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The determination of penicillin G by isotope dilution, using C^{14} isotope as a tracer

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THE DETERMINATION OF PENICILLIN G BY ISOTOPE DILUTION, USING C^{14} ISOTOPE AS A TRACER

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CHAPTER I

THE PROBLEM AND DEFINITIONS OF TERMS USED

One of the major problems which has confronted researchers in the development or improvement of any process, especially in the recovery of antibiotics from fermentation broths, has concerned the availability of a reliable, precise, and accurate analytical method to account for losses and determine yields in each step of the process.

For many years in the penicillin industry there has been a scarcity of any sort of absolute assay method for determining the amount of penicillin G in fermentation broths and in various extracts and concentrates obtained in the recovery process. There have been a number of assay methods developed for determining penicillin G in crystalline samples. The utility of such methods, however, was limited because these methods were confined to relatively pure samples. In the evaluation of a recovery process, these methods could be used only for the analysis of final products and they have proved to be indispensable for this purpose.

Obviously, differentiation between penicillins in each step of a recovery process is highly desirable particularly when attempts have been made to separate penicillin G from the undesirable penicillins during the recovery and

to control the production of penicillin G in the broth through the use of precursors or through the selection of molds.

I. THE PROBLEM

Statement of the problem. It was the object of this research undertaking (1) to develop an absolute assay method for the determination of penicillin G in fermentation broths by applying the principle of isotope dilution using the radioactive isotope, C^{14}, as a tracer; (2) to compare the assay results obtained by this method with results obtained by other methods available for the determination of total penicillins; (3) to show the application of the isotope method to the penicillin industry through its use in determining the rate of production of penicillin G during the fermentation cycle.

Importance of the study. The only method employed for the production of penicillin has been the fermentation method. During the fermentation process many different types of penicillins are produced. The various types of penicillins are commonly called penicillin F, G, K, X, flavicin, and dihydro F. The general structure of each type will be described later in this chapter under "Definitions of Terms Used". Only penicillin G has been

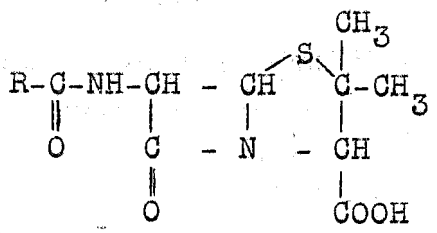
acceptable by the Food and Drug Administration for use in medicine and in the pharmaceutical trade. It has been, therefore, of paramount importance that industrial fermentations be designed to produce as near 100 per cent penicillin G as possible through the selection of proper precursors and molds. Likewise, it has been of equal importance that the recovery process be designed to separate penicillin G from the undesirable penicillins when small amounts of the latter were produced during the fermentation.

In order to design and successfully control either a fermentation or recovery process selective for the production of penicillin G, it has necessitated the availability of a reliable assay method to determine penicillin G content. Prior to this study the assay methods at hand were few and were either impractical or were limited as to precision and accuracy. In this study an attempt was made to develop and employ an entirely new penicillin G assay method which was precise, accurate, and practical.

II. DEFINITIONS OF TERMS USED

Penicillin. The word penicillin was derived from the Latin term "Penicillium Notatum" which is the name of the mold that produces the antibiotic. This mold was so-named because of the resemblance of the hyphae, with their

terminal spores, to a brush or pencil (penicillus). Penicillin can best be defined by writing the basic structural formula:[1]



To date there have been six known types of natural penicillin. These penicillins differ in structure only in the nature of the side-chain, R. The structure and names of these side-chains are given in Table I.

Isotope dilution analysis. The isotope dilution method of analysis consists of a simple set of successive operations. An isotopically labeled compound is added to an unknown mixture. This is allowed to come to equilibrium; then the same compound is isolated from the system. The isotopic content of the original compound is then compared with that of the final one. The ratio of the two specific activities depends upon the relative amounts of the substance added and that already present.[2]

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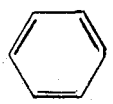
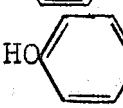
[1]G. W. S. Andrews and J. Miller, Penicillin and Other Antibiotics (London: Todd Publishing Group, Ltd., 1949), p. 60.

[2]George K. Schweitzer and Ira B. Whitney, Radioactive Tracer Techniques (New York: D. Van Nostrand Company, Inc., 1949), p. 147.

TABLE I

TYPES OF NATURAL PENICILLIN[3]

Penicillin Name (American)	Structure of the R Group	Name of the R Group
Penicillin F	$CH_3-CH_2-CH=CH-CH_2-$	Δ^2-pentenyl
Penicillin Dihydro F	$CH_3-CH_2-CH_2-CH_2-CH_2-$	amyl
Flavicin	$CH_3-CH=CH-CH_2-CH_2-$	Δ^3-pentenyl
Penicillin G	 $-CH_2-$	benzyl
Penicillin X	HO  $-CH_2-$	p-hydroxybenzyl
Penicillin K	$CH_3-(CH_2)_5-CH_2-$	n-heptyl

[3]Andrews, op. cit., p. 61.

C^{14} isotope. C^{14} is a radioactive isotope of carbon. This important isotope was made from nitrogen by N^{14} (n,p) C^{14} in the Oak Ridge, Tennessee chain reactor. The isotope was purchased from the Atomic Energy Commission at the price of fifty dollars per millicurie. One millicurie of pure C^{14} would weigh very nearly twenty-five hundredths of a milligram if the half-life were taken as 6400 years. In many ways this isotope has been a very excellent tracer and since it emits no gamma rays and only soft beta rays, it has been easily shielded during chemical operations. The beta rays emitted from this isotope have been found to have an energy of seventeen-hundredths millielectron volts.[4]

III. ORGANIZATION OF THE REMAINDER OF THE THESIS

Chapter II has included a discussion of a review of the literature concerning other methods which have been used for the assay of penicillin G as well as a survey of a number of applications of the isotope dilution method of analysis. Chapter III was composed of a complete description of the preparation of the C^{14}-labeled penicillin G which was used as the radioactive standard in the analysis herein described. Chapter IV has included the complete procedure which was worked out for the analysis of penicillin G in

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[4]G. Friedlander and J. W. Kennedy, Introduction to Radiochemistry (New York: John Wiley and Sons, Inc., 1949), p. 293.

broth samples using C^{14}-labeled penicillin G as a tracer. Chapter V has contained all the experimental results obtained in the study. The entire research project has been summarized in Chapter VI.

CHAPTER II

REVIEW OF THE LITERATURE

Much has been written in regard to methods of analysis for penicillin G. At the very outset of research to develop processes for the isolation of penicillin, before it became known that several different kinds of penicillin were produced in the fermentation, only those analytical methods which determined total penicillins were in demand. After the structure of the various types of penicillin became known and each type had been clinically tested for its effectiveness in destroying bacteria and infections, it was found that penicillin G was the most effective, the most stable, and easiest to isolate on an industrial basis. All these factors led to the acceptance of penicillin G by the Food and Drug Administration as the type to be marketed for medical use. Needless to say, the acceptance of penicillin G necessitated the development of assay methods which would distinguish this type from all the other forms of penicillin.

The various methods of analysis have been grouped into three catagories: microbiological, chemical, and isotope tracer methods.

I. MICROBIOLOGICAL METHODS

The high cost of analytical instruments for use in chemical methods of assay seems to have inspired researchers to develop other methods which did not require the use of instruments for the analysis of penicillin G. Among these methods was the microbiological assay. In general, this type of assay has been quite satisfactory for estimating "G" content in broth samples but they have all lacked the desirable precision and accuracy to have been accepted as absolute, thoroughly reliable methods.

Differential assay. Higuchi and coworkers[5] developed a technique for the estimation of the relative amounts of three penicillins in a mixture by means of a microbiological differential assay. This method was limited by the assumption that only three penicillins were present in any sample. Beyond three, errors made practical application difficult. This technique merely gave an indication of those penicillins present in a sample and was not meant to be a precise and accurate method.

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[5]K. Higuchi and W. H. Peterson, "Estimation of Types of Penicillins in Broths and Finished Products," Analytical Chemistry, 19:68, January, 1947.

Chromatographic absorption. Johnson and Thorn[6] developed an assay for the estimation of penicillin G in broth samples by chromatographic absorption. This technique was based on the fact that, on a column of Super Filtrol (an acid-treated bentonite), penicillin G was more strongly absorbed under given conditions than any other known penicillins and could be eluted as a separate fraction. Its application was to fermentation broths. This assay was also based on the assumption that about seventy-nine per cent of the penicillin G was absorbed and all values were corrected by this factor. The values obtained were generally within ten per cent of the theoretical value.

Paper chromatography. The third microbiological method has been quite widely used. This assay, called paper chromatography, was developed by Kluener[7] in 1949. A total time of twenty-four hours was required for a complete assay by this technique and was based on the principle that various penicillins possessed different distribution coefficients between ether and phosphate buffer at a given pH; hence, could be separated by paper strip partition chromatography.

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[6]J. A. Thorn and M. J. Johnson, "Direct Estimation of Penicillin G in Small Broth Samples," Analytical Chemistry, 20:614, July, 1948.

[7]R. G. Kluener, "A Paper Chromatographic Method for the Quantitative Estimation of Penicillin Entities," Journal of Bacteriology, 57:101-9, January, 1949.

A quantitative estimation of activity percentage was obtained by measuring the minor axis of the individual zones made on an agar plate, reading the equivalent penicillin percentage from a standard curve and dividing the units of each penicillin by the total units found. The precision of the method was estimated at plus or minus five per cent at best, with extremely good technique, and required a high-quality technician to produce desirable results.

II. CHEMICAL METHODS

Practically all of the chemical methods reported in the literature for the assay of penicillin G have been designed for the assay of crystalline samples of relatively high purity. They have not been adapted to broth samples.

N-ethylpiperidine method. Sheehan and coworkers devised a chemical method for the analysis of penicillin G in crystalline samples called the N-ethylpiperidine or N.E.P. method.[8] This analysis was an accurate one for crystalline products of relatively high purity and it depended on the sparing solubility of the N-ethylpiperidine salt of penicillin G in amyl acetate-acetone mixtures.

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[8]J. C. Sheehan, W. J. Mader, and D. J. Crane, "A Chemical Assay Method for Penicillin G," Journal of the American Chemical Society, 68:2407, November, 1946.

This assay has continued to be widely used in the industry; however, it has been applied only to crystalline samples.

Ultraviolet absorption. Herriott published a spectroscopic method for "G" determination in crystalline samples by measuring its ultraviolet absorption in an acetate buffer solution.[9] Grenfell and coworkers also worked out a similar spectroscopic technique, but both of these types of analysis could be applied only to crystalline samples.[10]

Infrared absorption. Barnes, et. al, contributed another spectroscopic method which depended upon infrared absorption of crystalline samples.[11] He found, however, that certain impurities in some samples interfered with the infrared analysis. The breakdown products and cogeneric impurities of penicillin G which could interfere included such monosubstituted phenyl bearing molecules as the sodium salt of phenylacetic acid. It was also found that penicillin X interfered to some degree.

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[9]R. M. Herriott, "A Spectrophotometric Method for the Determination of Penicillin," Journal of Biological Chemistry, 164:725, August, 1946.

[10]T. C. Grenfell, J. A. Means, and E. V. Brown, "A Study on the Naturally Occurring Penicillins, An assay for Penicillin G," Journal of Biological Chemistry, 170:527, October, 1947.

[11]R. B. Barnes, R. C. Gore, E. F. Williams, S. G. Linsley, and E. M. Peterson, "Infrared Analysis of Crystalline Penicillin," Analytical Chemistry, 19:620, September, 1947.

Modified colorimetric method. Boxer adapted a colorimetric method to the analysis for penicillin G in broth and crystalline samples which had been previously applied to total penicillin determinations.[12] This method, however, did not become very widely used. A number of interfering factors frequently presented themselves.

All of the herein described chemical methods have been quite useful in industry, but have all had the same limitations in that only crystalline samples could be assayed by these techniques. The industry was still in dire need for an absolute, accurate, and precise assay for the determination of penicillin G in broth samples and in other samples containing large amounts of impurities.

III. ISOTOPE TRACER METHODS

C^{13} isotope dilution method for penicillin G. Craig and coworkers presented a new technique for the assay of penicillin G based on the principle of isotope dilution using the stable isotope, C^{13}, as a tracer.[13] This method

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[12] G. E. Boxer and Patricia M. Everett, "Colorimetric Determination of Benzylpenicillin," Analytical Chemistry, 21:670-3, June, 1949.

[13] John T. Craig, John B. Tindall, and Murry Senkus, "Determination of Penicillin G Using C^{13} Isotope as a Tracer," Analytical Chemistry, 23:332-3, February, 1951.

employed the same basic principle as the method herein described. However, because of the fact that a stable isotope was used, the isolation of a pure penicillin G sample and the determination of the isotope ratio was entirely different. In the C^{13} method, the isolated sample had to be hydrolyzed to phenylacetic acid which contained both labeled and unlabeled carbon at the carboxyl group. The acid was then decarboxylated with quinoline and copper chromite, thereby releasing carbon dioxide containing both the labeled and unlabeled carbon. The resulting carbon dioxide was entrained into a gas bulb, then passed into the mass spectrometer to determine the isotope ratio. Although very reproducable and accurate results were obtained by this method, it was somewhat cumbersome, time consuming, and involved the use of a very expensive instrument for determining the isotope ratios.

The C^{13} method proved to be an invaluable tool because it was the only chemical method developed up to that time for assaying penicillin G in broth samples, penicillin extracts, and penicillin crystals.

It was the success of this method that led to the development of a very closely related technique, although quite different, the radioactive C^{14} isotope dilution analysis for penicillin G, herein described in the main text of the thesis.

S^{35} isotope dilution analysis of mixtures of sulfide, sulfoxide, and sulfone compounds. The isotope dilution analysis has been a valuable tool in the field of analytical chemistry. Information has been obtained by this type of tracer application which no other analytical procedure has been able to supply.

Rittenberg and Foster, both pioneers in the field of isotope dilution, in 1940 worked out an isotope dilution method with application to the determination of amino acids and fatty acids using the stable isotope, deuterium.[14]

Later, Henriques and Margnetti again demonstrated the practicality and efficacy of this technique and found that radioactive isotope dilution was a powerful tool applicable to analytical problems that could not be solved readily by any other means.[15] They showed that mixtures of dibenzyl sulfide, sulfoxide, and sulfone could be analyzed using S^{35} as a tracer. An average error of less than one per cent and a maximum error of two per cent was achieved.

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[14]D. Rittenberg and G. L. Foster, "A New Procedure for Quantitative Analysis by Isotope Dilution, with Application to the Determination of Amino Acids and Fatty Acids," Journal of Biological Chemistry, 133:737, May, 1940.

[15]F. C. Henriques, Jr. and Charles Margnetti, "Radioactive Studies," Industrial and Engineering Chemistry, Analytical Chemistry, 18:476-8, August, 1946.

Analysis of benzene hexachloride for gamma isomer using deuterium as a tracer. Trenner developed an isotope dilution method for the analysis of the gamma isomer content in benzene hexachloride using deuterium-labeled gamma isomer as the tracer which was quite successful.[16] The chief objection to this application was the necessity of using the mass spectrometer to determine the isotope ratios.

Analysis of benzene hexachloride for gamma isomer using Cl^{36} as a tracer. Very recently, Craig and coworkers also developed an isotope dilution method for the analysis of the gamma isomer content in benzene hexachloride using the radioactive isotope, Cl^{36}, as a tracer.[17] Again, very high precision and accuracy of plus or minus less than one per cent was achieved.

Mechanism of isotope dilution. The mechanism of the principle of isotope dilution was described by Yankwich.[18]

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[16]N. R. Trenner, "Determination of Gamma Isomer of Benzene Hexachloride," Analytical Chemistry, 21:285-90, February, 1949.

[17]John T. Craig, Philip F. Tryon, and Weldon G. Brown, "Determination of the Gamma Isomer of Benzene Hexachloride Using Cl^{36} as a Tracer," (in publication in Analytical Chemistry; will probably appear in 1953).

[18]Peter Yankwich, "Radioactive Isotopes as Tracers," Analytical Chemistry, 21:318-21, March, 1949.

He pointed out that the only requirements to be met were that an amount of pure compound sufficient for radioactivity measurement be isolated and that the labeled atom be located in an unreactive position. He also stated that when a small amount of labeled compound was added to an unlabeled mixture, followed by isolation and purification of a sample and determination of the decrease in isotope concentration, contamination by small amounts of impurities of high specific activity was absent.

There were a number of other applications of isotope dilution in the literature with practically all resulting in a precision of plus or minus one to two per cent. This basic principle, however, has not been applied to the analysis of penicillin G except for the work of Craig, et. al., previously described, wherein the stable isotope, C^{13}, was used.

CHAPTER III

PREPARATION OF THE C^{14}-LABELED PENICILLIN G

The first step in an isotope dilution analysis consists of preparing a chemically pure labeled compound identical in chemical structure to the compound to be determined in the unknown mixture. The compound which had to be prepared for the method described herein was C^{14}-labeled penicillin G. This compound was prepared by first conducting a microbiological fermentation designed to produce penicillin G. Radioactive carbon instead of ordinary carbon was introduced into the penicillin G molecule through the addition of C^{14}-labeled sodium phenylacetate to the fermentation medium. Unlabeled sodium phenylacetate has been widely used as a precursor for the microbiological production of penicillin G.

I. MICROBIOLOGICAL FERMENTATION TO PRODUCE PENICILLIN G

Description of the fermentation apparatus. The fermentation was conducted in thirty-six 500 milliliter Erlenmeyer flasks mounted on a rotary shaker in a fermentation room maintained at a temperature of twenty-five degrees centigrade. During the progress of the fermentation, the penicillin G precursor breaks down to

some degree and carbon dioxide is evolved as a decomposition product. Since a radioactive precursor was used to prepare the labeled penicillin, it was necessary to provide a means for eliminating a radiation hazard. This was accomplished by sealing each flask in a tin can containing an inlet and outlet port in the lid. Figure 1 shows a view of the can and cover. A foam glass collar was placed around the neck of the flask in order to reduce the possibility of flask breakage while shaking. A square of asbestos was placed in the bottom of each can to act as a cushion for the fermentation flask. Each can was bolted to the shaker head to prevent shifting during the shaking. The flasks were then inoculated and inserted into the cans after addition of the fermentation medium. The lids were placed on the cans. Each can was connected in series by means of short lengths of rubber tubing so that a stream of air could be pulled through the entire system to provide aeration for the fermentation. The air stream carried radioactive carbon dioxide into a barium hydroxide trap placed between the end flask and the vacuum outlet. The radioactive carbon dioxide was held in the trap by the formation of insoluble barium carbonate which was removed by filtration and dried for disposal. Figures 2 and 3 were included to illustrate the arrangement of the flasks on the shaker-head.

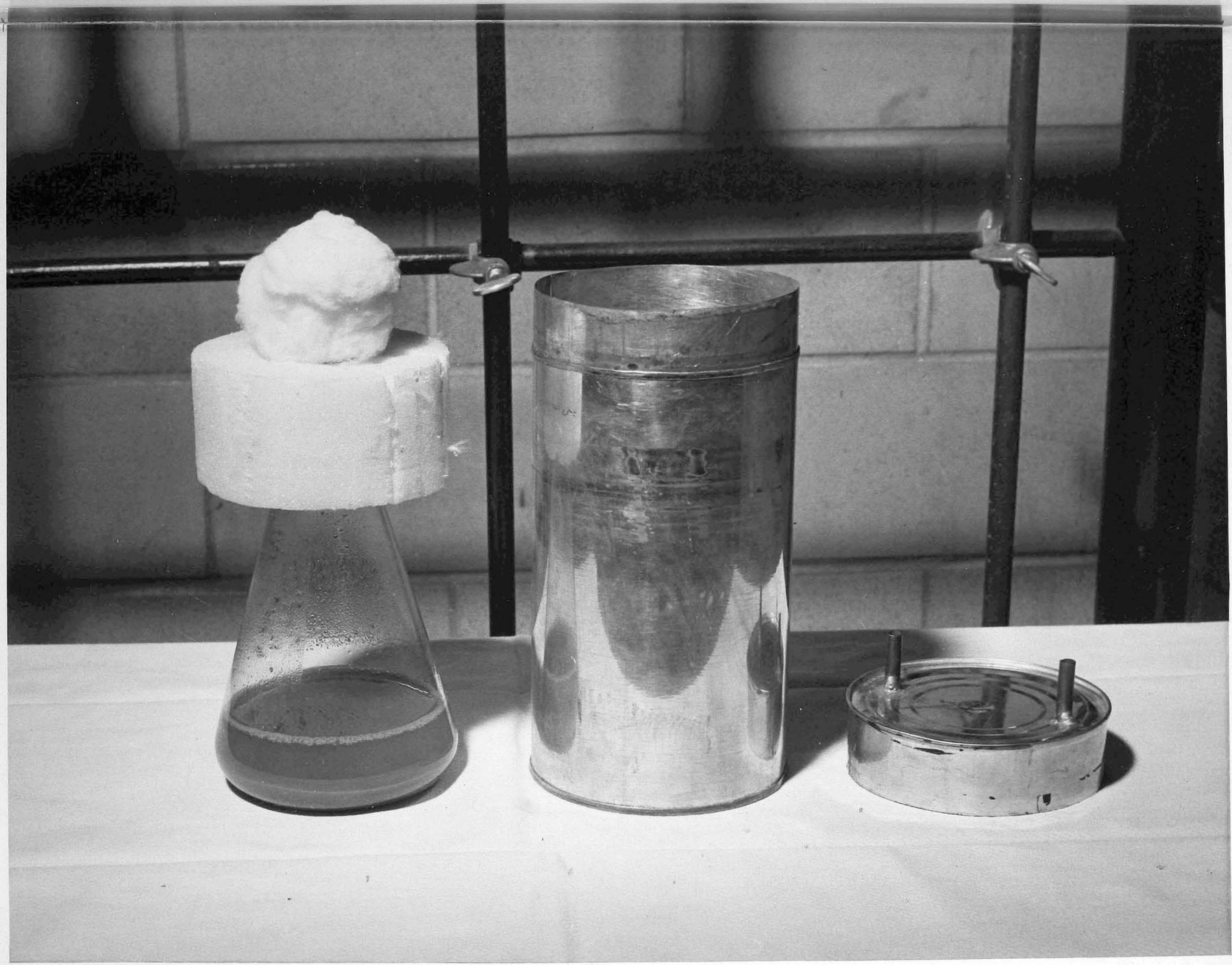


FIGURE 1 FERMENTATION FLASK, CAN, AND COVER

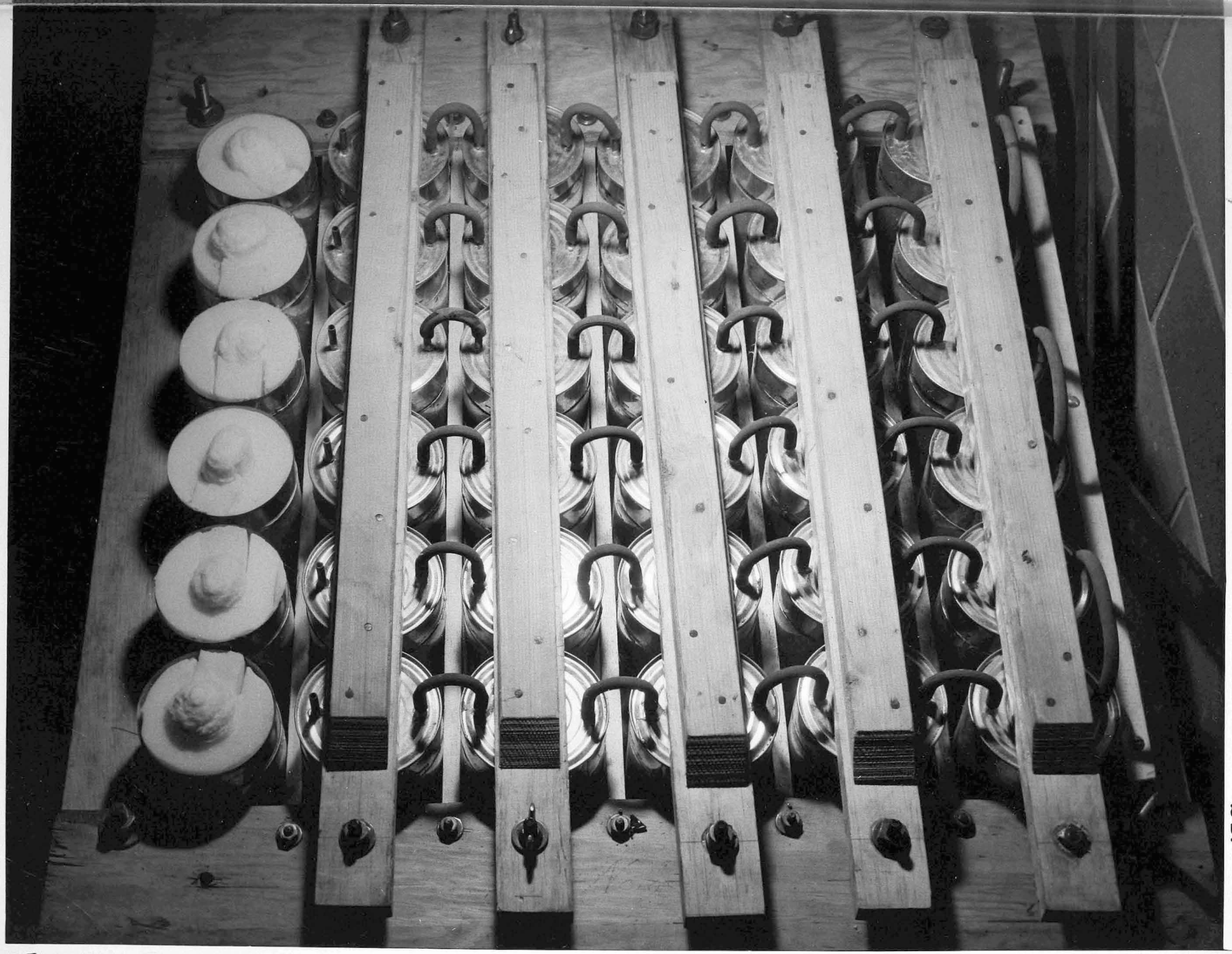


FIGURE 2 PARTIALLY ASSEMBLED FERMENTATION APPARATUS

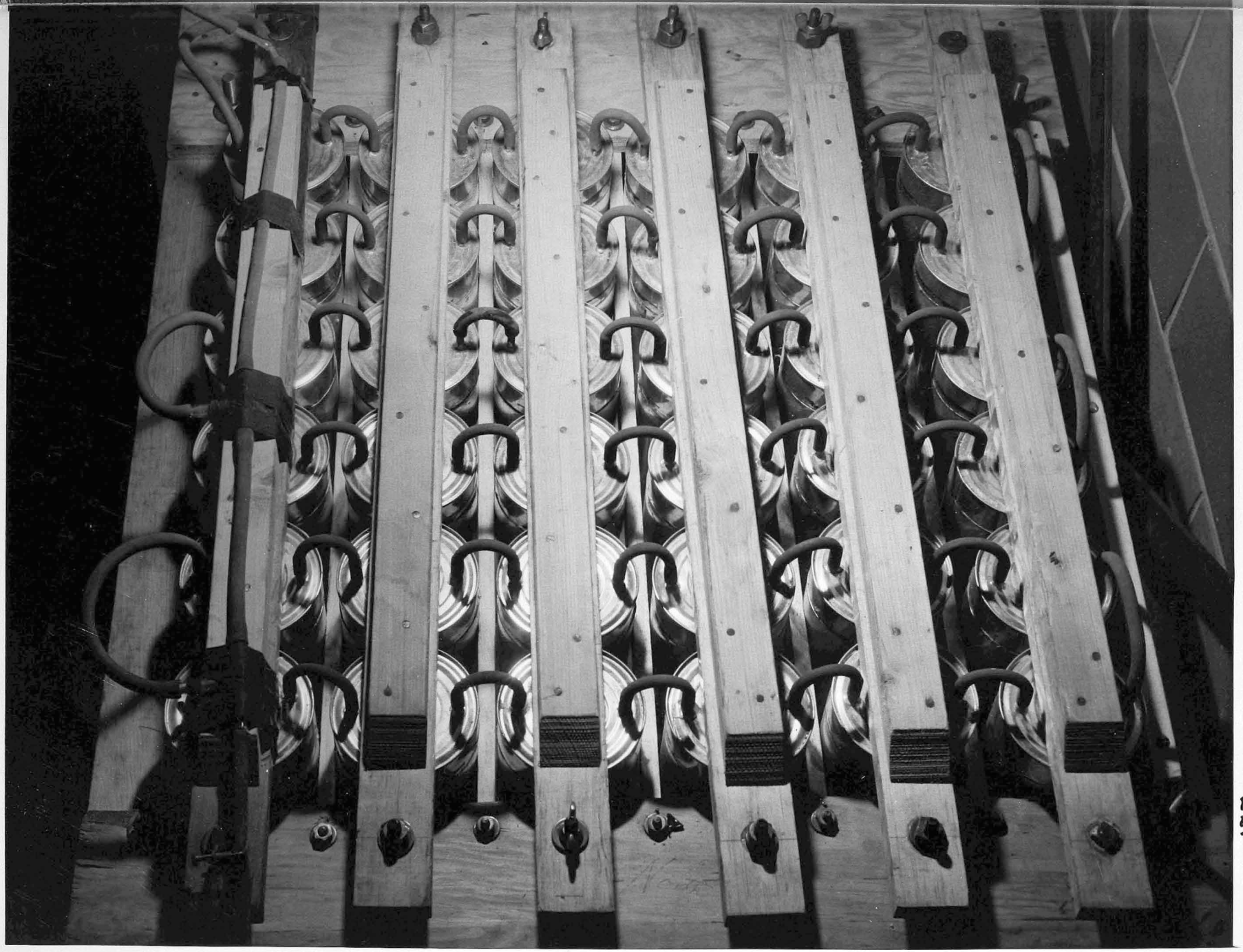
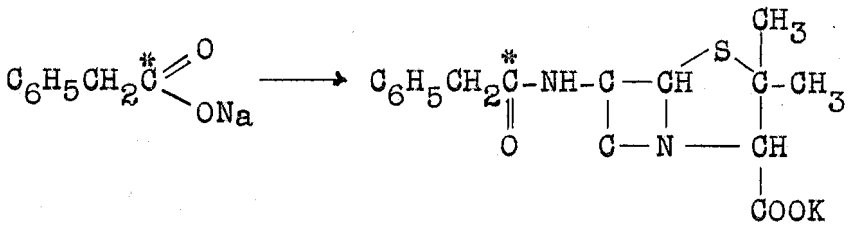


FIGURE 3 ASSEMBLED FERMENTATION APPARATUS

Microbiological fermentation to produce C^{14}-labeled penicillin G. The labeled precursor used in the fermentation was sodium phenylacetate labeled with C^{14} at the carboxyl group, prepared by a Grignard synthesis at the University of Chicago, under the direction of Dr. Weldon G. Brown. The position of the labeled carbon in the precursor and in the resulting penicillin G produced is shown below.



The fermentation medium favorable for the production of penicillin G was prepared. Approximately one millicurie of the labeled precursor was added to each fermentation. Preliminary experiments showed that the best conversion of precursor to penicillin occurred when a total of two-hundredths per cent precursor (based on the weight of the medium) was used. This was determined by conducting a low-level tracer fermentation (i. e., a fermentation containing a small amount of tracer material). It was found that the addition of larger amounts of precursor would produce much higher titres of penicillin in the broth. However, when using more than two-hundredths

per cent precursor, most of the precursor was wasted. The primary goal in planning the high-level fermentation was not to obtain high titres but to obtain low titres of very high C^{14} concentration in the penicillin produced.

Two fermentations were run to produce the desired quantity. The following calculations were made to determine the volume of C^{14} precursor in solution needed in each fermentation to introduce one millicurie of C^{14} at a precursor concentration of two-hundredths per cent:

Concentration of precursor solution	53 mg/ml.
Specific activity of precursor (solid)	1 mc/g.
Fermentation capacity (150 ml per flask x 36)	5400 ml.
Wt. of solid precursor for 0.02% conc.	1.08 g.
Volume of precursor solution containing 1.08 grams	20.4 ml.

Since there were thirty-six flasks used in the fermentation, the volume of precursor solution was diluted to thirty-six milliliters so that one milliliter could be placed in each flask. The flasks were autoclaved, cooled, and placed in the cans on the shaker head after the addition of the precursor. The cans were sealed in the manner described previously.

The fermentation was allowed to proceed for sixty-eight hours. The apparatus was disassembled after the completion of the fermentation cycle and the penicillin broth harvested. The final broth assay was 305 units per milliliter.

A second fermentation was conducted in the same manner as the first. The final assay of this broth was 552 units per milliliter.

II. ISOLATION OF CRUDE CRYSTALLINE C^{14}-LABELED PENICILLIN

The broth from each flask from the first fermentation was composited and filtered to remove the mycelia. The filtered broth was then divided into two nearly equal portions. Each portion was extracted separately according to the following procedure. The total volume of filtered broth and mycelia cake wash in each half was 2800 milliliters. From previous trial runs it was found that when filtered penicillin broth was extracted with an organic solvent at a low pH, an emulsion immediately formed which made it difficult to separate the spent broth layer from the solvent layer after the two had been agitated together. It was also found in preliminary work that the addition of diatomaceous clay to the filtered broth at a pH of five, followed by filtration, eliminated the formation of an emulsion to the extent that the two layers could be separated easily by centrifuging. Two per cent diatomaceous clay was added to the filtered broth in preparation for the extraction. The pH of the broth containing the clay was adjusted to five during vigorous agitation. The slurry was then filtered immediately to

remove the clay and some proteinaceous material which precipitated at the lowered pH. The treated broth was extracted with 600 milliliters of butyl acetate by adjusting the mixture to a pH of two with phosphoric acid with vigorous agitation. The mixture was transferred to a separatory funnel immediately after the proper pH was reached. The mixture remained in the funnel about ten minutes to allow the lower water layer to separate. The upper, slightly emulsified acetate layer containing the bulk of the penicillin was then centrifuged to separate the remaining water which became trapped in the emulsion. A total volume of 540 milliliters of clear acetate extract was obtained.

The butyl acetate extract was next extracted with one-eighth of its volume of potassium phosphate buffer solution by agitating for fifteen minutes. The two layers were separated in a separatory funnel. A sharp, rapid separation resulted.

The lower buffer extract now containing the penicillin was extracted with one-fourth of its volume of n-butanol by agitating for fifteen minutes. The layers were separated in a separatory funnel as before. This n-butanol extract was held until the second half of the broth from the first fermentation was processed to this same stage in the recovery process.

The second half of the filtered broth from the first fermentation was processed as previously described to the n-butanol extract.

The two n-butanol extracts obtained above were composited. Penicillin crystals were isolated by merely concentrating the extract at a reduced pressure of twenty millimeters of mercury and a temperature of thirty degrees centigrade. A water-butanol azeotropic mixture was distilled during the concentration until all but about one per cent water remained. Penicillin crystals, which are insoluble in nearly dry n-butanol, began to appear. The concentration was continued for ten minutes after the first appearance of crystals to allow enough time for maximum crystallization to take place. The resulting potassium penicillin was filtered, washed with dry n-butanol, and air dried. A yield of 0.5092 gram was obtained.

The broth from the second fermentation was processed according to the foregoing procedure. A yield of 0.8678 gram of crude potassium penicillin was isolated.

III. DILUTION AND PURIFICATION OF C^{14} CRUDE PENICILLIN

Preliminary dilution and initial purification. Both lots of crude penicillin from the first and second fermentations were composited. The crude labeled penicillin

underwent an initial dilution by dissolving with eighteen grams of highly purified unlabeled potassium penicillin G in water at a concentration of four grams per liter. The mixture was agitated for fifteen minutes.

An initial purification step was accomplished by extracting this water solution of labeled and unlabeled penicillin by following the same procedure as that used to isolate the labeled penicillin from the broth. A total yield of sixteen and five-tenths grams of partially diluted and partially purified C^{14} potassium penicillin was obtained.

Further purification of the diluted C^{14} penicillin. The penicillin obtained above was further purified by again dissolving in water and extracting. This procedure was repeated twice until constant specific activity of the crystals was reached.

Table II was included to show the yield and specific activity of each crop of crystals isolated from each of the three purification runs.

TABLE II

SPECIFIC ACTIVITY AND YIELD OF C^{14} PENICILLIN AFTER EACH PURIFICATION STEP

Purification run number	Crystal yield (grams)	Specific activity* (counts/minute)
1	16.5	30,240
2	13.5	28,620
3	12.3	28,350

*Counting procedure for determining the specific activity of the penicillin will be described in Chapter IV, page thirty-seven.

The data shown in Table II indicated that essentially all high specific activity impurities had been removed as demonstrated by the attainment of nearly constant specific activity after the second purification.

Final dilution of the C^{14} labeled penicillin. The potassium penicillin which was used to make the final dilution of the labeled penicillin was highly purified according to the following general procedure. One hundred-ninety grams of commercial recrystallized potassium penicillin G was first recrystallized from acetone three times by dissolving in seventy-five per cent acetone-water at a concentration of two and seven-tenths grams per

milliliter. The solution was agitated until all the penicillin dissolved. The solution was filtered through a sintered glass funnel. The funnel was washed with three milliliters per gram of ninety per cent acetone-water mixture. The filtrate and wash were diluted with nine times its volume of dry acetone with agitation at which time penicillin crystals immediately began to form as the per cent water became decreased by the addition of dry acetone. The crystal slurry was cooled to five degrees centigrade for two hours in order to induce maximum crystallization. The crystals were removed by filtration.

One hundred-sixty grams obtained above were dissolved in water at a concentration of four grams per liter. The dissolved penicillin was extracted according to the same procedure described on page twenty-five for isolating crystals from filtered broth.

The resulting crystals, 130 grams, were again recrystallized from acetone as described above. A final yield of 105 grams of highly purified potassium penicillin G was obtained.

Twelve and three-tenths grams of labeled penicillin, shown in Table II, were diluted with the above 105 grams of unlabeled penicillin by dissolving the mixture in aqueous acetone with thorough agitation, followed by dilution with

dry acetone, as described on page thirty. Crystals appeared upon the addition of the dry acetone.

The recrystallization was repeated twice more which finished the final dilution and purification of the labeled potassium penicillin G.

Table III was included to show the yield and specific activity of each crop of crystals isolated from each of the three final recrystallizations described above.

TABLE III

SPECIFIC ACTIVITY AND YIELD OF C^{14} PENICILLIN AFTER EACH FINAL PURIFICATION STEP

Purification run number	Crystal yield (grams)	Specific activity* (counts/minute)
1	107	2913
2	91	2691
3	83	2743

*Counting procedure for determining the specific activity of the penicillin will be described in Chapter IV, page thirty-seven.

The final purification, described above, concluded the preparation of the labeled penicillin standard.

CHAPTER IV

ANALYSIS OF PENICILLIN SAMPLES

Having completed the isolation, purification, and standardization of the C^{14}-labeled penicillin G, it was then possible to begin the analysis of penicillin samples.

The primary concern in designing an analytical procedure involving the use of the isotope dilution technique is to isolate a pure sample from the mixture of the labeled and unlabeled compound in large enough quantity for radioactive measurement. The desirable size of counting cup for solid counting was found to be two centimeters in diameter and three millimeters in depth. It was also found that in order to fill a cup having these dimensions, approximately 150 to 175 milligrams were needed. Knowing the minimum quantity of crystals needed to fill this cup, it was then possible to calculate the amount of unknown sample required, after an average yield had been established, from which 150 milligrams of pure compound could be isolated.

A laboratory procedure for the isolation of penicillin from the fermentation broth had been developed previous to the outset of this research project. It was known that a yield of crude penicillin of about sixty-five per cent could be obtained without difficulty. It

was also known from previous experiments that two recrystallizations were necessary to obtain a counting sample of the proper purity. A yield of about seventy per cent could be expected across the two recrystallization steps. The expected overall yield from broth to final purified crystals was estimated to be about forty per cent.

It was decided from an economic standpoint that 100 milligrams of labeled penicillin G could be used in each analysis so that the cost per analysis would not be prohibitive. The specific activity of the labeled penicillin G standard was sufficiently high to permit a dilution of at least a factor of three. From these facts and observations it was decided that a broth sample of 300 milliliters containing a microbiological activity ranging from 1500 units per milliliter to 4000 units per milliliter would be the ideal sample size from which could be obtained a final isolated counting sample of 150 to 200 milligrams.

I. ADDITION OF LABELED PENICILLIN G TO BROTH AND ISOLATION OF PENICILLIN G SAMPLE

Addition of labeled penicillin G to broth sample. The first step in the analysis of a penicillin broth sample was to obtain 500 milliliters of whole broth containing the mycelia. The mycelia was filtered through filter paper using a porcelain Buchner funnel inserted into a suction filter flask which was connected to the vacuum line.

Exactly 300 milliliters of the filtered broth was measured in a graduated cylinder and transferred to a 500 milliliter beaker. One hundred milligrams of the labeled penicillin G (weighed accurately to the nearest one-tenth milligram on an analytical balance) was weighed into a tared five milliliter beaker.

The accurately weighed labeled penicillin G was then transferred to the filtered broth sample while agitating. This was accomplished by dumping the material into the broth then rinsing the beaker a number of times by dipping it into the broth sample to remove all traces of the labeled penicillin from the beaker.

The C^{14}-enriched broth was allowed to agitate fifteen minutes so that a homogeneous solution was obtained.

Isolation of crude penicillin crystals from the mixture. The second step in the analysis was to isolate from the broth sample crude penicillin crystals containing a mixture of labeled penicillin and ordinary penicillin from the sample. The broth was first extracted with butyl acetate at a low pH. Previous experience had shown that during this extraction an emulsion formed because of the precipitation of proteinaceous materials which occurred

at an acid pH. Two per cent by weight of diatomaceous earth was added to the broth with agitation for demulsification purposes. The pH was adjusted to about five then the broth was filtered.

Sixty milliliters of butyl acetate was then added to the treated broth. The pH of the mixture was adjusted immediately with agitation to two, followed by continued agitation for one minute.

The mixture was transferred to a separatory funnel and allowed to stand five minutes. Two layers were formed which included a top cloudy butyl acetate extract layer and a clear spent broth bottom layer. The bottom layer was drawn off, discarded, and the top layer containing the bulk of the penicillin was centrifuged to separate the residual water. A clear butyl acetate extract remained.

The butyl acetate extract was transferred to a 150 milliliter beaker, eight milliliters of potassium phosphate buffer solution was added and this mixture was agitated for ten minutes. The two layers were again allowed to separate in a separatory funnel. A clean separation occurred in five minutes.

The bottom buffer extract containing the penicillin was drawn off into a thirty milliliter separatory funnel containing two milliliters of n-butanol. This mixture was shaken for ten minutes then allowed to separate.

The upper n-butanol extract was transferred to a five milliliter pear-shaped distilling flask connected to a water condenser. A vacuum take-off receiver was connected to the other end of the condenser. The extract contained a small amount of water which became dissolved in the n-butanol. The presence of the water caused the penicillin in the extract to remain in solution.

The extract was then distilled thereby removing the water as an azeotropic mixture with the n-butanol. As the last trace of water became removed, penicillin crystals appeared because of its insolubility in relatively dry n-butanol.

The crystals were removed by filtration and washed with acetone then siphoned dry.

Purification of the crude penicillin crystals. The crude crystals were purified by recrystallizing from acetone. The recrystallization was performed by first dissolving the crystals in two and seven tenths milliliters per gram of a three to one mixture of acetone and water. A 15 x 50 millimeter shell vial was used for this first step. Two per cent Darco was added to remove the bulk of the color and to aid in the purification. After about one minute of stirring, the Darco was removed by filtration. The filter was washed with three milliliters per gram (original crystal weight) of a nine to one mixture of acetone and water.

The combined filtrate and wash was diluted with ten times its volume of anhydrous acetone, thereby reducing the per cent water content. White crystals resulted immediately.

The crystal slurry was placed in the refrigerator for a short time to complete the crystallization. The crystals, after cooling, were removed by filtration, washed with acetone and siphoned dry.

The above recrystallization was repeated once more, omitting the Darco treatment. The final crystals were dried in a fifty-five degree vacuum oven for two hours after which time they were ready for radioactivity measurement.

II. DETERMINATION OF THE SPECIFIC ACTIVITY OF THE CRYSTALS

The second phase of the analysis was relatively simple. This step consisted of the determination of the specific activity of the isolated, purified, and dried penicillin G counting sample. Figure 4 was included to aid in the description of the counting apparatus.

Description of the counting apparatus. It has been previously stated that radioactivity measurement was accomplished by counting the material in the solid form. The counting shield into which was inserted the counting cup containing the sample was shown at the left in Figure 4. The iron shield weighed about 200 pounds and was purchased

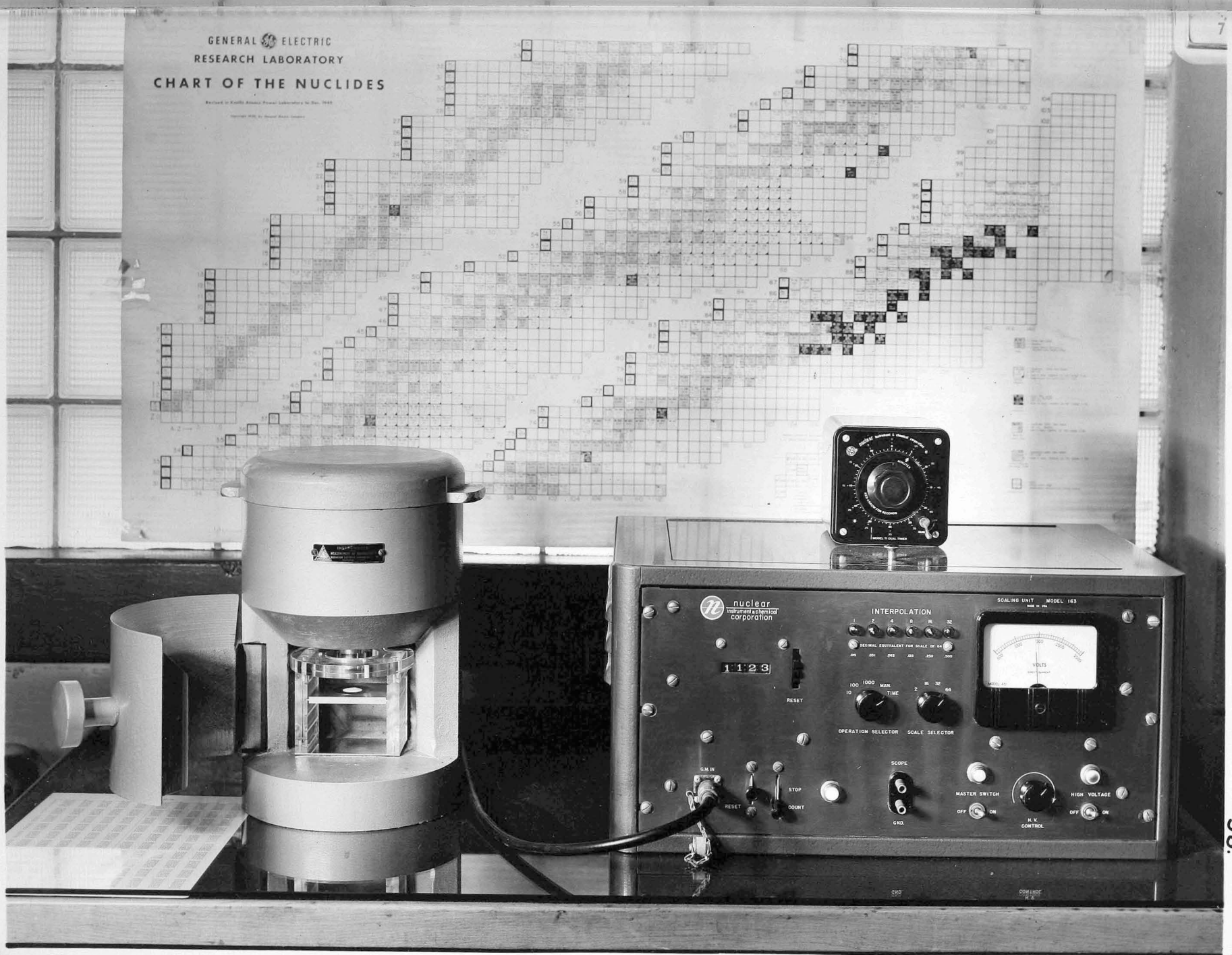


FIGURE 4 GEIGER COUNTER AND SCALER

from Radiation Counter Laboratory. The sample holding rack was fabricated from Leucite and was arranged with five shelves equally spaced. A Tracerlab Geiger tube, Model TGC-2, having a mica window thickness of one and five tenths milligrams per square centimeter thickness and a diameter of one inch was mounted in a stationary position one eighth inch from the surface of the sample cup mounted on the top shelf.

The counting cup and counting cup holder were machined out of aluminum. The cup holder was sixty-three millimeters wide, eighty-three millimeters long, and six millimeters deep. A circular hole forty millimeters in diameter was drilled four millimeters deep in the exact center of the sample holder. This hole lacked two millimeters going through the holder. This inset was used as a seat for the counting cup. Shoulders were machined into each edge of the holder to fit the groove in the rack.

The circular counting cup was fabricated by first machining a circular aluminum disc to fit exactly the inset in the sample holder. An inset was then drilled into the center of the disc which was twenty millimeters in diameter and one and one-half millimeters deep. This inset was the area into which was placed the counting sample.

A stainless steel ring was machined to the exact circumference as the outer circumference of the counting

cup. The inner hole was of the exact size as the inset of the counting cup. This ring was used to screen out any particles of penicillin which may have been spilled on the edge of the counting cup during the filling operation. Counting at constant diameter was accomplished when the ring was placed on the counting cup.

The scaler was shown at the right in Figure 4. This instrument was purchased from Nuclear Instruments and Chemical Corporation and had a code number of Moder 163. A Model T1 automatic time clock was also purchased from the same company.

Preparation of the sample for counting. The sample was prepared for counting by merely filling the cup with a spatula. Small portions were placed in the cup and packed down. After the cup became completely filled, the surface was smoothed by sliding the spatula over the surface with pressure until an even appearance was obtained. The sample was placed in the holder then slid into the counting rack on the top shelf. The shield door was closed and the sample was allowed to count for a total of 15,000 counts.

In a like manner the original labeled penicillin G standard was counted. Finally, ordinary penicillin was counted to determine background count. This background

count had to be subtracted from the sample and standard counts.

III. CALCULATION OF RESULTS

Isotope dilution formula. The conventional isotope dilution formula was used for calculating the results.[19] The formula and explanation of the symbols are shown below.

$$X = A\left(\frac{B}{C} - 1\right)$$

where:

X = Wt. of penicillin G in sample (milligrams).
A = Wt. of labeled penicillin G added to sample (milligrams).
B = Specific activity of labeled penicillin G added to sample (counts per minute).
C = Specific activity of isolated penicillin G mixture (counts per minute).

Example of a typical calculation. For purposes of illustration, the following sample calculation was included. A 300 milliliter sample of filtered penicillin broth was analyzed. One hundred milligrams of labeled penicillin G was added. The labeled penicillin G standard counted 4,192 counts per minute, net count. The

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[19] Ibid., p. 319.

isolated mixture counted 744 counts per minute net count. These experimental values were substituted into the isotope dilution formula as follows:

$$X = 100 \left(\frac{4,192}{744} - 1\right)$$

$$= 460 \text{ milligrams penicillin G.}$$

To convert milligrams to units of penicillin the following calculations were made:

One milligram of potassium penicillin G contains 1595 units. Therefore, 460 x 1595 = 733,700 units in the sample.

To calculate the penicillin G content in the sample in terms of units of penicillin G per milliliter of broth, the following calculations were made:

$$\frac{733,700 \text{ units}}{300 \text{ milliliters}} = 2446 \text{ units per milliliter.}$$

All of the experimental values reported in Chapter V were calculated according to the above formula.

CHAPTER V

EXPERIMENTAL RESULTS

The experimental results discussed in this chapter were divided into three sections. The first section concerned the results obtained when the labeled standard was assayed for penicillin G content by the reverse isotope dilution technique to determine whether or not the labeled penicillin G contained any impurities.

The second section included the results obtained when penicillin broth was inactivated with sodium hydroxide to destroy all of the penicillin contained therein, then neutralized, followed by the addition of a known weight of pure penicillin G, then assayed. This series of experiments was conducted in order to determine both the precision and the accuracy of the isotope dilution method. It was felt that if correct results could be obtained by assaying these samples containing more impurities than ordinary penicillin broth, because of the presence of excess decomposition products, then the method would be well grounded and the accuracy and precision well established.

The third section of the experimental results consisted of a series of analyses of broth samples. These samples were taken at different time intervals during the course of the microbiological fermentation cycle.

The analyses served a dual purpose: (1) to study the progressive production of penicillin G during the fermentation cycle, and (2) to use the C^{14} analysis as a standard method for evaluating the accuracy of the iodometric chemical assay and the microbiological assay methods for the determination of total penicillins.

I. ANALYSIS OF THE LABELED STANDARD FOR PER CENT PENICILLIN G

Object. The first step in any isotope dilution analysis is to try to determine whether or not the labeled standard contains any radioactive or other impurities. This was accomplished by performing what was called reverse isotone dilution.

Procedure. The analysis was made by dissolving a known weight of the labeled standard in 300 milliliters of water then adding a known weight of highly purified unlabeled penicillin G. Crystals were isolated by the same procedure used to isolate crystals from broth, previously described in the preceding chapter. The resulting crystals were purified, dried, and the specific activity determined.

Results. Results of five of these determinations are shown in Table IV. It should be pointed out that the

isotope dilution formula described in Chapter IV, page forty-one, had to be rearranged slightly to arrive at a formula for the reverse isotope dilution procedure.

TABLE IV

ANALYSES TO DETERMINE PURITY OF THE C^{14}-LABELED PENICILLIN G

Weight labeled penicillin assayed (grams)	Weight unlabeled penicillin G added (grams)	Specific activity of labeled penicillin (c.p.m.)*	Specific activity of isolated sample (c.p.m.)*	Weight penicillin G found (grams)	Per cent penicillin G found (%)
0.2000	0.2000	1532	765	0.2000	100.0
0.1000	0.2000	1680	555	0.1005	100.5
0.1000	0.2000	1664	560	0.1010	101.0
0.1000	0.2000	2721	906	0.1000	100.0
0.1000	0.3000	4097	1027	0.1005	100.5

*c.p.m. is the abbreviation for counts per minute.

The formula used for calculating the penicillin G content in the C^{14}-labeled penicillin is shown below:

$$A = \frac{X}{\left(\frac{B}{C} - 1\right)}$$

where:

A = Weight of penicillin G in the labeled penicillin.
X = Weight of unlabeled penicillin G added.
B = Specific activity of the labeled penicillin.
C = Specific activity of the isolated penicillin G sample.

It should be pointed out that the variation in the specific activity of the labeled penicillin G shown in column three of Table IV was the result of using Geiger counter tubes of varying window thickness. The distance between the sample and tube window was also varied. In each analysis, however, the isolated sample was counted with the same tube and in the same position as that used for counting the labeled penicillin G.

Conclusion. It was concluded from the experiment that the labeled penicillin G was free from impurities. In all cases a purity of 100 per cent, plus or minus one per cent, was obtained.

II. DETERMINATION OF THE ACCURACY OF THE ISOTOPE DILUTION ANALYSIS

Object. The purpose of the next series of experiments was to establish the accuracy of the C^{14} method for the determination of penicillin G.

Discussion. The original thought was that the accuracy of the method could be determined by analyzing water solutions of pure penicillin G. This plan was not followed for the reason that the information obtained would not demonstrate the true efficacy of the method, since impurities would be absent from the sample to be analyzed.

It was decided that samples characteristic of penicillin broth could be prepared by inactivating regular broth samples then adding to the inactivated broth a known weight of pure potassium penicillin G. By this technique a large quantity of impurities were introduced upon decomposing the penicillin in the sample. These impurities were in greater quantity than in regular broth. The penicillin G content in the inactivated broth was known since a weighed amount was added.

It was postulated that if accurate analyses of these synthetic broths could be achieved, then, certainly analyses of regular broth samples would be likewise accurate.

Procedure. Four experiments were conducted to test the accuracy of the method according to the following procedure.

Samples of regular filtered penicillin broth were inactivated by adding two per cent, by weight, of sodium hydroxide and agitating for twenty minutes. Preliminary assays had shown that all penicillin activity was destroyed during this period.

The inactivated broth was then neutralized. A known amount of pure potassium penicillin G and a known amount of C^{14}-labeled penicillin G were added.

Crystals were then isolated, purified, dried, and the radioactivity content determined according to the procedure described in Chapter IV.

Results. Table V shows the results obtained on the four experiments. The formula for calculating the results was given in Chapter IV.

Conclusions. The results obtained in these experiments revealed that although three of the four determinations were slightly high, all of the values were within plus or minus two per cent of the theoretical values. These results were considered to be quite good since there was present in the broth sample such a high concentration of decomposition

impurities. Any impurities carried through to the final counting sample would depress the count slightly, resulting in a correspondingly high final value.

TABLE V

ASSAY OF KNOWN SOLUTIONS OF PENICILLIN G IN INACTIVATED BROTH

Weight unlabeled penicillin G added (grams)	Weight labeled penicillin G added (grams)	Specific activity of labeled penicillin (c.p.m.)*	Specific activity of isolated sample (c.p.m.)*	Weight penicillin G found (grams)	Per cent of theory found (%)
0.2000	0.1000	1680	555	0.2020	101.0
0.3500	0.1200	1508	388	0.3470	99.0
0.3500	0.1200	1605	402	0.3580	102.0
0.3500	0.1200	1475	374	0.3540	101.0

The data in both Table IV and Table V supplied sufficient evidence that the C^{14} method was accurate and precise to at least plus or minus two per cent. This high degree of accuracy and precision has never been obtained by any other analytical method for the analysis of penicillin G in broth samples.

III. ANALYSIS OF PENICILLIN BROTH SAMPLES

Object. Having established the purity of the labeled standard and the accuracy of the method, analysis of broth samples was begun. The purpose of this series of analyses was to study the progressive production of penicillin G during the fermentation cycle and to determine the accuracy of two assay methods for total penicillins.

Discussion. During the production of penicillin by fermentation, several types of penicillin are produced. These different types were discussed in Chapter I. The normal fermentation usually runs for about 115 hours before the broth is harvested. It was of interest to know the amount of penicillin G produced at different time intervals of the fermentation as compared to total penicillins produced during the same time interval.

The penicillin G content, therefore, was determined on a number of broth samples taken at sixty-six hours,

ninety hours, and 114 hours fermentation time. Total penicillins were assayed by a chemical method called the iodometric assay, a routine method used in the industry, and a microbiological method called the bioassay.

The per cent penicillin G in the broth samples was calculated by dividing the units per milliliter of penicillin G, determined by the C^{14} method, by units per milliliter of total penicillins obtained by either the iodometric or bioassay methods multiplied by one hundred.

A paper chromatographic assay for the determination of per cent penicillin G, described in Chapter II, was conducted simultaneously with the other assay. This assay method was independent of any of the other methods and the results were expressed in relative per cent with respect to the other penicillins.

It should be emphasized that the paper chromatographic method did not give the amount of penicillin G in units per milliliter but as a ratio of penicillin G to other penicillins. These ratios were calculated on the basis of the relative diameter of the zones of inhibition made by the various penicillins present.

The accuracy of the iodometric and bioassay methods could then be determined by comparing the paper chromatographic per cent "G" with the per cent "G" as calculated by:

$$\frac{\text{units per milliliter of total penicillins}}{\text{units per milliliter of penicillin G by } C^{14}} \times 100$$

If the calculated per cent "G" were low in comparison to the paper chromatographic assay, then the assay for total penicillin would be high. Conversely, a high calculated per cent "G" value would indicate a low assay for total penicillins.

Procedure. To assay the broth sample for penicillin G content, 100 milligrams of the C^{14}-labeled penicillin were added to 300 milliliters of filtered broth sample. As described in Chapter IV, a pure penicillin G sample was isolated, dried, and the radioactivity content determined and compared with the original in the labeled penicillin G added.

Results. The results of these assays were shown in Tables VI, VII, and VIII. The assays of the sixty-six hour broth were tabulated in Table VI; assays of the ninety hour broth in Table VII; and assays of the 114 hour broths in Table VIII.

TABLE VI

ASSAYS OF SIXTY-SIX HOUR PENICILLIN BROTH

Fermentor number	Penicillin G assay by C^{14} method (units/ml)	Total penicillins by iodometric (units/ml)	Per cent penicillin G C^{14} / iodometric	Total penicillins by bioassay (units/ml)	Per cent penicillin G C^{14} / bioassay	Per cent penicillin G by paper chromatography
185	922	1031	89	1092	84	
186	835	1060	79	882	94	
187	763	983	77	830	92	
189	840	881	95	906	92	
190	811	875	92	879	92	96
192	880	968	91	1036	85	97
193	896	987	91	886	101	98
MEAN	850	969	88	930	92	97

TABLE VII

ASSAYS OF NINETY-HOUR PENICILLIN BROTH

Fermentor number	Penicillin G assay by C^{14} method (units/ml)	Total penicillins by iodometric (units/ml)	Per cent penicillin G C^{14} / iodometric	Total penicillins by bioassay (units/ml)	Per cent penicillin G C^{14} / bioassay	Per cent penicillin G by paper chromatography
185	1754	1927	91	2004	88	
186	1603	1795	89	1766	91	
187	1399	1548	90	1498	93	
188	1555	1630	95	1729	90	
189	1806	1855	97	1831	98	98
190	1507	1653	91	1578	95	98
192	1360	1571	87	1452	94	96
193	1667	1836	91	1622	103	98
MEAN	1520	1728	92	1685	94	97

TABLE VIII

ASSAYS OF ONE HUNDRED-FOURTEEN HOUR PENICILLIN BROTH

Fermentor number	Penicillin G assay by C^{14} method (units/ml)	Total penicillins by iodometric (units/ml)	Per cent penicillin $\frac{G_{C^{14}}}{\text{iodometric}}$	Total penicillins by bioassay (units/ml)	Per cent penicillin $\frac{G_{C^{14}}}{\text{bioassay}}$	Per cent penicillin by paper chromatography
184	1755	1805	97	1870	94	
185	2371	2642	89			
186	2089	2310	87	2227	90	
187	1914	2204	87	2053	93	
188	2073	2257	92	2100	99	96
189	2472	2680	92	2657	93	97
190	2058	2370	86	2245	91	93
191	1972	2010	98	2053	96	97
192	1643	2168	81	1688	97	98
193	2047	2153	95	2113	97	99
MEAN	2039	2260	90	2112	94	97

Conclusion. The results based on the C^{14} method of analysis for penicillin G and the iodometric and bioassay methods for total penicillins revealed that the rate of production of penicillin G, in respect to the other penicillins, increased slightly between sixty-six hours and ninety hours. After ninety hours the rate leveled off and the per cent penicillin G remained more or less constant.

When the calculated per cent "G" values, based on the C^{14} method, were compared with paper chromatographic results, the data indicated that iodometric values were running about seven per cent high while the bioassays were within about three per cent of the paper chromatographic values.

An investigation was immediately begun, by the personnel conducting the iodometric assay, to determine the reason for slightly high values. The cause was determined and the method was revised so that more accurate results could be obtained.

CHAPTER VI

SUMMARY AND CONCLUSIONS

I. SUMMARY

It was the purpose of the research project herein described (1) to develop a precise and accurate assay method for the analysis of penicillin G in broth samples; (2) to use the method so developed as a tool in establishing the accuracy of routine assay methods for the determination of total penicillins; and (3) to apply the method to the study of the progressive production of penicillin G during the microbiological fermentation.

The assay method developed was based on the principle of isotope dilution, using C^{14}-labeled penicillin G as a tracer.

To reach the above objectives, the research was divided into four phases: preparation of C^{14}-labeled penicillin G by microbiological fermentation; establishment of the purity of the C^{14}-labeled penicillin G by applying the reverse isotope dilution technique; establishment of the accuracy of the method by assaying known solutions of penicillin G in inactivated penicillin broth; and application of the method to the assay of broth samples.

II. CONCLUSIONS

A new isotope dilution method was developed for the assay of penicillin G in broth using C^{14}-labeled penicillin G as a tracer.

The precision and accuracy of the method was found to be at least plus or minus two per cent, with one series of experiments showing plus or minus one per cent.

Such precision and accuracy has never before been achieved by any other method for the analysis of penicillin G in broth samples.

This method could be applied not only to the assay of broth samples, but also the assay of extracts, liquors, and crystals throughout the penicillin recovery process with the same degree of precision and accuracy as that found in the assay of broth samples.

The accuracy of the iodometric and microbiological assay methods for total penicillins in broth was determined, using a combination of the C^{14} isotope dilution method and the paper chromatographic method for per cent penicillin G assay. It was found that the iodometric method gave average values which were about seven per cent high, while the average microbiological assay was within three per cent of the correct value. Steps were taken by the group preparing the iodometric assays to determine the cause of the high values. The cause was found and the difficulty was eliminated.

The method herein described was also applied to the study of the production of penicillin G during the microbiological fermentation. It was found that between sixty-six and ninety hours, the rate of production of penicillin G increased slightly in respect to the other penicillins, while between ninety and one hundred-fourteen hours, both rates were essentially the same.

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