Production of a new lysergic acid derivative in submerged culture by a strain of *Claviceps paspali* Stevens & Hall

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[Plate 1]

1. The production of a new lysergic acid derivative, identified as D-lysergic acid α-hydroxyethylamide, in submerged culture and in yields up to 1 mg/ml. and above, by a strain of *Claviceps paspali* Stevens & Hall is reported; this substance can be converted in high yields into D-lysergic acid amide.

2. The morphological properties of this strain under different culture conditions are examined and it is shown that it grows in a form resembling natural sclerotia.

3. The biochemical culture conditions for the production of the new lysergic acid derivative in shake flasks and in stirred fermenters, the course of the fermentation, the methods for the isolation and purification of the substance and its transformation products and some of its chemical and physical properties are described.

Introduction

The production of the pharmacologically important lysergic acid derivatives by different strains of *Claviceps purpurea* (Fr.) Tul. in surface or in submerged culture has been attempted by a number of authors (McCrea 1931; Jaretzky 1935; Baldacci 1946; Abe 1951; Sim & Youngken 1951; Stoll, Brack, Hofmann & Kobel 1957; Taber & Vining 1957, 1958, 1960). However, none has so far been obtained in more than trace amounts by this method. Some alkaloids, belonging to the clavin series which are structurally closely related to the lysergic acid derivatives, but do not possess the carboxyl function and are pharmacologically much less interesting, have been obtained in submerged culture from *C. purpurea* (Fr.) Tul. strains after incubation periods of several weeks. (Abe, Yamano, Kozu & Kusamoto 1952; Abe, Yamano & Kusamoto 1955; Stoll *et al.* 1954).

The present paper reports the production in submerged culture and in reasonable yields of lysergic acid α-hydroxyethylamide, a new simple lysergic acid derivative, by a strain of *C. paspali* Stevens & Hall after incubation periods of 6 to 9 days; from this derivative lysergic acid amide can be obtained readily and in high yield. A preliminary note on this work has been published (Arcamone, Bonino, Chain, Ferretti, Pennella, Tonolo & Vero 1960).

Methods and materials

I. *Microscopic observations*

These were carried out mainly on fresh unstained mycelium. In some cases the mycelium was stained with cotton-blue in lactophenol (Langeron & Vanbreuseghem 1952), but the stain did not take very well and therefore results were not

very satisfactory. The lipids in the hyphae were revealed by staining with sudan III in lactophenol. For the study of the internal structure of the synnemata these were embedded in paraffin wax after fixing in a chromic acid-formalin fixative ('Craf III' (Sass 1951): chromic acid 30%, acetic acid 20%, formaline 10%) and dehydrating in *n*-butanol. Microtome sections of 5 to 8μ were prepared and stained with ferric haematoxylin.

II. *Culture media*

Surface cultures

For all surface cultures a glucose-potato agar medium was used in slants or Petri dishes. It had the composition: glucose 20 g, potato infusion 300 g, agar 15 g, tap water 1 l., pH 7. The potato infusion was prepared by boiling a 30% suspension of finely cut potatoes in water for 20 min.

Submerged cultures

Two standard culture media, both modifications of the medium of Abe *et al.* (1952), were used for the submerged fermentations, one (*A*) for the preparation of the seed cultures and the other (*B*) for the fermentations proper, both in shake flasks and fermenters. The composition of the media was as follows:

Medium A. Mannitol 4%, succinic acid, neutralized to pH 5·2 with ammonia, 1%, chick pea meal 0·1%, KH_2PO_4 0·1%, $MgSO_4.7H_2O$ 0·03%, tap water.

Medium B. Mannitol 5%, succinic acid neutralized to pH 5·2 with ammonia, 3%, KH_2PO_4 0·1%, $MgSO_4.7H_2O$ 0·03%, tap water.

Medium C. Mannitol 5%, succinic acid neutralized to pH 5·2 with sodium hydroxide, 3%, $NaNO_3$ 1%, KH_2PO_4 0·1%, $MgSO_4.7H_2O$ 0·03%, tap water.

The culture media were sterilized for 20 min at 100 °C, followed by a further period of 20 min at 120 °C.

III. *Fermentation methods*

Shake flasks

Cotton-wool plugged 500 ml. Erlenmeyer flasks containing 100 ml. of culture medium were used on a rotary shaker at 24° C (Paladino 1954); under the conditions of agitation (220 rev/min, eccentric throw 10 cm) the aeration rate in the absence of mycelium was about 28 ml. O_2 per 100 ml. of sulphite solution per hour.

Inoculation

The shake flasks containing medium *A* for the seed cultures were inoculated with a vegetative mycelium grown on an agar slant for 5 to 10 days. The mycelial mat was scraped off the slant with a spatula and suspended in the culture medium *A*. After growing for 5 to 7 days the mycelial suspension formed was used to prepare the seed culture. The contents of one shake flask were homogenized in a Waring blendor for 5 to 10 s and 10 ml. of the suspension used to inoculate shake flasks containing 100 ml. of medium *A*. The shake flasks containing the fermentation medium *B* were inoculated with 10 ml. of the seed suspension grown for 48 h.

Stirred fermenters

Jacketed stainless-steel fermenters of 500 l. total capacity, constructed in the workshop of this Institute, were used with 300 l. of culture medium. Aeration was effected through a ring sparger with an air flow varying from 200 to 300 l./min at an overpressure of 1·4 atm. The oxygen concentration in the culture medium during the fermentation was measured and recorded continuously by means of the rotating brush electrode (see below) inserted into the fermenter, and the air flow suitably adjusted to give an oxygen level of about 70 to 80 % saturation. The fermenter was provided with a top-driven stirrer rotating at 280 rev/min and fitted with an eight-bladed turbine propeller (ratio diameter of fermenter to diameter of propeller 3:1). When the electrode indicated that the oxygen level had fallen below 20 to 30 % saturation (usually about the seventh day) agitation was started to raise the oxygen level to the initial value of about 80 % saturation. The 500 l. fermenters were inoculated with 30 l. of a mycelial suspension grown in a 90 l. fermenter (Paladino, Ugolini & Chain 1954) containing 40 l. of culture medium in the absence of mechanical agitation with an air-flow of 40 l./min and an overpressure of 1 atm. The 90 l. fermenters in turn were inoculated with 1 l. of vegetative mycelium grown in a 3 l. shake flask for 2 to 3 days.

IV. *Analytical methods*

1. *Lysergic acid derivatives*

The mixture of lysergic acid derivatives (abbreviated in the following text as *LAD*) was estimated quantitatively by the colorimetric method of Voigt (1959) based on the formation of a blue colour with the Ehrlich–van Urk reagent (*p*-dimethylaminobenzaldehyde in 65 % H_2SO_4). A calibration curve was prepared with B.D.H. ergometrine maleate as standard which was linear in the range of 5 to 20μg/ml. For estimation in the culture filtrates the *LAD* were extracted from 40 to 50 ml. with an equal volume of a mixture of chloroform and *iso*butanol 4:1 at pH 7·5 to 8 and retransferred to one-fifth volume of 1 % sulphuric acid. The results were expressed in μg of ergometrine (free base).

2. *Mannitol*

Mannitol was estimated in the culture filtrates by the polarimetric method of Nelson & Hudson (1951) based on the large optical rotation ($[\alpha]_D^{20}140°$, $c = 1$) of mannitol in presence of molybdate. To 5 ml. of culture fluid 0·5 ml. of 10 N-H_2SO_4 and 4·5 ml. of a 10 % solution of ammonium molybdate were added. The phosphomolybdate formed from the inorganic phosphate present in the culture filtrates after addition of ammonium molybdate was removed by extraction with half a volume of *iso*butanol saturated with water (Berenblum & Chain 1938). The optical rotation of the aqueous phase was read in a 20 cm tube and the mannitol concentration determined from a standard curve, after subtraction of a blank value obtained by determination of the optical rotation of the culture fluid after addition of 0·5 ml. of 10 N-H_2SO_4 and 4·5 ml. of water, instead of the ammonium molybdate solution. The standard curve was linear in the concentration range of 0·4 to 4 % of mannitol. The blank value was very small at all stages of the fermentation.

3. *Succinic acid*

Succinic acid was determined directly in the culture filtrates (0·1 ml.) by the enzymic manometric method of Cohen (1951). Ether extraction proved unnecessary, since the culture filtrates did not contain interfering substances reacting with the enzyme preparations and added succinic acid was recovered quantitatively.

4. *Phosphate*

The method of Berenblum & Chain (1938) was used.

5. *Nitrogen*

Ammonium nitrogen was determined by the method of Conway (1957), total nitrogen by a modified Kjeldahl method.

6. *Total lipids*

The amount of total lipids in the mycelium was determined by mixing in a mortar a weighed quantity of wet filtered mycelium (about 10 g) with about five times its weight of anhydrous sodium sulphate and extracting the cake with petrol ether (b.p. 40 to 70 °C) in a Soxhlet extracter for 10 h. The amount of lipids remaining after evaporation of the solvent was weighed. The values are expressed as percentage of dry weight (about 20 % of wet weight).

7. *Oxygen uptake*

The oxygen uptake of the mycelium was determined in conventional Warburg vessels using 3 ml. of a mycelial suspension, when necessary suitably diluted with its own culture medium, to give a wet weight of 15 to 20 mg per flask.

The results are expressed as Q_{O_2} (μl. mg^{-1} h^{-1}).

8. *Dissolved oxygen*

The amount of dissolved oxygen in the culture filtrates during the fermentation was determined amperometrically using the methods of Gualandi, Morisi, Ugolini & Chain (1959), in shake flasks by means of the collodion-coated electrode, in the stirred fermenters using the rotating brush electrode.

9. *Spectophotometric methods*

The ultra-violet spectra were obtained in a Cary spectophotometer, the infra-red spectra in a double-beam Perkin–Elmer instrument. The optical rotary dispersion curve was determined in a Rudolph spectropolarimeter with a xenon lamp.

The ultra-violet fluorescence spectra were determined with a Farrand spectrofluorometer.

10. *Dry weight*

An aliquot of the mycelial suspension (usually 20 to 100 ml.) was filtered through paper on a Buchner funnel, washed thoroughly with three volumes of water, and dried at 85 °C for 24 h.

11. *Chromatographic methods*

Analytical

For the chromatographic analysis of the *LAD* obtained in submerged culture these were spotted in amounts of about 5 μg in 1 to 10 μl. on Whatman No. 1 filter paper and a *n*-butanol-acetic acid-water mixture 40:10:50 (solvent *a*) was used for development.

For chromatographic analysis of the alkaloid mixture from natural ergot alkaloids a chloroform-anhydrous formic acid mixture 9:1 (solvent *b*) was used.

Preparative

For preparative purposes band chromatography was performed on Whatman no. 1 or 3 MM filter paper. In order to avoid contamination of the products with material coming off the paper it was found necessary to subject the paper to a preliminary washing by percolation with 10% acetic acid for 72 h and methanol for a further period of 72 h; the paper was then thoroughly dried in an air stream.

The material to be chromatographed was spotted in methanol-pyridine 1:1 or dimethyl formamide solution from a capillary tube continuously along the line of origin so that amounts of 0·2 or 0·4 mg were delivered for each 1 cm distance on Whatman No. 1 or Whatman 3 MM paper, respectively. In this way the total amount of material chromatographed per paper sheet was about 2 or 4 mg, and normally about ten sheets were used.

The chromatograms were developed with solvent *a* and the fluorescent bands cut out and eluted with methanol. All operations were carried out with minimum exposure to daylight. The methanol solution was evaporated in a desiccator covered with black paper. The acetates thus obtained were converted into the free bases by suspending in a minimum of water, bringing the pH to 7·5 with sodium bicarbonate, extracting with chloroform and evaporating the chloroform extracts to dryness.

12. *Preparation of rac.* iso*lysergic acid hydrazide from crude ergot alkaloids* (for reference purposes)

Crude ergot alkaloids were prepared by the method of Stoll (1945) from commercial powdered partially defatted ergot. 1000 g of powdered ergot were mixed with a solution of 100 g aluminium sulphate in 150 ml. H_2O and extracted in a percolator with benzene until the benzene extracts gave no residue on evaporation. The ergot-aluminium sulphate mixture was then alkalinized with gaseous ammonia and the alkaloids exhaustively extracted with benzene. The benzene extract was concentrated to 18 ml. *in vacuo* and the alkaloids precipitated by petroleum ether; 1·4 g were obtained.

A further amount of 0·63 g of alkaloid bases was obtained by continuing the extraction with chloroform, concentrating the chloroform extract to 10 ml. and precipitating with petroleum ether. Chromatographic analysis with solvent *b* revealed seven well-separated spots giving the Ehrlich–van Urk reaction and showing the typical fluorescence in ultra-violet light.

From the crude mixture of alkaloid bases (50 mg) rac. *iso*lysergic acid hydrazide was obtained (10 mg after recrystallization) by the method of Stoll & Hofmann (1943).

13. *Preparation of* iso*lysergic acid amide* (*ergine*) *from ergotamine tartrate* (for reference purposes)

*Iso*lysergic acid amide (ergine) was prepared by mild alkaline hydrolysis from ergotamine tartrate following the method of Smith & Timmis (1932). From 490 mg ergotamine tartrate 40 mg *iso*lysergic acid amide were obtained.

V. Chemicals

The mannitol used was of commercial pure grade. All the other chemicals were of pure or analytical grade.

Morphological observations

By A. Tonolo

1. *Origin of strain*

A large number of strains, freshly isolated and from culture collections of sclerotia of *Claviceps purpurea* (Fr.) Tul. and *C. litoralis* Kawatani, were screened in shake-flask cultures in medium *B* for the production of lysergic acid derivatives, without success. Not only was there no indication of the production of lysergic acid derivatives, even in traces, but the morphological appearance of the mycelium showed no similarity to that of typical sclerotia. In view of these failures it was decided to study in greater detail the mechanism of sclerotia formation *in vivo*. For this purpose rye embryos (var. Rosen 4n) were infected with different strains of *Claviceps* Tul. and then grown on agar. (For the techniques used for the infection and the culture media see Tonolo (1959).) Under these conditions some of the *Claviceps* strains tested were non-infective, some caused infections, but grew in the form of vegetative mycelium without sclerotia formation, whereas some gave rise to both infection and sclerotia formation in different parts of the plant. Among the latter groups was one isolated from a sclerotium found on an infected plant of *Paspalum distichum* L. on a hill in the neighbourhood of Rome and identified as *Claviceps paspali* Stevens & Hall. This strain, F-97, was able to infect about 20 % of the embryos and formed sclerotia. One of these, formed on a leaf of the plant, was isolated, made to germinate on agar and hyphal subcultures were made from the mycelium. One of these, F-140, proved to be more infective to the rye embryos than the parent strain, infecting about 90 % of embryos and giving rise to ample sclerotia formation. This strain, when grown in submerged culture in shake flasks, produced substances in the culture medium, giving the typical blue colour for lysergic acid derivatives with the Ehrlich–van Urk reagent. The amounts formed were small, corresponding to about 10 to 20 μg per ml. of ergometrine, but reproducible.

2. *Improvement of strain by selection*

Strain F-140 does not produce either conidia or artrospores under any conditions studied, hence hyphal subcultures had to be made. When mycelium grown in shake flasks for 7 days, after inoculation from a 7-day-old agar slant, was homogenized in a Waring blendor for 5 to 10 s, the ensuing hyphal suspension contained only single hyphal filaments or simple hyphal units with their apical ramifications.

TABLE 1. *LAD* PRODUCTION AFTER FIRST STRAIN SELECTION

colour of mycelium	percentage distribution	average yield (μg/ml. after 9 days)
white	28	10
brown	69	44
violet	3	120

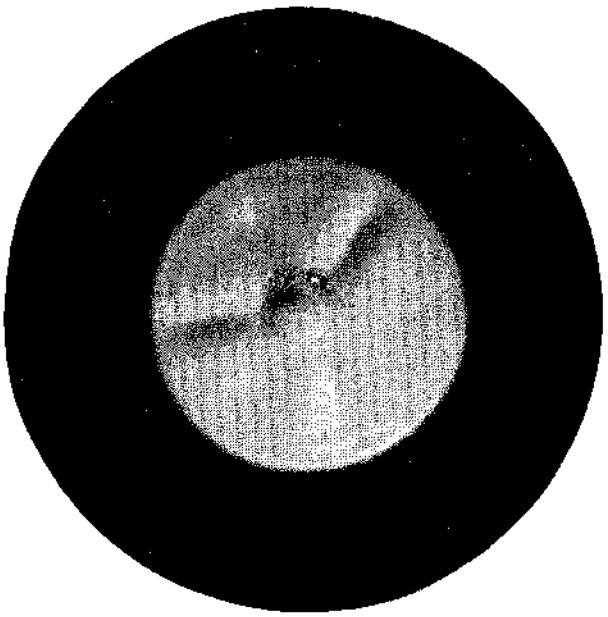
TABLE 2. *LAD* PRODUCTION AFTER SECOND STRAIN SELECTION

colour of mycelium	percentage distribution	average yield (μg/ml. after 9 days)
white	20	5
brown	70	55
violet	10	230

TABLE 3. *LAD* PRODUCTION AFTER THIRD STRAIN SELECTION

colour of mycelium	percentage distribution	average yield (μg/ml. after 9 days)
white	9	50
brown	29	204
violet	69	450

0·1 ml. of the hyphal suspension, containing about 300 hyphal elements, was spread over the surface of potato-glucose-agar Petri dishes. About thirty colonies developed. A number of these were transferred to agar slants and after 7 to 9 days growth were tested for the production of *LAD* in submerged culture. While the colonies developed on the agar plates did not show marked macroscopic differences in their appearance, the submerged cultures showed colour differences. Some were white, the majority brown and a small percentage had a violet colour, the pigment being present both in the mycelium and the culture filtrates. From table 1 it is clear that the highest production of lysergic acid derivatives occurred in the violet cultures. A further selection was therefore made from one of these, using the same technique. Table 2 shows that production of *LAD* was increased and maximal production again associated with the production of the violet pigment. A third selection by the same technique was then made from one of the violet cultures with the results reported in table 3. It will be seen that both the



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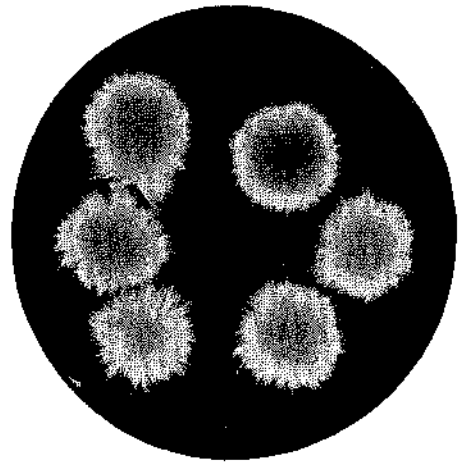
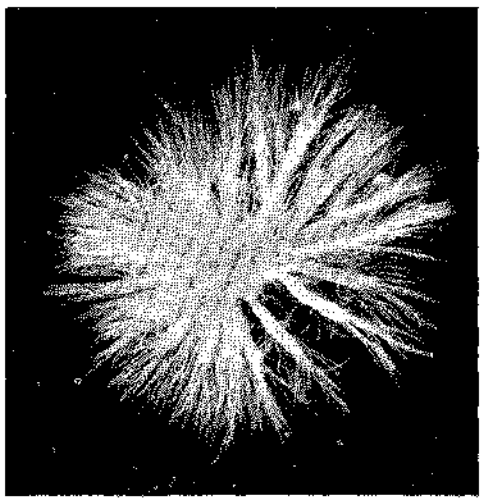


2*a*



2*b*

2*c*

FIGURE 1. Colony of strain F-550 of *Claviceps paspali* 7 days old, grown on potato-glucose agar. (Magn. × 1.)

FIGURE 2. Morphological appearance of mycelium of *Claviceps paspali* strain F-550 grown in submerged culture in shake flasks containing medium *B*. (*a*) Synnematic pellets, 5 days old. (*b*) Detail of (*a*): group of synnemata. (*c*) Detail of (*b*): single synnemata.

(*Facing p.* 33)

number of violet mutants and the production of lysergic acid derivatives was considerably increased. One of the high-yielding strains from the third selection, designated as F-550, was used for all the subsequent experiments.

3. *Morphological appearance of F-550 on agar*

The colonies grown on potato-glucose agar plates (figure 1, plate 1) reached a diameter of 2 to 3 cm after 10 to 15 days growth. They were round with an even border, had a smooth surface and a whitish-grey cottony or fluffy aerial mycelium, constituted of simple hyphae or hyphae grouped into synnemata of 3 to 4μ diameter, with septa 20 to 50μ long and containing some fat droplets. The vegetative mycelium developed on the agar surface lost the hyphal structure and was transformed into a pseudoparenchyma of leathery consistency consisting of isodiametric cells of 10 to 15μ diameter filled with fat droplets and in general resembling the structure of a sclerotium (see also Tonolo, Scotti & Vero 1960). The mycelium growing inside the agar layer, termed by Taber (1960) 'assimilative', consisted, on the other hand, of interlaced dark-brown hyphae, 3 to 4μ in diameter. No conidia or artrospores could be observed under any of the cultural conditions tried.

It was noticed that the marginal hyphae often showed a broken apex, with extruding cytoplasm.

4. *Morphological appearance of F-550 in submerged culture*

(*a*) *In shake flasks*

Without homogenization of the inoculum in the Waring blendor the mycelium of F-550 developed in form of very large pellets or agglomerated in one large mass. In this case diffusion of the culture medium and of oxygen into the cells was greatly impeded and consequently the fermentation proceeded in an abnormal and irreproducible manner.

For this reason the homogenization of the inoculum in a Waring blendor for 5 s was adopted as standard procedure. Under these conditions reproducible results were obtained. The mycelium developed always in oblong or pellet-shaped aggregates (figure 2, plate 1), the former having a length of 0·5 to 3 mm and a diameter of 0·3 to 0·7 mm, the latter a diameter of 1 to 3 mm. Isolated hyphae were encountered only very rarely.

The colour of the mycelium varied with its age and the culture medium. In the seed medium *A* it had a light-yellow to dark-yellow colour in the first 2 to 3 days and became green in the later stage of the fermentation. On cessation of the agitation the yellow as well as the green pigments were decolorized but regained their colour by shaking, indicating a reversible oxidation-reduction process. In the fermentation medium *B* the mycelium had a light-brown coloration at the beginning of the fermentation and became dark-brown violet after 8 to 10 days.

Microscopically the aggregates were proved to be composed of bundles of closely packed parallel straight or slightly undulated hyphae, 3 to 4μ in diameter (figure 3) with septa 30 to 70μ long, vacualized to various degrees and containing many fat droplets right from the beginning of their development. In young hyphae these

fat droplets were very small and distributed throughout the cytoplasm, in older hyphae they coalesced and often reached the same diameter as that of the hyphae. Lateral ramification was very rare. The growth of the hyphae was almost completely apical, the production of new hyphae occurring by dicotomy of the apex

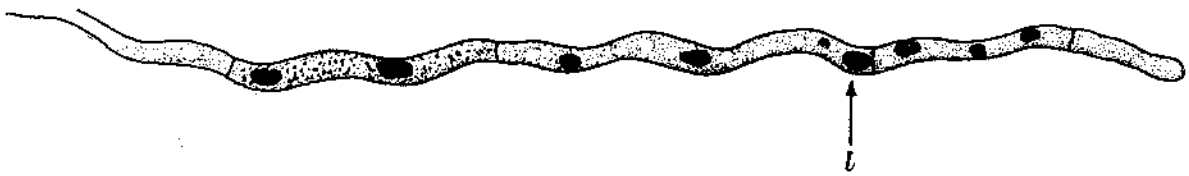


FIGURE 3. Microscopic appearance of single hypha of mycelium of *Claviceps paspali*, strain F-550, grown as above; *l* = lipid droplet (magn. × 2000).

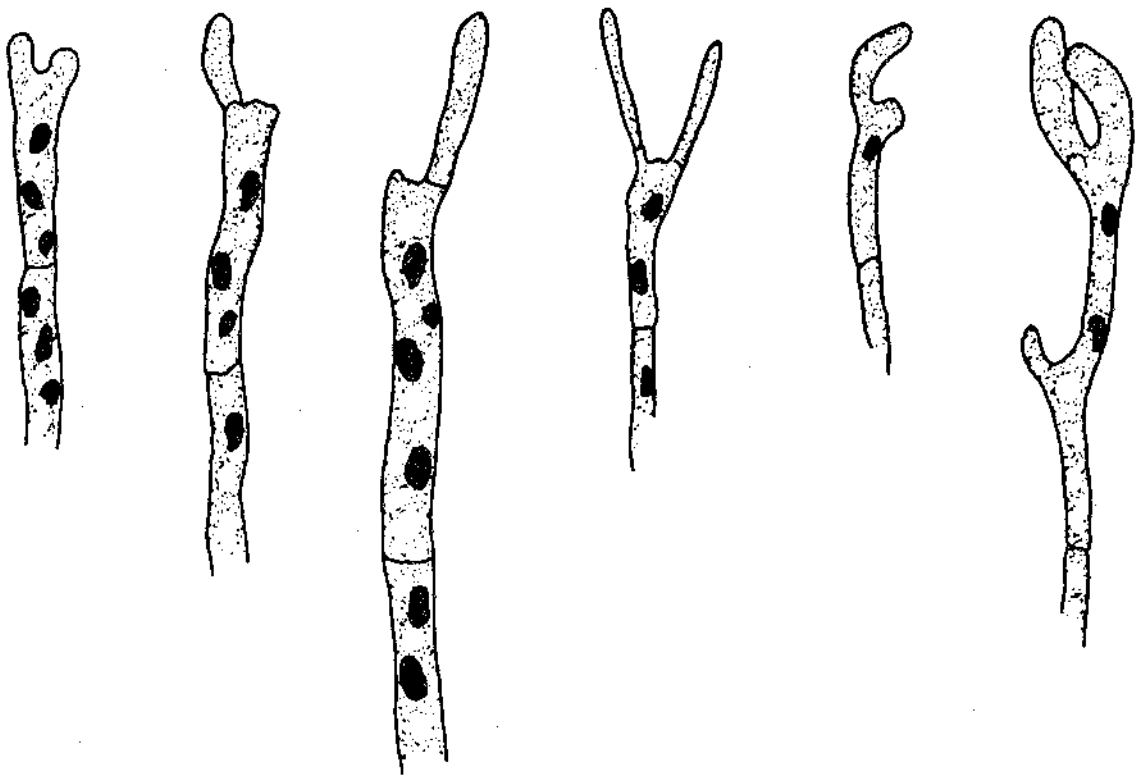


FIGURE 4. Different stages of dicotomy division of hyphal apex (magn. × 2000).

which at the time of division became enlarged and emitted two new hyphae (figure 4). The internal structure of the synnemata had all the features characteristic of sclerotia, and in this the strain of *Claviceps paspali* Stevens & Hall differed from all other strains of *Claviceps* Tul. previously examined.

The formation of a synnema appears to occur by one hypha beginning to emit a large number of lateral ramifications, some of which form new hyphae (figure 5). These arrange themselves parallel to each other in bundles consisting at the

FIGURE 5. Stages in the formation of a synnema. (*a*) Beginning of lateral ramifications. (*b*) to (*d*) formation of the first hyphae of a young synnema. (*e*) Parallel bundles of hyphae forming a young synnema (magn. × 1000).

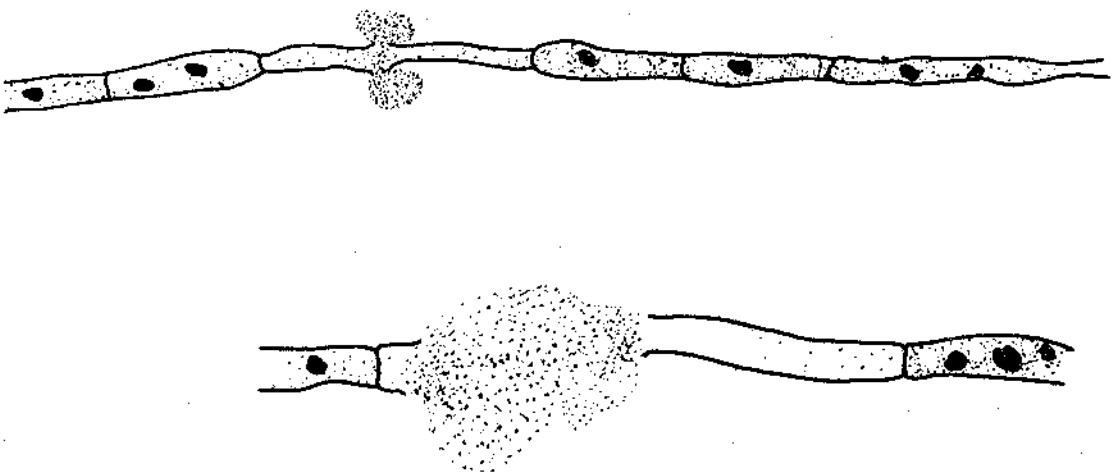


FIGURE 6. Detail of broken hypha with exuding cytoplasm (magn. × 2000).

beginning of ten to fifteen hyphae and having a diameter of 30 to 50μ. Further growth of the synnema occurs by apical growth and division of the primary hyphae.

The cell walls of the hyphae were found to be very fragile; the hyphae could, for instance, be broken by simple squashing with a cover-glass on a microscope slide (figure 6). Broken apices with cytoplasm exuding, such as were observed in agar culture, were also often noticed in submerged culture.

When mannitol was substituted by glucose as carbon source the morphological appearance of the mycelium changed; the hyphae of the synnemata were not so closely packed and pellets therefore less compact than those grown in the presence of mannitol. In addition swollen apical and intercalar forms frequently appeared (figure 7) which resembled the previously described clamidospores occurring in some strains of *C. purpurea* (Fr.) Tul. (Tonolo *et al.* 1960). In general the mycelium in the presence of glucose lost its sclerotial appearance.

(*b*) *In stirred fermenters*

In fermenters, where aeration in the first days was effected by bubbling only, synnematous aggregates formed as they did in the shake flasks, but they were much smaller in size and more numerous. This is probably due to the fact that more growth centres were formed through the detachment of hyphal elements from the synnemata by the action of the turbulence created by the air flow. Microscopically the synnemata formed in stirred fermenters had the same structure as those formed in shake flasks. Agitation in the latter stages of the fermentation did not affect morphological appearance of the synnemata, but in the early stage of the fermentation led to far reaching mechanical damage; most of the hyphal apices were broken under these conditions.

In some fermentations complete autolysis and re-growth of the mycelium took place without any obvious reasons, in some cases autolysis and growth were observed to occur several times. A similar phenomenon had been observed previously in penicillin fermentations in synthetic media (Dion, Carilli, Sermonti & Chain 1955).

Fermentations*

By E. B. Chain, F.R.S., A. Minghetti, P. Pennella, A. Tonolo and Lidia Vero

1. *Effect of temperature*

At temperatures above 24 °C the yield of *LAD* diminished sharply while the mycelial growth was not influenced significantly (table 4). The fermentations were carried out routinely at 23 °C.

2. *Effect of inoculum size*

The number of pellets per ml. increased with increasing inoculum while their diameter remained constant (table 5); in the latter respect the *C. paspali* Stevens & Hall culture behaved differently from *Penicillium chrysogenum* Thom (Camici,

* Unless otherwise stated the fermentations were carried out in shake flasks.

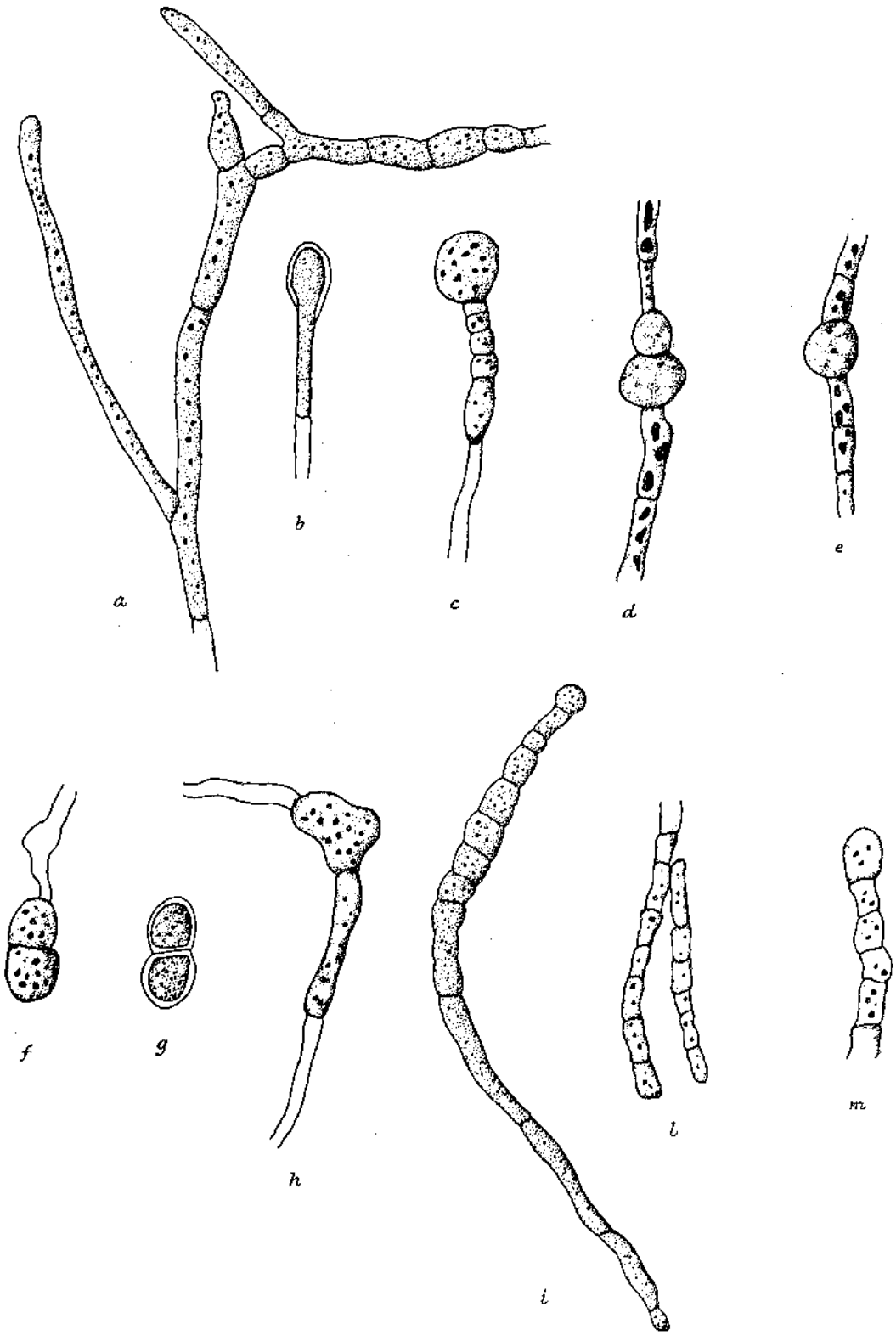


FIGURE 7. Morphological appearance of hyphae of mycelium of *Claviceps paspali*, strain F-550, grown in submerged culture in shake flasks in medium *B* containing glucose instead of mannitol. (*a*) Hyphal unit showing ramification. (*b*) To (*h*) different forms of clamidospores. (*i*) To (*m*) single hypha showing narrow septa resembling artrosporoid forms (magn. × 2000).

Sermonti & Chain 1952) and *Aspergillus flavus* Link (Testi-Camposano 1959). The production of *LAD* too was, within wide limits, independent of the inoculum size.

3. *Effect of age of inoculum on* LAD *production*

The production of *LAD* decreased sharply with the age of the inoculum though the dry weight reached was not significally influenced (table 6).

TABLE 4. EFFECT OF TEMPERATURE ON THE PRODUCTION OF *LAD*

temperature of incubator (°C)	yield (μg/ml.) after 9 days	dry weight (g/100 ml.)
21*	530 ± 15	1·43
24†	420 ± 13	1·36
27†	270 ± 9	1·40
30†	20 ± 3	1·34

* One experiment in triplicate. † Three experiments, each in triplicate.

TABLE 5. EFFECT OF INOCULUM SIZE ON GROWTH AND *LAD* PRODUCTION*

amount of inoculum (ml./100 ml. of medium)	number of pellets/ml.	average daimeter of pellets (mm)	dry weight (g/100 ml. after 9 days)	yield (μg/ml. after 9 days)
1	13	6·3	0·33	728 ± 69
2	21	5·8	0·88	1058 ± 74
5	23	6·2	1·05	1241 ± 30
10	47	5·7	1·19	1186 ± 40

* Two experiments, each in triplicate.

TABLE 6. EFFECT OF THE INOCULUM AGE ON *LAD* PRODUCTION*

age of inoculum (days)	yield of *LAD* (μg/ml.) after 9 days	dry weight (g/100 ml.) after 9 days
2	650 ± 24	1·65
4	214 ± 20	1·55
6	118 ± 4	1·67
8	66 ± 5	1·30
10	36 ± 2	1·52

* Two experiments, each in triplicate.

4. *Effect of number of transfers on* LAD *production*

The capacity of *LAD* production of the mycelium diminished on repeated transfer into fresh culture medium (using always a 10% inoculum) (table 7), while its capacity of growth was not impaired under these conditions.

5. *Influence of* pH *on* LAD *production*

In medium *B* where ammonium ion was the only nitrogen source *LAD* production proceeded between pH 4·5 and 5·5, optimal values being obtained around pH 5 (table 8), At pH 6 there was a sharp drop in growth and *LAD*

production suggesting that free ammonia had a toxic effect. In medium *C* where sodium nitrate was the only nitrogen source the pH plateau for *LAD* production was much larger, between the values of 4·5 and 6·5, but growth and production were much lower (table 9).

TABLE 7. EFFECT OF REPEATED TRANSFER OF MYCELIUM AFTER 9 DAYS GROWTH INTO FRESH CULTURE MEDIUM*

number of transfers	yield of *LAD* (μg/ml.) after 9 days	dry weight (g/100 ml.) after 9 days
1	610	1·27
2	440	1·5
3	430	1·0
4	280	1·1
5	110	—

* Two experiments, each in triplicate.

TABLE 8. EFFECT OF pH ON *LAD* PRODUCTION IN MEDIUM *B**

pH	yield (μg/ml.)	dry weight (g/100 ml.)
4·1	47	0·1
4·5	600	1·1
5·0	680	1·3
5·2	760	1·3
5·5	710	1·25
6	180	0·35
6·5	0	0

* Three experiments, each in triplicate.

TABLE 9. EFFECT OF pH ON *LAD* PRODUCTION IN MEDIUM *C**

pH	yield (μg/ml.)	dry weight (g/100 ml.)
4	0	0·05
4·5	220	0·5
5·2	230	0·59
5·5	230	0·57
6·0	210	0·51
6·5	210	0·51
7·0	170	0·26
7·5	0	0·16

* Two experiments each in triplicate.

6. *Effect of different concentrations of ammonium succinate on* LAD *production*

The influence of different concentrations of ammonium succinate in presence of a constant quantity of mannitol on *LAD* production and growth is shown in table 10. The production reached maximal values at a 4% concentration of ammonium succinate.

While the succinate consumption remained constant at about 1% at all ammonium succinate concentrations tested except the highest, the mannitol consumption, and, in consequence, growth decreased with increasing ammonium succinate concentration.

TABLE 10. EFFECT OF DIFFERENT CONCENTRATIONS OF AMMONIUM SUCCINATE ON *LAD* PRODUCTION IN PRESENCE OF A CONSTANT AMOUNT OF MANNITOL*

succinic acid (neutralized with NH_4OH to pH 5·2) (g/100 ml.)	yield (μg/ml.)	dry weight (g/100 ml.)	final pH	consumption of	
				mannitol (g/100 ml.)	succinate (g/100 ml.)
1	129 ± 11	2·22 ± 0·1	5·7	4·20	0·92
2	345 ± 15	1·4 ± 0·05	5·4	2·00	0·80
3	540 ± 52	1·2 ± 0·02	5·3	1·80	1·07
4	780 ± 30	1·2 ± 0·03	5·4	1·65	0·87
5	802 ± 2	1·00 ± 0·01	5·2	1·45	1·42
6	750 ± 30	0·7 ± 0·01	5·2	1·25	0·44

The mannitol concentration was kept constant at 5%.

* Two experiments, each in triplicate.

TABLE 11. EFFECT OF DIFFERENT CONCENTRATIONS OF SUCCINATE IN PRESENCE OF A CONSTANT AMOUNT OF AMMONIUM ION AND MANNITOL*

succinic acid concentration (%)	yield of *LAD* (μg/ml.)	dry weight (g/100 ml.)
1	192 ± 75	1·9
2	412 ± 12	1·7
3	725 ± 75	1·7
4	850 ± 25	1·3

* Two experiments, each in triplicate.

7. *Effect of different concentrations of succinate in presence of a constant amount of ammonium ion and mannitol*

Similar results with regard to production yields and growth were obtained when the succinate concentration was increased, but the ammonia content was kept constant at 1·45 mg/ml., the pH being adjusted to 5·2 with sodium hydroxide (table 11).

The ammonium ion concentration was kept constant at 1·45 mg per ml., and the mannitol concentration at 5%.

8. *Effect of* LAD *yield of varying the concentration of mannitol*

As will be seen from table 12 the yields of *LAD* increased to optimal level with increasing mannitol concentration up to 3% and then remained constant up till mannitol concentrations of 6%; with higher mannitol concentrations both growth and *LAD* production decreased.

9. *Substitution of mannitol by other carbon sources*

Table 13 shows that mannitol could be substituted by glucose and sucrose without impairment of the *LAD* yield provided these sugars were not sterilized by heat, but by Seitz filtration. Sorbitol, used instead of mannitol, gave about half the yields, whereas the other carbon sources listed were ineffective for *LAD* production, though they supported growth.

TABLE 12. EFFECT OF MANNITOL CONCENTRATION ON *LAD* YIELD*

initial mannitol concentration (g/100 ml.)	yield (μg/ml.) after 9 days	dry weight (g/100 ml.) after 9 days	mannitol consumption (g/100 ml.) after 9 days
1	210	1·2	1·0
2	400	1·0	1·3
3	670	1·0	1·4
4	670	1·0	1·8
5	660	1·0	2·0
6	650	1·0	1·5
7	470	0·6	1·4
8	210	0·4	1·4

* Two experiments, each in triplicate. The other constituents of the culture medium were kept at standard concentrations.

TABLE 13. EFFECT OF DIFFERENT CARBON SOURCES ON *LAD* PRODUCTION*

carbon source (all at 5% concn.)	yield (μg/ml.) after 9 days	dry weight (g/100 ml.)	final pH
mannitol	750	1·57	5·2
sorbitol	325	1·45	5·2
glucose	120†	1·06	5·2
	600‡	1·06	5·2
sucrose	160†	1·64	5·2
	620‡	1·64	5·2
maltose	110	1·50	7·0
galactose	80	0·97	5·2
lactose	50	0·50	7·6
dextrin	57	1·16	7·2
starch	55	0·99	7·3
glycerine	35	0·95	6·5

* Three experiments, each in triplicate.
† Separately sterilized by heat.
‡ Seitz filtered.

10. *Effect of substituting succinic acid by other organic acids*

Succinic acid can be substituted by malic acid and tartaric acid, the latter giving somewhat lower yields (table 14). Citric, lactic, acetic and oxalic acids did not support growth.

11. *Effect of aeration on* LAD *formation*

Measurements of the concentration of dissolved oxygen in culture media contained in wide-necked cotton-plugged shake flasks (Gualandi *et al.* 1959) showed that it was near saturation level during the whole course of fermentation; this is

to be expected in view of the low oxygen demand. Under these conditions *LAD* production was optimal. Reduction of the oxygen content of the atmosphere in the shake flasks to 5% led to a marked reduction of both growth and *LAD* production, and in the presence of 1% oxygen both growth and *LAD* production practically stopped. In narrow-necked tightly plugged Erlenmeyer shake flasks *LAD* production was only half that obtained in wide-necked loosely plugged shake flasks where aeration conditions were adequate (table 15).

TABLE 14. EFFECT OF SUBSTITUTING SUCCINIC ACID BY OTHER ORGANIC ACIDS ON *LAD* PRODUCTION*

acid†	yield (μg/ml.) after 9 days	dry weight (g/100 ml.)
succinic	630 ± 5	1·00 –
malic	650 ± 3	0·94
tartaric	420 ± 10	0·70
citric	215 ± 5	0·38
lactic	8 ± 1	0·01
acetic	13 ± 2	0·07
oxalic	14 ± 2	0·01

* Two experiments, each in triplicate.

† All acids were used in 0·25 M concentrations (equimolar to 3% succinic acid) and neutralized to pH 5·2 with ammonium hydroxide.

TABLE 15. INFLUENCE OF AERATION ON *LAD* PRODUCTION IN SHAKE FLASKS*

conditions used to obtain different aeration rates	% oxygen in $O_2 + N_2$ mixtures	yield (μg/ml.) after 9 days	dry weight (g/100 ml.)	final pH
narrow-neck (diam. 2·4 cm) Erlenmeyer flasks, closed with tight cotton plug	—	429	1·36	5·4
wide-neck (diam. 4 cm) Erlenmeyer flasks, closed with loose cotton plug	—	750	1·66	5·4
wide-neck Erlenmeyer flasks, closed with bored rubber bungs provided with tubes through which a continuous stream of $O_2 + N_2$ gas mixtures is passed over the surface of the culture medium	20	870	1·46	5·4
	5	204	0·85	5·2
	1	15	0·16	5·2

* Two experiments, each in triplicate

In stirred fermenters the production of *LAD* came to a stop when the oxygen concentration was allowed to remain at the low level to which it had fallen after the period of 6 to 7 days during which air dispersion was effected by bubbling only, without mechanical agitation.

12. *Course of fermentation*

The course of a typical *LAD* fermentation in shake flasks in medium *B* is shown in figure 8, in a stirred fermenter of 500 l. capacity in figure 9. The picture is very similar, but the Q_{O_2} of the old mycelium in the stirred fermenters had lower values

than mycelium of the same age grown in shake flasks. There was an excess of ammonia present in the culture medium at the end of the fermentation, but all the phosphate had disappeared. (For aeration conditions in stirred fermenters see above under Methods.)

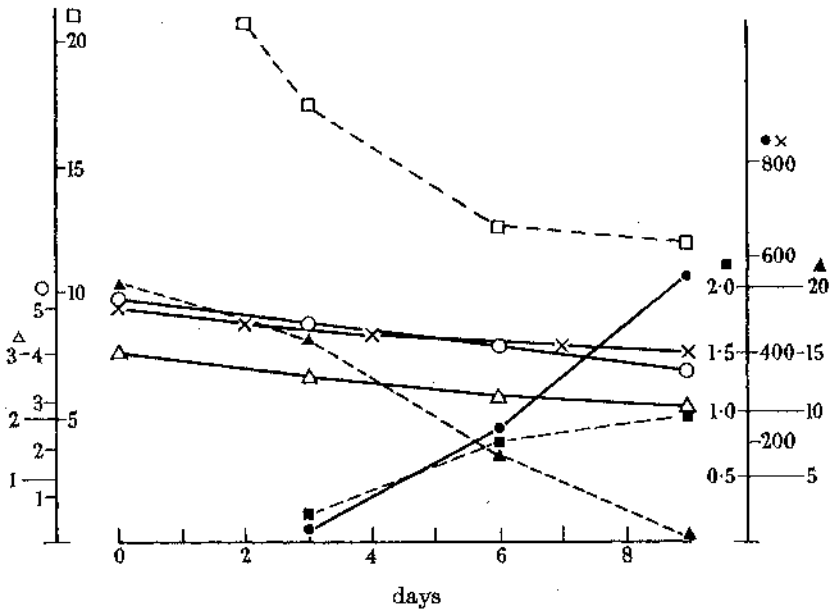


FIGURE 8. Course of a typical *LAD* fermentation by *Claviceps paspali*, strain F-550, in shake flasks in medium *B*. △, Succinic acid, g %; ○, mannitol, g %; □, Q_{O_2}; ■, dry weight, g/100 ml.; ▲, inorganic phosphate, mg P %; ●, *LAD* μg/ml.; ×, ammonia, mg N %.

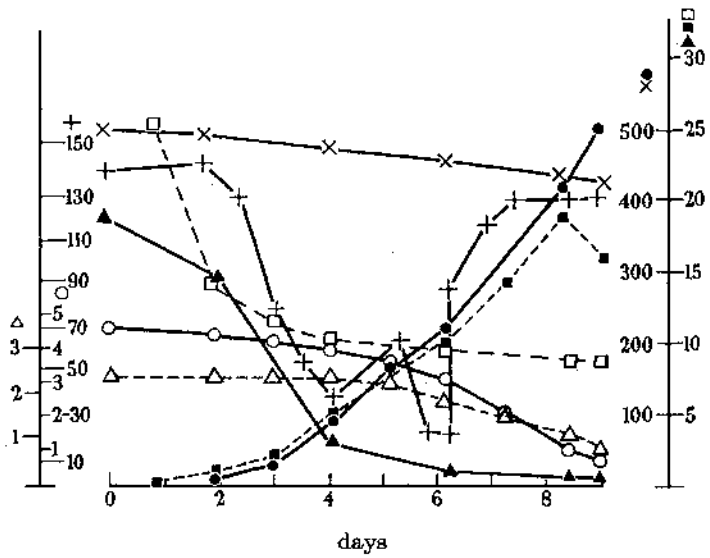


FIGURE 9. Course of a typical *LAD* fermentation by *Claviceps paspali*, strain F-550, in a 500 l. stirred fermenter in medium *B*. △, Succinic acid, g %; ○, mannitol, g %; □, Q_{O_2}; ■, dry weight, mg/ml.; ▲, inorganic phosphate, mg P %; ●, *LAD*, μg/ml.; × ammonia, mg N %; +, dissolved oxygen, per cent saturation at 1 atm over-pressure of air.

13. *Replacement cultures*

It was found when after 9 days the mycelium grown in shake flasks was sterilely filtered and resuspended in fresh medium, the *LAD* production was very much accelerated and reached higher levels. Altogether, the culture behaved as one initiated with a very large inoculum would be expected to behave; it was metabolically more active in all respects: succinate and mannitol consumption as well as growth (figure 10). The total ammonia and phosphate consumption were very similar to that in the first culture, but compressed into a shorter time period.

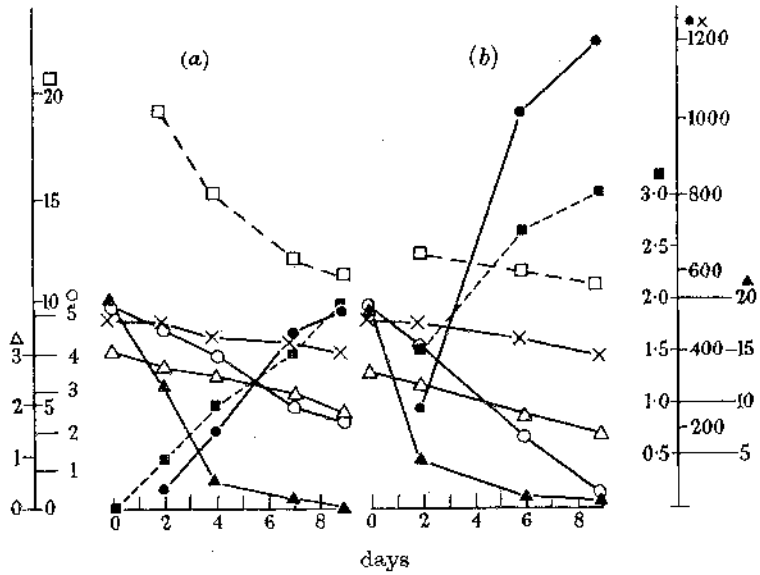


FIGURE 10. Course of a typical replacement fermentation of *Claviceps paspali*, strain F-550, in shake flasks in medium *B*. (*a*) First fermentation; (*b*) replacement fermentation. △, Succinic acid, g %; ○, mannitol, g %; □, Q_{O_2}; ■, dry weight, g %; ▲, inorganic phosphate, mg P %; ●, *LAD*, μg/ml.; ×, ammonia, mg N %.

14. *Composition of the mycelium*

(*a*) *Nitrogen*

The nitrogen content of the mycelium at different age is given in table 16.

TABLE 16. NITROGEN CONTENT (%) OF MYCELIUM OF *CLAVICEPS PASPALI* AT DIFFERENT AGE

age (days)	grown in shake flasks	grown in stirred fermenters
2	7·35	6·74
3	7·34	6·83
4	6·80	6·79
6	6·85	6·58
8	—	6·19
9	6·59	6·76

(*b*) *Phosphorus*

The phosphorus content of the mycelium and its distribution as free phosphate, readily hydrolyzable (poly) phosphate and organic phosphate is given in table 17.

TABLE 17. PHOSPHORUS CONTENT (%) OF MYCELIUM OF *CLAVICEPS PASPALI* AND ITS DISTRIBUTION

age (days)	total	free phosphate	readily hydrolyzable phosphate	organic
		grown in shake flasks		
2	2·24	0·25	1·41	0·58
4	2·18	0·24	1·40	0·54
6	1·95	0·21	1·34	0·40
8	—	—	—	—
9	2·08	0·10	1·81	0·17
11	—	—	—	—
		grown in stirred fermenters		
2	1·95	0·18	1·26	0·51
4	2·15	0·20	1·48	0·47
6	2·08	0·25	1·30	0·53
8	1·80	0·14	1·45	0·21
9	—	—	—	—
11	1·17	0·79	0·26	0·12

TABLE 18. LIPID CONTENT (%*) OF MYCELIUM OF *CLAVICEPS PASPALI*

age (days)	grown in shake flasks	grown in stirred fermenters
2	—	17·0
3	6·5	—
4	—	10·2
6	4·7	9·0
9	3·7	9·4

* Average of three experiments.

(*c*) *Lipid content*

As can be seen from table 18 the mycelium of *Claviceps paspali* has an unusually high lipid content, and the lipid content of the mycelium grown in stirred fermenters was much higher than that of mycelium grown in shake flasks. The lipid content of other filamentous fungi, such as *Penicillium chrysogenum* and *Aspergillus niger* was found to be around 0·2 to 0·3%.

15. *Stimulation of* LAD *production by tryptophane*

Tryptophane has been shown to be a precursor of ergot alkaloids (Taber & Vining, 1959, 1960; Gröger 1959; Gröger, Wendt, Mothes & Weygand 1959). When this amino acid was added to culture medium *B* at the beginning of the fermentation a marked stimulation of *LAD* production was noted (table 19).

Of all amino acids tested (*dl*-glycine; *dl*-alanine; *dl*-valine; *dl*-leucine; *dl-iso*-leucine; *dl*-serine; *dl*-cysteine; *dl*-cystine; *dl*-aspartic acid; *dl*-asparagine; *dl*-histidine; *dl*-proline) only tryptophane had this stimulating effect.

TABLE 19. STIMULATION OF *LAD* PRODUCTION BY TRYPTOPHANE*

quantity of *dl*-tryptophane added (μg/ml.)	*LAD* production (μg/ml.)	dry weight (g/100 ml.)
0	504	1·7
400	1430	1·6
800	1800	1·2

* Three experiments, each in triplicate.

TABLE 20. APPROXIMATIVE PARTITION COEFFICIENTS OF *LAD* PRESENT IN THE CULTURE FILTRATES AT DIFFERENT pH WITH DIFFERENT SOLVENTS

	pH		
solvent	7·5	5·5	3·5
n-butanol	> 24	3·8	1·6
iso-butanol	14·5	1·25	0·9
chloroform-*iso*butanol 4:1	9·0	1·25	< 0·05
chloroform	3·7	0·4	< 0·05
methylene chloride	3·4	0·2	< 0·05
n-butylacetate	3·7	0·2	< 0·05
benzene	0·3	0·2	< 0·05

CHEMICAL STUDIES

By F. Arcamone, E. B. Chain and A. Ferretti

Extraction of LAD *by solvents from the culture medium*

The approximate partition coefficients of the *LAD* present in the culture filtrates at different pH and with different solvents are given in table 20.

From these values it was evident that the *LAD* were weak bases, and therefore could be shuttled between solvent and water by appropriately changing the pH.

The *LAD* were insoluble in petroleum ether and carbon tetrachloride.

Chromatographic behaviour

When small aliquots of the culture filtrates (a few hundred ml.) were immediately extracted after the end of the fermentations with an equal volume of chloroform at pH 7, the solvent layer spotted without concentration on Whatman filter paper No. 1 in amounts containing about 5μg of *LAD* and chromatographed in solvent *a*, one main spot (substance B_1) with an R_F of 0·64 to 0·7 appeared in ultraviolet light or after spraying with the Ehrlich reagent, with traces of three spots having R_F values of 0·52 to 0·60 (substance A_1), 0·59 to 0·67 (substance A_2) and 0·73 to 0·77 (substance B_2).

When larger amounts of culture media were worked up and the chloroform extracts either evaporated to dryness or concentrated to a small volume and the

LAD precipitated with petroleum ether, so that the preparation of the samples for chromatographic analysis took a longer time period, the appearance of the chromatograms, prepared and developed as above, changed. The intensity of the spot corresponding to substance B_1 diminished while that of the spots corresponding to substances A_1, A_2 and B_2 greatly increased, suggesting that the latter products originated through transformation of the former. Substances A_2 and B_1, because of the proximity of their respective R_F values in the solvent used, could not be properly separated and coalesced into one single elongated spot. The slight scatter of the R_F values of the four substances encountered in different experiments was probably due to differences in the filter paper; when pure ergometrine was chromatographed on the same paper as a standard, a constant ratio of R_F *LAD* over R_F ergometrine was found for all four substances: 0·83 for substance A_1, 0·93 for substance A_2, 0·97 for substance B_1, and 1·1 for substance B_2.

In some batches small amounts of a *LAD* with a R_F of 0·9 were present, but this product has not yet been studied further.

Crystallization and identification of substances A_1 *and* A_2

For the crystallization of substances A_1 and A_2 the following procedure was adopted.

The culture medium, usually batches of 40 to 300 l., was filtered through large Buchner funnels or a filter press, and the culture filtrate, containing from 200 to 1000 μg *LAD*/ml., after adjusting the pH to 8, was extracted with an equal volume of chloroform: *iso*butanol mixture 4:1. The extraction was effected in stainless-steel containers with stirring. The solvent phase was separated by centrifuging through a Sharples centrifuge. The spent culture filtrate contained less than 5 % of its original *LAD* content.

From the solvent phase the *LAD* were retransferred into water by shaking three times with $\frac{1}{10}$ volume of water, adjusting the pH to 3·5 with sulphuric acid. About 75 to 80 % of *LAD* appeared in the aqueous phase while the solvent phase contained about 4 % of material giving the Ehrlich–van Urk reaction but of much weaker basic strength than the rest of the material; it was not further studied.

The aqueous extracts were brought to pH 8 with dilute soda and, after addition of 1 mg versene for each 10 to 15 mg *LAD*, extracted three times with half a volume of chloroform. The chloroform extract was evaporated *in vacuo* at 30 °C to $\frac{1}{50}$ or $\frac{1}{100}$ of its volume, avoiding exposure to day light as much as possible during the operations, and kept at +2 °C for 24 h.

A white crystalline substance, A_1, separated in yields of 10 to 30 % depending on the batch. The remaining mixture of *LAD* bases could be precipitated from the chloroform extract either in form of the free bases by adding 3 to 5 volumes of petroleum ether (b.p. 60 to 80 °C) or, preferentially, in form of maleates by addition of an ethereal solution of maleic acid. The latter procedure gave a purer product. The crude mixture of bases or maleates, a powder of off-white colour, was freed from solvent on a Buchner funnel, care being taken to exclude daylight as much as possible during all operations.

The products were stored in a desiccator covered with black paper. On exposure to light they darkened and gradually became transformed into intractable dark-brown resins. This transformation occurred much more rapidly if the addition of versene was omitted from the procedure, indicating that it was catalyzed by traces of metals.

The maleates were readily transformed into the free bases by suspending in water and adding a saturated solution of sodium bicarbonate to bring the pH to 8. The free bases were extracted with chloroform and, after concentration of the chloroform extract, recovered by precipitation with petroleum ether.

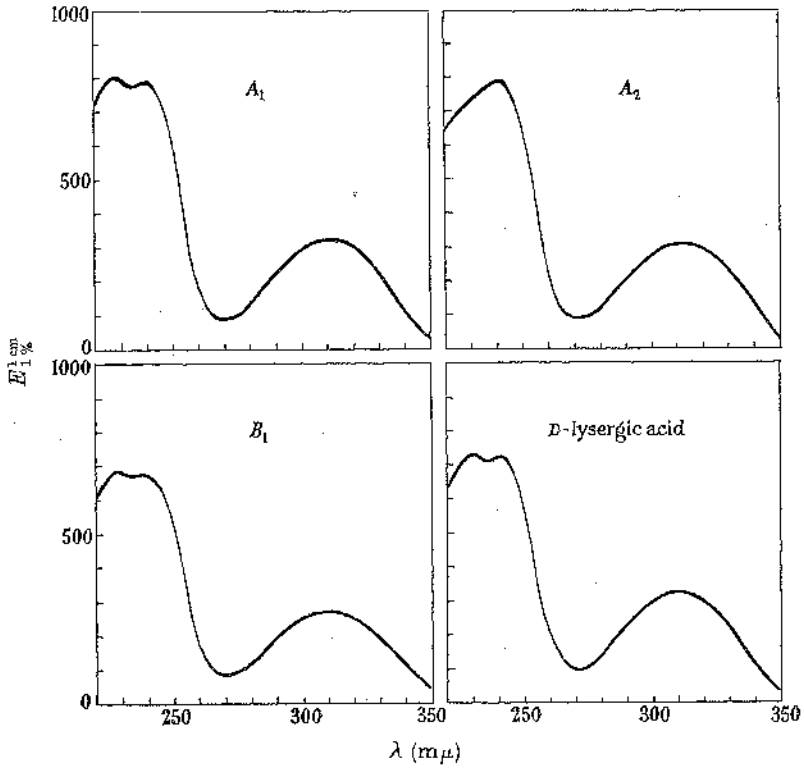


FIGURE 11. Ultraviolet spectra of A_1, A_2, B_1 and D-lysergic acid. (*c* 20 μg/ml., in 95% ethanol.)

On treating the free bases with chloroform (2 g with 50 ml.) a part (0·9 g) remained insoluble. This part was mainly substance A_1 which could be crystallized from boiling ethanol. The chloroform solution was evaporated to dryness and the residue taken up in 50 ml. methanol. On standing in the dark at +2 °C 275 mg of crystalline A_2 was deposited which was recrystallized from boiling methanol.

Identification of A_1 *and* A_2 *as* D-*lysergic acid amide and* D-iso*lysergic acid amide*

A_1. *Elementary analysis*

Found—C: 71·53, H: 6·53, N: 15·86%. Calc. for $C_{16}H_{17}N_3O$ (mol. weight 267·32, C: 71·88, H: 6·41, N: 15·72%).

Equivalent weight (determined by back titration with 0·1N-NaOH of a solution of 52·2 mg in 5·0 ml. 0·1N-HCl) 267.

Optical rotation: $[\alpha]_D^{20}$ in pyridine $+11° \pm 2 (c = 1)$, in dimethylformamide $[\alpha]_D^{23} + 63° \pm 1 (c = 1)$, ($[\alpha]_D^{20} + 10° (c = 0{\cdot}5)$ in pyridine reported by Smith & Timmis (1936)).

Rotary dispersion (in dimethylformamide $c = 1$) $[\alpha]_{700}^{22} + 27{\cdot}4°$; $[\alpha]_{589}^{22} + 57{\cdot}0°$; $[\alpha]_{500}^{22} + 132{\cdot}9°$; $[\alpha]_{400}^{22} + 514{\cdot}2°$.

Ultra-violet spectrum. The u.v. spectrum (figure 11) had the characteristic peaks of lysergic acid derivatives at 312 and 242 mμ. The u.v. fluorescence spectrum was identical with that of B_1 (see below).

Infra-red spectrum. The infra-red spectrum (figure 12) had many of the characteristic bands of lysergic acid and its derivatives; the bands of the $CONH_2$ group at 1680 and 1645 cm^{-1} were evident.

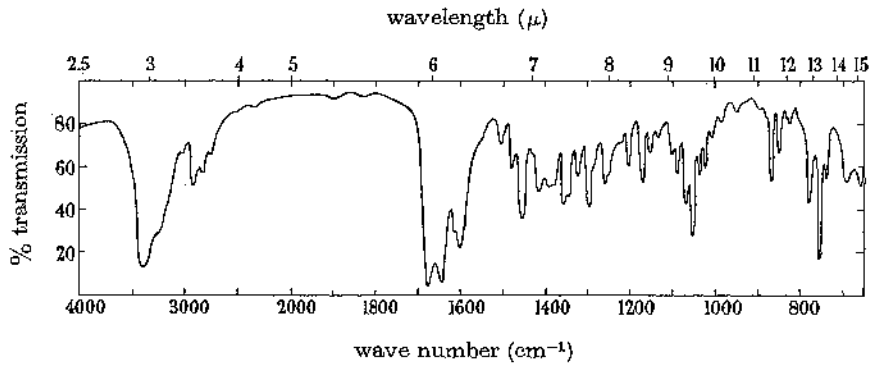


FIGURE 12. Infra-red spectrum of A_1. (c 0·3 % in KBr pellets.)

Formation of D-*lysergic acid and ammonia on alkaline hydrolysis*

On hydrolysis with boiling 1·2N methanolic potash (10 ml.) 206·5 mg of A_1 (0·772 mM) liberated 13·1 mg of ammonia (0·767 mM). On acidification with 4N acetic acid to pH 4 a crystalline product (121 mg) precipitated. This was identical with D-lysergic acid in all its properties (u.v. spectrum, infra-red spectrum, optical rotation, elementary analysis).

Formation of hydrazide of rac. iso*lysergic acid*

1 g of A_1, heated with 1 g of anhydrous hydrazine according to the method of Stoll & Hofmann (1943) gave, after recrystallization, 390 mg of the hydrazide of rac. *iso*lysergic acid. This substance was identified by its infra-red absorption spectrum which was identical with that of a specimen prepared by the same method from ergot alkaloids extracted from natural ergot (figures 13 *a*, *b*).

A_2. Elementary analysis

The product crystallized with 1·5 mol. of firmly bound methanol.

Found—C: 66·74, H: 7·13, N: 13·39%. Calc. for $C_{16}H_{17}N_3O.-\frac{3}{2}-CH_3OH$; C: 66·64, H: 7·35; N: 13·32%.

Optical rotation

$[\alpha]_D^{20}$ in chloroform (c = 0·88) + 448°. Rotary dispersion (in dimethylformamide c = 0·1) $[\alpha]_{700}^{22}$ + 216° ; $[\alpha]_{589}^{22}$ + 350°; $[\alpha]_{420}^{22}$ + 1269°; $[\alpha]_{360}^{22}$ + 3421°; (in 95% ethanol c = 0·1) $[\alpha]_{700}^{22}$ + 230°; $[\alpha]_{589}^{22}$ + 365°; $[\alpha]_{420}^{22}$ + 1299°; $[\alpha]_{360}^{22}$ + 3225°.

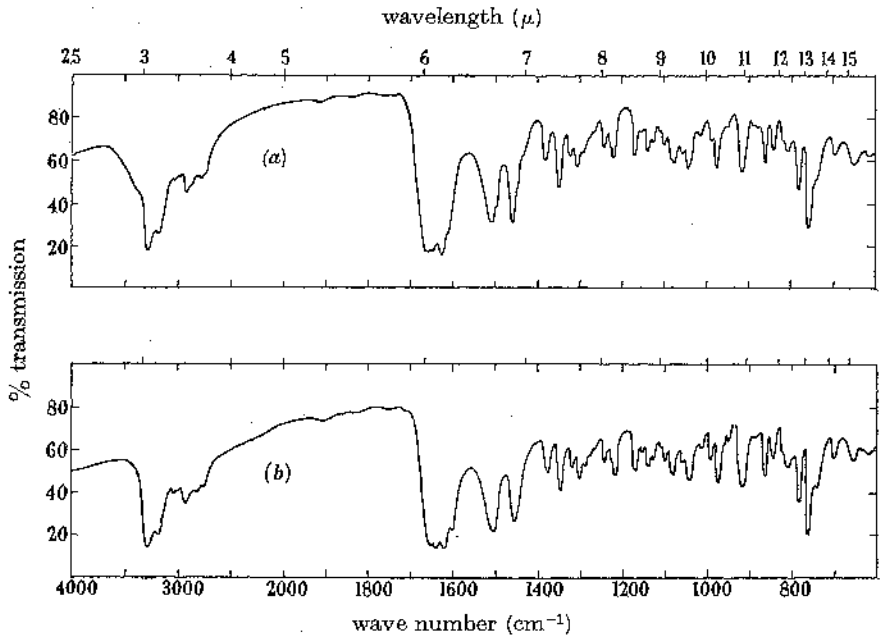


FIGURE 13. Infra-red spectrum of rac. *iso*lysergic acid hydrazide. (*a*) From A_1; (*b*) from ergot alkaloids.

U.v. spectrum and u.v. fluorescence spectrum

The u.v. spectrum is shown in figure 11. The u.v. fluorescence spectrum was identical with that of A_1

Infra-red spectrum

The infra-red spectrum of A_2 was identical with that of a specimen of D-*iso*lysergic acid amide prepared from ergotamine tartrate by mild alkaline hydrolysis following the method of Smith & Timmis (1932) (figure 14).

Chromatographic analysis

A_2 and D-*iso*lysergic acid amide prepared from ergotamine tartrate had the same R_F in solvent *a*.

Isomerization of A_1 *and* A_2

A_1 could be isomerized into A_2, and A_2 into A_1, by heating in 10 to 15% acetic acid for 1 h at 80 to 100 °C. The isomerization was revealed chromatographically in solvent *a*. A_2 was obtained by preparative chromatography from A_1 after isomerization by the above method. 100 mg of A_2 were heated for 1 h at 80 °C in

2 ml. of 15 % acetic acid, the solution brought to pH 8 and extracted three times with 10 ml. of chloroform and dried over Na_2SO_4. On concentration to 5 ml. and standing 17 mg pure A_1 crystallized.

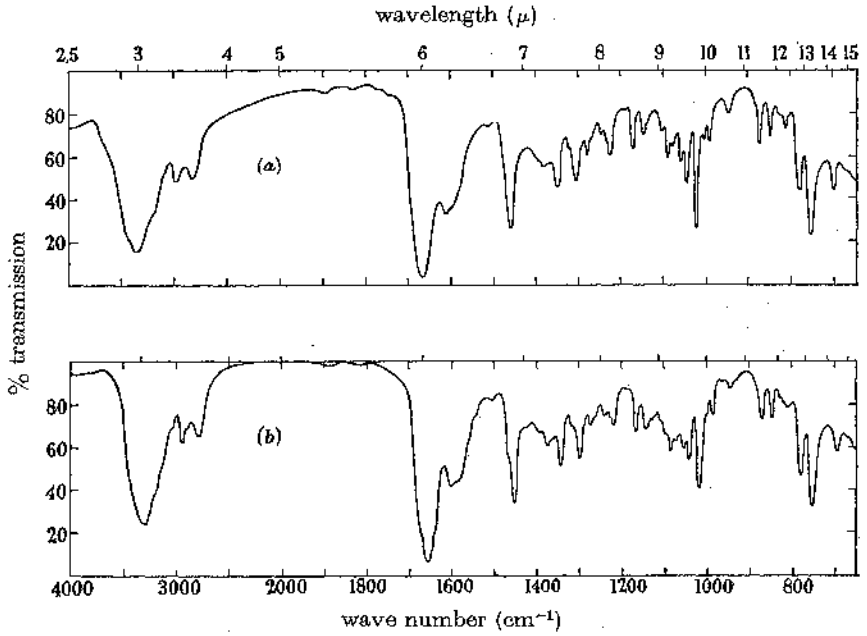


FIGURE 14. Infra-red spectrum of A_2 (*a*) and reference *iso*lysergic acid amide (*b*). (*c* 0·3 % in KBr pellets.)

Crystallization of B_1

As it had become evident that B_1 was the only alkaloid produced by the strain of *Claviceps paspali* used in these studies, but was transformed into A_1 and A_2 during the working-up operations of the culture filtrates when these were carried out over a prolonged time period at room temperature, the isolation of B_1 was attempted at a lower temperature and with the minimum of delay. The procedure was as follows: 175 l. of culture filtrate, containing 640 μg of *LAD* per ml., were adjusted to pH 7·2 with 2·5N-NaOH cooled to 10 °C, and extracted with 50 l. of cold *n*-butanol. The *n*-butanol extract was separated by decantation, an equal volume of *n*-hexane added and the *LAD* transferred into 5 l. of 10 % solution of tartaric acid cooled to +2 °C, the pH being adjusted to 3·5 by adding a 10 % solution of tartaric acid with vigorous stirring. The water layer was separated, brought to pH 5 and stored overnight in the refrigerator.

The aqueous phase was then brought to pH 7·4 by adding solid sodium bicarbonate and extracted three times with 1·5 l. of cold chloroform.

During the chloroform extraction a crystalline precipitate of B_1 (6·7 g) formed at the chloroform/water interface which was filtered off. On standing in the refrigerator a large amount of crystalline B_1 (26·2 g) separated from the combined chloroform extracts and was removed by filtration. The chloroform mother liquors were re-extracted with 780 ml. of a 10 % solution of tartaric acid, the aqueous solution

brought to pH 7·5 and extracted three times with 500 ml. of chloroform. On standing overnight in the cold room this solution deposited a further amount of B_1 (7·41 g). The chloroform mother liquors were evaporated to dryness and an amorphous residue (37·75 g) obtained which was shown to consist almost completely of B_2.

Properties of B_1

Melting point: the product began to decompose at 135 °C on the microscopic hot stage.

Elementary analysis. Found: C: 69·3; H: 6·9; N: 13·7 %. Calc. for $C_{18}H_{21}N_3O_2$; C: 69·4; H: 6·8; N: 13·5 %. Mol. weight 311·37. Equivalent weight (determined by back titration with 0·1N-NaOH of a solution of 38·3 mg in 2 ml. 0·1N-HCl) 314.

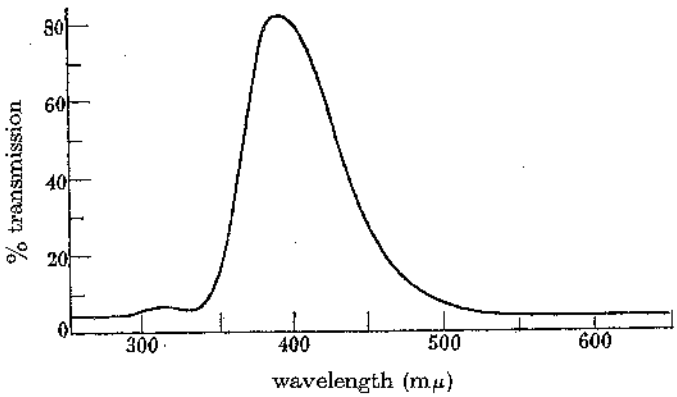


FIGURE 15. Ultra-violet fluorescence spectrum of B_1. (c 5 μg/ml. in 95 % ethanol; exciting wavelength 320 mμ.)

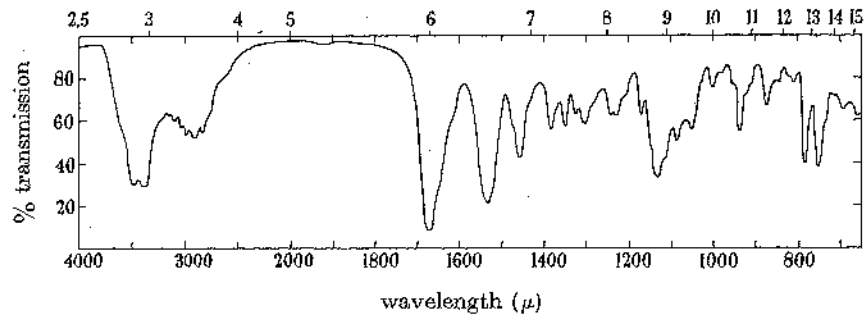


FIGURE 16. Infra-red spectrum of B_1. (c 0·3 % in KBr pellets).

Optical rotation. $[\alpha]_D^{20}$ in dimethylformamide + 29 ± 2 (c = 1); in pyridine no measurable rotation. Rotary dispersion (in dimethylformamide c = 1) $[\alpha]_{700}^{22}$ + 13·1°; $[\alpha]_{589}^{22}$ + 34·0°; $[\alpha]_{450}^{20}$ + 180·2°; $[\alpha]_{400}^{22}$ + 435·1°.

U.v. spectrum. The u.v. spectrum (figure 11) showed the two characteristic maxima of lysergic acid and its derivatives at 312 and 241 mμ. The u.v. fluorescence spectrum (figure 15) had a maximum at 390 mμ.

Infra-red spectrum. The infra-red spectrum (figure 16) was similar, but not identical with that of lysergic acid amide. The first amide band, at 1668 cm^{-1},

was very near the corresponding band of lysergic acid amide, the second amide band, at 1522 cm^{-1}, was shifted to that characteristic for mono substituted amides.

Cleavage into acetaldehyde and lysergic acid amide

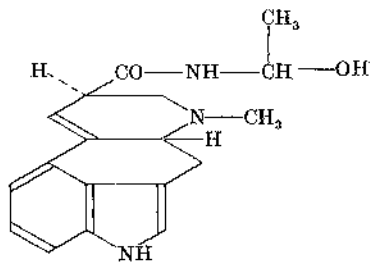
B_1 readily undergoes cleavage to acetaldehyde and lysergic acid amide under different conditions, such as boiling on the water bath for 20 min, or heating with 1M-H_3PO_4 at 100 °C for 60 min, as demonstrated by the following examples:

(1) The free base (62 mg) was suspended in 60 % aqueous ethanol (6 ml.) and heated on the boiling water bath for 20 min in a stream of nitrogen which was passed through two tubes each containing 25 ml. of a 0·5 % solution of 2,4-dinitrophenylhydrazine in 2N-HCl. The 2,4-dinitrophenylhydrazone of acetaldehyde, identified by melting point, mixed melting point and elementary analysis, precipitated (43 mg = 96 % of theory) in the tubes, while in the reaction vessel pure lysergic acid amide crystallized (41 mg = 77 % of theory). No CO_2 was developed during the reaction.

(2) The free base (100 mg) was dissolved in 1M phosphoric acid (3 ml.) and the solution heated in the boiling water bath for 60 min in a stream of nitrogen which was passed through two 2,4-dinitrophenylhydrazine traps as in the previous example. 50 mg of acetaldehyde 2,4-dinitrophenylhydrazone (= 70 % of theory) precipitated. Chloroform extraction of the solution in the reaction vessel at pH 8 yielded 50 mg of a mixture of lysergic and *iso*lysergic acid amide (58 % of theory) which were identified chromatographically.

Structure

On the basis of these facts the structure of B_1 is thought to be that of the following carbinol amide:



Isolation and properties of B_2

Treatment of B_1 at 80 °C for 20 min in 10 % acetic acid led to the formation of a mixture of A_1, A_2, B_1 and B_2. B_2 was isolated by preparative chromatography, but has so far not been obtained in a crystalline state. Its infra-red spectrum was very similar to, but not identical with that of B_1. B_2 could be transformed almost quantitatively into *iso*lysergic acid amide by allowing a 6·5 % solution in 66 % aqueous methanol to stand for 1 h after addition of a few drops of 0·1N-NaOH.

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