to bentonite-absorbed serum, failed to increase the bactericidal index[11,12].

Zusammenfassung. Das natürliche an Bentonit adsorbierbare «Co-opsonin», welches für eine optimale Phagocytose virulenter Streptokokken der Gruppe A nötig ist, erweist sich als verschieden von Lysozym. Es liegen Hinweise vor, dass das «Co-opsonin» in der β-Lipoprotein-Fraktion des Serums vorliegt.

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[12] From Research Project MR 005.12-1102, Bureau of Medicine and Surgery, Navy Department, Washington (D.C. USA). The opinions expressed herein are those of the authors and cannot be construed as reflecting the views of the Navy Department or of the Naval Service at large. Usage of commercially available materials cannot be construed as implying endorsement or preference for those products over other similar products available on the market. Requests for reprints should be addressed to: Officer in Charge, Naval Medical Research Unit No. 4, U.S. Naval Hospital, Great Lakes (Illinois USA).

Reaction of 6-Aminopenicillanic Acid with Frequentin

When 6-aminopenicillanic acid (6-APA) (I) was added to fungal fermentations it was possible to detect the occurrence of at least three different types of reaction other than those associated with penicillinase activity[1], its reactivity with other molecules of 6-APA[2,3] or with CO_2[4–6]. The first was found to be independent of the nature of the micro-organism and was in fact, shown to be the reaction of 6-APA with reducing sugars[7]. The second reaction is the synthesis of penicillins from added 6-APA by micro-organisms which do not necessarily produce penicillins in the absence of 6-APA. This is probably due to enzymic acylation as described by numerous workers with a wide range of micro-organisms[8–11]. The third type of reaction involves the formation of complexes between 6-APA and mould metabolites liberated into the medium. One such metabolite reacting with 6-APA was identified as frequentin, both from a consideration of its properties and by direct comparison with an authentic sample kindly given to me by Dr. W. B. Turner.

Thus when 6-APA was added to a growing culture of an organism producing frequentin a new iodine absorbing[12], penicillinase sensitive zone was detected on chromatograms of the fermentation medium. Chromatography was carried out in butanol/pyridine/water (1:1:1) using Whatman No. 1 paper. Although the new compound was chromatographically similar to the penicillins it had very little activity against *Bacillus subtilis*, and such activity as was observed appeared to increase somewhat variably,

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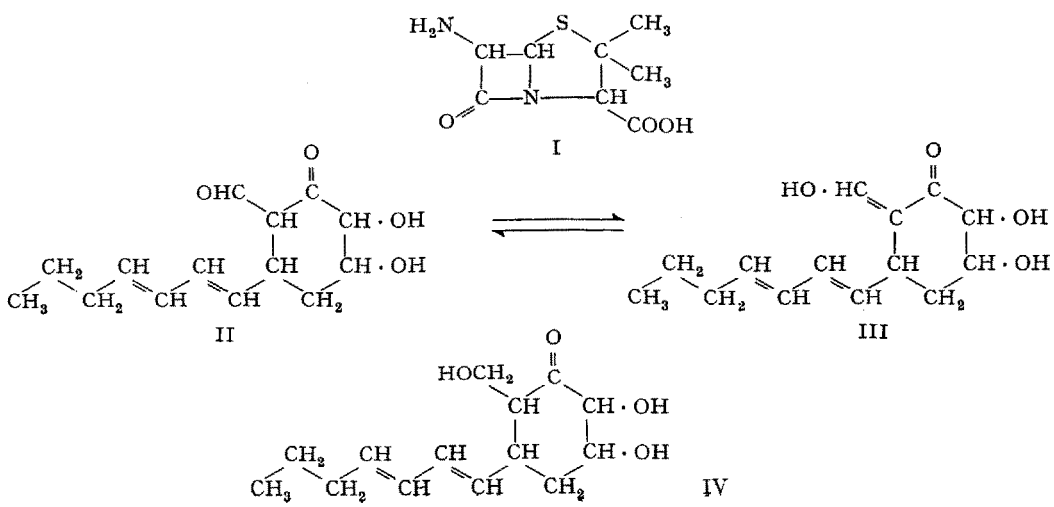


after spraying the chromatograms with sodium bicarbonate and phenylacetyl chloride[13].

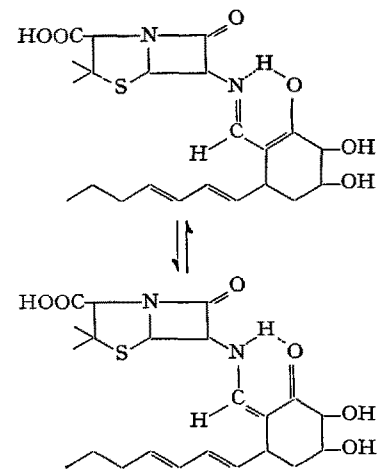
The relationship between the biological activity of the new derivative before and after phenylacetylation was confused by two factors. Firstly it was shown in subsequent experiments that the organism sometimes synthesized a small amount of penicillin from the added 6-APA which accounted for most of the biological activity before phenylacetylation. Secondly, when the nature of the complex was established, it seemed most likely that the reaction with phenylacetyl chloride involved the breakdown to free 6-APA which was subsequently phenylacetylated to give benzylpenicillin. The initial breakdown to free 6-APA occurs more readily at lower pH and the variable results obtained at first could be due to poor control of the bicarbonate buffer and hence varying conditions of acidity during the spraying procedure. More consistent results were later obtained by first spraying the tapes with acetic acid, thus deliberately breaking down the new complex to 6-APA, and then phenylacetylating in the usual way.

The new compound of 6-APA is solvent soluble at low pH and readily back extracted from the solvent with sodium bicarbonate solution. Like frequentin itself it gives a red colour with ferric chloride and an orange colour with diazotized *para*-nitro aniline. The absorption at 285 mμ in the UV-spectrum of frequentin is shifted to 330 mμ after reaction with 6-APA.

Attempts to isolate a completely pure preparation of the new derivative free from 6-APA itself have been unsuccessful for it readily breaks down to 6-APA and frequentin and it is probable that in aqueous solution an equilibrium becomes established between the complex and its constituents. After treating the complex with penicillinase a new compound is formed which runs slower on chromatograms and absorbs iodine more readily, thus behaving like a penicilloic acid, but the 6-APA/frequentin complex is at least twice as stable to *B. cereus* penicillinase as is 6-APA. It is even possible that penicillinase has no direct action on the complex but only attacks the free 6-APA, the penicilloic acid of which recombines with frequentin.

The structure of frequentin has been confirmed as the tautomeric mixture of a β-keto aldehyde (II) and its enolic form (III) in studies using nuclear magnetic resonance[14,15]. The closely related compound palitantin (IV) does not react with 6-APA; neither do penicillins such as benzylpenicillin react with frequentin. The formation of the complex would thus seem to involve the aldehyde group of frequentin and the primary amino group of 6-APA.

It has been shown in these laboratories and elsewhere that 6-APA will react with certain aldehydes and ketones under very mild conditions of temperature and pH. Thus a mixture of 6-APA and salicylaldehyde in phosphate buffer fairly rapidly becomes yellow and it is possible to isolate a new compound behaving chromatographically like the frequentin complex. In this instance it is suggested that the reaction involves the formation of a Schiff's base which is stabilized by hydrogen bonding and resonance with the ortho-quinonoid structure. The compound of 6-APA and 2-hydroxynaphthaldehyde has been isolated by WOLFE[16] and the preparation of Schiff bases of free amino acids using *ortho* hydroxy aromatic aldehydes was described by MCINTIRE[17], such derivatives being used in peptide synthesis by SHEEHAN and GRENDA[18]. The enamine-enolimine equilibrium has been investigated in some detail by HEINERT and MARTELL[19]. They suggest, from a study of infra-red data, that the enamine tautomer plays a large part in the structure of amino acid derivatives both of aliphatic β-dicarbonyl compounds and *ortho* hydroxy aromatic compounds. DEAN et al.[20] have also reported the reaction of amino acids with β-dicarbonyl compounds to form 'azo-methines'. The derivatives of benzoyl acetone and acetyl acetone are quite stable and they suggest that these derivatives exist mainly in the enamine form stabilized by strong hydrogen bonding. From these considerations it is possible to postulate the structure of the frequentin-6-APA complex as:



V

Undoubtedly, a number of fungi, under certain cultural conditions, will produce carbonyl compounds which could interact with 6-APA. Using the more sensitive technique of first breaking down these complexes with acid and subsequently phenylacetylating the liberated 6-APA it has been possible to detect such compounds on chromatograms of the fermentation media of several fungi to which 6-APA had been added, but the identity of these compounds is not known[21].

Zusammenfassung. 6-Aminopenizillansäure setzt sich mit Frequentin, einem Schimmelmetaboliten, unter gewissen Temperatur- und pH-Bedingungen um. Der entstandene Komplex stellt wahrscheinlich eine Tautomerenmischung aus Enamin und Enolimin dar, die beide durch Wasserstoffbrücken stabilisiert werden.

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