THE EFFECT OF CERTAIN CHEMICALS ON PENICILLIN PRODUCTION AND MOLD METABOLISM IN SHAKE FLASK FERMENTATIONS [1, 2]

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Any chemical compound capable of increasing penicillin formation by *Penicillium notatum* and *Penicillium chrysogenum* can serve at least two important functions. The first, obviously, is to give higher yields in the commercial production of penicillin. The second is to serve as a tool in the study of the mechanism of penicillin formation. That is, the metabolism of *Penicillium* grown on a medium which favors penicillin accumulation and that of *Penicillium* grown on the same medium with the added chemical stimulant can be compared. If certain physiological differences could be demonstrated between the "normal" and the "stimulated" mold, these differences might disclose information on the nature of penicillin formation.

A survey was undertaken on a series of chemical compounds, many of them respiratory inhibitors, to find substances that would increase penicillin yields. Only boron was studied to any extent as to its effect on the metabolism of the mold.

METHOD

The general procedure for producing penicillin by the shake flask method has been described elsewhere (Koffler, Emerson, Perlman, and Burris, 1945) and will not be repeated here.

To test whether a substance was capable of enhancing penicillin yields, the following technique was employed: Two series of 500-ml Erlenmeyer flasks containing 100 ml of one of the media given in table 1 were employed in each experiment. Both series of flasks received an inoculum of spores of *P. notatum* NRRL832 or *P. chrysogenum* NRRL1951–B25 at the outset of the experiment. Solutions or suspensions of chemicals, sterilized in an autoclave for 15 minutes at a pressure of 15 pounds, were added aseptically to one series of flasks (three or more flasks for every compound) to give the desired concentration. The flasks without added chemical served as controls. The shake flask fermentation proceeded thereafter in the usual manner. On the 6th, 7th, and 8th day—often also on the 5th day—aliquots of the fermentation liquor were taken aseptically from each individual flask and assayed for penicillin by the Oxford cup method

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with *Staphylococcus aureus* FDA209P as the test organism (Foster and Woodruff, 1944; Schmidt and Moyer, 1944). To test whether the chemical had an inhibiting effect on the assay organism, penicillin assays were also made on the sterile medium containing the chemical. In no case could such an inhibition of the test organism be observed. The pH of the broth was determined with a glass electrode.

According to observations in these laboratories, results obtained in shake flask fermentations are not always indicative of results that might be obtained in commercial tank fermentations. Since commercial tank fermentations are not practicable for routine surveys of this kind, reliance must be placed upon shake flask fermentations for the selection of stimulating substances, with the hope that experiments conducted in shake flasks may be reproducible in tanks.

In the metabolism studies reported in this paper, the analytical methods employed were those outlined by Koffler, Emerson, Perlman, and Burris (1945); the boron analyses were made by the quinalizarin procedure described by Berger and Truog (1944).

TABLE 1

Composition of media employed

Weights are expressed in g per L

REAGENT	MEDIUM A	MEDIUM B	MEDIUM C
$NaNO_3$	3.00	3.00	3.00
KH_2PO_4	0.50	0.50	0.50
$MgSO_4 \cdot 7H_2O$	0.25	0.25	0.25
$ZnSO_4$	0.025	0.00	0.00
Corn steep solids	20.00	40.00	30.00
Lactose	20.00	40.00	20.00

RESULTS

Chemical Compounds with Established Ability to Stimulate Higher Penicillin Yields

Among the 49 chemical compounds and mixtures of compounds investigated only boric acid and borax, which are equivalent in a solution of a given pH, and sodium citrate favored increased penicillin yields. Tables 2 and 3 present some typical protocols. Table 2 indicates that citric acid in 0.01 M, 0.001 M, and 0.0005 M concentration not only stimulated higher maximum yields, but usually also caused the maximum yields to be reached earlier than in the "normal" fermentation. A molar concentration of 0.0005 proved to be the most stimulatory of the levels tried. Table 3 presents some typical data on "boron" stimulation. A level of 0.2 per cent borax or an equivalent boric acid concentration of 0.13 per cent plus 0.04 per cent NaOH favored higher yields than a concentration of either 0.1 or 0.3 per cent borax. Boric acid, in addition to raising the value of the penicillin maximum, also caused an acceleration of the production of penicillin. The stimulatory action of sodium citrate could be reproduced consistently; the action of boron, however, was occasionally erratic.

TABLE 2

Penicillin production by P. notatum NRRL832 in medium B containing citric acid

Each figure is the average of the number of flasks indicated in the third column

CHEMICAL	FINAL CONCENTRATION OF CHEMICAL IN MOLES	NO. OF FLASKS	OXFORD UNITS PENICILLIN PER ML			
			Days 5	6	7	8
Sodium citrate	0.01	3	60	50	50	55
Control	0	3	42	50	47	44
Sodium citrate	0.001	3	44	66	76	79
Sodium citrate	0.0005	3	59	68	91	55
Control	0	3	36	41	60	69
Sodium citrate	0.001	5	37	61	43	25
Control	0	10	36	43	29	28

TABLE 3

Penicillin production by P. chrysogenum NRRL1951-B25 in medium B containing borax, sodium hydroxide, or boric acid

Each figure is the average of four flasks

EXP. NO.	CHEMICAL	PER CENT ADDED	INITIAL pH	pH			OXFORD UNITS PENICILLIN PER ML		
				Days 6	7	8	Days 6	7	8
I	Control	0.00	4.21	7.24	7.49	7.89	80	75	85
	Borax	0.10	4.26	7.25	7.51	7.88	84	101	101
		0.20	4.40	7.33	7.65	7.98	109	103	120
	NaOH	0.021	4.25	7.39	7.49	7.91	75	74	78
		0.042	4.35	7.40	7.67	7.95	78	81	82
	Boric acid	0.065	4.14	7.26	7.58	7.96	88	105	90
		0.130	4.11	7.07	7.47	7.87	100	102	97
	Boric acid plus NaOH	0.065 + 0.021	4.20	7.20	7.55	7.82	107	114	98
	Boric acid plus NaOH	0.130 + 0.042	4.36	7.35	7.57	7.95	99	132	105
II	Control	0.00	4.20	4.52	4.80	4.60	61	80	83
	Borax	0.10	4.26	4.84	4.80	4.70	60	82	105
	Borax	0.20	4.40	4.83	4.78	4.68	69	98	125
	Borax	0.30	4.54	4.80	4.76	4.58	72	98	109

Experiment II, table 4, illustrates the exception to the general effect of borax indicated in experiment I. The experiments were run at different times; media of the same composition and identical conditions were employed in each case. The controls of experiment I gave penicillin levels which were usual for *P. chrysogenum* NRRL1951–B25 grown on medium B. The stimulation in yields obtained through the addition of either 0.1 or 0.2 per cent borax was typical. The normal fermentation of experiment II, however, produced penicillin in amounts which were higher than average; when borax was added to this fermentation, maximum penicillin levels were practically the same in the borax-treated and in the normal fermentation. Variations of penicillin yields in replicate experiments are commonly observed by workers engaged in penicillin research; periods in which consistently high penicillin yields are obtained alternate with those in which penicillin yields are below average. The cause of these variations has not yet been determined but cannot be ascribed to experimental error in sampling or assaying. The experiments just described indicate

TABLE 4

The effect of borax on penicillin production by P. chrysogenum NRRL1951-B25 in medium B

Each figure is the average of three flasks

EXP. NO.	CHEMICAL	PER CENT ADDED	OXFORD UNITS PENICILLIN PER ML		
			Days		
			6	7	8
I	Control	0	63	71	63
	Borax	0.1	62	73	70
	Borax	0.2	61	67	83
II	Control	0	77	98	99
	Borax	0.1	86	92	106
	Borax	0.2	71	82	102

such a variation and also make clear that the stimulation of borax is marked in fermentations which give low or average penicillin yields and absent in fermentations in which much penicillin is produced. However, the experiments illustrate extremes. In general, the following relationship holds: Borax can stimulate yields beyond any control yields; however, in cases in which stimulation of penicillin production by borax cannot be observed, control yields are almost always high. As will be pointed out, this relationship also holds for other compounds. These observations explain why the term "stimulation" must be used with reservation; actually, the "stimulatory" substance might not "stimulate" the "normal" fermentation but bring the penicillin level of the "subnormal" fermentation up to "normal."

Table 5 exemplifies another interesting observation, namely, that borax stimulated penicillin formation by *P. chrysogenum* NRRL1951–B25, but failed to increase the penicillin levels obtained from *P. notatum* NRRL832; in fact, 0.2 per cent borax caused a decrease in penicillin yields. Similarly, citric acid in molar concentration of 0.01, 0.001, and 0.0005 gave only slight stimulation

to penicillin production by strains R-38 and NRRL1951–B25, whereas they increased the penicillin yields with strain NRRL832. This would indicate more intrinsic metabolic differences between these molds than those described previously (Koffler, Emerson, Perlman, and Burris, 1945).

An attempt was made to elucidate the question of whether boron or trace elements were responsible for the stimulation of penicillin yields, or of whether a pH effect was the cause of the enhanced penicillin level. The addition of borax raised the initial pH of the medium (table 3). However, when NaOH was used to increase the initial pH to similar values, no stimulatory effect could be observed. Solutions of boric acid, containing boron levels equivalent to those of the corresponding borax solution, when added to medium B lowered the pH somewhat, but had a stimulatory power which was only slightly lower than that of borax. When the initial pH of the medium treated with boric

TABLE 5

Comparison of yields with P. chrysogenum NRRL1951-B25 and P. notatum NRRL832 in medium B containing borax

Each figure is the average of two flasks

PER CENT OF BORAX ADDED	STRAIN OF MOLD	OXFORD UNITS PENICILLIN PER ML					
		Days					
		3	4	5	6	7	8
0.0	832	12	31	66	74	89	97
	1951-B25	6	26	49	58	59	60
0.01	832	10	26	52	77	80	87
	1951-B25	4	23	55	92	85	86
0.1	832	9	18	47	73	78	95
	1951-B25	7	42	66	110	122	131
0.2	832	11	16	29	54	67	56
	1951-B25	6	31	56	76	102	140

acid was adjusted with NaOH to the same pH as the one obtained in the borax-treated medium, the stimulation of boric acid was greater than that of borax. These data seem to indicate that either boron, or trace elements contained in boric acid and borax, act as agents which stimulate increased penicillin production. The compounds employed were U.S.P. reagents; the borax used, according to information kindly furnished by the Pacific Coast Borax Company, Los Angeles, contained the following impurities: Cl, 0.04 per cent; SO_3, 0.04 per cent; CO_2, 0.06 per cent; Fe, 7 ppm; As_2O_3, 16 ppm. These assays represent maximum values. Even if it is assumed that there were other trace elements in the salt which escaped analysis, it seems very unlikely that they were responsible for the stimulation of penicillin yields because of their low concentration in the medium. In fact, it is doubtful whether U.S.P. reagents would furnish more trace elements to the medium than do the corn steep solids. Thus, there

is every indication that the stimulatory effect of borax or boric acid is specifically an effect of boron.

Chemical Compounds with Doubtful Ability to Stimulate Higher Penicillin Yields

In addition to the compounds discussed, other substances were observed to be capable of raising penicillin levels. However, their stimulatory effect could not always be proved. In some runs, especially when control runs were low, they would stimulate yields; in other cases, especially when control runs were high, they would even depress yields. This might be indicative of conditions fairly similar to those existing when the addition of borax did not result in the expected higher penicillin yields. In some cases, the compounds were not studied sufficiently to claim stimulatory abilities for them.

The following compounds tested in the concentrations given fall in the group of doubtful stimulants: a mixture of 0.0005 M sodium citrate, 0.0005 M sodium formate, and 0.0005 M sodium glycolate; a mixture of 10 ppm choline and 0.001 M sodium taurocholate; 0.001 M phloridzin; a mixture of 0.001 M sodium sulfide[3] and 0.001 M 2,4-dinitrosoresorcinol; 10 ppm choline; 0.01 M phenylacetic acid; 0.01 M malonic acid; 0.01 and 0.001 M sodium taurocholate; 0.01 M sodium glycolate; 0.001 M 2,4-dinitrosoresorcinol; 0.01, 0.002, 0.001, and 0.0005 M resorcinol; 0.002 M furfural; 0.002 M sodium fluoride.

Chemical Compounds with No Ability to Stimulate Higher Penicillin Yields

The majority of compounds tested caused no enhanced penicillin accumulation; many of them had a depressing effect on penicillin yields. A list of these compounds is given below. The following were used in 0.01 M and 0.001 M concentrations: 1-amino-8-naphthol-4-sulfonic acid, sodium azide, iodoacetic acid, hydroxylamine hydrochloride, *m*-nitrophenol, strychnine nitrate, *p*-aminophenol, 8-hydroxyquinoline, diphenylacetic acid, phloroglucinol, naphthol, catechol, 1-amino-2-naphthol-4-sulfonic acid, orthotolidine, ethyl carbamate, tartaric acid, sodium acetate, glycine, 2,4-nitrophenylhydrazine, and brucine. The following were used in the concentrations indicated: sodium pyrophosphate, 0.05 M, 0.02 M; ethyl-*n*-phenyl carbamate, 0.02 M, 0.01 M; diglycolic acid, 0.02 M, 0.01 M; 2,4-dinitrophenol, 0.001 M, 0.0001 M; sodium arsenite, 0.01 M, 0.002 M; choline, 20 ppm, 100 ppm; maleic acid, 0.01 M; picric acid, 0.01 M; sodium sulfite, 0.01 M; phenol, 0.001 M; oxalic acid, 0.01 M; a mixture of 0.01 M sodium formate and 0.01 M sodium citrate; a mixture of 0.001 M sodium formate and 0.001 M sodium citrate; a mixture of 0.0005 M sodium formate and 0.0005 M sodium citrate.

The Effect of Boron on the Metabolism of P. chrysogenum NRRL1951–B25

Since borax and boric acid enhanced penicillin yields so conspicuously, experiments were designed to study the effect of boric acid on mold metabolism. Some information has been obtained by these laboratories on the chemical changes in submerged penicillin fermentations. The approach described by

[3] This salt was about two years old and unquestionably contained some polysulfides.

Koffler, Emerson, Perlman, and Burris (1945) was employed in this investigation. Shake flask fermentations of *P. chrysogenum* NRRL1951–B25 were carried out on medium B. One series of normal shake flask fermentations was set up; to the other series 0.13 per cent sterile boric acid was added before inoculation with spores.

Tables 6 and 7 present the data on the chemical changes occurring in the normal and the boron-treated fermentations. The pH values were practically identical in both media. The maximum penicillin yield of the boron medium was considerably higher and was reached one day later than that of the normal medium. The stimulation of penicillin production by the boron occurred characteristically after the control had had a head start.

The oxygen uptake of the boron-fed mold expressed on a dry weight or ml basis was greater on the 2nd, 3rd, and 4th day than that of the normal mold.

TABLE 6

Chemical changes induced by P. chrysogenum NRRL1951-B25 on a 4 per cent lactose, 4 per cent corn steep medium

ANALYSIS	DAYS								
	0	1	2	3	4	5	6	7	8
Penicillin (Oxford units)				27	45	52	72	63	49
pH	4.10	4.17	6.48	6.98	6.83	7.55	7.85	8.09	8.23
QO_2			17.4	29.0	8.8	4.8	3.0	1.8	
QO_2(N)			219.9	386.0	135.8	81.4	54.3	35.3	
QO_2 (ml)			130.5	342.0	97.6	56.6	35.1	17.4	
Lactose*	2,000		1,750		75		75	25	
Dry weight†			0.75	1.18	1.19	1.18	1.17	0.97	0.89
Total soluble N*	155.0	124.7	77.8	57.6	58.4	65.4	87.4	78.9	72.9
Nonammonia Kjeldahl N*	144.5	104.2	30.6	26.9	47.2	40.1	53.2	35.8	24.3
Ammonia N*	10.5	20.5	47.2	20.7	11.2	25.3	34.2	43.1	48.6
Per cent N in mycelium			7.92	7.51	6.48	5.90	5.53	5.10	4.32
Boron*	0.08	0.06	0.08	0.06	0.04	0.01	0.01	0.01	0.02

* mg per 100 ml.

† g per 100 ml.

This difference became especially marked when the oxygen uptake was expressed on the basis of nitrogen, i.e., active protoplasm. These QO_2 (N) (μl O_2 uptake per hr per mg N content of suspension) values were higher for the boron-fed mold throughout the entire fermentation, but more markedly so on the 2nd, 3rd, and 4th day. The more rapid lactose utilization by the boron-fed mold in the early stages of the fermentation occurred with a higher uptake of oxygen. There was a higher residual lactose concentration after the 4th day in the boron medium than in the normal medium. The mycelial weight of the control was somewhat higher than that of the boron-fed mold through most of the run, but the maximum values were equal.

The nonammonia Kjeldahl nitrogen was utilized more rapidly on the first day by the control than by the boron-fed mold; later the levels were about equal.

The lowest total soluble nitrogen content was reached near the 3rd and 4th days; this agrees closely with the data on the highest mycelial growth which was obtained near that time. However, while mycelial weights in both fermentations stayed relatively constant from the 3rd to the 6th day, the total soluble nitrogen increased, indicating that some of the nitrogenous constituents were autolyzed and replaced by nonnitrogenous constituents. This is in agreement with the percentage nitrogen values of the mycelium; the nitrogen content of the mycelium decreased at a nearly linear rate. The percentage of nitrogen content in the control mycelium was higher than that in the boron-fed mold. The ammonia levels showed a maximum at the 2nd day, a minimum on the 4th day, and another rise thereafter, which continued to the end of the fermentation.

TABLE 7

Chemical changes induced by P. chrysogenum NRRL1951-B25 on medium A containing 0.2 per cent borax

Each figure is the average of three flasks

ANALYSIS	DAYS								
	0	1	2	3	4	5	6	7	8
Penicillin (Oxford units)				23	44	62	87	95	71
pH	4.10	4.18	6.66	7.00	6.88	7.57	7.86	8.10	8.25
QO_2			21.0	31.0	13.8	4.8	3.2	1.9	
QO_2 (N)			276.0	441.0	237.0	91.4	65.2	46.6	
QO_2 (ml)			161.7	362.8	144.9	49.4	32.7	18.2	
Lactose*	2,000		1,400		175		100	25	<25
Dry weight†			0.77	1.17	1.05	1.03	1.02	0.96	0.81
Total soluble N*	155.0	144.3	78.8	61.7	60.5	68.0	88.4	85.4	73.0
Nonammonia Kjeldahl N*	144.5	132.1	37.0	40.6	46.5	48.0	59.5	55.2	42.0
Ammonia N*	10.5	12.2	41.8	21.1	14.0	20.0	28.9	30.2	31.0
Per cent N in mycelium			7.60	7.03	5.83	5.25	4.90	4.08	3.98
Boron*	22.0	22.0	21.0	18.0	16.0	9.5	12.0	15.0	20.0

* mg per 100 ml.

† g per 100 ml.

These changes are typical. Although the slopes were similar in both fermentations, there were some differences in detail. The ammonia content of the normal medium was higher than that of the boron medium except on the 4th day. This was especially marked during active penicillin production and thereafter. Whereas the ammonia concentration in the control rose relatively rapidly and constantly after the 4th day, the ammonium slope in the boron medium rose less rapidly and flattened out after the 6th day. This experiment corroborates earlier observations on the interrelationship between ammonia levels and penicillin yields.

In both fermentations the boron in solution decreased until the 5th day, after which it increased. These changes were large in the boron medium and insignificantly small in the normal medium. The uptake of boron by the mold

is of significance in the antiseptic use of borax and boric acid in the production of penicillin; the effectiveness of these boron compounds in counteracting bacterial contaminants is described by Knight and Frazier (1945). At times during the fermentation, boron levels are reached in the medium which are much less antiseptic than the original levels. Growth of contaminants is more likely to occur at such times.

Since the metabolism of the normal and boron-fed mold differed in certain aspects, it seemed appropriate to study these differences on another medium. As previously, a normal series and a boron-treated series of shake flask cultures were set up on medium C. The conclusions to be drawn from this experiment are the same as the ones described earlier with the following exceptions: The pH of the boron medium was somewhat lower than that of the control on the 7th and 8th day (8.02 and 8.20 for the control as compared with 7.84 and 8.08 for the boron-fed mold), probably because of the lower ammonia content of

TABLE 8

The relationship between ammonia levels and penicillin yields

Each figure is the average of three flasks

ANALYSIS	DAYS								
	0	1	2	3	4	5	6	7	8
I Penicillin*.....				20	46	70	75	67	66
Ammonia N†.....	24.1	21.7	43.0	26.1	33.5	37.8	45.2	62.0	70.6
II Penicillin.....				14	43	63	90	103	105
Ammonia N.....	24.1	24.7	21.9	46.8	36.2	42.2	34.2	38.7	47.2

Shake flask cultures of *P. chrysogenum* NRRL1951-B25 were grown on medium C (I) and on medium C containing 0.2 per cent borax (II).

* Oxford units per ml.

† mg per 100 ml.

the boron-treated fermentation (see below). The penicillin yields of the boron fermentation were higher than those of the previous experiment (compare tables 6, 7, and 8) and did not fall off so rapidly. The fact that the penicillin level was still increasing on the 8th day in the case of the boron-fed mold was probably due to the lower pH. Less lactose was utilized in this experiment than in the last, very likely because of the higher corn steep liquor concentrations employed. The mycelial weights in this experiment were considerably higher than those of the previous experiment (maximum weight 1.91 g per 100 ml on the 8th day for the control and 1.79 g per 100 ml for the boron-treated mold). The differences in weight between the control and boron-fed mold were also more marked, the control weighing consistently more except on the 7th and 8th day when autolysis was much more conspicuous in the control than in the boron fermentation.

Table 8 summarizes the information on ammonia changes. The ammonia concentrations of the control showed a characteristic curve with 2 maxima; the ammonia levels of the boron fermentation, however, gave a rather unusual

curve with 3 peaks. The ammonia levels in both the control and the boron fermentation were considerably higher in this experiment than in the last. Nevertheless, the penicillin yields were better in this experiment. One could surmise on the basis of these data that the absolute level of ammonia does not control penicillin levels. However, the relatively high level of ammonia in the boron-free medium associated with low yields again shows a relationship between ammonia and penicillin yield.

The fact that different penicillin levels, as obtained by the addition or absence of a stimulatory substance, can be associated with definite metabolic changes indicates that such a substance can be advantageously used for further studies on the metabolism of *Penicillium*.

SUMMARY

Of 49 chemical compounds and mixtures tested for their ability to raise penicillin levels in shake flask cultures, only citric acid and borax or boric acid proved to be stimulatory. The degree of stimulation varied with strains. Citric acid showed an optimum stimulatory action at a concentration of 0.0005 M with strain NRRL832, but not with strains R-38 and NRRL1951–B25. Borax and boric acid were most stimulatory at a level of 0.2 per cent and 0.13 per cent, respectively, with strain NRRL1951–B25, but not with strain NRRL832. The stimulatory effect of borax and boric acid was indicated as due to the boron and was not attributable to a pH effect or to impurities of the reagents. There was a general tendency for borax and for compounds with doubtful stimulatory power to stimulate runs with low-yielding but not with high-yielding control fermentations. A list of chemicals that might stimulate penicillin production but whose action was irregular was given; compounds lacking in stimulatory power or possessing depressing powers were also enumerated.

The effect of boric acid on the metabolism of *Penicillium chrysogenum* NRRL-1951–B25 was studied. The boron-fed mold differed from the control in these aspects: it utilized lactose more rapidly, had a higher rate of respiration and nitrogen utilization, had a less abundant mycelium which contained less nitrogen, and had an internal balance which was reflected outside the organism in lower ammonia levels and higher penicillin yields.

This investigation pointed out that stimulatory substances might be of importance commercially, and also indicated how these compounds could be used as tools in the study of the mechanism of penicillin production.

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