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On Iodometric and Polarimetric Determination of Penicillin

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THE PRINCIPLE AND EARLIER FORM OF THE IODOMETRIC DETERMINATION

It was early found that certain products of inactivated penicillin, but not the pure active substance, show a marked consumption of iodine. Alicino [1] made use of this fact for the determination of penicillin. The principle is the following: Two identical samples are taken from a solution of penicillin. The one is inactivated with alkali (or penicillinase) and then neutralized with acid. The same amount of iodine is added to both samples and, after a time, they are titrated with thiosulphate. The difference in the consumption of iodine is a function of the amount of penicillin. Careful analytic descriptions have been given by, for example, Mundell, Fischbach and Eble [2]. In principle, the same iodometric determination is also included in the analytical descriptions of penicillin issued by the Food and Drug Administration, Washington, D. C. The methods, however, do not always give reproduceable values.

A new modification of the iodometric determination

Reagents. Sodium hydroxide 1 *N*, acid (hydrochloric or sulphuric acid) 1 *N*, buffer (0.3 *M* phthalate) pH 4.5, iodine solution 0.01 *N*, and standardized 0.01 *N* sodium thiosulphate.

The hydroxide and the acid need not be exactly normal, but they should, on the other hand, be equivalent to each other. A solution of 60 g potassium biphthalate and 80 ml 1.00 *N* sodium hydroxide diluted to 1 l is a suitable buffer, but any buffer with pH about 4.5, or, in most cases, one with pH between 4 and 5 or even pH 4—6, can be used if it has sufficient strength and does not react with other components in the reaction mixture. A iodine solution containing 12.7 g iodine and 20 g potassium iodide in 1 l

water is diluted 1 : 10 before use. The thiosulphate solution may be prepared by diluting 0.1 *N* sodium thiosulphate solution with boiled distilled water. The solution may be standardized after dilution and then frequently checked. Canbäck *et al.*[3] has stated that for certain reasons it is desirable to substitute an iodine solution which contains 1.27 g iodine and 8.3 g potassium iodide in one liter for the above-mentioned solution.

Procedure. From a *weakly* buffered (pH 5.5—7.5) or unbuffered water-solution, which contains about 2.5 mg sodium benzyl penicillin (approximately 4000 units; minimum 800, maximum 6000) per 5 ml or an equivalent amount of other penicillin, two samples, A and B, of 5 ml each are pipetted into 100 ml Erlenmeyer flasks with glass stoppers.

To A, add 5 ml buffer and 10 ml iodine. Close the flask immediately, keep it for 20 minutes in darkness at room-temperature, and then titrate with (v_a ml) thiosulphate.

To B, add 1 ml sodium hydroxide and, after 20 minutes in room-temperature, 5 ml buffer, 1 ml acid and 10 ml iodine. Close the flask immediately and keep it a further 20 minutes in darkness at room-temperature, and then titrate with (v_b ml) thiosulphate.

For the determination of procaine penicillin, a phthalate buffer, with pH 4.5, is satisfactory but one with pH 4.3 is to be prefered.

If the determination is carried out on an impure solution which has a high consumption of iodine at pH 4 to 5, not due to penicillin, and a strong buffer effect, the prescribed amount of iodine may be increased to 15 or 20 ml, and the amounts of hydroxide and acid to 2 or 3 ml. In this case it is also desirable to control the pH in the titrated solutions.

Calculations. If (v_a—v_b) is converted to 0.01000 *N* thiosulphate and divided by 100, the result gives the number of milligram equivalents of iodine, that have been consumed by the penicillin in the reaction-mixture. Concerning the conversion from equivalents of consumed iodine to mg or units of penicillin, see p. 528.

The effect of changes in the concentration of certain reagents, times, and temperatures

Pedersen [4] has systematically varied the alkali-concentration used by the destruction of the penicillin, the time for hydrolysis with the sodium hydroxide, the iodine-concentration, the time for the oxidation with iodine, the temperature, and the pH of the reactionmixture. In the oxidation with iodine, the pH is of the greatest importance, while moderate variations in the other factors have less effect. Pedersen recommends pH 5.8 to 6.3 in the reaction-mixture and Wild [4] a pH between 6 and 7.

Table 1. The effect of variations in the reaction times with alkali and iodine solution, in the amounts of these reagents, and in the amount of penicillin on the iodometric determination of sodium penicillin "Pc VI" (Table 3).

mg Na-benzyl-penicillin (Pc VI) in react.-mixture	Treatment with alkali for minutes	Treatment with iodine for minutes	Recovered penicillin (%) after treatment with: 1 ml 1 *N* sodium hydroxide and iodine solution			3 ml 1 *N* sodium hydroxide and iodine solution			Recovered penicillin %		
			10 ml	15 ml	20 ml	10 ml	15 ml	20 ml	average	ε	σ
0.5	20	20	103.1	100.5	100.5	100.4	99.7	103.7	101.3	± 0.7	± 1.7
1.6	20	20	101.1	102.1	101.8	103.1	102.0	101.8	102.0	± 0.3	± 0.7
2.5	20	20	100.8	98.0	100.2	103.2	99.6	99.2	100.2	± 0.7	± 1.8
2.5	40	20	100.3	100.0	99.2	103.6	99.2	100.0	100.4	± 0.7	± 1.7
2.5	40	40	101.1	101.5	103.5	101.1	103.5	102.8	102.3	± 0.5	± 1.2
2.5	20	40	99.2	101.1	101.9	100.3	100.3	101.9	100.8	± 0.4	± 1.1
3.5	20	20	99.8	101.3	103.1	101.7	102.0	99.7	101.3	± 0.5	± 1.3
Recovered % penicillin	average		100.8	100.6	101.5	101.9	100.9	101.3	101.2	± 0.3	± 0.8
	ε		± 0.5	± 0.5	± 0.6	± 0.5	± 0.6	± 0.6	± 0.2	—	—
	σ		± 1.2	± 1.3	± 1.6	± 1.4	± 1.6	± 1.7	± 0.5	—	—

A long series of determinations carried out according to Pedersen did not, however, give satisfactory results. This necessitated a careful investigation of the iodometric method.

The effect of increased amounts of sodium hydroxide and iodine and prolonged reaction times is, as may be seen from Table 1, not greater than the experimental error of single determinations. Moreover, the average 101.2 ± 0.2 %, obtained from all the values in Table 1, agrees well with the value 100.8 ± 0.2 % that has been obtained below for penicillin Pc VI. It is thus possible to vary the amount of sodium benzyl penicillin in the reaction-mixture between 0.5 and 3.5 mg, the amount of sodium hydroxide between 1 and 3 ml, the amount of iodine-potassium iodide solution between 10 and 20 ml, and the reaction time between 20 and 40 minutes without any significant effect upon the analytic results.

Thus, if the concentrations of iodine and potassium iodide used are in the same proportion, it is possible to increase them at least 50 % without the consumption of iodine being significantly changed. Canbäck *et al.*, who were informed of our results, found that an increase in the concentration of potassium iodide alone leads to an increase in the iodine consumption of the penicillin in the pH region immediately below 4. There is, however, no difficulty in keeping the concentration of potassium iodide constant. In all our experiments, this concentration has been maintained unchanged.

The iodometric penicillin determinations do not appear to be greatly affected by small temperature differences, but the values do show a tendency to increase with the tem-

Table 2. The effect of variations in temperature on the iodometric determination.

Added Pc VI mg	Temp. on treatment with iodine and alkali °C	Recovered amount of sodium penicillin %	
2.6	+ 10	97.4 97.4	97.4
2.6	+ 20	98.5 99.3	98.9
2.6	+ 30	99.3 101.2	100.3

perature. Accompanying treatments with iodine and sodium hydroxide were always carried out at the same temperature (Table 2). In normal determinations, a variation in the room-temperature, *e. g.* from + 15° to + 25° C, scarcely had any measurable effect.

Thus, none of the above-mentioned variables can cause any significant deviations in the iodometric determination of penicillin. Temperature, reaction times, and concentrations of reagents should, however, be kept as constant as practical working conditions permit.

The influence of pH

In the subsequent experiments the pH was varied in the iodine reaction-mixture. Acetate, citrate, phosphate, phthalate, and tartrate buffers (0.3 *M*) were used. The composition of the buffer solutions does not, however, play any rôle as long as they are sufficiently strong and do not react with other components in the reaction-mixture. In certain cases the pH of the solution showed slight displacements in relation to that of the buffer.

The investigation covers altogether eleven penicillin preparations. Four are ordinary commercial preparations of crystalline benzyl penicillin sodium, and seven are special preparations. In Figs. 1 and 2 the pH of the titrated solutions have been plotted as abscissae, while the number of equivalents of iodine that have been consumed per mole penicillin (if the sample is considered 100 % pure) have been taken as the ordinates.

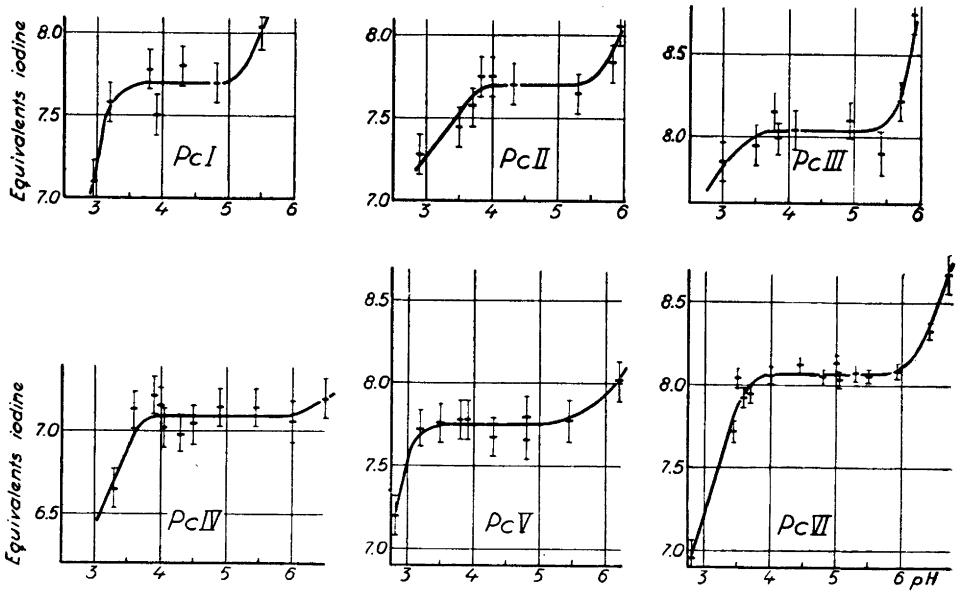

Fig. 1. The number of iodine equivalents consumed by 6 penicillin preparations are plotted against pH. The penicillins are described in Table 3. The errors in the determinations are represented by lines parallel with y-axis. Errors in pH max. ± 0.03.

Pc I: Eeach point represents one determination.
Pc II: Each point represents one determination.
Pc III: Each point represents one determination. except that at pH 3.85 which represents two.
Pc IV: Each point represents one determination.
Pc V: Each point represents one determination.
Pc VI: From the left (pH = 2.8) to the right (pH = 6.7) the points represent 1, 6, 4, 4, 10, 7, 5, 10, 4, 6, 4, 4, 8, 10, and 1 determinations, respectively.

The pH region where the curves run parallel with the pH axis is rather great, and a slight displacement in the pH is thus of subordinate importance. Where several determinations have been carried out at the same pH, the mean value and the corresponding standard error (ε) have been plotted. In those cases in which a certain value of a curve corresponds to only one determination, the mean value for σ (= 1.5 %) obtained in Table 3 has been introduced as the limits of error of the determination.

The curves of the preparations examined here are rather similar and do not agree with that given by Pedersen. The middle part of all curves is parallel with the pH axis in the region about pH 4—5, but the pH where the curves

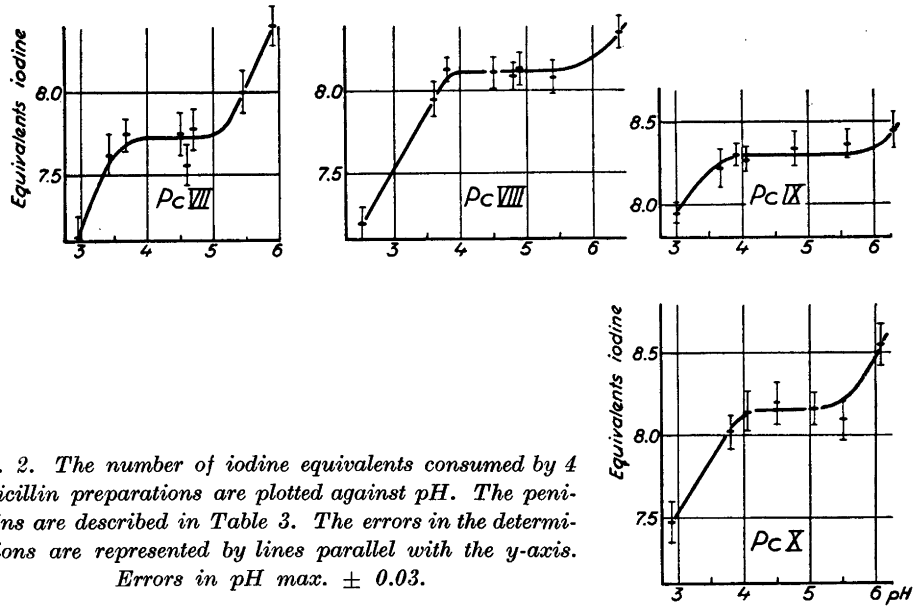

Fig. 2. The number of iodine equivalents consumed by 4 penicillin preparations are plotted against pH. The penicillins are described in Table 3. The errors in the determinations are represented by lines parallel with the y-axis. Errors in pH max. ± 0.03.

Pc VII: Each point represents one determination, except that at pH 3.7, which represents two.

Pc VIII: From the left (pH = 2.5) to the right (pH = 6.4) the points represent 2, 2, 4, 3, 3, 2, 2, and 2 determinations, respectively.

Pc IX: From the left (pH = 3.0) to the right (pH = 6.3) the points represent 4, 1, 6, 3, 2, 4, and 2 determinations, respectively.

Pc X: From the left (pH = 2.9) to the right (pH = 6.1) the points represent 1, 2, 1, 1, 2, 1, and 1 determinations, respectively.

begin to rise or fall are somewhat different for the penicillin preparations examined here.

In the pH range 4 to 5 or 6, where according to Brodersen [6] the penicillin keeps rather well for 20 minutes, constant values are obtained for the difference between the iodine consumption of the penicillin before and after the treatment with sodium hydroxide. At a lower pH, where the penicillin is quickly inactivated even in samples not treated with alkali, the difference in the consumption of iodine is less. At a higher pH (5 or 6—7), where, certainly, the penicillin is not disintegrated but where the presence of iodate may be conceived to give rise to a stronger oxidation, one obtains greater differences in the consumption of iodine.

For the iodometric determination of penicillin the pH in the iodine reaction-mixture should thus in ordinary cases be kept at about 4.5. In some cases, procaine seems to interfere with the determination at a pH higher than 4.6.

Table 3. Iodometric and polarimetric determination of certain penicillin preparations.

Penicillin preparation	Of the total penicillin content biolog. type determ. shows				Iodomet. determination					Polarimetric determination; content	Diff. in iodomet. and polarim. content	Remarks
	%G	%F	% H_2F	%K	pH-region	Number of determ.	Content %	Content ε	Content σ	%		
Pc I; cryst. sodium penicillin (Bristol)	99	1	—	—	3.8—4.8	4	96.2	±0.8	±1.6	95.1±0.6	+1.1	Ordinary commercial prep.
Pc II; cryst. sodium penicillin (Kärnbol.)	98	1.5	0.4	0.3	3.8—5.3	4	96.5	±0.8	±1.6	97.1±0.6	−0.6	» »
Pc III; cryst. sodium penicillin (Novo)	94	4	2	—	3.8—5.4	6	100.3	±0.6	±1.5	99.9±0.6	+0.4	» »
Pc IV; cryst. sodium penicillin (Astra)	97	2	0.7	0.3	3.8—5.4	7	88.7	±0.6	±1.5	89.4±0.6	−0.7	Less pure prep. included for comparison.
Pc V; cryst. sodium penicillin (Astra)	97	2	0.3	—	3.8—4.8	5	96.8	±0.7	±1.5	96.7±0.6	+0.1	Ordinary commercial prep.
Pc VI; cryst. sodium penicillin (Astra)	98.4	1.6	—	—	4.0—5.5	40	100.8	±0.2	±1.4	100.4±0.3	+0.4	Specially purified sodium benzylpenicillin
Pc VII; cryst. sodium penicillin (Kärnbol.)	99.5	0.5	—	—	3.7—4.7	5	96.5	±0.7	±1.6	97.3±0.6	−0.8	Specially purified sodium penicillin. Determination on remains in earlier opened flask (moisture?)
Pc VIII; benzylpenicillin di-isopropyletherate (Astra)	99	0.6	—	—	3.8—5.4	14	101.4	±0.3	±1.2	101.4±0.6	±0.0	The high content has for IX been verified by N_2 determ. which gave 102.7 %. Both propyletherates seem to lose ether on drying
Pc IX; benzylpenicillin di-isopropyletherate (Kärnbol.)	98	1.5	0.5	—	3.7—4.8	12	103.7	±0.5	±1.8	103.2±0.6	+0.5	
Pc X; N-ethylpiperidinpenicillin	98	2	—	—	4.1—5.5	5	102.0	±0.2	±0.6	98.8±0.6	+3.2	Precipitate obtained by G-determ.
PcXI; procaine penicillin (Astra)	95	4	1	—	4.2—4.6	8	99.8	±0.4	±1.2	99.5±0.6	+0.3	Ordinary commercial prep.
Total:						110	(1082.7)		—	(1078.8)	+3.9	
Average:						—	98.4±0.2		±1.4	98.1±0.2	+0.3	
Total Pc I to Pc IX and Pc XI						105	(980.7)		—	(980.0)	+0.7	
Average Pc I to Pc IX and Pc XI						—	98.1±0.2		±1.5	98.0±0.2	+0.1	

Table 4. Effect of the »blank iodine time» on the iodine consumption obtained at different pH.

Penicillin sodium "Pc V"					Penicillin sodium "Pc VI"					
pH in the reaction mixt. with iodine	Iodine consumption in equiv./mol. if the blank sample has been treated with iodine				pH in the reaction mixt. with iodine	Iodine consumption in equiv./mol. if the blank sample has been treated with iodine				
	0 min	5 min	10 min	20 min		0 min	5 min	10 min	20 min	30 min
2.3	8.31	7.85	7.28	6.82	2.5	8.53	8.38	8.07	6.80	6.58
3.2	7.83	7.76	7.76	7.72	3.3	8.40	8.23	7.98	7.63	7.82
3.9	7.79	7.84	7.81	7.79	4.0	8.08	8.05	7.86	8.02	8.02
4.3	7.73	7.73	7.75	7.68	4.4	8.00	7.96	7.78	7.92	7.88
4.8	7.80	7.72	7.72	7.67	5.0	8.11	8.16	8.20	8.11	8.08
5.5	7.73	7.69	7.67	7.63	5.5	8.16	8.08	8.09	8.10	8.05
6.2	8.14	7.99	8.06	7.99	6.4	8.45	8.39	8.34	8.40	8.27
Mean for the values corresponding to pH 3.2—5.5	7.78	7.75	7.74	7.70	Mean for the values corresponding to pH 4.0—5.5	8.09	8.06	7.98	8.06	8.01
ε	±0.02	±0.03	±0.02	±0.03	ε	±0.04	±0.04	±0.09	±0.04	±0.04
σ	±0.05	±0.07	±0.05	±0.06	σ	±0.07	±0.08	±0.20	±0.09	±0.08

The blank test in the iodometric determination

As has already been mentioned (p. 519), "blanks" (= A) have been carried out in such a way that the sample not treated with alkali has been allowed to react with iodine for as long a period as the sample treated with alkali, *i. e.* for 20 minutes. This is in opposition to earlier prescriptions [2, 4, 5], but agrees with general analytical practice, *i. e.* to treat the blank and the sample identically as far as possible. Within the pH range used, no destruction of the penicillin has been found to occur. If there were a measurable destruction, then the part of the pH curves now parallel with the pH axis should incline slightly upwards with rising pH; and no such tendency has been observed.

Investigations relating the change of the pH curve with the time of the iodine treatment of the blank have been carried out on the sodium benzyl penicillin preparations Pc V and Pc VI. The reaction time, referred to below

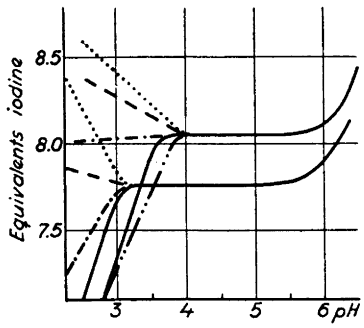

Fig. 3. Iodine equivalent curves for Pc V and VI from different "blank iodine times" (. . . . . 0 minutes, — — — — 5 minutes, — · — · — 10 minutes, ——— 20 minutes, and — · · — · · — 30 minutes).

as "blank iodine time", was varied from zero to 30 minutes, while the reaction time of the alkaliinactivated samples remained unchanged at 20 minutes. The values for the iodine equivalent obtained may be seen in Table 4. The "blank iodine time" does not play a very great rôle in the pH range 6.5—4 or 3. The mean values, which correspond to the part of the pH curve parallel with the pH axis, do, however, show a falling tendency with increased time. This tendency is confirmed by 12 parallel determinations carried out on Pc VI and 8 on Pc V, which were performed with both 5 and 20 minutes' "blank iodine time". The mean values give the contents 101.4 and 100.8, and 97.3 and 96.7 %, respectively. The differences between the corresponding values amount, however, to only about 1 %, and are within the experimental error of a single determination. At low pH (less than 3), on the other hand, one obtains considerable differences. Schematic curves for Pc V and VI can be graphically represented as in Fig. 3. The "blank iodine time" thus is not of too great importance when the iodometric determinations are carried out at a suitable pH. If, however, less pure samples are analysed, impurities which react slowly with iodine may be present. If in this case the "blank iodine time" is short, the impurities will not have time to react completely, and will be included in the determination as penicillin.

Experimental errors of the iodometric method

The limits of error in Table 1, and other such errors given for iodometric determinations in this paper, have been calculated throughout according to the usual formulae. The standard error of the average is thus denoted by ε and is calculated from $\varepsilon = \pm \sqrt{\frac{\Sigma \Delta^2}{n\,(n-1)}}$, and the standard deviation for a single determination is denoted by σ and calculated from $\sigma = \pm \sqrt{\frac{\Sigma \Delta^2}{(n-1)}}$.

The deviations in the iodometric determinations are considerably greater than the actual titration errors. Thus, ten determinations of Pc VI at pH 4.8 gave, for the mean value, a standard error (ε) of $\pm$ 0.5 %. The standard deviation (σ) for a single determination was $\pm$ 1.5 %. In all the other series, errors of approximately the same magnitude were obtained, even if the series included not only determinations carried out at exactly the same pH, but all those on the part of the pH curve running parallel with the pH axis. Examples of such values are given in Table 3. Thus, if single iodometric determinations are carried out on fairly pure substances, the deviation from the correct value in about 65 per cent of the cases should not exceed $\pm$ 1.5 % and in 95 per cent should not exceed $\pm$ 3 %.

The main cause of the great irregularities in iodometric determination of penicillin according to earlier prescriptions

Mundell, Fischbach, and Eble stated that the blank, *i. e.* the nonalkali inactivated sample, must be retitrated immediately, and should not be too acid (not below pH 3). The alkali inactivated sample, on the other hand, is purposely acidified to pH 2 to 3 with a slight excess of acid. Iodine is then added and the sample is titrated after 15 minutes. Experiments show that if the blank on pure sodium penicillin is titrated *immediately*, a pH displacement from 2 to 6 is of small importance. In consequence of the disintegrating of the penicillin, however, the iodine consumption increases rapidly in blanks whose pH is less than 3. In the determination according to Mundell *et al.* or the Food and Drug Administration Washington D. C., the pH is unsuitable because in this range the pH curve inclines very strongly. Compare the dotted parts of the curves in Fig. 3. The determination in the pH range 2—3 is therefore strongly affected even by variations of the pH and the time for which the iodine is allowed to react with the alkali-inactivated penicillin. The limits 2 to 3, which are given for pH, are too wide, and the way of adjusting pH without buffer is unsuitable. The rising pHcurve also explains the necessity of the low factor that is introduced. This factor is valid only for a point corresponding to a definitely established pH. As may be seen from Fig. 3, differences of 8 to 9 % may be obtained in the determination when the pH is changed from about 2 to 3. By carrying out the determination at a definitely established and more suitable pH (4—5), one can obtain 2 to 4 times better reproduceability. For some penicillin preparations (Figs. 1 and 2 Pc I, II, III, VI, VII, and X) the pH chosen by Pedersen is also rather unsuitable, because the pH curves begin to rise. At pH 5.8 and 6.3, which are the limit

values Pedersen indicates, determinations of Pc VI *e. g.* give the average values 101 and 104 % and single determinations of Pc VII at pH 5.8 and 6.3 give 10 % difference.

CONVERSION FACTOR

The part of the pHcurves parallel with the x-axis gives, as may be seen, y-values of about 8. This indicates that, after inactivation with alkali, one mole penicillin consumes about 8 equivalents of iodine. If we assume the value for pure penicillin to be exactly 8, the percent content of penicillin may be calculated. The difference of 1.00 ml between the consumption by the blank and the sample of 0.01 N thiosulphate then corresponds to 742 units of benzyl penicillin or 0.445 mg benzyl penicillin sodium, and 0.712 mg water-free benzyl penicillin procaine. The iodometric contents given in Table 3 have been calculated in this way from the mean values (with corresponding ε) for the determinations on the part of the curves parallel with the pHaxis.

POLARIMETRIC DETERMINATIONS

In order to confirm the correctness of the assumption that the equivalent weight of the penicillin on iodine reaction is one-eighth part of its molecular weight, the content of the preparation should also be determined according to some other method than the iodometric. Biological determination or colorimetric methods, *e. g.* according to Boxer and Everet [7] or Henstock[8], require standard substances and entail rather considerable errors. They are thus not suitable as comparative methods for the determination of purer preparations. Another conceivable method is one in which potassium ferricyanide is used instead of iodine. Such a method has been published by Hiscox [9]. This method has also been tested and found to be very unsatisfactory. Its greatest drawback probably lies in the fact that the penicillin must be treated with the reagent at a high temperature and an unsuitable pH. Thus, during the determination a non-reproduceable destruction of penicillin occurs which causes large errors. For fairly pure substances, like the crystalline preparations included in Table 3, there remains, however, the possibility of carrying out polarimetric determinations of content.

The specific rotation for pure substance must then be known. Available data for sodium benzyl penicillin vary between + 290° and + 305°. Leigh [10] gives the specific rotation, in sodium light, for *n*-amyl penicillin in 1 % neutral water solution at + 23° C as + 319°, and Salivar, Howard and Brown [11] measured a 1 % solution of benzyl penicillin procaine in 50 % aqueous acetone and found the rotation to be + 173° at 25° C. If these values are converted to benzyl penicillin sodium, one obtains the rotations + 301° in water and + 286° in 50 % aqueous acetone. The specific rotation for Pc VI, which does not contain any moisture, is + 302° ± 0.7. This value is the average of seven determinations, which gave $\varepsilon = \pm 0.7$ and $\sigma = \pm 1.8$ (*i. e.* ± 0.6 %), and were carried out in 1 % water-solutions with pH between 5.1 and 7.2 in sodium light at room-temperature. In this pH range it was not possible to observe any systematic change in rotation with pH. A single determination on a 1 % solution in a mixture of equal volumes of water and

acetone gave for Pc VI $[\alpha]_D^{20} = + 289°$. As may be seen from Table 3, however, the preparation contains 1.6 % penicillin F. The specific rotation for pure benzyl penicillin sodium would then be somewhat lower, *viz.* + 301° ± 1 in the water-solution and + 288° ± 2 in 50 % aqueous acetone.

The values + 301° for the specific rotation in sodium light for the water-free sodium salt of benzyl penicillin in 1 % water-solution with pH 5 to 7 and + 173° for procaine penicillin with one mole of water of crystallization in 50 % aqueous acetone has been used below for calculation of contents. From the first value, a specific rotation of + 240° has been calculated for the N-ethyl piperidine salt of the benzyl penicillin. For benzylpenicillin Diisopropyl Etherate, Nelson, Trenner, and Buhs [12] give the value + 241°, which corresponds to + 296° for the sodium salt. They consider that it is in satisfactory agreement with the value of + 290° for crystalline sodium benzyl penicillinate. The agreement is just as good, however, if 290 is replaced by 301.

In Table 3 the polarimetric contents are calculated from the quotients between rotations observed in the single cases and the above specific rotations. This is of course theoretically correct only if the optical rotation of the substances is derived exclusively from active penicillin. For the preparations in Table 3, this condition is probably satisfactorily complied with. In those cases in which the polarimetric contents are based upon only one determination, the same percentual error, ± 0.6 %, has been ascribed to them, as has been calculated for a single determination of specific rotation. Both the iodometric and the polarimetric contents have been calculated as if benzyl penicillin were the only penicillin component. If the actual composition is taken into aocount, the values may be changed by a few tenths of one per cent.

If, however, the polarimetric method of determination is used for a penicillin preparation that has been partly disintegrated, the penicillin-content can not be calculated as the quotient between the observed and the theoretical specific rotation.

When the inactivation takes place in strongly alkaline solution (about 0.3 *N* sodium hydroxide), the specific rotation at room-temperature is rapidly reduced, as may be seen from Table 5, to about half the original value. The continued inactivation, on the other hand, takes place very slowly. For Pc VI, for example, 39 % of the rotation remains after twenty-four hours, 20 % after 3 days, 14 % after 5 days and still 7 % after 12 days. Thus, if for a penicillin partially inactivated in a basic solution, the specific rotation is + $B°$, and for the corresponding 100 % preparation + $A°$, it is probably more correct to calculate the content of penicillin (x %) that remains in the preparation from the equation $X = \frac{100\ (B—0.5\ A)}{A—0.5\ A}$ than from $X = \frac{100\ B}{A}$. The

Table 5. Inactivation of crystalline penicillin preparations. 1 % penicillin preparation, 0.5 N sodium hydroxide, + 20° C.

Inactivation period in minutes	Remanent specific rotation in % of original				Remaining activity accord. to iodomet. determ.			
	PcI	PcIV	PcVI	PcIX	PcI %	PcIV %	PcVI %	PcIX %
Approx. 5	58 (= 18 % pc undestroyed)	62 (= 25 % pc undestroyed)	56 (= 12 % pc undestroyed)	66 (= 29 % pc undestroyed)	17	22	—	26
10	51	52	—	55	0	0	—	1
20	50	52	54	53	0	0	0	0
40	49	50	52	52	0	0	0	0

polarimetric contents given in parentheses in Table 5 have been calculated according to the first formula. On disintegrating in strongly acid solution (about 0.3 N sulphuric acid), the rapid reduction of the rotation, as may be seen from Table 6, seems to terminate when the value has diminished approx. 25 %. Within parentheses is given the remanent amount of penicillin calculated on analogy with the above from the equation $X = \frac{100\ (B—0.73\ A)}{A—0.73\ A}$.

Table 6. Inactivation of crystalline sodium penicillin. 0.3 % Pc VI, 0.2 normal sulphuric acid. + 20° C.

Inactivation period in minutes	Remanent specific rotation in % of original	Remaining penicillin in % of original		
		calculated from rotation	determined	
			iodomet.	biolog.
2 1/4	92	(70)	73.4	70
7	84	(41)	41,2	39
10	81	(30)	32.5	—
15	79	(22)	17.2	17
20	77	(15)	10.5	—
90	70	—	1.2	—
18 × 60	64	—	0	—

Table 7. Inactivation of crystalline sodium penicillin. 0.3 % Pc VI, 0.4 normal sodium carbonate pH 10.6, + 20° C.

Inactivation period in hours	Remanent specific rotation in % of original	Remaining penicillin in % of original		
		calculated from rotat.	determined	
			iodomet.	biolog.
1	85	(70)	71.6	79
3	71	(42)	46.0	50
4.5	61	(22)	33.2	—
6	52	—	21.4	22
8	43.4	—	10.7	—
20	31.4	—	5.5	—

Even disintegration at pH 10.6 (in 0.4 *N* carbonate) evidently takes place in at least two distinct steps at different rates. If the content of penicillin remaining is calculated according to the same formula as for the inactivation in strongly basic solution, the values given in parentheses in Table 7 are obtained.

The above observations concerning the reduction of the rotation permit a quantitative calculation of remanent penicillin only if the pH is constant and known during the disintegration. In practice, however, the majority of penicillin preparations are either not at all or only weakly buffered, and thus the pH will vary during the disintegration.

Polarimetric determination of the content of a penicillin solution should thus only be carried out if it is known that the penicillin in the solution exists in the active form, and that the solution does not contain other optically active compounds. A rotation value that is lower than the theoretical can scarcely be regarded as anything other than a qualitative test for disintegration. If the rotation amounts to only 50 % of the theoretical value, it is, in general, probably justifiable to assume that the preparation is biologically completely inactive. The polarimetric determination also suffers from the drawback that it requires relatively large amounts of substance.

AGREEMENT AND SPECIFICITY OF THE METHODS OF DETERMINATION

As has been mentioned, the polarimetric method is very unspecific, and may be used only for pure substances. Table 3, however, shows that the agreement between iodometric and polarimetric determinations on pure prepara-

Table 8. Comparison between iodometric and polarimetric determinations of commercial crystalline sodium and procaine benzyl penicillin and of N-ethylpiperidin penicillin.

Preparation type	Number of determinations	Iodometric content : polarimetric content				
		highest value	lowest value	average	ε	σ
Sodium penicillin	15	1.043	0.953	1.001	± 0.006	± 0.02
Procaine penicillin	15	1.016	0.983	0.998	± 0.003	± 0.01
N-ethyl piperidin penicillin	15	1.055	0.996	1.020	± 0.005	± 0.02

tions is very satisfactory. This has been further confirmed by a pair of experimental series carried out on Astras commercial sodium and procaine salts of benzyl penicillin. In Table 8 are given the number of parallelly determined substances, the highest and lowest values for the quotients between the contents, the mean values of the quotients, as well as ε and σ. The table also gives the results of some determinations on N-ethyl-piperidine preparations.

The quotient (iodometric content) : (polarimetric content) for ethyl-piperidine penicillin is 1.020, which indicates that the high iodometric value in Table 3 is not due to chance. As the iodometrically determined contents for ethyl-piperidine penicillin are sometimes considerably over 100 %, the error is probably to be sought in this determination. Ethyl piperidine itself, however, does not consume iodine under the experimental conditions used, and in four determinations of Pc VI, in the presence of an equivalent amount of ethyl piperidine, the same content was obtained (100.6 *W* 0.7 %) as earlier. All the ethyl-piperidine penicillins tested have, however, been produced in the same way, and approximately unchanged amounts of the same impurities may be conceived to occur in all the preparations. Experiments with highly purified ethyl-piperidine penicillin have not been made.

Iodometric determinations on partially disintegrated penicillin preparations, give good values. The Tables 5, 6, and 7 include a comparison between biological, iodometric and polarimetric determinations after disintegration in sodium hydroxide, sulphuric acid, and sodium-carbonate solution at pH 10.6.

The iodometric determinations so far discussed have been carried out on rather pure penicillin preparations. In many cases, however, the method is

Table 9. Comparison between biological and iodometric determination on two different substrate series.

Substrate	l_1	b_1	c_1	d_1	e_1	f_1	g_1	Mean of the quotient	ε
Biol. assay / Direct iodom.	0.80	0.85	1.11	0.83	1.01	1.13	1.17	0.97	± 0.05
Biol. assay / Iodom. after extraction	0.86	0.85	1.07	0.85	1.15	0.83	1.07	0.95	± 0.05
Direct iodom. / Iodom. after extraction	1.08	1.00	0.96	1.03	1.14	0.74	0.92	0.98	± 0.05
Substrate	a_2	b_2	c_2	d_2	e_2	f_2	g_2	—	—
Biol. assay / Direct iodom.	0.85	0.77	0.66	0.72	0.86	0.54	0.79	0.74	± 0.04
Biol. assay / Iodom. after extraction	0.91	0.91	0.93	0.88	1.04	0.94	0.88	0.93	± 0.02
Direct iodom. / Iodom. after extraction	0.94	0.84	0.70	0.82	0.82	0.65	0.90	0.81	± 0.04

usable also for very impure preparations, even for broth. The amount of iodine is then increased to 15 or 20 ml, while the sodium hydroxide and acid amounts are increased to 2 or 3 ml. Iodometric determinations were carried out in this way, in parallel with biological determinations, on a number of broths of a certain type. The agreements were satisfactory. For certain other broths the agreement was poor, and deviations of ± 25 % or more were not uncommon. After a simple shaking with an organic solvent (chloroform, pH about 2) and subsequent transfer to a buffer (pH 7—7.5), however, it was possible to determine the penicillin even in these broths with satisfactory reproducability. This does *not*, however, mean that the iodometric method is, in general, especially well suited for determinations of the penicillin content in broth. The quotients between the biological and iodometrical contents of the series may be seen in Table 9.

The comparison between the iodometric determinations and other determinations thus shows that the iodine method is surprisingly specific, and it also supports the assumption that under the above-described conditions one mole of penicillin consumes 8 or very near 8 equivalents of iodine.

SUMMARY

A modified iodometric method for the determination of penicillin is described.

The accuracy of the modified method is greater than that of the earlier methods. The standard deviation for a single determination is about $\pm$ 1.5 %.

The pH value in the iodine reaction mixture is of great importance and should be approx. 4.5.

The blank is treated with iodine for the same time, 20 minutes, as the alkaliinactivated sample.

The alkaliinactivated penicillin consumes, under the conditions described, 8 equivalents of iodine per mole.

Iodometric determinations on partially disintegrated penicillin agree well with biological determinations of potency.

The iodometric determination can, in certain cases, be used directly on filtered broth.

Polarimetric determinations are satisfactory if active penicillin of a known type is the only unknown rotating component.

Only under special conditions is it possible to calculate quantitatively the inactivation of penicillin from the reduction of rotation.

With neither the iodometric nor with the polarimetric methods is it possible to distinguish between the different types of penicillin, unless they are present as pure substances.

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REFERENCES

1. Alicino, J. F. *Ind. Eng. Chem. Anal. Ed.* **18** (1946) 618.
2. Mundell, M., Fischbach, H., and Eble, T. E. *J. Am. Pharm. Ass. Sci. Ed.* **35** (1946) 373.
3. Canbäck, T., Ehrlén, I., Ilver, K., Reimers, F., and Wester, S. *J. Pharm. Pharmacol.* **2** (1950).
4. Pedersen, V. *Arch. Pharm. Chemi* **55** (1948) 625.

5. Wild, A. M. *J. Soc. Chem. Ind. (London)* **67** (1948) 90.
6. Brodersen, R. *Acta Pharmacol. Toxicol.* **3** (1947) 345.
7. Boxer, G. E., and Everett, P. M. *Anal. Chem.* **21** (1949) 670.
8. Henstock, H. I. *Nature* **164** (1949) 139.
9. Hiscox, D. J. *Anal. Chem.* **21** (1949) 658.
10. Leigh, T. *Nature* **163** (1949) 95.
11. Salivar, Ch. J., Howard, F., and Brown, E. V. *J. Am. Chem. Soc.* **70** (1948) 1287.
12. Trenner, N. R., and Buhs, R. P. *J. Am. Chem. Soc.* **70** (1948) 2897.

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