Biochemical

Step-Photometric Determination of Histidine. K. Schmid (*Helv. Chim. Acta*, 1946, **29**, 226–228)—The method, which can be used to determine histidine in impure solutions, such as urine, is a modification of that of Kapeller - Adler (*Biochem. Z.*, 1933, **264**, 131) in which a slight excess of bromine is added to the histidine-containing solution and the resulting dye is developed by means of an ammonia - ammonium carbonate mixture, and determined photometrically. In the present method, the excess of bromine is removed by addition of arsenious acid (Conrad and Berg, *J. Biol. Chem.*, 1937, **117**, 350), the urine is strongly diluted before test, and the bromination is carried out in presence of an excess of acid.

Procedure—Dilute 24-hr. rat urine to 100 ml. with water. Treat 1-ml. portions of this diluted urine with increasing volumes of a histidine solution containing 100 mg. of histidine and 2 ml. of 2 *N* sulphuric acid per 100 ml. Also, prepare 0·04 *N* sulphuric acid and use this to dilute to 2 ml. urine samples containing histidine. Add dropwise a solution containing 2·5 ml. of bromine, 250 ml. of glacial acetic acid, and 750 ml. of water, until the slight yellow colour of the liquid after 10 min. standing shows that there is a slight excess of bromine present. After 10 min., add 2 drops of 10 per cent. aqueous ammonia solution saturated with arsenious acid and 2 ml. of an ammonia - ammonium carbonate solution (2 parts of concentrated ammonia + 1 part of 19 per cent. ammonium carbonate solution). Heat in boiling water for 5 min., cool, and allow to stand at room

temperature for 10 min. to develop the colour. Dilute to 10 ml. with the ammonia - ammonium carbonate solution, and determine the intensity of the colour with a step-photometer, using an S50 filter and a 5-mm. cell. As the compensating liquid use urine treated in the same way. For 0·4 to 1·6 mg. of histidine hydrochloride per millilitre in the diluted urine there is a linear relationship between the histidine content and the extinction.

The method is ten times less sensitive than that of Edlbacher *et al.* (*Z. physiol. Chem.*, 1941, **270**, 158), but has the advantage of being specific for histidine. E. M. P.

Method for the Estimation of Barbituric and Thiobarbituric Acids in Biological Materials. J. Raventos (*Brit. J. Pharmacol.*, 1946, **1**, 210–214)—In order to study the fate and distribution of barbiturates in the animal body, several methods of estimation were examined, but none was completely satisfactory. Using the methods of Levvy (*Biochem. J.*, 1940, **34**, 73), Delmonico (*Proc. Mayo Clin.*, 1939, **14**, 113), and Anderson and Essex (*Anaesthesiology*, 1943, **4**, 113) the recoveries of known amounts of barbiturates added to blood or tissues were low. A method was developed for the estimation of barbituric acid based upon Koppanyi's colour reaction (*J. Amer. Pharm. Assoc.*, 1934, **22**, 1076), and for the estimation of thiobarbituric acids on a colour reaction described by Cowan.

Procedure—A. *Extraction*—Using blood, take 10 to 20-ml. volumes of oxalated blood and mix with equal volumes of water and of a 10 per cent. solution of sodium dihydrogen phosphate. Extract with ether in a continuous extractor at 45° to 50° C. for 8 to 10 hr. Evaporate the ether extract to dryness. For urine, acidify to *p*H 5 with concentrated hydrochloric acid and extract with ether in a similar manner. When using tissues, take samples of 10 to 20 g., grind in a mortar with sand, and then mix with 10 per cent. of solid sodium dihydrogen phosphate. Allow to stand for 5 to 10 min., add 20 g. of anhydrous sodium sulphate for every 10 g. of tissue with continuous grinding. Transfer to a desiccator containing calcium chloride for one hr. Extract the dry powder for 2 to 3 hr. with 50 ml. of benzene in a well-stoppered flask. Filter and wash three times with 10 to 15 ml. of benzene. Concentrate the filtrate and washings to about 5 ml. by distillation at 50° C. under reduced pressure.

B. *The separation of barbituric and thiobarbituric acids*—The extracts must first be purified by chromatography. Dissolve the residues obtained from the extraction of urine or blood in 5 ml. of chloroform and dry with 1 to 2 g. of anhydrous sodium sulphate. Add the solutions to columns of activated alumina $\frac{3}{8}$ in. × 4 in. The activated alumina used was grade O supplied by Messrs. Peter Spence of Manchester, and was treated by boiling 1200 g. with 1800 ml. of 10 per cent. acetic acid for 2 hr., filtering and washing with at least 20 litres of hot, distilled water. The alumina was dried, heated until the temperature reached 360° C., and then partly de-activated by adding $2\frac{1}{2}$ per cent. w/v of water. Filter the chloroform solutions on to the columns and wash the flask and filter 3 times with 5 ml. of chloroform, pouring the washings on to the column. Wash the column with chloroform until the eluate is free from pigment. When tissues are used, pass the benzene extract through alumina columns with slight suction, washing the flasks with benzene and using benzene to elute the pigment from the columns, and finishing with 20 ml. of chloroform. The chloroform and benzene washings are discarded. Thiobarbituric acids are recovered from the columns by elution with 50 ml. of 2 per cent. methanol in chloroform, the eluates being kept for estimation. To elute the barbituric acids, use 50 ml. of 10 per cent. methanol in chloroform. Under these conditions separation is complete, and the recoveries of both acids are almost theoretical.

C. *Estimation*—Evaporate the eluates to dryness under reduced pressure at 40° to 50° C. and dissolve the residues in chloroform.

Thiobarbituric acids—Take an aliquot of the final chloroform solution in a test tube, and for every 2 ml. add 0·2 ml. of a 10 per cent. solution of diethylamine in methanol followed by 0·5 ml. of a saturated solution of anhydrous copper sulphate in methanol. A green colour develops at once and is stable for about 2 hr. Compare the samples in a suitable colorimeter with a series of similarly treated standard solutions of the thiobarbituric acid to be estimated, containing 0·03 to 0·5 mg. per ml. Tissues such as brain and liver give a blank value sometimes as high as 1 mg. per 100 g. of tissue. The reaction is fairly selective, not being given by malonic acid, theophylline, theobromine, thiourea, caffeine, guanine, uric acid, urea, creatinine, oxamide, succinic acid, lecithin, cholesterol, cystine, or glutathione.

Barbituric acids—For every 2 ml. of the final chloroform solution add 0·6 ml. of 5 per cent. *iso*propylamine in methanol followed by 0·1 ml. of a 1 per cent. solution of cobalt acetate in methanol. A reddish colour is produced and can be compared in a colorimeter with a series of similarly treated standards containing 0·1 to 1 mg. of the appropriate barbituric acid per ml. of chloroform. The recovery of known amounts of barbituric or thiobarbituric acids added to blood and tissues is approximately complete except when the amount from a 10-ml. sample is less than 0·3 mg.; recovery may then fall below 95 per cent. R. H. T.

Chemical Assay of Penicillin. M. Mundell, H. Fischbach, and T. E. Eble (*J. Amer. Pharm. Assoc., Sci. Ed.*, 1946, **35**, 373–378)—Methods for the assay of penicillin are reviewed, and the occurrence of the four types of penicillins G, K, F, and X, is pointed out. Data are given of the relative activities of these substances against *Staphylococcus aureus in vitro*. The following chemical methods were investigated:—the colorimetric method of Scudi (*J. Biol. Chem.*, 1946, **164**, 183), the penicillinase method (Murtaugh and Levy, *J. Amer. Chem. Soc.*, 1945, **67**, 1042), the methods proposed by the Chas. Pfizer Co. based upon alkali inactivation and hydrogen peroxide inactivation, a spectrophotometric method (Herriott, *J. Biol. Chem.*, 1946, **164**, 725), and the iodimetric method proposed by the Squibb Institute for Medical Research (Alicino, *Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 619; *Abst.*, ANALYST, 1947, **72**, 68).

It was found that the penicillinase method and the iodimetric method gave the most consistent results when applied to diverse samples of commercial penicillin. The paper describes both methods in full, but points out that the iodimetric method gives better results generally and is technically simpler and more straightforward. The authors examined the factors involved in the iodimetric reaction of the alkaline products of inactivation of sodium penicillin G and found that 2·52 to 2·53 ml. of 0·01 *N* iodine are consumed by the inactivation product from 1 mg. of crystalline sodium penicillin G. The following method is proposed for the assay of ampoules of penicillin.

Procedure.—Place 5-ml. aliquots of a solution of penicillin (approximately 1000 units/ml.) in each of two iodine flasks. To one, add an equal volume of *N* sodium hydroxide and allow to stand at room temperature for 15 min. and then add 5 ml. of 1·1 *N* hydrochloric acid and 15 ml. of 0·01 *N* iodine. After 15 min., titrate the excess of iodine with 0·01 *N* sodium thiosulphate, approaching the end-point with the use of starch indicator and the addition of 5 ml. of carbon tetrachloride. To the second flask add 15 ml. of the 0·01 *N* iodine and titrate immediately to serve as a blank determination. The difference between the two titration values divided by 2·52 gives the number of milligrams of penicillin present. The use of the 1·1 *N* solution of hydrochloric acid results in a medium of low *p*H which has been shown to be advantageous for iodimetric titrations. No adjustment of *p*H is made to the blank determination since this would result in the formation of degradation products that would react with iodine and yield a correspondly low value for the penicillin potency.

A series of batches of sodium penicillin G was assayed by this procedure and recoveries ranging from 99 to 101·6 per cent. of the theoretical were obtained. A few samples of penicillins F, X, and K were assayed both by the iodimetric and penicillinase methods, and the results indicate that a similar number of equivalents of iodine react with equimolecular quantities of the respective penicilliates resulting from alkaline inactivation. The work of the Squibb group suggested that penicillin X might give abnormally high values, but the results obtained did not show this. Comparative data obtained for samples of crystalline penicillin G using the iodimetric, penicillinase, and bio-assay methods are given. The results show good agreements between the chemical methods, the average recoveries being 99·8 per cent. for the penicillinase, 99·2 per cent. for the iodimetric, and 98·2 per cent. for the bio-assay method. The standard deviations from the known weight of penicillin were, respectively, 1·5 per cent., 1·2 per cent., and 4·8 per cent. Good agreement was similarly obtained with samples of commercial penicillin.

The results of the chemical methods are most accurately expressed in milligram-equivalent weights of penicillin without any reference to the type of penicillin involved. Since the molecular weights of the various known penicillins are of the same order, little error is to be anticipated by converting the results to milligrams by multiplying by the average of the molecular weights of the four penicillins. The results cannot be accurately translated to the unit basis without knowing the proportions of the four penicillins present. Hence, comparisons of the accuracy of the chemical and biological methods must be made upon material of known composition. The penicillinase method requires the use of special equipment, and is difficult to apply to some preparations of penicillin. It is probably safe to assume that it is highly specific, and methods based upon its action are specific for penicillin. The iodimetric method requires no special apparatus or reagents, and can be successfully applied to most preparations of penicillin, but is not specific for penicillin, so that in the absence of information on the composition of the sample, supplementary analysis might be required.

R. H. T.

Three-Hour "Physical Development" Cup-Plate Assay for Penicillin. F. M. Goyan, J. Dufrenoy, L. A. Strait, R. Pratt, and J. Juntunen (*J. Amer. Pharm. Assoc., Sci. Ed.*, 1947, **36**, 65–68)—A practical method for the assay of penicillin depending on the fact that cells of *Staphylococcus aureus* differ in their affinity for silver, supplied in the form of an aqueous solution of silver nitrate, according to whether they are under the influence of penicillin or not, is described. It has been found that the silver-impregnated plates are analogous to photographic emulsions, and the application of principles of photographic development produces images that show a marked difference

between zones of inhibition and non-inhibition with a clearly defined boundary between the zones after only 5 hr. incubation. If the seeded plates are incubated for 3 hr. before the beginning of the assay, satisfactory images are produced after only 3 hr. incubation, and normal results have been obtained using such "pre-incubated" plates after storage for 5 days in a refrigerator.

Procedure—Prepare and seed the plates as for the standard cup-plate method of assay of penicillin, using *Staph. aureus*, NRRL Strain No. 313 (F.D.A. Strain No. 209P), incubate at 38° C., and store in a refrigerator until needed. Transfer the solutions to be tested to cylinders on the plates in the usual manner and re-incubate at 38° C. for 3 hr. After removal from the incubator, flood each plate with about 30 ml. of a nearly neutral, 0·1 per cent. aqueous solution of silver nitrate, expose for 2·5 to 3 min. to the illumination from two 40-watt daylight fluorescent lamps mounted in a reflector at a distance of about 35 cm. (this is equivalent to about 350 foot-candles). Remove the excess of silver nitrate solution by means of a suction device and then add 30 ml. of physical developer prepared as described below. After allowing the developer to act for 7 to 10 min., measure the diameters of the zones of inhibition in the conventional way.

Preparation of Physical Developer. Stock Solution A—Dissolve 80 g. of sodium thiosulphate and 20 g. of anhydrous sodium sulphite in 300 ml. of distilled water and add to this solution, slowly with constant stirring, a solution of 8 g. of silver nitrate in 200 ml. of distilled water. *Stock Solution B*—Dissolve 20 g. of sodium sulphite in 500 ml. of distilled water and, when dissolution is complete, add 3·8 g. of 2 : 4-diaminophenol hydrochloride and stir until dissolved. Store both stock solutions in a refrigerator and, just before use, mix equal volumes of solutions *A* and *B* and dilute their combined volume with 3 volumes of distilled water. The dilution of the developer and the time of development should be determined empirically for each laboratory, to allow for varying conditions of light intensity, temperature of development, etc. The concentration of silver nitrate used for impregnating the plates must be not less than 0·1 per cent. nor greater than 0·13 per cent., whilst the time of exposure must be adjusted empirically, as is customary in adjusting the time of exposure of photographic materials. The curve relating concentration of penicillin with diameters of zones of inhibition is linear from 1·0 unit per ml. to 8·0 units per ml. when plotted on log-log paper. Figures indicating that penicillins of varying degrees of purity are satisfactorily assayed by the proposed method are quoted, the results obtained being compared with those determined by the standard 18-hr., cup-plate method. J. A.