The Production of Penicillin Using Fractions Obtained from Aqueous Extracts of Pea (*Pisum sativum*)

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Aqueous extracts of air-dried ground peas can provide a good medium for the growth and production of penicillin by *Penicillium notatum* (Cook & Tulloch, 1944). The experiments to be described were carried out to determine what constituents of the pea were responsible for this effect. Although other vegetable extracts give a similar effect the pea has been chosen as it is a readily available material and one on which methods may be standardized.

EXPERIMENTAL

Raw material. Dried green peas obtained from a local seedsman were used; the variety of seed was found not to affect the results. The dried peas were ground in a coffee-mill and the pea-meal kept in a cool place. No deterioration in the activity of extracts was found after storage. Dundee mains water was used to prepare extracts; distilled water was used to reconstitute evaporated fractions.

Fractionation of the pea extract. Both hot and cold water extracts were prepared and fractionated as shown in Fig. 1.

Chemical methods of analysis used

Dry weight. Materials were dried to constant weight in an air oven at 100°. **Ash.** The dried material was ashed at 1000° in an electric furnace.

Estimation of mineral constituents. After the ash was dissolved in HCl, K was estimated using the Na salt of 6-chloro-5-nitrotoluene-3-sulphonic acid (Davies & Davies, 1923). Ca was estimated as oxalate; P_2O_5 as the molybdate complex; SO_4^{2-} as $BaSO_4$ (Cumming & Kay, 1942).

Nitrogen. (i) *Total-N.* The micro-Kjeldahl method was used with the digestion mixture recommended by Chibnall, Rees & Williams (1943). Triplicate determinations were made, each sample being digested for 4 hr. after clearing. (ii) *Non-protein N* was determined in the 15% (w/v) trichloroacetic acid filtrate. (iii) *Amide-N* was determined after hydrolysis with 5% HCl. (iv) *Amino-N* by the Sorensen formal titration method. (v) *Basic-N* was determined on the phosphotungstic acid precipitate.

Carbohydrate. The amount of carbohydrate present, before and after hydrolysis by 2N-HCl and after fermentation with yeast, was estimated as glucose by the Hagedorn and Jensen method. Although the carbohydrates are not only glucose, nor convertible into glucose, they are expressed as such as a convenient basis of comparison.

Galactose. The mucic acid method of Van der Haar (1917) was used.

Biological methods

Preparation of media. Although the pea extracts alone will support growth and permit some penicillin formation (Cook & Tulloch, 1944), this simple medium by itself does not always give consistent results. It has now been found that both growth and penicillin formation may be stabilized by the addition of certain other substances. The most interesting of these is NaCl, the presence of which enhances the yield of penicillin. Other salts ($NaNO_3$, KH_2PO_4 , $MgSO_4 \cdot 7H_2O$) also appear to have some effect in stabilizing the yield. The addition of lactose also exerts a similar though less marked effect and is now included in the medium. A basal medium is made up to contain (as g./l.): NaCl, 10; $NaNO_3$, 3; KH_2PO_4 , 0.5; $MgSO_4 \cdot 7H_2O$, 0.25 and lactose, 30. Fractions of material from the pea are added to the basal medium approximately in the proportion in which they occur in a '10% extract' (see Fig. 1).

As some of these fractions were added as concentrates the volume was made up with distilled water to that corresponding to the original. In general, no adjustment of pH was made, the values usually ranging between pH 5-6. In cases where ashed or acid fractions were added the pH was adjusted to 5.5. The basal medium itself did not support growth. We are not completely satisfied that this basal medium is the most suitable, but until further work has been done it has been convenient. The basal medium, with added fractions from the pea extract, was made up to 40 ml. and all experiments have been carried out using this amount in 250 ml. conical Pyrex flasks. The flasks containing the complete medium were autoclaved for 15 min. at 15 lb. pressure.

Seeding culture. The flasks were seeded by means of a platinum loop from cultures of *P. notatum* grown on Sabouraud's medium, upon which the stock cultures were kept. The strain used throughout was Peoria 1249 B 21. After seeding, the flasks were incubated at $24^\circ \pm 1^\circ$.

pH. The pH of the culture medium was determined from the seventh day of growth onwards using a spiral glass electrode and valve potentiometer (Unicam Instruments Ltd.).

Measurement of mould growth. At the end of an experiment the fluid was decanted off, the mycelial felt drained, pressed between absorbent papers until practically dry and weighed.

Penicillin assay. The activity has been determined by Brodie's method (1945). We should like to stress that, although the error of this method may be high ($\pm 25\%$), our results are comparative. We have repeated with different batches of material all our series of experiments and no marked variation in the results has been observed.

RESULTS

Typical results obtained for growth and penicillin production obtained when various fractions of material from hot-water extracts of pea meal were tested are shown in Table 1. Similar results were obtained using cold-water extracts; in general growth and penicillin production reached a peak earlier with the latter although the maximum yields were the same as those given by hot-water extracts.

residue by itself hardly supported growth and the yield of penicillin was low (2). Although not shown in Table 1, further extracts made from the pea residue (fraction II) were relatively inactive. Using 80% ethanol as a fractionating agent a fair separation of active constituents was obtained. Thus fraction V had an activity much greater than fraction VI; but on combining these two fractions the activity was greater than that of either fraction alone (4, 9 and 13). This indicated that there were

Fig. 1. Scheme of fractionation of pea meal to separate material active in stimulating penicillin production.

Table 1. *The growth-promoting and penicillin-producing activities of pea-meal fractions shown in Fig. 1*

(Text number (column 1) is referred to in text. The fraction numbers (column 2) are those used in Fig. 1.)

Text no.	Fraction in Fig. 1	Dry matter (g./100 ml.)	Ash (g./100 ml.)	Total N (g./100 ml.)	Wet wt. of mycelium (g.)	Penicillin production units/ml. days in ()	pH*
1	I	4.0	0.27	0.19	2.27	225 (12)	8.4 (14)
2	III	2.0	0.02	0.09	0.85	25 (12)	7.7 (12)
3	IV	2.0	0.26	0.09	1.92	225 (12)	8.2 (14)
4	V	1.2	0.19	0.05	2.03	175 (12)	7.9 (14)
5	Va	0.2	0.02	0.01	1.04	20 (12)	7.5 (12)
6	Vb	1.03	0.18	0.05	1.60	140 (12)	7.4 (12)
7	Vc	0.31	0.05	0.03	1.42	0	—
8	Vd	0.18	0.18	0	0	0	—
9	VI	1.0	0.06	0.02	1.27	15 (12)	7.4 (14)
10	VIa	0.2	0	0	0	0	—
11	VIb	0.8	0.06	0.02	1.73	15 (12)	7.5 (12)
12	Vic	0.06	0.06	0	0	0	—
13	V + VI	2.2	0.25	0.07	1.99	225 (12)	8.5 (14)
14	Vb + VI	2.03	0.24	0.07	2.22	180 (12)	7.6 (12)
15	Vc + VI	1.31	0.11	0.05	0.84	20 (10)	7.7 (10)
16	Vd + VI	1.18	0.24	0.02	1.08	80 (12)	7.9 (12)

* Time at which determination was made (days) given in parentheses.

In the following text Roman figures and letters refer to fractions in Fig. 1; the arabic numerals in brackets refer to Table 1, where these text-numbers in column 1 refer to the activities, etc., of the corresponding fractions or combinations of fractions.

The results show that the clarification process, in which the dry matter and N content were reduced by half, had no effect on growth and penicillin production (Table 1; text nos. 1 and 3). The clarification

essential constituents in fraction VI. Further fractionation of fraction V showed that fraction Va, which consisted mainly of starch and insoluble protein, could be removed without affecting the yield, although it had some slight activity (5). Fraction Vb alone had marked activity; this was further increased by the addition of fraction VI (6 and 14). Fraction Vc was inactive; even tested in presence of fraction VI the activity was not greater than that

of VI alone (7 and 15). Fraction Vd alone was inactive, but fractions (Vd + VI) together gave five times the value for either fraction alone (8, 9 and 16). These results indicated that in fraction V important constituents were contained in the ash, but that the protein exerted no marked effect. Further fractionation of fraction VI showed that fraction VIa did not support growth and that fraction VIb behaved like fraction VI (10 and 11). Fraction VIc did not support growth (12), nor did its addition to fraction Vb increase the activity of the latter. It appears that in fraction VI ether-soluble acids and mineral constituents of the ash are unimportant.

Analyses of different batches of the two most active fractions (Vb and VI) show that although there are variations in their composition certain constituents are always present. Typical analyses are presented below.

Analyses of fraction Vb

The solution was slightly dextrorotatory. The carbohydrate was non-reducing and gave a blue colour with iodine. That it contained carbohydrate other than starch was suggested by the formation of mucic acid after treatment with nitric acid. The mucic acid was identified by: m.p. 204°, mixed m.p. 205° (slow heating); equivalent 105 (theory 105). The galactose thus assumed to be present is apparently in the form of the actual sugar in combination, as tests for uronic acids were negative. The amount of carbohydrate present (as glucose) after acid hydrolysis was 40 % of the dry weight, 5 % of which was galactose.

Nitrogen. The total N was 8 % of the dry weight. The distribution of N (as % total) was: non-protein-N, 71; amino-N, 8; amide-N, 10; basic-N, 73. The small amount of protein or proteose present (fraction Vc) was precipitated by protein reagents and gave the usual colour tests with the biuret, xanthoproteic, Millon and glyoxylic reagents. The ether-extractable material obtained by extracting the acidified fraction was 28 % of the dry weight. This material was dark in colour, but distinct droplets of an oily substance could be seen. This oil was soluble in chloroform and had an equivalent of 234. **Ash.** This was 14 % of the dry weight. Analysis of the ash gave the following values (as % total ash): K₂O, 24; CaO, 5; P₂O₅, 15; SO₄, 17. In addition to these, trace elements were found in considerable variety, the following spectrographic analysis of the ash being obtained by Dr R. Mitchell: Mo, 44; Ni, 10; Sn, 30; Pb, 200; Mn, 1000; Rb, 1000; Ba, 200; Sr, 200; Cr, 10 p.p.m. and Cu, 1 %. The Mo determination was made quantitatively but the other values are approximations. The Mo content of fraction Vb itself was 4.5 p.p.m.

Analysis of fraction VI

The solution was slightly dextrorotatory. The carbohydrate estimated as glucose before acid hydrolysis was 16 % of the dry weight and after hydrolysis with 2N-HCl it was 56 %; galactose was 30 %. After acid hydrolysis the following values (as % total carbohydrate) were found: fermentable sugar 60, non-fermentable sugar 40. Tests indicated the presence of glucose and fructose. Ether extraction after acidification yielded 2 % of material and had an equivalent of 250.

Nitrogen. Total N: 4 %; the distribution of N (as % total) was: non-protein-N, 100; amino-N, 16; amide-N, 11; basic-N, 59. **Ash.** 5 %, of which 50 % was K₂O, 20 % P₂O₅. Tests for phenolic compounds were strongly positive.

Trace elements

As mentioned above, the mineral portion of the ethanol-precipitable fraction (V) appeared to be important. The migration of the trace elements from the medium into the mould mycelium has been followed by Dr Mitchell. In Table 2 are shown semi-quantitatively the distribution of trace elements in

Table 2. *The migration of trace elements from media to mycelium*

Spectrographic analysis of the ashes of pea medium and Coghill medium before growth, and of mycelium of *P. notatum* grown thereon.

+, ++, etc. indicate relative amounts present in the ash; - indicates that the presence of the element was not demonstrable. The sign → indicates that the element migrates into the mycelium.

	Pea medium before growth	Mycelium	Coghill medium before growth	Mycelium
Ag	+		-	
Pb	++	→ +	+	→ +
Sn*	-		+++	→ +++
Mo	++	→ ++	+	→ +
Cr	+		-	
Ni	+++	→ +	+++	→ +
Co	-		-	
Zn	+	→ +	+	→ ++
V	-		+	→ +
Ba	++	→ +	+	→ +
Sr	++	→ +	+	→ +
Li	-		-	
Rb	++	→ +	++	→ +

* The result for Sn is for a batch of medium made by Prof. J. Cruickshank. Our medium gave Sn +.

the pea-medium, in Coghill corn-steep medium, and in the mycelium. The result for Sn is from a batch of Coghill medium prepared by Prof. J. Cruickshank; our Coghill medium showed only traces of this metal.

DISCUSSION

Precipitation with ethanol was used to obtain fractions by a mild method. With hot-water extracts a large amount of material, containing much mineral matter and two-thirds of the total N, was precipitated by ethanol. From cold-water extracts ethanol precipitated much less material. The precipitated material contains carbohydrate, ether-soluble compounds, N-containing compounds and mineral constituents.

The carbohydrate consists of starch and a carbohydrate apparently containing galactose. The presence of galactose in hemicellulose obtained from ungerminated peas was shown by Schulze, Steiger & Maxwell (1890) and Schulze (1892). The ether-soluble matter is present in large amount. The presence of lecithin in the pea has been demonstrated by Winterstein & Hiesstand (1908). The distribution of N shows that a large proportion of it is present in compounds of a basic nature. The trace elements of the large mineral portion of the fraction are of interest, particularly the Mo, previously demonstrated by Ter Meulen (1932) and by Bertrand (1939).

In the ethanol-soluble fraction carbohydrate forms a large proportion of the dry weight but the ether-soluble portion is small in amount. The N is apparently present mainly in compounds of a basic nature. The inorganic moiety appears to consist mainly of potassium phosphate and is not important in penicillin production.

Although both the ethanol-soluble and ethanol-insoluble fractions support growth of the mould, it is the ethanol-precipitable fraction which stimulates penicillin production. There are, however, com-

ponents in the ethanol-soluble fraction which increase its activity, particularly if the extracts have been prepared with cold water or if germinated or fresh pea extracts are used.

Routine determinations of pH showed that although an increase in pH of the medium was often associated with increased penicillin production this is not always so. A marked increase in pH was always shown early in media in which the protein and starch contents were high, as for example, in unclarified extracts.

SUMMARY

1. A scheme of preliminary fractionation of aqueous extracts of ground dried peas to separate the constituents responsible for penicillin production is described.

2. 80% ethanol precipitates a fraction which is very active. The ethanol-soluble portion, although it supports growth, has only slight activity in affecting penicillin production, but in the presence of the precipitable fraction increases the latter's activity.

3. Preliminary analyses of the 80% ethanol-soluble and ethanol-insoluble fractions are given.

4. The inorganic constituents of the precipitable fraction are important. The migration of certain trace elements from the medium to the mycelium has been demonstrated.

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REFERENCES

- Bertrand, D. (1939). *C.R. Acad. Sci., Paris*, **208**, 2024.
 Brodie, J. (1945). *J. Path. Bact.* **57**, 257.
 Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
 Cook, R. P. & Tulloch, W. J. (1944). *J. Path. Bact.* **56**, 555.
 Cumming, A. C. & Kay, S. A. (1942). *Quantitative Chemical Analysis*, 8th ed. London: Gurney and Jackson.
 Davies, H. & Davies, W. (1923). *J. chem. Soc.* **123**, 2976.
 Schulze, E. (1892). *Hoppe-Seyl. Z.* **16**, 387.
 Schulze, E., Steiger, E. & Maxwell, W. (1890). *Hoppe-Seyl. Z.* **14**, 227.
 Ter Meulen, H. (1932). *Nature, Lond.*, **130**, 966.
 Van der Haar, A. W. (1917). *Biochem. Z.* **81**, 263.
 Winterstein, E. & Hiesstand, O. (1908). *Hoppe-Seyl. Z.* **54**, 288.

The Glucose Metabolism *in vitro* of *Trypanosoma rhodesiense*

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It was first shown by Biot, Biot & Richard (1911) that glucose is favourable to the continued life and motility *in vitro* of the rat-trypanosome *T. lewisi*; this effect of glucose has been confirmed by many authors using different species of trypanosomes.

Kudicke & Evers (1924) showed that other sugars and glycerol could be used to maintain *T. brucei* in culture media. The rates of glucose decomposition, oxygen consumption, and acid production by certain trypanosomes have been determined (Nauss &