CHEMICAL CHANGES IN SUBMERGED PENICILLIN FERMENTATIONS[1, 2]

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To control a fermentation it is helpful, if not necessary, to understand its mechanism; these studies of the various factors influencing penicillin production have therefore included an investigation of the chemical changes occurring during the fermentation. Since the body of observations is so large that an adequate presentation of the data would require an inordinate amount of space, much of the material presented here will consist of generalizations, followed by representative illustrative data.

METHODS

The Production of Penicillin in Submerged Cultures

Other reports from these laboratories will describe the production of penicillin in submerged cultures and the factors involved. Since the same procedures were followed in this investigation, a brief presentation of methods will suffice.

Cultures. The following cultures were employed:[3] Northern Regional Research Laboratories—*Penicillium notatum* NRRL832 and *Penicillium chrysogenum* NRRL1951-B25; University of Minnesota, Departments of Plant Pathology and Botany—*Penicillium* R-38 isolated from dry soil, *Penicillium* strain 15-U-1 from ultraviolet-irradiated conidia of R-13, another soil isolate, and strain X-1612 (strain X-1612 was selected by the University of Minnesota group from X-ray-treated cultures of *P. chrysogenum* NRRL1951-B25 which were isolated at the Department of Genetics of the Carnegie Institution, Cold Spring Harbor, New York); Stanford University, Department of Biology—strains 35347 and 35217 obtained from ultraviolet irradiation of *P. chrysogenum* NRRL1951-B25; University of Wisconsin, Department of Botany—strain 174, a non-penicillin-producing mutant from the treatment of *P. notatum* NRRL832 with chloral hydrate, 181-A, a nonconidial race from *P. notatum* NRRL832 treated with camphor, and 314-C, from the irradiation of *P. notatum* NRRL832 with ultraviolet light. The cultures were carried as spore stocks on soil.

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Preparation of inoculum for shake flask fermentations. Two loopfuls (loop moistened) of a soil stock culture were suspended in 10 ml of sterile water. One ml of this suspension was transferred to a bottle plate (170-ml prescription bottle) containing 15 ml of the following medium: $NaNO_3$, 3.0 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; sucrose 20.0 g; agar, 20.0 g; distilled water to make 1 L. The pH of the medium was not adjusted. The bottle plates were incubated at 23 C until the cultures had sporulated; they could be stored in the refrigerator if not used immediately. To prepare the inoculum for fermentations, 50 ml of sterile water were added to each bottle plate and the spores were suspended by scraping with a sterile pipette or sterile wire needle. One ml of this dark green suspension of spores served as inoculum for a 500-ml Erlenmeyer flask containing 100 ml of the medium.

Preparation of inoculum for aerated bottle fermentations. Shake flask cultures were inoculated with spores as described and shaken for 48 hours on a reciprocating shaker. A quantity of culture corresponding to 5 per cent of the volume of the aerated bottle culture was added as inoculum. Inocula were grown on the same medium that was under test in the aerated bottles.

Preparation of inoculum for tanks. To 68 L of a glucose, corn steep medium in a 115-L aerated and stirred tank were added the spores from two 170-ml bottle cultures. After 48 hours of growth at 23 C, 20 L of the culture were used as inoculum for each fermentation tank (10 per cent inoculum, GCS 4-2 [no salts] medium).

Media. Subsequently in this paper media will be referred to by letters giving the constituents (L, lactose; G, glucose; S, sucrose; CS, corn steep liquor) and figures giving the respective percentages of these components on a dry basis. Unless otherwise indicated parenthetically following the medium designation, the medium contained the basal salt mixture.

Basal salt mixture: (quantities in all cases g per L of medium)

$MgSO_4 \cdot 7H_2O$	0.25 g
$NaNO_3$	3.00 g
KH_2PO_4	0.50 g
$ZnSO_4$	0.025 g

Medium, LCS 2-2

Lactose	20.00 g
Corn steep solids	20.00 g
Basal salts	

Medium LCS 2-2 (no nitrate) was the same as LCS 2-2 except that the sodium nitrate was omitted from the basal salts. Media LCS 3-1, LCS 4-4, LCS 3-3, and LCS 1-3 contained the percentage of lactose designated by the first number and the percentage of corn steep solids designated by the last number of their symbol. Medium GCS 4-2 (no salts) contained only 4 per cent glucose and 2 per cent corn steep solids. Similarly, medium GCS 4-4 (no salts) consisted of 4 per cent glucose and 4 per cent corn steep solids.

Aeration. When the term *shake flask* appears it refers to a 500-ml Erlenmeyer flask containing 100 ml of medium aerated by shaking on a reciprocating shaker completing 90 to 95 four-inch cycles per minute. Whenever the rotary shaker, rather than a reciprocating shaker, was used, that fact is specifically mentioned; the rotary shaker imparted a motion to 500-ml Erlenmeyer flasks such that all points on the flask described a circle of 1-inch diameter; the speed was 325 rpm.

Bottle fermentations were conducted with 4 L of culture medium in a 2.5-gallon pyrex solution bottle. These bottles were supplied with air at a rate of 2 L per minute, and were stirred mechanically at approximately 450 rpm.

Tank fermentations were run in two 80-gallon tanks, containing 220 L of medium; air was supplied to a tank at a rate of 200 L per minute through a sparger containing 54 holes of $\frac{1}{32}$-inch diameter. The fermentation medium was agitated by a 2-bladed propeller revolving at 270 rpm. For foam control a 3 per cent solution of octadecanol in lard oil was added as needed.

Temperature. All fermentations were conducted at 23 to 24 C. Shakers were placed in constant temperature rooms, the bottle cultures were grown in constant temperature water tanks, and the fermentation tanks were jacketed for temperature control.

The Analysis of Cultures

Dry weight. Four shake flask cultures were pooled daily and filtered on a Buchner funnel; the filtrate was preserved for analysis. The residue was washed with distilled water and dried at 100 C for 12 hours before weighing.

Penicillin. The culture filtrate was assayed for penicillin by the Oxford cup method with *Staphylococcus aureus* FDA209P as the test organism (Foster and Woodruff, 1944; Schmidt and Moyer, 1944). A preparation from the Food and Drug Administration, Washington, was used as a reference standard.

pH. The pH was determined with a glass electrode immediately after filtration of the broth.

Ammonia nitrogen. A 5-ml aliquot of the penicillin broth filtrate was made alkaline (approximate pH 11 to 12), and the ammonia was aerated into standard acid and titrated as described by Umbreit and Bond (1936). Ammonia analysis of tank fermentation broth was performed by aerating the ammonia from a 0.1-ml alkaline aliquot into acid in a standardized colorimeter tube and measuring the color developed with Nessler's reagent (Johnson, 1941).

Nitrate nitrogen. DeVarda's alloy was added to another alkaline 5-ml aliquot of broth; the ammonia originally present and that arising from the reduction of nitrate was aerated and titrated. Subtraction of the ammonia nitrogen value from the value obtained in the presence of DeVarda's alloy gave the amount of nitrate nitrogen present in the sample.

Inorganic phosphorus. The method of Fiske and Subbarow (1925) was used for phosphorus determination.

Sugars. Glucose and sucrose were determined on filtered broth samples by the method of Shaffer and Somogyi (1933). Lactose was hydrolyzed in 1 N

HCl solution in the autoclave at 15 pounds' pressure for 30 minutes. Standard curves were prepared for each carbohydrate to relate titration values to the amount present.

Lactic acid. The method of Friedemann and Graeser (1933) was used for the determination of lactic acid on the ether extract of acidified (pH 2.0) filtered broth.

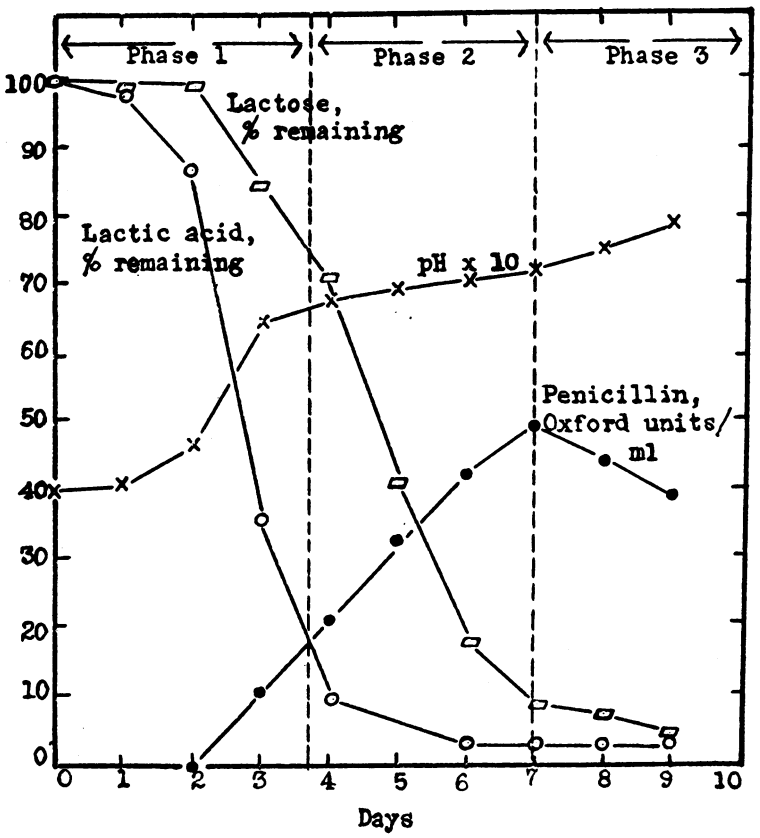

FIG. 1. TYPICAL CHANGES IN SHAKE FLASK CULTURES OF P. NOTATUM NRRL832 GROWN ON A 2 PER CENT LACTOSE, 2 PER CENT CORN STEEP SOLIDS MEDIUM

CHEMICAL CHANGES IN SHAKE FLASK FERMENTATIONS AND AERATED BOTTLE FERMENTATIONS

The changes which accompany the growth of *P. notatum* and *P. chrysogenum* and their production of penicillin were followed in deep aerated cultures and shake flasks, with attention being directed to the effect of the composition of the medium and the strain of organism on the course of the fermentation. The cultures, shaken in the reciprocating shaker, were grown on 100-ml portions of medium in 500-ml Erlenmeyer flasks; the contents of four of these flasks were pooled for daily analysis. Fifty-ml portions of medium in 500-ml Erlenmeyer

flasks were used for fermentations conducted in the rotary shaker; a single flask was analyzed at the indicated intervals.

General Considerations of the Chemical Changes in a Lactose, Corn Steep Medium

In order to facilitate discussion, representative curves for a shake flask experiment with *P. notatum* 832 on a LCS 2-2 medium are presented in figure 1; this will serve as a basis for comparison with changes occurring when strains,

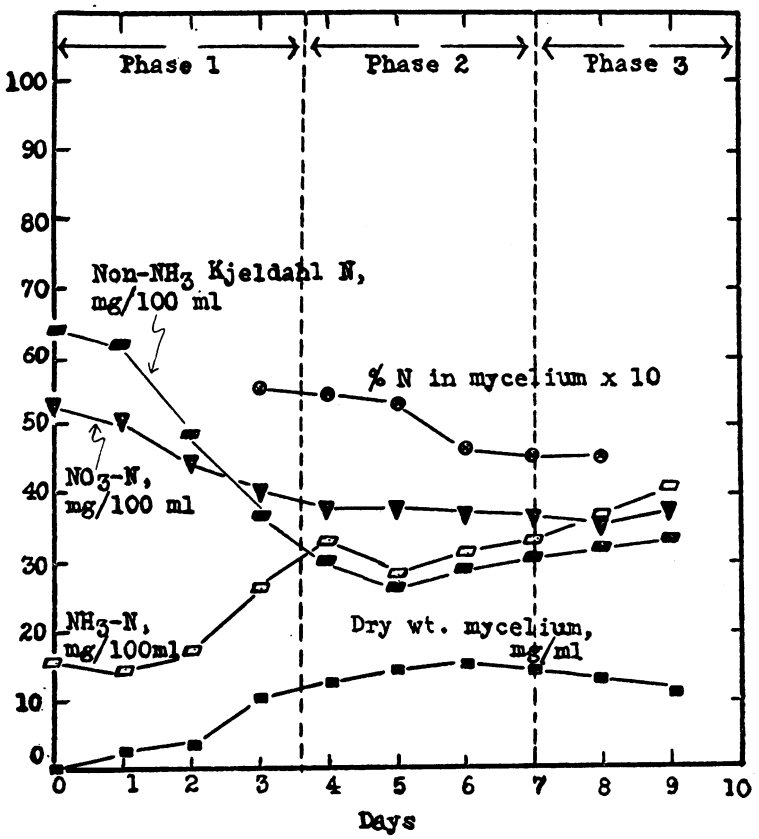

FIG. 1A.

medium, and cultural conditions were altered. More specific examples will supplement the general discussion given first.

Fermentation phases. The penicillin fermentation can be clearly divided into three phases as distinguished by pH rise, varying respiratory intensity, change in mycelial weight and composition, penicillin production, and shift in the composition of the medium. The course of the fermentation is normally more rapid in aerated bottles than in shake flasks, but the changes observed are very similar. Table 1 summarizes the general trends which characterize the three phases.

Penicillin. The maximum concentration of penicillin, which averages between 50 and 90 Oxford units per ml, usually appears on the 6th day in shake flasks, but may appear from the 3rd to the 6th day in aerated bottle cultures, and even earlier in flask cultures agitated in the rotary shaker. Penicillin yields are, of course, influenced by a variety of factors, which will be discussed in other reports from these laboratories.

pH. The pH rises to a maximum after initiation of growth, and then remains constant or drops somewhat before showing a secondary rise during the final phase of the fermentation. Such changes uniformly accompany satisfactory penicillin production; a slow pH rise is associated with slow accumulation of penicillin, and poor yields also are expected with an early rise to pH 8 or above.

TABLE 1

Changes characterizing the three phases of penicillin formation

	PHASE 1	PHASE 2	PHASE 3
Penicillin.....	Slight production	Maximum rate of production	Concentration falls
pH.....	Sharp rise	Plateau or slight drop	Rise
Mycelium.....	Rapid growth, high N content	Slow growth, N content lower	Decrease in weight and N content
Lactose.....	Used slowly	Used more rapidly	Small remaining amount exhausted
Lactic acid.....	Exhausted rapidly		
Ammonia.....	Released into medium	Utilized	Released into medium
Nitrate..... ...	Used at maximum rate, although slowly	Slow use	Slow use, not exhausted
Nonammonia Kjeldahl N....	Used extensively	Concentration rather stable	Concentration increases
Inorganic phosphorus....	Used at maximum rate, although slowly	Slow use	No use or liberation
$Q_{O_2}(N)$.....	Maximum	Decreases	Minimum

Chemically, the pH changes can be correlated with a shift in the composition of the medium: the first pH rise in phase 1 is accounted for by an accumulation of ammonia, by liberation of sodium ions after the utilization of nitrates, and by a rapid breakdown of the lactic acid which is always present in corn steep liquor. The plateau of phase 2 occurs during a period of relative chemical stability; the small drop often observed usually is associated with a drop in the ammonia content at that time. The final rise apparently is attributable to the liberation of ammonia and basic nitrogen from the mycelium. A strictly quantitative correlation between pH and chemical changes is difficult to establish. Theoretical values for pH, based on the concentration of lactic acid, nitrate, and ammonia, were calculated from electrometric titration curves of the media; these were higher than observed values (LCS 2-2, table 2). This indicates the production of acid ions not accounted for by our analyses. However, in a

medium with no added nitrate, predicted and observed pH values agreed well until the final autolytic phase. During this period the observed pH was higher than predicted, the high pH resulting, perhaps, from the release of basic organic nitrogen compounds.

Dry weights of mycelium. The speed with which the mycelium reaches a maximum weight depends upon the substrate and type of the inoculum, a vegetative inoculum giving more rapid growth than a spore inoculum. Glucose, sucrose, or dextrin support more rapid mycelial growth than does lactose. Usually a maximum level of penicillin is present shortly after the mycelium weight starts to decrease. The mycelium may be undergoing decomposition prior to a drop in total weight, for the percentage of nitrogen in the mycelium changes from an early value of 8 to 10 per cent to a final value of 4 to 5 per cent. The drop in mycelial weight occurs near the time of carbohydrate exhaustion.

Carbohydrates. Lactose is used slowly by the mold, and a 2 per cent level may not be exhausted in 6 days in shake flasks. Sucrose, invert sugar, glucose, or dextrin support more rapid growth and respiration, and as a result they are

TABLE 2

Observed and calculated pH values during penicillin production

AGE OF CULTURE IN DAYS.....	0	1	2	3	4	5	6	7	8	9	10
Observed pH LCS 2-2 (without NO_3).....	3.75	3.83	6.41	6.69	6.10	5.99	6.10	6.25	7.11	7.14	7.96
Calculated pH LCS 2-2 (without NO_3).....		3.92	6.37	6.48	6.05	5.92	5.90	6.20	6.25	6.30	6.31
Observed pH LCS 2-2..	4.02	4.01	4.59	6.88	7.30	7.35	7.40	7.66	7.81	7.89	
Calculated pH LCS 2-2.		4.27	6.20	>8.4*	>8.4	>8.4	>8.4	>8.4	>8.4	>8.4	

* Our titration curve extended only to pH 8.4.

more quickly exhausted at a given level. The attack on native starch is initially slow, probably because of the necessity of amylolytic breakdown. The relation of the carbohydrate supplied to the aeration rate necessary is discussed in another section of this report. A large amount of the carbon metabolized is furnished by the corn steep liquor rather than by added carbohydrate. The rapid liberation of ammonia as well as the slow initial disappearance of lactose seems to indicate utilization of corn steep carbon in preference to lactose carbon.

Lactic acid. In a medium containing 2 per cent corn steep solids of the types employed, lactic acid is at a level of about 350 mg per 100 ml. The mold uses lactic acid as a carbon source in preference to lactose but apparently not in preference to sucrose or glucose.

Nonammonia Kjeldahl nitrogen. Organic nitrogen compounds from corn steep liquor furnish a much greater amount of nitrogen for growth than does the nitrate supplied. As pointed out, the early liberation of ammonia indicates that the nitrogenous compounds from corn steep liquor are serving as carbon sources as well as being incorporated into mycelial protein. In a high corn

steep liquor, low carbohydrate medium much ammonia is liberated and little nitrate is used; in a high carbohydrate, low corn steep medium very little ammonia appears and nitrate is used extensively.

Ammonia. A small amount of ammonia may be used early in the fermentation, this utilization being followed by a liberation of ammonia as organic nitrogen compounds are metabolized. Some of this may be used before the final release of ammonia that accompanies autolysis of the mycelium. The relative levels of carbohydrate and corn steep liquor, and the relative availability of the carbohydrate, govern the amount of ammonia appearing in the medium. For example, a high level of lactose (4 per cent) and a low concentration of corn steep (1 per cent) will foster a very rapid utilization of the ammonia formed in phase 1; nitrate utilization also will be more marked in such a fermentation. Readily available nitrogen sources are needed as building units for protoplasmic synthesis, which is increased in the presence of greater amounts of sugar. Similarly, the addition of 0.5 per cent of readily available glucose to the normal LCS 2-2 medium results in conspicuously low ammonia levels during phase 2. On the other hand, a medium that furnishes an abundance of lactose (3 per cent) and also a high corn steep level (3 per cent) will contain much ammonia, because there is more than enough nitrogen in the corn steep to satisfy the requirements for synthesis. High ammonia levels are especially apparent in low lactose (1 per cent) and high corn steep (3 per cent) media. Indications of a definite interrelation between ammonia concentration and penicillin accumulation will be discussed later.

Nitrate. Nitrate nitrogen is not exhausted when it is supplied at a level of 3 grams of $NaNO_3$ per L in a LCS 2-2 medium; generally about a third of it is used. When corn steep is at a 1 per cent level, up to 5 g of $NaNO_3$ per L may be used.

Inorganic phosphorus. The changes in inorganic phosphorus in the medium have not been studied extensively, but the available data show, as might be expected, a utilization of phosphorus compounds during phase 1, a slower utilization during phase 2, and no change or actual liberation during phase 3.

Pigmentation. The yellow pigment produced by strain NRRL832 and the non-penicillin-producing mutant strain Wisconsin 174 was extracted from acidified (pH 0.7) culture filtrates with butyl alcohol and measured for light absorption at 4,500 A. The results showed that the non-penicillin-producing strain gave approximately as much yellow pigment as strain 832.

Chemical Changes As Affected by the Composition of the Medium

Lactose, glucose, corn steep medium. In a normal LCS 2-2 medium to which 0.5 per cent glucose has been added, the presence of readily available glucose causes a very rapid sugar oxidation during phase 1. Simultaneously with the increased utilization of glucose, a more rapid utilization of the ammonia formed in phase 1 occurs, resulting in very low ammonia levels in phase 2; considerable ammonia is liberated in phase 3. In a typical run the level of ammonia nitrogen

was 4.4 mg per 100 ml of fermentation liquor on the 5th day (phase 2) and 37.9 mg per 100 ml on the 8th day (phase 3). Other changes in this medium closely resembled those observed in the LCS 2-2 medium.

Medium without nitrate. When nitrate is omitted from the normal medium, the following changes occur: The relatively large amount of ammonia liberated (e.g., 34.7 mg per 100 ml on the second day) is very rapidly used during phase 2, since only ammonia and the nitrogen of the corn steep are available. The ammonia level remains low from the 6th to the 10th day (e.g., 2.6 mg per 100 ml on the 6th day; 9.9 on the 10th day).

In a medium containing $NaNO_3$ the use of the nitrate ion releases sufficient sodium ions to account for a considerable rise in pH; however, in a medium without added nitrate the pH reached for the plateau level of phase 2 is relatively low (e.g., 6.69 on the 3rd day to 6.10 on the 6th day). Only during phase 3 does the pH rise to values optimal for penicillin formation; consequently, penicillin formation continues into phase 3. As mentioned before, the observed pH in this medium is very close to the values calculated from the known buffer capacity of the medium and the basic and acidic groups liberated during the fermentation.

Low corn steep medium. The medium used in the bottle fermentation summarized in figure 2A is a LCS 3-1 medium. The amount of corn steep carbon present is small; hence, considerable lactose is used in phase 1, and the limited amount of ammonia formed is utilized before the end of phase 1. In addition, nitrate nitrogen is utilized rapidly. Liberation of nonammonia Kjeldahl nitrogen from the mycelium is marked in phase 3. Reduction in the corn steep level gives a medium of lowered buffer capacity, and hence the pH rises more rapidly than in the LCS 2-2 medium. Similar changes occur in shake flask fermentations employing the same medium or one composed of 4 per cent lactose and 1 per cent corn steep solids.

High corn steep medium. Since medium LCS 1-3 contains insufficient lactose to supply the carbon requirements of the mold, the corn steep must furnish a large share of the carbon. The extensive utilization of corn steep as a carbon source is accompanied by the liberation of considerable ammonia (e.g., 50 mg ammonia nitrogen per 100 ml on the 4th day). The early exhaustion of lactose and the later complete reliance upon corn steep as a carbon source give little chance for a reduction in the ammonia levels during phases 2 and 3. Penicillin yields are low (36 Oxford units at the maximum). About half the nitrate is utilized rapidly during phase 1. Figure 2B presents a bottle fermentation on medium LCS 3-3. Lactose fermentation begins the 1st day, and the large amount of ammonia accumulated in phase 1 decreases rapidly during the period of rapid lactose fermentation. During this period of low ammonia, penicillin production is most active. Nitrate utilization is low because readily available ammonia and corn steep nitrogen are plentiful. It will also be noted that much of the nonammonia Kjeldahl nitrogen remains unused.

Glucose, corn steep medium. Fermentations employing glucose as the car-

bohydrate were run in the reciprocating shaker with the medium GCS 4-2 (no salts). No nitrogen analyses are available for the reciprocating shaker fermentation. Glucose is fermented more rapidly than lactose, being consumed by the 5th day even when supplied at the 4 per cent level; lactic acid is utilized to a lesser extent than glucose and is exhausted by the 6th day. Since the lactic acid disappears slowly and the medium contains no added sodium nitrate, the pH rises slowly with no marked plateau region until a pH of about 8 is established

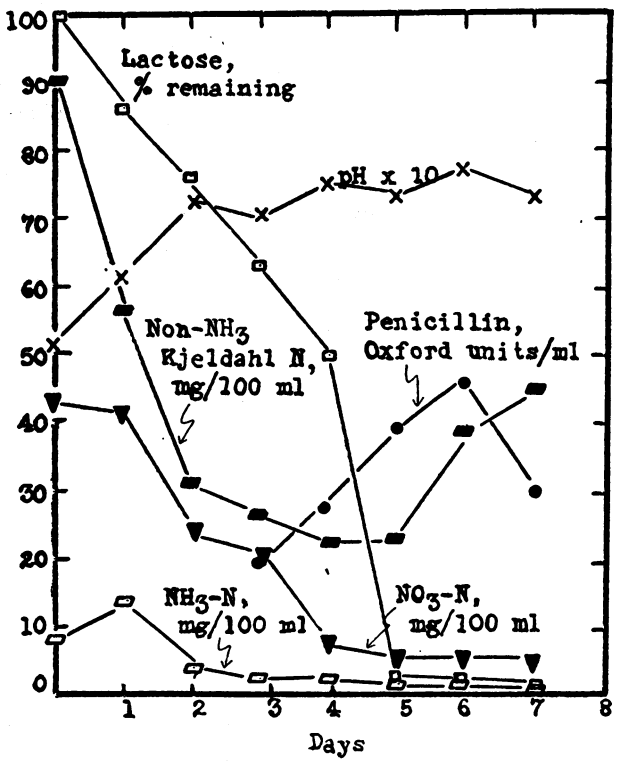

FIG. 2A.

FIG. 2. CHEMICAL CHANGES IN BOTTLE FERMENTATIONS WITH P. NOTATUM NRRL832 GROWN ON HIGH LACTOSE, LOW CORN STEEP SOLIDS, AND HIGH LACTOSE, HIGH CORN STEEP SOLIDS, MEDIA

A. Three per cent lactose, 1 per cent corn steep solids.
B. Three per cent lactose, 3 per cent corn steep solids.

from the 7th to the 9th day. As Foster, Woodruff, and McDaniel (1943) suggested, the accumulation of sugar acids which cannot be immediately broken down because of insufficient oxygen supply might be another factor responsible for this slow rise. The yields are very low (21 Oxford units maximum on the 6th day), for optimal pH conditions (7 to 8) are present for a period of 2 days only.

When aeration is increased by the use of a rotary shaker, with a GCS 4-4

(no salts) medium that has been adjusted to pH 6.25 before autoclaving, the metabolic picture differs considerably from the one of the "slow" glucose fermentation previously described. Figure 3 shows a graph of the rotary shaker fermentation. The first striking observation is the speed of fermentation; the maximum penicillin yield is obtained in 54 hours. In spite of this acceleration of metabolic processes, phasic division can still be recognized. If plotted with the same dimensions as figures 1 and 2, for example, this "fast" glucose fermentation becomes a compressed normal lactose fermentation differing

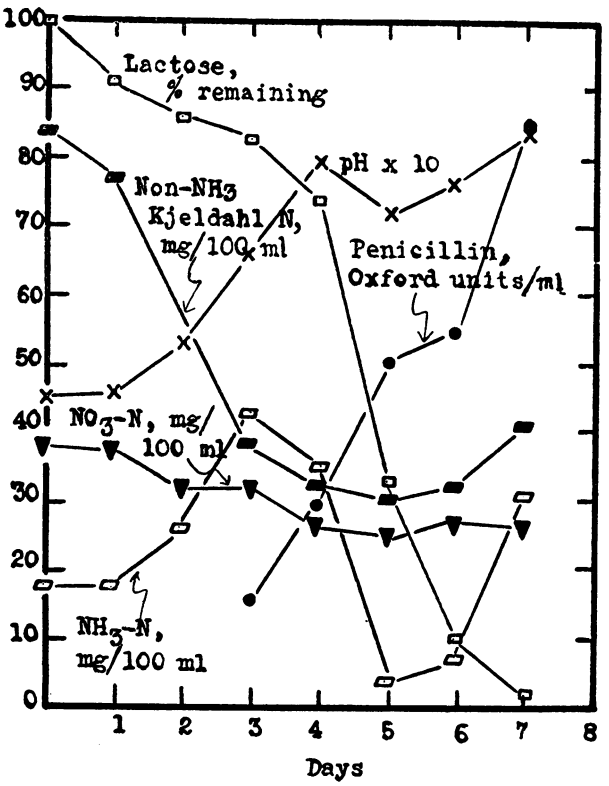

FIG. 2B.

only in minor aspects. Indications are that the rotary shaker furnishes approximately 3 times as much oxygen to the mold as agitation in the reciprocating shaker. The combination of the initial pH adjustment and rapid utilization of lactic acid in the presence of an abundant oxygen supply gives a pH favorable for penicillin accumulation. The first pH drop might be explained by an accumulation of sugar acids which are soon thereafter oxidized. Ammonia levels are very low, but nevertheless show a characteristic dip near the maximum penicillin formation of 64 Oxford units.

Metabolism of Strains Other than P. notatum NRRL832

The most extensive work reported here was conducted with *P. notatum* NRRL-832. After observing that this strain would consistently produce the same chemical changes in a given medium during its growth and penicillin production, it was considered desirable to compare, at least in a limited way, its established metabolism with that of other strains showing some notable difference.

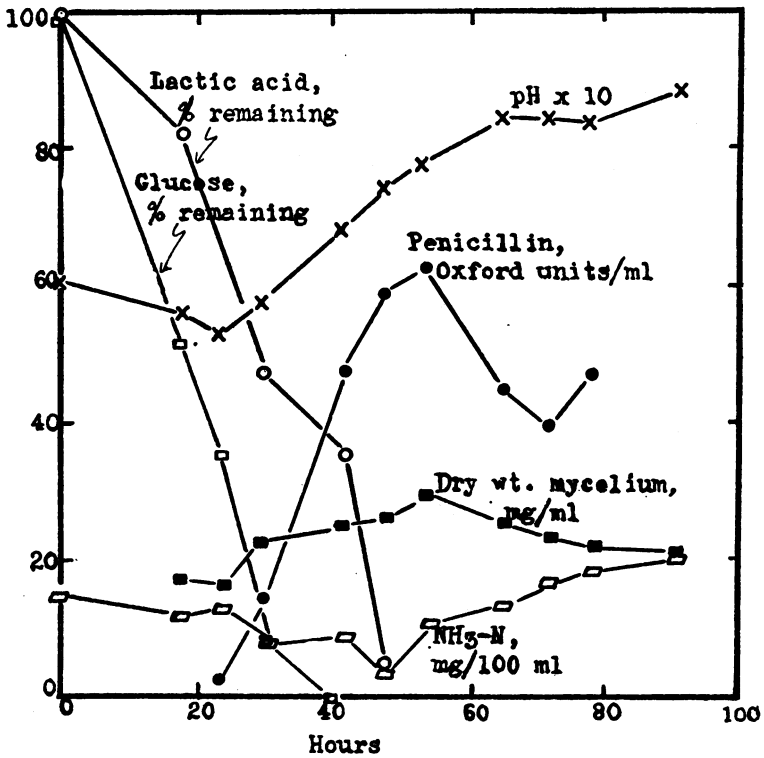

FIG. 3. CHANGES PRODUCED BY P. NOTATUM NRRL832 IN A 4 PER CENT GLUCOSE, 4 PER CENT CORN STEEP SOLIDS MEDIUM WITHOUT ADDED SALTS

The pH adjusted to 6.25 before autoclaving, 2 per cent preformed inoculum grown for 22 hours on 2 per cent glucose, 2 per cent corn steep solids medium; culture volume 50 ml in 500-ml Erlenmeyer flasks, aeration with a rotary shaker giving 325 rpm.

P. notatum NRRL832 and *P. chrysogenum* NRRL1951-B25 are hardly distinguishable metabolically, though in general 1951-B25 cultures show a more conspicuous drop in ammonia during phase 2, a more rapid utilization of nitrate during phase 3 (see figure 4 and compare with figure 1 for 832), and a higher penicillin production under most conditions.

Culture 181-A, the nonsporeforming race derived from strain 832 by treatment with camphor, must be vegetatively propagated, and hence preformed inoculum was employed for shake flask cultures. These cultures differed from strain 832 in their unusually high levels of ammonia nitrogen in the late phase of the

fermentation, in their very limited utilization of nitrate nitrogen, and in their slower utilization of lactic acid.

The non-penicillin-producing strain 174, derived from strain 832 by chloral hydrate treatment, is indistinguishable macroscopically and microscopically from the parent strain, but under all the conditions tested it produced no trace of penicillin. Strain 174 differed from strain 832 in its virtual nonutilization of nitrate, in showing a more striking drop in ammonia concentration during phase 2, and in its preferential utilization of lactose, rather than lactic acid, during the

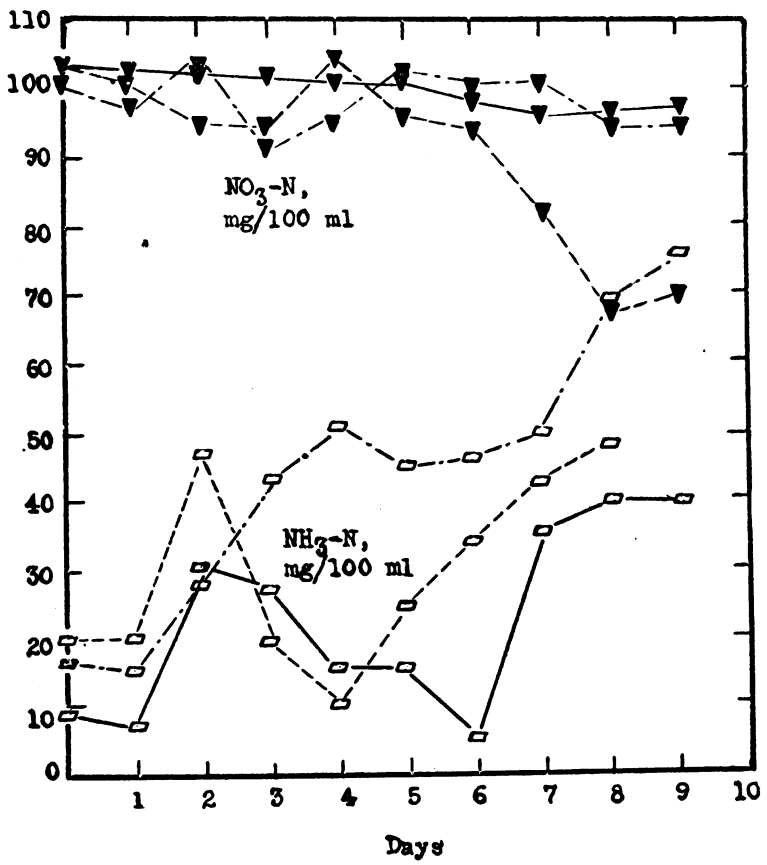

FIG. 4. NITRATE-NITROGEN AND AMMONIA-NITROGEN METABOLISM OF DIFFERENT P. NOTATUM AND P. CHRYSOGENUM STRAINS

Solid line, *P. notatum* 174; broken line, *P. chrysogenum* NRRL1951-B25; dash-dot line, *P. notatum* 181-A.

1st day of the fermentation. None of these differences are such as to suggest an explanation for the lack of penicillin formation by strain 174.

The similarities in the metabolism of the mold strains studied were perhaps more notable than their differences, though each strain did exhibit minor variations from the metabolic picture of *P. notatum* NRRL832.

THE INTERRELATIONSHIP BETWEEN AMMONIA LEVELS AND PENICILLIN PRODUCTION

It has been a general observation that among fermentations set up under similar conditions a correlation exists between ammonia levels and penicillin yields.[4] Tank fermentations have shown this effect most conspicuously; this is not necessarily attributable to an inherent difference in the fermentation in tanks, aerated bottles, and shake flasks, but may result merely from a difference in experimental procedures, namely, that replicate tanks were analyzed separately and replicate shake flasks were pooled for analysis and compared only with pooled replicates of another treatment. Replicate tanks often show differences in ammonia levels, and the one with the higher ammonia level during the phase of active penicillin production rather regularly gives the lower penicillin yield. In addition to the general consideration of ammonia at this point, specific treatment will be given in the section on tank fermentations and in other sections of the paper; additional shake flask data will be covered by Koffler, Knight, Emerson, and Burris (1945).

In the case of two or more replicate shake flasks or aerated bottle fermentations, the one containing less ammonia during phase 2 will commonly yield more penicillin. In a study of the stimulatory effect of boron on penicillin production in shake flasks, Koffler *et al.* (1945) found that the fermentations giving higher yields contained less ammonia throughout the fermentation, strikingly so during the period of maximum penicillin accumulation. This investigation involved two different media. One was the ordinary LCS 2-2 medium and the other a LCS 2-3 medium. As expected, the medium containing less corn steep released less ammonia. The addition of boric acid caused a relative decrease of ammonia, i.e., the LCS 2-3 fermentation, to which boric acid had been added, had an ammonia concentration which was considerably lower than that of the "normal" fermentation on the same medium, but higher than that of the "normal" LCS 2-2 fermentation. The "normal" LCS 2-2 medium, on the other hand, produced larger amounts of ammonia than the corresponding "boron treated" medium. In both cases the "boron treated" fermentation yielded more penicillin than the "normal" fermentation. The "boron treated" LCS 2-3 medium, despite its higher ammonia levels than the "normal" LCS 2-2 medium (average values for 8 days 35.1 and 29.0 mg of ammonia nitrogen per 100 ml, respectively), supported greater penicillin yields (maximum values 105 and 72 units, respectively). This would indicate that the *absolute* level of ammonia is not the controlling factor in penicillin production; however, the relatively high levels of ammonia in the absence of boron accompanied by lowered yields show a relationship between ammonia and penicillin yields.

Figure 5 serves to illustrate this point further. Figure 5A presents a "fast" fermentation in aerated bottles; the maximum penicillin yield is obtained on the 3rd day. The level of ammonia is characteristically low, especially at the

[4] Workers at the Northern Regional Research Laboratories early noted that inhibition of penicillin production accompanied high ammonia levels and suggested the formation of ammonium carbamate in the medium as a possible cause of this inhibition.

time of maximum penicillin production. In contrast, the fermentation presented in figure 5B shows a higher ammonia concentration over a more extended period and a delayed arrival at peak penicillin production.

Up to this point, no indication was obtained as to whether high ammonia is the cause of low yields or the effect of some other factor which is the primary cause of the suppression of penicillin production. It was therefore necessary to test the effect of ammonia on penicillin formation. Table 3 summarizes

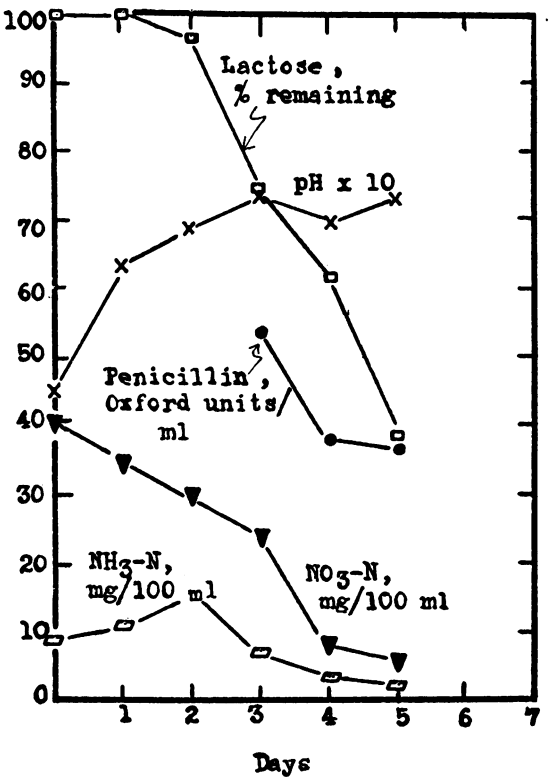

FIG. 5A.

FIG. 5. CHEMICAL CHANGES ACCOMPANYING "FAST" AND "SLOW" PENICILLIN PRODUCTION BY P. NOTATUM NRRL832 IN AERATED BOTTLE FERMENTATIONS

A. "Fast" fermentation. Each value is the average of 3 bottles.
B. "Slow" fermentation. Each value is the average of 2 bottles.

the information obtained. The implications are self-evident: ammonia concentration in itself does not affect penicillin production even in concentrations higher than we have met in actual experience (80 to 168 mg of ammonia N per 100 ml of fermentation liquor).

On the basis of these observations the following interpretation might be offered: The physiological condition of the mold mycelium favoring maximum penicillin production is reflected by a certain ammonia level in the mold environment. This level will vary with the organic nitrogen content of the medium and

the relationship of carbohydrate to corn steep in the medium, but need not vary with the strains involved. Deviations from this "optimal" level in either direc-

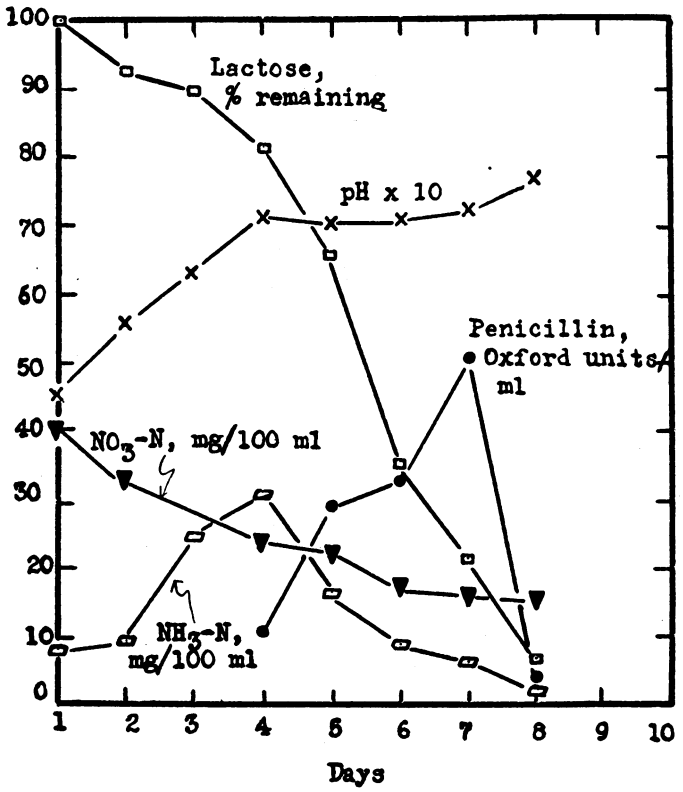

FIG. 5B.

TABLE 3

Effect of high levels of ammonia nitrogen on penicillin production in shake flasks
(Each figure is the average of two flasks)

AMMONIUM SULFATE ADDED (G/L) TO LCS 2-2	NH_3-N CONTENT OF MEDIUM (MG/ML)							PENICILLIN PRODUCTION (OXFORD UNITS/ML)					
	Days							Days					
	0	2	3	4	5	6	7	3	4	5	6	7	8
0	0.19	0.27	0.39	0.56	0.56	0.44	0.40	7	12	26	40	74	80
0.15	0.22	0.28	0.40	0.63	0.49	0.44	0.37	7	13	31	40	65	75
0.5	0.30	0.32	0.46	0.69	0.69	0.58	0.55	8	24	43	48	81	86
1.5	0.53	0.56	0.63	0.86	0.88	0.81	0.70	14	30	28	36	90	73
3.0	0.90	0.96	1.04	1.19	1.12	1.18	1.00	11	21	24	36	88	85
6.0	1.45	1.65	1.21	1.44	1.67	1.68	1.58	17	16	30	56	72	81

tion accompany lower yields. The association between abnormally high ammonia levels and lowered penicillin yields is particularly pronounced, but there

are indications, especially in tank fermentations to be discussed later, that in addition a correlation between abnormally low ammonia levels and corresponding lower yields exists. The exact nature of this interconnection is not well understood and is difficult to resolve, since ammonia plays the dual role of metabolic waste product and readily available source of nitrogen.

CHEMICAL CHANGES IN TANK FERMENTATIONS

Periodic chemical analyses furnished a comparison of the metabolic picture in tank fermentations and shake flask cultures. Only a representative summary of the data obtained will be presented, and details of the apparatus and procedure followed in the tank fermentation will be described in later reports from these laboratories.

When 3 per cent lactose is initially present in the medium, the utilization of the lactose varies from less than half to nearly complete utilization. There is no obvious correlation between yields and sugar fermented.

In almost all fermentations, the ammonia content of the medium increases during the first 24 hours. The ammonia peak generally occurs at 24 hours, but is sometimes delayed, especially in poor runs. There is a definite correlation between ammonia level and rate of penicillin accumulation. When the ammonia content is greater than 30 mg ammonia nitrogen per 100 ml, penicillin production is slow; as pointed out in the previous section, ammonia per se does not seem to suppress yields, but it rather seems to be the effect of another causative factor, of which penicillin formation is also an effect. The decrease in ammonia concentration occurs simultaneously with lactose utilization; also, if the pH rises to 8.0 or more, some ammonia may be carried out with the air. A rapid fall in ammonia concentration is accompanied by a fall in pH. A decrease in pH of 0.5 pH units has occurred only when relatively large amounts of ammonia were present and were later utilized.

Data from some representative runs are given in figures 6, 7, and 8. In run 75 (figure 6A) a high ammonia level persisted for a long period. Very little lactose was used and ammonia utilization was slow. Penicillin production was slow and the final yield was low. In run 71 (figure 6B) a high ammonia level was reached, but with the onset of rapid sugar utilization the ammonia content decreased rapidly. Penicillin production was slow during the period when the ammonia content was high, but the rate of penicillin production increased after the ammonia level had fallen. Fermentations giving good to excellent penicillin yields were almost always "low ammonia" runs, as the one illustrated in figure 7. This fermentation gave the highest penicillin yields obtained in these laboratories to date. The ammonia level was low during active penicillin accumulation although the medium contained a high level of corn steep solids.

It was thought that the addition of glucose to the medium would decrease the ammonia content of the fermentation, since the rapid utilization of the glucose would result in simultaneous ammonia uptake. Four tests were made to determine the effect of glucose addition. The results are given in figure 8. It will be noted that in every case the presence of glucose resulted in a decrease in ammonia content. In only one case (runs 88 to 89), however, was the ammonia

content without glucose above 40 mg of ammonia nitrogen per 100 ml. In this case, glucose addition resulted in a somewhat higher yield of penicillin. In the other three cases, where ammonia levels without glucose were very low, the presence of glucose appeared to delay the fermentation, and lower yields were obtained. In all cases in which the addition of glucose was responsible for the lowering of yields, there was a definite decrease in ammonia in the first

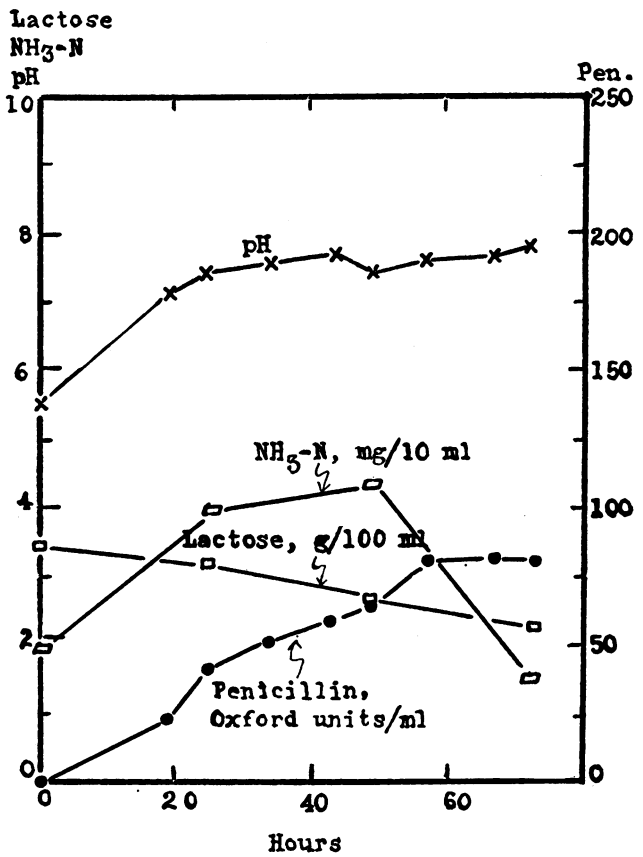

FIG. 6A.

FIG. 6. TYPICAL CHANGES IN TANK FERMENTATIONS ON MEDIA CONTAINING 3 PER CENT LACTOSE, 4 PER CENT CORN STEEP SOLIDS, AND 1 PER CENT CALCIUM CARBONATE BUT NO OTHER SALTS

A. Run 75, culture R-38.
B. Run 71, culture 1951-B25.

stages of the fermentation instead of the usual rise in the first 24 hours. It is difficult to draw conclusions on the basis of a few experiments, but we regard this effect of abnormally low ammonia levels as another indication of the delicate physiological balance which exists within the cell and is reflected *outside* the cell by a definite ammonia pattern. These observations form a basis for reconciling the views of those commercial producers of penicillin who find that the

addition of glucose to their medium favors high yields and the opposing group whose data consistently show that glucose lowers yields. The indications are that the addition of glucose to a high corn steep medium, which has a considerable ammonia content in the initial fermentation phase, will lead to metabolic conditions responsible for a diminished ammonia, and an enhanced penicillin, level; in contrast, with a low corn steep medium, the introduction of glucose will condition a physiological state which is reflected in the lowering of the already low ammonia level and of the "normal" penicillin yield. It must be remembered

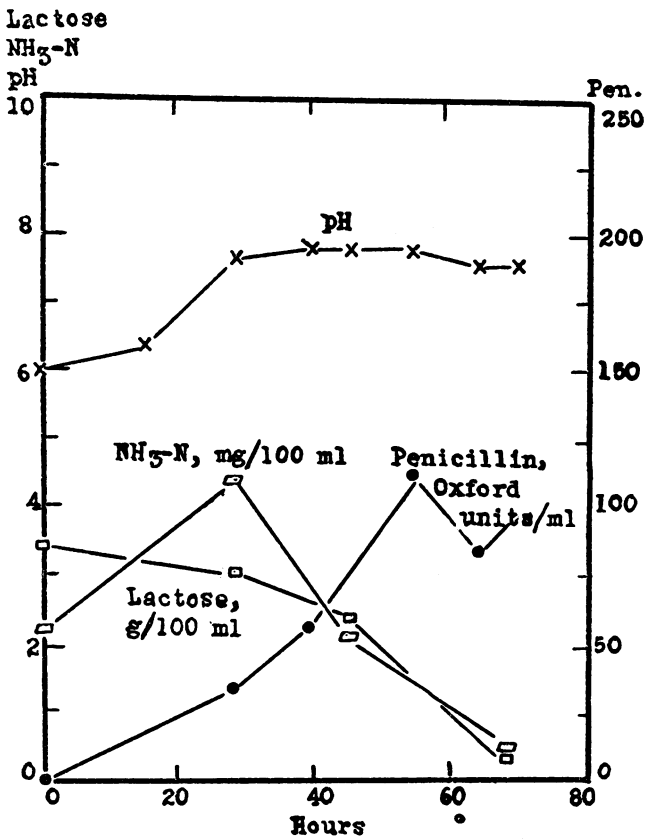

FIG. 6B.

that "high" and "low" corn steep levels are relative terms and that a considerable range in the concentrations must be expected in commercial media.

GASEOUS METABOLISM

Oxygen Consumption of Shake Flask Cultures

Optimal aeration is of critical importance in successful penicillin production. No quantitative data, however, have been available to supplement the results of trial and error methods employed to determine the optimum oxygen

concentration for penicillin production. Our experiments relating to this complex problem have been limited, but they present a general picture of the oxygen requirements of the mold.

Three Erlenmeyer shake flask cultures were pooled daily for the usual chemical analysis, and 3-ml samples of the fermentation liquor containing mold pellets were transferred to Warburg respirometers. Oxygen uptake was followed

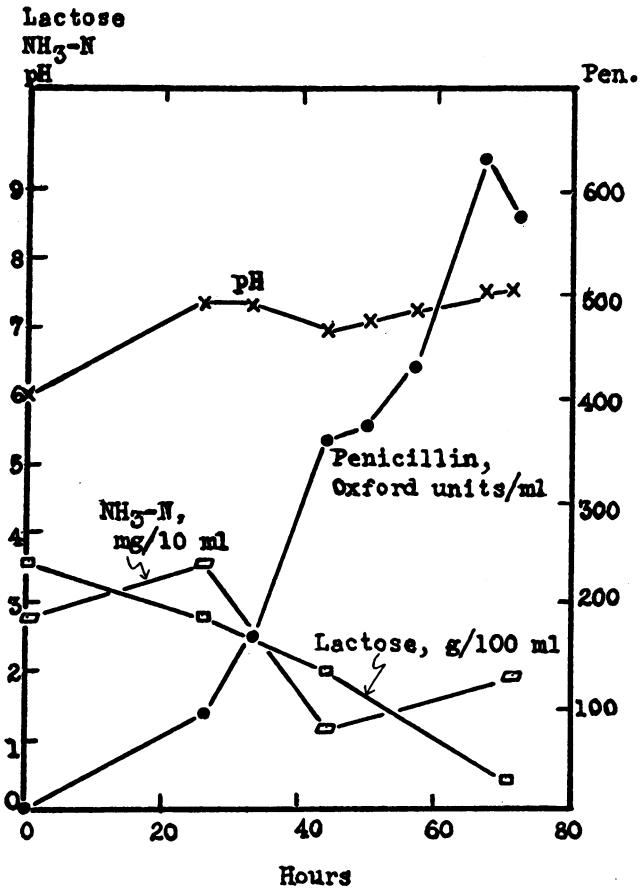

FIG. 7. CHEMICAL CHANGES IN A TANK FERMENTATION WITH HIGH PENICILLIN YIELDS
Culture X-1612 grown on a medium containing 3 per cent lactose, 6 per cent corn steep solids, 1 per cent calcium carbonate, but no other salts.

for 90 to 120 minutes; it remained linear during this period. After the respiration measurement the mold pellets from each flask were washed and their dry weight determined. Q_{O_2} values (on a dry weight basis) were determined, but only Q_{O_2}(ml) values are reported here. The Q_{O_2}(ml), which is defined as oxygen uptake in μl per hr per ml of actual fermentation liquor plus mycelium, is of particular interest, for it provides a direct measure of the oxygen requirements of the mold in its normal culture medium.

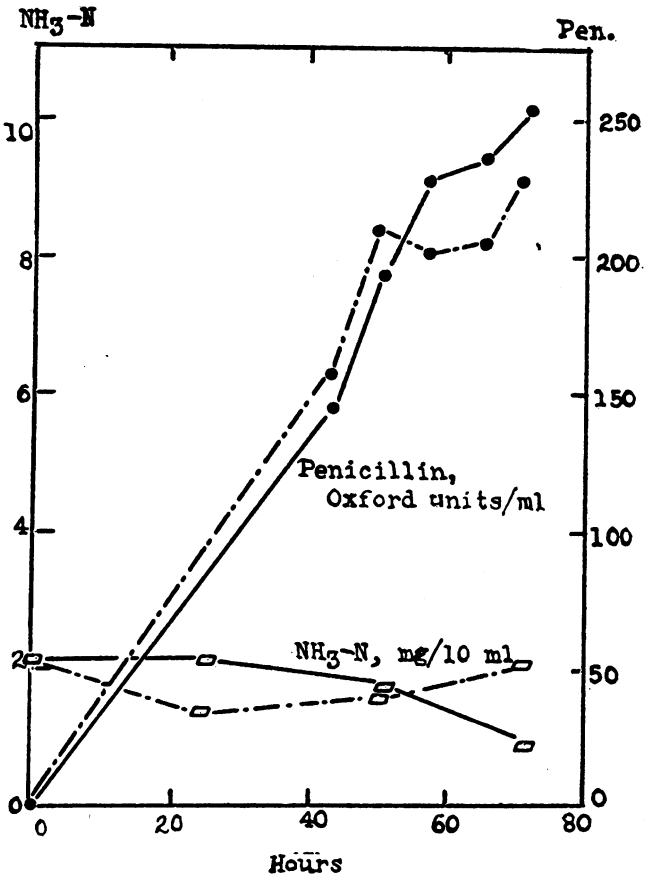

FIG. 8A.

FIG. 8. THE EFFECT OF GLUCOSE ADDITION ON PENICILLIN YIELDS AND AMMONIA LEVELS IN TANK FERMENTATIONS

Solid line, culture grown on a medium containing 3 per cent lactose, 4 per cent corn steep solids, 1 per cent $CaCO_3$, but no other salts; broken line, culture grown on above medium plus 0.5 per cent glucose.

A. Runs 94 to 95, culture 35347.
B. Runs 96 to 97, culture 35217.
C. Runs 98 to 99, culture X-1612.
D. Runs 88 to 89, culture 1951-B25.

The results are summarized in table 4 and figure 9. As might be expected, the most rapidly utilized sugars gave rise to the highest rate of oxygen uptake. The data below are taken from figure 9:

Carbohydrate	*Maximum rate of sugar utilization g per L per hr*	*Maximum O_2 uptake ml per L per hr*
Lactose	0.32	109
Sucrose	0.46	150
Glucose	0.71	300

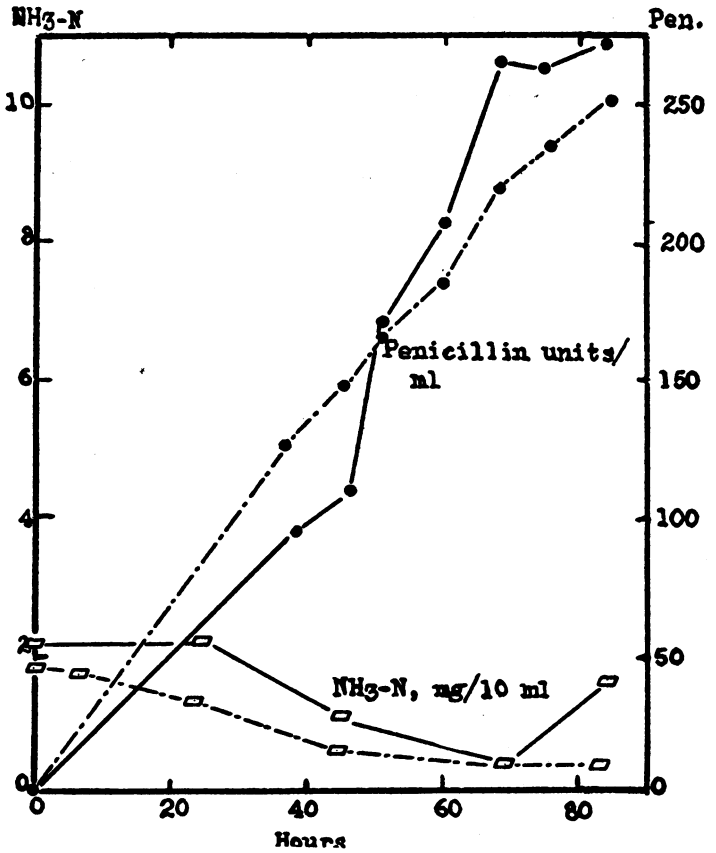
NH3-N
Pen.
10
8
6
4
2
0
250
200
150
100
50
0
Penicillin units/ml
NH3-N, mg/10 ml
0
20
40
60
80
Hours

FIG. 8B.

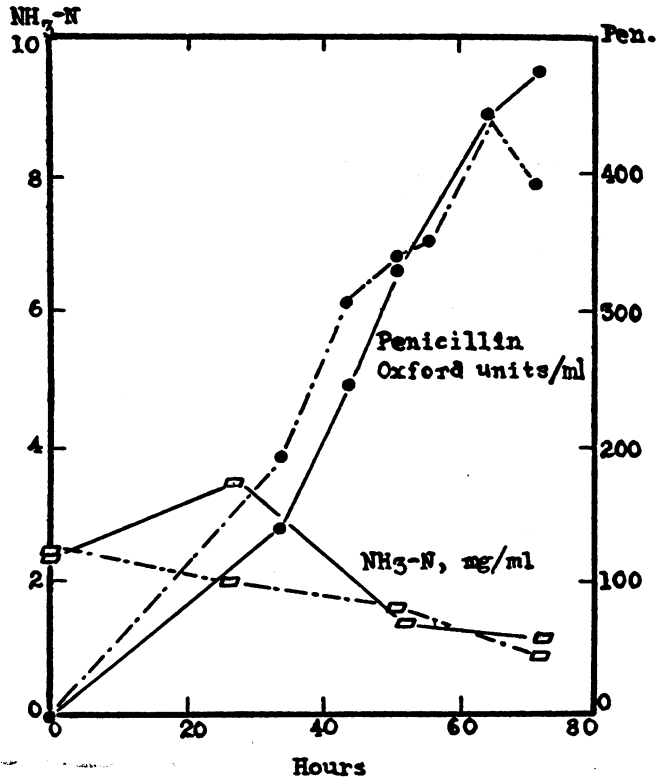
NH3-N
Pen.
10
8
6
4
2
0
400
300
200
100
0
Penicillin Oxford units/ml
NH3-N, mg/ml
0
20
40
60
80
Hours

FIG. 8C.

Since all flasks were shaken at the same rate, the oxygen actually available in all fermentations was the same. It will be noted that the penicillin yields were progressively greater on glucose, sucrose, and lactose, indicating that only in the case of the lactose was the oxygen supply sufficiently close to the oxygen demand in shake flasks to result in conditions for good penicillin production.

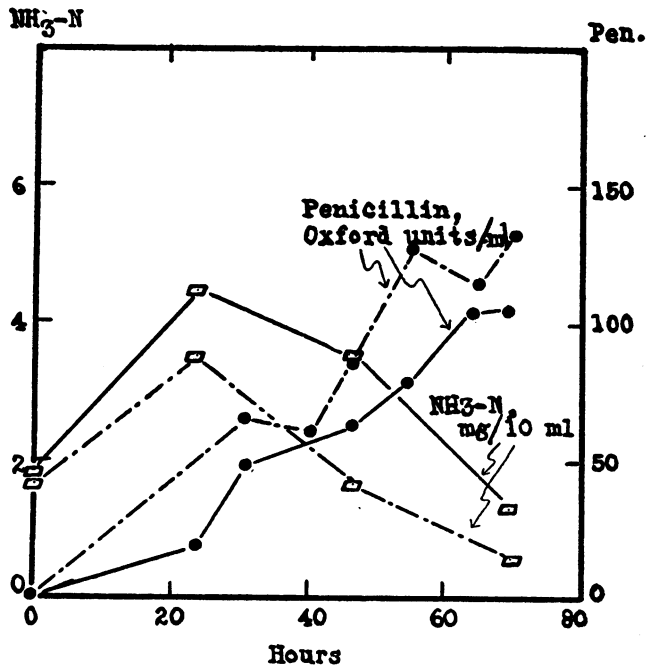

FIG. 8D.

TABLE 4

Oxygen uptake by different strains of Penicillium notatum and Penicillium chrysogenum

MEDIUM	STRAIN	Q_{O_2} (ML) Days							
		1	2	3	4	5	6	7	8
LCS 4-2	314-C			108.0	48.0	51.0	63.0	60.0	30.0
LCS 2-2	181-A	7.8	6.5	12.8	53.5	132.0	53.3	42.6	14.4
LCS 2-3	1951-B25		130.5	342.0	97.6	56.6	35.1	17.4	
LCS 2-2	1951-B25		147.0	256.0	116.0	104.5	60.7	38.4	

A comparison of the curves for the LCS 2-2 medium with those for the LGCS 2-2-2 medium shows that the addition of glucose increased both the amount of mycelium produced and the oxygen demand of the culture. The penicillin produced was no more than in the lactose medium.

Maximum Q_{O_2}(ml) values coincide with or occur before maximal mycelial

weights are reached; in either case they coincide with the highest concentration of active mold protoplasm and fall generally on the 3rd, 4th, or 5th day. It will be remembered from the discussion on nitrogen metabolism that the nitrogen content of the mycelium decreases with time. After the Q_{O_2}(ml) values have passed their maximum, their decline is not only due to smaller amounts of active protoplasm present but is also due to decreased respiratory activity per unit

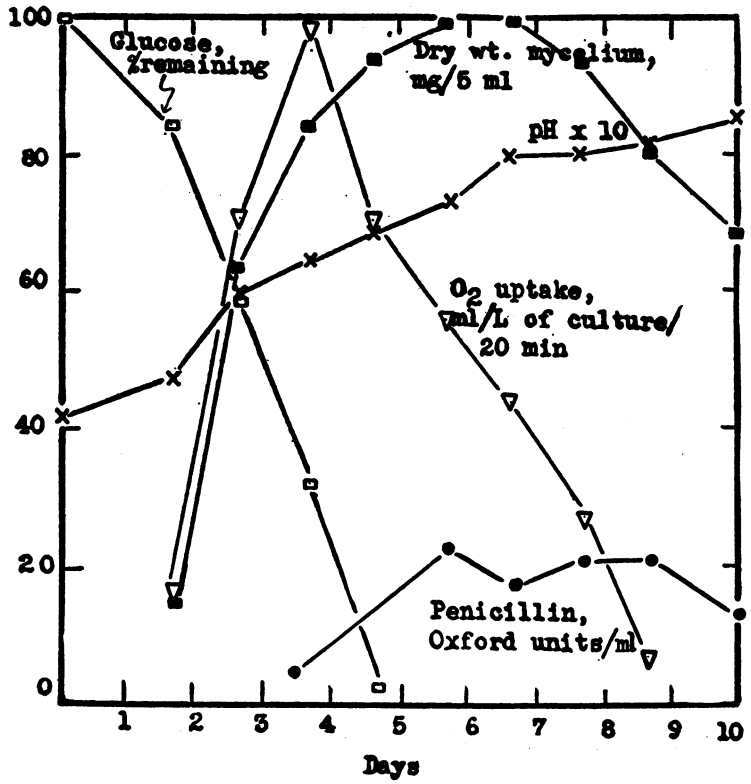

FIG. 9A.

FIG. 9. OXYGEN UPTAKE BY P. NOTATUM NRRL832 GROWN IN SHAKE FLASK CULTURES ON VARIOUS CARBOHYDRATE, CORN STEEP SOLIDS MEDIA

A. Four per cent glucose, 2 per cent corn steep solids.
B. Two per cent lactose, 2 per cent glucose, 2 per cent corn steep solids.
C. Four per cent glucose, 2 per cent corn steep solids.
D. Two per cent lactose, 2 per cent corn steep solids.

of protoplasm. From the rates observed in the microrespirometers it would appear that a given fermentation medium plus mycelium is capable of taking up oxygen at a rate of 21 to 368 ml per L per hr in shake flasks; however, to obtain maximum penicillin yields in tank fermentations oxygen is furnished at a rate of 6 to 12 L per L per hr. Even if it is considered that some of the oxygen supplied serves to remove CO_2 and ammonia, and aids in agitation, the discrepancy between actual oxygen requirement and oxygen supply is very large; this

discrepancy indicates how very difficult it is to design an efficient aeration system for industrial use, and emphasizes the fact that such design must as yet be based on empirical observation.

If the oxygen demand of the mold grown on glucose is really greater than the oxygen supply (as furnished by shaking the flasks on a reciprocating shaker), a more abundant and effective supply of oxygen should satisfy the mold's need

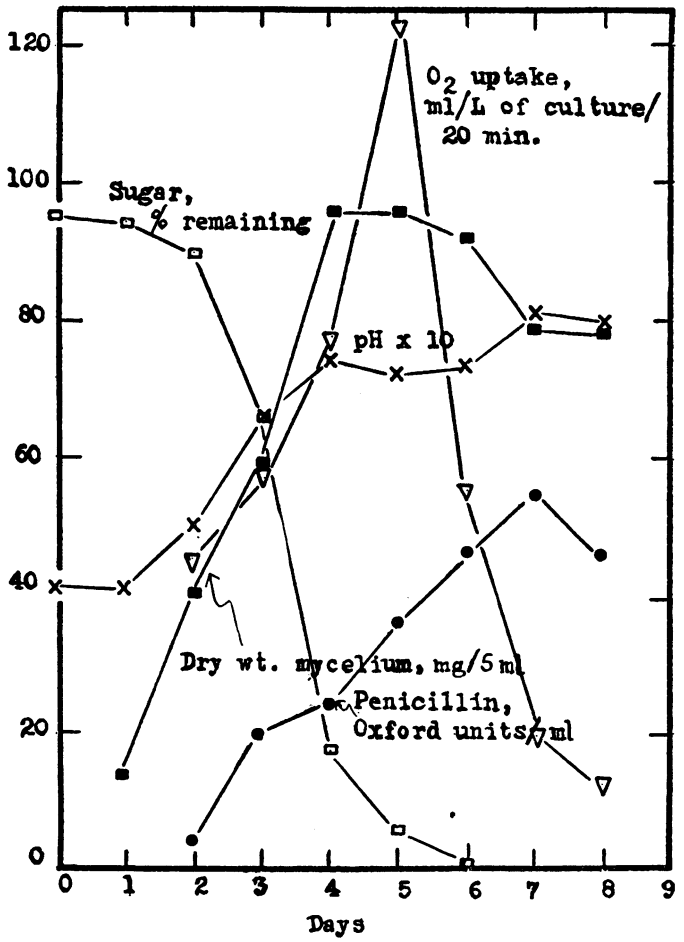

FIG. 9B.

and consequently penicillin yields should increase, provided penicillin yields are limited by the rate of glucose oxidation. A rotary-type shaker was employed in our laboratories to furnish more oxygen to the mold. As mentioned previously, the quantity of oxygen supplied to the medium (as measured by Winkler's reagent) in a flask on the rotary shaker was approximately 3 times that available to flasks on the reciprocating shaker. With the rotary shaker penicillin yields of 60 Oxford units per ml were obtained in 3 days on a glucose medium as com-

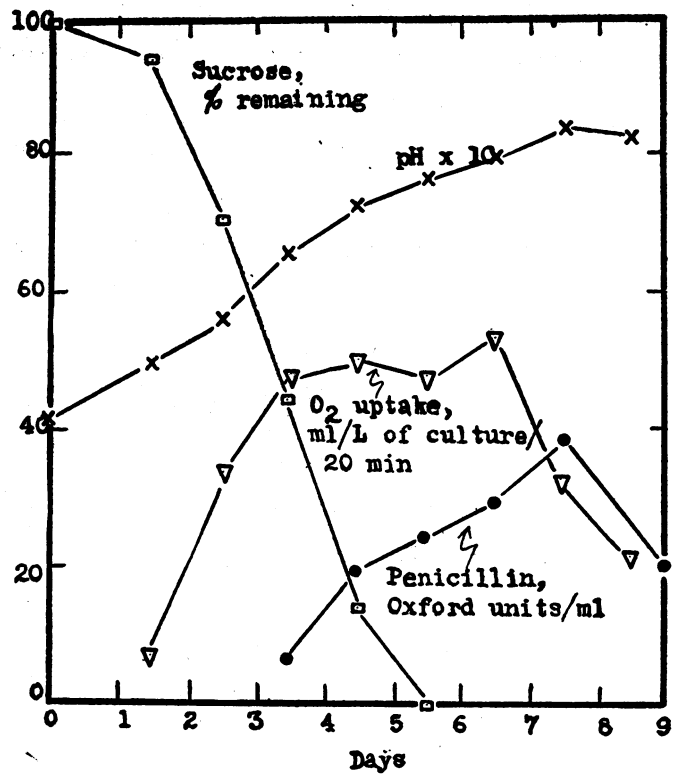
Sucrose,
% remaining
pH x 10
O_2 uptake,
ml/L of culture/
20 min
Penicillin,
Oxford units/ml
Days

FIG. 9C.

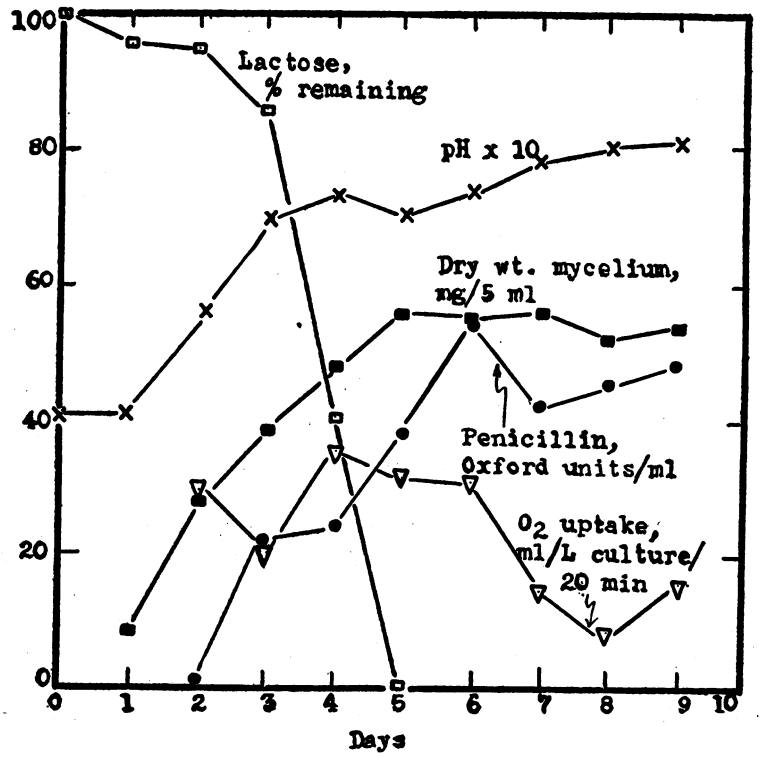
Lactose,
% remaining
pH x 10
Dry wt. mycelium,
mg/5 ml
Penicillin,
Oxford units/ml
O_2 uptake,
ml/L culture/
20 min
Days

FIG. 9D.

pared to 20 units in 6 days on the reciprocating shaker. The chemical changes occurring under these different conditions of oxygen supply were described earlier in the paper. There were indications that increased aeration of the mold grown on the ordinary lactose medium lowered penicillin production somewhat. Experiments employing different methods of obtaining increased aeration will be reported by other members of these laboratories. Microrespiration data on a particular strain with a particular medium are helpful in estimating relative aeration requirements.

As indicated above, shake flask cultures give good results on lactose, which does not require a high aeration rate, but give poor yields on rapidly fermenting carbohydrates such as glucose. Flask cultures, agitated on the rotary shaker, and larger aerated and agitated cultures, on the other hand, do give good yields on rapidly fermenting carbohydrates. Because of this difference in behavior, the usual tests of new cultures or new media made on a reciprocating shaker appear of questionable value; aeration conditions in shake flask cultures are not entirely comparable to those of bottle or tank fermentations.

The Effect of Various Oxygen Tensions in the Absence of Carbon Dioxide

Since the effect of aeration on metabolism and penicillin production was of evident importance, studies of some of the metabolic reactions taking place under various pO_2's were undertaken. Two Erlenmeyer flasks, containing the ordinary LCS 2-2 medium and KOH absorption tubes, formed an interconnected closed system and were filled with a gas mixture of the desired oxygen concentration. Four such closed systems were prepared for each level of oxygen. The pO_2's of the gas mixtures were 0.2 (I), 0.4 (II), 0.6 (III), and 1.0 (IV) atmosphere. The CO_2 formed was absorbed by the concentrated KOH, and the oxygen which was respired by the mold was continuously replaced with tank oxygen from a supply carboy. Two controls were employed: first, an open system Erlenmeyer flask (i.e., an Erlenmeyer flask plugged with cotton) containing a KOH absorption tube (V); and, second, an open system flask without KOH (VI). The results reported in table 5 represent the average of two flasks, except in the case of V and VI where triplicate flasks were averaged.

The mold produced more penicillin when grown in the presence of CO_2 than in its absence (table 5). This does not seem to be the result of a pH effect but actually appears to proceed from a CO_2 requirement for optimum penicillin production. The nature of this need is not yet understood.

The penicillin yields of closed flasks were fairly uniform; there was no indication that the pO_2 influenced penicillin yields in shake flask fermentations on a lactose medium. Maximum yields, with the exception of III, fell on the 5th day. The open system flask containing KOH produced essentially the same amount of penicillin as the other CO_2-free flasks.

The relationships between ammonia and yields and ammonia and lactose were especially interesting because they supported a generalization which was made earlier. Table 5 shows that III had a rather high ammonia content on the 5th day, whereas IV and VI had a much lower ammonia level. Correspond-

ingly, high yields for IV and VI and low yields for III were observed. In the same manner, this relationship is conspicuous for VI on the 7th day. This corroborates the generalization made before, that in flasks of the same series, containing the same medium and receiving similar treatment, a negative correlation between ammonia content and penicillin yields is apparent.

TABLE 5

Chemical changes in shake flask fermentations of Penicillium notatum NRRL 832 grown under various oxygen tensions on a 2 per cent lactose, 2 per cent corn steep solids medium

DAYS	I	II	III	IV	V	VI
			Penicillin (Oxford units/ml)			
3	22	20	20	22	17	19
5	41	42	15	52	40	46
7	38	34	34	28	28	62
8	18	33	27	40	32	72
			pH			
0	4.41	4.41	4.41	4.41	4.41	4.41
3	7.74	7.50	7.75	7.21	7.21	7.21
5	7.92	8.12	8.01	8.00	7.82	7.12
7	8.22	8.50	8.50	8.55	8.49	7.94
8	8.20	8.64	8.49	8.32	8.32	7.85
			Lactose (mg/100 ml)			
0	2240	2240	2240	2240	2240	2240
3	680	934	934	934	1960	1672
5	408	378	414	414	308	324
7	258	258	248	198	162	162
8	224	248	198	190	162	150
			NH_3-N (mg/100 ml)			
0	9.4	9.4	9.4	9.4	9.4	9.4
3	1.7	3.5	5.3	3.5	32.6	24.6
5	22.4	19.4	26.1	13.4	15.0	2.6
7	29.9	31.2	32.8	32.1	29.0	22.0
8	38.2	30.7	31.9	30.4	30.8	18.9

See text for experimental details. The pO_2's employed were: I—0.2 atm, II—0.4 atm, III—0.6 atm, IV—1.0 atm, V—open system flask with KOH, and VI—open system flask without KOH.

Ammonia levels remained high in treatments V and VI for the first 3 days of the fermentation when the rate of sugar utilization was small. As soon as lactose utilization became more vigorous, ammonia disappeared, probably serving for synthesis of more complex nitrogenous compounds. The slower lactose utilization may be attributed to the lower mycelial weights of V and VI, at the beginning of the fermentation, as compared to the mold weights in the closed flasks.

The Effect of CO_2 on Penicillin Production

The foregoing experiments suggested that the virtual elimination of CO_2 suppresses penicillin production. Two approaches were employed to obtain data regarding the effect of the pCO_2: (1) the CO_2 levels prevailing in the atmospheres of shake flasks during the fermentation were determined, and (2) the CO_2 concentration was controlled at different levels in closed flasks to find the concentration optimum for penicillin production.

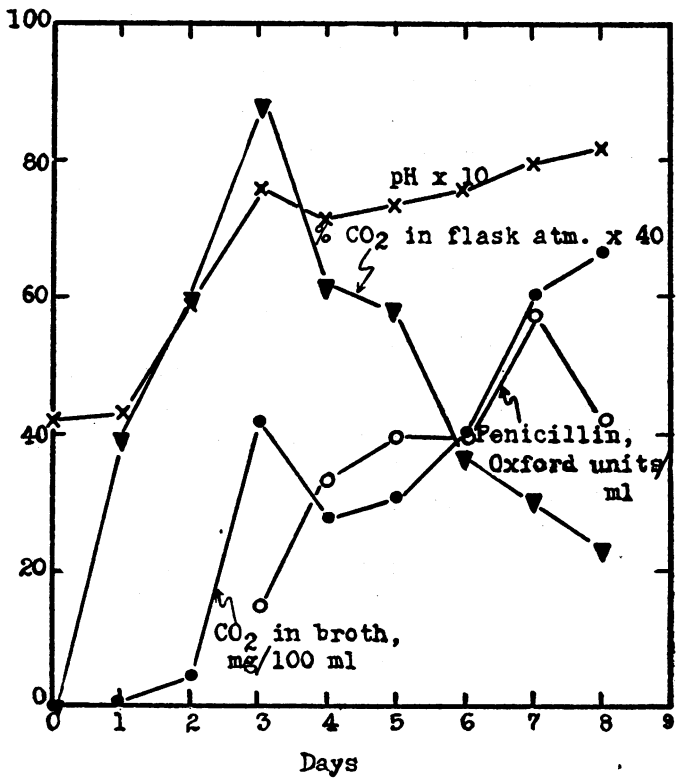

FIG. 10. CARBON DIOXIDE IN SOLUTION AND IN THE ATMOSPHERE ACCOMPANYING THE PENICILLIN FERMENTATION

Average values from duplicate cotton-plugged shake flask cultures of *P. notatum* 832 grown on 2 per cent lactose, 2 per cent corn steep solids medium.

The CO_2 concentration in the atmosphere of cotton-plugged shake flasks was determined by displacement of the gas through $Ba(OH)_2$ and titration of the residual alkali to the phenolphthalein end point. This determination was made daily on two shake flasks. The CO_2 content of the fermentation liquor was determined by cold aeration of the acidified liquor and absorption of the gas by $Ba(OH)_2$. This analysis was made daily in duplicate on 2 pooled flasks other than those used for the gas analyses. The pH, the penicillin content, and in some cases the Q_{O_2}(ml) were also determined.

Figure 10 gives a representative picture of the CO_2 levels obtained in cotton-

plugged flasks. The CO_2 concentration was highest on the 3rd day when the pH of the liquor was only slightly alkaline. This high CO_2 level was noted at almost the same time as the maximum Q_{O_2}(ml) values reported previously; in fact, the two functions showed parallel trends over most of the course of the fermentation. The shake flask atmosphere at times contained up to 4.7 per cent CO_2, although concentrations near 2.5 per cent were more common as maximum values. The lower CO_2 concentration in the atmosphere on the days after the maximum was chiefly a function of decreased respiration.

In the next series of experiments, constantly flowing gas mixtures containing the desired CO_2 concentration were supplied to the mold grown in Erlenmeyer flasks which were stoppered but had an outlet for the escape of the gas mixture. The mold thus could be exposed to an atmosphere of known CO_2 concentration. As a control, the mold also was grown in constantly flowing air and in cotton-plugged shake flasks. The cultures in flowing air produced very little penicillin; the constant flow of air (70 to 100 ml of air per min per 500-ml Erlenmeyer flask) prevented any appreciable accumulation of metabolic CO_2. It seems that the CO_2 level of the air is insufficient for optimum penicillin production; mycelial weights were lower in the flasks supplied flowing air. That the influence of CO_2 on the pH was not a controlling factor of the penicillin yield was apparent from the similarity of the pH values for the flowing air and cotton-plugged controls. When the air flow was reduced to 40 ml per min per flask, thus allowing accumulation of more metabolic CO_2, penicillin yields were increased. Flowing gas mixtures containing 0.1, 1.0, and 5.0 per cent CO_2, whether supplied throughout the run or only from the 2nd or 5th day until the completion of the run, suppressed penicillin yields as compared to the cotton-plugged controls. In a flowing gas mixture containing 0.25 per cent CO_2 (supplied from the 2nd day on), penicillin yields were the same as in cotton-plugged controls. These results, which were obtained on 2 to 5 flasks for each gas mixture, would indicate that a level of 0.25 per cent CO_2 is optimal *if maintained throughout the entire fermentation*, and that lower or higher levels of CO_2 under the same conditions definitely decrease yields. However, since the yields obtained at a constant CO_2 pressure of 0.25 per cent were no better than those found in cotton-plugged flasks where the CO_2 level varied from 0.03 to 4.7 per cent, this observation remains unexplained.

SUMMARY

The chemical changes induced by *Penicillium notatum* and *Penicillium chrysogenum* during submerged penicillin fermentations follow a predictable pattern. The penicillin fermentation can be divided conveniently into 3 phases as distinguished by pH rise, varying respiratory intensity, change in mycelial weight and composition, penicillin production, and shift in the composition of the medium. On a medium containing 2 per cent lactose and 2 per cent corn steep solids, the metabolic changes proceed in the following manner: The lactic acid and nitrogenous compounds of corn steep, being more readily available than lactose as carbon sources, are rapidly attacked during fermentation phase 1.

As the soluble organic nitrogen compounds of corn steep are utilized, the nitrogen they contain is partially liberated in the form of ammonia and partially used in building mycelium. When the bulk of the readily available carbon is exhausted, phase 2 begins. Lactose is now rapidly oxidized by the mycelium which has been formed in phase 1, and oxidation of this sugar is usually completed by the end of phase 2. Coincident with the rapid oxidation of lactose, ammonia is utilized more rapidly than it is formed and its concentration falls. The nonammonia Kjeldahl nitrogen furnished by the corn steep decreases rapidly during phase 1 but remains without great change through phase 2. In phase 3 marked liberation of soluble nitrogen from the mycelium is apparent; ammonia often is freed and nonammonia Kjeldahl nitrogen always is freed. The nitrate nitrogen in the medium is utilized slowly; if large amounts of nitrate are added or if the level of corn steep is low, more nitrate is utilized. The initial rise in pH during phase 1 can be accounted for largely by an accumulation of ammonia, by liberation of sodium ions from the utilization of nitrates, and by a rapid breakdown of lactic acid. Phase 2 is characterized by a pH plateau, or some depression in the pH resulting from ammonia utilization. A plateau near a neutral reaction is most favorable for penicillin accumulation. Penicillin concentrations decrease during the period of the final pH rise that accompanies the liberation of ammonia and basic nitrogenous compounds from the mycelium.

The effect of the composition of the medium on metabolic changes has been illustrated by specific examples, including fermentations carried out on a lactose, glucose, corn steep medium, on a medium without nitrate, on low corn steep media, on high corn steep media, and on a glucose, corn steep medium. The different strains investigated have shown but slight differences in their metabolism.

Data concerning the oxygen requirement of various strains on different media have been obtained and related to oxygen supply. For example, it has been found that because the oxygen requirement of the mold is high when grown on glucose, the oxygen supply with a reciprocating shaker is insufficient to favor good penicillin yields on this medium. Increased oxygen supply changes, not only the level of penicillin and the time necessary to achieve this peak, but also the associated metabolic picture; data on fermentations conducted in aerated bottles and tanks have been given as additional examples of the influence of aeration. There has been no indication that the pO_2 of the flask atmosphere influences penicillin yields in shake flask fermentations employing a lactose, corn steep medium; the pCO_2, however, seems to affect penicillin production.

Among fermentations set up under similar conditions a correlation exists between ammonia levels and penicillin yields; this interrelationship has been discussed.

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