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HYDROLYSIS OF BETA LACTAM LINKAGE BY PENICILLINASE

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1 Claim. (Cl. 195—30)

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This invention relates to an enzymatic substance and more particularly to an enzymatic substance from a species of actinomyces now identified as *Actinomyces candidus*, which acts as an inhibitor or antagonist of penicillin, an antibiotic agent.

In the sterility control of penicillin and penicillin-containing substances it has been standard practice to mix a solution containing the penicillin with solutions of antagonists, such as cysteine-HCl, or Hydroxylamine-HCl, allowing them to remain in contact sufficient time before inoculation into various media to insure destruction of the antibiotic activity of the penicillin. In certain instances a taka-diastase preparation commercially available as "Clarase" is used for the inactivation of penicillin. The penicillin inhibiting materials in "Clarase" are derived from bacterial contaminants of the subtilis-mesentericus group during the manufacture of the taka-diastase and are in no way associated with the metabolic products of the mold itself, *Aspergillus cryzae*. The penicillin inactivating materials produced in the preparation of clarase vary widely in the amount present and in certain lots, depending upon the other organisms present, may be entirely absent.

When penicillin has been inactivated by any of the above procedures the inactive material is distributed into portions of culture media and observed for a period of time for signs of growth. Sensitive organisms, which may be capable of resisting the action of penicillin while in the sporulating stage, may thus be grown out. This is also true of penicillin-resistant organisms which may remain dormant in the penicillin preparations but become viable in the body. Thus the preparation to be described has decided value in the sterility control of this antibiotic and its various preparations.

During the therapeutic application of penicillin preparations, it at times becomes necessary to determine whether the infecting organisms has been eliminated from the patient. The organism may have been inhibited by the penicillin but not destroyed. In this condition it may remain virulent and produce a relapse as soon as penicillin therapy is withdrawn. Blood must be obtained from the patient to determine whether the infectious agent has been eliminated. This is difficult at times during the therapy since there is penicillin in the blood stream, often in concentrations high enough to inhibit the infecting organisms in the blood withdrawn. The enzyme to be described here rapidly destroys this residual blood penicillin when added to the culture flasks and allows viable organisms present to grow out. Thus the

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substance is of value in diagnostic procedures.

In the chemical assay of penicillin one method employs the specific reactivity of an enzyme preparation specific for the beta-lactam linkage which exists in penicillin. This enzyme acts by forming a new carboxyl group which is subject to measurement, the molar concentration affording an extremely accurate method of assaying the amount of penicillin present. When the enzyme to be described is purified by alcohol and acetone precipitations to concentrate the enzyme and remove interfering buffer systems, it may be employed directly in this type assay. The assay results obtained agree very closely with all known chemical methods for assay of penicillin. The assay of crystalline penicillin G by this method, for instance, gives a value within 4% of the theoretical as a maximum deviation.

In the identity tests for antibiotic agents it often becomes necessary to use a specific enzyme to differentiate two closely related substances. The penicillinase to be described is specific for the beta-lactam structure which in antibiotics occurs only in the penicillins. Thus this enzyme serves as a reliable method for the identification of penicillin.

The substances now in use for accomplishing the above have several inherent disadvantages. Cysteine and hydroxylamine are toxic to many bacteria unless used with certain qualifications which detract from their value. "Clarase," the commercial taka-diastase is not bacteriostatic but the production of the penicillin inactivating materials is haphazard depending upon contamination with poorly defined bacterial agents. Thus a desirable preparation of penicillinase is one that can be readily produced from an identifiable micro-organism and one which can be produced consistently and in a yield which will permit practical application.

Briefly restated the penicillinase to be described concerns an enzymatic substance which specifically reacts with penicillins and in so doing affords a means for determining sterility, efficacy of therapeutic application, identity and potency of penicillin and penicillin-containing preparations. Accordingly, an object of this invention is to provide a novel penicillinase in such form that it possesses practical value. This substance is obtained as a metabolic product of growth of an air borne micro-organism which was isolated in the following manner:

Petri dishes were prepared with sterile nutrient agar containing sufficient penicillin added just before hardening to give a final concentration of 1000 units per milliliter. These plates were then exposed in various places like dusty attics, on ledges of buildings, in storerooms, etc.

After thirty minutes to two hours the plates were covered and allowed to incubate. Any colonies that grew were removed and subjected to further study. A typical experiment is detailed in Table 1.

Table 1

Tentative Identification of Organisms growing on plate containing 1,000 u. Penicillin per ml.	Date 9.17.1945	
	Assigned No.	Type of Growth
Aspergillus, species	M-1	Luxuriant.
Do	M-2	Do.
Actinomyces, species	M-101	Poor.
Do	M-102	Luxuriant.
Penicillium, species	M-200	Do.
Do	M-203	Do.

It was assumed that organisms of this nature were capable of either destroying the penicillin in its immediate area or of growing in relatively high concentrations. Bacteria, many of which may produce penicillinase, were not included in this study because past experience showed technical difficulties, particularly filtration problems, preclude large scale production techniques. Only fungi were studied further.

The next step involved determination of whether the fungi actually destroyed the penicillin or merely grew in its presence. Liquid culture media containing 1000 units of penicillin per milliliter were prepared and inoculated with mycelial transplants of the fungi. These were incubated at room temperature which is optimum for most fungi, and when growth appeared the culture media was assayed to determine amount of penicillin remaining. This assay was repeated on the second and third day to determine rate of destruction. The experiment, using the fungi described in Table 1, is described in Table 2.

Table 2

Organism No.	Organ. Identified as:	Per Cent Penicillin remaining: No. of days after growth appeared.		
		1 Day	2 Days	3 Days
M-1	Aspergillus oryzae	73	27	0
M-2	Aspergillus terrus	55	45	0
M-101	Actinomyces viridochromogenus.	50	20	0
M-102	Actinomyces candidus	0	0	0
M-200	Penicillium citrineum	83	50	27
M-203	Penicillium lilacium	60	45	30

All six of the fungi, it should be noted, destroy penicillin to some extent. The outstanding inactivator is M-102, identified as actinomyces candidus. An agar slant culture of this particular strain of actinomyces has been deposited in a permanent collection in the Northern Regional Research Laboratories at Peoria, Illinois, under the number NRRL 2280. This is the fungus employed and is the one with which this patent application is concerned. In accordance with this invention this particular strain of actinomyces is used in production of the penicillin according to the following procedure:

Any good nutrient broth is prepared and sterilized in large containers of fermentation tanks. A source of sterile air is supplied in such a manner that it continuously bubbles through the fermentation mixture. The media is inoculated with the fungi, the air supply adjusted and growth allowed to proceed at room temperature for five days or until the medium attains a pH of 8.0–8.1, whichever occurs first. The resulting solution may be sterilized by passing through a bacteria retaining glass filter or it may be dried and retained in the dehydrated state from which it is reconstituted before use.

This preparation may be concentrated by saturating the fermentation medium after removal of the mycelia with ammonium sulfate and collection of the precipitate in a small volume of water from which it is precipitated by the addition of two volumes of alcohol or acetone. This precipitate is then resuspended in water and filtered through glass to sterilize or dehydrated and held in the dry state. This purification step is necessary in preparing the penicillinase for use in the enzymatic assay of penicillin, since the buffering effect must be at a minimum to allow accurate measurement of the fall in pH.

As illustration of the possible uses for this novel penicillinase a series of experiments follow.

Table 3 presents data secured using the filtered fermentation medium from a run made exactly as described above.

Table 3

Units of Penicillin	Ml. broth added	Inactivation in One Hour
10,000	1.0	Complete.
20,000	1.0	Do.
30,000	1.0	Do.
40,000	1.0	Nearly complete.
50,000	1.0	Partial.

The consistency with which this penicillinase was produced was determined by examining five consecutive production batches. These data are set forth in Table 4.

Table 4

Lot #	Final pH	Units Penicillin	Time	Inactivation
			Hours	
1	8.0	35,000	1	Complete.
2	8.0	35,000	1	Do.
3	8.1	35,000	1	Do.
4	8.0	35,000	1	Do.
5	8.2	35,000	1	Do.

This crude material which consisted of the penicillinase, in the presence of other metabolic products and remnants of culture media constituents, was tested for stability. At 37° C. deterioration was rapid, but at room and at refrigerator temperatures the material was quite stable.

When the crude fermentation broth was dried and reconstituted as a 10% aqueous solution and filtered, it was employed in the routine method for determining the sterility of penicillin preparations with the following results. (Table 5.)

Table 5

Sample #	Size	Penicillin Product	Inactivation
	u.		
1	100,000	Amorph. Sod. Penicillin	Complete.
2	200,000	Amorph. Sod. Penicillin	Do.
3	100,000	Cryst. Sod. Penicillin G	Do.
4	200,000	Cryst. Sod. Penicillin G	Do.
5	500,000	Amorph. Sod. Penicillin	Do.
6	500,000	Cryst. Sod. Penicillin G	Do.
7	500,000	Cryst. Pot. Penicillin G	Do.
8	1,000,000	Amorph. Calcium Penicillin	Do.
9	1,000,000	Amorph. Sod. Penicillin	Do.
10	1,000,000	Cryst. Sod. Penicillin G	Do.
11	1,000,000	Cryst. Pot. Penicillin G	Do.

When this 10% suspension was used in the inactivation of penicillin in oil and wax, a somewhat more difficult procedure, the following results were obtained.

Table 6

Sample	Containing	Amount of Penicillinase Used			
		0.05 ml.	0.1 ml.	0.2 ml.	0.5 ml.
	units				
1	30,000	(1)	(1)	(1)	(1)
2	30,000	(1)	(1)	(1)	(1)

[1] Inactivated.

When the penicillinase was used in blood cultures, inactivation of penicillin present was complete. When employed in the purified state for the assay of penicillin the results were identical with those obtained by the sodium hydroxide-iodometric titration method.

The invention described herein may be manufactured and used anywhere by or for the Government of the United States for governmental purposes without the payment to me of any royalty thereon in accordance with the provisions of the act of April 30, 1928 (ch. 460, 45 Stat. L. 467).

I claim:

In a process involving the quantitative destruction of the beta-lactam linkage of the penicillin nucleus in a delicate vehicle containing penicillin, and selected from the group consisting of blood and bacterial media without substantial injury to said vehicle, the improvement which comprises introducing into such vehicle a solution of filtrable, sterile penicillinase enzyme produced during the growth of the fungus *Actinomyces candidus.*

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