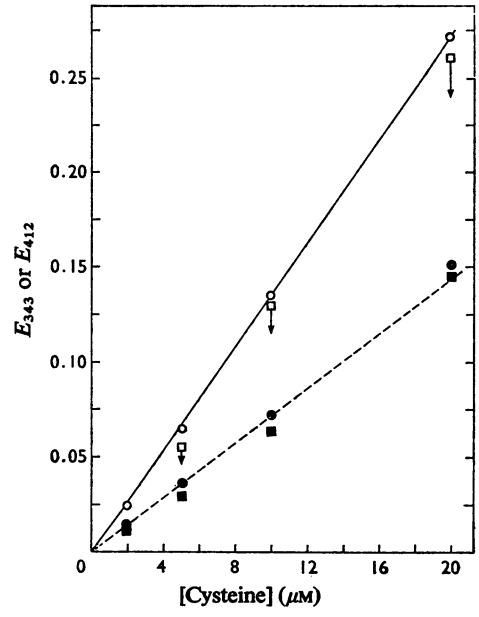

Fig. 5. *Effect of physostigmine on the extinction when 5,5'-dithiobis-(2-nitrobenzoate) and aldrithiol-2 are used as coupling disulphides at pH 7.4*

The extinctions indicated are those observed a few seconds after addition of cysteine to the buffered solutions. The phosphate buffer (pH 7.4), in which the extinction was observed, also contained: ○, 0.35mM-5,5'-dithiobis-(2-nitrobenzoate); □, 0.35mM-5,5'-dithiobis-(2-nitrobenzoate) and 1.0mM-physostigmine sulphate; ●, 0.35mM-aldrithiol-2; ■, 0.35mM-aldrithiol-2 and 1.0mM-physostigmine sulphate. The arrows indicate the decrease in extinction during the first 5min after the addition of cysteine.

Even the immediate effect of physostigmine in the presence of 5,5'-dithiobis-(2-nitrobenzoate) was greater than that observed in the presence of aldrithiol-2. In the presence of another carbamate, prostigmine bromide, a decrease in extinction was also observed immediately after the addition of cysteine to the 5,5'-dithiobis-(2-nitrobenzoate)–carbamate solution, but on standing no further decrease appeared. In the presence of prostigmine, no significant effect on the molar extinction coefficient of 2-thiopyridine was observed.

Discussion

The results presented in this paper show that 5,5'-dithiobis-(2-nitrobenzoate) at the concentrations tested does not have any effect on the cholinesterase activity, and that aldrithiol-2 inhibits both types of cholinesterases investigated in a competitive way. These conclusions contradict those reported by Brownson & Watts (1973) although they are not inconsistent with their data. We suggest that this difference can be explained by the fact that the molar extinction coefficient is lowered in the presence of physostigmine, more so with 5,5'-dithiobis-(2-nitrobenzoate) than with aldrithiol-2 as coupling disulphide.

That effect probably depends on a carbamoylation of the liberated chromophore, which can be expected to be stronger with 5,5'dithiobis-(2-nitrobenzoate) owing to the stronger nucleophilicity of the ion liberated. This hypothesis is further emphasized by the fact that the effects of prostigmine were weaker, probably owing to the weaker electrophilicity of its carbamoyl carbon. A spectral investigation was also made with 5,5'-dithiobis-(2-nitrobenzoate)–physostigmine and it was observed that the extinction around 410nm decreased with time and that the

extinction increased in the region 290–340nm, as expected.

The results reported here suggest a continued use for 5,5′-dithiobis-(2-nitrobenzoate) in esterase determinations, especially where the quantitative aspects are of importance. Aldrithiol-2 could have limited usefulness when highly coloured (e.g. haemoglobin-containing) solutions are used. However, even with 5,5′-dithiobis-(2-nitrobenzoate) caution should be exercised if interfering compounds are included in the assay mixture. The possibility that these might arise in crude blood samples etc. should not be neglected.

This investigation was supported by grants (to K.-B.A.) from the Swedish Natural Science Research Council. We thank Mrs. Inga Andersson for her valuable advice and assistance with the Warburg technique.

References

Augustinsson, K.-B. (1957) *Methods Biochem. Anal.* **5**, 1–63

Augustinsson, K.-B., Axenfors, B. & Elander, M. (1972) *Anal. Biochem.* **48**, 428–436

Brownson, C. & Watts, D. C. (1973) *Biochem. J.* **131**, 369–374

Ellman, G. L., Courtney, K. D., Andres, V., Jr. & Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7**, 88–95

Grassetti, D. R. & Murray, J. F., Jr. (1967) *Arch. Biochem. Biophys.* **119**, 41–49