

STUDIES ON ASPERGILLUS FLAVUS

II. THE PRODUCTION AND PROPERTIES OF A PENICILLIN-LIKE SUBSTANCE—FLAVACIDIN

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In the previous paper of this series Jones, Rake and Hamre (1943) have reported on the biological properties of an antibiotic substance, aspergillic acid, produced by the mold *Aspergillus flavus*. The culture used in this work was obtained from White (1940) who had reported that this antibiotic substance, active against both gram-positive and gram-negative organisms, was produced when the culture was grown on certain liquid media. A more detailed account of the biological and chemical properties of this substance has recently been published by White and Hill (1943). Glistler (1941) reported an antibiotic substance obtained from a mold which was later identified as *Aspergillus flavus*, which inhibited gram-negative as well as gram-positive organisms, and suggested that it might be similar to the substance described by White. Experiments by Menzel, Rake and Wintersteiner (1943), in which both White's and Glistler's strains of *Aspergillus flavus* were used, have shown that identical substances are produced by both. It has been shown elsewhere (McKee and MacPhillamy 1943) that the same variant of White's strain of *Aspergillus flavus*, used previously for the production of aspergillic acid, is capable of producing under different methods of cultivation an antibiotic substance similar to penicillin. This has been called flavacidin. Bush and Goth (1943, a, b), Heatley (Florey, 1943), and Waksman and Bugie (to be published) have also obtained from *Aspergillus flavus* antibiotic substances similar to penicillin with properties differing from aspergillic acid.

In previous work (Rake, McKee and Jones, 1942; and Jones, Rake and Hamre, 1943) in which *Aspergillus flavus* was cultivated for the production of aspergillic acid a rapid test was used for determining potency which depended upon the inhibition of the bioluminescence of luminescent bacteria. It was this test which first disclosed the fact that a new antibiotic substance unlike aspergillic acid was being produced by the strain under altered conditions. Filtrates in which aspergillic acid is present cause disappearance of luminescence in dilutions even as high as 1-256. On the contrary, filtrates containing flavacidin, even when highly active against streptococci or staphylococci, show slight and irregular inhibition of luminescence. In the present paper further data on this new antibiotic substance are presented.

METHODS OF CULTIVATION

Since deep culture methods had proved of value in the production of other antibiotic substances, it was thought of interest to try these methods for the

cultivation of strains of *Aspergillus flavus*. The first strain used was the variant of the culture supplied by Dr. White which had been used by Jones, Rake and Hamre (1943) for the production of aspergillic acid. Erlenmeyer flasks of two-liter capacity containing one liter of medium were inoculated with spore suspensions prepared by placing spores, scraped from the surface of Sabouraud agar plates, in distilled water. These dry spores tended to clump and float on the surface of the water, but uniform suspensions were obtained by shaking vigorously in tightly stoppered bottles for one half hour. It was at first thought that best results would be obtained by using a heavy inoculum but later experiments, in which a series of fifteen flasks were inoculated with a spore suspension used in three different concentrations, undiluted, 10^{-1} , and 10^{-2} , showed that better results were obtained with the lighter inocula. The average titer of five flasks inoculated with the undiluted spore suspension was 3.5 O.U. per ml., with the 10^{-1} dilution 5.8 O.U., and with the 10^{-2} dilution 6.4 O.U. Possibly a very heavy inoculum exhausts the nutrient elements in the medium before maximal production of flavicidin can be obtained. The inoculated flasks were placed in a shaking machine and subjected to moderate agitation sufficient to keep the medium well aerated. Growth occurred either in the form of small balls or as a formless mass. A light inoculum usually produced the former type of growth. With either light or heavy inocula there was heavy mycelial growth within 48 hours.

The shaking device, an adapted sifter, consisted of a carriage sliding on an angle iron platform, the motion being given by a variable speed motor driving a cam shaft which in turn operated a horizontal eccentric wheel to which the carriage was connected. This gave a circular motion at one end of the carriage and a back and forth motion at the other end. The carriage supported two trays 20 x 48 inches one above the other in which the flasks were placed and separated by suitable packing.

In common with our experience with other molds, considerable variation occurred in titers of individual flasks which contained the same medium and which had received like inocula. The difference in motion in different parts of the shaker seemed to account in some degree for the variable titers obtained.

Since the shaker was operated in a laboratory without special temperature control the optimal temperature for flavicidin production has not been determined. Temperatures varied between 23° and 28°C. Although *Aspergillus flavus*, unlike *Penicillium notatum*, grows well at temperatures as high as 37°C. this may not be best for flavicidin production since inactivation occurs more rapidly at higher temperatures. Titers of flavicidin obtained varied with type and size of flasks and volume of media. Two-liter Erlenmeyer flasks containing 500 ml. of media, 250-ml. Erlenmeyer flasks containing 125 ml. of media and two-liter Florence flasks containing 1 liter of media were all less satisfactory than the two-liter Erlenmeyer containing 1 liter of medium. It is evident that optimal production can be obtained only by ascertaining the best conditions for growth and adhering strictly to those conditions, since slight variations give widely divergent results.

MEDIA

Various media were tried such as tryptone, amigen, casein hydrolysate and beef-heart infusion broths, with the addition of brown sugar as first suggested by Hobby, Meyer and Chaffee (1942) or other adjuvant substances. Flavacidin was produced in these media but the best results were obtained with a modified Czapek-Dox medium. Different basic formulae were tried with the addition of varying amounts of brown sugar and other adjuvant substances. Slight differences in the basic formula apparently made less difference than variation in the adjuvant substances added.

In the medium finally adopted the initial pH was from 5 to 5.5. As growth progressed, alkalinity increased steadily. Unlike penicillin there was no early

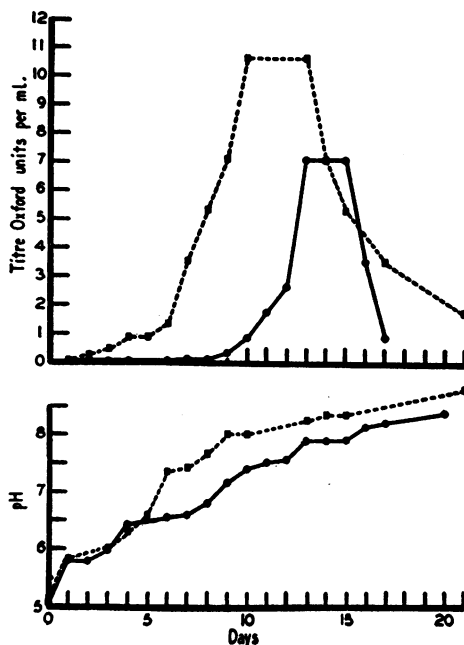


CHART 1

rise in pH with subsequent fall and secondary rise. Appearance of activity was somewhat irregular and bore no direct relation to pH change. In some cases there was a slow and steady rise in activity while at other times there was a more rapid rise, with some activity demonstrable as early as the second day after inoculation. Maximal activity occurred usually about 10 to 14 days after inoculation at a time when the medium had reached a pH of 8 to 8.4. Longer cultivation resulted in loss of activity. The best filtrates obtained had a potency of 22 Oxford units per ml., more frequently however 7 Oxford units was the maximal titer. In several cases in which the medium was adjusted to pH 6.8 before inoculation there was a more rapid production of flavacidin. However, final titers were about the same as the best obtained with the unadjusted medium. Chart I

shows the development of activity in relation to pH change in two flasks which illustrate the different activity curves obtained.

Methods of assay

A standard serial dilution test (McKee, Rake and Menzel, in press) has been used for determining the potency of flavacidin filtrates. The medium used was a beef-extract broth and the test organism a *Staphylococcus aureus* culture obtained from Dr. N. G. Heatley which has been widely used in testing the potency of penicillin filtrates. In order to have some basis of comparison with penicillin a standard which was used in determining Oxford unitage of penicillin was adopted as the standard for flavacidin. All tests with flavacidin filtrates and purified preparations have been compared with this standard which was tested at the same time. It is thus possible to express flavacidin potency in terms of Oxford units. Another more accurate test (McKee, Rake and Menzel, in press), which has been used in estimating the activity of penicillin preparations when the approximate potency is known, has also been used in testing flavacidin preparations. In this test an appropriate preliminary dilution of flavacidin is made in broth and amounts varying from 1 to 0.1 ml. are added to a series of ten tubes. The volume in each tube is then brought up to 1 ml. by the addition of sterile broth and an equal volume of culture diluted 10^{-6} is added. Readings are made in comparison with the standard which is tested in the same manner.

Flavacidin filtrates have shown great irregularity in their response to the antiluminescent test mentioned previously. Some were completely inactive, some were active in dilutions of 1-4 or 1-8, and others showed an inactive prozone with inhibition of luminescence occurring in dilutions ranging from 1-16 to 1-64, or 1-32 to 1-128. We are at present unable to explain these irregularities. It seemed possible, however, that inhibition of luminescence, at least in the higher concentrations, might have been due to the presence of small amounts of aspergillic acid. It has been shown that flavacidin filtrates contain aspergillic acid in small amounts. Forty-one mg. of a substance identified chemically as aspergillic acid were obtained from 1 liter of a deep culture filtrate containing 7 O.U. per ml.¹ This substance caused the same inhibition of luminescence as other purified preparations of aspergillic acid. When *Aspergillus flavus* is cultivated under the best conditions for the production of aspergillic acid the average yield is 250 mg. from one liter.

The antiluminescent test has also been useful in indicating the presence of aspergillic acid in partially purified preparations of flavacidin. Three sodium salts of flavacidin having potencies of 50, 107 and 214 Oxford units per mg. have shown antiluminescent activities respectively of 13, 25 and 4 R. units when compared with an aspergillic acid standard to which has been ascribed the value of 256 R. units. It is probable that this activity is caused by aspergillic acid present as an impurity in these comparatively crude preparations of flavacidin since the methods used for the extraction of flavacidin from crude filtrates would also

¹ We are indebted to Dr. J. D. Dutcher of the Division of Organic Chemistry of the Squibb Institute for the isolation and identification of the compound.

extract any aspergillic acid which might be present. It may be noted that the preparation of highest purity had the least antiluminescent activity.

RESULTS

The ability of *Aspergillus flavus* to produce, under different methods of cultivation, two antibiotic substances of such diverse character as aspergillic acid and flavacin suggested that a mutation might have occurred in the organism. In order to test this possibility cultures on Sabouraud agar were made from two flasks in which flavacin had been produced by deep culture methods. The resultant cultures had a morphology typical of the original culture which had been used for the production of aspergillic acid. Spores from these cultures were inoculated on the surface of tryptone brown-sugar medium in Blake bottles for the production of aspergillic acid and into the same medium in Erlenmeyer flasks for the production of flavacin by shaking. Typical yields of aspergillic acid were obtained from the Blake bottles and flavacin from the shaken cultures in Erlenmeyer flasks.

The production of flavacin instead of aspergillic acid by *Aspergillus flavus* appears to be a function both of type of medium and method of cultivation, thus when tryptone brown-sugar medium was used aspergillic acid was produced in static cultures and flavacin in shaken culture and the method of cultivation was the determining factor. However, when a modified Czapek-Dox medium in shallow layers in Blake bottles was used flavacin was produced. In this case the medium was unsatisfactory for aspergillic acid production but satisfactory for flavacin. It is probable that all crude filtrates of *Aspergillus flavus* cultures contain an admixture of the two antibiotic substances, the predominating substance being determined by method of cultivation. The activity of flavacin produced in static culture even with the best medium was not as great as that produced in shaken culture. In one instance maximal activity, 2 Oxford units per ml., was reached on the tenth day when the pH was 7.8 and had begun to decline by the twelfth day when the pH was 8.

Production of flavacin has also been obtained in larger volumes of media. A 40-liter capacity container equipped with an electric stirrer and a tube for the introduction of air was filled to one-half capacity and inoculated with a spore suspension of *Aspergillus flavus*. Growth occurred rapidly and within 6 days a titer of 7 Oxford units was obtained. When air was bubbled through the medium without added agitation little activity resulted.

Two other strains of *Aspergillus flavus*, the Glistler strain² and one obtained from Dr. Waksman,² as well as variants of the White strain have been tested for ability to produce flavacin. The Glistler strain produced flavacin but in somewhat lower titer than the White strain. The Waksman strain 137 produced filtrates having approximately the same maximal potency as that produced by the White strain; the activity, however, appeared and declined earlier. Two variants of

² We are indebted to Dr. Selman A. Waksman of the New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, New Jersey for strain 137 and to Dr. H. W. Florey, Department of Pathology, University of Oxford, England, for the Glistler strain.

the White strain which sporulated less readily than the variant used proved comparatively inactive.

A crude flavacidin filtrate having a potency of 3.5 Oxford units per ml. was passed through a Seitz filter and divided into six portions which were adjusted to pH values between 8.2 and 4.13. These were stored in the icebox and potency tests made at intervals. The results of five tests made from 7 to 80 days after storage at 0°C., and of two tests made after subsequent storage at 26°C. are shown in table 1.

The range of greatest stability is thus shown to be between pH 6 and 6.6. On the acid side there is rapid loss and on the alkaline side slower loss. Penicillin filtrates are most stable at a pH of 5.5 to 6.5 and they too show a more rapid loss of activity at pH 4 than at 8.

Flavacidin shows a somewhat greater toxicity than penicillin, which may be due to small amounts of contaminating aspergillitic acid. Because of the limited supply of flavacidin available only small numbers of mice were used for toxicity

TABLE 1
Effect of pH on stability of flavacidin

DAYS	0°C.					26°C.	
	7	14	24	40	80	8	16
pH 8.2	2.5*	2.5	1.7	0.9	0.9	0.15	0.06
7.6	3.5	2.5	2.5	1.7	0.66	0.6	0.11
6.6	3.5	3.5	3.5	3.5	2.5	2.5	1.78
6.35	3.5	3.5	3.5	3.5	3.5	2.5	1.78
6	3.5	3.5	3.5	3.5	2.5	2.5	1.78
4.13	0.9	0.44	0.16	0.1	0.05		

* The figures represent the number of Oxford units per ml. at the different time intervals. The original potency was 3.5 Oxford units per ml.

tests. In the case of two sodium salts of flavacidin with potencies respectively of 134 and 214 Oxford units per mg. which were tested for toxicity by intravenous injection into 20-gram mice the $L_D/50$ ranged from 280 mg. per kilogram for the less pure to 560 for the purer preparation. When these figures are compared with those for penicillin and aspergillitic acid the toxicity of flavacidin is found to be intermediate between the two. The $L_D/50$ for a sodium salt of penicillin having 100 Oxford units per mg. was >1000 mg. per kilogram and for one having 500 Oxford units was >2000. On the other hand, aspergillitic acid has a toxicity for mice of 48.5 mg. per kilogram when given by the intravenous route. Since it has been shown that flavacidin filtrates contain small amounts of aspergillitic acid and since the methods used for purification may not have removed the aspergillitic acid, the greater toxicity of flavacidin over penicillin is readily explained. Purer preparations of flavacidin than are now available are necessary before adequate toxicity studies can be made.

The similarity in spectrum of activity of flavacidin and penicillin is very marked. As is well known penicillin, while very active against most gram-posi-

tive organisms, has little activity against gram-negative bacilli. This is likewise true of flavacidin. Three sodium salts of flavacidin of potencies of 35, 50 and 107 Oxford units per mg. were tested for activity against four gram-positive and four gram-negative organisms. Table 2 gives the results obtained with these three sodium salts of flavacidin as compared with results obtained with a calcium salt of penicillin having a potency of 30 Oxford units per mg. and two sodium salts having potencies respectively of 105 and 890. A purified preparation of aspergillic acid is also included.

It has been shown (Rake, McKee, Hamre and Houck, in press) that the potency of penicillin against gram-positive organisms, as determined *in vitro*, is a reliable guide for efficacy *in vivo*. A comparison of the efficacy of penicillin and flavacidin against a Type I pneumococcus infection in mice (McKee and Mac-

TABLE 2
Comparison of activity of flavacidin, penicillin and aspergillic acid

ANTIBIOTIC SUBSTANCE	AC-TIVITY OXFORD UNITS	STA-PHYLO-COCCUS AUREUS	STREP-TOCOCCUS PYO-GENES	PNEU-MOCOCCUS TYPE III	PNEU-MOCOCCUS, TYPE I	SALMON-ELLA ENTERI-TIDIS	AERO-BACTER AERO-GENES	KLEB-SIELLA PNEU-MONIAE	ESCHE-RICHIA COLI
Flavacidin.....	35	0.39	0.195	0.195		125	250	125	250
	50	0.156	0.313	0.234	0.234	78	312	624	312
	107	0.078	0.156	0.078	0.039	15.6	>250	>250	>250
Penicillin.....	30	0.39	0.39	0.39		78	1560	1870	1870
	105	0.091	0.136	0.195		53.2	1666	1562	468
	890	0.156	0.156	0.156	0.019	1.95	62.5	125	62.5
Aspergillic acid.....		8	4	4	2	23.5	31	8	31

The figures show the smallest amount in micrograms per ml. which inhibited growth of the test organisms. Cultures of the staphylococcus and gram-negative organisms were diluted 10^{-6} and the pneumococcus and streptococcus cultures 10^{-2} .

Phillamy, 1943) has shown that the two substances give equal protection when comparable amounts in terms of Oxford units as determined *in vitro* are given.

Since equal protection was achieved with flavacidin and penicillin when comparable doses were given, it seemed probable that excretion by the kidneys might occur at about the same rate. This proved to be the case. A solution of a sodium salt of penicillin was prepared with a potency comparable to that of a crude flavacidin filtrate which had 5 O.U. per ml. The two preparations were injected subcutaneously into groups of mice. Urine was collected at intervals from each group. Pooled samples taken at various time intervals were tested *in vitro* for activity. Table 3 shows the comparative activity in Oxford units per ml. of urine of six pooled samples from the two groups taken from $\frac{1}{2}$ to 5 hours after injection.

In order to investigate further possible points of similarity between flavacidin and penicillin a Type III pneumococcus culture which had been rendered resistant

to penicillin by passage *in vitro* in increasing concentrations of penicillin (McKee and Houck, 1943) was compared with its parent culture for resistance to flavacidin. At the same time the cultures were tested with three other antibiotic substances, aspergillic acid, gramicidin, and gliotoxin. It was found that the penicillin-resistant culture was likewise resistant to flavacidin, but not to the other three antibiotic substances. The figures given in table 4 show that while there was a 32-fold increase in resistance to penicillin there was a 16-fold increase to resistance to flavacidin. The apparent greater sensitivity of the penicillin-resistant culture to the action of gramicidin may be explained by the fact that cultures which have been rendered resistant to penicillin have a slower rate of growth than the parent cultures and are possibly more sensitive to an agent with such marked bacteriostatic action.

It has also been shown that a *Staphylococcus aureus* culture when subjected to serial passage *in vitro* in increasing concentrations of flavacidin readily became resistant to flavacidin and also to penicillin. Sixteen passages resulted in 30-fold increase in resistance to both flavacidin and penicillin. As is true with

TABLE 3
Amounts of flavacidin and penicillin in urine of mice at various time intervals after subcutaneous injection

TIME OF INJECTION	FLAVACIDIN	PENICILLIN
hours	O.U./ml.	O.U./ml.
$\frac{1}{2}$	14	14
1	14	14
$1\frac{1}{2}$	7	3.5
$2\frac{1}{2}$	1.7	0.8
$3\frac{1}{2}$	0.2	0.1
5	<0.05	

penicillin-resistant cultures, loss of virulence for mice accompanied increased resistance to flavacidin.

Another point of similarity between flavacidin and penicillin was in their susceptibility to the destructive action of an enzyme which was inactive against aspergillic acid, gliotoxin and tyrothricin. A *Staphylococcus aureus* which appeared as a contaminant in a culture of *Aspergillus flavus* was found to be resistant to the action of both flavacidin and penicillin. This resistance was due to the destructive action for both substances of an enzyme elaborated by the culture. Seitz filtrates of broth cultures when mixed with either flavacidin or penicillin destroyed activity. The length of time necessary for complete destruction of activity was proportional to the potency of the flavacidin or penicillin, more time being required to inactivate the preparations of higher potency. Destruction of activity occurred at 25° and 37°C. Table 5 shows the degree of inactivation of flavacidin and penicillin obtained after 24 hours at 37°C. as compared with the stability of preparations of aspergillic acid, gliotoxin and tyrothricin. It will be seen that in the flavacidin and penicillin solutions of higher concentration (10 mg. per ml. and 2 mg. per ml. respectively) no inactivation was apparent after 24

TABLE 4

Comparative action of five antibiotic substances on penicillin-resistant and control cultures of a type III pneumococcus

TYPE III PNEUMOCOCCUS CULTURE	ANTIBIOTIC SUBSTANCE	DILUTIONS IN MICROGRAMS PER ML.									
		25	12.5	6.25	3.125	1.56	0.78	0.39	0.19	0.097	
Control Penicillin resistant	Flavacidin	25	12.5	6.25	3.125	1.56	0.78	0.39	0.19	0.097	
		-	-	-	-	-	-	-	-	-	+
Control Penicillin resistant	Penicillin	12.5	6.25	3.125	1.56	0.78	0.39	0.19			
		-	-	-	-	-	-	-	+		
Control Penicillin resistant	Aspergillie acid	250	125	62.5	31.25	15.6	7.8	3.9	1.9	0.9	
		-	-	-	-	-	-	-	-	-	+
Control Penicillin resistant	Gliotoxin	25	12.5	6.25	3.125	1.56	0.78	0.39	0.19	0.097	
		-	-	-	-	-	-	-	-	-	+
Control Penicillin resistant	Gramicidin	0.025	0.012	0.006	0.003	0.0015	0.00075	0.00037			
		-	-	-	-	+	-	-	+		

TABLE 5

Comparative destructive action of an enzyme on five antibiotic substances

ANTIBIOTIC SUBSTANCES WITH EQUAL VOLUME OF ENZYME FILTRATE OR BROTH. 24 HRS. AT 37°		STARTING DILUTION OF ANTIBIOTIC SUBSTANCES TESTED	TEN TWO-FOLD DILUTIONS										
			1	2	3	4	5	6	7	8	9	10	
Flavacidin	10 mg. + Enzyme + Broth	2 mg.*	-	-	-	-	-	-	-	-	-	-	+
		200 γ	-	-	-	-	-	-	-	-	-	+	
	200 γ + Enzyme + Broth	200 γ	+	-	-	-	-	-	-	+			
		20 γ	-	-	-	-	-	-	+				
Penicillin	2 mg. + Enzyme + Broth	2 mg.*	-	-	-	-	-	-	-	-	-	-	-
		20 γ	-	-	-	-	-	+					
	200 γ + Enzyme + Broth	200 γ	+	-	-	-	-	-	+				
		20 γ	-	-	-	-	-	+					
Aspergillie acid	200 γ + Enzyme + Broth	200 γ	-	-	+								
		200 γ	-	-	+								
Gliotoxin	20 γ + Enzyme + Broth	20 γ	-	-	-	-	-	+					
		20 γ	-	-	-	-	-	+					
Tyrothricin	20 γ + Enzyme + Broth	20 γ	-	-	+								
		20 γ	-	-	+								

* When subjected to a further 24 hours incubation activity was completely destroyed.

hours, while the preparations of lower potency were inactivated. However, after a further incubation of 24 hours all activity in the higher concentrations had been destroyed.

DISCUSSION

As has been indicated in the foregoing, one particular variant of one particular strain of *Aspergillus flavus* has proven especially active in the production not only of aspergillic acid (White 1940; Jones, Rake, and Hamre, 1943) but also of a substance closely resembling penicillin which we have called, provisionally, flavacidin. That one strain of an organism should produce two different antibiotic substances is not a new observation, nor is it particularly surprising. Thus *Penicillium notatum* produces penatin as well as penicillin (Kocholaty, 1942). If, however, flavacidin is identical with penicillin, and all the facts point to the conclusion that it is very closely related, it is perhaps more surprising that similar substances should be produced by molds believed to belong to different genera. In at least one other case it has been possible to show that two molds belonging to different genera, namely, *Gliocladium fimbriatum* and *Aspergillus fumigatus* both produce the same antibiotic substance—gliotoxin (Menzel, Wintersteiner, and Hoogerheide, to be published).

As should be emphasized, it is possible to produce aspergillic acid or flavacidin, at least in preponderance, if not to the exclusion of the other, by certain definite manipulations. These may be changes in medium while maintaining other conditions of cultivation (agitation, aeration, etc.) unaltered; or changes in method of cultivation while using the same medium. This fact draws attention to the great importance of studying any new mold species in more than one medium and under more than one physical condition of cultivation. Earlier surveys for new antibiotic substances which have omitted this precaution, may prove to have been inadequate.

The presence of a substance other than aspergillic acid in the shaken cultures was indicated in the present instance through the use of the antiluminescent test (Rake, McKee and Jones, 1942). This fact emphasizes the importance of carrying out at least two different types of biological assay on culture filtrates from molds of unknown potentiality in order that the presence of more than one substance may be detected.

The possible identity of flavacidin with penicillin has been based up to the present mainly on biological grounds. Since pure flavacidin is not yet available, chemical means of identification may be lacking for some time although indications of chemical similarity do already exist (McKee and MacPhillamy, 1943). However, at least two of the biological tests, namely, that of susceptibility of penicillin to a flavacidinase and also the fact that induced resistance of organisms to penicillin enhanced resistance to flavacidin and vice versa, are of such a nature that one can safely predict the close chemical similarity if not identity of the two substances.

SUMMARY

The mold, *Aspergillus flavus*, produces under certain conditions a substance flavacidin which resembles penicillin.

Submerged growth in a modified Czapek-Dox medium with agitation and aeration is the most suitable of these conditions.

The biological characteristics of flavacidin and penicillin are similar: (a) both are highly active against gram-positive organisms and relatively inactive against gram-negative bacilli, (b) both protect mice in equal degree against pneumococcus infection, (c) both are highly soluble and hence are readily absorbed after parenteral inoculation and are quickly excreted by the kidneys, (d) cultures resistant to the action of penicillin are resistant also to flavacidin but not to other antibiotic substances, (e) an enzyme active against penicillin is active also against flavacidin but not against other antibiotic substances.

We wish to express our indebtedness to Dr. H. B. MacPhillamy of the Division of Organic Chemistry of the Squibb Institute for Medical Research for the partially purified sodium salts of flavacidin and penicillin used in this work.

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