The Production of Penicillin in Surface Culture, using Chemically Defined Media

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SUMMARY: The production of penicillin by *Penicillium notatum* in surface culture on a number of chemically defined media is described. Good results were obtained with media containing lactose, glucose, acetic, citric and phenylacetic acids, ammonium sulphate and inorganic salts. The yield of penicillin was raised by further addition of starch and an aliphatic base such as ethylamine. In the latter instance the penicillin yield was about the same as that obtained with media containing corn-steep liquor.

A suitable addition of mineral salts should include magnesium, potassium, iron, zinc, copper, manganese, cobalt, phosphate and fluoride.

The addition of starch and ethylamine increased the growth of the mould and produced crinkled felts similar to those obtained with corn-steep liquor. These media have a composition which is in many ways comparable with that of media containing corn-steep liquor, and it seems likely that corn-steep liquor owes its effectiveness to the fortuitous presence in it of several types of substances rather than to one substance.

Although media containing lactose and corn-steep liquor (hereafter referred to as CSL) are usually used for the preparation of penicillin, considerable interest has been shown in chemically defined media. Apart from their possible technical value, such media may be a means of throwing light on the mechanism of penicillin formation.

A number of chemically defined media has already been described which are claimed to give high yields of penicillin in surface culture. White, Krampitz & Werkman (1945) described a medium containing lactose, glucose, arginine, histidine and glutamic acid; this recipe was based on analyses made on CSL. Cook & Brown (1946, 1947) subsequently described media containing gelatine or leucine and sugars, and also used media containing a variety of simple and complex nitrogen sources. More recently a patent specification (Glaxo Laboratories Ltd., British Drug Houses Ltd., Bide, Mead, Smith & Stack, 1947), has been published describing a number of more or less chemically defined media, which contained cystine or protein hydrolysates, in addition to phenylacetic acid or some similar substance with a suitably substituted benzyl group. A group of workers at Wisconsin has described media for the production of penicillin in submerged culture (cf. Jarvis & Johnson, 1947; Higuchi, Jarvis, Peterson & Johnson, 1946); these media contained, in addition to carbohydrates, lactate, acetate, ammonia and frequently phenylacetamide or a similar substance. The reason for the high productivity of media containing CSL has not yet been completely explained; current opinion is that it may be due to the presence of certain amino-acids in conjunction with substances like

β -phenylethylamine, which CSL is known to contain (Glaxo Laboratories Ltd. *et al.* 1947), and which enter directly into the penicillin molecule, thus stimulating production.

When the present work was begun we thought that the amino-acids were not perhaps of specific importance, and that they could probably be replaced by a suitable organic acid or by a mixture of acids. The stimulation of growth of *Phycomyces blakesleeanus* by the addition of organic acids to the medium had already been described by several groups of workers (e.g. Leonian & Lilly, 1940), while the Wisconsin workers mentioned above had already described their use for submerged cultures of *Penicillium*. Marloth (1931) had also shown that the addition of citrate to media containing sucrose increased the growth of two species of *Penicillium*.

Preliminary experiments showed that media containing fairly high concentrations of citric acid gave fairly good yields of penicillin, and a basal medium was devised containing lactose, glucose, acetic acid, citric acid, ammonium salts, phenylacetic acid and metallic salts. This medium gave yields of 70–90 units of penicillin/ml., but these were lower than those obtained with media containing CSL, which usually give 100–140 units/ml. under our conditions of culture. The weight of mycelium produced was also less, and the felts were smooth and wavy rather than crinkly and without the cracked and pitted undersurface usually obtained with CSL. In order to enrich the medium without the use of protein hydrolysates or amino-acids, ethylamine or other aliphatic bases together with starch were added. This resulted in the production of thick, crinkly felts with pitted undersurface which are characteristic of CSL growths. The yield of penicillin was also raised to levels obtained with CSL. In the course of our experiments tests were made of the effects of varying the concentrations of the various ingredients, carbohydrates, organic acids, ammonium concentration, etc. Difficulties which occurred at one period also led to a brief investigation of the inorganic requirements of the mould.

EXPERIMENTAL

Cultural conditions and methods. The organism used throughout this work was derived from the original Peoria strain *Penicillium notatum*, 1249 B21 (the culture used is labelled by us M2). For the inoculation of cultures the mould was grown on glycerol-molasses-peptone agar (cf. Frank, Calam & Gregory, 1948), and the spores brought into aqueous suspension by milling with glass beads. One ml. of suspension, containing 5×10^8 spores, was added to each flask of medium under test. On occasion we have also used suspensions containing 5×10^7 spores/ml.; these have given equally satisfactory results.

Media under examination were put up in 250 ml. Pyrex conical flasks containing 100 ml. of medium. The depth of medium is thus 25 mm., which is similar to that used in commercial practice. Three or four replicate flasks were normally employed. All flasks, etc., were sterilized by autoclaving for 20 min. at 15 lb./sq.in., and after inoculation were incubated, stationary, at 23–24°.

Penicillin was assayed by the cylinder-plate method normally used in these laboratories, using *B. subtilis* as test organism (cf. Foster & Woodruff, 1944). For assay, metabolism solutions were diluted twenty-fold with water. Differences of 5–10 % are distinguishable by this procedure.

Media. Preliminary experiments indicated that a medium of the following composition was likely to be of interest, and experiments were therefore based on it (all figures as % w/v): lactose, 3; glucose, 1; citric acid, 1; acetic acid, 0.25; phenylacetic acid, 0.05; ammonium sulphate, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; KH_2PO_4 , 0.1; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.001; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.001; $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 0.002; NaF, 0.001; pH adjusted to 5.6–5.9 with potassium hydroxide; water to 100. This recipe will be referred to as Basal Medium. Throughout, the figures given represent the total concentration in the medium and are given as % w/v. As far as possible A.R. or B.P. materials were used for medium preparation. Shortages necessitated the use of glucose chips instead of A.R. glucose in many experiments.

RESULTS

Inorganic components

There is considerable evidence in the literature that the penicillin-producing *Penicillia* are fairly exacting in their mineral requirements. Knight & Frazier (1945) have shown that with their chemically defined medium, penicillin titres were increased when ash from CSL was added. Pratt (1945) showed that there were optimal concentrations for MgSO_4 , KH_2PO_4 and NaNO_3 when added to media containing CSL, and Cook, Tulloch, Brown & Brodie (1945) found that the ash from certain pea fractions was beneficial to penicillin production. More recently Koffler, Knight & Frazier (1947) made a further study, using *P. chrysogenum* X-1612, of the causes of the stimulatory effect of the ash of CSL mentioned above. They concluded that Fe and phosphate were the active constituents of the ash. The presence of more than 2 p.p.m. Cu prevented the formation of penicillin, but the addition of 1 p.p.m. Fe antagonized this effect and good yields of penicillin were obtained. Pratt & Dufrenoy (1947) have also shown that *P. chrysogenum* X-1612 gave improved yields of penicillin when traces of Cr and Al were added to chemically defined media.

In our earliest experiments additions of inorganic salts to the media consisted of small quantities of KH_2PO_4 and the sulphates of Mg, Fe, Zn, Cu and Mn (referred to as GA salts). It appeared later that these were not adequate and that we were relying on casual impurities in the ingredients to supply the requirements of the organism.

In some of our cultures poor growth of the mould was accompanied by low titres; 30–50 units/ml. instead of 70–90 units/ml. Dried felts weighed 0.4–0.5 g./flask instead of 0.7–0.8 g./flask. This behaviour suggested that the mould was being deprived of necessary inorganic nutrients or trace elements, especially because there was usually an increase in the titre when the ash from CSL was added to the medium. Thus in one experiment titres were raised from 51 to

78 units/ml. on the 10th day by addition of the ash from the equivalent of 0.67 % (w/v) of CSL solids.

Spectrographic analyses of ash of a sample of British CSL showed that it contained considerable quantities of Mg, K, Na and P. There were small amounts of Fe, Mn and B and traces of Al, Cu, Zn, Si, Sn and Ga. On the other hand, Sc, Cb, Co, Ni and Mo were absent. Spore suspensions, as used for inoculation, contained Na, Mg, and lesser quantities of Si, Mn, Fe, and probably B; Sn, W, Mo, Cb, Sc, Co and Ni were absent.

These results for the analysis of CSL ash do not differ substantially from those described by Koffler *et al.* (1947), except that the American workers covered a somewhat wider range of elements. The American CSL contained traces of Co and Ni, whereas these were absent in the British materials. Quantitative analyses of four other batches of British CSL gave the following results: Mg, 0.12–0.38 %; K, 2–2.4 %; phosphate, 2.5–3.6 %; Cu, 4–40 p.p.m.; Zn, 44–65 p.p.m.; Fe, 250–325 p.p.m., based on the liquid which contained 35 % (w/v) solids.

Experiments were carried out in which the effect of adding various elements was tested. The elements tested included Cu, Zn, Fe, Mn, Mg, Mo, W, F, I, Co, Sn, Rb, Ag and Al. Of these all except the last four gave results of interest. The results of a typical experiment are given to illustrate the methods used. To Basal Medium, with no salt addition except Na_2SO_4 , NH_4NO_3 , KOH and KH_2PO_4 , there were added all combinations of 1 % (v/v) of solutions which contained the following salts in 1 l. of water:

(a) CuSO_4 , ZnSO_4 , MnSO_4 , 1 g.; FeSO_4 , 2 g.	(d) NaF, KI, 1 g.
(b) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 g.	(e) $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 1 g.
(c) Na molybdate and Na tungstate, 1 g.	

Groups of ingredients were used in this way to reduce the number of treatments, which was 32. The mean titre was 57 units/ml., the group with no additions having a titre of 48 units/ml. The mean effects of the five treatments were: (a) +2 units/ml.; (b) +6; (c) –3; (d) +5; (e) +4. There were some positive interactions, so that with suitable additions titres were of the order of 70–75 units/ml.

On the basis of this and similar experiments a salt addition was devised in which the following supplements (quoted above in the recipe for the Basal Medium) were made: KH_2PO_4 , 0.1 %; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 %; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.001 % each; $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 0.002 %; and NaF, 0.001 %. The concentrations of the various elements were determined as a result of separate experiments. Iodide was found to be deleterious. When media were made up with this salt the ash of CSL ceased to have a stimulatory effect.

With most batches of ingredients, media containing the original GA salt addition gave results similar to those quoted for the Basal Medium, which contains the above salt additions. This is no doubt due to the accidental presence of the necessary elements in the various ingredients. Thus in a certain experiment the addition of 5 p.p.m. fluorine had no effect; analysis showed, however, that the unsupplemented medium already contained 8 p.p.m.

The experiments described above do not define exactly the inorganic requirements of the mould, but show that growth and penicillin production are satisfactory when Mg, K, P, Fe, Cu, Mn, Zn, Co and F are added to the medium. It is possible that the elements in the ash of CSL that produce the stimulatory effect are different from these, but that one of them is a satisfactory substitute. Our results differ from those of Koffler *et al.* (1947), especially in that low titres were obtained even in the presence of Fe. However, it is possible that the two strains of *Penicillium*, 1249 B21 and X-1612, differ in their mineral requirements.

Organic components

Citric and acetic acid. The effect of varying the concentration of citric acid in the Basal Medium was first studied. The use of citric acid in chemically defined media had already been described by Clayton, Hems, Robinson, Andrews & Hunwick (1944), but in that instance low concentrations of the acid (0.2 % sodium citrate) were used. Higher concentrations than this are necessary if high yields of penicillin are to be obtained. Thus, in one experiment

Table 1. *Penicillin production with citric and acetic acid*

The Basal Medium was used with 0.5 % NH_4NO_3 instead of $(\text{NH}_4)_2\text{SO}_4$ and sugars as indicated.

Citric acid (%)	Mean titres (units/ml.) on 6th and 7th days Acetic acid (%)				Citric acid (%)	Mean titres (units/ml.) on 8th and 9th days Acetic acid (%)			
	0	0.125	0.25	0.5		0	0.125	0.25	0.5
	1. With 2 % glucose and 2 % lactose.								
0	0	0	25	25	0	0	0	19	35
0.5	28	44	60	76	0.5	33	48	62	71
1.0	56	72	64	51	1.0	51	68	76	76
1.5	59	61	70	44	1.5	74	74	79	73
Mean of 6 best titres 67 units/ml.					Mean of 6 best titres 75 units/ml.				
2. With 1 % glucose and 3 % lactose.									
0	0	0	21	21	0	0	tr	40	29
0.5	17	23	38	53	0.5	24	37	49	61
1.0	37	34	61	61	1.0	42	50	78	70
1.5	45	50	58	57	1.5	65	74	78	84
Mean of 6 best titres 58 units/ml.					Mean of 6 best titres 75 units/ml.				

the titres at 9 days were 23, 62, 83 and 91 units/ml. with 0.2, 0.5, 0.8 and 1.2 % respectively of citric acid. Table 1 illustrates another experiment, in which various concentrations of acetic and citric acid were employed. It will be noted that in the test of citric acid, titres rose slightly more rapidly with the 2 % glucose 2 % lactose mixture, but that otherwise the two sets gave similar results. The highest titres were obtained with 1.0–1.5 % citric acid.

No acid or mixture of acids has so far been found that is quite so effective as citric acid in chemically defined media, although some give quite good

results. The acids tested included glutamic, succinic, malic, lactic and tartaric acids, as well as mixtures of glutamic acid and either succinic acid or butyleneglycol.

Many substances can replace acetic acid. These include: succinic acid, oxalic acid, nucleic acid, acetylcholine, glutamic acid, diethylamine, sarcosine, glycerol, ethanol, *iso*-propanol, *n*-butanol, aspartic acid, hydroxypropionic acid, glutamic acid, oxalic acid. Benzylalcohol, glycine, glycollic acid, methanol, alanine, lactic acid and *iso*-caprylic alcohol were unsatisfactory.

Carbohydrates. The use of lactose follows current practice in penicillin work (Moyer & Coghill, 1946). The addition of glucose was found necessary by us if rapid growth were to be obtained. The concentrations used, lactose 3% and glucose 1%, were chosen empirically, and such tests as have been carried out suggest that these concentrations are as good as any, though in Table 1, where media are compared containing two different mixtures of the sugars, there is a suggestion that penicillin production is slightly more rapid with the 2:2 than with the 3:1 combination. Initially A.R. glucose was used, but this later became unobtainable and commercial glucose chips were used thereafter. Comparative experiments showed that both grades of glucose gave satisfactory results. Glycerol can replace glucose in synthetic media, but ethyleneglycol and 2:3-butyleneglycol are unsuitable for this purpose. The use of dextrans or starches as carbohydrates is discussed below. In another experiment the effect of varying the quantity of glucose in the presence and absence of acetic acid was tested. In the presence or absence of acetic acid an increase in the concentration of glucose from 0.5 to 1% raised the titre on the 8th day by about 40%. A further increase to 2% produced no improvement. Media containing starch and ethylamine give a different effect (see below, Table 5).

Ammonia, nitrate, sulphate and the use of potassium and sodium for neutralization. In a survey experiment all combinations of these factors were tested using three concentrations of each. The Basal Medium contained 2% lactose, 2% glucose, 1.5% citric acid, 0.125% acetic acid, 0.03% phenylacetic acid and salts. To these were added all combinations of:

Sulphuric acid (%)	0.5	1.0	2.0
Nitric acid (%)	0	0.5	1.0
Ammonia (as NH ₃ , %)	0.08	0.16	0.5

The media were adjusted to pH 5.7 with mixtures of sodium and potassium hydroxides in the ratios 1:0, 1:1, 0:1. The experiment was thus a $3 \times 3 \times 3 \times 3$ factorial design with 81 combinations.

The average penicillin titres for the different concentrations of ammonia and for the different neutralization mixtures were:

NH ₃ (%): 0.08, 0.16, 0.5; 38, 71, 31 units/ml.
Na/K:: 1:0, 1:1, 0:1; 43, 49, 47 units/ml.

The ammonia concentration of 0.16% was thus optimal, and potassium or a mixture of sodium and potassium was better for neutralization than sodium alone. Taking the results for the media containing 0.16% NH₃ neutralized with potassium only, the effect of using different concentrations of nitrate and

sulphate is shown in Table 2. Sulphuric acid was best at 0.5 %, and nitric acid was better at 0.5 % than at 0 or 1 % with this concentration of sulphuric acid.

The two basal media commonly used, incorporating 0.5 % of either ammonium sulphate or ammonium nitrate, contained 0.124 and 0.106 % NH_3 respectively. As it was thought that the former gave slightly higher titres the matter was investigated in two experiments, over a range of 0.05–0.3 % NH_3 . The best titres were obtained with about 0.13 % NH_3 . Mycelial weights were highest with rather more ammonia (c. 0.22 %). The presence or absence of nitrate (0.5 %) did not affect these optima, nor did a change in the concentration of citric acid from 0.9 to 1.5 %. In one experiment titres were the same with or without nitrate, in the other nitrate increased the average from 70 to 83 units/ml.

Table 2. *Effect of concentration of nitrate and sulphate on penicillin production*

Nitric acid (%)	Sulphuric acid (%)		
	0.5	1.0	2.0
	Titres (units/ml.)		
0	73	72	61
0.5	92	78	59
1.0	70	78	59

From these experiments it was concluded that the addition of about 0.5 % ammonium sulphate is suitable and that neutralization with potassium is advantageous. The concentration of ammonia is rather sharply optimal at 0.13 %. Nitrate usually increased the production of penicillin slightly, generally about 10 %.

Phenylacetic acid and β -phenylethylamine. The great increase in penicillin production which follows the addition of phenylacetic acid or other suitable compounds containing the benzyl radical is now well known (cf. Glaxo Laboratories Ltd. *et al.* 1947). When phenylethylamine is used it is probably oxidized before utilization. It was thought that this substance or a combination of it with phenylacetic acid might give better results than the acid alone, since its action might be more prolonged. An experiment showed that although the titres obtained with β -phenylethylamine were slightly higher than those with phenylacetic acid, the difference between the two was insignificant.

Sulphur sources. In our experiments sulphate has normally been used as a source of sulphur. Cystine and methionine are also satisfactory sources of sulphate, though in our hands they have not proved superior to sulphate. A detailed study of the sulphur metabolism of *P. notatum* has recently been made and will be published soon.

Starches. The idea of adding starches and organic bases arose from a consideration of CSL, which, it was thought, might well contain substances of these types. A considerable number of different types of starches and dextrans exist, and an experiment was carried out in which several different kinds were added to the Basal Medium (with 0.5 % NH_4NO_3 and 1.5 % citric acid). Of

the starches 1 or 3 % were added with 3 % lactose and 1 % glucose respectively. The two sets were inoculated and incubated side by side. The results of this experiment are shown in Table 3.

Table 3. *Penicillin production by media containing dextrans instead of glucose or lactose*

Carbohydrate	Days			Felt wt./flask (g.)
	8	9	10	
	Titre (units/ml.)			
(a) With 3 % lactose and 1 % carbohydrate.				
Soluble starch (B.D.H. Ltd.) A.R.	46	22	82	—
White maize dextrin	35	57	79	—
Yellow maize dextrin	15	27	35	—
British gum	39	54	63	—
British gum (dark)	24	41	58	—
Chlorinated gum 250	51	84	77	—
Chlorinated gum 300	56	63	67	—
Wet acid dextrinized gum	55	71	77	—
Alkali treated gum	37	64	68	—
Glucose (crude) chips	68	82	88	0.92
Pure glucose	68	84	88	—
(b) With 3 % carbohydrate and 1 % glucose.				
Soluble starch (B.D.H. Ltd.) A.R.	84	89	92	0.98
White maize dextrin	59	71	77	1.12
Yellow maize dextrin	38	50	70	—
British gum	45	58	65	—
British gum (dark)	39	52	59	—
Chlorinated gum 250	28	63	71	1.03
Chlorinated gum 300	29	55	78	—
Wet acid dextrinized gum	47	72	76	—
Alkali treated gum	61	72	92	—
Lactose B.P.	68	84	88	0.84

It was apparent from this experiment that soluble starch and the alkali-treated gum could be substituted for lactose. The second set of media gave higher titres than the first. It was noticed that with most of the media containing 3 % polysaccharide the growth habit of the mould resembled that on CSL to such an extent that it was almost impossible to distinguish mycelial felts of similar age grown on the chemically defined medium containing the chlorinated gum 250 from those grown on a normal CSL medium. With many of the starches at the 3 % level the mycelial felt weights were also higher than with lactose. Taken together the results of these experiments show that, with suitably chosen mixtures, starch or dextrin can replace lactose in this type of chemically defined medium, but that the titres are not much enhanced by these additions.

Organic bases. A preliminary experiment with some organic bases indicated that the addition of ethylamine to media as a source of nitrogen might be useful. It also showed that ethanolamine, nitrate and urea were less satisfactory, while triethylamine, hexamethylenediamine, octadecylamine and dibutyl-

amine-propylamine failed to support growth. Ethylamine was therefore selected for further trial.

A large experiment was set up in which three mixtures of sugars and three mixtures of citric and acetic acid were tested with the addition of ammonia (0, 0.06 and 0.13 % as NH_3) ethylamine (0, 0.3 and 0.6 %) and starch (0, 1 and 2 %). The Basal Medium containing 2 % glucose and 2 % lactose was the best of those tested, and addition of citric acid in the usual way seemed advisable. Ammonia enhanced the titres as starch did, 1 or 2 % giving about the same results. Ethylamine was best at 0.3 %.

On the basis of these and a few other experiments it was concluded that as a basis for investigation the Basal Medium given above, with the addition of 0.3 % ethylamine and 1.5 % starch, would be suitable. This medium contains too many components for a trial of every combination, so the effect on penicillin titre of varying the composition of the medium was tested in a number of small experiments dealing with only a few of these components. The factors investigated included the effect of varying the concentrations of ethylamine, starch, glucose, acetic acid and ammonia. A number of other organic bases were also tested. The results of these experiments are shown in Tables 4-6.

Table 4. *Penicillin production with different concentrations of starch and ethylamine*

Basal Medium but with 0.5 % glucose instead of 1 %.

Starch (%)	Ethylamine (%)		
	0.15	0.3	0.45
	Titres (units/ml.) 10th day		
0	78	56	38
1	81	67	49
2	100	105	72

With this basal medium the best results were clearly given by 2 % starch and not more than 0.3 % ethylamine. In another test with 2 % glucose and no acetic acid, the highest titre was 100 units/ml. with 0.45 % ethylamine and 1 % starch.

Table 5. *Effect of concentration of glucose and acetic acid on penicillin production with Basal Medium plus starch and ethylamine*

Acetic acid (%)	Glucose (%)		
	0.5	1.0	2.0
	Titres (units/ml.) 10th day		
0	99	68	19
0.25	85	111	120

The results quoted in Table 5 show the effect of addition of glucose and acetic acid to Basal Medium containing starch and ethylamine. They were confirmed in repeat experiments, and should be compared with the results for

the same basal medium but without the starch and ethylamine. These showed that in the absence of starch and ethylamine penicillin production increased with glucose concentration whether acetic acid was present or not. Table 5, however, shows that when starch and ethylamine were present, increased concentration of glucose reduced production, unless acetic acid was present, in which case it increased it.

Table 6. *Effect of differing concentrations of starch, ethylamine and ammonia on yield of penicillin*

Ethylamine (%)		Basal Medium					
		No starch			1.5 % starch		
		0	0.15	0.3	0	0.15	0.3
		Titres (units/ml.) means of 8-10 days					
NH ₃ (%)	0.6	38	85	87	25	64	84
NH ₃ (%)	0.13	76	94	78	70	89	102

An examination of Table 6 reveals marked effects from the addition of ammonia and ethylamine and practically no effect from the addition of starch except with 0.3 % ethylamine and 0.13 % ammonia where the titre is markedly higher in the presence of starch.

When taken together the above results indicate that the media containing starch and ethylamine should give good yields in practice. This has, in fact, proved to be the case. With a simpler chemically defined medium, titres were usually about 75-90 units/ml. With the addition of starch and ethylamine, peak titres (10-11 days) were 134, 110, 105, 102, 113, 120, 103, 125 and 137, 112 and 113 units/ml. on different occasions. The mean is 119 units/ml., and compares favourably with the titres obtained with CSL media which usually reach 100-140 units/ml.

These results were all obtained with ethylamine. A number of other substances containing amino-groups has been tested. *n*-Butylamine and aspartic acid gave good results, ethanolamine gave poor results, and various other substances such as *isobutylamine*, glycine, alanine and propylamine were intermediate. Table 7 gives the results of one experiment with some of these compounds.

Table 7. *Effect on penicillin titres of addition of organic nitrogen to the Basal Medium (with 1.5 % starch)*

Addition (0.3 %)	Penicillin (units/ml. at 11 days)
Nil	82
Methylamine	79
Ethylamine	106
<i>n</i> -Propylamine	100
<i>n</i> -Butylamine	112
<i>n</i> -Amylamine	58
Dimethylamine	91
Glutamic acid	113
Aspartic acid	120
Hydrolysed casein	105

DISCUSSION

When the experiments were begun it was hoped to obtain chemically defined media which would give results comparable with those obtained with media containing CSL. The media which we have described resemble the latter in respect of the yield of penicillin and the macroscopic appearance of the mycelium they produce.

Suitable chemically defined media appear to require the simultaneous presence of several groups of components if the best results are to be obtained. Lactose is essential but is not satisfactory alone; glucose, or a similar carbohydrate such as glycerol, must also be added to ensure quick growth. Nor are these two carbon sources sufficient; the presence of an organic acid is also necessary in a fairly high concentration. For this we have found citric acid best, and a further addition of acetic acid is advantageous. The presence of a substance like phenylacetic acid, which enters the penicillin molecule directly, is also essential. It is also evident that the inorganic requirements must be carefully fulfilled if satisfactory results are to be obtained.

Table 8. *Comparison of corn-steep liquor and chemically defined medium*

Important constituents	CSL medium	Chemically defined medium
(1) Principal carbohydrate sources	Lactose 3-4 %	Lactose 3 %, glucose 1 %
(2) Auxiliary carbohydrate source	Non-reducing carbohydrate including polysaccharide	Starch 1.5 %
(3) Organic acids	Acetic acid <i>c.</i> 0.05 %, lactic acid <i>c.</i> 0.5 %	Acetic acid 0.25 %, citric acid 1.0 %
(4) Special compounds	β -Phenylethylamine and hydroxyphenylethylamine	Phenylacetic acid 0.05 %
(5) Principal nitrogen sources	Mixture of amino-acids, amines and ammonia	Ammonium sulphate 0.5 %
(6) Secondary nitrogen source		Ethylamine 0.3 %
(7) Total solutes	8-9 %	<i>c.</i> 8.5 %
(8) Total nitrogen	0.15-0.20 %	<i>c.</i> 0.20 %

In certain respects the best chemically defined media show a marked similarity of composition to CSL media. The groups of ingredients which seem to be essential, and the substances representing them in CSL and in the chemically defined media, are shown in Table 8. It appears that each group of components in the one medium has its counterpart in the other. Nitrate is often added to both media, and may increase the yields slightly.

Media prepared on the above lines can, in absence of starch and ethylamine, be expected to yield 70-90 units/ml. of penicillin. The addition of starch or certain dextrans together with short-chain aliphatic amines such as ethylamine increased the penicillin yield to an average of 119 units/ml.; this is of the

same order as the yields given by media containing CSL under our conditions of growth.

The reasons for the specific advantages of each component of the medium are not known. It seems possible that lactose and starch act by reason of their slow assimilation which is perhaps dependent upon a prior hydrolysis. The other nutrients, such as acetic acid, citric acid, amino-acids and primary amines, may act as carbon sources or assist in the uptake of nitrogen or affect the regulation of pH.

It is noteworthy that there is little or no interaction between the various substances in the media: the introduction of each constituent, citric acid, acetic acid, ethylamine, etc., usually produced an increase in titre, the effect of each being additive. The only exception so far encountered lies in the effect of adding acetic acid and glucose in the presence or absence of ethylamine and starch together. In the absence of the last two, penicillin yield is about the same with increased addition of glucose, with or without acetic acid. In presence of ethylamine and starch, however, increase of glucose depresses production of penicillin unless acetic acid is present at the same time, in which instance it increases it. There is no obvious explanation of this effect, but it suggests that as the media become more effective and more complex, the interrelation between their components becomes more critical.

The new media and CSL media both have a high content of total solutes. They have also about the same concentration of nitrogen. In addition to these similarities, the complex nature of the new media serve to illustrate the convenience conferred by using CSL. In so far as one can base any hypotheses on the above results, it would appear that CSL owes its particular suitability for penicillin production to the fortuitous presence therein of several classes of inorganic and organic substances necessary for penicillin production by *P. notatum* rather than to the presence of a particular essential substance. Our experiments show that the effects observed with CSL can also be produced by means of suitable chemically defined mixtures. They do not, however, exclude the possibility that CSL may owe some of its usefulness to an unidentified nutrient or growth factor.

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REFERENCES

- CLAYTON, J. C., HEMS, B. A., ROBINSON, F. A., ANDREWS, R. D. & HUNWICK, R. F. (1944). Preparation of penicillin. Improved method of isolation. *Biochem. J.* **38**, 452.
- COOK, R. P. & BROWN, M. B. (1946). Synthetic media for penicillin production. *Biochem. J.* **40**, xlix.
- COOK, R. P. & BROWN, M. B. (1947). Effects of the source of nitrogen in the medium on the formation of penicillin by surface cultures of *Penicillium notatum*. *Nature, Lond.*, **159**, 376.

- COOK, R. P., TULLOCH, W. J., BROWN, M. B. & BRODIE, J. (1945). The production of penicillin using fractions obtained from aqueous extracts of pea (*Pisum sativum*). *Biochem. J.* **39**, 314.
- FOSTER, J. & WOODRUFF, H. B. (1944). Microbiological aspects of penicillin. VI. Procedure for cup assay of penicillin. *J. Bact.* **47**, 43.
- FRANK, M. C., CALAM, C. T. & GREGORY, P. H. (1948). The production of spores by *Penicillium notatum*. *J. gen. Microbiol.* **2**, 70.
- Glaxo Laboratories Ltd., British Drug Houses Ltd., BIDE, A. E., MEAD, T. H., SMITH, E. L. & STACK, M. V. (1947). Improvements in or relating to the manufacture of penicillin. Brit. Pat. No. 586,930.
- HIGUCHI, K., JARVIS, F. G., PETERSON, W. H. & JOHNSON, M. J. (1946). Effect of phenylacetic acid derivatives on the types of penicillin produced by *Penicillium chrysogenum* Q 176. *J. Amer. chem. Soc.* **68**, 1669.
- JARVIS, F. G. & JOHNSON, M. J. (1947). The role of constituents in synthetic media for penicillin production. *J. Amer. chem. Soc.* **69**, 3010.
- KNIGHT, S. G. & FRAZIER, W. C. (1945). The effect of corn-steep liquor ash on penicillin production. *Science*, **102**, 617.
- KOFFLER, H., KNIGHT, S. G. & FRAZIER, W. C. (1947). The effect of certain mineral elements on the production of penicillin in shake flasks. *J. Bact.* **53**, 115.
- LEONIAN, H. & LILLY, W. G. (1940). Studies on the nutrition of fungi. IV. Some factors influencing the growth of some thiamine requiring fungi. *Amer. J. Bot.* **27**, 18.
- MARLOTH, R. H. (1931). The influence of hydrogen-ion concentration and of sodium bicarbonate and related substances on *Penicillium italicum* and *Penicillium digitatum*. *Phytopathology*, **21**, 169.
- MOYER, A. J. & COGHILL, R. D. (1946). Penicillin. VIII. Production of penicillin in surface cultures. *J. Bact.* **51**, 53.
- PRATT, R. (1945). Influence of the proportions of KH_2PO_4 , MgSO_4 and NaNO_3 in the nutrient solution on the production of penicillin in surface cultures. *Amer. J. Bot.* **32**, 528.
- PRATT, R. & DUFRENOY, J. (1947). Untitled. *Science*, **105**, 574.
- WHITE, A. G. C., KRAMPITZ, L. O. & WERKMAN, C. H. (1945). Synthetic medium for the production of penicillin. *Arch. Biochem.* **8**, 303.

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