CCXVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XL. THE CRYSTALLINE PIGMENTS OF SPECIES IN THE *ASPERGILLUS GLAUCUS* SERIES.

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The species in the *Aspergillus glaucus* series constitute the most common group of all the *Aspergilli* and are characterised by quite definite morphological and biochemical characters. According to Thom and Church [1926, p. 102] the general morphological characters are: "Heads green; stalks smooth (not rough or pitted) septate (articulate); sterigmata 1 series; conidia predominantly elliptical, more than 5μ in long axis (with few exceptions), mostly with roughly pitted wall, rarely smooth; most races or species producing sessile, globose, cleistocarpic yellow to orange perithecia with membranaceous walls, borne above the surface of the substrata and producing disk-shaped ascospores, colourless or nearly so."

The conidial heads are always some shade of green, but the green colour is often masked by the abundant growth of coloured perithecia or by the production of red incrustations on the aerial hyphae by certain species in the series. Macroscopically the colony colour varies widely from brilliant yellow through red to deep chocolate-brown. Blòchwitz [1929] indeed divided the series into 4 pigment groups based on the macroscopic appearance of the colony, *viz. Aspergillus glaucus*, myc. *aureo*, myc. *ferrugineo*, myc. *rubro*, myc. *violaceo*.

Biochemically, the *A. glaucus* series of species forms a distinct group among the *Aspergilli*. Because of their ability to tolerate extremely high osmotic concentrations the organisms are often found on what would generally be called dry matter. They are a common cause of "mildew" in textiles. They tolerate extremely high concentrations of glucose (50 %). The optimum hydrogen ion concentration for growth is in the vicinity of neutrality with little or no growth above p_H 8 and below p_H 4. The optimum temperature range for growth is between 18 and 27° with growth most luxuriant for most species at 24°. Raistrick *et al.* [1931] carried out studies on the biochemistry of various species in the series by the "carbon balance sheet method" and concluded after an analysis of the various metabolic products that, "The *Aspergillus glaucus* group is of particular biochemical interest since the species included in it have a well marked type of metabolism...."

As early as 1897, Meissner reported that the red-orange coloration produced in the mycelium of cultures of a new unnamed species in the *A. glaucus* series was due to the formation of a coloured substance separating from the surface of the mycelial filaments. The later researches of Mangin [1909] led him to conclude that the colouring matters produced by a large number of species in the series

are characteristic. Bainier and Sartory [1911; 1912] emphasised the production of red and yellow colouring matters as diagnostic characters for various species in this series. In these early attempts to isolate the pigments no crystalline products were obtained, the conclusion that the pigments were the same in the various species being based solely on the colour reactions of a mycelial extract. Research along these lines has been sporadic in recent years. Blochwitz [1929] attempted to use the solubilities of the various colouring matters present as a means of separation and claims that there are four mycelial pigments—yellow, red, brown and violet—none of which was, however, obtained crystalline or in a pure condition.

In the present research a successful attempt has been made to isolate these colouring matters, so apparently characteristic of the *A. glaucus* series, in the crystalline state and to determine whether what has hitherto been regarded as a preliminary diagnostic and confirmative character may not actually be an absolute character either of separate species or of the group as a whole.

Three crystalline pigments have been isolated by solvent extraction of the dried mould substance of 25 species of the *A. glaucus* series:

(1) A yellow pigment, which we propose to call *flavoglaucin*, has the empirical formula $C_{19}H_{28}O_3$, is probably derived from the perithecia and is readily soluble in light petroleum. It is elaborated by all 25 species and is therefore series-specific.

(2) An orange pigment, which we have named *auroglaucin*, is less soluble in light petroleum and has the empirical formula $C_{19}H_{22}O_3$. This pigment is elaborated by 15 of the species examined, and these organisms therefore form a definite sub-group in the series.

(3) A red pigment, which we have termed *rubroglaucin*, is least soluble in light petroleum, has the formula $C_{16}H_{12}O_5$ and displays the characteristics of a polyhydroxyanthraquinone. It has been isolated accompanied by flavoglaucin from 4 species.

Two species which produce flavoglaucin, auroglaucin and a second red pigment distinct from rubroglaucin form a transition group between the two groups previously described. Certain other species give rise to dark-coloured pigments which have not yet been obtained crystalline or in a pure condition.

To prove the specificity of flavoglaucin, auroglaucin and rubroglaucin for the species of the *A. glaucus* series, 19 different species, representing practically all the groups in the genus *Aspergillus*, were investigated, and in no case could any of the *glaucin* pigments be isolated or detected.

In order to determine the effect on pigment production of variations in the period and temperature of incubation, p_H and nitrogen and carbon sources, a number of experiments were carried out and are discussed in a later section of this paper. As a result of these experiments a medium has been found on which markedly increased growth and pigment production are obtained, and large quantities of flavoglaucin (191 g.) and auroglaucin (65 g.) have been isolated for future work on the molecular constitution of these pigments.

EXPERIMENTAL.

Species of the Aspergillus glaucus *series used.*

For the purpose of this research a large number of species in the series was collected, so that the study could be made as comprehensive as possible. The 25 species investigated represent practically all known in culture, or identifiable

from previous descriptions, as well as a number of newly isolated species. To Prof. Abbé Biourge of the University of Louvain we are grateful for the following species: *A. profusus* (Bainier) Hann. (London School of Hygiene and Tropical Medicine Catalogue, No. A 27); *A. oriolus* Biourge (A 30); *A. lovaniensis* Biourge (A 31); *A. Dierckxii* Biourge (A 33); *A. argillaceus* Biourge (A 34); *A. mongolicus* Biourge (A 36); *A. fumigatoides* Bainier and Sartory (A 38); *A. echinulatus* (Delacroix) Thom and Church (A 28); and *A. albidus* Spegazzini (A 35). We are grateful to Dr Charles Thom, U.S.A. Department of Agriculture, for a culture of *A. mutabilis* Bainier and Sartory (A 40). From the Centraalbureau voor Schimmelcultures at Baarn, Holland, we have purchased the following well-defined species: *A. glaucus* mut. *alba* Blochwitz (A 16); *A. repandus* Bainier and Sartory (A 17); *A. umbrosus* Bainier and Sartory (A 18); *A. pseudoglaucus* Blochwitz (A 19); *A. glaucus* Link (Ac 36); *A. ferrugineus* Fuckel (Ac 39); *A. mollis* Bainier and Sartory (Ac 58); *A. Scheelei* Bainier and Sartory (Ac 60); and *A. disjunctus* Bainier and Sartory (Ac 61). In addition we are indebted to Mr G. Smith, one of our colleagues, for the contribution of the following species isolated by him from infected cotton: *A. ruber* (Spieckermann and Bremer) Thom and Church (No. 6); *A. repens* (Corda) Sacc. (No. 13); *A. Chevalieri* (Mangin) Thom and Church (No. 50); *A. Amstelodami* (Mangin) Thom and Church (No. 75); and *A. herbariorum* series *minor* (Mangin) Thom and Church (No. 96). *A. novus* Wehmer (Ac 46) was purchased originally in 1923 by Raistrick from Collectio microbiologica, Příbram, Vienna, and is placed by Thom and Church [1926] in the *A. flavus-oryzae* group, but both Blochwitz [1929] on purely morphological grounds, and Raistrick *et al.* [1931] on biochemical grounds, contest the classification and place this organism in the *A. glaucus* series. We have included this species as a member of the *A. glaucus* series, and by the subsequent isolation of both flavoglaucin and auroglaucin can further substantiate the claims of both Blochwitz and Raistrick *et al.*

Species other than those of the A. glaucus *series.*

To show that the pigments isolated are specific for the *A. glaucus* organisms a group of 19 organisms representative of practically all the other types of *Aspergilli* was investigated. These cultures consisted of well-defined strains of the following organisms obtained from the stock collection of fungi of the Division of Biochemistry of the London School of Hygiene and Tropical Medicine. Where possible, strains known to produce yellow, orange or red pigmented growths were employed. The organisms were: *A. fumigatus* Fresenius (No. 47); *A. versicolor* (Vuillemin) Tiraboschi (No. 35 and A 14); *A. ustus* (Bainier) Thom and Church (Ac 84); *A. nidulans* (Eidam) Winter (No. 79); *A. flavipes* (Bainier and Sartory) Thom and Church (No. 89); *A. Wentii* Wehmer (Nos. 2 and 41); *A. ochraceus* Wilhelm (Nos. 69 and 92); *A. auricomus* (Guéguen) (A 41); *A. vitellinus* (Ridley) (A 42); *A. ostianus* Wehmer (Ac 35); *A. terricola* Marchal (Ac 22); *A. tamarii* Kita (No. 72); *A. oryzae* (Ahlburg) Cohn (A 11); *A. flavus* Link (A 12); *A. terreus* Thom (Nos. 3 and 37).

All the cultures used in the investigation were kept on wort agar slopes and before use in the experiments were plated out; single colony isolations were made, and these were used for growing pure cultures for subsequent inoculations.

Preliminary growth experiments on synthetic media.

The species in the *A. glaucus* series are characterised by extremely poor growth on the usual synthetic media but grow luxuriantly on natural media. A series of experiments was therefore carried out to determine the rate of growth

on various synthetic media. For this purpose each of the following six nitrogen sources: sodium nitrate, ammonium sulphate, ammonium nitrate, asparagine, ammonium tartrate and peptone was employed with each of the following five carbon sources: glucose, maltose, sucrose, glycerol and soluble starch.

A quantity of the usual Czapek-Dox medium was made up without carbon or nitrogen source, having the following composition: KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4$, $7H_2O$, 0·5 g.; $FeSO_4$, $7H_2O$, 0·01 g.; distilled water, 1 litre. 50·0 g. of the carbon source and an amount of nitrogen source equivalent in nitrogen to 2·0 g. $NaNO_3$ were added, and the media were solidified by the use of 2 % agar-agar. Plates were poured and single colony inoculations of the five test organisms, *A. Chevalieri* (No. 50); *A. Amstelodami* (No. 75); *A. repens* (No. 13); *A. ruber* (No. 6); and *A. herbariorum* (No. 96) were made. The plates were incubated at 24° and observed daily for rate of growth, as determined by measuring the colony, and for pigment production. It was found that all the sources of carbon used except soluble starch were quite comparable. The combination of glucose and diammonium tartrate gave a growth which was highly pigmented, and whilst not as spreading as some of the others was far more dense. On the glucose-peptone medium growth was abundant but floccose and consisted of an abundance of conidia with few perithecia. Pigmentation on the sodium nitrate and on the asparagine media was much less marked. The ammonium sulphate and ammonium nitrate media produced very poor growths which consisted mainly of conidia. The medium chosen, therefore, for the cultivation of the *A. glaucus* species on a large scale was a modified Czapek-Dox medium containing 5 % glucose as a source of carbon and 0·216 % diammonium tartrate as a source of nitrogen.

Growth of the organisms and isolation of the crude pigments.

For each organism investigated a modified Czapek-Dox medium was made up of the following composition: glucose, 1750 g.; KH_2PO_4, 35·0 g.; KCl, 17·5 g.; $MgSO_4$, $7H_2O$, 17·5 g.; $FeSO_4$, $7H_2O$, 0·35 g.; distilled water, 34 litres. 340 ml. quantities were distributed in each of 100 one litre conical flasks and sterilised by steaming on 3 successive days. A solution containing 75·6 g. diammonium tartrate per litre was sterilised at the same time and after sterilisation of the separate solutions 10 ml. of the diammonium tartrate solution were added, aseptically, to each of the flasks. This precaution was taken to prevent discoloration of the medium, which occurs when the diammonium tartrate is sterilised along with the rest of the medium. Each flask was then sown with a spore suspension of the chosen organism grown on wort agar slopes. Usually 4 flasks were sown from each slope. The flasks were then incubated at 24° until no further apparent decrease in the sugar concentration took place, usually from 4–8 weeks. At the end of this period the mycelium (including perithecia and conidia), which with different species varied in colour from a pure yellow or green to a red-brown, was filtered off, washed carefully with cold distilled water, pressed and dried in a vacuum-oven at 35–40°. The dried mycelium was ground to a fine powder in a coffee-mill, mixed with ether-extracted pumice and exhaustively extracted with light petroleum (B.P. 40–50°) in a Soxhlet apparatus. Light petroleum (B.P. 40–50°) was, in general, found to be the most suitable solvent for the extraction of the pigments, since the crude product contains fewer impurities than after extraction with ether, methyl alcohol, chloroform or acetone. The pigments are more readily soluble in these latter solvents and in the case of organisms of Group I, which will be discussed later, ether serves as the most efficient solvent. Using light petroleum (B.P. 40–50°) the extraction

time varies from 1–2 working days with those organisms producing flavoglaucin and auroglaucin only, to 3–5 working days for those species which produce these pigments together with rubroglaucin or the unnamed red pigment. The total yields of crude pigments varied from 1·27 g. per 100 flasks with *A. Dierckxii* to 23·80 g. per 100 flasks with *A. mongolicus*. The yield varied from 1·69 % to 15·6 % of the weight of the dry mycelium produced.

COMPOSITION OF THE CRUDE PIGMENTS AND ISOLATION FROM THEM OF THE PURE PIGMENTS.

A. Group I.

In this group are included 13 species which produce a crude pigment consisting entirely of a mixture of flavoglaucin and auroglaucin. The yields and characteristics of these pigments are given in Table I.

Table I. *Organisms of Group I.* (*Producing flavoglaucin and auroglaucin.*)

Organism		No. of flasks	Incubation period days	Dry mycelium per flask g.	Total crude pigment mixture g.	Total crude flavoglaucin g.	M.P.* of flavoglaucin °C.	Total crude auroglaucin g.	M.P.* of auroglaucin °C.
Aspergillus Chevalieri	(No. 50)	98	24	0·839	2·52	2·42	105	0·100	151
„ *Amstelodami*	(No. 75)	99	28	1·010	2·75	2·65	103–105	0·100	150–151
„ *novus*	(Ac 46)	98	36	1·280	15·20	11·815	102–103	3·385	152
„ *repens*	(No. 13)	98	40	0·928	4·18	3·285	102–103	0·900	152
„ *glaucus*, Link	(Ac 36)	98	33	1·225	9·47	7·80	101–103	1·670	151
„ *Scheelei*	(Ac 60)	96	39	1·031	8·10	7·525	99–102	0·575	151–151·5
„ *mollis*	(Ac 58)	98	31	1·306	6·11	5·78	98–100	0·330	151–152
„ *argillaceus*	(A 34)	99	48	0·879	5·63	4·72	99–101	0·910	148–150
„ *Dierckxii*	(A 33)	95	41	0·789	1·27	1·175	97	0·095	147–149
„ *profusus*	(A 27)	98	48	1·104	5·41	4·760	101	0·650	148
„ *fumigatoides*	(A 38)	97	32	1·010	5·05	4·20	100	0·850	149·5–151
„ *disjunctus*	(Ac 61)	99	42	1·032	3·02	2·28	102	0·740	151
„ *pseudoglaucus*	(A 19)	99	39	1·025	2·67	2·49	105	0·180	146

* Mixed melting-points not depressed on admixture with pure sample.

An average sample of the crude pigment sintered at 85–90° and melted indefinitely from 120° upwards. Its solubilities in various solvents showed that it consisted of flavoglaucin which is insoluble in water, sparingly soluble in cold but moderately soluble in hot light petroleum, and freely soluble in cold ether, methyl alcohol, ethyl alcohol, chloroform, acetone, benzene and ethyl acetate; and auroglaucin which is almost insoluble in cold light petroleum, slightly soluble in cold, but readily in hot, methyl or ethyl alcohol, freely soluble in ether, acetone or ethyl acetate.

In order to obtain pure samples of flavoglaucin and auroglaucin the difference in their solubilities in light petroleum was first utilised as a means of preliminary separation. For example, 2·75 g. of the crude pigment mixture from *A. Amstelodami* were suspended in 85 ml. hot (B.P. 50–60°) light petroleum and boiled. The greenish yellow solution was filtered from the orange residue (0·10 g.). The first crop of crystals consisted of 0·65 g. of brilliant yellow feathery needles, M.P. 95–97°. These were recrystallised twice from 90 ml. and 50 ml. (B.P. 50–60°) light petroleum yielding 0·215 g. of lemon-yellow long feathery needles of flavoglaucin, M.P. 105° (sharp, without decomposition), which was not altered by further recrystallisation.

The crude orange pigment remaining after boiling with light petroleum was recrystallised from hot methyl alcohol. 0·77 g. of crude material from *A. disjunctus* (sinters at 88°, melts indefinitely from 120° upwards) was crystallised from 50 ml. methyl alcohol. The first crop consisted of 0·65 g. of slender needles melting at 143–144°; second crop, 0·09 g., M.P. 146°. Recrystallisation of the first crop from 40 ml. methyl alcohol yielded 0·40 g. of slender orange hairy needles of auroglaucin, M.P. 152° (sharp, without decomposition), which after repeated crystallisation from methyl alcohol still melted at 152°.

It was often found extremely difficult to separate the last traces of auroglaucin from the flavoglaucin and to crystallise the flavoglaucin by the method described above. The following procedure was therefore utilised to effect complete separation. 2·27 g. of a crude mixture of flavoglaucin and auroglaucin (M.P. sinters 85–90°, melts indefinitely at 120° upwards) was dissolved in 25 ml. of hot methyl alcohol. The first crop of crystals obtained consisted of 0·65 g. of auroglaucin, M.P. 148°. The mother-liquor was cooled in ice-water and a second crop consisting of 0·055 g., M.P. 142–144°, was collected. Distilled water was added until the mother-liquor was turbid, and the solution was warmed until the precipitate dissolved. The process was repeated. The residual auroglaucin separated as a golden oil. When no further oil separated out the aqueous methyl alcohol solution was allowed to cool slowly, the flavoglaucin separating out on standing.

Analysis and properties of flavoglaucin. Flavoglaucin crystallises in lemon-yellow, long feathery needles and melts at 105° without decomposition. Found: C, 74·87, 75·07; H, 9·00, 8·91%; mol. wt. (cryoscopic in camphor), 299, 306. Contains no methoxyl or nitrogen. $C_{19}H_{28}O_3$ requires C, 75·00; H, 9·22%; mol. wt. 304. (Analyses by Schoeller, Berlin.) It dissolves sparingly in cold, and moderately in hot, light petroleum to give a brilliant yellow-green solution. It dissolves readily in ether, acetone, methyl and ethyl alcohols, glacial acetic acid and ethyl acetate. It is insoluble in hot or cold water but moderately soluble in hot 80 % aqueous methyl or ethyl alcohol.

It gives the following colour reactions: With cold concentrated sulphuric acid a beautiful intense red colour is produced which blackens on heating. The solid is not immediately soluble in cold aqueous NaOH but dissolves slowly to give an orange-coloured solution which fades to pale yellow.

With cold concentrated nitric acid the solid dissolves slowly to give an orange solution which is unchanged on heating. If the nitric acid solution is treated with aqueous NaOH a yellow solution is obtained which turns orange on the addition of an excess of NaOH.

An alcoholic solution of flavoglaucin was used for the following tests:

(*a*) The addition of an aqueous solution of NaOH gives an orange-red colour which rapidly fades to yellow; sodium carbonate gives a transient pale orange; $NaHCO_3$ gives a faint orange and borax solution no coloration. Ammonia (concentrated) gives a deep red colour which fades slowly to orange-brown.

(*b*) Aqueous ferric chloride gives only a faint yellow-orange colour.

(*c*) No coloration is produced upon the addition of aqueous solutions of nickel sulphate, potassium alum or magnesium chloride.

(*d*) Dilute aqueous permanganate solution is decolorised, more slowly than with an alcoholic solution of auroglaucin.

(*e*) With 2:4-dinitrophenylhydrazine in aqueous HCl a red precipitate is immediately produced.

Analysis and properties of auroglaucin. Auroglaucin crystallises in slender orange needles, M.P. 152°, without decomposition. (Found: C, 76·52, 76·40;

H, 7·57, 7·43 %; mol. wt. (cryoscopic in camphor), 297, 298; contains no methoxyl or nitrogen. $C_{19}H_{22}O_3$ requires C, 76·50; H, 7·44 %; mol. wt. 298.) It dissolves very sparingly in cold and sparingly in hot light petroleum. It is easily soluble in ether and acetone, sparingly soluble in cold and moderately soluble in hot methyl or ethyl alcohol to give a yellow solution. It is insoluble in hot or cold water.

The solid gives the following colour reactions: With concentrated sulphuric acid in the cold auroglaucin is charred. It is not immediately soluble in cold aqueous NaOH but dissolves slowly to give an orange-red solution. With concentrated nitric acid the solid dissolves slowly to give an orange-brown solution which turns yellow on warming and upon the addition of NaOH turns pale straw-yellow.

A dilute alcoholic solution of auroglaucin which is brilliant yellow in colour gives the following colour reactions:

(*a*) With aqueous NaOH a deep red coloration with a tinge of violet is produced which fades to an orange-brown on standing. Ammonia (concentrated) gives an intense violet which fades slowly; dilute ammonia (2*N*) gives a transient violet. Sodium carbonate gives an orange solution which turns red-violet on standing; $NaHCO_3$ gives a pale yellow colour which turns pink and then fades. Aqueous borax solution produces an orange colour which turns to brilliant red-violet on standing and then slowly fades to pale brown.

(*b*) Aqueous ferric chloride solution gives an orange-brown colour.

(*c*) No colour is produced by aqueous solutions of nickel sulphate, magnesium chloride or potassium alum.

(*d*) No precipitate is formed on the addition of an aqueous HCl solution of 2:4-dinitrophenylhydrazine.

(*e*) A dilute aqueous solution of permanganate is rapidly decolorised.

Further extraction of the mycelia of Group I organisms. The mycelia of the 13 organisms of Group I after exhaustive extraction with light petroleum in the process of extracting flavoglaucin and auroglaucin were then extracted successively with ether, acetone, chloroform, methyl alcohol, ethyl alcohol and ethyl acetate, but in no case could any further pigment extracts be obtained. These organisms constitute therefore a definite biochemical sub-group in the *A. glaucus* series characterised by the presence of only the two pigments—flavoglaucin and auroglaucin.

B. Group II.

A. oriolus (A 30) and *A. albidus* (A 35) are unique among the organisms investigated in that they alone produce in perceptible amounts, in addition to flavoglaucin and auroglaucin, an unnamed red pigment which has not yet been obtained in a pure condition.

A batch of 100 flasks of *A. oriolus* was sown on the modified Czapek-Dox medium given on p. 1643. The flasks were incubated at 24° for 29 days. The weight of washed, vacuum-dried mycelium was 132·5 g. The mycelium was ground in a coffee-mill and extracted with light petroleum for approximately 24 hours. The yield of crude pigment thus obtained was 13·53 g., corresponding to 10·2 % of the mycelium formed or 2·42 % of the sugar metabolised. The crude mixture was suspended in 100 ml. of cold methyl alcohol and filtered from the red insoluble residue which consisted of 0·60 g. of a pale red product, obviously a mixture of red and colourless material. These two substances have not been satisfactorily separated owing to their similar solubilities in all solvents. The methyl alcohol solution was evaporated to about 20 ml. at atmospheric pressure and allowed to

cool slowly. The first crop of crystals consisted of 0·13 g. of orange needles, M.P. 143–145°. Recrystallisation from 7 ml. of methyl alcohol gave 0·075 g. of feathery needles, M.P. 150–151°, which on admixture with pure auroglaucin (M.P. 152°) melted at 150–152°. Auroglaucin is thus one of the constituents of the crude pigment of *A. oriolus*. The methyl alcohol mother-liquors from the first crop of auroglaucin were evaporated to dryness and the residual flavoglaucin was crystallised from light petroleum, yield about 12 g.

A. albidus was treated in a similar fashion. From 97 flasks incubated for 43 days, 123·2 g. of mycelium were obtained which yielded 7·45 g. of crude pigment, from which 7·24 g. of flavoglaucin and 0·21 g. of auroglaucin were isolated. Owing to the small amount present, the red pigment could not be separated but its presence was detected by the formation of characteristic lakes with aluminium and magnesium oxides.

Unlike the organisms of Group I, the mycelia of *A. oriolus* and *A. albidus*, after exhaustive extraction with light petroleum, still retain their original red-orange colour, and upon further extraction with either methyl alcohol, ethyl alcohol, chloroform or acetone give a deep cherry-red extract which upon evaporation gives a black amorphous material which has evaded all attempts at crystallisation.

These two organisms therefore constitute a second group in the pigment classification of the *A. glaucus* series, forming a transition group between groups I and III.

C. Group III.

The remaining ten organisms investigated fall into a group characterised by the presence of at least one red pigment soluble in light petroleum associated with the presence of flavoglaucin and the absence of auroglaucin. The yields and characteristics of these pigments are given in Table II.

Table II. *Organisms of Group III. (Producing flavoglaucin and a red pigment.)*

Organisms		No. of flasks	Incubation period days	Mycelium per flask g.	Total crude pigment mixture g.	Total crude flavoglaucin g.	M.P.* of flavoglaucin °C.	Total red pigment g.	M.P.* of rubroglaucin °C.
Aspergillus ruber	(No. 6)	97	28	0·878	4·200	3·950	99–100	0·250	172–173
„ *ferrugineus*†	(Ac 39)	95	51	0·958	3·340	3·280	97	Trace	173–174
„ *glaucus* mut. *alba*	(A 16)	91	51	1·412	20·050	18·370	104	1·680	173–175
„ *umbrosus*	(A 18)	96	41	1·860	5·120	Trace	102	Trace	170–172
„ *mongolicus*	(A 36)	99	57	2·120	23·800	21·700	103–105	2·100	Mixed M.P. 167–170
„ *echinulatus*	(A 28)	100	52	0·880	1·240	0·570	95–96	0·670	—
„ *lovaniensis*	(A 31)	93	62	1·230	7·690	7·430	99–100	0·260	—
„ *herbariorum*	(No. 96)	100	56	1·360	1·750	0·750	98	1·00	—
„ *mutabilis*	(A 40)	98	33	1·620	3·920	0·870	104–105	3·050	—
„ *repandus*†	(A 17)	98	58	0·809	3·000	2·940	96	0·060	—

* Mixed melting-point not depressed on admixture with authentic flavoglaucin or rubroglaucin.
† Produces a red pigment, M.P. 193–195°.

The red pigment of *A. ruber* (No. 6), *A. ferrugineus* (Ac 39), *A. glaucus*, mut. *alba* (A 16) and probably *A. umbrosus* (A 18) has been isolated in the pure crystalline condition and has been named *rubroglaucin*.

In the case of *A. umbrosus*, as with most of the members of this group, the bulk of the light petroleum extract consists of flavoglaucin, together with small

amounts of a colourless impurity and of a red pigment. By slow crystallisation of the mixture of red and colourless products of *A. umbrosus* from ethyl acetate, a mixture of almost colourless needles and ruby-red crystals was obtained. The melting-point of the mechanically separated red crystals was 170–172°, but upon admixture with a pure sample of rubroglaucin, M.P. 172–173°, the melting-point was depressed to 167–170°. This may have been due to the presence of a small amount of the colourless product.

A. ferrugineus (Ac 39) produces in addition to flavoglaucin and rubroglaucin a third pigment, also red, which differs from rubroglaucin in melting-point, 193–195°, and in colour reactions. The melting-point of a mixture of the two shows a depression. The same red pigment, as indicated by similar melting-point, non-depression of the melting-point upon admixture of the two and similarity of colour reactions, was isolated from *A. repandus* (A 17), but in this case no rubroglaucin could be detected. As was the case with *A. ferrugineus*, only a trace could be isolated and after purification there was insufficient material for analysis. It is hoped that in a later communication the isolation and properties of this second red pigment will be described.

The remaining species produce flavoglaucin and a red pigment in each case, but it has been impossible to isolate these red pigments in a state of purity, owing either to the small amounts present or to the presence of large amounts of impurities which could not be selectively removed. It is quite certain, however, that the red pigment in all these remaining organisms is not rubroglaucin since the colour reactions vary from species to species, especially the coloured lakes produced with aluminium and magnesium oxides. Group III represents therefore a general group which contains, probably, a number of sub-groups.

For the isolation of pure rubroglaucin the mycelium from 100 flasks of *A. ruber* grown on the modified Czapek-tartrate medium and amounting to 85·2 g. was extracted thoroughly with light petroleum (B.P. 40–50°) for approximately 40 hours, and the reddish yellow mixture of pigments was collected. 4·20 g. of crude material were suspended in 25 ml. methyl alcohol in which flavoglaucin is readily soluble and rubroglaucin only very sparingly soluble. The yellow solution was filtered off and the red residue, 0·25 g., M.P. 162–164°, was collected and crystallised from 20 ml. of ethyl acetate. The first crop consisted of 0·15 g. of short, blunt, ruby-red needles, M.P. 169–170°. This was then twice recrystallised from ethyl acetate and once from butyl alcohol, the melting-point remaining unchanged at 172–173°.

Analysis and properties of rubroglaucin. Rubroglaucin crystallises in short, blunt, ruby-red needles, M.P. 172–173°. (Found on material dried *in vacuo* at 60–65°: C, 67·16, 67·16; H, 4·34, 4·30 %; mol. wt. 336, 340, cryoscopic in camphor; $C_{16}H_{12}O_5$ requires C, 67·58; H, 4·25 %; mol. wt. 284·1 Schoeller, Berlin.)

Rubroglaucin is slightly soluble in methyl alcohol, ethyl alcohol and ether; moderately soluble in cold butyl alcohol, benzene, acetone and ethyl acetate, and readily soluble on heating. It is very slightly soluble in light petroleum and insoluble in hot or cold water. Its solution in organic solvents is orange-coloured with a slight green fluorescence. An alcoholic solution gives the following colour reactions:

(*a*) The addition of an aqueous solution of ammonia or sodium hydroxide produces an intense red-violet colour which is destroyed on the addition of aqueous sodium hypochlorite solution.

(*b*) The addition of aqueous nickel sulphate, magnesium chloride, potassium alum, lead acetate or borax solution produces a rose-pink colour.

(*c*) Aqueous ferric chloride solution produces a faint green colour.
(*d*) Aqueous copper sulphate solution produces a violet-pink colour.
(*e*) Upon shaking an aqueous alcoholic solution of rubroglaucin with magnesium oxide a deep blue-violet colour is produced; with calcium oxide a violet colour; with aluminium oxide a blue colour.

The solid produces an intense violet solution in concentrated H_2SO_4 and a beautiful red-violet in NaOH. It is insoluble in aqueous $NaHCO_3$ and aqueous Na_2CO_3.

Experiments with Aspergilli *other than members of the* A. glaucus *series.*

In order to show that flavoglaucin, auroglaucin and rubroglaucin are characteristic of and specific for the species in the *A. glaucus* series, an experiment was undertaken in which nineteen species of *Aspergillus* other than members of the *A. glaucus* series were examined. The organisms were selected so as to be fully representative of the entire genus *Aspergillus,* and where possible, species producing yellow to red coloured colonies were employed. The organisms, listed on p. 1642, were grown in batches of 10 flasks on the usual modified Czapek-ammonium tartrate medium as were the *A. glaucus* organisms, and under the same conditions. The mycelia were filtered off, washed, pressed, dried *in vacuo* and thoroughly extracted with light petroleum (B.P. 40–50°). Each of the extracts was treated in the same manner as with species in the *A. glaucus* series. As a further test, since in no case was any pigmented precipitate obtained on evaporating the light petroleum extract to a small volume, the evaporation was carried to dryness, and the residue, if any, was dissolved in methyl alcohol. To a portion of the methyl alcohol solution was added an aqueous HCl solution of 2:4-dinitrophenylhydrazine, which would produce a red precipitate if flavoglaucin were present. Water was added to another portion in order to precipitate any flavoglaucin or auroglaucin. None of the 19 organisms appears to produce any of the glaucin pigments, and it seems certain therefore that the pigments are specific for the species in the *A. glaucus* series.

Production of flavoglaucin and auroglaucin under varying cultural conditions.

I. *Period of incubation.*

To follow the quantitative production of flavoglaucin and auroglaucin 150 flasks, each containing 350 ml. of the usual Czapek-ammonium tartrate medium, were made up, sterilised and sown with equal amounts of a spore suspension of heavily sporing cultures of *A. novus* (Ac 46). This species was chosen since it rapidly gives a good growth, and the only pigments it produces are flavoglaucin and auroglaucin, both of which are formed in good yield. The flasks were incubated at 24° in the dark. Batches of 8 flasks in the early stages of growth and of 5 flasks in the later stages were removed periodically, and the mycelium was filtered off, washed, pressed, dried *in vacuo* and weighed. The dry mycelium was ground to a fine powder and thoroughly extracted with ether. The ether extract was evaporated to dryness and its weight determined after drying to constant weight *in vacuo* over concentrated H_2SO_4. This product is termed the *total crude extract.* This was then rubbed up with light petroleum (50–60°) and the volume made up to approximately 70 ml. of light petroleum per g. of extract. After boiling on a hot bath for 8–10 minutes the orange residue was filtered off, dried *in vacuo* and weighed. This is called *crude auro-*

glaucin. The difference between the total crude extract and the crude auroglaucin is taken as the *crude flavoglaucin*. It contains, of course, in addition to flavoglaucin a certain amount of fat and other products soluble in light petroleum.

The results of analyses carried out over a period of 75 days are given in Table III. It will be seen that growth increases until the forty-sixth day and

Table III. *Growth and pigment production by* Aspergillus novus (*Ac 46*) *on the Czapek-tartrate medium**.

Type of growth	Period of incubation days	Weight of mycelium g. per flask	Crude extract g. per flask	Crude extract per g. mycelium mg.	Crude flavoglaucin per flask g.	Crude auroglaucin per flask g.	Ratio flavoglaucin/auroglaucin
Groundwork of hyphae	5	0·074	0·0074	99·3	0·0036	0·00375	0·96
Formation of perithecia	7	0·128	0·0203	190·9	0·0155	0·005	3·1
	9	0·148	0·0365	247·5	0·030	0·0066	4·55
	11	0·189	0·064	338·3	0·052	0·012	4·33
Formation of conidia	13	0·313	0·066	211·1	0·066	Trace	—
Complete normal growth	15	0·476	0·091	192·4	0·071	0·020	3·55
	17	0·679	0·126	185·1	0·095	0·031	3·07
	19	0·862	0·155	179·4	0·110	0·044	2·50
	23	0·965	0·205	212·5	0·148	0·057	2·60
	27	1·063	0·217	204·5	0·160	0·058	2·76
	33	1·230	0·234	189·9	0·178	0·056	3·18
	35	1·287	0·255	197·9	0·185	0·070	2·64
	38	1·242	0·250	201·4	0·163	0·087	1·87
	42	1·366	0·259	189·9	0·183	0·076	2·41
	46	1·372	0·270	198·4	0·193	0·078	2·47
Period of autolysis	50	1·172	0·228	194·8	0·168	0·060	2·80
	54	1·028	0·206	200·8	0·154	0·052	2·96
	58	1·066	0·206	190·5	0·140	0·063	2·22
	65	1·093	0·212	190·4	0·153	0·059	2·59
	75	1·005	0·210	208·9	0·149	0·061	2·44

* Temperature of incubation 24° in all cases.

production of crude pigment increases correspondingly. During the first 5 days of growth when the groundwork of hyphae is being laid down, there is formed only a small percentage of crude pigment. From the fifth to eleventh days—during the period of abundant perithecia formation—the amount of pigment increases to almost 34 % of the total weight of mould substance. After this period, from the eleventh to thirteenth days, when conidia formation takes place almost exclusively, there is no additional production of pigment. From the thirteenth day onwards the percentage of pigment in the mycelium remains at a fairly constant level over the entire period of the experiment, being between 179·4 and 212·5 mg. per g. of mycelium, the average being 196·0 mg.

During the period when growth falls off (after 46 days) and there is a decrease in the weight of mould substance, due probably to autolysis, there is a corresponding decrease in the total crude extract so that the percentage amounts of pigment in the mycelium remain about the same as for the earlier figures.

During the whole period of growth there is a steady increase in the amounts of both flavoglaucin and auroglaucin, the latter being produced only very slowly and in smaller quantities. In the early stages of growth the ratio of flavoglaucin to auroglaucin rises very rapidly from about 1·0 to 4·5, but soon the ratio falls to a figure between about 2·0 and 3·0. It is interesting to note that from the eleventh to the thirteenth days, when abundant conidia formation began abruptly,

there was apparently no change in the total pigment production but practically all of the auroglaucin had disappeared. It is thus possible that auroglaucin has some function connected with conidia formation. The analytical figures seem to give very little indication as to whether the pigments are produced independently or whether one is the precursor of the other.

II. *Temperature of incubation.*

To determine the optimum temperature for pigment production by *A. novus* (Ac 46), a quantity of the usual Czapek-ammonium tartrate medium was made up and distributed in one litre flasks in 350 ml. quantities. The flasks were sterilised and sown with equal amounts of a heavy spore suspension of the organism. Batches of 5 flasks were incubated at room temperature (17–19°), 24°, 27°, 32° and 37°. After 35 days' incubation the growths were filtered off, washed, dried and weighed, ground to a fine powder and extracted, and the pigments were isolated as described on p. 1649. From the results of the experiment given in Table IV it will be seen that optimum growth as well as optimum pigment production is given at 24°. There is no growth at 32° and 37° but after 35 days at these temperatures, upon further incubation at room temperature, the spores germinated quite readily. It appears that incubation temperatures above 24° impede growth and pigment production to a greater extent than temperatures below 24°.

Table IV. *Effect of temperature on the growth and pigment production of* Aspergillus novus (*Ac 46*)*.

Temperature of incubation °C.	Weight of mycelium per flask g.	Weight of crude extract per flask g.	Crude extract per g. of mycelium mg.	Crude flavo-glaucin per flask g.	Crude auro glaucin per flask g.
17–19 (room temp.)	1·091	0·189	173·2	0·132	0·060
24	1·287	0·255	197·9	0·185	0·070
27	0·807	0·078	96·6	0·078	Trace
32	No growth	—	—	—	—
37	No growth	—	—	—	—

* Period of incubation in all cases was 35 days.

III. p_H *of the medium.*

350 ml. quantities of the usual Czapek-tartrate medium were distributed among each of 40 one litre conical flasks and sterilised. After sterilisation the appropriate amounts of sterile solutions of either *N* HCl or *N* NaOH were added to bring the p_H values to 3·0, 4·0, 6·0, 7·0, 8·0 and 9·0. The p_H of the unadjusted medium was 5·4. The flasks were sown with a heavy spore suspension of *A. novus* (Ac 46) and incubated at 24° for 35 days.

Upon analysis of growth and pigment production in the manner described previously the results given in Table V were obtained. It appears that the unadjusted medium is the best in so far as pigment production is concerned. The yield of mould substance is greater at p_H 6 and p_H 7 but the pigment yield is smaller. Below p_H 5·4 and above p_H 8 growth and pigment production are very poor. At p_H 4 the formation of perithecia is greatly impeded, the growth being predominantly conidial.

The final p_H values of the media were determined, and it was found that in every case where growth occurred the medium tended to become acid and seemed to approach a fairly constant figure of about p_H 3·2–3·5.

Table V. *Effect of hydrogen ion concentration upon the growth and pigment production of* Aspergillus novus (*Ac 46*)*.

Initial p_H	Weight of mycelium per flask g.	Weight of crude pigment extract per flask g.	Crude extract per g. mycelium mg.	Crude flavoglaucin per flask g.	Crude auroglaucin per flask g.	Final p_H
3·0	No growth	—	—	—	—	—
4·0	0·795	0·058	74·8	0·058	Trace	3·0
5·4	1·287	0·255	197·9	0·185	0·070	3·2
6·0	1·494	0·198	132·4	0·150	0·048	3·6
7·0	1·476	0·233	157·6	0·167	0·066	3·5
8·0	1·260	0·160	127·6	0·122	0·038	3·5
9·0	0·206	0·035	171·2	0·032	0·004	6·0

* Period of incubation in all cases was 35 days at 24°.

IV. *Nitrogen concentration of the medium.*

To determine the effect of varying the total available nitrogen upon growth and pigment production, quantities of the following two media without a source of nitrogen were made up: (*a*) glucose, 50 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.; water to a final volume of 1 litre after addition of ammonium tartrate solution: (*b*) as (*a*) with 100 g. glucose in place of 50 g. 30 one litre conical flasks, each containing 340 ml. of medium (*a*) and 30 flasks containing 340 ml. of medium (*b*) were made up and sterilised. Each group was divided into 6 lots of 5 flasks each and to each were added 10 ml. of the appropriate sterile diammonium tartrate solution to give nitrogen concentrations equivalent to 1·0, 2·0, 4·0, 6·0, 8·0 and 10·0 g. of $NaNO_3$ per litre (1·0 g. $NaNO_3$ equivalent to 1·08 g. diammonium tartrate). The flasks were sown with equal amounts of a heavy spore suspension of *A. novus* (Ac 46) and incubated at 24° for 35 days. After this period the mycelia were filtered off, washed, dried *in vacuo* and weighed. The dried, powdered mycelium was thoroughly extracted and the pigments were separated as in the previous experiments. The results are given in Table VI.

Table VI. *Effect of variation in available diammonium tartrate upon the growth and pigment production of* Aspergillus novus (*Ac 46*)*.

Ammonium tartrate per litre of medium g.	Weight of mycelium per flask g.	Weight of crude pigment extract per flask g.	Crude extract per g. mycelium mg.	Crude flavoglaucin per flask g.	Crude auroglaucin per flask g.	Apparent residual glucose %
		1. Glucose content 5 %.				
1·08	0·807	0·097	119·7	0·070	0·027	3·27
2·16	1·287	0·255	197·9	0·185	0·070	2·21
4·32	1·587	0·303	190·9	0·224	0·079	1·29
6·48	2·043	0·538	263·5	0·427	0·112	0·368
8·64	2·185	0·813	371·9	0·672	0·140	0·368
10·80	2·310	0·997	431·7	0·764	0·233	0·368
		2. Glucose content 10 %.				
1·08	0·909	0·160	176·3	0·106	0·054	6·84
2·16	1·636	0·244	149·4	0·169	0·075	5·48
4·32	2·575	0·350	135·8	0·275	0·075	3·86
6·48	3·175	0·526	165·9	0·358	0·168	2·61
8·64	3·417	0·691	202·1	0·489	0·202	1·93
10·80	3·591	0·990	275·7	0·830	0·160	1·65

* Period of incubation in all cases was 35 days at 24°.

The most striking result obtained is that by increasing the available diammonium tartrate there occurs a very marked increase in growth, pigment production and glucose metabolised. With 10 % glucose, growth increases to a much greater extent than with 5 % glucose, the added increase in weight being essentially due to conidia formation since there is not a correspondingly great increase in pigment production. Since the weights of the total crude extracts are practically the same using 5 % or 10 % of glucose, it is obviously far more economical to use the lower concentration of sugar for the preparation of the pigments.

Using 5 % glucose and 10·8 g. per litre of diammonium tartrate the yield of pigment corresponds to 43·2 % of the total weight of mould substance and is equivalent to 5·64 % of the glucose metabolised. In spite of this remarkable increase in pigment production the ratio of flavoglaucin to auroglaucin still remains in the vicinity of 2·5–3·0. On this medium conidia formation is almost entirely absent, the growth being practically entirely perithecial.

A comparison of the growth obtained in these experiments with that obtained by Raistrick *et al.* [1931] on ordinary Czapek-Dox medium, containing 2·0 g. $NaNO_3$ as a source of nitrogen and using the identical organism in each case, shows that by the present modification in the medium, growth has been increased almost elevenfold, and three times the quantity of glucose has been metabolised.

V. *Concentration of glucose in the medium.*

Batches of flasks of the usual Czapek-tartrate medium containing respectively per batch 2·5, 5·0, 7·5, 10·0, 12·5, 15·0 and 20 % of glucose were made up and sterilised. Five flasks from each batch were sown with a heavy spore suspension of *A. novus* (Ac 46) and incubated at 24° for 35 days. The mycelia were then filtered off, washed, dried *in vacuo*, weighed, powdered and thoroughly extracted with ether and the pigments separated as previously described. From the results of the experiment given below in Table VII it appears that whilst growth

Table VII. *Effect of variation in glucose concentration upon the growth and pigment production of* Aspergillus novus *(Ac 46)**.

% of glucose	Weight of mycelium per flask g.	Weight of crude pigment extract per flask g.	Crude extract per g. mycelium mg.	Crude flavoglaucin per flask g.	Crude auroglaucin per flask g.	Apparent residual glucose %
2·5	0·901	0·190	210·8	0·141	0·049	0·00
5·0	1·287	0·255	197·9	0·185	0·070	2·21
7·5	1·274	0·206	170·3	0·137	0·069	4·41
10·0	1·636	0·244	149·4	0·169	0·075	5·48
12·5	1·697	0·231	136·3	0·158	0·074	8·46
15·0	1·740	0·250	143·7	0·166	0·084	10·26
20·0	2·179	0·348	159·5	0·287	0·061	14·34

* Period of incubation in all cases was 35 days at 24°.

is increased when the glucose concentration is above 7·5 %, the total pigment production does not increase until 20 % glucose is available. It appears that with an increase in the available glucose there is a corresponding decrease in the percentage of pigment formed, due to the relative increase in conidia formation with the increase in glucose concentration.

VI. *Large scale preparation of flavoglaucin and auroglaucin.*

From the results of the previously described experiments on growth and pigment production by *A. novus* (Ac 46) the following modified medium was adopted for large scale production of flavoglaucin and auroglaucin: Solution I, glucose, 1750·0 g.; KH_2PO_4, 35·0 g.; KCl, 17·5 g.; $MgSO_4, 7H_2O$, 17·5 g.; $FeSO_4, 7H_2O$, 0·35 g.; distilled water, 32·5 litres. Solution II, diammonium tartrate, 378·0 g.; water, 2·5 litres. The solutions were made up separately, and 325 ml. of solution I were distributed in each of 100 one litre conical flasks and sterilised by steaming on 3 successive days at 100° for 1 hour. The diammonium tartrate solution was sterilised in the same way, and after sterilisation 25 ml. were added aseptically to each of the flasks. 385 flasks were made up in this manner and sown with a heavy spore suspension of *A. novus* (Ac 46). After incubation at 24° for from 30 to 35 days the flasks were removed, and the mycelium was filtered off, washed, dried and weighed. 767 g. of dried mycelium so obtained were powdered and thoroughly extracted with ethyl ether. The crude dry extract, 315 g., was then boiled with light petroleum (50–60°) and the insoluble crude auroglaucin filtered off (65 g.). The light petroleum solution was evaporated, and from this there separated 191 g. of crude flavoglaucin. The light petroleum mother-liquors on evaporation gave 59·0 g. of "crude fat."

DISCUSSION.

Qualitative nature of pigment production. Earlier investigators have been of the opinion that pigment production by species in the *A. glaucus* series is quantitative as well as qualitative in the sense that any particular species in the series always produces the same pigments in the same final amounts. An analysis of Tables I and II might possibly lead one to conclude that there is a quantitative as well as a qualitative basis for the differentiation of the separate species in the series. It was found, however, in the present investigation that a comparison of the figures obtained in preliminary small scale experiments with those obtained in the final large scale experiments on the same organisms and under the same conditions showed no marked correlation. Some of the organisms gave in both cases comparable figures, whilst others showed an equally striking difference. It appears, therefore, that the present investigation indicates only a qualitative specificity of the pigments for the entire series and groups within it.

Final p_H *and pigment production.* In the experiment on the effect of p_H on the growth of and pigment production by *A. novus* (Ac 46), p. 1651, it was found that the optimum p_H was 5·4 and that growth and pigment production were very poor below p_H 5·4 and above p_H 7·0. It was also found that the final p_H in all cases where growth occurred tended to become lower than the initial p_H. In view of this finding the final p_H of most of the media used in the growth and pigment production experiments with *A. novus* (Ac 46) was determined, and it was found that most of the final p_H values fell within the limits 3·2–3·6, the exceptions being in those cases where growth and pigment production were markedly high. It seems, therefore, that one of the most important limiting factors for growth and pigment production is the p_H of the medium, and it appears that the proper buffering of the medium to maintain it at the initial p_H 5·4 is essential for increased growth and pigment production.

Earlier investigators were of the opinion that the walls of the stalks and aerial hyphae become encrusted with granules, yellow in the young colony when conditions are acid, and becoming red or ferruginous in age when the reaction

changes to alkaline. They assumed that the pigment behaved as an indicator to acid and alkali, yellow in the acid phase and red when alkaline. From the results of the present investigation it seems quite evident that both flavoglaucin and auroglaucin occur together and independently of the p_H. In the case of group III organisms, flavoglaucin and the red pigment occur together in spite of the acid nature of the medium. In growing *A. novus* (Ac 46) at various p_H values, both of the pigments could be isolated in every case where growth occurred, and the medium became acid after incubation rather than alkaline. Where the initial p_H was above 7·0 auroglaucin was present in lesser quantity than at the lower p_H values, which is contrary to what would be expected from the assumptions of earlier workers. It seems therefore that the colour of the colony is not an indicator of the reaction of the medium.

Site of auroglaucin and flavoglaucin formation. To show that the perithecia are the site of flavoglaucin and auroglaucin formation, attempts have been made to inhibit entirely the formation of perithecia, which appear to be the seat of pigment production because of their bright sulphur-yellow colour. In no case could their formation be completely suppressed, but when *A. novus* (Ac 46) was grown on the Czapek-tartrate medium at p_H 4 perithecia formation was greatly retarded. After 35 days' incubation at 24° the yield of crude pigment was 58·0 mg. per g. of mycelium as compared with the normal average obtained at p_H 5·4 of 197·9 mg. per g.

That the pigments originate in the perithecia seems to be indicated also by an analysis of pigment production during the early stages of growth. During the first five days of growth, when the foundation of hyphal threads is being laid down, and perithecia formation is scanty, the total crude pigment percentage of the mould substance is 9·9 %. During the following six days of growth perithecia formation is abundant and the growth becomes brilliant yellow. Correspondingly the total crude pigment increases to 33·8 % of the mould substance. During this period of abundant perithecia formation one-half the total increase in weight of the mould substance is accounted for by the increase in weight of pigment. Moreover, using the newly modified medium, the growth is practically purely perithecial and, correspondingly, the yield of pigment is 43·2 % of the weight of mould substance. That the pigment is not associated with the conidia seems quite definite, since during the period of abundant conidia formation, between the eleventh and thirteenth days, the total crude pigment extract per flask is practically constant, being 0·064 g. per g. of mould substance after 11 days and 0·066 g. after 13 days. It seems quite evident, therefore, that flavoglaucin and auroglaucin formation takes place in great part or entirely in the perithecia.

Rubroglaucin appears as red incrustations on the walls of the aerial hyphae.

The investigation has shown, therefore, that what has hitherto been considered a preliminary diagnostic or a confirmative character of species in the *A. glaucus* series is, actually, an absolute qualitative character of the series as a whole and of separate groups of species within the series.

SUMMARY.

In a study of 25 species in the *Aspergillus glaucus* series, three hitherto undescribed crystalline pigments have been isolated from the dried mould substance: (*a*) flavoglaucin, $C_{19}H_{28}O_3$, in lemon yellow needles; (*b*) auroglaucin, $C_{19}H_{22}O_3$, in golden orange needles, and (*c*) rubroglaucin, $C_{16}H_{12}O_5$, in ruby-red short rods. These pigments are not produced by 19 other coloured species of *Aspergillus* examined and are believed to be specific for species in the *A. glaucus*

series. On the basis of these results the entire *A. glaucus* series has been divided into three distinct pigment groups. Flavoglaucin has been isolated from all 25 species in the *A. glaucus* series and is, therefore, a series-specific pigment. Group I contains thirteen species which produce flavoglaucin and auroglaucin but no other pigments. Group II contains two species which produce flavoglaucin, auroglaucin and an unidentified red pigment. Group III contains 10 species which do not produce auroglaucin but produce, in addition to flavoglaucin, a red pigment. In four species in this group this pigment has been shown to be rubroglaucin. In the remaining six species the red pigment has not been identified. The optimum cultural conditions for the production in quantity of flavoglaucin and auroglaucin by *Aspergillus novus* Wehmer have been determined.

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