CCXXVII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXVI. THE FORMATION FROM GLUCOSE BY MEMBERS OF THE *PENICILLIUM CHRYSOGENUM* SERIES OF A PIGMENT, AN ALKALI-SOLUBLE PROTEIN AND PENICILLIN—THE ANTIBACTERIAL SUBSTANCE OF FLEMING.

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IN a recent paper, Fleming [1929] showed that a mould which appeared as an air contaminant in laboratory bacterial cultures, and which resembled *Penicillium rubrum* Grasberger-Stoll, produced when grown on nutrient broth a very powerful antibacterial substance. Filtrates of the broth cultures were named "penicillin" and it was found that the active substance affected different bacteria in different degrees. The action was very marked on the pyogenic cocci and the diphtheria group of bacilli, whilst the coli-typhoid and the influenza bacillus groups were quite insensitive to it. Staphylococci, *Streptococcus pyogenes* and the pneumococcus were completely inhibited in a 1 in 800 dilution of the filtrate, which did not affect the growth of *H. influenzae* (Pfeiffer's bacillus), and a method was described for the isolation of *H. influenzae* by incorporating penicillin in a suitable solid medium.

Examination of Fleming's organism by Dr Charles Thom at our request showed that it was not *Penicillium rubrum* but a strain closely related to *P. notatum* Westling in the *P. chrysogenum* Thom series. Dr Thom writes as follows: "I have cultivated Fleming's organism under several different conditions and cannot agree with the nomenclature as *P. rubrum* either in the sense of my 1910 paper or in the sense of Biourge's monograph. In fact, I believe this culture, although showing some divergencies in culture reactions, to be closer to *P. notatum* of p. 264 in my book than to the groups discovered on pp. 249-50 as indicated by the nomenclature used."

In his book [1930, pp. 261-2] Dr Thom says that the type strain of *P. chrysogenum* (No. 26) rapidly digests milk and liquefies gelatin giving a yellow colour. "Studied on a series of culture media, it changes colour with the composition of the substratum and with the age or stage of metabolism

of the colony itself: colonies grown on Czapek's solution in presence of abundance of fermentable sugar long remain green, without such sugar little or no yellow appears in the substratum." The production of this yellow pigment on milk appears to be characteristic of species in the *P. chrysogenum* series. One of the results of this work was the isolation and investigation of this pigment to which the name chrysogenin is given.

Although Fleming's organism appears, on the usual systematic criteria and from the fact that it produces chrysogenin, to belong to the *P. chrysogenum* series, the fact (see below) that we have never been able to demonstrate the production of the bactericidal substance, penicillin, by *P. chrysogenum* or by *P. notatum* renders the identification of Fleming's organism with either of these species doubtful from a physiological point of view.

Working both with Fleming's strain and the type strain of *P. chrysogenum* (No. 26), we have frequently experienced difficulty in repeating our experiments. Thus single spore cultures gave colonies sometimes with a white and sometimes with a yellow reverse, and when used for sowing flasks of our medium the former gave greater yields of chrysogenin than the latter. Moreover, with both strains after continued subcultivation on our medium, the mycelial felts tended to become less green, to show white patches, floccose in character, and this was always accompanied by decrease in the production of both chrysogenin and penicillin. From such cultures, by single spore technique, both floccose and green colonies were re-obtained, and the latter, but not the former, on using for sowing gave considerable chrysogenin formation (with all the strains) and penicillin formation (with Fleming's strain).

Eight cultures, for which we are indebted to our colleagues Miss L. Farrell and the late Mr J. H. V. Charles, have thus been employed.

Catalogue No.	Strain	Source
589	<i>P. chrysogenum</i> Thom (No. 26)	British National Collection of Type Cultures, Lister Institute
P 19 W	„ white reverse	Isolated from culture No. 589 at the London School of Hygiene and Tropical Medicine
P 19 Y	„ yellow reverse	Do.
P 19 Y ₂	„ white floccose form	Do.
57	Fleming's strain (a)	British National Collection of Type Cultures, Lister Institute
152	„ (b)	Dr Fleming
I.D.	Fleming's strain, white floccose form	Isolated from culture No. 57 at the London School of Hygiene and Tropical Medicine
P 45	<i>P. notatum</i> Westling	Dr C. Thom

With all these strains (except the two giving a floccose type of mycelium, Nos. P 19 Y₂ and I.D.), the metabolism solutions gradually assumed a yellow colour and a negative rotation and gave a characteristic olive-brown colour with ferric chloride. The metabolism solutions on acidifying to p_H 3.5 gave a

yellow flocculent precipitate which was shown to consist chiefly of the yellow pigment chrysogenin, and a protein. Only the two green strains, cultures Nos. 57 and 152, and to a much smaller extent, the floccose culture I.D. from Fleming's culture, showed any antibacterial activity.

This activity, although arising more slowly on this synthetic medium, was as great or greater than that recorded by Fleming using a broth medium. This should be of considerable practical importance since the isolation of penicillin from a synthetic medium should be much simpler than from broth.

The purpose of the present paper is to describe the isolation of the pigment chrysogenin, and the protein, using the type strain of *P. chrysogenum* Thom No. 26, and the preliminary attempts at isolation of penicillin, using Fleming's culture.

Preparation of the three chief metabolic products.

A quantity of modified Czapek-Dox medium was made up having the following composition, for which we are indebted to our colleague Dr H. G. Turley:

NaNO ₃	...	...	...	3.0 g.
KH ₂ PO ₄	...	...	...	1.0 g.
KCl	...	...	...	0.5 g.
MgSO ₄ , 7H ₂ O	...	...	...	0.5 g.
FeSO ₄ , 7H ₂ O	...	...	...	0.01 g.
Glucose	...	...	...	40.0 g.
Distilled water to 1000 cc.				

400 cc. quantities of this medium were placed in 100 1-litre conical flasks which were plugged with cotton wool, and sterilised by steaming for half an hour on each of three consecutive days. Each flask was heavily sown with a suspension in the above medium of spores of a 6-day culture of either the type strain of *P. chrysogenum* or of Fleming's organism, grown on Czapek-Dox agar at 25°. The flasks were shaken and then incubated at 25° for 21–28 days, until the sugar had almost entirely disappeared, when a sample of the filtered metabolism medium from the type strain of *P. chrysogenum* gave in a 2 dm. tube a rotation of – 0.3 to – 0.5°. With Fleming's strain the rotation with optimum antibacterial activity was – 0.03 to – 0.08° but increased to – 0.36° during the subsequent few days.

The contents of the flasks were now filtered, the mycelium pressed and the combined filtrates (about 38 litres) were acidified to p_H 3.6 with about 480 cc. 2N H₂SO₄. With careful acidification a yellow precipitate was obtained which quickly flocculated, but if excess of acid was rapidly added the precipitate remained in an unfilterable dispersed form. It was found with each organism that the crude precipitate consisted of about equal weights of protein and chrysogenin. In the case of Fleming's organism this precipitate could be filtered off almost without loss of antibacterial activity. A further small amount of chrysogenin can be precipitated from the filtrate by saturating

with salt and filtering. From a typical batch of 100 flasks (using the type strain of *P. chrysogenum*) 13 g. of crude precipitate were obtained containing 4.2 % of nitrogen by Kjeldahl. This material was then extracted exhaustively with ether in a Soxhlet apparatus and was thus separated into two fractions: (a) an ether-insoluble fraction containing 8.53 % N, (b) an ether-soluble pigment containing only traces of N.

PURIFICATION OF THE NITROGENOUS SUBSTANCE (PROTEIN).

The crude nitrogenous substance readily dissolved in dilute NaOH and could be roughly titrated by adding excess *N*/10 alkali and back-titrating using phenolphthalein as indicator. Thus 0.1903 g. of the crude material required 4.3 cc. *N*/10 NaOH. For purification, 7 g. of the substance were dissolved in excess dilute (160 cc. *N*/10) NaOH, diluted to 300 cc. and 200 cc. of absolute alcohol added. After filtering, 16 cc. of *N* HCl were added with vigorous shaking and the solution centrifuged. The supernatant highly pigmented layer was poured off. The process was repeated. The protein was then repeatedly shaken with 50 % alcohol (increasing gradually to absolute) and again centrifuged until all the colouring matter had been removed. The material was finally pressed on a Büchner funnel, washed with alcohol and ether and dried. The dried residue weighed 4.1 g., and had an ash content (Schoeller) of 0.54 %. The following figures were obtained by micro-combustion (Schoeller): C, 51.51, 51.76 %; H, 6.85, 6.95 %; N, 12.75, 12.95 %; S, 1.35, 1.32 %. A macro N determination also gave 12.95 % and this figure was used for the subsequent Van Slyke analysis of the protein.

0.3613 g. of this purified material required 3.6 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 1004.

Van Slyke analysis of the nitrogenous product (protein).

The above figures suggest that the substance is either a polypeptide or protein and since, so far as we are aware, no similar nitrogenous product has been isolated from mould metabolism solutions, a detailed study of the nature of the product was embarked on, by means of the Van Slyke nitrogen distribution method [1911; 1915].

Hydrolysis of nitrogenous product. 3.3439 g. of purified and dried product (containing 12.95 % N and 0.433 g. of total N) were boiled in a tared flask under reflux with 61.1620 g. of 20 % hydrochloric acid for 6 hours, the flask being heated in an oil-bath at 130–135°. The hydrolysis was then stopped, the flask cooled and its weight adjusted to the original weight by addition of water.

1 cc. of the fluid was then removed, made up to 10 cc. in a graduated flask and retained for the amino-nitrogen determination by the Van Slyke method. The remainder was re-weighed and the hydrolysis continued for a further period of 3 hours. The hydrolysis was then stopped, the weight adjusted, a

1 cc. sample removed and made up to 10 cc. for the amino-nitrogen determination as before, and the residue re-weighed.

Analysis of hydrolysis fluid. A small amount (2 cc.) of the diluted solution after 6 and 9 hours' hydrolysis gave 2.14 and 2.13 cc. amino-nitrogen respectively. It appeared therefore that the protein was readily hydrolysed, the hydrolysis being complete in 6 hours or less. In two other experiments the hydrolysis was almost complete in the same time using 2*N* H₂SO₄ instead of 20 % HCl. The amino-nitrogen figure (2.14 cc.) corresponds with a total amino-nitrogen of 0.3441 g., and the ratio after hydrolysis of amino/total nitrogen = 79.45 %. After removal of the samples for these determinations, the residue calculated on a weight basis corresponded with the presence of 3.224 g. hydrolysed protein or a total N content of 0.4176 g. This solution of hydrolysed protein was washed into a Claisen flask and evaporated to dryness *in vacuo* at 45°. Water was added and the evaporation continued, thus removing most of the hydrochloric acid. The residue was then dissolved in water and made up to 250 cc.

The total N was determined on two samples, each of 15 cc., by Kjeldahl and was 0.4176 g., agreeing exactly with the value obtained by calculation on a weight basis.

After removal of these samples, the residual solution corresponded to 2.837 g. of hydrolysed protein or a total N content of 0.3675 g., and the remaining determinations were made on this solution.

Ammonia was first determined in the hydrolysate by distillation with lime under reduced pressure. The melanin formed during hydrolysis and absorbed on the excess lime during the ammonia determination was filtered off and the nitrogen content obtained by Kjeldahl. The bases were then precipitated with purified phosphotungstic acid, filtered and washed, following the modified technique of Hartley [1914] and Van Slyke [1915]. The phosphotungstic acid precipitate of the bases was then decomposed by the hydrochloric acid-amylic alcohol-ether method [Van Slyke, 1915], the arginine being determined in the usual way, and the cystine by the Denis modification [1910] of the Benedict method.

Results. Ammonia. The amount of *N*/10 acid neutralised was 17.6 cc. indicating 0.0246 g. amide-nitrogen = 6.69 % of the total N.

Melanin. The amount of *N*/10 acid neutralised was 11.7 cc. indicating 0.0164 g. melanin-nitrogen = 4.46 % of the total N.

Cystine. The BaSO₄ weighed 0.043 g. indicating 0.0129 g. cystine-nitrogen = 3.51 % of the total N.

Arginine. The amount of *N*/10 acid neutralised was 5.8 cc. indicating 0.0324 g. arginine-nitrogen. Applying the cystine correction this reduces to 0.0302 g. arginine-nitrogen = 8.22 % of the total N.

Total nitrogen of bases. The amount of *N*/10 acid neutralised in the Kjeldahl determinations was 23.5 cc. Added to the amounts neutralised by the ammonia evolved in the arginine determination (5.8 cc.) the total acid

neutralised = 29.3 cc. indicating a total N of bases of 0.0820 g. N = 22.31 % of the total N.

Amino-nitrogen of bases. The mean amount of N gas evolved with 2 cc. (of 50 cc.) of solution was 2.73 cc. at 20° and 754 mm. indicating a total amino-nitrogen of bases of 0.0402 g. = 10.93 %.

Total nitrogen of filtrate from bases. The mean amount of N/10 acid neutralised per 25 cc. (of 150 cc.) was 29.5 cc. indicating 0.2478 g. N = 67.42 % of the total N.

Amino-nitrogen of filtrate from bases. The mean amount of nitrogen gas evolved with 2 cc. (of 150 cc.) was 5.3 cc. at 20° and 754 mm. indicating a total amino-nitrogen of filtrate of 0.2282 g. = 62.09 % of the total N.

The results are summarised in Table I.

Table I. *Results of protein analysis in percentages of total nitrogen.*

	Found g.	% of total nitrogen	Corrected for solubility of bases g.
Ammonia-N	0.0246	6.69	—
Melanin-N	0.0164	4.46	—
Cystine-N	0.0129	3.51	0.0155
Arginine-N	0.0302	8.22	0.0334
Histidine-N	0.0313	8.51	0.0351
Lysine-N	0.0076	2.07	0.0081
Amino-N of filtrate from bases	0.2282	62.09	0.2230
Non-amino-N of filtrate from bases	0.0196	5.33	0.0147
Total N	0.3708	100.88	—

The mould protein appears therefore to resemble fairly closely a typical alkali-soluble leaf protein. The following table gives the corresponding figures for the mould protein and the alkali-soluble protein of the alfalfa leaf [Dowell and Menaul, 1921].

Nitrogen content	Protein of alfalfa leaf %	Mould protein %
Total nitrogen	13.6	12.95
NH ₃ -N	6.8	6.69
Melanin-N	7.8	4.46
Arginine-N	11.01	8.22
Histidine-N	6.26	8.51
Cystine-N	0.85	3.51
Lysine-N	5.26	2.07
Monoamino-N	53.53	62.09
Non-amino-N	8.48	5.33

Action of trypsin and pepsin on the nitrogenous product.

The protein nature of the nitrogenous product was finally confirmed by its hydrolysis by trypsin in alkaline and by pepsin in acid medium.

A solution of 1 % protein in 0.5 % Na₂CO₃ was incubated at 37° after addition of trypsin along with controls containing on the one hand similar amounts of protein and carbonate, and on the other of trypsin and carbonate. 1 cc. of solution was removed for determination of amino-nitrogen by Van Slyke's method. The increase of amino-nitrogen (as cc. of nitrogen gas) per cc. of fluid due to the action of trypsin on the protein was from the blank value

of 0.38 cc. nitrogen to 1.08 cc. after 20 hours, and 1.40 cc. after 48 hours. A similar series of values was obtained from the action of pepsin in $N/30$ HCl, but the action was much slower. Thus the blank value of 0.43 cc. nitrogen increased to 0.75 cc. after 48 hours and to 1.05 cc. after 96 hours.

ISOLATION OF CHRYSOGENIN.

The ether-soluble pigment chrysogenin, on removal of the ether was obtained as a transparent glaze on the side of the containing vessel. It was extremely soluble in most organic solvents except light petroleum in which it was insoluble. Attempts to crystallise it by slow evaporation of mixtures of solvents failed, the material always separating on the vessel as a glaze. It could, however, be precipitated from ether solution by light petroleum as a non-crystalline light yellow powder. This powder consisted chiefly of chrysogenin, together with a small amount of a reddish-brown dehydration product, the pigment readily losing water to form this compound. The dehydrated product was, however, not very soluble in benzene, and could be separated by shaking the yellow powder with benzene, filtering and reprecipitating the pigment with light petroleum. After repeating this treatment the product from several batches appeared homogeneous and gave the same analytical figures.

The following values were obtained on micro-combustion (Schoeller): C, 64.75, 65.06 %; H, 6.58, 6.51 %; OCH_3 , nil. $\text{C}_{18}\text{H}_{22}\text{O}_6$ requires C, 64.69 %; H, 6.58 %.

This purified preparation was obtained in micro-crystalline form by dissolving in a very slight excess of $N/10$ NaOH and precipitating by passing a stream of CO_2 . After precipitating in this way twice, filtering, washing and drying, a sample gave the following figures on micro-combustion (Schoeller): C, 64.82, 64.84 %; H, 6.52, 6.54 %; $\text{C}_{18}\text{H}_{22}\text{O}_6$ requires C, 64.69 %; H, 6.58 %.

The analytical figures agree very closely amongst others with those required by the formulae $\text{C}_{15}\text{H}_{18}\text{O}_5$, $\text{C}_{18}\text{H}_{22}\text{O}_6$. The molecular weight (Schoeller) was returned as 470, 490, but the results of titration and preliminary methylation experiments favour the formula $\text{C}_{18}\text{H}_{22}\text{O}_6$ (mol. wt., 334) and in view of possible errors in molecular weight determination by the Rast camphor method this latter value is provisionally adopted.

Properties of chrysogenin.

Chrysogenin is very soluble in ether, benzene, acetone, ethyl alcohol, ethyl acetate and chloroform, but insoluble in light petroleum and water. It gives a characteristic olive-brown colour with FeCl_3 . It is very strongly *l*-rotatory, the specific rotation being $[\alpha]_{5461} - 762^\circ$, $[\alpha]_{5790} - 570^\circ$, and it is thus responsible for the development of the negative rotation in the metabolism medium. It possesses two very weakly acidic groups, and probably does not contain a free carboxyl group. Thus a solution containing 0.1320 g. of pigment, on

titration with $N/10$ NaOH to phenolphthalein, became more and more deeply reddish-brown until it was impossible to see the indicator colour, and the titration was continued using the indicator externally. After addition of 4.0 cc. $N/10$ NaOH (corresponding with an equivalent of 330, $C_{18}H_{22}O_6$ requires 334) the colour of the solution suddenly changed again from dark reddish-brown to light yellow. Further addition of NaOH led to a second increase of the red-brown colour and the end-point came after an addition of 8.22 cc. (corresponding to an equivalent of 160.6, $C_{18}H_{22}O_6$ assumed dibasic requires 167). On passing CO_2 through this neutralised solution the pigment again separated in microcrystalline form. Electrometric titration, kindly carried out by Dr Kekwick in Prof. Drummond's laboratory, showed that two stages of buffering were present, but the groups responsible were so weakly acidic that reliable dissociation constants could not be obtained, and only a rough value, 193, for the lower equivalent was given. The absence of a carboxyl group is also indicated by the fact that the substance does not esterify with ethereal diazomethane.

Chrysogenin on warming with dilute NaOH gives amongst other unidentified products acetaldehyde and sorbic acid. It does not methylate with ether-diazomethane, but gives with ether-nitrobenzene-diazomethane a monomethyl derivative which no longer gives the characteristic olive-brown colour with $FeCl_3$. The pigment reduces readily with Zn and HCl giving a colourless compound dihydrochrysogenin, $C_{18}H_{24}O_6$ (found: C 64.48 %; H 7.10 %; $C_{18}H_{24}O_6$ requires C 64.27 %; H 7.20 %) which readily re-oxidises in air, gives a wine-red colour with $FeCl_3$, and with ether-diazomethane gives a dimethyl derivative which shows a green colour with $FeCl_3$. The above facts suggest that chrysogenin contains a quinol nucleus. Investigation of its structure is reserved for a separate communication.

ANTIBACTERIAL ACTIVITY OF THE METABOLISM FLUID OF FLEMING'S ORGANISM.

A few preliminary experiments confirmed the observations of Fleming [1929] that *Bact. coli* and *H. influenzae* were resistant to the action of "penicillin" whilst *Staph. albus*, *Strept. haemolyticus*, *Strept. pneumoniae* and *N. meningitidis* were susceptible. The pneumococcus (*Strept. pneumoniae*) and Pfeiffer's influenza bacillus (*H. influenzae*) were selected for further experiments, the latter being used as a control.

Technique for determination of antibacterial power.

The metabolism fluid, after passing through a Seitz filter, was incorporated in both fluid and solid media.

Fluid media. Dilutions of the filtrate from 1/10 to 1/5120 falling in geometrical progression were made, using sterile precautions, in 1 cc. quantities of the test media. For the pneumococcus, Hartley's trypsinised horse muscle

broth was used, and for the influenza bacillus heart broth to which had been added 5 % of Fildes's extract [Fildes, 1920]. In each case a control tube was included containing the test media without the addition of metabolism solution.

After the preparation of the media, all the tubes were seeded with one loopful (approx. 1/250 cc.) of an 18 hours' broth culture of the test organism. All were then incubated at 37° for 24 hours and opacity readings taken. No difference between the early and late readings was apparent in a few experiments in which incubation was prolonged for 48 and 72 hours.

Solid media. In each experiment Petri dishes containing 15 cc. of solid media with the metabolism solution in a concentration of 1 in 20 were included, as a check on the detailed titrations in fluid media. The medium used for the pneumococcus was 5 % horse blood agar, and for the influenza bacillus 5 % Fildes's agar. Controls containing no metabolism solution were always included. The plates were streaked with one loopful of the test cultures.

Results of experiments.

The maximum yield of antibacterial substance produced by Fleming's culture in a broth medium appeared about the eighth day, which corresponds with the findings of Fleming. On the other hand, the yield in the synthetic medium was greater and reached its maximum concentration about the 16th to the 20th day. Further incubation resulted at first in a slight loss and later in a rapid loss of antibacterial activity. The following table gives the result of testing the yield over a short period.

Period of growth of Fleming's culture	Dilution of metabolism fluid inhibiting growth of	
	<i>Strept. pneumoniae</i>	<i>H. influenzae</i>
15 days	1/640	1/10
16 "	1/1280	1/10
18 "	1/640	—
22 "	1/640	1/10
24 "	1/160	1/10
31 "	1/160	1/10

In each experiment the control tubes gave a good growth of the test organism. On solid media, the pneumococcus was completely, and the influenza bacillus not at all inhibited at 1/20. It should be noted that this inhibition of *H. influenzae* in low dilutions occurred in relatively few experiments.

All subsequent work was carried out with filtrates from the growth of Fleming's culture on synthetic media.

Storage of filtrate.

Untreated filtrates taken when showing a high concentration of the antibacterial substance can be stored for quite long periods without great loss. A filtrate active against the pneumococcus in a dilution of 1/1280 was stored

in the ice-chest at 0° for 16 weeks without taking precautions as to sterility. Table II shows the reduction observed during that period.

Table II.

Period of storage in ice-chest at 0°	Dilution inhibiting growth of <i>Strept. pneumoniae</i>
Before storage	1 in 1280
After 5 days	1 in 1280
" 7 "	1 in 640
" 13 "	1 in 640
" 14 "	1 in 640
" 21 "	1 in 640
" 12 weeks	1 in 320
" 16 "	1 in 320

This method of storage was, however, inadequate as in two experiments the fluid became infected and antibacterial power was immediately lost. When the fluid was adjusted to p_H 5·6, passed through a sterilised Seitz filter into sterile flasks, and stored at 0°, the antibacterial power remained unchanged for 3 months. In all the experiments the p_H was readjusted to 7·2 before testing for antibacterial activity.

Antibacterial inactivity of chrysogenin and protein.

It has already been shown that careful adjustment of the p_H of the filtered metabolism solution to 3·6 caused the flocculation of most of the protein together with a large portion of chrysogenin. The filtrate, after removal of this precipitate and adjustment of the p_H to 7·2 still contained most of the antibacterial substance, whilst the precipitate on redissolving in dilute NaOH and readjusting the p_H showed no activity at all. Samples of dihydrochrysogenin, purified chrysogenin and protein also showed no activity.

Effect of evaporation of the metabolism fluid on the antibacterial power.

In order to deal successfully with the large bulk of fluid it was necessary to evaporate the fluid without serious loss of activity. Evaporation *in vacuo* at 40–45° of the filtered metabolism fluid to 1/20 its volume at p_H 2 and 3·5, with subsequent readjustment of volume and p_H resulted in complete loss of antibacterial power. Evaporation at p_H 7·2 also led to a fall of inhibitory power from a dilution of 1:1280 to 1:80. Between p_H 5 and 6, however, there appeared to be a region of greater stability and loss of activity was only small (1/640 to 1/320). It was, therefore, possible to adjust the filtered metabolism solution to p_H 3·6, to filter off most of the chrysogenin and protein, to readjust to p_H 5·5 and to evaporate *in vacuo* at this p_H , without great loss of activity.

Extraction of the antibacterial substance with ether.

Fleming regarded penicillin as insoluble in ether, but this was due to the fact that his filtrates were alkaline in reaction.

Extraction with ether at p_H 7·2 partly, and at p_H 2 completely, removes the antibacterial substance. Thus when the metabolism solution (150 cc.) was

extracted three times with ether (100 cc.) at p_H 7.2, the inhibitory power after removal of ether *in vacuo* at 40–45° fell from 1/1280 to 1/320, and after extraction at p_H 2 from 1/1280 to 1/10. When the ether of the ethereal extracts was removed in a current of air, the residue was inactive, but if the ethereal extracts together with 50 cc. water were evaporated *in vacuo* at 40–45°, the residual aqueous phase, after adjusting its volume and p_H showed a considerable portion of the original activity. Thus a solution originally inhibiting the growth of the pneumococcus in a dilution of 1/640 gave an extract showing inhibition at 1/160 ($\pm$ 1/320).

From the above experiments it appears that the antibacterial substance is not identical with either chrysogenin or the protein, and that the greater part of these substances can be removed by suitable adjustment of p_H and filtering without serious loss of antibacterial activity. The antibacterial substance is extremely labile, becoming inactivated during evaporation of an ether solution in a stream of air and by evaporation *in vacuo* at 40–45° in both fairly acid and alkaline solutions. It is, however, more stable at p_H 5–6 and may be extracted with ether from acid solution. The investigation of the isolation and chemical nature of penicillin is being continued.

SUMMARY.

The production, in synthetic media containing glucose and mineral salts with sodium nitrate as a source of nitrogen, of a new yellow pigment, chrysogenin, a new alkali-soluble protein and Fleming's antibacterial substance "penicillin" by several strains in the *P. chrysogenum* series is investigated.

Chrysogenin and the alkali-soluble protein are produced by normal strains of *P. chrysogenum* Thom, *P. notatum* Westling and by Fleming's organism. Neither of these substances is produced by the floccose forms of these organisms. Penicillin is produced only by Fleming's organism and not by the type strains of *P. chrysogenum* Thom and *P. notatum* Westling.

Chrysogenin, $[\alpha]_{5461} - 762^\circ$, is responsible for the *l*-rotation and the olive-brown $FeCl_3$ reaction of the metabolism solution. It contains two very weakly acidic groups and is probably a quinol. The empirical formula $C_{18}H_{22}O_6$ is provisionally adopted. It does not show any antibacterial activity.

The protein is readily soluble in dilute alkali and a Van Slyke analysis shows that it closely resembles a typical alkali-soluble leaf protein. It does not show any antibacterial activity. This protein has thus been synthesised by the fungi from glucose as the sole source of carbon, and sodium nitrate as sole source of nitrogen.

Penicillin, previously obtained by growth of Fleming's species of *Penicillium* only in broth cultures, has now been prepared by growing this organism in a synthetic medium consisting solely of glucose and inorganic salts, thus permitting the hope of readier isolation. A technique for following the antibacterial power is described, and by its means the conditions for storage and

evaporation of active solutions without serious loss of activity, for separation of most of the pigment and protein and for extraction of the active material with ether, are investigated. Penicillin is an extremely labile substance, very readily inactivated by oxidation, evaporation *in vacuo* at 40–45° in both acid (p_H 2 and 3.5) and alkaline (p_H 8) solutions, but is moderately stable at p_H 5–6.

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