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The Modification of Cholinesterase Activity by 5,5′-Dithiobis-(2-nitrobenzoic Acid) included in the Coupled Spectrophotometric Assay

EVIDENCE FOR A NON-CATALYTIC SUBSTRATE-BINDING SITE

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1. Compared with the acetylcholinesterase assay carried out in the absence of a dithiol, the presence of 5,5′-dithiobis-(2-nitrobenzoic acid) caused marked activation, 6,6′-dithiodinicotinic acid and 2,2′-dithiobis-(5-nitropyridine) less so and 2,2′-dithiodipyridine (aldrithiol-2) had no effect at all. Measurements are further complicated in that the 5-thio-2-nitrobenzoate ion also appears to interact with the enzyme, resulting in slightly lowered absorbance values. 2. Acetylthiocholine competes for the 5,5′-dithiobis-(2-nitrobenzoic acid)-binding site so that activation is essentially eliminated by saturating concentrations of substrate. The presence of the dithiol decreases the K_m value of acetylthiocholine. 3. Similar results were obtained with pseudocholinesterase. However, with butyrylthiocholine clear activation was still observed under $V_{max.}$ conditions in addition to K_m being lowered. 4. All the data yielded Hill coefficients of 1 and analysis of the results leads to the conclusion that activation results from the dithiol being bound to a site on the subunit that is actively catalysing ester hydrolysis. 5. The use of aldrithiol-2 is recommended for kinetic work where absolute quantitative measurements are required.

At the present time there are two popular methods for assaying for cholinesterase activity. The first is by titration of protons liberated in the reaction by using a pH-stat assay (Rosenberry *et al.*, 1972). The second uses a modified substrate, acetylthiocholine, so that thiocholine liberated during catalysis may be estimated by disulphide exchange with 5,5′-dithiobis-(2-nitrobenzoic acid), which yields the coloured 5-thio-2-nitrobenzoate ion (Ellman *et al.*, 1961). The attraction of the spectrophotometric assay is its ease of use, requiring only conventional laboratory equipment, its freedom from maintenance problems that usually attend titrimetric equipment and its potential versatility.

The work reported here arose from our interest in extending the useful pH range of the dithiol-coupled assay. It was found that Nbs_2* interacts with both acetylcholinesterase and pseudocholinesterase in a way that renders this assay largely valueless for quantitative, mechanistic investigations. An alternative, though less sensitive, assay system is suggested.

Experimental

Materials

Chemicals. Acetylthiocholine chloride and butyrylthiocholine chloride were obtained from Sigma (London) Chemical Co. Ltd., London W.6, U.K. Dithiols were obtained from Ralph N. Emanuel Ltd., Wembley, Middx. HA0 1PY, U.K. Lubrol W flakes were a gift from I.C.I. Ltd., London S.W.1, U.K. *NN*-Di-(2-hydroxyethyl)glycine (bicine) was prepared as described previously (Milner-White & Watts, 1971). Other chemicals were of the best grade obtainable from British Drug Houses Ltd., Poole, Dorset, U.K.

All solutions were made up in double glass-distilled water.

Buffers. Sodium phosphate buffer (50mM) was prepared by adjusting to pH7.4 a solution of NaH_2PO_4 with 1M-NaOH before it was made up to the final volume. Bicine buffer (50mM), pH8.5, was prepared in the same way. All pH measurements were made at room temperature with a Radiometer pH-meter 25 fitted with a scale expander.

Enzymes. Human erythrocyte 'ghosts' were used, prepared by a slight modification of the method of Dodge *et al.* (1962).

Preparation of erythrocyte 'ghosts'

Whole blood was collected into tubes containing lithium–heparin. The plasma and buffy coat were removed after centrifugation in the usual way at room temperature and the erythrocytes washed three times in iso-osmotic saline at room temperature. 5mM-Sodium phosphate buffer, pH7.4 (10vol. relative to the packed erythrocyte volume), was added to the washed cells and the resulting suspension

* Abbreviation: Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid).

was stirred for 30min at room temperature. After centrifuging for 1h at 25000*g* at 4°C in the MSE High-Speed 18 centrifuge the supernatant was discarded and the lysed cell 'ghosts' were washed a further three or four times until they were a milky-white colour. The wash in salt solution used by Dodge *et al.* (1962) was not found to be necessary.

Enzymic methods

Solubilization of acetylcholinesterase. The protein concentration of the 'ghosts' in the final suspension was measured by the method of Lowry *et al.* (1951) and usually adjusted to 4mg/ml. Enzyme release from the membranes was obtained by adding an equal volume of a solution of Lubrol W flakes, containing sufficient of the detergent to give an absorbance = 0.1 at 233nm (equal to $\lambda_{max.}$) for each mg of protein/ml in the original 'ghost' suspension. The mixture was incubated for 30min at 30°C and then centrifuged at 100000*g* for 1h in the 5ml swing-out head of the Christ Omikron ultracentrifuge at 4°C. For an initial protein concentration of 4mg/ml in the washed 'ghost' suspension a final protein concentration of approx. 1mg/ml in the enzyme-containing supernatant was obtained.

Pseudocholinesterase. A suitably diluted solution of the plasma obtained in the preparation of acetylcholinesterase was used as the source of this enzyme.

Enzyme assays. All measurements were made with a Cary 16S recording spectrophotometer thermostatically controlled at 30°C and by using a final volume of 0.5ml in quartz semi-micro cells of 10mm path length.

(a) Assay in the presence of a dithiol. All dithiols were present in a final concentration of 0.35mM, as determined from the appropriate extinction coefficients.

For the determination of acetylcholinesterase activity acetylthiocholine was used at five or six concentrations in the range 0.02–0.1mM. Assays were carried out in duplicate with 10 or 20μl of enzyme solution containing approx. 0.1mg of protein/ml to start the reaction. The change in absorbance was followed at the wavelength appropriate to the dithiol being used.

In the first series of experiments, because of the insolubility of 2,2′-dithiobis-(5-nitropyridine), all the dithiols were dissolved in buffer containing 10% (v/v) formamide. This concentration of formamide affected neither the rate of catalysis nor the extinction coefficient of the thiol. In subsequent assays the insoluble dithiol was not used and the formamide was omitted from the assay mixtures.

(b) Assay in the absence of a dithiol. With a final assay volume of 2ml, acetylthiocholine was added to test tubes to give eight different concentrations in the range 0.02–0.1mM in the appropriate buffer. The tubes were equilibrated at 30°C and the enzyme was added to initiate the reaction. Efficient mixing was achieved by means of a Vortex mixer. After appropriate time-intervals samples (0.2ml) were transferred to a stopping mixture, containing, in 0.3ml, dithiol (0.5mM) and physostigmine sulphate (1.5mM), and mixed as before. The absorbance of the sample was then measured at the wavelength appropriate for the dithiol used. With Nbs_2 the sampling times were 1.5, 3.5, 5.5 and 7.5min and with aldrithiol-2 were 2, 4, 6 and 8min.

With both assay procedures the progress curves were linear over the measured range. Initial velocities were measured as the slopes of these plots and are expressed as μmol of substrate hydrolysed/min per ml of spun Lubrol-treated enzyme.

For the Lineweaver–Burk plots the lines of best fit to the data were determined by using an Olivetti P203 Computer with a program for linear regression analysis placing equal emphasis on all points.

Results

Absorption maxima and molecular extinction coefficients of dithiol chromophores

Because of the importance of knowing the exact molecular extinction coefficients of the different dithiols for calculating enzyme reaction rates it was essential to determine these under the conditions used in the present work. With a standard cysteine solution in phosphate buffer, pH7.4, ten determinations were carried out by adding known amounts to an excess (0.35mM) of dithiol. The liberation of chromophore was a linear function of the cysteine concentration over the range measured (0.02–0.12mM). Table 1 compares the values found with the previous literature values at the absorption maxima used and it can be seen that there are no significant differences. The coefficients for Nbs_2 and aldrithiol-2 were also determined without formamide and the same results obtained. Oxidation of the accumulated free thiol in the second assay procedure was found to be 5% of the total. This has not been corrected for in the figures as it does not significantly affect the results and because a small measure of oxidation, that is not readily measurable, also probably occurs even in the presence of the dithiol.

It should be pointed out that the term molecular extinction coefficient is, strictly speaking, an erroneous description of the constants obtained. Rather, they are calibration constants representing the amount of chromophore liberated from the dithiol upon addition of a known amount of cysteine. As this is an equilibrium reaction the amount of chromophore liberated may not equal the amount of added thiol, so a known concentration of pure chromophore would not necessarily give the theo-

Table 1. *Absorption maxima and molecular extinction coefficients of dithiols*

Dithiol	$\lambda_{max.}$ (nm)	Molecular extinction coefficient (M) Present work	Previous work	Reference
5,5′-Dithiobis-(2-nitrobenzoic acid)	408	1.36×10^4	1.36×10^4	Ellman (1959)
2,2′-Dithiodipyridine (aldrithiol-2)	343	7.06×10^3	7.06×10^3	Grassetti & Murray (1967)
2,2′-Dithiobis-(5-nitropyridine)	392	1.35×10^4	1.4×10^4	Grassetti & Murray (1969)
6,6′-Dithiodinicotinic acid	342	1.00×10^4	1.0×10^4 at λ344nm	Grassetti *et al.* (1969)

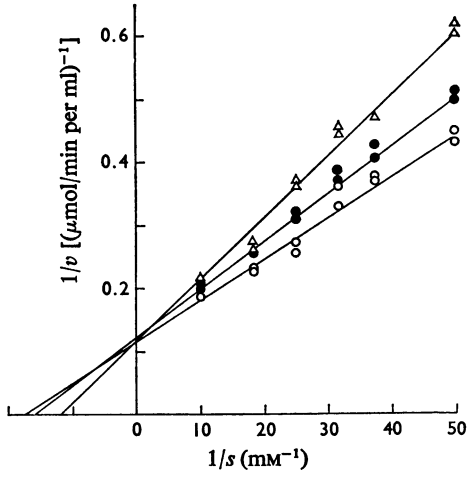

Fig. 1. *Effects of various dithiols on the activity of human erythrocyte acetylcholinesterase at pH* 7.4 *and* 30°*C*

The acetylthiocholine concentration was varied over the range 0.02–1.0mM in the presence of: ○, 5,5′-dithiobis-(2-nitrobenzoic acid); ●, 6,6′-dithiodinicotinic acid; △, aldrithiol-2. The final concentration of dithiol in the assay mixture was 0.35mM.

retical absorbance calculated from the extinction coefficients quoted here. This does not matter for the type of assay described here so long as the equilibrium of the dithiol of the exchange reaction does not alter or, alternatively, an appropriate calibration curve is used. In fact, the linearity of the determined calibration curve indicates that the equilibrium does not shift over the range of thiol measured in the cholinesterase assay. The term molecular extinction coefficient has been retained here, because this has been firmly established by past usage, as the description of the values listed in Table 1 obtained by other workers using the general procedure described above.

Effect of dithiols on the reaction of acetylcholinesterase

The effects of equimolar concentrations of different dithiols on the reaction of acetylcholinesterase at various substrate concentrations are shown as Lineweaver–Burk plots in Fig. 1. It can be seen that at low substrate concentrations maximum activity is obtained with Nbs_2, minimum activity with aldrithiol-2 and that with 6,6′-dithiodinicotinic acid falls between the two. As the substrate concentration is raised the difference in activity becomes progressively less and $V_{max.}$ is essentially the same regardless of the dithiol used. The difference in rates appears to be largely an effect on K_m, that obtained in the presence of Nbs_2 being 0.055mM as compared with 0.06mM in 6,6′-dithiodinicotinic acid and 0.083mM in aldrithiol-2.

The plot with 2,2′-dithiobis-(5-nitropyridine) is not shown in Fig. 1, because the data showed considerable scatter, owing to difficulties in obtaining a satisfactory solution of the dithiol even in the presence of 10% formamide. However, in other experiments not directly comparable with Fig. 1, the results were essentially the same as those obtained with 6,6′-dithiodinicotinic acid.

Subsequent investigations were carried out with Nbs_2 and aldrithiol-2 only.

Comparison of acetylcholinesterase activity in the presence and absence of dithiols

The same fresh enzyme preparation was used for these experiments so that the data in Figs. 2 and 3 are directly comparable. Fig. 2 shows that at pH7.4 the presence of Nbs_2 in the assay mixture causes a marked activation of the enzyme compared with the same mixture without the dithiol. In contrast, when aldrithiol-2 is used as the coupling dithiol at pH7.4, the Lineweaver–Burk plot is superimposable on that obtained without dithiol in the assay mixture (Fig. 3*a*).

As was explained in the Experimental section, measurement in the absence of dithiol was made by transferring samples at measured time-intervals into physostigmine solution, to inhibit the enzyme. The dithiol used for the linked assay is also present to react with acetylthiocholine liberated. Since in these experiments the dithiol is being used only to determine acetylthiocholine after the reaction has been

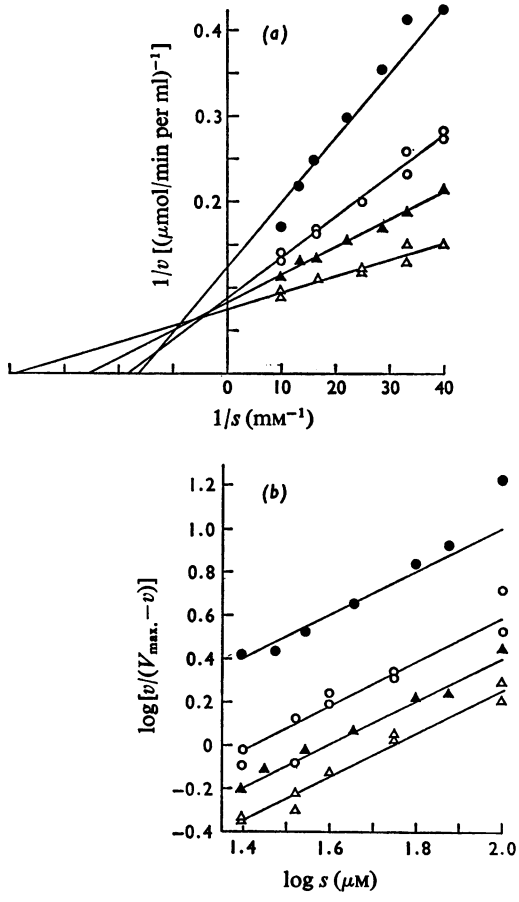

Fig. 2. *Effect of 5,5′-dithiobis-(2-nitrobenzoic acid) on the activity of human erythrocyte acetylcholinesterase at 30°C*

The results are expressed (*a*) as Lineweaver–Burk plots and (*b*) as Hill plots. The acetylthiocholine concentration was varied over the range 0.025–1 mM at pH7.4 in the absence (●) and the presence (○) of 5,5′-dithiobis-(2-nitrobenzoic acid) and at pH8.5 in the absence (▲) and the presence (△) of 5,5′-dithiobis-(2-nitrobenzoic acid). Details of the assay procedure are given in the Experimental section.

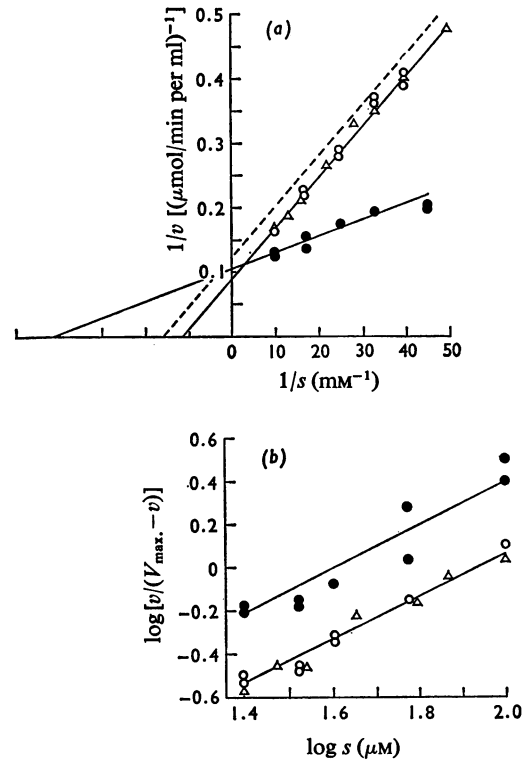

Fig. 3. *Effect of aldrithiol-2 on the activity of human erythrocyte acetylcholinesterase at 30°C*

The results are expressed (*a*) as Lineweaver–Burk plots and (*b*) as Hill plots. The acetylthiocholine concentration was varied over the range 0.02–1 mM at pH7.4 in the presence (○) and the absence (△) of aldrithiol-2 and at pH8.5 in the presence (●) of aldrithiol-2. Details of the assay procedure are given in the Experimental section. The broken line shows the plot, taken from Fig. 2(*a*), in which 5,5′-dithiobis-(2-nitrobenzoic acid) is not added until the enzyme reaction has been stopped.

stopped it might be expected that Nbs_2 and aldrithiol-2 would give identical Lineweaver–Burk plots. In fact, as indicated in Fig. 3(*a*), the two plots are not superimposable, with that for Nbs_2 appearing to give less activity by approximately the same amount at all substrate concentrations. This is also apparent in Fig. 2(*a*) as an increase in $1/V_{max.}$ as compared with the value obtained in the presence of Nbs_2. The kinetic constants derived from these data are listed in Table 2.

At pH8.5, the optimum value for enzyme activity under these conditions, the pattern of activity in the presence and absence of Nbs_2 is the same as that found at the lower pH (Fig. 2*a*). Again the Lineweaver–Burk plot in the presence of aldrithiol-2 (Fig. 3*a*) is similar but not quite identical with that obtained by adding Nbs_2 after the reaction had been stopped. The kinetic constants from these data are also listed in Table 2.

Table 2. *Kinetic constants for acetylthiocholine with human erythrocyte acetylcholinesterase*

The data are calculated from Figs. 2 and 3.

pH	Dithiol present: In reaction mixture	Dithiol present: In assay mixture	$V_{max.}$ (μmol/min per ml)	K_m (μM)
7.4	Nil	Aldrithiol-2	11.3	88
7.4	Nil	Nbs_2	8.0	38
7.4	Aldrithiol-2	Aldrithiol-2	10.9	84
7.4	Nbs_2	Nbs_2	11.5	54
8.5	Nil	Nbs_2	12.0	38
8.5	Nbs_2	Nbs_2	14.0	25
8.5	Aldrithiol-2	Aldrithiol-2	9.4	23

Effects of dithiols on pseudocholinesterase

Fig. 4(*a*) shows that, with acetylthiocholine as substrate, Nbs_2 markedly activates pseudocholinesterase, as compared with assay in the presence of aldrithiol-2. As with acetylcholinesterase, the effect decreases as the substrate concentration is raised and the $V_{max.}$ values, 4.08 and 4.36 μmol/min per ml, are not significantly different. The K_m values obtained were 0.032mM in the presence of Nbs_2 and 0.12mM in the presence of aldrithiol-2.

With butyrylthiocholine as substrate both K_m and $V_{max.}$ are altered (Fig. 5*a*). In the presence of Nbs_2 the values for $V_{max.}$ and K_m were 6.12 μmol/min per ml and 0.033mM respectively, whereas with aldrithiol-2 as the coupling agent they were 4.08 μmol/min per ml and 0.059mM.

Discussion

From the results presented here it is clear that Nbs_2 activates both acetylcholinesterase and pseudocholinesterase. With acetylcholinesterase the degree of activation is greater at pH7.4 than at pH8.5 and at both pH values the effect is progressively decreased as the substrate concentration is increased.

The activation by Nbs_2 might occur in one of two possible ways. It might bind only to a subunit that is not hydrolysing acetylcholine. Activation would then be mediated by a conformational change in the subunit binding the Nbs_2 being transmitted to the active subunit. This hypothesis would be compatible with the substrate displacing the Nbs_2 as it progressively saturates the enzyme. However, there is no evidence for changes in the subunit organization of either enzyme from Hill plots, which remained with a slope of unity under all the conditions tested for both enzymes (Figs. 2*b*, 3*b*, 4*b* and 5*b*). This is in contrast to other compounds such as atropine that induce sigmoid kinetics (albeit using the Nbs_2-coupled assay) and change the Hill coefficient (Kato *et al.*, 1972).

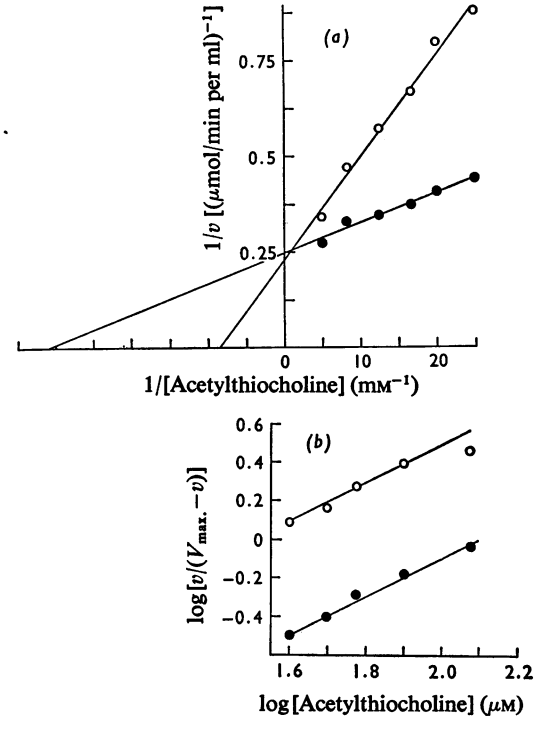

Fig. 4. *Effects of dithiols on the acetylthiocholinesterase activity of human pseudocholinesterase at pH7.4 and 30°C*

The results are expressed (*a*) as Lineweaver–Burk plots and (*b*) as Hill plots. ○, 0.35mM-Aldrithiol-2 present; ●, 0.35mM-5,5′-dithiobis-(2-nitrobenzoic acid) present.

Further, this hypothesis cannot readily explain the finding that butyrylthiocholine is unable to displace Nbs_2 from pseudocholinesterase so that the enzyme is

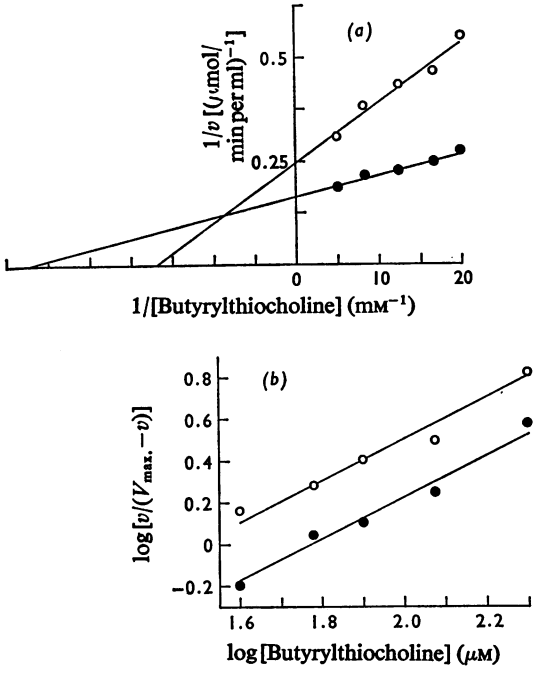

Fig. 5. *Effects of dithiols on the butyrylthiocholinesterase activity of human pseudocholinesterase at pH 7.4 and 30°C*

The results are expressed (*a*) as Lineweaver–Burk plots and (*b*) as Hill plots. ○, 0.35mM-Aldrithiol-2 present; ●, 0.35mM-5,5′-dithiobis-(2-nitrobenzoic acid) present.

still activated, as compared with the activity in the presence of aldrithiol-2, even under $V_{max.}$ conditions. In the light of this evidence the more probable explanation is that Nbs_2 binds to the catalytically active subunit and either directly influences the catalytic process or causes a limited conformational change that does not affect subunit interactions. The Nbs_2 would be displaced from this site by high concentrations of acetylthiocholine, but not completely, for pseudocholinesterase, by butyrylthiocholine. This is compatible with the finding that eel acetylcholinesterase also binds acetylcholine at sites on the enzyme other than the catalytic site (Changeux, 1966).

Another problem with Nbs_2 is that in the presence of the acetylthiocholine assay mixture the 5-thio-2-nitrobenzoate ion appears to possess a lower molecular extinction coefficient than it does in the presence of a simple thiol alone. This can lead to a significant change in $V_{max.}$ as indicated by Figs. 2(*a*) and 3(*a*) and Table 2. Since the molecular extinction coefficients of the dithiols used in this work were very carefully checked (Table 1), we consider that this error probably arises from interaction between the 5-thio-2-nitrobenzoate ion and the enzyme.

Thus although the coupled assay with Nbs_2 is both sensitive and easy to use the nature of the interactions with both human acetylcholinesterase and pseudocholinesterase, and probably cholinesterase from other sources as well, makes it quite unsuitable for reliable kinetic investigations. Aldrithiol-2 on the other hand appears to be free from any of these difficulties and has the added advantage that it can be used over a much wider range of pH values. The disadvantage of aldrithiol-2, apparent from Table 1, is that its molecular extinction coefficient is only about half that of Nbs_2. For this reason, although aldrithiol-2 becomes the dithiol of choice for pure kinetic work, Nbs_2 will still remain of considerable value for comparative work such as characterizing genetic variants of cholinesterase that may only be available in small amounts and where the extra sensitivity of the assay becomes the more important factor.

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