PENICILLIN

X. The Effect of Phenylacetic Acid on Penicillin Production[1]

ANDREW J. MOYER and ROBERT D. COGHILL[2]

Northern Regional Research Laboratory, Peoria, Illinois[3]

Received for publication December 13, 1946

As penicillin chemistry unfolded during the early days of production development, it soon became evident that there existed more than one compound having the pharmacological and bactericidal properties associated with the name "penicillin." American workers had isolated a pure crystalline penicillin (G) which was characterized by the benzyl radical and which yielded phenylacetic acid as a degradative product, whereas the crystalline penicillin isolated by the English workers (penicillin F) contained the Δ-2-pentenyl radical and yielded no phenylacetic acid on degradation, Δ-3-hexenoic acid being obtained in its place (Committee on Medical Research, Office of Scientific Research and Development, Washington; and the Medical Research Council, London). It was of interest, therefore, to ascertain the effect of adding phenylacetic acid to the culture medium in or upon which the mold was grown. Two possibilities were anticipated, both based on the assumption that the mold could use the phenylacetic acid as a building stone for the penicillin molecule. If this supposition were true, it would follow that (a) yields would be increased if synthesis of phenylacetic acid by the mold were the bottleneck in its penicillin production, and (b) the penicillin G:penicillin F ratio would be increased.

The experiments described below show that the total penicillin yield was increased in both surface and submerged cultures, but no marked change in the ratio of penicillin types could be demonstrated as being due to phenylacetic acid.[4]

METHODS AND MATERIALS

All of the surface production cultures were grown at 24 C, in 200-ml pyrex Erlenmeyer flasks containing 50 ml of medium. The submerged cultures were also grown at 24 C, but in 300-ml Erlenmeyer flasks containing 125 ml of medium and shaken on a Ross-Kershaw machine.

[1] The expenses of the work described in this paper were met in part from Contract OEMcmr-100 with the Office of Scientific Research and Development, recommended by the Committee on Medical Research.

[2] Present address: Abbott Laboratories, North Chicago, Ill.

[3] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

[4] The effect of phenylacetic acid on penicillin production was communicated in a restricted monthly report, no. 16, November 20, 1943, to Dr. A. N. Richards, Chairman, Committee on Medical Research, Office Scientific Research and Development, who in turn sent copies to all penicillin producers and to many research groups in this country and abroad. The phenomenon here described has been generally applied industrially. Owing to the strategic significance of penicillin, publication of this research has been delayed.

The assays were conducted by the cylinder plate method of Abraham *et al.* (1941), as modified by Schmidt and Moyer (1944). Some assays were made by the same procedure, except that *Bacillus subtilis* NRRL B-558 and *Staphylococcus aureus* NRRL B-313 were used in parallel as the test organisms. Schmidt *et al.* (1945) demonstrated that a comparison of assay values obtained with *S. aureus* and *B. subtilis* gives an indication of the type of penicillin present.

The inoculum for the submerged production cultures consisted of a suspension of tiny pellets, about 1 mm in diameter, which were obtained after 3 days' growth in the following medium: lactose monohydrate, 40.0 g; glucose monohydrate, 4.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KH_2PO_4, 0.50 g; $NaNO_3$, 3.0 g; $ZnSO_4 \cdot 7H_2O$, 0.080 g; corn steep liquor, 36.0 g; and distilled water to make 1 liter. Portions of 125 ml of this medium were dispensed in 300-ml Erlenmeyer flasks. One gram of sterile, dry $CaCO_3$ was added to each flask just before inoculation. Inoculations were made with 10-ml portions of a heavy suspension of ungerminated spores. These cultures were incubated at 24 C on the Ross-Kershaw shaking machine.

The production media contained the corn steep liquor and lactose which have been shown by the authors (1946) to be essential to good penicillin yields in both surface and submerged cultures.

The experimental error of the cup plate assay on crude culture filtrates was frequently of sufficient magnitude under routine conditions to render difficult a very accurate evaluation of units per ml based on the dual assays. The variation in the assays appeared to be greater with the crude penicillin filtrates than with highly purified samples of the F and G types of penicillin.

Three fungus strains, *Penicillium notatum* NRRL 1249.B21 (Moyer and Coghill 1946*a*), *P. notatum* NRRL 832 (Moyer and Coghill 1946*b*), and *Penicillium chrysogenum* NRRL 1951.B25 (Raper and Alexander, 1945) were employed in these investigations.

EXPERIMENTAL RESULTS

Surface cultures. Concentrations of phenylacetic acid varying from 0.025 to 0.8 g per liter, added at the time of inoculation, were first employed in surface cultures of *P. notatum* NRRL 1249.B21. On the second day a concentration of phenylacetic acid of 0.2 g per liter had caused a definite inhibition of growth; at 0.4 g per liter there was a marked toxicity; and at 0.8 g per liter growth was completely inhibited. As the cultures aged and the pH increased, the toxicity of phenylacetic acid appeared to decrease. There was some increase in penicillin yields apparently due to phenylacetic acid. The cultures containing 0.2 g per liter of phenylacetic acid, although slower in growth and pH change, gave as high yields of penicillin as the cultures containing only 0.025 g of phenylacetic acid per liter. This result suggested that higher yields of penicillin might be obtained if the toxic effect of such concentration of phenylacetic acid were eliminated during the stage of early growth.

The first attempt to eliminate such toxicity was made by adding the phenylacetic acid to 2-day-old surface cultures. Under these conditions a marked in-

crease in penicillin yields was obtained by the addition of 0.1 to 0.6 g of phenylacetic acid per liter of medium. The tolerance for this compound was much greater when it was added to 2-day-old cultures than when it was added at the time of inoculation with ungerminated spores. This difference in phenylacetic acid tolerance was due either to a greater resistance of the 2-day-old mycelium or to a close relationship between toxicity and the pH of the medium, similar to that known to exist for acetic and benzoic acids.

To test the effect of adding phenylacetic acid at different ages of the culture, three series of surface cultures were prepared. To reduce the toxic effect associated with low pH, the initial pH of the medium was raised from 4.0 to 4.6 by adding KOH. Phenylacetic acid was then added to one series of cultures at the time of inoculation, and to another series when the cultures were 2 days old. At the initial pH of 4.6, it was found that 0.4 g per liter of phenylacetic acid was just slightly toxic, as shown by a slower growth and pH change than occurred in the control cultures (table 1). The presence of phenylacetic acid in both culture series caused a marked increase in the penicillin yield. The assay values based on *B. subtilis* were not significantly different from those based on *S. aureus*, regardless of phenylacetic acid, indicating that the penicillin was of the G type even in the control cultures of *P. notatum* NRRL 1249.B21.

Since these results showed that some advantage was gained by raising the initial pH of the medium from 4.0 to 4.6, another series of cultures was prepared with the initial pH values at 4.2, 4.7, 5.2, and 5.8, with and without phenylacetic acid (0.30 g per liter) added at the time of inoculation with ungerminated spores. At an initial pH of 4.2, moderate toxicity of phenylacetic acid was again encountered (table 2). With the initial pH at 5.2 and 5.8, there was no indication of toxicity due to the phenylacetic acid. A penicillin yield of 266 units per ml was obtained in those cultures containing phenylacetic acid with an initial pH of 5.2 or 5.8.

The best penicillin yields with phenylacetic acid were obtained in a medium containing a special steep liquor nutrient prepared commercially by a starch manufacturing company. The initial pH was adjusted to 5.6 with NaOH, and 0.40 g per liter of phenylacetic acid was sterilized in the basal medium. Under these conditions, a penicillin yield of 316 units per ml was obtained in 7 days (table 3). The superiority of this special steep liquor nutrient over the ordinary corn steep liquor has been demonstrated by comparative tests in other surface culture experiments.

Submerged-shaker cultures. The effect of phenylacetic acid on penicillin yield was studied concurrently in both surface and submerged cultures. In the submerged cultures of *P. notatum* NRRL 832, the potency value in terms of units obtained from *B. subtilis* assays was 20 to 30 per cent lower than from *S. aureus* assays. This difference in assay values was believed to be due to the presence of penicillin F as the predominant type. Hence, attention was directed not only to the possible increase in total penicillin yield but also to possible changes in the type of penicillin found in these submerged cultures.

The submerged-shaker production flasks were inoculated with small portions of a preformed pellet type growth of *P. notatum* NRRL 832. The initial pH

TABLE 1

Effect of phenylacetic acid (0.40 g per liter) on penicillin yields, added at time of inoculation and after 2 days to surface cultures of P. notatum NRRL 1249.B21

PHENYLACETIC ACID ADDITIONS	CULTURE AGE, DAYS				
	3	4	5	6	7
Penicillin, units per ml (*S. aureus* NRRL B-313)					
None	47	106	126	143	130
At start	36	90	160	191	194
At 2 days	50	128	170	189	178
Penicillin, units per ml (*B. subtilis* NRRL B-558)					
None	—	111	126	150	132
At start	—	96	164	191	200
At 2 days	—	134	176	200	196
pH of filtrates					
None	6.3	7.1	7.5	7.7	8.0
At start	5.4	6.5	7.2	7.5	7.9
At 2 days	6.0	6.8	7.5	7.7	8.1
Dry weight of fungus growth, g per culture					
None	0.73	0.97	1.11	1.20	1.17
At start	0.41	0.81	0.98	1.18	1.23
At 2 days	0.67	0.96	1.10	1.23	1.19

Culture medium: Lactose monohydrate, 55.0 g; glucose monohydrate, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KH_2PO_4, 0.50 g; $NaNO_3$, 3.0 g; $ZnSO_4 \cdot 7H_2O$, 0.022 g; corn steep liquor, 90.0 g; and distilled water to make 1 liter.

Initial pH: 4.6.

TABLE 2

Effect of initial pH of medium on the value of phenylacetic acid in penicillin production by P. notatum NRRL 1249.B21 in surface cultures

INITIAL pH	PHENYLACETIC ACID PER LITER	CULTURE AGE, DAYS				
		3	4	5	6	7
Penicillin, units per ml (*S. aureus* NRRL B-313)						
	g					
4.2	0.0	45	78	102	130	159
4.2	0.3	21	67	109	138	188
4.7	0.0	54	85	125	150	194
4.7	0.3	47	106	177	200	224
5.2	0.0	45	80	127	157	178
5.2	0.3	48	106	159	200	266
5.8	0.0	40	80	123	150	178
5.8	0.3	51	106	168	200	266

Culture medium: Lactose monohydrate, 44.0 g; glucose monohydrate, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KH_2PO_4, 0.50 g; $NaNO_3$, 3.0 g; $ZnSO_4 \cdot 7H_2O$, 0.022 g; corn steep liquor, 78.0 g; and distilled water to make 1 liter. KOH used to make pH adjustments.

of the production medium was 4.2. In the preliminary experiments, phenylacetic acid was added at the time of inoculation and to 1-day-old production cultures. Under these conditions, phenylacetic acid at 0.4, 0.8, and 1.2 g per liter resulted in no increase in penicillin yield. There was a marked toxicity with phenylacetic acid at 0.6 and 1.2 g per liter.

It was known that the pH of these production cultures rose rapidly, and it seemed likely that, as in the surface cultures, the toxicity of phenylacetic acid would be less if additions were made to 2- or 3-day-old cultures. A series of cultures was prepared in which phenylacetic acid (0.8 g per liter) was added

TABLE 3

*Effect of phenylacetic acid on penicillin yields by P. notatum NRRL 1249.B21 in surface cultures using a special corn steep liquor nutrient**

PHENYLACETIC ACID PER LITER	CULTURE AGE, DAYS			
	4	5	6	7
	Penicillin, units per ml (*S. aureus*)			
g				
0.0	98	146	189	194
0.4	132	233	263	313
	Penicillin, units per ml (*B. subtilis*)			
0.0	—	160	192	189
0.4	—	222	250	316
	pH of filtrates			
0.0	7.2	7.5	7.7	7.9
0.4	7.2	7.6	7.7	7.9
	Dry weight of fungus growth, g per culture			
0.0	0.70	0.89	0.92	0.88
0.4	0.68	0.85	0.90	0.89

Culture medium: Same as given in table 2, except that 70.0 g per liter of special steep liquor nutrient was employed instead of ordinary corn steep liquor. The initial pH of the medium was adjusted to 5.6 with NaOH.

* Special corn steep liquor nutrient 14 Ea supplied by a commercial firm.

to 1-, 2-, and 3-day-old production cultures (table 4). Phenylacetic acid added to the 1-day-old cultures was quite toxic, inhibiting both growth and penicillin production, but additions to the 2- or 3-day cultures showed no toxicity and a significant increase in penicillin yield as compared with the control cultures. On the third day, 0.40 g of $CaCO_3$ was added to one of the control cultures. As a result of this treatment, a rapid growth occurred and a good yield of penicillin was obtained on the seventh day. This was evidence that the inhibitory or toxic effect of phenylacetic acid could largely be overcome by raising the initial pH of the medium or by making the addition to 2- or 3-day-old

cultures. The comparative assay values with *B. subtilis* and *S. aureus* at 6 and 7 days did not indicate any change in type of penicillin produced, although the total yield of penicillin had been increased by the addition of phenylacetic acid.

In one culture series the initial pH was raised from 4.1 to 5.0 by the addition of KOH. After 1-day incubation, the pH of these cultures had arisen to 6.6 in the case in which 0.8 g of phenylacetic acid per liter had been added to the cultures. There was no evidence of toxicity due to the phenylacetic acid, and there was a significant increase in the penicillin yield (table 5). Again assays

TABLE 4

Effect of 0.8 g per liter of phenylacetic acid on penicillin yields by P. notatum NRRL 832, when added to 1-, 2-, and 3-day-old submerged production cultures

PHENYLACETIC ACID PER LITER	CULTURE AGE, DAYS					
	2	3	4	5	6	
	Penicillin, units per ml					
g						
Control	11	22	36	57	65, (51)	68, (51)
1-day add'n	2	3	4	4	4	—
1-day add'n.*	2	3	8	30	59	95, (88)
2-day add'n	15	35	50	71	80, (69)	85, (76)
3-day add'n	14	32	45	71	85, (72)	86, (63)
	pH of filtrates					
Control	7.1	7.7	7.3	7.2	7.9	8.1
1-day add'n	4.4	4.7	4.9	4.9	5.2	—
1-day add'n.*	4.4	4.7	6.0	7.6	7.8	7.4
2-day add'n	7.2	7.5	7.4	7.4	7.8	7.9
3-day add'n	7.2	7.3	7.4	7.3	7.9	8.0

Culture medium: Amounts per 1 liter: Lactose monohydrate, 30.0 g; $NaNO_3$, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.125 g; KH_2PO_4, 0.250 g; $ZnSO_4 \cdot 7H_2O$, 0.022 g; corn steep liquor, 50.0 g.

All cultures inoculated with 4 ml of a 3-day-old pellet suspension.

Initial pH: 4.2.

Assays values in parentheses determined with *B. subtilis* NRRL B-558; all others with *S. aureus*.

* 0.40 g $CaCO_3$ added per culture on third day.

with both *S. aureus* and *B. subtilis* showed that phenylacetic acid, although increasing the total yield of penicillin, did not suggest a change in the type of penicillin produced. Other experiments showed there was no advantage in raising the initial pH of the medium much above 5.2.

Several investigations were made to determine the concentration of phenylacetic acid required to give the maximum penicillin yield. It was not possible to determine clearly such an optimum concentration of phenylacetic acid, possibly because of some uncontrolled factors, such as activity or uniformity of the inoculum, foaming, variations in shaker speed, etc., which varied

to some extent from week to week. A comparison of 0.2 g and 0.8 g of phenylacetic acid per liter (added at 30 hours) on penicillin yield is shown in table 6.

TABLE 5

The effect of phenylacetic acid when added to 1-day-old submerged production cultures of P. notatum NRRL 832 for which the initial pH was raised to 5.0 with KOH

PHENYLACETIC ACID PER LITER	CULTURE AGE, DAYS					
	2	3	4	5	6	7
	Penicillin units per ml					
0.00	22	48	76, (54)	80	65	58
0.80	26	60	90, (69)	100	95	80
	pH of filtrates					
0.00	8.0	7.6	7.6	8.0	8.2	8.3
0.80	7.9	7.6	7.7	8.0	8.2	8.3

Culture medium: Amounts per 1 liter: Lactose monohydrate, 30.0 g; $NaNO_3$, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.125 g; KH_2PO_4, 0.250 g; $ZnSO_4 \cdot 7H_2O$, 0.022 g; corn steep liquor, 50.0 g.

Cultures inoculated with 6 ml of a 3-day-old pellet inoculum.

Assay values in parentheses determined with *B. subtilis*, NRRL B-558; all others with *S. aureus*.

TABLE 6

Effect of phenylacetic acid on penicillin yields by P. notatum NRRL 832, when added to 30-hour-old, pellet-inoculated submerged production cultures

PHENYLACETIC ACID PER LITER	CULTURE AGE, DAYS				
	2	3	4	5	6
	Penicillin, units per ml				
g					
0.00	11	23	50	84, (66)	95, (75)
0.20	18	36	80, (66)	102, (88)	125, (105)
0.80	12	34	83, (68)	113, (101)	141, (120)
	pH of filtrates				
0.00	7.7	7.6	7.3	7.5	7.9
0.20	7.7	7.6	7.3	7.4	7.8
0.80	7.6	7.6	7.3	7.4	7.8

Culture conditions same as given in table 4.

Cultures inoculated with 6 ml of a 3-day-old pellet suspension.

At 30 hours the pH was 6.3.

Assays values in parentheses determined with *B. subtilis* NRRL B-558; all others with *S. aureus*.

There was no significant difference in the pH change between the control cultures and those receiving phenylacetic acid. These and other experiments showed that a maximum increase in penicillin yield could be attained by sup-

plying phenylacetic acid at 0.2 to 0.8 g per liter. No further increase in penicillin yield was ever obtained by increasing the concentration of phenylacetic acid beyond 0.8 g per liter of medium.

Part of the results of the foregoing experiment is presented in figure 1. At 30 hours the pH had risen from 4.1 to 6.3 when 0.8 g per liter of phenylacetic acid was added. During the 6-day incubation period, 5-ml samples were removed once daily for assay and pH determinations. The amount

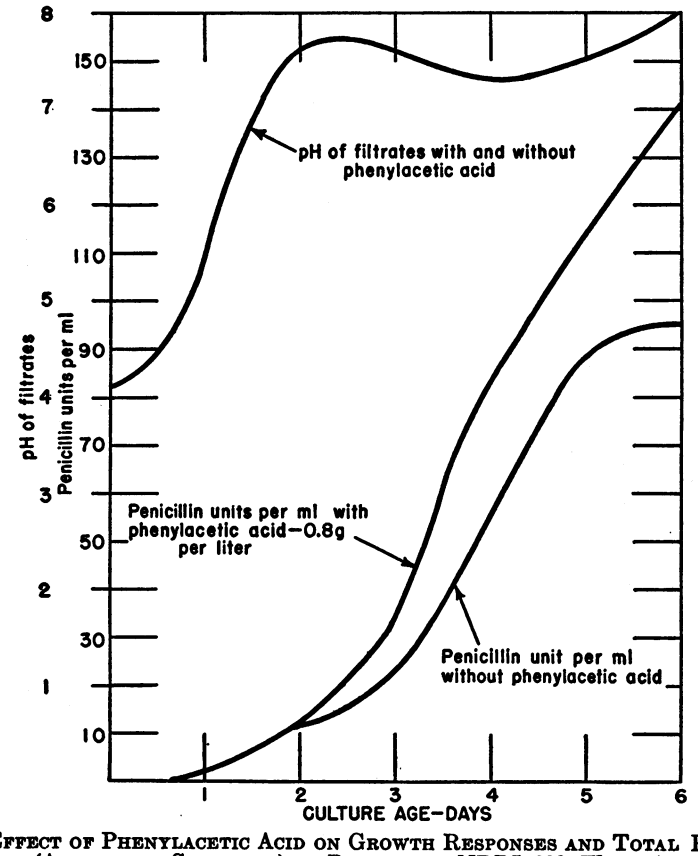

FIG. 1. EFFECT OF PHENYLACETIC ACID ON GROWTH RESPONSES AND TOTAL PENICILLIN YIELD (ASSAY WITH S. AUREUS) BY P. NOTATUM NRRL 832, WHEN ADDED TO 30-HOUR-OLD PELLET-INOCULATED PRODUCTION CULTURES

of total growth was based on a visual score. After 4 days no increase in the amount of fungus growth could be detected. After 3 days there was a marked increase in the formation of a yellow pigment which diffused from the pellet growth out into the medium. The peculiar type of pH curve has already been discussed by the authors (1946*b*).

A strain of *P. chrysogenum* NRRL 1951.B25 was employed in a series of cultures in which the initial pH was raised to 5.3 by the addition of $CaCO_3$ or to pH 5.6 with NaOH. Phenylacetic acid (0.4 g per liter) was added to the

nutrient medium before sterilization. These cultures were inoculated with a suspension of ungerminated spores. In the presence of phenylacetic acid, approximately the same levels of penicillin were produced whether NaOH or $CaCO_3$ was used as the neutralizing agent. It is worth noting that less penicillin was produced in the absence of phenylacetic acid when NaOH was employed as the neutralizing agent than when $CaCO_3$ was used. At the fifth day, penicillin yields with *S. aureus* were 106 units in both NaOH and $CaCO_3$ cultures (table 7). Again no effect of phenylacetic acid on the type of penicillin produced could be shown by the differential assays.

TABLE 7

Effect of phenylacetic acid, 0.4 g per liter, on penicillin production by P. chrysogenum NRRL 1951.B25 in submerged cultures

INITIAL pH OF MEDIA	PHENYLACETIC ACID PER LITER	CULTURE AGE, DAYS				
		3	4	5	6	7
Penicillin units per ml						
	g					
NaOH to pH 5.6	0.0	26	40, (29)	60, (48)	68, (52)	54, (41)
	0.4	36	75, (69)	106, (78)	102, (87)	80, (69)
$CaCO_3$* pH 5.3	0.0	53	80, (63)	95, (82)	100, (84)	68, (46)
	0.4	50	85, (73)	106, (80)	106, (71)	71, (54)
pH of filtrates						
NaOH to pH 5.6	0.0	8.0	8.1	7.7	8.0	8.2
	0.4	8.0	7.9	8.0	8.4	8.3
$CaCO_3$ pH 5.3	0.0	8.0	8.1	7.9	8.4	8.5
	0.4	8.0	8.1	7.9	8.3	8.6

Culture medium: Lactose monohydrate, 27.5 g; glucose monohydrate, 5.5 g; $MgSO_4 \cdot 7H_2O$, 0.125 g; KH_2PO_4, 0.25 g; $ZnSO_4 \cdot 7H_2O$, 0.022 g; corn steep liquor, 50.0 g; and distilled water to make 1 liter.

All cultures inoculated with ungerminated spores.

Phenylacetic acid added before medium sterilization.

Assay values in parentheses determined with *B. subtilis* NRRL B-558; all others with *S. aureus*.

* $CaCO_3$ 5.0 g per liter, added to cool, sterile medium.

Sodium benzoate, used at 0.8 g per liter in both surface and submerged culture, caused no increase in penicillin yield, nor was there any indication of a change in type of penicillin produced.

Various concentrations of phenylacetic acid were employed in both surface and submerged cultures grown on a synthetic medium. In no case was it possible to demonstrate clearly an increase in total penicillin yield or a change in type of penicillin due to the effect of phenylacetic acid.

The addition of small portions of a finely ground wheat bran (about 50 per cent starch) and phenylacetic acid to submerged cultures of *P. chrysogenum* 1951.B25 with a nonpellet type of inoculum resulted in a marked increase in

the speed of penicillin accumulation (table 8). The particles of bran seemed to act as focal points for the germinated spores, resulting in soft "fuzzy" pellets. The addition of wheat bran in the presence of phenylacetic acid caused not only an increase in total penicillin yield but also caused a change in the assay ratio which might be interpreted as indicating greater accumulation of penicillin G over that obtained in the absence of bran. This effect of bran in combination with phenylacetic acid has been repeated in many experiments. The pH of the cultures receiving 5.0 to 10.0 g of wheat bran per culture was always lower up to the fourth day than the pH observed in the control cultures. After

TABLE 8

Effect of finely ground wheat bran on total yield and type of penicillin produced in submerged culture of P. chrysogenum 1951.B25

	CULTURE AGE, DAYS		
	3	4	5
Penicillin, units per ml			
Control	34 (32)	59 (41)	66 (47)
+ 0.5 g bran per culture	79 (84)	89 (96)	95 (95)
pH of filtrates			
Control	7.4	7.7	7.8
+ 0.5 g bran per culture	6.5	7.6	7.8
Bulk of growth, score			
Control	3.2	3.5	3.6
+ 0.5 g bran per culture	4.7	4.7	4.7

Culture medium: Amounts per 1 liter—corn steep liquor, 36.0 g; lactose monohydrate, 27.5 g; $MgSO_4 \cdot 7H_2O$, 0.125 g; KH_2PO_4, 0.25 g; $NaNO_3$, 1.50 g; $ZnSO_4 \cdot 7H_2O$, 0.088 g; phenylacetic acid, 0.30 g.

Culture size: 200 ml in 1-liter Erlenmeyer flasks. Bran and phenylacetic acid sterilized in presence of medium; 1.2 g sterile $CaCO_3$ added per culture just before inoculation with a nonpellet type of inoculum (10 ml per culture). Culture incubated at 25 C on a rocking cradle.

Assay values in parentheses determined with *B. subtilis* NRRL B-558; all others with *S. aureus*.

the fourth day there was usually little difference in pH between cultures with or without bran. In a large number of experiments, penicillin assays by use of *S. aureus* and *B. subtilis* were nearly identical up to 3 days in cultures receiving only phenylacetic acid, if the pH was not higher than 7.6. Identical results could not be obtained by adding wheat flour in an amount equivalent to that occurring in the bran or by the addition of bran in the absence of phenylacetic acid.

DISCUSSION

The effectiveness of phenylacetic acid in increasing penicillin yields in both surface and submerged cultures is closely related to its toxicity. The degree

of this toxicity depends both on the concentration of the phenylacetic acid and the pH of the culture medium. The toxicity of phenylacetic acid is believed to be due to the undissociated molecule, as has been demonstrated with respect to acetic acid by other investigators. Kiesel (1913) observed that formic and acetic acids were more toxic for *Aspergillus niger* than were mineral acids. He attributed the toxicity of these organic acids to the undissociated molecule. Cruess and Irish (1932) found that *P. glaucum* could tolerate 160 times more acetic acid at pH 7.0 than at pH 2.5. Kirby *et al.* (1937) showed that the toxicity for *A. niger* at a fixed concentration was a function of the pH of the medium. At pH 5.5 to 6.0 acetic acid slightly inhibited germination, but it had only a slight effect, ranging from slight inhibition to a slight stimulation, on the ultimate growth of several molds. It was claimed that the undissociated molecule, not the acetate ion, was responsible for toxicity. The increased antiseptic efficiency of weak acids has been proved in the majority of cases to be due to the undissociated acid molecules. Such was found to be true for acetic, propionic, butyric, choroacetic, bromopropionic, oxalic, selenious, nitrous, benzoic, salicylic, sulfurous, and hypochlorous acids, and also for phenol (Huntington and Rahn, 1945). Therefore, it is believed that the toxicity of phenylacetic acid is probably due to the undissociated molecule.

A steady rise in the pH of the medium made it possible to add phenylacetic acid in effective amounts to 2-day-old surface cultures without serious toxicity. It was found to be a more convenient and equally effective procedure to raise the initial pH of the medium to 5.0 to 5.8, and to sterilize the phenylacetic acid in the culture medium. Using a pellet inoculum in the submerged cultures, better penicillin yields were obtained by adding the phenylacetic acid during the fermentation, when the pH had risen to approximately 5.5, than were obtained by adjusting the initial pH to 5.5. When ungerminated spores were used for inoculum, the toxicity of phenylacetic acid could be avoided by adjusting the initial pH to a suitable level by means of $CaCO_3$ or a soluble alkali.

The quantitative relationship between the amount of phenylacetic acid added and the increase in penicillin yield was not always apparent in these experiments. Approximately 5 mg of phenylacetic acid, as based on the data in tables 2 and 3, gave a 1-mg increase in the penicillin yield. A slight increase in penicillin yield was obtained with 0.025 g of phenylacetic acid per liter. If all the phenylacetic acid added (tables 2 and 3) was utilized directly in penicillin synthesis, then the penicillin yield should have been several times that actually obtained. Thus it appears that factors other than the amount of phenylacetic acid limit the amount of penicillin that accumulates in the fermented liquor. The optimal amount of phenylacetic acid over a wide range of pH has not been systematically determined; however, best penicillin yields were obtained in the surface cultures with phenylacetic acid at 0.3 to 0.4 g per liter and an initial pH of 5.0 to 5.8.

The role of whole-wheat bran in bringing about a marked increase in the rate of penicillin accumulation is not clearly understood. The fungus growth surrounded the bran particles to form soft, "fuzzy" pellets. This bran contained about 50 per cent starch, but the addition of an equivalent amount of starch

or aqueous bran extracts had little, if any, effect on penicillin yields. The bran cultures gave a much more rapid growth than the control cultures and a near maximum yield of penicillin was obtained before the medium became very alkaline. The apparent change in the type of penicillin was encountered only upon addition of bran to cultures containing the phenylacetic acid. It seems likely that a difference in the stability of the various types of penicillin at the various pH levels in the culture medium would have as much effect on the assay ratio as might result from alteration in the proportion of types of penicillin actually produced.

Phenylacetic acid has been regarded as a "building block" in the synthesis of penicillin G. Thus phenylacetic acid would not be effective in the synthesis of the F and K types which do not contain the benzenoid ring structure. At the time these investigations were made, all differential assay ratios were interpreted in terms of penicillins F and G only. It is now generally known that penicillin X is present in fairly large amounts in surface cultures and to some extent in submerged cultures. Penicillin X gives a high ratio in the differential assay. Since no quantitative separations of the various types of penicillin were made during the course of these investigations, all deductions as to penicillins present were originally based on the differential assay (Schmidt *et al.*, 1945) with *S. aureus* and *B. subtilis*. No significant change in type G penicillin produced in culture could be directly attributed to phenylacetic acid with the possible exception of those cultures containing wheat bran. In both surface and submerged cultures there was a pronounced increase in the total penicillin yield due to the addition of phenylacetic acid to the culture medium. The failure, normally, to show a change in the proportions of the types of penicillin produced in the presence of phenylacetic acid may be due to the high concentrations of corn steep liquor employed, to certain inadequacies of the differential assay, and to possible differences in stability of the penicillins at various pH levels in the culture medium.

SUMMARY

Marked increases in total penicillin yield were obtained in surface cultures of *Penicillium notatum* NRRL 1249.B21 and in submerged cultures of *P. notatum* NRRL 832 and *Penicillium chrysogenum* 1951.B25 by the addition of phenylacetic acid.

The toxicity of phenylacetic acid was closely associated with the initial acidity of the culture medium. This toxicity could be sufficiently overcome by raising the pH prior to inoculation or by adding the phenylacetic acid during the fermentation after the pH of the medium has risen above the critical level.

Only in the presence of whole-wheat bran in submerged culture was it possible to obtain evidence that phenylacetic acid had an effect on the type of penicillin produced.

The optimum concentration of phenylacetic acid was not clearly determined, but it appears to be between 0.2 g and 0.8 g per liter of culture medium.

REFERENCES

Committee on Medical Research, Office of Scientific Research and Development, Washington; and the Medical Research Council, London. 1945 Chemistry of penicillin. Science, **102**, 627.

CRUESS, W. V., AND IRISH, J. H. 1932 Further observation on the relation of pH value to toxicity of preservatives to microorganisms. J. Bact., **23**, 163–166.

HUNTINGTON, G. IONE, AND RAHN, O. 1945 Acidity controlling antisepsis by weak acids. J. Bact., **49**, 655–659.

KIESEL, A. 1913 Recherches sur l'action de divers acides et sels acides sur le development de l'*Aspergillus niger*. Ann. inst. Pasteur, **27**, 391–420.

KIRBY, G. W., ATKINS, L., AND FREY, C. N. 1937 Growth of bread moulds as influenced by acidity. Cereal Chem., **14**, 865–878.

MOYER, A. J., AND COGHILL, R. D. 1946*a* Penicillin. VIII. Production of penicillin in surface cultures. J. Bact., **51**, 57–78.

MOYER, A. J., AND COGHILL, R. D. 1946*b* Penicillin. IX. The laboratory scale production of penicillin in submerged cultures by *Penicillium notatum* Westling (NRRL 832). J. Bact., **51**, 79–93.

RAPER, K. B., AND ALEXANDER, DOROTHY F. 1945 Penicillin. V. Mycological aspects of penicillin production. J. Elisha Mitchell Sci. Soc., **61**, 74–113.

SCHMIDT, W. H., AND MOYER, A. J. 1944 Penicillin. I. Methods of assay. J. Bact., **47**, 199–208.

SCHMIDT, W. H., WARD, G. E., AND COGHILL, R. D. 1945 Penicillin. VI. Effect of dissociation phases of *Bacillus subtilis* on penicillin assay. J. Bact., **49**, 411–412.