A prominent effect of salicylate on the incorporation of ^{14}C from $[3-^{14}\text{C}]$ pyruvate into the soluble intermediates of rat tissues is the increased accumulation of ^{14}C in glutamate (Huggins *et al.* 1961*b*). This suggests that salicylate interferes with the further metabolism of glutamate. The more important pathways involved in glutamate metabolism include protein synthesis, conversion into α -oxoglutarate by glutamate dehydrogenase and various transamination reactions, decarboxylation to γ -aminobutyrate and the formation of glutamine. Salicylate inhibits the incorporation of ^{14}C -labelled amino acids into protein (Manchester, Randle & Smith, 1958). It also inhibits both glutamate-pyruvate transaminase (Steggle *et al.* 1961) and glutamine synthetase (Messer, 1958). The present work suggests that the conversion of glutamate into α -oxoglutarate by glutamate dehydrogenase and into γ -aminobutyrate by glutamate decarboxylase may also be inhibited by salicylate. In the radioactivity experiments of Huggins *et al.* (1961*b*) labelled α -oxoglutarate was detected in the isolated brain preparation and its formation was decreased in the presence of salicylate. However, this finding cannot be interpreted as direct evidence for an inhibitory effect of salicylate on glutamate dehydrogenase because a similar action on the glutamate-pyruvate transaminase would also cause a decreased incorporation of ^{14}C from labelled glutamate into α -oxoglutarate. More specific evidence for an interference by salicylate with glutamate-decarboxylase activity was a decreased formation of labelled γ -aminobutyrate from $[3-^{14}\text{C}]$ pyruvate.

SUMMARY

1. Salicylate inhibits glutamate-dehydrogenase and glutamate-decarboxylase activities *in vitro*.

2. The mechanism of the inhibition of glutamate dehydrogenase is reversible but does not involve competition with either glutamate or NAD; the inhibition of glutamate decarboxylase is irreversible. Preincubation of glutamate decarboxylase with pyridoxal phosphate reduces the degree of inhibition by salicylate.

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Biosynthesis of Griseofulvin

OBSERVATIONS ON THE INCORPORATION OF $[^{14}\text{C}]$ GRISEOPHENONE C AND $[^{36}\text{Cl}]$ GRISEOPHENONES B AND A

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Inhibition of griseofulvin (I) biosynthesis enabled Rhodes, Boothroyd, McGonagle & Somerfield (1961) to isolate three methylated benzophenone intermediates designated griseophenone C (2,4',6-trihydroxy-2',4-dimethoxy-6'-methylbenzo-

phenone) (II; R = R' = H), griseophenone B (5-chloro-2,4',6-trihydroxy-2',4-dimethoxy-6'-methylbenzophenone) (II; R = H; R' = Cl) and griseophenone A (5-chloro-4',6-dihydroxy-2,2',4-trimethoxy-6'-methylbenzophenone) (II; R = CH₃;

(I)

(II)

$R' = \text{Cl}$). They concluded that chlorination of griseophenone C gave griseophenone B, which is then methylated to yield griseophenone A: it was suggested that an oxidation-reduction reaction dependent on folic acid then completed the biosynthesis of griseofulvin.

We have now tested this suggested biosynthetic sequence by observing the incorporation of [^{14}C]griseophenone C and [^{36}Cl]griseophenones B and A.

EXPERIMENTAL

Rhodes *et al.* (1961) have given detailed descriptions of several materials and methods used in this work: they include the standard fermentation of griseofulvin in shake-flasks with mutant strains of *Penicillium patulum* Bainier-Thom C.M.I. 39,809, the assay of griseophenone intermediates in fermentation broths by paper chromatography, the determination of melting points and of ultraviolet- and infrared-absorption spectra, the extraction of griseofulvin and griseophenones, a solvent system and method for countercurrent distribution, and the alumina used for adsorption chromatography. Radioactivity was usually determined in the manner described by Barnes & Boothroyd (1961). This was supplemented when ^{14}C was used by scintillation-counting of a solution in toluene containing a scintillator or, when ^{36}Cl was used, by heating the compound with a mixture of $\text{K}_2\text{Cr}_2\text{O}_7$ and H_2SO_4 . The liberated chlorine was absorbed in an alkaline solution of H_2O_2 and chloride was precipitated from the boiled acidified solution with AgNO_3 . The AgCl was collected on a filter paper mounted in a perforated planchet (cf. Popják, 1950) and its radioactivity was determined after drying.

Isolation of [^{14}C]griseophenone C. During the investigation of the inhibition by aminopterin of griseofulvin fermentations and of the distribution of radioactivity resulting from the use of [^{14}C]acetate, a small quantity of [^{14}C]griseophenone C was isolated. Broth from mutant strain C4/30 in a chloride-deficient but otherwise standard fermentation medium, to which aminopterin ($2\text{ }\mu\text{g./ml.}$) and sodium [^{14}C]acetate ($0.5\text{ mc; } 549\text{ mg.}$) had been added at

the beginning of fermentation, was processed at the end of the ninth day. The alkali-soluble fraction of the combined CHCl_3 extract was purified by alumina chromatography (Rhodes *et al.* 1961) to yield crude [^{14}C]griseophenone C, contaminated with griseophenone A and griseoxanthone C. This mixture was separated by countercurrent distribution (50 transfers) and fractions 8–22 then gave, after slow evaporation of a concentrated acetone solution, brown gummy needles of [^{14}C]griseophenone C (613 mg.). Two-way paper chromatography in solvent systems used by Rhodes, McGonagle & Somerfield (1962) showed the presence of a small quantity of 6-methylsalicylic acid, and this was removed by extraction of the CHCl_3 solution with m-NaHCO_3 solution. Evaporation of the CHCl_3 solution gave brown gummy needles (550 mg.); these were crystallized several times from benzene, to give pale-yellow prisms (280 mg.) of griseophenone C, m.p. $183\text{--}185^\circ$, specific radioactivity $0.38\text{ }\mu\text{C/m-mole}$.

Incorporation of [^{14}C]griseophenone C into griseofulvin. Mutant strain C4/30 was grown in ten conical flasks (250 ml.) containing standard fermentation medium (40 ml./flask) supplemented with folic acid ($10\text{ }\mu\text{g./ml.}$) on the fourth day. On the fifth day of the fermentation period, broth from each flask was filtered separately through sterile Whatman no. 54 paper, the filtrate being discarded and the mycelium washed three times with sterile water. The washed mycelium was transferred to another conical flask (250 ml.) containing sterile phosphate buffer, pH 8.0 (40 ml.), plus 0.1% of KCl. An aqueous solution of the sodium salt of [^{14}C]griseophenone C (80 mg.) was distributed among the flasks to give a concentration of $200\text{ }\mu\text{g./ml.}$, and the ten flasks were placed on a rotary shaker at 25° . A sample (0.5 ml.) was removed from each of four flasks before adding the radioactive substrate and again at 10 min. and at 24, 32, 48, 56, 72 and 80 hr. after the addition. The samples obtained during the incubation with substrate were extracted with CHCl_3 and assayed by paper chromatography for griseophenone intermediates. After 14 days the mycelial suspensions remaining in the ten flasks were combined for extraction, and a sample of the combined broth was taken for chromatographic analysis.

Extraction of the suspension of mycelium in buffer (360 ml.) yielded a concentrated CHCl_3 solution (20 ml.) containing 85% of the theoretical radioactivity remaining in the broth after allowance for material removed in samples; the residual aqueous solution contained 12%. The CHCl_3 solution was extracted with N-NaOH ($3 \times 20\text{ ml.}$), washed with water, dried and evaporated to yield a brown solid (840 mg.; 33% of extracted radioactivity). This solid was dissolved in benzene (25 ml.) and placed on a column of alumina (grade V) (25 ml.). Elution of the column with benzene and with benzene containing ethanol (2%, v/v) was followed by observation under ultraviolet light; the fraction containing a bright-blue fluorescent band was collected. Evaporation of the eluent yielded griseofulvin (758 mg.), which was recrystallized twice from ethanol to give colourless prisms (350 mg.), m.p. and mixed m.p. $222\text{--}223^\circ$, $[\alpha]_D^{25} + 320^\circ$ (c 1.49 in acetone). The crystals had specific radioactivity $0.011\text{ }\mu\text{C/m-mole}$ (corresponding to about 25% of the radioactive substrate added) and $0.0076\text{ }\mu\text{C/m-mole}$ by scintillation-counting (corresponding to about 17% of the radioactive substrate added).

The alkaline extract of the CHCl_3 solution was acidified before extraction with CHCl_3 . The concentrated CHCl_3 solu-

tion (10 ml.), which contained 65% of the extracted radioactivity, was shown by paper chromatography to contain griseophenones A, B and C, griseoxanthone C and 3-chlorogriseophenone A (Rhodes *et al.* 1962).

In another experiment the standard fermentation medium (40 ml./flask) (ten flasks) was inoculated with vegetative inoculum of mutant strain C4/30; [^{14}C]griseophenone C (80 mg.) was added directly to the fermentation broth on the fifth and sixth days, when griseofulvin was forming rapidly, to give a total concentration of 200 $\mu\text{g./ml.}$ The broths were sampled only after they had been combined on the twelfth day and were processed in the usual manner to yield a CHCl_3 solution that contained 85% of the radioactive material initially supplied to the broth. The CHCl_3 solution was extracted with N-NaOH ($3 \times 25 \text{ ml.}$), washed with water, dried and evaporated to yield a brown solid (2.62 g.), which contained 30% of the extracted radioactive material. After alumina chromatography and crystallization from ethanol, griseofulvin (2.45 g.), m.p. 219–221°, was obtained and had specific radioactivity 0.0035 $\mu\text{C/m-mole}$ (at least 25% of the radioactive material added as substrate).

The griseofulvin was recrystallized twice from ethanol to yield colourless crystals, m.p. 220.5–222°, $[\alpha]_D^{25} + 330^\circ$ (c 1.06 in acetone). The crystals had the same specific radioactivity as before and 0.003 $\mu\text{C/m-mole}$ by scintillation-counting (at least 21% of the radioactive material added as substrate).

The alkaline extract was acidified and extracted with CHCl_3 ; this solution contained 60% of the extracted radioactive material. Paper chromatography showed the presence of a large amount of griseophenone A together with some 3-chlorogriseophenone A and griseophenones B and C. The chromatograms were scanned for radioactivity, and this showed that the spots corresponding to all these compounds were radioactive.

Preparation of [^{36}Cl]griseophenones A and B. Standard shake-flask fermentation medium (40 ml./flask) (ten flasks), but prepared from substantially chloride-free corn-steep liquor (*Brit. Pat.* 773,453) and K^{36}Cl , was inoculated with vegetative inoculum of mutant strain GA 766 and 'harvested' on the fourteenth day. The broth was extracted, by the method described by Rhodes *et al.* (1961) for the isolation of griseophenones A and B, to yield an alkaline extract of the combined CHCl_3 solution. This was acidified and extracted with ethyl acetate, and the dried ethyl acetate solution was evaporated to yield a brown gum. Countercurrent distribution gave crude [^{36}Cl]griseophenone A (200 mg.) and crude [^{36}Cl]griseophenone B (145 mg.).

The crude [^{36}Cl]griseophenone A was crystallized from benzene to yield brown needles (75 mg.), m.p. 200–207°, specific radioactivity 4.87 $\mu\text{C/m-mole}$. Griseophenone A (40 mg.) was dissolved in the hot benzene residual liquors, and brown needles (15 mg.) separated in the cold. Both batches of brown needles were mixed with griseophenone A (20 mg.) and crystallized from benzene to yield [^{36}Cl]griseophenone A (52 mg.), m.p. and mixed m.p. with griseophenone A 213–214°. These crystals had specific radioactivity 3.48 $\mu\text{C/m-mole}$. Paper chromatography showed that only griseophenone A was present.

The crude [^{36}Cl]griseophenone B did not crystallize from benzene, and some benzene-insoluble material was discarded. Griseophenone B (50 mg.) was dissolved in a hot benzene solution of the [^{36}Cl]griseophenone B, but crystals

failed to form after several days. Slow evaporation of the benzene solution gave brown sticky crystals: these were washed with benzene (0.5 ml.), and the residual brown crystals (99 mg.; specific radioactivity 1.76 $\mu\text{C/m-mole}$) were shown by paper chromatography to contain only [^{36}Cl]griseophenone B.

Incorporation of [^{36}Cl]griseophenone B into [^{36}Cl]griseophenone A and [^{36}Cl]griseofulvin. [^{36}Cl]Griseophenone B (80 mg.) was added as its sodium salt to give a concentration of 200 $\mu\text{g./ml.}$ in ten flasks containing mycelium of mutant strain C4/30 that had been grown for 8 days in fermentation medium and then resuspended in phosphate buffer, pH 8.0, as described above. Samples (1 ml.), removed from each flask before the addition of the radioactive substrate, and again at 10 min. and at 24, 32, 48, 56, 96 and 144 hr. after the addition, were assayed for griseophenone intermediates by paper chromatography. The mycelial suspensions remaining in the ten flasks after 14 days' incubation at 25° on a rotary shaker were combined for extraction.

Extraction of the suspension of mycelium in buffer (330 ml.) yielded a concentrated CHCl_3 solution (50 ml.) containing 85% of the theoretical radioactivity of the broth after allowance for material removed in samples: the residual aqueous solution contained 18%. The CHCl_3 solution was extracted with N-NaOH ($3 \times 20 \text{ ml.}$), washed with water, dried and evaporated to yield a brown solid (980 mg.; 76% of extracted radioactivity): this was purified on a column of alumina (grade IV) (15 ml.). Evaporation of the eluent yielded griseofulvin (804 mg.; 64% of extracted radioactivity; 51% of radioactive material added), which crystallized from benzene as colourless plates (437 mg.), m.p. undepressed on admixture with an authentic specimen 220.5–222°, $[\alpha]_D^{25} + 323^\circ$ (c 1.87 in acetone). The crystals had specific radioactivity 0.13 $\mu\text{C/m-mole}$ (corresponding to about 71% of the radioactive substrate added). A sample (35.42 mg.) was converted into AgCl (13.22 mg.; 91.5%), and then counted at infinite thinness (area of cross-section, 1.2 cm.^2) to give 3310 counts/min., corresponding to a specific radioactivity of griseofulvin of 0.12 $\mu\text{C/m-mole}$. A replicate determination gave the same specific radioactivity, corresponding to about 67% of the radioactive substrate added.

The alkaline extract of the CHCl_3 solution was acidified and extracted with CHCl_3 , and the solvent was evaporated to yield a brown solid (219 mg.; 24% of extracted radioactivity): this was separated by countercurrent distribution and fractions 16–50 yielded crude griseophenone A (94 mg.; 9% of extracted radioactivity). Paper chromatograms showed that only griseophenone A was present: the solid was crystallized from benzene to yield brown needles, having m.p. 205–209° (207–211° when mixed with griseophenone A) and specific radioactivity 0.12 $\mu\text{C/m-mole}$ (corresponding to 8% of the radioactive substrate added). The infrared-absorption spectrum (CHBr_3) of the needles was identical with that of griseophenone A.

Evaporation of fractions 1–15 yielded a brown gum (7% of extracted radioactivity): paper chromatograms showed that this contained griseoxanthone C, 3-chlorogriseophenone A, a very small quantity of griseofulvin and small amounts of other substances. The griseofulvin was removed in the usual manner, and a benzene solution of the acidic residue (5% of the extracted radioactivity) was placed on a column of alumina (grade IV) (5 ml.). Elution with

benzene-ethyl acetate mixtures (19:1 and 9:1, v/v) yielded a small quantity of a yellow gum giving a radioactive ultraviolet-quenching spot on paper chromatograms that corresponded to 3-chlorogriseophenone A.

Paper chromatograms showed that fractions 51-62 (1.5% of the extracted radioactivity) were mixtures of griseophenones A and B and that fractions 63-98 (3.5% of the extracted radioactivity) contained only griseophenone B.

Attempted incorporation of [³⁶Cl]griseophenone A into griseofulvin. The labelled substrate was added either to a suspension of mycelium in buffer or directly to fermentation broths under a variety of experimental conditions.

In an experiment identical with that described for the incorporation of [³⁶Cl]griseophenone B, the sodium salt of [³⁶Cl]griseophenone A (48 mg.; specific radioactivity 3.48 μ C/m-mole) was added to give a concentration of 200 μ g./ml. in six flasks containing 8-day-old mycelium of strain C4/30 that had been resuspended in phosphate buffer, pH 8.0. The contents of the flasks were combined after 16 days' incubation at 25° on a rotary shaker and then extracted to yield a CHCl₃ solution containing 82% of the theoretical radioactivity remaining in the broth: the residual aqueous solution contained a further 10%. The CHCl₃ solution was fractionated to yield crude griseofulvin (340 mg.), which crystallized from benzene as colourless plates (130 mg.), m.p. undepressed on admixture with an authentic specimen 220.5-222°, specific radioactivity 0.0023 μ C/m-mole. A sample (35.67 mg.) was converted into AgCl (12.96 mg.; 89.3%), which was counted at infinite thinness (area of cross-section, 1.2 cm.²) to give 11.4 counts/min.; (specific radioactivity of griseofulvin 0.00040 μ C/m-mole), corresponding to less than 0.1% of the radioactive substrate added. A replicate determination showed the griseofulvin to have a specific radioactivity of 0.00042 μ C/m-mole.

Countercurrent distribution of the alkali-soluble fraction (162 mg.) yielded a brown gum containing griseophenone A (120 mg.; specific radioactivity 0.72 μ C/m-mole). Two-way paper chromatography showed that griseophenone A was the only phenolic substance present. This material was used again in the experiments described below.

In a second experiment the washed mycelium was transferred earlier (third day) to the phosphate buffer, pH 8.0, and the [³⁶Cl]griseophenone A was added on both the third and the fourth days to give 100 μ g./ml. on each occasion. In three other experiments [³⁶Cl]griseophenone A was added to fermentation broths to give a total concentration of 200 μ g./ml. The additions were made on both the sixth and seventh days when griseofulvin biosynthesis was proceeding rapidly. There was no incorporation of [³⁶Cl]griseophenone A into griseofulvin in any of the five experiments.

There remains the possibility that [³⁶Cl]griseophenone A failed to penetrate the mycelium. This seems unlikely because of its close structural similarity to griseophenones B and C, both of which were readily incorporated. This aspect was investigated by studying the distribution between mycelium and filtrate of unlabelled griseophenone A added to 8-day-old mycelium that had been resuspended in phosphate buffer, pH 8.0. Chromatograms clearly showed that nearly all the added substrate was associated with the mycelium extract throughout the experiment.

Confirmation of this distribution was obtained from a similar experiment but with [³⁶Cl]griseophenone A as substrate. The mycelium was separated from the broth (33 ml.),

washed with water (40 ml.) and extracted with acetone (2 $\times$ 20 ml.). This extract contained 70% of the recovered radioactivity. The filtrate and washings were acidified, extracted with ethyl acetate (3 $\times$ 15 ml.) and the extract was shown to contain 31% of the recovered radioactivity.

RESULTS

Incorporation of [¹⁴C]griseophenone C into griseofulvin. Assay by paper chromatography of extracts of fermentation broth or of the suspension of mycelium in buffer, taken before the addition of substrate, showed griseophenones C, B and A to be present together with griseofulvin. The initial increment in griseophenone C assay on the addition of [¹⁴C]griseophenone C as substrate led within 24 hr. to an increased amount of griseophenone B and thereafter, until the end of the experiment on the fourteenth day, to a gradual decrease in griseophenone C, as might be expected if the substrate were being metabolized.

Metabolites extracted from the combined suspension of mycelium in buffer accounted for approx. 85% of the added radioactivity (corrected for material removed in sampling), and a further 12% was found in the residual aqueous solution. The extract was fractionated, and at least 17% of the added radioactivity was isolated as griseofulvin. When [¹⁴C]griseophenone C was added directly to the fermentation broth, the isolated griseofulvin contained at least 21% of the added radioactivity.

Scintillation-counting to determine the radioactivities of the substrate used in these experiments proved to be impracticable. The results obtained for [¹⁴C]griseophenone C were much lower than expected, and this may have been due to 'poisoning' of the scintillator by griseophenone C or, alternatively, the weak-yellow colour of the dilute solution (0.43 mg./ml.) of griseophenone C may have interfered with the scintillations: a stronger solution (0.78 mg./ml.) of griseophenone C produced an even greater discrepancy. Confirmation that the error was due solely to the griseophenone C was obtained when solutions of this substance were mixed with similar solutions containing different amounts of a highly radioactive but colourless compound: the resultant radioactivities were always much lower than the theoretical value for the mixtures. Scintillation-counting of griseofulvin, on the other hand, presented no problem, and this method was used to confirm the presence of ¹⁴C in the isolated antibiotic.

Incorporation of [³⁶Cl]griseophenone B into griseofulvin and griseophenone A. Assay by chromatography of an extract of a mycelial suspension in buffer, taken before the addition of substrate, showed the presence of small amounts of griseophenones B and A together with griseofulvin. The

Table 1. *Summarized results from experiments relating to incorporation of griseophenones C, B and A into griseofulvin*

Experimental details are given in the text.

Substrate	Radioactivity extracted (% of theoretical added)	Extracted radioactivity on fractionation with aq. NaOH (%)	
		Insoluble (griseofulvin)	Soluble (griseophenones)
$[^{14}\text{C}]$ Griseophenone C	85	33	65
	85	30	60
$[^{36}\text{Cl}]$ Griseophenone B	85	76	24
$[^{36}\text{Cl}]$ Griseophenone A	82	2	95
	85	2	93
	80	5	90

initial increase in griseophenone B assay on the addition of $[^{36}\text{Cl}]$ griseophenone B as substrate led within 24 hr. to a diminution in griseophenone B and the decrease continued, so that at 48 hr. its concentration was less than it was before the addition of the substrate. Thereafter, griseophenone B remained consistently at low concentration until the completion of the experiment at 144 hr. Corresponding changes in the concentration of griseophenone A could not be observed because of the large amount of this substance present from the beginning of the experiment.

Circles (1.5 cm. diam.) punched from the centre of the griseophenone B spot on each paper chromatogram were counted for radioactivity on a planchet; the results showed a rapid loss of radioactivity reaching completion within 32 hr.

The chloroform extract of the combined suspension of mycelium in buffer contained approx. 85 % of the added radioactivity (corrected for material removed in sampling); the remainder was present in the residual aqueous solution. The broth extract was fractionated and the percentage distribution of radioactivity was: griseofulvin, 64; griseophenone A, 9; 3-chlorogriseofulvin A, 5; griseophenone B, 3.5. The incorporation of $[^{36}\text{Cl}]$ griseophenone B into griseofulvin and griseophenone A was approx. 71 and 8 % respectively.

Attempted incorporation of $[^{36}\text{Cl}]$ griseophenone A. Five experiments in which $[^{36}\text{Cl}]$ griseophenone A was added either to fermentation broths that were rapidly synthesizing griseofulvin or to resuspended mycelium in phosphate buffer, pH 8.0, showed no incorporation into griseofulvin. It seemed unlikely that failure to utilize $[^{36}\text{Cl}]$ griseophenone A as substrate for the biosynthesis of griseofulvin could be due to lack of penetration into mycelium because $[^{36}\text{Cl}]$ griseophenone B and $[^{14}\text{C}]$ griseophenone C were rapidly converted into griseofulvin under similar conditions. Support for this belief was obtained in two further experiments in which the mycelium and filtrate were extracted separately. It

was shown that the griseophenone A was associated with the mycelium and that 70 % of the recovered radioactivity was present in the mycelium extract.

The results from incorporation of the three griseophenones into griseofulvin are summarized in Table 1.

DISCUSSION

The incorporation into griseofulvin of $[^{14}\text{C}]$ griseophenone C to the extent of 17–21 % and of $[^{36}\text{Cl}]$ griseophenone B to the extent of 67–71 % clearly confirms the conclusion of Rhodes *et al.* (1961) that both these benzophenones are intermediates in biosynthesis of griseofulvin. The incorporation of $[^{36}\text{Cl}]$ griseophenone B into griseophenone A, together with the chromatographic evidence indicating labelling of griseophenone A by $[^{14}\text{C}]$ griseophenone C, supports the biochemical sequence previously found: griseophenone C $\rightarrow$ griseophenone B $\rightarrow$ griseophenone A.

The complete failure to utilize $[^{36}\text{Cl}]$ griseophenone A for the biosynthesis of griseofulvin in the five experiments reported here suggests, on the other hand, that this benzophenone, in the free state, may not be an intermediate, although Rhodes *et al.* (1961) have shown that the biosynthesis of both griseofulvin and griseophenone A proceed simultaneously and that the formation of each is blocked by any interference with biosynthesis of griseophenone B or C.

Barton & Cohen (1957) have proposed that, in the biogenesis of griseofulvin, one-electron-transfer oxidizing agents oxidize griseophenone A (II; $\text{R} = \text{CH}_3$, $\text{R}' = \text{Cl}$) to the diradical (III) and that this is coupled to give the intermediate dehydrogriseofulvin and finally griseofulvin (I) by enzymic reduction. Day, Nabney & Scott (1961) provided a laboratory analogy for this concept by the total synthesis of $(\pm)$ -griseofulvin. Chemical reactions known to proceed by free-radical coupling of phenolic precursors have been reproduced with a phenolase obtained from *Polyporus versicolor*

(III)

(Bocks, Brown & Todd, 1962). The same enzyme has been used to convert griseophenone A into $(\pm)$ -dehydrogriseofulvin (B. R. Brown, personal communication).

Rhodes (1963), reviewing investigations on the biosynthesis of griseofulvin before the work of Brown and his co-workers, concluded that the free-radical mechanism proposed for the final stages of griseofulvin biosynthesis was supported by much of the available evidence, but that there were a few observations to suggest that it may be inadequate. Thus the theory did not account for the observed aminopter-in-induced block leading to accumulation of griseophenone A and did not offer an explanation for the inefficiency of dechlorogriseofulvin biosynthesis under chloride-deficient conditions (Rhodes *et al.* 1961).

Further evidence of the inadequacy of the free-radical mechanism is now supplied by the work of Brown and his collaborators, showing that the enzymic free-radical coupling of griseophenone A yields $(\pm)$ -dehydrogriseofulvin: the product of the normal griseofulvin fermentation is $(+)$ -griseofulvin, and free griseophenone A is not utilized in this biosynthesis.

We have therefore formulated an alternative mechanism compatible with our results from labelled griseophenone substrates and with the additional advantage that it gives natural griseofulvin, which is only one of the four possible isomers (Brossi, Baumann, Gerecke & Kyburz, 1960).

Our hypothesis is based on a concept of Bentley & Neuberger (1952), according to which the primary product of many biological oxidations may have a carbonium-ion structure. We suggest that the final stage in biogenesis of griseofulvin may begin with the adsorption of griseophenone B to a multi-enzyme complex that can effect a concerted reaction involving more or less simultaneous oxidation-reduction and methylation. It is postulated that, when this process operates smoothly, all intermediates are 'enzyme-bound', so that none occurs in a free state before the end product, griseofulvin, is finally released from the enzyme complex. Free griseophenone A and free $(-)$ -dehydrogriseofulvin are thus regarded as by-products released when their 'enzyme-bound' homologues are not utilized in perfect unison.

The detailed enzymic reactions might then be considered to proceed as indicated in Scheme 1. The chlorine atom in ring A of griseophenone B (IV) might be expected to strengthen the hydrogen bond between the 6-hydroxyl group and the carbonyl bridge (Pauling, 1960) and to aid loss of a proton from the *p*-hydroxyl group (Bennett, Brooks & Glasstone, 1935). It is therefore further suggested that griseophenone B may give an anion of structure (V), in which the orientation of rings A and B is unchanged.

The reaction begins when the griseophenone B anion (V) is adsorbed to three specific centres in the multi-enzyme complex. The methoxyl groups of the griseophenone B anion (V) are assumed to form two points of attachment: the third is formed by attraction of the negatively charged oxygen atom at position 2 in the benzophenone anion to a positive centre situated at a geometric point in the multi-enzyme complex. Formation of this ionic bond re-orientates ring A relative to ring B and thereby ruptures the hydrogen bond with the carbonyl bridge. The two rings of the adsorbed anion (VI) are so disposed that the 6-hydroxyl group is now close to C-1' and may form a hydrogen bond with the 5-chlorine atom: this anion has the desired configuration for subsequent asymmetric biosynthesis of the coumaranone ring.

Transfer of two electrons from anion (VI) to oxygen produces the carbonium ion (VII). A triphosphosphate (PPP_i) group is orientated at a fixed position in the multi-enzyme complex so that the carbonium ion (VII) is approached from the open side of ring B, thereby facilitating stereospecific closure of the coumaranone ring by attack of the 6-oxygen atom on the C⁺-1'. Structural models show that presence of the PPP_i group in the anion (VIII) completely blocks one side of ring B, thus directing the stereospecific *cis*-hydrogenation across positions 5' and 6' now assumed to occur with simultaneous methylation of position 2 to give the negatively charged ion (IX). In the final step, removal of phosphate closes the coumaranone ring, and natural griseofulvin (I) is released from the surface of the enzyme complex, thus completing the concerted reaction.

We suggest that 5-methyltetrahydrofolate (5MeFH₄) (Larrabee, Rosenthal, Cathou & Buchanan, 1961) may play a key role by reacting with ATP on the surface of our hypothetical multi-enzyme complex to form a quaternary amine (A5MeFH₄⁺) by addition of an adenosyl group to the nitrogen atom in position 5 (cf. Sakami & Uktins, 1961) and to leave 'enzyme-bound' PPP_i, which is fixed in an orientation determined by its origin from ATP (cf. Mudd, 1962). This provides the positively-charged centre we have postulated for the third point of attachment of the griseophenone

B anion (VI), brings position 2 of this benzophenone anion into juxtaposition with the 5-methyl group of the activated prosthetic group, and at the same time provides the orientated PPP₁ group that directs *cis*-hydrogenation of ring B and activates closure of the coumaranone ring. Transfer of the methyl group from A5MeFH₄⁺ accompanied by dehydrogenation gives adenosyldihydrofolate and results in methylation of position 2 in anion (VIII) with simultaneous saturation of the 5'-6' double bond. Tripolyphosphatase associated with the multienzyme complex splits off inorganic phosphate from the tripolyphosphate 'bridge' between enzyme and structure (VIII), with the result that the coumaranone ring is immediately closed by elimination of H₂PO₄⁻ ions to release natural griseofulvin (I). Finally, adenosine, dihydrofolate (FH₂) and PP₁ are removed and replaced by ATP and 5MeFH₄ (cf. Mudd, 1962).

When the enzymes of the complex do not act in unison, griseophenone A (XI) and (-)-dehydrogriseofulvin (X) can arise as by-products. Free (-)-dehydrogriseofulvin formed in this manner might eventually be hydrogenated to griseofulvin; but the griseophenone A would be expected to accumulate because methylation of position 2 would prevent reorientation of rings A and B, and would thereby interfere with adsorption to the three specific centres in the multienzyme complex. The griseophenone A accumulated may then presumably be chlorinated to give 3-chlorogriseophenone A (XII) (Rhodes *et al.* 1962).

The continued operation of our multienzyme complex would depend on the maintenance of a suitable pH, the continuous provision of ATP, NADPH₂ and oxygen, and the regeneration of 5MeFH₄ from the mol. of FH₂ formed in each cycle. The inhibition by aminopterin of dihydrofolate reductase would be expected to prevent regeneration of 5MeFH₄ by trapping the intermediates at the dihydrofolate level (cf. Jukes & Broquist, 1961). A transmethylation side reaction might then intervene to form griseophenone A: this could account for the accumulation of griseophenone A observed when griseofulvin biosynthesis was inhibited by aminopterin (Rhodes *et al.* 1961).

Biosynthesis by different mechanisms of the last-formed methoxyl group of griseofulvin and of griseophenone A (Rhodes *et al.* 1961) at position 4 would still permit labelling of the methoxyl group from [Me-¹⁴C]choline chloride (Huennekens & Osborn, 1959) in the pattern observed by Hockenhull & Faulds (1955). The suggestion is consistent with a report from Jackson *et al.* (1962), who found that DL-ethionine (labelled with ¹⁴C in the terminal carbon of the ethyl group), which is able to participate in reactions analogous to transmethylation, produced a small amount of the 2'-ethoxy analogue

of griseofulvin when added to broths of *Penicillium griseofulvum* on the third day of the fermentation period: if both positions were susceptible to trans-ethylation, the expected product would be the 2',4-diethoxy analogue of griseofulvin.

Our hypothesis might also account for the relative inefficiency of dechlorogriseofulvin biosynthesis (Rhodes *et al.* 1961) because griseophenone C, lacking the chlorine-assisted loss of proton from position 2 in ring A and having less stabilization of the enzyme-bound benzophenone anion, would be expected to be a less effective substrate than griseophenone B for attachment to the multienzyme complex.

SUMMARY

1. [¹⁴C]Griseophenone C and [³⁶Cl]griseophenone B added to shake-flask fermentation broths of mutant strains of *Penicillium patulum* Bainier-Thom C.M.I. 39 809 were incorporated into griseofulvin, thus confirming that both these benzophenones are intermediates in the biosynthesis of griseofulvin.

2. Similarly, labelling of griseophenone A from both [¹⁴C]griseophenone C and [³⁶Cl]griseophenone B confirmed the biochemical sequence previously found: griseophenone C → griseophenone B → griseophenone A.

3. Complete failure to utilize [³⁶Cl]griseophenone A for the biosynthesis of griseofulvin, under a variety of experimental conditions, suggested that this benzophenone in the free state may not be an intermediate, although it is closely concerned with griseofulvin biosynthesis.

4. Consideration of these results indicates that the free-radical mechanism postulated for the final stages of griseofulvin biosynthesis is inadequate: an alternative hypothesis for the biosynthesis of (+)-griseofulvin from griseophenone B is put forward.

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Differential Metabolism of Various Brain Regions

BIOCHEMICAL HETEROGENEITY OF MITOCHONDRIA

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Brain is an organ characterized by a high oxidative metabolism, the oxygen consumption on the basis of DNA content being the highest of any organ in the rat (Mandel, Bieth & Weill, 1957). Several reports have appeared on the distribution of the respiratory enzymes in the brain which broadly run parallel to the q_0 values of these areas. According to Meyer (1958), the cortical vulnerability to hypoxia is related to the concentration of cytochrome oxidase. Hess & Pope (1960), employing quantitative histochemical techniques, have shown that the highest activity of cytochrome oxidase is in layers II, III and IV of human cerebral cortex. The enzyme thus would be principally located in the cell bodies, in dendrites of the neurons and in axon terminals. As in other tissues this enzyme is almost exclusively mitochondrial (Brody, Wang & Bain, 1952; Friede & Pax, 1961). The activity of cytochrome oxidase varies in different areas of the brain; thus that in corpus callosum, populated with only non-neuronal glial cells, is significantly lower than the activity in cortical areas (Tolani & Talwar, 1961). These results are in line with the electron-micrographic observations indicating higher densities of mitochondria in most neurons and their larger processes than in oligodendroglia or astrocytes (Windle, 1958). Allen (1957) also observed low cytochrome-oxidase activity in well differentiated human gliomas. However, Hyden & Pigon (1960),

employing microchemical techniques and working on isolated neuronal and oligodendroglial cells, found that the cytochrome-oxidase activity in oligodendroglia was twice as high as in an equal volume of nerve cells. The methods of assay employed by various workers have differed in some respects, which can introduce extraneous factors in the measurement of enzyme activity. For example, the addition of exogenous cytochrome *c* to the medium can raise the cytochrome-oxidase activity of rat brain, an observation that led Elliott & Greig (1938) to postulate that the concentration of cytochrome *c* is the rate-limiting factor in rat brain. The human-cortex enzyme, on the other hand, does not show any such limitation (Hess & Pope, 1960). By taking into consideration these factors and employing an optimally constituted system, it was observed that the cytochrome-oxidase activity/mg. of mitochondrial protein is not the same in different brain regions. This could imply (a) a heterogeneity of the physical composition of the mitochondrial pellet obtained from different regions under the standard experimental conditions, (b) that the amount of enzyme in analogous particles is different, indicating a constitutional heterogeneity of the mitochondria, or (c) that the nature of the enzyme protein could be qualitatively different.

The present paper describes a few experiments to inquire into these points.