PREPARATION OF PURIFIED PENICILLIN

BY

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This communication describes a simple and inexpensive method of preparing purified penicillin suitable for systemic use. The method can be carried out with the equipment available in most bacteriological laboratories. Production of penicillin in bacteriological laboratories would augment the supplies and make penicillin available to patients who otherwise could not be treated by this valuable drug.

Cultivation of the Mould

The mould *Penicillium notatum* was obtained from Sir Alexander Fleming. It was grown on a modified Czapek-Dox medium, using lactose instead of glucose (Taylor, 1943), corn-steep liquor instead of yeast extract (Clifton, 1943), and adding traces of zinc (Foster *et al.*, 1943). Yields of penicillin of 30 to 40 units per ml. were regularly obtained on this medium. The mould was grown at room temperature. The antibacterial activity was assayed by the cylinder-plate method (Heatley, 1944) and the tube-dilution method (Fleming, 1942).

Penicillin for Systemic Administration

Methods for extraction and purification of penicillin are all based on the observation that this substance can be extracted from strongly acidified aqueous solutions into ether, amyl acetate, or chloroform, and re-extracted from the organic solvent into water at pH 7 (Clutterbuck *et al.*, 1932; Abraham *et al.*, 1941; Meyer *et al.*, 1942). Further purification is achieved by chromatographic methods (Abraham and Chain, 1942; Catch *et al.*, 1942).

These methods proved very laborious and uneconomical, and therefore quite unsuitable for routine laboratory use. Meyer *et al.* (1942) claimed for their method a yield of over 50% of the original potency. Abraham *et al.* (1941) obtained in a form suitable for intravenous injection only about a third of the penicillin which had been present in the crude culture filtrate. The considerable losses of penicillin during purification by these methods were chiefly due to the rapid destruction of penicillin in strongly acid solutions, particularly when extraction was done at room temperature. To minimize losses, extraction had to be carried out in the ice-chest. Other features of these methods which have been found inconvenient were the large bulk of the solvents required and the long period of time (4 to 12 hours) which had to be allowed for complete separation of the amyl acetate-culture fluid mixture. Chromatographic methods for further purification of penicillin were too laborious and wasteful for routine purposes.

A search was made for a solvent which would extract penicillin from aqueous solutions at a pH not harmful to penicillin. It was found that most of the antibacterial substance could be extracted by *n*-butyl alcohol from culture filtrates adjusted to pH 6.4 (Berger, 1944), at which penicillin is most stable. Before extraction with butyl alcohol most of the inactive pigments and other impurities could be removed from the culture fluid by precipitation with ammonium sulphate. The salting out of impurities with ammonium sulphate did not result in any loss of antibacterial potency of the filtrate, and incidentally caused still more complete extraction of penicillin by butyl alcohol. Further experiments have shown that the solubility of penicillin in butyl alcohol is considerably greater than in the culture fluid at pH 6.4, thus allowing extraction with relatively small amounts of solvent. Penicillin dissolved in butyl alcohol was quite stable. Two samples of sodium penicillin obtained from the same butyl alcohol extract—one immediately after extraction and the other after having been kept in butyl alcohol for 48 hours in the ice-chest—contained the same amount of penicillin. The butyl alcohol-culture fluid mixture separated well, separation being complete after 10 to 15 minutes.

Penicillin was precipitated from butyl alcohol by light petroleum ether and obtained as an aqueous solution of the sodium salt by addition of a suitable quantity of sodium

bicarbonate solution. During this procedure further purification of penicillin was achieved as certain impurities were left behind in the organic solvents. At this stage a fifteenfold to fortyfold concentration of penicillin could be obtained, and although extraction was carried out at room temperature the losses of penicillin were never greater than 15%, and were as a rule as small as 5%, of the amount present in the culture fluid.

Penicillin solutions were further purified by extraction into chloroform at pH 3.6 in the presence of ammonium sulphate (Meyer *et al.*, 1942). The addition of sodium bicarbonate solution to the chloroform extract again yielded an aqueous solution of sodium penicillin. The losses during this stage of purification were small, and usually less than 5% when the penicillin solution was well chilled before acidification. A further tenfold concentration was achieved by the chloroform extraction, and solutions of sodium penicillin containing 3,000 to 4,500 units per ml. were finally obtained.

Technical Details

The technical details of the method were as follows: The culture fluid was adjusted with 20% phosphoric acid to pH 6.4. The Lovibond comparator, with bromothymol blue as indicator, was used for this purpose. The culture fluid was then cooled in the ice-chest and 40% of ammonium sulphate was dissolved in it. The precipitate which formed was filtered off in the ice-chest through a Chardin filter paper. Five volumes of the filtrate were then extracted by shaking in a separating-funnel with one volume of butyl alcohol, and after separation the extracted culture fluid was discarded. To the butyl alcohol extract were then added an equal volume of light petroleum ether and one-eighth of the total volume of the mixture of 2% sodium bicarbonate solution. The butyl alcohol-petroleum ether mixture was extracted for a second time with a fresh portion of the 2% sodium bicarbonate solution.

The first extract of sodium penicillin, after having been chilled to approximately 4° C., was adjusted to pH 3.6 with 20% phosphoric acid, using the capillator method with bromophenol blue as indicator. Again 40% of ammonium sulphate was dissolved in the acidified penicillin solution, which was then extracted with an equal volume of chloroform. After separation penicillin was extracted into sodium bicarbonate solution by shaking ten volumes of the chloroform extract with one volume of 2% sodium bicarbonate solution. The chloroform was then extracted for a second and third time with fresh portions of sodium bicarbonate solution. The accompanying table gives the results obtained in a typical experiment.

Table showing Results obtained with the Butyl Alcohol Extraction Method followed by Further Purification and Concentration of the First Extract by the Chloroform Method

No.	Sample	Units per ml.	Volume of Fluid in ml.	Total Units	Percentage of Units Recovered
1	Culture fluid	31.6	14,000	442,400	—
2	1st extract from butyl alcohol	384.5	980	376,810	85.1
3	2nd extract from butyl alcohol	55.0	850	46,750	10.5
4	Extracted culture fluid	0.5	14,000	7,000	—
5	1st extract from chloroform made from No. 2	3,156.6	100	315,660	83.7
6	2nd extract from chloroform made from No. 2	489.7	100	48,970	12.9
7	3rd extract from chloroform made from No. 2	40.7	100	4,070	1.0
8	No. 2, after chloroform extraction	3.5	980	3,430	—

The aqueous solutions of penicillin were then adjusted to approximately pH 6.6 with dilute phosphoric acid. When kept in the ice-chest they retained their full potency for at least two months. The penicillin solutions were sterilized by passing through a Seitz filter; they were then stored in sealed ampoules and used for systemic treatment of patients. These chloroform-free penicillin solutions retained their full antibacterial potency at 4° C. for at least a further six weeks. The penicillin solutions could be freeze-dried without difficulty.

Second extracts from the butyl alcohol and the second and third extracts from chloroform were further purified and concentrated by the chloroform method to give a product assaying 250 to 500 units per ml. These solutions were incorporated into creams or used as drops for treatment of infections of the eye.

Before dispensing, the penicillin solutions were tested for sterility. Suitable amounts of penicillase, prepared according to Harper's (1943) method, were added to 1-ml. quantities of the penicillin solution and incubated aerobically and anaerobically in heart broth for three days. The penicillin solutions appeared to be non-toxic.

Rabbits injected intravenously with 20 ml. of a solution containing 60,000 units of sodium penicillin did not show any immediate or delayed reactions. Intracutaneous injections into the abdominal wall of rabbits and guinea-pigs did not cause any local inflammatory reactions, and the cutaneous swelling arising from the injection rapidly disappeared.

Morgan *et al.* (1944) when administering penicillin intravenously by the continuous-drip method noticed the development of thrombosis or thrombophlebitis in a large proportion of their cases. In view of these experiences intravenous application was not tried; the intramuscular route was employed exclusively. Usually 4 to 6 ml. of the penicillin solution (about 12,000 to 18,000 units) was injected every three hours. The continuous-intramuscular-drip method was also used in suitable cases, giving 80,000 to 100,000 units in 24 hours. Up to the present more than 40 patients suffering from various diseases due to penicillin-sensitive organisms were successfully treated in this way. Details of the clinical results will be published later. The injections of penicillin did not cause any immediate or delayed painful sensations, and no irritative or inflammatory reactions were observed.

Penicillin was also repeatedly injected intrathecally in two cases of pneumococcal meningitis and one case of streptococcal meningitis. The injections were therapeutically effective, were well tolerated, and did not cause any untoward symptoms.

Penicillin for Local Treatment

Crude culture filtrates containing as little as 2 to 10 units of penicillin per ml. have been used successfully by Pulvertaft (1943), Alston (1944), and Hobson (1944) for treatment of many localized infections. The clinical use of crude culture filtrates appears inadvisable and potentially dangerous for two reasons. First, such filtrates always contain large amounts of protein, which is also produced by the mould and secreted into the culture fluid. The repeated application of such fluids to large raw surfaces is likely to lead to sensitization—a point also made by Raper and Coghill (1943) in connexion with "home-made" penicillin. Secondly, culture filtrates from media which do not contain yeast extract or corn-steep liquor may contain notatin (Kocholaty, 1942; Coulthard *et al.*, 1942; Roberts *et al.*, 1943), a potent antibacterial substance of extreme toxicity.

Clutterbuck *et al.* (1932) found that by adjusting the fluid to pH 3.6 most of the protein and a considerable proportion of pigment could be flocculated and filtered off from the culture fluid without loss of penicillin. This method has been successfully employed for this purpose, and incidentally the gummy substances formed from the corn-steep liquor during autoclaving were precipitated out at the same time. Kocholaty (1943) found that notatin is not formed in cultures of *P. notatum* which contain corn-steep liquor.

The protein-free penicillin solutions were not toxic. Two rabbits, weighing about 1,600 g. each, were injected with 50 ml. intravenously in 10-ml. portions in the course of two days. The animals did not show any immediate or delayed reactions of any kind. The protein-free penicillin solutions have been used successfully in the treatment of extensive infected burns, wounds, abscesses, ulcers, discharging sinuses, throat and ear infections, empyemata, and gonococcal vulvovaginitis, and in selected cases of osteomyelitis and compound fractures. The penicillin solutions proved non-irritating and did not cause untoward symptoms in any case. Manifestations of allergy were not observed even when application of penicillin was continued for a month or longer.

Summary

A simple and efficient method of preparing purified penicillin for systemic treatment is described.

Intramuscular injections of penicillin solutions obtained with this method were therapeutically effective, were painless, and did not cause untoward effects of any kind.

This work has been done under the direction of Prof. P. L. Sutherland, to whom I am indebted for many valuable suggestions and for help and advice throughout the course of the investigation. Thanks are due to Sir Alexander Fleming for a subculture of the mould; to Dr. J. W. Trevan, of the Wellcome Research Laboratories, for a sample of standardized penicillin; and to Dr. D. Engel, medical superintendent of Staincliffe County Hospital, Dewsbury,

for clinical application of the penicillin. Acknowledgments are also due to Mrs. B. Berger, Mr. G. Denton, and Mr. H. Schofield for technical assistance.

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THYROID HYPERPLASIA AFTER PROLONGED EXCESSIVE DOSAGE WITH THIOURACIL

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During the past year we have treated with thiouracil a total of 31 patients suffering from thyrotoxicosis. The results obtained are very similar to those noted by other workers. They are on the whole most encouraging, and comparable to those produced by successful thyroidectomy. Only one of the patients developed serious toxic reactions to the drug, consisting of leucopenia and signs of acute sensitivity. Another appeared relatively drug-resistant, and, in spite of prolonged treatment, thyrotoxic symptoms and signs persisted. With the exception of the case noted below no significant changes in the size of the goitres occurred as the result of treatment, though they usually became softer, and a transient slight increase in size was often noted at the menstrual period. It seems to be the general experience of other workers that thiouracil in therapeutic doses does not as a rule affect the size of the goitre significantly, but Himsworth (1944) has reported three cases in which the goitre increased considerably in size as the result of treatment with thiouracil—an effect which he attributes to overdosage.

There seems to be general agreement that the optimum dosage during the initial period of treatment should be 0.6 g. daily. This dosage should be kept up for from three to four weeks, at which time a fall in the basal metabolic rate has usually become significant. No increased effect is obtained by doubling the dose, as any additional quantity appears to be immediately excreted in the urine. There is as yet no definite consensus of opinion as to the dose required for maintenance treatment. There can be little doubt that 0.2 g. daily is adequate. We have never noticed any evidence of "escape" from the control of the drug when a reduction in the dosage has been made from 0.6 to 0.2 g. daily. There is no definite evidence, however, that 0.2 g. is the minimum dose required. We have latterly treated a number of patients with a maintenance dose of 0.1 g. daily, and it is our impression that this dose is quite sufficient. One case, through a misunderstanding, received an excessively large maintenance dose for a period of five and a half months.

Case Record

A female patient, aged 33, suffering from classical thyrotoxicosis, had experienced her first symptoms one year before admission to hospital. A small primary goitre was present. She was treated