THE EFFECT OF CERTAIN MINERAL ELEMENTS ON THE PRODUCTION OF PENICILLIN IN SHAKE FLASKS[1, 2]

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In earlier publications from these laboratories (Knight and Frazier, 1945*b*; Koffler, Knight, Frazier, and Burris, 1946) attention was directed to the fact that the addition of corn steep ash to certain synthetic media significantly increased the production of penicillin as well as the rate of other metabolic reactions. Although the importance of mineral elements in the production of penicillin by submerged mold cultures has been emphasized by Foster, Woodruff, and McDaniel (1946), Moyer and Coghill (1946), and Pratt and Hok (1946), no suggestions were offered as to which constituents of the corn steep ash were responsible for the higher penicillin levels in synthetic media. This paper attempts to answer that question.

METHODS

Penicillin was produced by submerged cultures of *Penicillium chrysogenum* X-1612 (agitated in a reciprocating shaker) in the manner outlined by Koffler, Emerson, Perlman, and Burris (1945). The following basal medium was used:

Lactose, U.S.P.	25.00 g
Starch	5.00 g
Dextrin, N.F.V.	5.00 g
Glacial acetic acid, cp	6.00 g
$(NH_4)_2SO_4$, reagent	5.00 g
KH_2PO_4, reagent	1.50 g
$MgSO_4 \cdot 7H_2O$, U.S.P.	0.25 g
$ZnSO_4 \cdot 7H_2O$, reagent	0.04 g

Distilled water to 1 L
pH adjusted to 6.3 with KOH before autoclaving
Supplements made and constituents removed, as indicated in the text, before autoclaving

This basal medium was only slightly different from the one used previously (Knight and Frazier, 1945*b*) and without supplements permitted excellent growth of the mold but no appreciable penicillin yields. Because this suggested that metallic contaminants, if they occurred at all, were not present in high enough concentration to affect penicillin production, it did not seem necessary to purify

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the medium. The medium used for the growth of the spores has been described by Knight and Frazier (1945*a*).

Corn steep ash was obtained by heating corn steep solids (University of Wisconsin no. 71, purchased on July 20, 1945, from the Staley Company, Decatur, Illinois) in an electric furnace at 760 C for 4 to 5 hours. No attempt was made to dissolve the ash before it was added to the medium.

Penicillin assays were made by the Oxford cup method with *Staphylococcus aureus* FDA209P as the test organism (Foster and Woodruff, 1944; Schmidt and Moyer, 1944). A penicillin preparation from the Food and Drug Administration served as the reference standard. The method of Fiske and SubbaRow (1925) was used for the determination of phosphorus, and the method of Saywell and Cunningham (1937) was used for the determination of iron.

EXPERIMENTAL

Subsequent to the demonstration by Knight and Frazier (1945*b*) that the addition of corn steep ash to a synthetic medium increased penicillin yields, experiments were designed to determine which ash constituent or combination of constituents was responsible for this stimulation. The general procedure was to study the chemical composition of the ash and then observe the effect of various ash components, added both singly and in pairs, on the accumulation of penicillin in the basal medium.

The chemical composition of the corn steep ash. Knight and Frazier (1945*b*) found that 500 mg of corn steep ash was required for maximum penicillin production in 100 ml of synthetic medium.[3] The following method was used to prepare soluble and insoluble fractions of the whole ash and to determine their effect on penicillin yields: Simulating the conditions during the preparation of the fermentation medium, 500 mg of ash were put into 100 ml of water, and the pH was adjusted to 6.3 with glacial acetic acid. After autoclaving for 15 minutes at 15 pounds, 213 mg (42.6 per cent) of ash were in solution while 287 mg (57.4 per cent) remained as granular sediment on the bottom of the flask.

Table 1 indicates that the ability of the ash to stimulate the biosynthesis of penicillin resided almost entirely in its insoluble fraction. The soluble portion was slightly stimulatory, but both fractions were necessary for maximum penicillin yields.

Table 2 presents a qualitative spectrographic analysis of the whole ash, and of its soluble and insoluble fractions.[4] The following metals have not been detected: Sb, Be, Bi, Cd, Cb, Ge, Au, La, Hg, Pt, Sr, Ta, Ti, V, and Zr.

The stimulatory action of compounds, added singly and in combinations, on penicillin production in the basal medium. Salts of all the elements listed in table 2, with the exception of Si, were added in various amounts to the basal medium, but none increased penicillin yields as markedly as did corn steep ash. Details of some of these experiments were presented by Knight (1946).

[3] This was approximately equivalent to the amount of ash in 3 per cent corn steep solids.

[4] We are grateful to Dr. Y. SubbaRow of the Lederle Laboratories and Mr. W. L. Dutton of the American Cyanamid Company for furnishing us with this analysis.

Since preliminary experiments indicated that combinations of Fe salts and phosphates enhanced penicillin yields as greatly as did corn steep ash, quantitative analyses for Fe and P were made on the whole ash and its two fractions. It was

TABLE 1

The effect of corn steep ash and of two ash fractions on the production of penicillin by Penicillium chrysogenum X-1612 in the basal medium

(Each figure is the average of three experiments)

BASAL MEDIUM PLUS (MG/100 ML)			PENICILLIN (OXFORD UNITS/ML)				
			Days				
Whole ash*	Soluble ash	Insoluble ash	4	5	6	7	8
			18	29	39	35	9
500			88	110	151	132	127
	213		32	48	57	75	62
		287	69	100	141	121	124
	213	287	91	106	152	118	125

* Five hundred mg of whole ash contained 213 mg of soluble constituents and 287 mg of insoluble constituents.

TABLE 2

Spectrographic analysis of corn steep ash and of two ash fractions

The ranges for qualitative estimates are as follows:

8 is 100 to 1%
7 is 10 to 0.1%
6 is 1.0 to 0.01%
5 is 0.1 to 0.001%
4 is 100 to 1.0 ppm
3 is 10 to 0.10 ppm
2 is 1.0 to 0.01 ppm
1 is less than 0.1 ppm
x is metal not detected
A is whole ash
B is soluble ash
C is insoluble ash

ELEMENT	A	B	C	ELEMENT	A	B	C
Al	3	3	2	Mg	8	4	8
As	4	4	4	Mn	6	4	6
B	6	5	6	Ni	4	3	4
Ca	7	7	7	P	8	5	8
Cr	6	3	6	K	8	8	7
Co	2	x	2	Si	7	7–	6
Cu	6	4	6	Ag	1	1	1
Fe	7	7	6	Sn	2	2	2
Pb	5	4	4	W	5	5	5
Li	2	2	1	Zn	5	2	4

found that the whole ash, the soluble ash, and the insoluble ash contained P in concentrations of 12.9 per cent, 5.3 per cent, and 18.3 per cent, respectively, and Fe in concentrations of 0.27 per cent, 0.01 per cent, and 0.47 per cent, respectively.

Table 3 gives the penicillin yields when the basal medium was supplemented with either corn steep ash, or Fe and P in concentrations in which these elements occur in corn steep ash. The insoluble P was added arbitrarily as $Ca_3(PO_4)_2$ (reagent grade), and the soluble phosphates were added as KH_2PO_4 (reagent grade). The stimulatory effect of Fe salts did not depend upon their relative solubility (for example, $Fe_2(SO_4)_3$ showed the same effect as an equivalent amount of $FePO_4 \cdot 2H_2O$), and Fe was usually supplied as $Fe_2(SO_4)_3$, although in the ash Fe was present almost entirely in an insoluble form.

Table 3 reveals that the addition of both Fe salts and phosphates to the basal medium raised penicillin yields as markedly as did the addition of corn steep ash. Fe was highly stimulatory by itself, but not to the same degree as was

TABLE 3

The effect of corn steep ash, soluble P, insoluble P, and Fe on the production of penicillin by Penicillium chrysogenum X-1612 in the basal medium

(Each figure is the average of three experiments)

BASAL MEDIUM PLUS (MG/100 ML)				PENICILLIN (OXFORD UNITS/ML)				
				Days				
Ash	Sol. P*	Insol. P†	Fe‡	4	5	6	7	8
				18	29	39	35	9
500				88	110	151	132	127
		50		17	23	32	37	32
	10			22	36	41	32	27
			1.4	75	101	118	138	129
	10	50		19	35	52	55	53
		50	1.4	62	105	138	112	119
	10		1.4	73	108	149	125	124
	10	50	1.4	71	102	142	115	120

The Fe and P content of 287 mg insoluble ash was 1.35 mg and 52.5 mg, respectively, and of 213 mg soluble ash, 0.02 mg and 11.3 mg, respectively.

* As KH_2PO_4.

† As $Ca_3(PO_4)_2$.

‡ As $Fe_2(SO_4)_3$.

whole steep ash; in the absence of Fe, phosphates showed scarcely any effect on penicillin production. Media containing both Fe and soluble phosphates supported penicillin production as well as did media with corn steep ash.

Table 4 corroborates the contention that the physiological function of corn steep ash can be attributed mainly to Fe and soluble phosphates. Insoluble ash (rich in Fe and insoluble phosphates but free from soluble phosphates) plus soluble phosphates enhanced penicillin production as greatly as did whole ash. The addition of soluble corn steep ash (rich in soluble phosphates but containing merely traces of Fe) caused maximum penicillin yields only when Fe was present in the medium.

The fact that corn steep ash contains a large number of constituents suggested that good penicillin yields might also be obtained in a simplified synthetic medium consisting of lactose, starch, dextrin, glacial acetic acid, $(NH_4)_2SO_4$, and ashed

corn steep. Experiments indicated that this assumption was justified. Corn steep ash—and any contaminants which might have been present as traces in the other components of the medium—satisfactorily served as a source of the mineral elements essential to maximum penicillin formation.

The importance of ion antagonism in the production of penicillin. It was observed that the addition of 0.2 mg Cu to 100 ml of the basal medium completely inhibited penicillin production without visibly affecting the growth of the mold. The addition of only 0.1 mg of Fe to such a fermentation minimized the "toxic" effect of Cu, and higher amounts of Fe neutralized the inhibition completely. Table 5, which contains the results of a few typical experiments, strikingly illustrates this relationship.

TABLE 4

The effect of soluble and insoluble corn steep ash on the production of penicillin by Penicillium chrysogenum X-1612 in the basal medium which was also supplemented with Fe or phosphates, or both

(Each figure is the average of three experiments)

BASAL MEDIUM PLUS (MG/FLASK)					PENICILLIN (OXFORD UNITS/ML)				
					Days				
Sol. ash	Sol. P*	Insol. ash.	Insol. P†	Fe‡	4	5	6	7	8
	10	287			92	112	147	128	118
213				1.4	75	105	145	126	112
213			50		23	41	55	62	52
213			50	1.4	78	102	143	134	128

* As KH_2PO_4.
† As $Ca_3(PO_4)_2$.
‡ As $Fe_2(SO_4)_3$.

The question arose whether this "antagonism," which found its expression in *yields* of penicillin, affected the *biosynthesis* or merely the *stability* of penicillin. The effect of Fe or Cu, or both, on the stability of penicillin was observed as follows: Enough commercial penicillin (Pfizer)[5] was dissolved in a K_2HPO_4—KH_2PO_4 buffer of the desired pH (5.8, 6.4, 7.0, and 7.6) to give a concentration of approximately 120 Oxford units per ml. One-hundred-ml portions of this solution were placed in 500-ml Erlenmeyer flasks, supplemented as indicated in table 6, and agitated by a reciprocating shaker under the same conditions as were shake flask cultures. The penicillin content of the solution was assayed at the start and after 8, 24, and 48 hours. Table 6 demonstrates that Cu decreased the stability of penicillin solutions; at pH 5.8 Fe appeared destructive. At pH values of 6.4, 7.0, and 7.6 the action of Cu was offset by Fe.

From these experiments it would appear as if the association between penicillin yields and Fe-Cu antagonism could be explained more satisfactorily by strictly chemical (i.e., penicillin destruction) than physiological (i.e., penicillin synthesis) reactions. Such an interpretation, however, would be based on the assumption

[5] Approximately 75 per cent of this sample consisted of penicillin G and 25 per cent was penicillin K. We are indebted to Mr. K. Higuchi for doing the differential assay.

that penicillin responds to the action of Fe and Cu in actual fermentation liquors as it did in phosphate buffer. This was tested in the following manner: *P. chrysogenum* X-1612 was grown in shake flasks on the basal medium (containing no added Fe or Cu) for 4 days; then the liquor was freed of pellets and sterilized

TABLE 5

The effect of Fe and Cu on the production of penicillin by Penicillium chrysogenum X-1612 in the basal medium

(Each figure is the average of three experiments)

BASAL MEDIUM PLUS (MG/100 ML)		PENICILLIN (OXFORD UNITS/ML)			
		Days			
Fe ($Fe_2(SO_4)_3$)	Cu ($CuSO_4 \cdot 7H_2O$)	4	5	6	7
0	0	40	23	16	10
0	0.01	43	21	16	8
0	0.05	30	15	5	0
0	0.2	0	0	0	0
0	1.0	0	0	0	0
0.1	0	81	89	94	90
0.5	0	85	97	123	118
2.0	0	88	100	122	119
10.0	0	83	90	103	109
0.1	0.01	83	90	92	91
0.5	0.01	84	99	125	115
2.0	0.01	83	96	121	109
10.0	0.01	83	86	97	110
0.1	0.05	79	69	58	42
0.5	0.05	73	96	119	121
2.0	0.05	80	95	121	123
10.0	0.05	83	89	108	118
0.1	0.2	12	13	15	0
0.5	0.2	63	42	36	28
2.0	0.2	89	108	132	121
10.0	0.2	76	95	109	115
0.1	1.0	0	0	0	0
0.5	1.0	28	32	84	96
2.0	1.0	80	88	92	90
10.0	1.0	79	102	113	123

by filtration through a Berkefeld filter. Commercial penicillin was dissolved in this sterile solution to give a concentration of 140 Oxford units per ml. After the addition of Fe or Cu, or both, aliquots were shaken as they had been in the previous experiment. Penicillin assays were done at the start, and after 8, 24, 48, and 76 hours. Table 7 shows that in the presence of organic matter, at pH

7.0, neither Cu nor Fe affected the stability of commercial penicillin. This would justify the assumption that the interaction between Fe and Cu was more likely connected with the formation than with the destruction of penicillin.

TABLE 6

The effect of Fe and Cu on the stability of penicillin in phosphate buffer at various hydrogen ion concentrations and 24 C

pH	BUFFER PLUS (MG/100 ML)		PENICILLIN (OXFORD UNITS/ML)			
			Hours			
	Fe ($Fe_2(SO_4)_3$)	Cu ($CuSO_4 \cdot 5H_2O$)	0	8	24	48
5.8			118	113	90	99
	10.0		118	96	45	26
		0.5	118	66	35	0
	10.0	0.5	118	67	20	0
6.3			120	123	86	88
	10.0		120	130	100	118
		0.5	120	56	20	15
	10.0	0.5	120	113	75	83
7.0			124	118	93	98
	10.0		124	118	96	90
		0.5	124	53	27	12
	10.0	0.5	124	123	75	79
7.6			120	103	95	81
	10.0		120	106	90	100
		0.5	120	45	0	0
	10.0	0.5	120	110	75	70

TABLE 7

The effect of Fe and Cu on the stability of penicillin in the fermentation liquor at pH 7 and 24 C

FERMENTATION LIQUOR PLUS (MG/100 ML)		PENICILLIN (OXFORD UNITS/ML)				
		Hours				
Fe ($Fe_2(SO_4)_3$)	Cu ($CuSO_4 \cdot 5H_2O$)	0	8	24	48	72
		144	144	140	142	132
	1.5	140	140	140	138	134
30.0		140	138	134	148	134
30.0	1.5	144	138	150	140	130

DISCUSSION

This paper has attempted to show that the increase in penicillin yields which was observed when corn steep ash was added to a basal medium was actually due to the Fe and phosphates in the ash. Considering the great number of minerals in the ash, the difficulty of such an attempt is apparent. Combinations of

elements other than Fe and P might eventually be shown to be as stimulatory as was corn steep ash. In fact, unpublished experiments indicate that combinations of Fe and Cr raised penicillin yields almost as much as did Fe and phosphates. Pratt and Dufrenoy (1945) must have noted the stimulating effect of Cr because they included this element in their synthetic medium.

Since media low in Fe never gave high penicillin yields, Fe can be regarded as the most indispensable constituent of corn steep ash. To obtain maximum penicillin production, however, the addition of both Fe and soluble phosphates was prerequisite. It should be noted that the level of Fe and soluble phosphates necessary for maximum penicillin production was greater than the level for maximum growth of the mold.

The function of Fe and P in the production of penicillin is unknown. These elements may either affect the formation or the stability of penicillin. Many enzymes carry Fe in their prosthetic groups, and such enzymes may be involved in the biosynthesis of penicillin. On the other hand, Fe may appear necessary in penicillin production because it protects penicillin from the destruction catalyzed by other elements. Hutner (1946) holds the general view that many elements are considered essential nutrients because they form precipitates in dilute media and thereby remove toxic elements by precipitation or adsorption. However, there is no reason to assume that Fe affected penicillin production in this manner because the basal medium that was employed in these studies already contained high levels of starch and dextrin, which are able to act as adsorbents.

It is even more difficult to explain the role of soluble phosphates, since KH_2PO_4 was present in the medium and increments of P neither caused additional mold growth nor changed the pH picture of the fermentation. Pulvertaft and Yudkin (1945) found it possible to stabilize penicillin solutions by the addition of phosphates, a protection which was shown not to be due to an effect of pH. The degree of stabilization depended upon the sample of penicillin, the concentration of penicillin in the solution, and the concentration of phosphate. Different samples differed in the amount of phosphate that gave maximal protection. The enhancement of penicillin yields by soluble phosphates in the presence of Fe might be explained, at least tentatively, in the light of Pulvertaft and Yudkin's experiments.

The observation that Cu impaired penicillin production but that Fe was able to offset this damage may be of practical as well as theoretical importance. Industrially, corn steeps with unusually high contents of Cu might be improved by supplements of Fe. Theoretically, a study of the counteraction between Fe and Cu might help to elucidate the mechanism of penicillin synthesis. Of course, such a study would be valuable only if Cu actually interfered with penicillin formation and not with the stability of penicillin already synthesized. The fact that Cu did not destroy commercial penicillin that had been added to fermentation liquors suggests that the interplay between Fe and Cu influenced more fundamental reactions than the destruction of penicillin.

It is probable that antagonistic relationships between elements other than Fe and Cu will be demonstrated. An incomplete survey made in this laboratory

has furnished evidence that Fe can also prevent the destruction of penicillin by Al.

It was realized that the inorganic constituents of the medium may affect not only the quantity of penicillin but also the type of penicillin produced; a study of this problem, however, was beyond the scope of this investigation.

SUMMARY

The ability of corn steep ash to increase penicillin production by *Penicillium chrysogenum* X-1612 in a basal synthetic medium was reproduced by the addition of Fe and soluble phosphates.

The presence of copper (>2 ppm) in the basal medium completely prevented the accumulation of penicillin; the addition of only 1 ppm of iron offset the effect of copper. Evidence indicated that this interaction between iron and copper affected the synthesis rather than the destruction of penicillin.

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