Table 11. *Stabilization of the blood pyruvate level by 'Cetavlon'-haemolysis in the presence of citrate buffer, pH 4, and fluoride*

Time (days)	Pyruvic acid (mg./100 ml.)	Time (days)	Pyruvic acid (mg./100 ml.)
0	1.15	7	1.16
1	1.15	10	1.18
2	1.18	14	1.13
3	1.12	21	1.14
4	1.15		

250 to give the pyruvic acid concentration in mg./100 ml. blood.

In an experiment using 10 ml. blood, the pyruvate level has been found to be quite constant for at least 3 weeks, as shown in Table 11. This pyruvate level was identical with that observed when the blood sample, in the absence of 'Cetavlon', was precipitated immediately with trichloroacetic acid.

SUMMARY

1. The changes in pyruvic acid level of blood samples treated with different anticoagulants, inhibitors and haemolytic agents have been investigated. The results obtained are in harmony with the occurrence of the two following reactions:

- (i) $\text{Pyruvate} + \text{triosephosphate} \rightleftharpoons \text{lactate} + \text{3-phosphoglycerate}$,
- (ii) $\text{3-Phosphoglycerate} \rightleftharpoons \text{2-phosphoglycerate} \rightleftharpoons \text{phosphopyruvate} \rightarrow \text{pyruvate}$.

2. The detailed procedure for stabilizing and estimating pyruvic acid in blood samples is given. Stabilization is brought about by haemolysis with cetyltrimethylammonium bromide in the presence of fluoride and a pH 4 citrate buffer; the estimation is by means of the 2:4-dinitrophenyl-hydrazone.

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Preparation of Penicillin. Improved Method of Isolation*

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(Received 24 January 1944; deposited with the Royal Society 23 May 1944-23 September 1944)

Fleming (1929) noticed that a mould which had accidentally contaminated a plate culture of *Staphylococcus aureus* was surrounded by a clear ring of agar, which suggested that the mould had produced an antibiotic substance in its immediate neighbourhood. He made no attempt, however, to isolate this substance, to which he gave the name penicillin, and the first attempt to do so was made three years later by Clutterbuck, Lovell & Raistrick (1932).

* This paper describes work completed by the end of 1942. Its publication has been delayed for security reasons. The authors are aware that considerable progress has been made in the preparation and isolation of penicillin since this preliminary work was carried out.

They confirmed Fleming's observations that penicillin was active against Gram-positive but not against Gram-negative organisms, and showed that it could be extracted by ether from aqueous solutions at pH 2. They gave details for growing the mould, now known as *Penicillium notatum* Westling, on a synthetic medium of Czapek-Dox type, instead of the broth media used by Fleming.

Widespread interest in penicillin was aroused by two papers published by Florey and his colleagues (Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings, 1941; Chain, Florey, Gardner, Heatley, Jennings, Orr-Ewing & Sanders, 1940), who described a method of preparing crude concen-

trates of penicillin from the metabolism solution produced by the growth of *P. notatum*, and showed that the product was of value in the treatment of certain infective conditions. These papers also described the bacteriostatic and bactericidal properties of penicillin, *in vitro* and *in vivo*, and the effects of serum and other biological materials on the activity. The effects of penicillin on leucocytes and tissues were also described, together with results of absorption and excretion experiments in man and laboratory animals.

The method of isolation now described differs radically from that recommended by the Oxford workers; it is more convenient and more efficient for large-scale working, and has been carried out on a substantial scale.

EXPERIMENTAL

Cultivation of the mould

Selection of strain. The first stage of the investigation comprised the selection of the best of nine different strains of *P. notatum* provided by Prof. H. Raistrick. The moulds were grown on a modification, introduced by Dr Turley, of the well-known Czapek-Dox medium; this had the following composition:

NaNO ₃	3.0 g.	FeSO ₄ .7H ₂ O	0.01 g.
KH ₂ PO ₄	1.0 g.	Glucose	40.0 g.
KCl	0.5 g.	Distilled water to	1000 ml.
MgSO ₄ .7H ₂ O	0.5 g.		

The solutions were decanted from the mould mycelia and tested for antibacterial activity against *Staphylococcus*, *Streptococcus* and *Pneumococcus*. From these preliminary experiments, several of the strains were selected for further examination, and the results indicated the marked superiority of strain B 592, originally obtained in 1929 from the Medical Research Council (National Institute for Medical Research).

Maintenance of stock cultures of Penicillium notatum. Stock cultures of *P. notatum* strain B 592 were grown on several different media, including Czapek-Dox agar, Czapek-Turley agar, Czapek-Dox agar containing sucrose instead of glucose, and malt or wort agar. It was found that those cultures grown on malt or wort agar gave the most reliable and morphologically stable moulds. The malt agar was made from a 5% solution of commercial malt extract and the wort agar from concentrated wort (sp. gr. 1.040). These stock cultures were held for at least 3 months before being subcultured, and it has been our custom throughout to inoculate our production batches from fresh subcultures prepared from an old stock culture, the parent culture being used for as long as possible, until growth was ex-

hausted. Care was taken, in making these subcultures, to transfer only the spores, avoiding transfer of the mycelium as far as possible, for it was found that mycelial cultures tended to give poor yields of penicillin. The subcultures, like the stock cultures, were made on malt agar slopes, which were incubated at 24° and used for sowing the batches when 7 days old.

Cultivation of the mould. The medium used for production of penicillin was Turley's modification of the Czapek-Dox medium described above. In the experimental stage of the work, 400 ml. portions were introduced into 1 l. conical flasks, which were autoclaved for $\frac{1}{2}$ hr. at a pressure of 15 lb./sq.in. It is known that autoclaving a solution of this type causes changes in the glucose, and the rotation of the autoclaved medium corresponded with about 2.5% instead of 4% of glucose; the copper reduction figure was equivalent to 4%. A suspension of spores, made by introducing 10 ml. of Czapek-Turley medium into each of the subcultures and dislodging the spores with a sterile stainless steel spatula, was used for inoculating the flasks, the contents of one culture tube being distributed as evenly as possible among four flasks, which were then shaken and incubated in the dark at 24°. Subsequently, however, it was found to be quicker and more convenient to transfer the spores directly from the slopes by means of a moistened platinum loop.

The growth of the mould was characteristic and has not varied greatly over a long period; only occasionally has serious trouble been experienced through a falling off in the yields of penicillin. The above precautions have, however, been rigidly followed; it is especially important to use a subculture obtained from an old stock culture. After 2-3 days the surface of the liquid in the flasks became covered with a white felt; this gradually formed folds, the crests of which were blue-green; orange-yellow transpiration drops accumulated in these folds. The reverse side was golden yellow in colour, and the colour of the solution gradually deepened to an intense yellow. Penicillin production was complete in 10-14 days. During this time the apparent concentration of glucose fell steadily, the optical rotation reaching a minimum value corresponding to about 0.5%. The pH of the solution fell to about 4 in the first 4-5 days and then rose to 7.0-7.6.

The method of assay

The testing of penicillin was carried out by the serial dilution method (Clutterbuck *et al.* 1932). Each test solution was sterilized by filtration through a small Seitz filter, and dilutions were made in tubes containing 4 ml. of digest broth medium of pH 7.2, the series forming a geometrical progression from 1 in 10 to 1 in 2560. Each tube of medium was

then sown with one loopful of a 24 hr. culture of the chosen *Staphylococcus aureus* strain, and the results were read after incubation for 18 hr. at 37°.

The medium was a papain-digest broth, employed by us for other purposes, and was made by mixing 1000 g. of minced horse muscle with 4 l. of distilled water and digesting the suspension with 10–15 g. of papain at 65–70° for 3 hr., the pH being maintained at 7.0 throughout. The digest was filtered, made alkaline to phenolphthalein and sterilized by steaming for 1 hr. on two successive days. The concentrated digest was filtered to remove precipitated phosphates, and then diluted with an equal volume of normal saline. The pH was adjusted to 7.4, and the solution tubed and autoclaved at a pressure of 20 lb./sq.in.

The strain of *Staph. aureus* used was isolated in these laboratories from a pathological specimen, and stock cultures of it were carried on agar. Test cultures were made from these by transference to the papain-digest broth and incubation for 24 hr., the culture so obtained then being used for inoculating the penicillin solution, and 0.1 ml. of the suspension being added to each 4 ml. tube.

Inoculations with a drawn-out pipette were made from a 24 hr. culture in the same medium. Once a week the culture was plated out on blood agar. Addition of glucose enhanced the growth in papain-digest broth, but the end-point was less sharply defined. Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick (1942) showed that notatin, another substance produced by *Penicillium notatum*, inhibits the growth of *Staphylococcus aureus* if the medium used contains glucose; in view of this it is essential that the medium used in testing metabolism solutions for penicillin should not contain glucose.

An interchange of samples with other workers has shown that it is possible to obtain good agreement between assay results from different laboratories. Occasional discrepancies have been encountered, however, and recently the practice has been adopted of comparing each batch of samples with a standard preparation.

Variations in composition of medium

The amount of penicillin produced in early batches was very variable, the dilutions necessary to inhibit the growth of *Staphylococcus* ranging from 1 in 200 to 1 in 1600 for different batches, and even for different flasks of the same batch. Moreover, if the metabolism solutions were allowed to stand, marked loss of activity occurred in a few days.

Stability of penicillin at various pH's. It was found that the solutions were more stable, and indeed showed very little loss after 14 days, if they had been boiled for 10 min. and then cooled. This is illustrated by an experiment in which a metabolism solution was tested at intervals and the activity compared with that of a solution which had been boiled for 10 min. The results are shown in Table 1. This increased stability was possibly due to the destruction of an enzyme capable of inactivating penicillin.

The stability of penicillin in metabolism solutions was found to be optimal at pH 7–8, and little or no

Table 1. *Effect of boiling the metabolism solution on the stability of penicillin during storage*

Pretreatment of solution	Titre of solution after storage for (days)			
	0	3	7	14
None	1/1280	1/160	1/40	—
Boiled for 10 min.	1/1280	1/1280	1/1280	1/640

activity was lost when such solutions were kept at 80° for 2 hr. A solution of pH 5, on the other hand, lost all its activity when heated at 60° for 4 hr., whilst a solution of pH 3 lost all its activity at room temperature (23°) in 1 day and in 3 days at 0°. Solutions of pH 10 and above were also unstable; most of their activity was lost on storage at room temperature for 3 days.

Modifications of medium for penicillin production. Towards the end of 1941, after the formation of the Therapeutic Research Corporation Ltd., we continued this work in collaboration with the Wellcome Physiological Research Laboratories (W.P.R.L.), where a new medium for growing the mould had been elaborated by Dr C. G. Pope. This consisted of the Czapek-Turley medium with added copper sulphate, sodium acetate and sodium citrate, and had the following composition: glucose, 4 %; NaNO_3 , 0.4 %; KH_2PO_4 , 0.1 %; KCl, 0.05 %; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 %; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 %; sodium citrate, 0.2 %; sodium acetate, 0.2 %; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.00175 %.

The B 592 strain on this medium gave more consistent yields of penicillin than on the Czapek-Turley medium, and the penicillin itself appeared to be more stable; titres averaging 1 in 640, with some variation on either side of this figure, were obtained.

The optimal depth of the medium in culture was found to be 3–4 cm. A greater depth of liquid gave lower titres, the rate of diffusion of oxygen from the atmosphere then being insufficient for complete fermentation; since the lower layers remained colourless, whereas the layer immediately below the mycelium was dark yellow in colour.

The effect of modifying the composition of the W.P.R.L. medium was tested by making up with tap water a basal medium containing 4 % glucose, 0.1 % KH_2PO_4 , 0.05 % KCl, 0.05 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 % $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and adding varying amounts of NaNO_3 , CuSO_4 , sodium citrate and sodium acetate. The titres obtained after growing the mould on these media for 11–12 days are shown in Table 2.

These preliminary results indicated that (a) the addition of copper alone either to the Czapek-Dox or to the Czapek-Turley medium had no effect on the penicillin production or was even harmful; (b) the addition of citrate and acetate had a limited effect; and (c) improved results were obtained by adding copper sulphate, citrate and acetate together..

Table 2. *Effect on penicillin production of adding various supplements to the basal medium*

Additions to basal medium (%)				Titre
NaNO ₃	CuSO ₄ ·5H ₂ O	Sodium citrate	Sodium acetate	
0.4	0.00175	0.2	0.2	1 in 640
0.4	0.00175	0	0	1 in 160
0.3	0.00175	0	0	1 in 80
0.3	0	0.2	0.2	1 in 320
0.3	0	0	0	1 in 160

Later experiments threw some doubt on the beneficial effect of copper; it proved difficult to obtain conclusive evidence on this point, for the variations from flask to flask were of the same order as those encountered between media with and without added copper. The conclusion reached after a large number of experiments was that with strain B 592 the addition of copper increased the maximum titre slightly or not at all, but rendered the yields of penicillin more consistent; it undoubtedly increased the rate and degree of sporulation. The presence of copper also appeared to inhibit the formation of the yellow pigment chrysogenin, first described by Clutterbuck *et al.* (1932); only negligible amounts of this pigment were isolated from metabolism solutions containing copper + acetate + citrate.

The effect of other substances on the yield of penicillin and on the morphology of the mould was also studied. Tyrosine up to 0.045% was without effect, but the addition of 0.01% of asparagine markedly increased the concentration of penicillin produced during 11 days' incubation. Ascorbic acid (0.1%) and nicotinic acid (0.1%) were without effect on the titre, but the former produced a bluer felt and the latter increased the growth rate. Aneurin (0.01%) and riboflavin (0.01%) had little or no effect. *dl*-Aspartic acid (0.01%) had no effect, but 0.1% of glutamic acid speeded up growth and led to a somewhat higher titre.

The simultaneous addition of aneurin, riboflavin, nicotinic acid, ascorbic acid and pimelic acid had no effect on the titre, the pH or the rate of glucose consumption. Guanidine hydrochloride (0.001%) had a marked effect on the appearance of the mould and resulted in the rapid formation of numerous transpiration drops, but did not materially increase the yield of penicillin. The effect of guanine and adenine on the titre was negligible, whilst the addition of uracil (up to 0.05%) gave a darker metabolism solution without affecting the titre.

Isolation of penicillin from metabolism solutions

(1) *Extraction methods.* The first attempts to isolate penicillin from the mould metabolism solution (Czapek-Turley medium) were made by the method of Abraham *et al.* (1941), using extraction with amyl

acetate at pH 2. Very troublesome emulsions were obtained, however, and aqueous extracts from the amyl acetate solutions were only slightly active. Better results were obtained by the ether extraction method of Clutterbuck *et al.* (1932).

Portions (50 ml.) of the solution were extracted with three 35 ml. portions of ether, and the ethereal extract, after concentration by distillation in the presence of water, was tested alongside the residual metabolism solutions. Extraction was optimal at pH 3, some loss of penicillin taking place at pH 2. Extraction was facilitated by acidification to pH 3.6 and filtration to remove protein and chrysogenin, as recommended by Clutterbuck *et al.* (1932). The resulting filtrate did not form emulsions as readily as did the original solution.

The ethereal extract obtained from this filtrate was extracted with dilute NaHCO₃, and the aqueous solution acidified and extracted with ether. The barium salt of penicillin was prepared by shaking this with a suspension of excess BaCO₃, separating off the aqueous phase, filtering and evaporating the filtrate in the frozen state. The solid so obtained inhibited growth at a dilution of 1 in 4 × 10⁶, and a 60% recovery of the original activity was obtained.

One disadvantage of this process was encountered when it was applied to metabolism solutions obtained from the W.P.R.L. medium. A considerable amount of acetic acid was extracted along with the penicillin, so that calcium acetate was present in the product, which was therefore less active per mg. than similar material obtained from Czapek-Turley medium.

(2) *Adsorption methods.* It was found that penicillin was quantitatively adsorbed from protein-free metabolism solutions of pH 2, 3, 4, 5 and 7 by activated charcoal or fuller's earth (20 g./l. of metabolism solution); the charcoal removed the colour as well as the antibacterial activity. Less charcoal, e.g. 10 g./l., did not remove the activity completely. The activity could be recovered quantitatively from the charcoal adsorbate by elution with 80% or absolute ethanol, or with methanol, acetone or pyridine, but the degree of concentration effected by adsorption and elution varied according to the pH at which the adsorption was carried out.

This is illustrated by an experiment in which portions of the same metabolism solution, adjusted to various pH values, were adsorbed on charcoal (20 g./l.), and the titres and total solids of the ethanol eluates determined. The results (Table 3) indicated that the best pH for adsorption

Table 3. *Effect of alterations in the pH of metabolism solution on recovery of penicillin and of total solids from charcoal adsorbates by elution with ethanol*

pH	Titre	Total solids (g./l.)
3	1/160	1.2
4	1/320	1.15
5	1/320	1.05
6	1/320	0.75
7	1/320	0.75
8	1/160	0.75
9	1/160	0.65

was 6-7. When a volume of ethanol equal to one-half that of the metabolism solution was used, most of the activity was contained in the first eluate; the amount of penicillin present in the third eluate was negligible. Elution was almost, but not quite, complete with a volume of ethanol one-quarter that of the metabolism solution. Aqueous acetone (20%), recommended by the W.P.R.L. workers, was found to be as good an eluant as ethanol, but aqueous ether proved to be less satisfactory, only 50% of the activity being recovered when a volume equal to that of the original solution was used.

A new method of working up metabolism solution was based on these results. The solution was acidified to pH 3.6 and precipitated protein and pigment were filtered off. The filtrate was neutralized (pH 7), stirred with 2% of its weight of charcoal (Sutcliffe and Speakman no. 5) and filtered. The charcoal was eluted on the filter with a one-half volume of ethanol and the filtrate diluted with a one-tenth volume of water and evaporated under reduced pressure, great care being taken to prevent the internal temperature from rising above 20°. After acidification to pH 3, the aqueous solution was extracted three times with two-thirds its volume of ether, the combined ethereal extracts were shaken with a suspension of excess BaCO_3 and the lower layer was separated off, filtered and evaporated in the frozen state. The solid so obtained inhibited completely the growth of *Staphylococcus aureus* at a dilution of 1 in 0.75×10^6 to 1 in 1.5×10^6 , and contained 20-40% of the activity of the original solution.

When this process was applied to 20 l. batches, the yields were considerably below 20%. It was then learned that Dr C. G. Pope at the W.P.R.L., using the same process, was obtaining much more satisfactory recoveries, and a comparison of the two methods showed that he added chloroform to the metabolism solution to prevent bacterial contamination. It was found that the addition of chloroform to our metabolism solution greatly facilitated the subsequent elution of activity from the charcoal adsorbate, whilst adsorption was in no way impaired. All the activity was then recovered from the charcoal by elution with a one-fifth volume of 20% acetone. Under these conditions, the charcoal adsorption method gave better yields of better material, needed less bulk of materials than extraction with ether or other solvent, and required less complicated plant. Moreover, when sodium acetate was used in the medium, it was advantageous to use charcoal adsorption because it did not result in contamination of the final product with calcium acetate or barium acetate, as did solvent extraction. Yields varied from 30 to 60%, and the material inhibited growth at dilutions of 1 in 3×10^6 to 1 in 5×10^6 .

Adsorption occurred more easily when the metabolism solution was filtered through a bed of charcoal instead of being stirred with charcoal as in the original process. In this way not only was the

amount of charcoal required reduced from 20 to 4 g./l., but elution of the adsorbate was easier; even aqueous ether gave complete recovery. The resulting penicillin preparation was more active, and barium salts which inhibited growth in a dilution of 1 in 15×10^6 , equivalent to 300 Oxford units/mg., were obtained. This observation has not been confirmed by the W.P.R.L. workers, who obtained only partial elution under these conditions. With the strain of *Penicillin notatum* used at the W.P.R.L. we also failed to obtain complete extraction; we can only conclude that other strains of mould produce impurities which affect adsorption and elution.

The effect of the presence of organic solvents and of inorganic salts in the aqueous solution on the adsorptive power of the charcoal was next studied. The penicillin was adsorbed when a solution saturated with ether was run through a bed of charcoal, but a solution containing in addition 1-2% of NaCl retained its activity under the same conditions. No penicillin was then adsorbed but a considerable reduction in the colour was effected, with a corresponding increase in the activity of the resulting calcium salt from 100 to 140 units/mg. The process gave smaller total yields, however, when applied to large batches, and was discontinued.

Large-scale production of penicillin

The general procedure described above is now being operated on a fairly large scale. The mould is grown in special glass flasks (Pl. 3). These flasks are so designed that several can be stacked during incubation; for filling and sterilization they are accommodated in specially designed wire baskets, each of which holds 12 flasks. This reduces the necessity for frequent handling of individual flasks.

Each flask holds 800 ml. of W.P.R.L. medium, which is introduced by means of a large tap attached to a measuring device. The flasks are then plugged with cotton-wool and autoclaved for 10 min. at a pressure of 10 lb./sq.in. After cooling overnight, the flasks—still in the wire baskets—are inoculated by means of a wire loop with spores of *P. notatum* grown on a wort-agar slope, the inoculation being carried out in a closed cabinet. The flasks are then taken from the baskets and stacked on racks, which are wheeled into an incubator room maintained at a constant temperature of $25 \pm 1^\circ$.

After 14 days' incubation, the liquid is filtered through muslin and then run through a bed of charcoal and kieselguhr (which facilitates filtration) prepared on a large stoneware funnel; 5 g. of charcoal are used for each litre of metabolism solution. When all the solution has filtered, the charcoal is washed with a little water and then saturated with chloroform. The penicillin is eluted by running through the charcoal a volume of 20% acetone equal to one-tenth that of the original metabolism solution. The eluate, acidified to pH 2.5-3.0 with 10% H_3PO_4 , is emulsified by rapid stirring with an equal volume of chloroform, and the emulsion then broken by means of an Alfa-Laval separator. The chloro-

Photograph by Bertram Park

Mould culture flasks in use for penicillin production.

form layer is collected separately and stirred with 0.5% NaHCO_3 . Alternatively, the chloroform solution is shaken with a suspension of CaCO_3 to give a solution of calcium penicillin. Both the sodium salt and the calcium salt, obtained from the solutions by drying at low temperatures (between -30° and -15°) *in vacuo*, are bright yellow amorphous powders, which generally inhibit growth at a dilution of about 1 in 10×10^6 ; this is equivalent to an activity of 200 Oxford units/mg.; 1000 l. of metabolism solution are produced and worked up each day, with an average titre of 15 Oxford units/ml., and 60–70% of the total activity is recovered as sodium or calcium salt. With a somewhat smaller installation producing 200 l. of metabolism solution each day, yields of 75–80% are obtained.

Attempted purification of crude penicillin

Various attempts were made to purify further the crude penicillin obtained from the metabolism solution by charcoal adsorption and elution.

(a) *Formation of salts of penicillin.* Several attempts were made to obtain sparingly soluble salts of penicillin. An aqueous solution of barium penicillin, on treatment with benzyl-pseudothiuronium chloride, gave a yellow precipitate, whilst the filtrate contained all the activity of the original solution. Since the amount of precipitate formed was very small, however, the degree of purification effected was insufficient to justify the routine use of this procedure.

None of the following quaternary ammonium salts gave insoluble salts with penicillin: pyridinium and quinolinium methiodide, benzyl-quinolinium chloride, *p*-nitrobenzyl-pyridinium chloride and *m*-nitrophenyl-trimethyl ammonium iodide.

(b) *Miscellaneous.* Catch, Cook & Heilbron (1942), by means of their chromatographic method, in which columns of silica gel mixed with alkaline earth carbonates are used, succeeded in increasing the activity of some of our material, containing 200 units/mg., to over 1000 Oxford units/mg.

Treatment with aluminium amalgam, recommended by Abraham *et al.* (1941), was applied to our material and resulted in almost complete decolorization of the solution; all the activity of the original salt was found in the supernatant liquid, and some degree of concentration was effected. The necessity for employing in this connexion the troublesome three-stage chromatographic treatment recommended by Abraham *et al.*, and the greater success of the chromatographic method of Catch *et al.* (1942), led us to abandon this line of attack. Penicillin can also be reduced without loss of activity by catalytic hydrogenation in presence of platinum oxide, and by electrolytic reduction by means of a mercury cathode and lead anode with a diaphragm.

Attempts to improve the purity of penicillin by electrodialysis in a simple cell or by foaming according to the method of Schütz (1937, 1942) were unsuccessful.

Potency. The best specimens of penicillin prepared by us are inhibitory to *Staphylococcus aureus* at a dilution of 1 in 15×10^6 to 1 in 20×10^6 , but are probably not more than 10–20% pure; no useful purpose would be served by a description of their chemical and physical properties.

Stability. In aqueous solution the activity of crude penicillin is unchanged on standing for several days at pH 8, but 50% of the activity is lost at pH 3.6 in 8 hr. at 20° or in 24 hr. at 0° . It is also destroyed by standing overnight in solution in ethanol or methanol.

The dry barium or calcium salt can be heated for 1 hr. at 100° *in vacuo* without loss of activity, though some of our batches proved unstable to heat and even to cool storage. An aqueous solution of the salts is inactivated by boiling for 10 min., in striking contrast to the stability of penicillin in metabolism solutions; the latter can be boiled in a copper-free medium (see above) for 10 min. without loss.

Penicillin solutions are inactivated by tin, zinc, lead and ferric salts, 0.01% destroying the activity completely in 24 hr. at 0° . Copper has an even more striking effect, 0.001% being sufficient to inactivate penicillin solutions in 24 hr. at 0° . Ferrous salts are comparatively harmless, whilst silver appears to be inert. The deleterious effect of copper was not inhibited by addition of 0.1% glycine or citrate. The effect of copper is rather surprising, for, whereas the presence of up to 4.5 p.p.m. in the mould metabolism solution apparently does no harm, somewhat larger amounts, e.g. 10 p.p.m., are sufficient to inactivate purified preparations of penicillin in a few hours. The presence of varying amounts of copper or ferric iron in our calcium salts probably accounts for the different stability of different batches.

SUMMARY

1. A satisfactory method of cultivating *Penicillium notatum*, and a method of isolating crude penicillin from the metabolism solution, are described.

2. Penicillin can be adsorbed from aqueous solutions by activated charcoal and can be eluted from the adsorbate by certain organic solvents, or mixtures of solvents with water.

3. The production of preparations of penicillin containing up to 200 units/mg., by an adsorption and elution process, is now being carried out on a large scale, the mould being grown in specially designed glass flasks.

We thank the Directors of the Therapeutic Research Corporation Ltd. for permission to publish this account of our work, and we also wish to record our gratitude to Prof. H. Raistrick for the very considerable help he has given us throughout our investigations. We have to thank too our colleagues in the Therapeutic Research Corporation, especially Dr J. W. Trevan and Dr C. G. Pope of the Wellcome Physiological Research Laboratories, whose collaboration has been particularly valuable; their results have been made freely available to us and have greatly facilitated our work. Finally, we have to acknowledge the help given, in designing the flasks, by Mr A. G. Howkins, and in the laboratory work by Messrs G. V. Cawthorne, H. H. Somerville, and the Misses A. M. Hill, J. A. Withycombe and J. Waldie.

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A Critical Examination of Lugg's Method for the Determination of *l*-Ascorbic Acid. 2

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(Received 11 August 1944)

Our previous contribution to the subject (Snow & Zilva, 1943) was devoted mainly to the study of the kinetics of the combination of pure ascorbic acid, reductic acid and reductone with HCHO, and the results were utilized for the consideration of the limitations of Lugg's method (1942). Further observations made us aware of the fact that certain indophenol-reducing substances which are formed on heating solutions containing glucose in the presence of alkali behaved differently in their combination with HCHO to pure reductone. It therefore became desirable to investigate in detail the problem that arose. The results which are reported in this communication, apart from raising points of theoretical interest, throw further light on the limitations of the method. A technique which we consider yields the most satisfactory results under the circumstances is described.

METHODS

Reagents

l-Ascorbic acid and reductic acid (see Snow & Zilva, 1941).

Reductone (see Snow & Zilva, 1938).

Formaldehyde (see Snow & Zilva, 1943).

Metaphosphoric acid. Laboratory reagent. Solutions prepared freshly each day.

Pectin. Commercial specimen prepared from lemons. Completely soluble in water, giving an almost colourless opalescent solution. pH of 1% w/v solution = 3.04. Free acid—equivalent to 0.98 ml. *N*-NaOH/g. (indicator—phenolphthalein).

Glycine. Anal. reagent twice recrystallized from water.

Ethylaminoacetate hydrochloride. Prepared from Anal. glycine and recrystallized from ethanol; m.p. 143°.

Methyleneaminocetonitrile. Dimeric form prepared by the method of Klages (1903); twice recrystallized from ethanol; m.p. 129°. The molar concentrations of this com-

pound given in this paper are calculated on the basis of the monomeric formula.

Methylamine. Dilute solutions prepared from a 33% aqueous solution. Concentration determined by titration with HCl.

Alanine (dl). Synthetic commercial specimen.

Glutamic acid. Commercial specimen containing a mixture of the enantiomorphs.

Other compounds employed were all Anal. reagents.

Indophenol titration. Solutions containing ascorbic acid or reductic acid were titrated directly with *mn*-2,6-dichlorophenolindophenol. With reductone or alkali-treated glucose solutions, however, direct titration was unsatisfactory owing to the slowness of the reaction. In these cases the reducing solution was run into an excess of acidified indophenol solution, allowed to react for 1 min., and the excess of the indicator back-titrated with stabilized *mn*-ascorbic acid solution.

Treatment of glucose solutions with varying concentrations of sodium hydroxide. 20 ml. 2.5% w/v glucose solution together with sufficient water to give a total volume of 25 ml. after the addition of the necessary quantity of NaOH were placed in a boiling tube in a water-bath at 91.5° so that the temperature inside the tube was 90°. After the addition of NaOH the solutions were mixed for a short time by a current of nitrogen; continuous bubbling of the gas was undesirable owing to evaporation. At the end of the required period the reaction was stopped by the addition of a small excess of HCl. The solutions were cooled in an atmosphere of nitrogen, diluted to a known volume, and the reducing value assessed by indophenol titration.

Buffers

For adjustment to ...	pH 3.5	pH 1.5
ml. 10% $\text{CCl}_3\text{CO}_2\text{H}$	417	417
g. NaCl	167	167
g. KH_2PO_4	23.3	23.3
g. citric acid	36	36
ml. 4 <i>M</i> -sodium acetate	123	30.4
Water	To 1 l.	To 1 l.