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THE POLAROGRAPHIC DETERMINATION OF SULFHYDRYL GROUPS

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A number of electrometric methods for determination of sulfhydryl (SH) groups have been evolved. Examples of these methods include: polarographic measurement of the SH anodic wave (Kolthoff and Barnum, 1940); amperometric titration with the rotated platinum electrode (Kolthoff, *et al.*, 1954); and measurement of the catalytic evolution of hydrogen produced by SH groups at the dropping mercury cathode (Heyrovsky and Babicka, 1930; Birdicka, 1933). The fact that p-chlormercuribenzoate is a highly specific reagent for mercaptide formation with SH groups and that Benesch and Benesch (1953) demonstrated that SH groups produce a diminution of the first polarographic reduction wave height of phenylmercuric hydroxide has focussed attention on the possible utility of p-chloromercuribenzoate (PCMB) as a reagent for polarographic determination of SH groups in amino acids, proteins, and enzyme homogenates. It was reasoned that any diminution of the first wave of the PCMB might be attributable to quantitative production of a mercaptide (from the SH compound and the PCMB) which would be more resistant to polarographic reduction (*i.e.*, reduced at a more negative potential) than the PCMB. Test of this proposition has been undertaken in the work reported here. Use of the method which has resulted from this work in connection with drug action mechanism studies will be discussed.

Materials

Amino acids and peptides used included cysteine hydrochloride, glycine, cystine, and reduced glutathione. All of these were products of Eastman Kodak Co. Stock solutions of cysteine hydrochloride, glycine, and glutathione were prepared at 10^{-3} M concentration in air-free water freshly distilled from a pyrex still. Stock solution of the cystine consisted of 10^{-3} M cystine in 0.1N sodium hydroxide made up with pyrex glass distilled water. Fresh stock solutions of these substances were prepared each day. Proteins used were crystalline bovine plasma albumin and crystalline zinc insulin, both of which were products of Armour Laboratories. The albumin was found to contain water (6-7%) and was always heated at 110° C. to constant weight before use. The activity of the insulin preparation was 25 units per mg. $\pm$ 5%. Parachloromercuribenzoic acid was synthesized according to the method of Whitmore and Woodard (1932). Reagent solutions of the PCMB were 8.4×10^{-4} M and were prepared by adding 3 ml. of 0.1 N potassium hydroxide to 30 mg. of the acid and 20 ml. of distilled water in a 100 ml. volumetric flask, shaking to dissolve the acid, and diluting to the mark

with distilled water. The buffer solution used in the determinations was a M/15 phosphate buffer, pH 7.4, made up according to the method of Hastings and Sendroy (1924: 706). All chemicals used were C. P. reagent products unless otherwise indicated.

A model XXI Sargent recording polarograph was used in all determinations. The capillary used for the dropping mercury electrode had the following characteristics: m = 3.76 mg./second, t= 2.56 seconds/drop at –0.4 V vs. the Hg pool. The mercury pool served as reference electrode. In most cases the current sensitivity used was 0.010 μa/mm.

Methods

Proportionality between PCMB concentration and the first reduction wave of the PCMB was ascertained by adding varying volume increments of the PCMB reagent solution to 5 ml. of the phosphate buffer, effecting final dilution to 10 ml. with distilled water in each case, and polarographing after oxygen had been removed by passing nitrogen through the solutions for 10 minutes. Determinations of the effect of amino acids, peptides, and proteins on the first polarographic reduction wave of the PCMB were carried out by adding aliquots of appropriately diluted stock solutions of these substances to 5 ml. of phosphate buffer and 2 ml. of the PCMB reagent solution, making the volume to 10 ml. with distilled water, and polarographing following deaeration as before. The effect of appropriate aliquots of homogenate solutions was determined in the same manner. The polarographic effect of all biochemical substances tested was compared with a polarogram from a control solution of equal PCMB concentration run at the same time.

Results

The two polarographic waves yielded from the reduction of the PCMB are shown in Figure 1. The first wave has a half-wave potential ($E_{1/2}$) of –0.28 V vs. the Hg pool, while the second wave $E_{1/2}$ occurred at –0.79 V vs. the Hg pool. Figure 2 shows that a straight line relationship exists when the PCMB first wave height is plotted against PCMB concentration under the polarographic conditions used. It was found that cysteine hydrochloride, in the concentrations tried (from 5 μg. to 25 μg./ml.), effected diminution of the first PGMB wave height and that this reduction in wave height was proportional to the cysteine concentration. This relationship is illustrated in Figure 3. Table 1 illustrates typical results obtained from SH determination in amino acids, peptides and proteins. These results indicate that fairly ac-

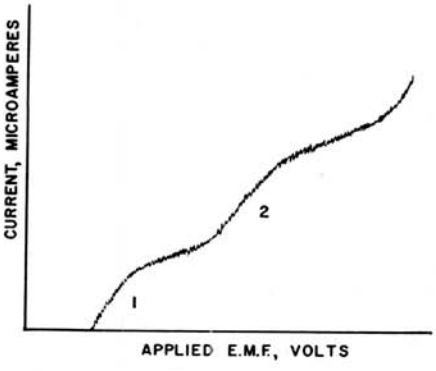

Figure 1

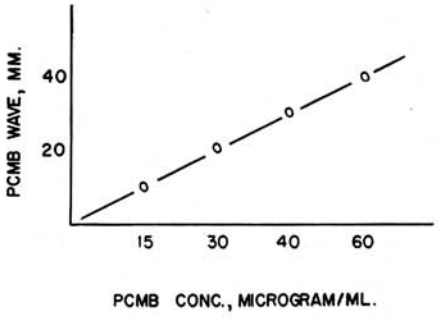

FIGURE 2

curate determination of the SH content of these compounds can be effected using the method and that disulfide compounds (*i.e.*, insulin, cystine) are not likely to interfere with the determination of SH by this procedure. Tissue homogenates of heart and kidney caused diminution of the first PCMB wave that was proportional to the homogenate volume increment added to the PCMB-buffer solution.

DISCUSSION

The results obtained would seem to indicate that the method evolved for SH determination is capable of a wide range of utility. It appears to be applicable to SH determination in large and small molecules with reasonable accuracy at low SH concentrations. The experiments demonstrate that in all probability the method is relatively free from interference due to likely contaminants.

Comparison with other available methods reveals that it is probably superior to indicator titration methods and equal in accuracy and utility to amperometric titration procedures. At its present stage of development the method is more accurate than the method employing use of catalytic evolution of hydrogen measurement (Birdicka, 1933), but it is less versatile than this method which can be used to measure disulfide as well as SH.

Use of the procedure being reported here has already been made in our laboratory in the course of studies of the mechanism of the action of drugs. We have advanced an hypothesis on the mechanism of cardiac glycoside action on the heart, the details of which are in press elsewhere (Proctor, *et al.*, 1955). This hypothesis postulates cardiac glycoside inhibition of heart adenosinetriphosphatase (ATPase) and acetylcholinesterase (AcHase), both of which require free SH groups for activity. Cardiac glycosides have an unsaturated lactone ring (located at C-17 in the steroid moiety of the molecule) which is capable of SH reactivity in two possible ways. Addition of SH to the carbon-carbon double bond can occur and the lactones can form peroxides (Kirkland, *et al.*, 1950) which could in turn oxidize SH to disulfide. Our work to date has demonstrated that digi-

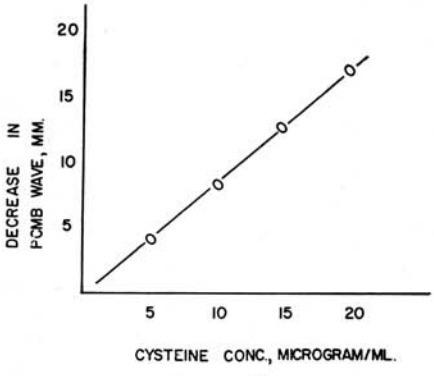

FIGURE 3

TABLE 1.—Sulfhydryl Determination in Various Compounds.

Compound	SH	1st PCMB wave depression	Calculated mole SH/mole compound	
			found by PCMB polarography	theoretical
cystine	absent	—	none	none
glycine	absent	—	none	none
glutathione[1]	present	+	1.02, 1.01, 1.00, 0.98	1.00
albumin[2]	present	+	0.60, 0.65, 0.66, 0.61	0.66
insulin	absent	—	none	none

[1] Reduced form, last two determinations made in the presence of cystine.
[2] Molecular weight assumed to be 70,000.

toxin, at concentrations approximating levels reached *in vivo* in therapeutic dosage, brings about *in vitro* inhibition of the two enzymes in heart muscle of dogs and rabbits. This inhibition is enhanced by effecting glycoside lactone ring peroxide formation (Proctor, *et al.*, 1955). Using homogenates from these experiments it has been ascertained by the PCMB method for SH determination that the inhibition observed correlates qualitatively with the degree of SH "lost" from the homogenate in the presence of the glycoside. While these findings do not definitely establish the SH-digitoxin mechanism postulated by the hypothesis (this must await similar study of digitoxin effect on purified enzymes) the qualitative correlation obtained gives preliminary support to the hypothesis. It is also of interest to note that the digitoxin enzyme inhibitions can be reversed by SH compounds.

SUMMARY

An indirect polarographic method for the determination of sulfhydryl groups in amino acids, peptides, and proteins has been described. Essentially the determination consists of measuring the quantitative depression of the wave height of the first polarographic reduction wave of p-chloromercuribenzoate caused by sulfhydryl compounds.

Accuracy and comparative advantage of the method in relationship to other methods has been discussed.

Application of the method in a pharmacodynamic study has been described.

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