STUDIES ON ASPERGILLUS FLAVUS

I. Biological Properties of Crude and Purified Aspergillic Acid

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Received for publication October 7, 1942

In 1940 White (1940) described a strain of *Aspergillus flavus* which showed antibacterial activity against both gram-positive and gram-negative bacteria. A culture of this strain was obtained through the kindness of Dr. White. The following report deals with certain biological experiments carried out with this mold, with crude filtrates containing soluble products obtained from its growth, and with active crystalline aspergillic acid (White and Hill, in press), obtained from these filtrates.

The culture as originally received was growing on Czapek-Dox agar and showed colonies covered with brown spores. On subculture in our laboratory on Czapek-Dox or Sabouraud[1] agar it proved to be a mixed culture of several distinct variants. On Sabouraud agar, which was used as the solid medium of choice, luxuriant growth occurred. All variants originally showed white hyphae but with the onset of sporulation, in those that did sporulate, the colonies took on a brown, green or yellow color. Many single colonies of these variants were picked, obtained in pure culture, grown on one or more of the better fluid media described below, and the crude filtrates tested for antibiotic activity. The variants bred true but new variants appeared in subcultures from time to time and had to be eliminated. Of the variants tested for antibiotic activity, some proved completely inactive, others gave results as good as, or better than, those of the original mixed culture, and one yellow variant proved to be particularly good. On further transfer a new and equally active variant appeared in this yellow substrain,—namely one giving greenish spores. The bulk of the work described below was carried out with the yellow substrain or with the greenish variant obtained from it. The results obtained with these latter two strains appeared identical.

MEDIA

For most purposes cultures of the mold were grown on liquid media of 1.5 cm. depth in Blake or similar bottles. Inoculation was carried out by floating on the surface of the fluid medium large numbers of spores from a 10- to 20-day culture on Sabouraud agar.

The media found to produce highest titers, and hence greatest yield of active crystalline material, were one which contained 2 per cent tryptone and 0.5 per cent NaCl, and others in which 1, 2 or 4 per cent dark brown sugar was added to this simple tryptone-salt base. Media containing 2 or 4 per cent brown sugar

[1] Glucose 4 per cent, peptone 1 per cent, agar 1.8 per cent, and distilled water.

gave, in many cases, titers, and yields, almost twice as high as those obtained with plain tryptone. Czapek-Dox fluid medium, even with addition of brown sugar, supported only scant growth and yielded almost no activity. A medium containing 4 per cent glucose and 1 per cent peptone produced very luxuriant surface growth with heavy sporulation, but again there was almost no activity.

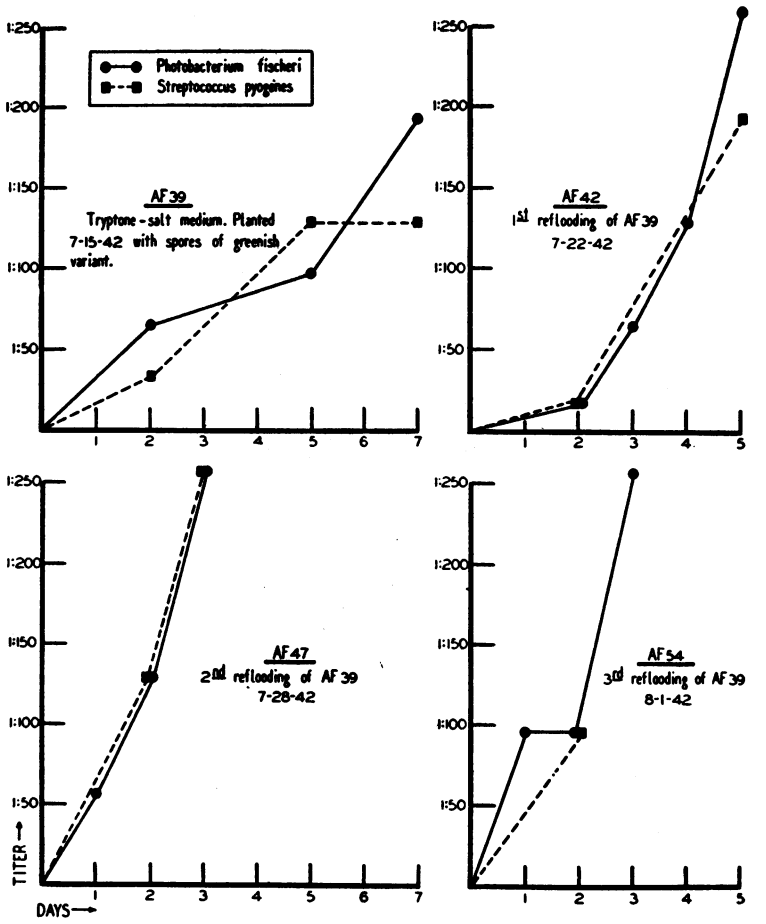

FIG. 1. PARALLEL TITRATIONS WITH ANTIBACTERIAL AND ANTILUMINESCENT TESTS SHOWING INCREASED SPEED OF ATTAINMENT OF MAXIMAL TITERS WITH REFLOODING

On the plain tryptone medium both the variants described above as giving highly active filtrates gave very scant if any sporulation. This is in contradistinction to the results of White and Hill (in press) who noted that, with their culture grown on this medium, good sporulation occurred in five days at which time maximal activity was usually obtained. Certainly, with our yellow or greenish variants on plain tryptone medium, no correlation was found between maximal activity and the presence or absence of even scanty sporulation. However, the

addition of brown sugar produced luxuriant yellow-brown spores within four days and maximal titers usually within the following 24 hours.

Yields of crystalline aspergillic acid obtained from plain tryptone filtrates varied between 120 and 253 mg. per liter. In accord with the higher titers obtained from filtrates of media with 2 or 4 per cent brown sugar, the yield of crystalline material was significantly higher, reaching as high as 400 mg. per liter. These yields are considerably higher than those obtained by White and Hill (in press) and probably result from the use of the two particularly active variants described above.

Reflooding of the mycelial mat on the tryptone-salt medium after harvesting of crude filtrates gave excellent results. Up to two or three refloods were usually made and in each successive case full activity tended to be reached sooner until it was obtained in three days as compared to about seven for the original inoculation run (fig. 1). The reflooding was rendered easy by the fact that contamination never appeared, due, no doubt, to the fact that aspergillic acid is active against almost all organisms (see below) and is produced sufficiently rapidly in the refloods to keep down any chance contaminant. The one difficulty encountered in reflooding arose from the fact that the mats produced on plain tryptone, unlike those obtained on tryptone and brown sugar, were delicate and tended to fold together during the manipulation of harvesting and reflooding. As might be expected, rapid appearance of full activity appeared only with unfolded mats.

Reflooding of the mycelial mat on tryptone brown sugar medium also gave excellent results for at least two refloods. Maximal titer was reached very rapidly, in some cases as early as the 3rd day, and this appeared to be correlated with the fact that in the refloods no initial fall in pH occurred. The difficulty noted in reflooding the mats on plain tryptone medium was not encountered in the case of the brown sugar medium where the mats were much thicker and stiffer and hence did not fold readily.

TESTS FOR ANTIBIOTIC ACTIVITY

For routine testing of antibiotic activity, in order to obtain results comparable to those in other laboratories, use has been made until recently of *Streptococcus pyogenes*—strain C203.

In setting up the test 0.5 ml. of broth is placed in each of ten 13 x 100 mm. tubes. To the first is added 0.5 ml. of the appropriate dilution of the solution to be tested. The contents of this tube are thoroughly mixed and 0.5 ml. transferred to the next tube. This process is continued throughout the series, thus giving two-fold dilutions. To each tube is now added 0.5 ml. of a 1:100 dilution of a six-hour culture of *Streptococcus pyogenes*.[2] The contents of the tubes are mixed thoroughly by shaking, and the test is read after being incubated for 18 hours at 37°C. In every case a standard solution of purified crystalline

[2] In testing for the spectrum of activity, as described below, *Diplococcus pneumoniae* type III, was used in a 1:25 dilution of a six-hour culture. For all other organisms, except the anaerobes, six-hour cultures were used at 1:1,000,000 dilution.

aspergillic acid has been used, and the results in all unknown solutions or filtrates read in comparison with this standard which has been given an arbitrary value of 256 units per mg.

Using this test, increase in activity has been followed in the filtrates. It has been found that after original inoculation the tryptone medium reached full activity in from five to nine days—usually in seven days. As stated above, full activity was reached more rapidly on reflooding, at least for the first three refloods. When brown sugar was added to the medium, full activity was sometimes reached more slowly, occasionally taking as long as ten days. In keeping with the remarkable stability of crystalline aspergillic acid, (Menzel, Wintersteiner and Rake, to be published), the active fraction in the crude filtrates has shown itself to be more stable than is, for example, penicillin (Florey, *et al.*, 1941). Thus in most instances there might be a slight decrease in activity against all organisms (of one tube dilution or less), which tended to occur directly after the peak of activity had been achieved. Following this decrease, if it occurred, the activity remained unaltered for as long as ten weeks, the longest period thus far studied.

Increase in titer was paralleled to some degree by changes in the pH of the medium. In the tryptone medium the pH was originally around 6.7. No initial drop was observed with this medium and by 42 hours an appreciable increase (up to about 7.4) was found. Activity started to appear at this point and maximal titers were achieved when the pH reached between 7.9 and 8.6 (average 8.3). When 2 per cent brown sugar was added to the medium the original pH was unchanged. In this case, however, an initial drop to about pH 6.0 occurred during the first 48 to 72 hours and was followed by a rapid rise. Maximal activity may be associated with a lower pH (pH 7.6 to 8.1) in the case of this medium.

USE OF LUMINESCENT BACTERIA

Following on the fundamental studies on the luminous bacteria (Harvey 1920), the inhibitory action of many substances on the bioluminescence of these organisms has been demonstrated, including such narcotics as the urethanes (Taylor 1934, van Schouwenburg 1938) and barbital (Johnson and Chambers 1939) and also such substances as para-aminobenzoic acid (Woods 1940) and sulfanilamide (Johnson and Moore 1941).

In connection with chemical studies on aspergillic acid it seemed important to develop a routine test for activity which could be read within an appreciably shorter time than the 18 hours required for the antibacterial test described above. It seemed possible that aspergillic acid might have the property of interfering with the luminescence produced by luminescent bacteria; that the degree of blacking out of the luminescence might be read within a few minutes of setting up the test; and that the figures so obtained might show a direct correlation with those obtained in the antibacterial test. With these points in view a culture of *Photobacterium fischeri* was obtained from the American Type Culture Collection. This organism grew well at room temperatures on a medium

consisting of ocean water to which had been added 0.2 per cent peptone or on Cole's artificial sea water containing 0.2 per cent peptone.[3] Using these media faint luminescence was visible in 6 hours, and maximal luminescence appeared in 18 to 26 hours.

The test is set up in the same way as the above antibacterial test already described save that dilutions are made with sea-water medium and that an undiluted 18- to 26-hour old culture of *Photobacterium fischeri* is used. As with the antibacterial test, a standard solution of crystals of aspergillic acid is included in every test, and readings obtained on unknown filtrates and solutions are referred to this standard. All tubes are shaken vigorously immediately before the test is read. Readings are made with the unaided dark-adapted eye and the degree of luminescence recorded as from 1+ to 4+, the latter figure representing the degree seen in the unaffected luminescent mixture. Tests are very clear cut; and, except in early filtrates of very low activity, there is at most only one tube between full luminescence and complete blacking out. In most instances no intermediate tube is found.

Preliminary experiments were read after standing at room temperature for 5, 10 and 30 minutes and also for 18 hours. It soon became apparent that the readings obtained at 30 minutes were the most reliable and significant. Earlier readings were unsatisfactory because interference with luminescence was incomplete. After 30 minutes, however, no further changes in the readings could be detected for several hours. After the test had stood 18 hours, further changes were apparent. New growth of the organism appeared in those tubes in which aspergillic acid was not present in concentrations sufficient to be antibacterial, and this resulted in reappearance of luminescence in some tubes which had been blacked out in the early readings. Not only did these later readings offer no advantage over the ordinary antibacterial test, but it was soon found that a closer correlation obtained with the results of the 30-minute reading and the antibacterial test than was obtained with the 18-hour reading.

Using the 30-minute reading, and comparing the titers so obtained with those obtained from the antibacterial test, it was found in 81 consecutive tests that 26 gave identical titers while of the other 55, 24 were lower with the luminescent test and 31 were higher. In none of the 55 tests in which there was a variation between the two values was this variation greater than one whole tube (i.e. two-fold) dilution and in only 6 of the 55 was it as great as this. The degree of parallelism is shown in figures 1, 2 and 3. This difference is no greater than is found in a number of antibacterial tests run in duplicate. In practice the results obtained by judging activity from the degree of interference with luminescence have been as satisfactory as those obtained with the antibacterial test.

[3] We are grateful to Dr. W. H. Cole of Rutger's University for suggesting the formula for artificial sea water which we have used, viz.:

NaCl	26.7 grams	$MgSO_4 \cdot 7H_2O$	6.81 grams
KCl	0.71 grams	Distilled water	1,000 ml.
$CaCl_2 \cdot 2H_2O$	1.52 grams		
$MgCl_2 \cdot 6H_2O$	5.11 grams		

This method of assay has been applied to the study of other antibiotic substances and to substances of simple chemical nature (Rake, McKee and Jones 1942, Rake, Jones and McKee 1943). Under the conditions of this assay, sulfanilamide was found to have comparatively little activity.

SPECTRUM OF ACTIVITY OF ASPERGILLIC ACID

The activity of aspergillic acid, whether in crude filtrates or in purified crystalline form, has been tested against other organisms than *Streptococcus pyogenes* and *Photobacterium fischeri*. In agreement with White, it has been found that the aspergillic acid has a wide range of activity. Thus, both the crude filtrates, after they have attained a reasonably high titer against *Streptococcus pyogenes*, and also solutions of crystalline material, have proven active against *Diplococcus pneumoniae*—Type III, *Staphylococcus aureus*, *Klebsiella pneumoniae*,

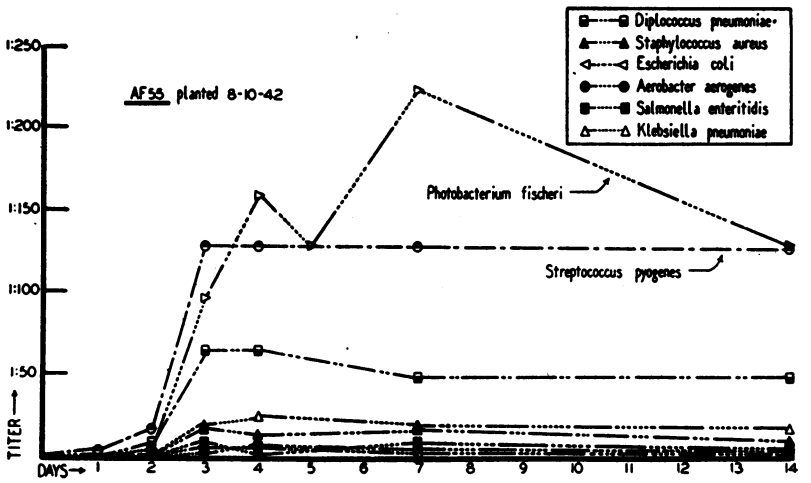

FIG. 2. SPECTRUM OF ACTIVITY OF ASPERGILLUS FLAVUS GROWN ON TRYPTONE-SALT MEDIUM

Salmonella enteritidis, *Escherichia coli*, and *Aerobacter aerogenes*. Crystalline preparations also were found active against the following organisms of the *Clostridium* group: *Clostridium perfringens*, *Clostridium sporogenes*, *Clostridium bifermentans*, *Clostridium septicum*, *Clostridium histolyticum* and *Clostridium novyi*. The degree of activity is summarized in table 1.

The activity against the anaerobes is tested in the following manner: amounts varying from 0.9 ml. to 0.1 ml. of a solution of aspergillic acid containing 400 μg. per ml. are placed in a series of 13 x 100 mm. test tubes, the volume in each tube brought up to 3.9 ml. with broth, and the tubes placed in boiling water for 15 minutes. After rapid cooling, 0.1 ml. of the dilution of culture in broth is added to each tube, followed by a small amount of vaseline-paraffin mixture. After incubation at 37°C. for 18 hours, the smallest amount of aspergillic acid inhibiting growth, as indicated by lack of turbidity, is taken as the endpoint.

The range of aspergillic acid tested was from 10 to 90 μg. per ml. For all

TABLE 1

ORGANISM	MICROGRAMS OF ASPERGILLIC ACID	MAXIMAL ACTIVITY ACHIEVED IN		NUMBER OF ORGANISMS INHIBITED
		Tryptone medium	Brown sugar medium	
Streptococcus pyogenes	2**	512*	784*	1,500,000
Diplococcus pneumoniae	4	256	256	30,000,000
Klebsiella pneumoniae	8	96	128	500
Staphylococcus aureus	10	64	192	500
Salmonella enteritidis	30	32	48	500
Escherichia coli	30	16	24	500
Aerobacter aerogenes	30	24	24	500
Clostridium novyi	20			1,000
Clostridium perfringens	40			5,000
Clostridium septicum	40			1,000
Clostridium sporogenes	60			10,000
Clostridium bifermentans	60			1,000
Clostridium histolyticum	60			1,000

* Figures in these columns are expressed as the reciprocal of the highest dilutions found active against the number of organisms indicated in the final column.

** Figures in this column express the smallest amount of aspergillic acid found active against the number of organisms indicated in the final column.

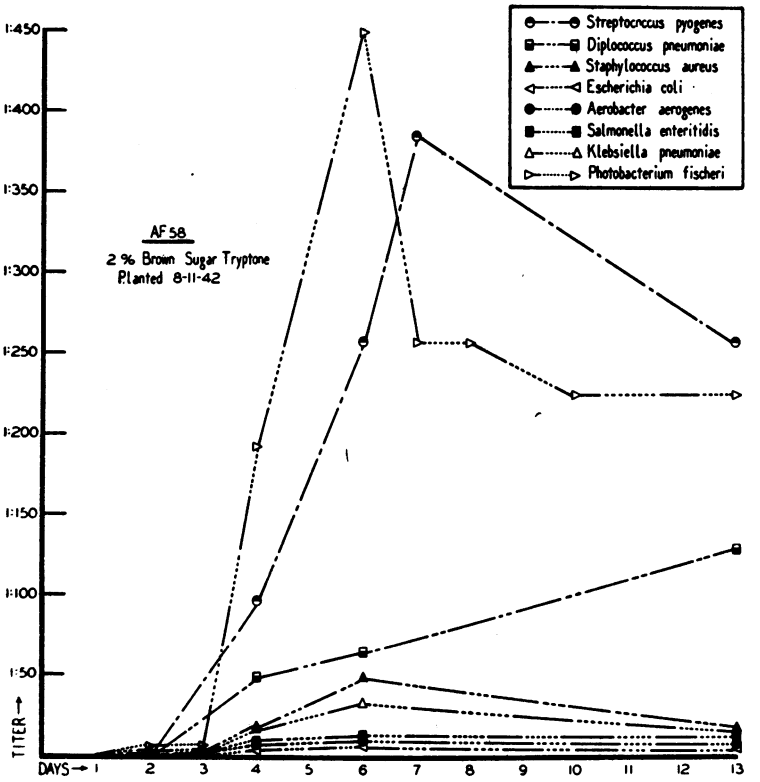

FIG. 3. SPECTRUM OF ACTIVITY OF ASPERGILLUS FLAVUS GROWN ON 2 PER CENT BROWN SUGAR TRYPTONE MEDIUM

cultures and tests the medium was composed of 2.0 per cent Difco tryptose, 0.2 per cent glucose, 0.1 disodium phosphate, 0.5 per cent sodium chloride, and 0.1 per cent sodium thioglycollate at pH 7.6. Six-hour cultures of all organisms were used, except in the case of *Clostridium histolyticum* and *Clostridium novyi*, where 20-hour cultures were employed. Repeated experiments with *Clostridium perfringens* showed that boiling did not alter the activity of aspergillic acid. This is in keeping with the results of Menzel, Wintersteiner and Rake (to be published) on the stability of this material.

In the crude plain tryptone filtrates, activity against other organisms appeared some 24 to 48 hours later than that against *Streptococcus pyogenes* or *Photobacterium fischeri*. From this time on, however, activity against the gram-positive cocci and the gram-negative bacilli appeared and increased concurrently with that shown against *Streptococcus pyogenes*, levelling off at about the same time (fig. 2). As shown in table 1 high titers against the gram-negative organism were not achieved. At no time has it been possible to obtain any significant indication of a different spectrum of activity in early as compared with older filtrates or in any of the filtrates as compared with solutions of the purified crystalline acid.

Similar results have been obtained in the tests on filtrates from the 2 per cent brown-sugar tryptone medium (table 1). In this case higher titers have been achieved in almost every case, but again no significantly different spectrum of activity is apparent in early as compared to later filtrates (fig. 3).

SUMMARY

A strain of *Aspergillus flavus*, previously described by White, has been studied. Careful study of the frequent variants which appeared has allowed the isolation of two which have given consistently far higher yields of the active antibiotic substance, aspergillic acid, than those reported by White, and confirmed by us, as coming from the mixed parent strain.

While good activity and yield were obtained on a 2 per cent tryptone, 0.5 per cent NaCl medium, the addition of 2 per cent brown sugar in many cases significantly increased the yield of crystalline aspergillic acid from 250 mg. to 400 mg. per liter. The length of time required to reach full titer in the brown-sugar medium was longer (average 10 days) than with the plain tryptone medium (average 7 days). Sporulation of the mycelial mats was not essential for production of the active material since practically no sporulation occurred on the plain tryptone medium.

In the early work a simple antibacterial test, using *Streptococcus pyogenes*—strain C203, was employed for the routine assaying of activity. This required a wait of 18 hours before the results could be read. Later, use was made of the interference of the aspergillic acid with the bioluminescence of *Photobacterium fischeri*. Assays by this method could be read within 30 minutes and were found to parallel so closely those obtained with the antibacterial test that the former could replace the latter. This fact may have important application in the study of other antibiotic agents.

Aspergillic acid was found to have wide activity, being very active against gram-positive cocci and less active against the anaerobes of gas gangrene and the gram-negative bacilli. No evidence of significant difference was found in the spectrum of activity shown by early filtrates, by later filtrates or by solutions of purified aspergillic acid.

REFERENCES

FLOREY, H. W., ET AL. 1941 Further observations on *Penicillin*. Lancet, **241**, 177–188.

HARVEY, E. N. 1920 The Nature of Animal Light. Lippincott, Philadelphia.

JOHNSON, F. H., AND CHAMBERS, E. L. 1939 Oxygen consumption and methylene blue reduction in relation to barbital inhibition of luminescence. J. Cellular Comp. Physiol., **11**, 263–267.

JOHNSON, F. H., AND MOORE, K. 1941 Sulfonamide inhibition of bacterial luminescence. Proc. Soc. Exptl. Biol. Med., **48**, 323–325.

MENZEL, A. E. O., WINTERSTEINER, O., AND RAKE, G. To be published.

RAKE, G., MCKEE, C. M., AND JONES, H. 1942 A rapid test for the activity of certain antibiotic substances. Proc. Soc. Exptl. Biol. Med., **51**, 273–274.

RAKE, G., JONES, H., and MCKEE, C. M. 1943 Antiluminescent activity of antibiotic substances. Proc. Soc. Exptl. Biol. Med., **52**, 136–138.

TAYLOR, G. W. 1934 The effect of narcotics on respiration and luminescence in bacteria, with special reference to the relation between the two processes. J. Cellular Comp. Physiol., **4**, 329–355.

VAN SCHOUWENBURG, K. L. 1938 On respiration and light emission in luminous bacteria. Thesis, Delft.

WHITE, E. C. 1940 Bactericidal filtrates from a mold culture. Science, **92**, 127.

WHITE, E. C., AND HILL, J. H. In press.

WOODS, D. D. 1940 The relation of p-aminobenzoic acid to the mechanism of the action of sulphanilamide. Brit. J. Exptl. Path., **21**, 74–90.