Summary. Evidence is presented in confirmation of the work of others that smallpox (vaccinia) vaccination gives rise to false positive reactions for syphilis. The incidence of positive reactions by the use of the Kolmer, Kahn and Mazzini tests was 11.8%. The false positive reactions are transitory but may persist for 2 months or more.

Addendum. The patients with positive reactions were retested every two weeks. There was a gradual diminution in the intensity of the reactions until all became negative by the end of 120 days.

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Growth of *Penicillium Notatum* on Various Media and the Development of an Antibacterial Substance.

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The antibacterial action of penicillium was first observed by Fleming,[1] and a further report was made by Chain and others.[2] Subsequently the preparation and the properties of this substance were reported in greater detail.[3,4]

The more recent studies have been primarily concerned with the isolation of a substance designated as penicillin and the mold was grown on a modified Czapek-Dox synthetic medium.[3] It seemed desirable to make some observations with this organism on other media. Various procedures were used to increase the growth of the penicillium and the effect of such procedures upon the production of an inhibitory substance was determined.

The data reported in this paper include (1) the growth of the mold penicillium on 3 different media (modified Czapek-Dox, infusion broth, and Amigen); and the relation of the pH of the media to the production of an antibacterial substance; (2) the influence of the addition of various sugars on the production of bacterial inhibitory substance; (3) the extraction of an antibacterial substance from the thallus.

Materials and Methods. Culture Media: (1) Modified Czapek-Dox synthetic media; this will be referred to as the Oxford Media. (2) Difco stock heart infusion broth; (3) an enzymic digest of purified casein and pork pancreas in which the proteins have been hydrolyzed to amino acids 75% and di- and tri-peptides 25%. This preparation will subsequently be referred to by its trade name of Amigen.* The pH range of all media was 4.4 to 5.0 when inoculated. All pH determinations were made with a glass electrode.

Cultures: A strain of *Penicillium notatum* was obtained from Dr. N. F. Conant, Durham, N.C. Stock cultures were grown on Sabouraud's media. The cultures for study were all grown in 225 cc Ehrlenmyer flasks. A strain of hemolytic *Staphylococcus aureus* isolated from a patient with osteomyelitis was used as the test organism. Dr. Gladys Hobby kindly determined the sensitivity of this culture to several preparations of penicillin. It was found to be about one-fourth as sensitive to the drug as the test organism, a strain of hemolytic streptococcus (C 203 MV).[5] This

[1] Fleming, A., *Brit. J. Exp. Path.*, 1929, **10**, 226.

[2] Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J., and Sanders, A. G., *Lancet*, 1940, **2**, 226.

[3] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **2**, 177.

[4] Abraham, E. P., and Chain, E., *Brit. J. Exp. Path.*, 1942, **23**, 103.

* The media made with Amigen contained 10% of Amigen powder by weight dissolved in distilled water. The Amigen was supplied by Mead Johnson Co., Evansville, Indiana.

[5] Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 277.

indicates that its sensitivity to penicillin is comparable to that of the strain of *Staphylococcus aureus* used by the Oxford workers.[6]

Methods of Titration: (1) Serial dilution method. The material to be tested was diluted with broth in dilutions of 1:10, 1:20, 1:40 to 1:1,280. The tubes each containing 0.5 cc were then inoculated with the test organism using a 3 mm platinum loop (containing approximately 300,000 organisms) and incubated for 24 hours at 37°. The titrations seldom varied more than one tube. (2) Plate method: Melted blood agar was inoculated with hemolytic *Staphylococcus aureus* culture and poured into Petri dishes. Plugs of agar were removed with a 4 mm metallic cork borer, and 0.05 cc of the material to be tested was carefully placed in the openings. The plates were incubated for 24 hours and the diameter of the zone in which no growth occurred was measured. Observations made during this study indicate that the serial dilution method was the more satisfactory of the 2 methods.

Growth on Different Media. The mold penicillium grew well on both Amigen and broth when the initial pH was adjusted to 4.5. The growth was accelerated when grown on Amigen as compared with the other 2 media. The antibacterial titers did not persist for longer than 7 days in Amigen whereas they remained for a considerably longer period in broth. Growth was more uniform on broth and Amigen than on the Oxford media. Growth was slow at pH of 3 and there was none at a pH below 2.3. No change in pH occurred until growth became grossly apparent. The rate of growth then paralleled the increasing pH until the media became neutral; as the pH continued to rise, growth diminished.

Although no relationship was observed between the size of the thallus and the titer of the antibacterial substance, there was apparently some relationship between the character of the growth and titer. A slow-growing homogeneous thallus with a deep green, fine early sporulation that subsequently became green-brown in color was usually associated with a persistent titer in the media. Maximum titers were obtained usually between the fifth and eighth day when the pH of the media was between 7.0 and 8.0. The addition of hydrochloric acid to a growing culture was a marked stimulus to growth but temporarily delayed the development of an inhibitory titer of the culture media. The addition of acid to an alkaline culture without a titer reinstituted growth with subsequent rapid development of an inhibitory titer. When such an alkaline culture was acidified it rapidly became alkaline and there was renewed growth of the pellicle. This procedure was repeated several times and in each instance the titer which had fallen would again rise to its original level but it never exceeded the original titer. Subsequently, when the media was allowed to remain alkaline the titer rapidly disappeared.

The inhibitory titers of broth cultures persisted considerably longer than those of Amigen cultures. Titers of broth cultures have persisted for 20 days at a temperature of 2°C, whereas the titer of the same material disappeared in 4 days at room temperature. The titer of frozen broth media persisted for as long as 60 days.

Titers were enhanced by concentrating the broth culture media in a cellophane bag and hastening evaporation by an electric fan. There was a fair relationship between the titer and the degree of concentration (Table I).

TABLE I.

Effect of Concentration of Infusion Broth Cultures of Penicillium on the Inhibitory Titer.

Culture	Titer
Unconcentrated	1:160
Conc. to 50% original vol.	1:640
" " 65% " "	1:1280
" " 90% " "	1:20,480

The Influence of the Addition of Various Sugars to the Media. Experiments were set up to determine the effect of the addition of carbohydrate to the media. When sugar was added to broth media the pH remained acid for a longer period as growth progressed. The addition of sugar to broth or Amigen produced an increase in growth of penicillium in all instances.

Dextrose, sucrose, maltose and lactose were added to broth at pH 4.5, in concentrations of 1, 2, 5 and 10%. The media was then

[6] Hobby, G. L., personal communication.

TABLE II.
Inhibitory Titers of Infusion Broth Cultures of Penicillium to Which Different Sugars Were Added.

	Days									
	4	6	8	10	12	14	18	22	26	30
Infusion Broth	80	320	80	80	80	10	—	—	—	—
" " + 5% Lactose	40	160	320	640	640	1280	1280	640	320	160
" " + 5% Maltose	0	0	80	160	640	80	80	—	—	—
" " + 5% Sucrose	0	0	0	80	320	160	20	—	—	—
" " + 5% Dextrose	0	20	80	40	20	—	—	—	—	—

inoculated and antibacterial titers and pH determined usually daily and occasionally every second day. The results will not be described in detail; however, of the various concentrations tested those with 5% sugar developed the highest inhibitory titers. A comparison of the different sugars is given in Table II. It is evident that of the sugars tried 5% lactose gave the maximum titer and that it persisted at a fairly high level for as long as 30 days. When lactose was added to Amigen or Czapek-Dox media it did not apparently enhance the inhibitory titers of penicillium cultures.

Bacteriostatic Substances Extracted from the Thallus. The thallus was removed from the culture media, dried carefully on filter paper and weighed. A small part was used for determination of dry weight and the remainder ground in a mortar with sea sand. The ground material was then immediately extracted with various solvents.

Extracts of the thallus in distilled water, ethyl ether, acetone or pyridine had no inhibitory effect upon the growth of *Staphylococcus aureus*. The ground thallus was extracted with small quantities of ethyl alcohol at 65°C. Much of the extracted material was inert and could be precipitated at 0°C. Equally active extracts were obtained at 25-40°C but they contained less inert material. When the thallus was dried to constant weight at 100°C prior to extraction, no bacteriostatic effect was noted in the alcoholic extracts.

The activity of such extracts was tested by the serial dilution method and it was found that 70 γ of crude alcohol soluble material was active against 300,000 organisms in 1 cc. Expressed in terms of Oxford units per mg the alcohol soluble material contained 0.095 Oxford units or approximately 0.1 of an Oxford unit per mg.

Alcohol extractions of the thallus were made successively from the 5th to the 11th day inclusive. The most potent extracts were obtained on thalli removed from 5- to 7-day-old cultures. The potency decreased on succeeding days and disappeared on the 10th day. No extracts of the thallus were active when the culture media on which the thallus grew failed to show some bacteriostatic property. At no time could a relationship be demonstrated between the amount of inhibitory substance in alcoholic extracts of the thallus and the titer of the culture media when the latter was active. There was no apparent relation between the quantity or rate of growth and the potency of the extracted material.

Conclusions. 1. *Penicillium notatum* grows well on nutrient broth and Amigen at pH 4.5. The growth is superior to that obtained on a modified Czapek-Dox medium. 2. The nature of the growth as well as the persistence of an antibacterial substance in the media is, in part, dependent upon the pH of the media. 3. Lactose added to broth cultures in a concentration of 5% considerably increased the inhibitory titer which persisted for as long as 30 days. 4. A bacteriostatic substance was extracted from the thallus of *Penicillium notatum* with ethyl alcohol.