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Polarographic Estimation of Chloramphenicol (Chloromycetin)

GLENN B. HESS, *Chas. Pfizer and Co., Inc., Brooklyn, N. Y.*

A polarographic method for the estimation of chloramphenicol (Chloromycetin) is presented. The reduction of chloramphenicol and of its hydrolysis product, 1-*p*-nitrophenyl-2-amino-1,3-propanediol, at the dropping mercury electrode has been studied at various pH values and the analysis of the polarographic waves is given. The reaction is irreversible and involves four electrons in the first wave, indicating preliminary reduction of the nitro group to the hydroxylamine.

THE development of fermentation and recovery processes for the production of chloramphenicol (Chloromycetin) for use as an antibiotic made necessary the concomitant development of rapid analytical methods for its determination in fermentation broths, concentrates, and solids. Before publication of the structure, it was known (*4*) that chloramphenicol contained covalently bound chlorine and a group giving an aryl amine on treatment with hydrochloric acid and zinc. This reaction, together with diazotization and coupling, has been used as a method for the colorimetric estimation of chloramphenicol (*4*). Because the compound could evidently be reduced and preliminary polarographic tests, without hydrolysis, showed well defined diffusion currents, a study was made of the behavior of chloramphenicol at the dropping mercury electrode, to determine the optimal conditions and to develop an assay method.

After publication of the structure, as *d*-threo-1-*p*-nitrophenyl-2-dichloroacetamido-1,3-propanediol (*5*), it was realized that the nitro group was probably the reactive portion of the molecule, inasmuch as nitro compounds are, in general, reducible at the dropping mercury electrode (*1*, *2*, *6*). For comparative purposes, the biologically inactive free amine, 1-*p*-nitrophenyl-2-amino-1,3-propanediol, obtained by hydrolysis of chloramphenicol with the splitting off of dichloroacetic acid, was prepared and studied under similar conditions to determine the effect of the dichloroaceto group on the polarographic reduction.

APPARATUS

A Sargent Model XXI recording polarograph was used for all the reported work. Two types of cells have been used, an H-type cell containing a saturated calomel electrode in one leg for investigative work, and a flask-type cell with a quiet mercury pool anode for routine use. Separate capillaries have been used with each cell; that for the H-type cell delivered at the rate of 2.588 mg. per second with an $m^{2/3}t^{1/6}$ value of 2.281, whereas that for the flask-type cells delivered at the rate of 2.321 mg. per second with an $m^{2/3}t^{1/6}$ value of 2.184. All determinations were made in a constant temperature room which was controlled at 24° ± 0.5° C.

EXPERIMENTAL

1-*p*-Nitrophenyl-2-amino-1,3-propanediol was prepared by hydrolyzing purified chloramphenicol in a slight excess of 0.5 *N* hydrochloric acid for 0.5 hour at reflux temperature. The solution was cooled, washed with ether and with chloroform to remove the dichloroacetic acid together with any unhydrolyzed chloroamphenicol, and evaporated to dryness under vacuum. The resultant amine hydrochloride was washed with a small amount of alcohol, then with ether, and dried.

For the investigation of the effect of pH on the reduction, master aqueous solutions, approximately 3 millimolar, of chloramphenicol and, later, of the 1-*p*-nitrophenyl-2-amino-1,3-propanediol hydrochloride were prepared. Bioassay potency was taken as the measure of purity of the chloramphenicol. Clark and Lubs buffers, hydrochloric acid–potassium chloride for pH 2, sodium hydroxide–potassium acid phthalate for pH 4 and 6, and sodium hydroxide–potassium chloride–boric acid for pH 8 and 10 were prepared in double strength solution. Just before use, equal volumes of master solution and double strength buffer solution were mixed, and the pH was checked by Beckman pH meter. Dissolved oxygen was removed by bubbling nitrogen through the solution. A trace of thymol was added to the chloramphenicol solutions as a maximum suppressor, because preliminary tests showed thymol to have less depressive effect on the diffusion current than gelatin. Methyl red was used as maximum suppressor for alkaline solutions of the amine hydrochloride and thymol in the acidic solutions.

Because chloramphenicol, buffered at pH 4, was found to give the best-defined and most reproducible first wave, and hydrolysis of the compound could be expected to be minimal at this pH, this buffer value was chosen for the routine determination of chloramphenicol. Potassium acid phthalate, monopotassium phosphate, and pyridine–pyridine hydrochloride buffers have been used with equally good results. The buffers have been prepared as concentrates so that upon dilution with chloramphenicol solutions, the proper pH is achieved. When the chloramphenicol concentrations were corrected for the bioassay potency, it was found that concentrations ranging from 100 to 1000 micrograms per milliliter gave values of the current-concentration ratio in excellent agreement, and that concentrations as low as 30 micrograms per milliliter could be assayed with only slight error.

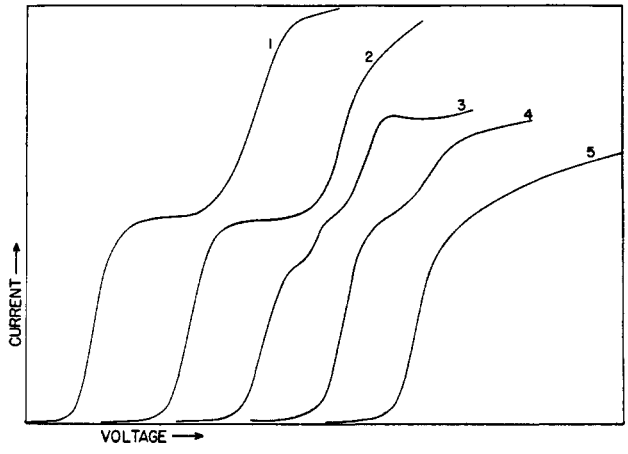

Figure 1. Polarograms of Chloramphenicol

1. In Clark and Lubs buffer at pH 2.2
2. At pH 4.2
3. At pH 6.4
4. At pH 8.1
5. At pH 10.1

Curves have been offset for clarity. For numerical values of $E_{1/2}$, see Table I

In routine assay of chloramphenicol, as solid material or aqueous solution, a solution is prepared 0.2 molar with respect to buffer and containing 300 to 500 micrograms of chloramphenicol when possible, but if necessary as low as 30 micrograms per milliliter. A portion of this solution is placed in the cell, a few crystals of thymol are added, and the solution is deaerated by bubbling for several minutes with wet oxygen-free nitrogen, and then electrolyzed between −0.4 and −1.1 volts versus the quiet mercury pool electrode. The diffusion current is determined by the method of midlines and the concentration of active material calculated from the current-concentration ratio, previously determined from the polarographic waves obtained with several concentrations of three biologically standardized lots.

DISCUSSION

Chloramphenicol and its hydrolysis product, 1-*p*-nitrophenyl-2-amino-1,3-propanediol, gave well defined primary waves which corresponded closely, both in half-wave potential and in diffusion current, in all buffers above pH 2.2. Because of this similarity, it would be impossible to differentiate between chloramphenicol and its hydrolysis product under the conditions used. However, it appears that the hydrolysis product is not a natural decomposition product or by-product, for in the polarographic assay of fermentation broths, concentrates, impure solids, and waste liquors, only a few samples of waste liquor have shown significant differences from the bioassay results. In the assay of 36 consecutive lots of finished material, assayed by polarograph, by the chemical method previously mentioned, and by biological methods, the polarographic assays showed a standard deviation of 3% from the biological assay and of 1.7% from the chemical assay. Polarographic assays were on the average slightly higher than bioassay results and slightly lower than chemical assay results.

The diffusion coefficient of chloramphenicol was calculated from the form of the Stokes-Einstein equation, $D = 3.32 \times 10^{-5}/V^{1/3}$ (*3*), where V is the molar volume. Determination of the density of crystalline chloramphenicol, calculation of the molar volume, and application of this relationship gave a value of 5.59×10^{-6} cm. sec.$^{-1}$ as the diffusion coefficient. Using the diffusion current values obtained at pH 4 in standardizing the routine method, application of the Ilkovič equation gave a value for N of 4.0 electrons. This value indicates that the nitro group of the compound is first reduced to the corresponding hydroxylamine, as has been found the case for other nitro compounds (*1*, *2*). A composite plot of $\log i/(i_d - i)$ versus E gave a straight line with a slope of 0.091, while the Heyrovský-Ilkovič equation requires a slope of 0.015 for a reaction involving 4 electrons. Consequently, the reduction of chloramphenicol is in all probability irreversible.

Representative polarographic curves obtained with chloramphenicol at the various pH values are shown in Figure 1 and the results obtained with both chloramphenicol and 1-*p*-nitrophenyl-2-amino-1,3-propanediol hydrochloride are combined in Table I. The values of the half-wave potentials versus the saturated calomel electrode have been corrected for the IR drop due to the resistance included in the circuit by the cell and the damping resistance. Diffusion currents are given in terms of the diffusion current constant, $I = i/cm^{2/3}t^{1/6}$, which is relatively independent of the characteristics of the dropping mercury electrode being used.

A perusal of the table or chart will show that the diffusion current of the first wave of chloramphenicol is practically constant at all pH values, while the half-wave potential shifts to more negative values at regular intervals with increasing pH. On the other hand, the half-wave potential of the final wave, which occurs in all buffers up to pH 10, is practically constant, while the diffusion current of this wave decreases markedly with increasing pH values. Because of this variation in half-wave potentials, the two waves interfere somewhat at pH values of 6 and 8. This interference, and lack of a well-defined diffusion current after the second wave, make these pH values unsuitable for analytical purposes.

The hydrolysis product of chloramphenicol, 1-*p*-nitrophenyl-2-amino-1,3-propanediol, shows a well-defined first wave, which corresponds closely, both in half-wave potential and in diffusion current, to the first wave of chloramphenicol at all pH values except 2.2, where this compound behaves anomalously. Poorly defined second waves, not included in the data of Table I, appear in the polarograms of this material, occurring at half-wave potential of about −1.0 volt at pH 2.2 and increasing in negative value with increasing alkalinity of the buffer even more rapidly than do the potentials of the first wave.

The occurrence of the break in the first wave of chloramphenicol at pH 6.4 has not as yet been explained. The wave in this buffer

Table I. Half-Wave Reduction Potentials (Volts *vs.* S.C.E.) and Diffusion Current Constants

pH	Chloramphenicol $E_{1/2}$	Chloramphenicol I	1-p-Nitrophenyl-2-amino-1,3-propanediol $E_{1/2}$	1-p-Nitrophenyl-2-amino-1,3-propanediol I
2.2	−0.35	5.84	−0.53	7.08
	−1.09	5.26	...	...
4.2	−0.48	5.75	−0.45	5.79
	−1.14	3.84	...	...
6.4	−0.60	4.42	−0.53	5.98
	−0.85	1.45	...	...
	−1.07	2.42	...	...
8.1	−0.69	5.85	−0.64	5.59
	−1.10	1.96	...	...
10.1	−0.79	5.90	−0.71	5.85

$E_{1/2}$ is corrected for IR drop.

I is $\frac{i}{Cm^{2/3}t^{1/6}}$.

has not been completely reproducible, as the maximum is difficult to suppress, and in some instances a double maximum has been observed. The behavior of 1-*p*-nitrophenyl-2-amino-1,3-propanediol in buffer of pH 2.2 with increased diffusion current and reversal of the pH-potential curve is thought to be due to the characteristics of the ionic amine hydrochloride, which would be formed in the hydrochloric acid–potassium chloride buffer.

SUMMARY

The behavior of chloramphenicol and of its hydrolysis product, 1-*p*-nitrophenyl-2-amino-1,3-propanediol, at the dropping mercury electrode has been studied, and the polarographic waves have been analyzed. The reaction is irreversible and involves 4 electrons, with preliminary reduction of the nitro group to the hydroxylamine. A method for the routine analysis of broths, concentrates, and solids in buffer of pH 4 has been shown to be accurate within the limits of the polarographic technique.

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Qualitative Scheme of Analysis for the Common Sugars

T. H. WHITEHEAD AND W. C. BRADBURY[1], *University of Georgia, Athens, Ga.*

A systematic scheme of qualitative analysis is given for mixtures of sucrose, glucose, fructose, maltose, and lactose in solid or liquid samples. The presence of starch and dextrin does not interfere. This scheme is applicable to mixtures of sugars and most food products. The procedures call for no reagents that are difficult to prepare or store, no specialized laboratory equipment, and no unusual techniques. The scheme of analysis is based upon removing fructose from the solid sample with 90% ethyl alcohol, in which it is soluble but other sugars and starches are not. Fructose is confirmed by a specific test. Lactose is next removed from the residue by dissolving it in 50% ethyl alcohol and its presence is confirmed with the formation of its osazone. Sucrose is detected by its color reaction with cobalt nitrate or by the Raybin test with diazouracil. Glucose and maltose are separated by forming the osazones and taking advantage of their difference in solubility at 87° C. The methods are capable of detecting 5 mg. of sucrose and fructose or 200 mg. of the other sugars in a sample.

CHEMISTS who are frequently required to make qualitative and quantitative analyses of natural and manufactured food products recognize the need for a simple, rapid, and reliable scheme for determining sucrose, glucose, maltose, lactose, and fructose in the presence of starch and dextrins. There is no lack of tests for individual sugars or groups of sugars, but mixtures containing three or more sugars are difficult to analyze. Most quantitative methods for the accurate analysis of sugar mixtures require a knowledge of the qualitative composition of the mixture before the analysis is begun. The practice of reporting reducing sugars "in terms of glucose" gives no indication of the true composition of the mixture. Unless attention is given to the presence of starch or dextrins, they may be converted to glucose during the analysis and introduce a large error.

The scheme of analysis given here is satisfactory for the sugars mentioned above in the presence of starch and dextrins. Because the high cost and low sweetening value of other sugars preclude their use in most food products in competition with the common sugars, this scheme is suitable for such products.

REAGENTS

1-Naphthol Solution. Dissolve 15 grams of 1-naphthol, melting point 95–96° C., in 100 ml. of chloroform.

Fehling's Solution. A. Dissolve 34.6 grams of cupric sulfate pentahydrate in 400 ml. of water, add 0.5 ml. of 36 *N* sulfuric acid, and make up to 500 ml. with water.

B. Dissolve 172 grams of Rochelle salt, U.S.P., in 300 ml. of water, add 62.5 ml. of saturated sodium hydroxide solution, and make up to 500 ml. with water.

Ethyl Alcohol, 90%. Dilute 95% ethyl alcohol, U.S.P., with water until the specific gravity of the mixture at 20° C. is 0.8305.

Ethyl Alcohol, 50%. Dilute 95% ethyl alcohol, U.S.P., with water until the specific gravity of the mixture at 20° C. is 0.9316.

Phenylhydrazine base. Redistill Eastman Kodak Company No. 329 under reduced pressure at frequent intervals. It becomes unreactive after long exposure to sunlight and air.

Dinitrosalicylic Acid Reagent. Dissolve 2.0 grams of 3,5-dinitrosalicylic acid (Eastman Kodak No. 1802) in 70 ml. of water at 80° to 90° C., and add 10 ml. of sodium carbonate solution containing 20 grams of sodium carbonate per 100 ml. of water. When this mixture is cool, dilute it to 100 ml. with water.

Sodium Hydroxide Solution. Dissolve 1.5 grams of C.P. sodium hydroxide in enough water to make 100 ml. of solution.

Cobaltous Nitrate Solution. Dissolve 5.0 grams of cobaltous nitrate hexahydrate in 50 ml. of water, add 1 drop of 15 *N* nitric acid, and make up to 100 ml. with water.

Acetic Acid Solution. Dissolve 50 ml. of glacial acetic acid in 50 ml. of water.

Potassium Hydroxide Solution, 50%. Dissolve 50 grams of C.P. potassium hydroxide in 50 ml. of water.

PROCEDURES

Preliminary Examination. If the sample is a liquid, use 3 ml. If the sample is a solid, shake 50 mg. with 3 ml. of water and filter if all of the sample does not dissolve. Wash the residue on the filter paper with 0.5-ml. portions of water until the filtrate has a volume of 3 ml. (All filtrations in this scheme which call for filter paper require Whatman No. 40 paper or its equivalent.)

Using 3 ml. of sample solution, perform the Molisch test (*2*) to determine if any carbohydrate is present. This is done by adding 3 drops of the 1-naphthol solution to the 3-ml. sample. Shake

[1] Present address, Upjohn Laboratories, Kalamazoo, Mich.