124. Aspects of Formation of the D-Penicillamine-Antigenic Determinant from Penicilloyl Compounds

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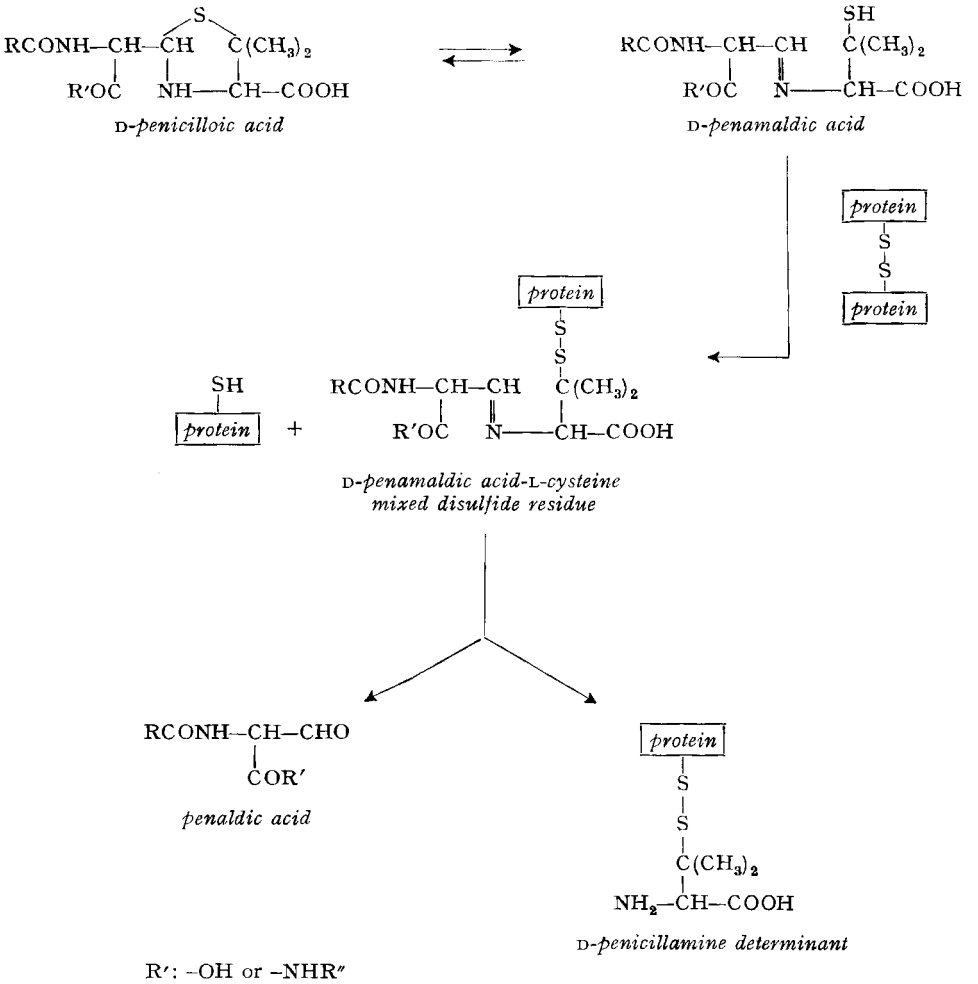
Summary. The formation of D-penicillamine-L-cysteine mixed disulfide from benzylpenicilloic acid, benzylpenilloic acid and benzylpenicilloyl amide derivatives in L-cysteine suspensions at pH 7,6 and 37° was studied. The reaction is faster than direct penicilloylation of proteins known to be a route to penicilloyl antigenic determinants. The production of S-bound penicillamine on protein from penicilloyl compounds must therefore definitely be considered as a potential antigen-forming step. The reaction may be partly if not fully blocked by acylation of the thiazolidine nitrogen of penicilloyl compounds. When formylation is applied a considerable reduction of the capacity of penicilloyl antigenic determinants to interact with anti-penicilloyl antibody is noted. A D-penicillamine-specific test antigen was prepared by binding D-penicillamine *via* thioether links to a partly succinylated poly-L-lysine. Clinical test reactions elicited with this conjugate and with penicilloic acid cannot be well correlated. Penicilloic acid probably detects reactions of undefined specificity in addition to D-penicillamine-specific ones.

In a number of patients hypersensitive to penicillin, allergic wheal and erythema reactions can be elicited by intracutaneous administration of relatively high doses of sodium benzylpenicilloate. These skin responses do not involve the penicilloyl antigenic determinant because, *inter alia*, they cannot be correlated with the penicilloyl-specific reactions elicited at the same time by penicilloylated polylysine[1]). It has been postulated that D-penicillamine-L-cysteine mixed disulfides may form from penicilloic acid and cysteine residues of host proteins and function as antigenic determinants. According to *Levine* [1], monosodium D-α-benzylpenicilloate reacts in aqueous solution at pH 7.5 with cystine and a product which seems to be D-penicillamine-cysteine mixed disulfide can be separated by paper chromatography. The mechanism could function *via* an initial disulfide exchange between cystine and penamaldate, present in small amounts in equilibrium with penicilloate. The penamaldic acid-cysteine mixed disulfide thus produced should decompose yielding the observed D-penicillamine-cysteine mixed disulfide (scheme 1).

Our interest in penicillamine determinants is due to the fact, demonstrated in this paper, that in addition to penicilloic acid, functional derivatives of its α-carboxyl group can produce penicillamine-cysteine mixed disulfides in the presence of cystine. Therefore, any penicilloylated carrier within the body has to be regarded as a potential source for the penicillamine determinant. Furthermore, this determinant may arise from low molecular weight penicilloyl derivatives such as ε-(benzylpenicilloyl)-α-formyl-L-lysine introduced into the body in large amounts in order to inhibit penicilloyl specific allergic manifestations during penicillin therapy [2].

[1]) The penicilloyl compounds in this paper are functional derivatives of the α-carboxyl group of penicilloic acid. Their isomeric form is D-α. Acid treated penicilloyl derivatives such as the formylated ones are isomeric mixtures.

Scheme 1



R': –OH or –NHR''

In a first series of experiments the formation of D-penicillamine-L-cysteine mixed disulfide from benzylpenicilloic acid as well as from ε-benzylpenicilloyl amidocaproic acid in the presence of L-cystine was confirmed. Thus, phosphate buffered solutions of benzylpenicilloic acid or ε-benzylpenicilloyl amidocaproic acid which were stirred at pH 7.6 with suspended L-cystine for one day at 37° gave rise to a new compound which moved at the same rate as an authentic sample of D-penicillamine-L-cysteine mixed disulfide on a paper chromatogram with phenol/water. Small amounts of the material were then isolated from cellulose thin layer plates on which centrifuged aliquots of the reaction mixtures had been chromatographed. The compound was identified as D-penicillamine-L-cysteine mixed disulfide by infrared spectrometry. The authentic sample of D-penicillamine-L-cysteine mixed disulfide was prepared by reacting L-cysteine with the S-monoxide of D-penicillamine disulfide [3]. A method based on a disulfide interchange reaction [4] proved unsatisfactory for this purpose.

The results suggest that under neutral aqueous conditions and body temperature, D-penicillamine determinants may indeed arise by reaction of cystine disulfide bonds with penicilloate and with penicilloyl derivatives as well. The over all reaction rate seemed small however, and its quantitative estimation and comparison with reactions known to be of immunological significance appeared desirable.

In order to obtain quantitative data on the rate of production of the mixed disulfide, solutions containing suspended L-cystine on one hand and either benzylpenicilloate, benzylpenilloate or amide derivatives of the penicilloate on the other, were reacted at 37°. Aliquots taken after various intervals were centrifuged and chromatographed on paper. After ninhydrin staining, the D-penicillamine-L-cysteine mixed disulfide spots were eluted and quantitated by photometry at 505 nm. The results are shown in Fig. 1. They show that the rate of production of D-penicillamine-L-cysteine mixed disulfide is indeed small, the second order rate constant calculated from the extent of reaction after 20 or 30 hours as an approximation, amounting to 0.2 $mol^{-1}h^{-1}$ for penicilloate and penilloate, to 0.08 $mol^{-1}h^{-1}$ for the penicilloyl caproate and to 0.04 $mol^{-1}h^{-1}$ for the penicilloyl lysine derivative. These values are between two and eight times higher than the second order rate constant found under approximately comparable conditions for the penicilloylation of ε-aminocaproic acid [5]. Since direct penicilloylation has been shown to be a route to penicilloyl antigenic determinants, the even faster production of S-bound penicillamine from penicilloyl compounds must definitely be considered as a possible antigen forming step.

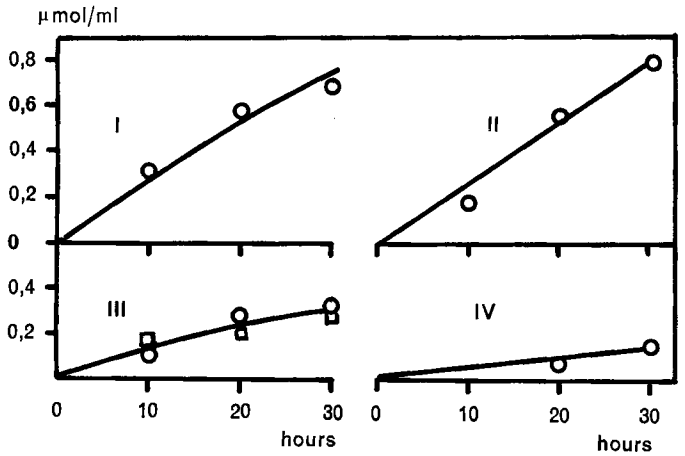


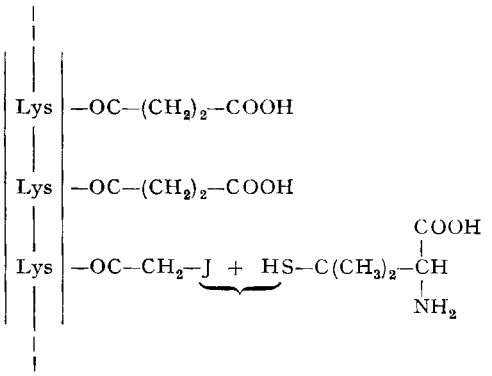
Fig. 1. *Rate of production of* D-*penicillamine*-L-*cysteine mixed disulfide from penicilloyl compounds in* L-*cystine suspensions*

I: benzylpenicilloic acid, II: benzylpenilloic acid, III: ε-benzylpenicilloyl amidocaproic acid, 2 different runs, IV: ε-(benzylpenicilloyl)-α-formyl-L-lysine

Obviously the *in vitro* reaction rates may or may not be related to the actual rates of conjugate production in the body and further investigation on penicillamine-specific sensitization depends on a more direct approach. More detailed evaluation of clinical sensitization seems mandatory and could be best performed with a non-immunogenic conjugate carrying stably bound penicillamine determinants. We felt

that a partly succinylated polylysine with a multitude of D-penicillamine determinants bound to the carrier by thioether links would be satisfactory. Therefore poly-L-lysine was treated with an amount of succinic anhydride sufficient to substitute maximally two thirds of the amino groups present. The remaining amino groups were reacted with iodoacetic anhydride and the product was finally treated with D-penicillamine (scheme 2). This procedure has been successfully used before, for linking *p*-(*p*-iodoacetylaminobenzeneazo)-hippuric acid to thiolated proteins [6]. The degree of penicillamine substitution was estimated by *van Slyke* nitrogen determinations. Usually

Scheme 2



one third of the lysine monomeric units are substituted with penicillamine, suggesting that on the average a polylysine chain of 20 lysines carries 6 to 7 penicillamine determinants. This material can be used clinically and has been found to elicit positive skin responses in a number of patients also responding to sodium benzylpenicilloate. However, very strong reactions were elicited by penicilloate in a number of individuals with only weak or negative responses to the penicillamine conjugate. These data – to be reported in full, elsewhere – could implicate that in addition to the D-penicillamine determinant yet another determinant may be involved in the skin reactions elicited by sodium benzylpenicilloate.

A final aspect to be dealt with here, is the suppression of the formation of D-penicillamine antigenic determinants from penicilloyl compounds by suitable chemical modification of the penicilloyl structure. If one accepts the penamaldic acid intermediate as the species attacking protein disulfides, one obviously should look for modified penicilloyl compounds that do not easily undergo transformation into penamaldates. Available evidence [7] [10] indicates that mercuric chloride does not produce penamaldates from penicilloates containing an acylated thiazolidine nitrogen although the reaction is rapidly accomplished in alcoholic or aqueous solution if the nitrogen is unsubstituted. Penicillin behaves as an N-acylated thiazolidine and readily forms penamaldate only after the β-lactam ring has been opened. It appears that in alcoholic solution, methylation of the thiazolidine nitrogen also protects penicilloates from attack by mercuric chloride but less fully than acylation.

It seemed appropriate therefore to prepare a ε-benzylpenicilloyl amidocaproic acid formylated at the thiazolidine nitrogen, to incubate it with suspended L-cystine and to locate and quantitate the eventual reaction product, N-formyl-D-penicillamine-L-

cysteine mixed disulfide by means of the chromatographic procedure used for measuring the formation of the unformylated disulfide. When the experiment was performed in this way no new reaction product was detectable. Furthermore, when the reaction solution was shortly treated with 1N HCl before chromatography in order to remove the formyl group from eventually formed product, no D-penicillamine-L-cysteine mixed disulfide could be detected.

These results suggest that indeed substitution of the thiazolidine nitrogen by a formyl group hinders penicilloyl derivatives to form, if not prevents them from forming D-penicillamine-L-cysteine mixed disulfide determinants in the presence of cystine. This information was applied in an attempt to enlarge the arsenal of reagents used for skin testing penicillin-allergic patients. Since penicilloylamide derivatives may – according to present results – form penicillamine-specific eliciting conjugates in the skin, it is to be expected that penicilloylated polylysine produces such conjugates in the skin and thus detects not only penicilloyl-specific skin reactions but also penicillamine-specific ones at least in individuals responding strongly to penicillamine antigens. The availability of a test conjugate which detects penicilloyl-specific reactions exclusively, would therefore be important in a variety of cases.

For this reason a penicilloylated polylysine conjugate was formylated under the conditions used for the formylation of ε-(benzylpenicilloyl)-amidocaproic acid. The product was used as a skin test reagent in a number of patients with strict penicilloyl-specific hypersensitivity. The wheal and erythema reactions elicited were much less pronounced than those elicited at the same time with penicilloylated polylysine. When used in guinea pigs for elicitation of penicilloyl-specific passive cutaneous anaphylaxis, the formylated conjugate was also much less efficient than unformylated penicilloyl polylysine. It appears therefore that the formyl group on the nitrogen of the thiazolidine ring considerably reduces the capacity of penicilloyl determinants to interact with specific antibody. A detailed account on this aspect of penicilloyl formylation will be reported later.

Experimental Part

General information. Melting points were determined in capillary tubes and are corrected. IR.-spectra were measured on a *Beckman*-IR 5 spectrometer. NMR.-spectra were obtained on a *Varian* A-60A spectrometer at 60 mHz with TMS as an internal standard (Institute of organic chemistry, University of Berne). Paper chromatography (PC.) (circular technique) was carried out on *Schleicher & Schuell* Nr. 2043 B mgl paper discs (radius 8,5 cm). The ninhydrin reagent used for the quantitative estimations and most qualitative detections contained 1,0 g ninhydrin in 112 ml of a solution obtained by mixing 0,1 g cadmium acetate, 10 ml water, 2 ml acetic acid and 100 ml acetone.

Materials. All phosphate buffers are according to *Sorensen*. Bio-Gel, a molecular sieve gel was obtained from *Calbiochem AG.*, Luzern. L-Cystine and D-penicillamine were from *Fluka* AG., Buchs. Peracetic acid was kindly supplied by *Degussa* AG, Frankfurt. ε-(Benzylpenicilloyl)-α-formyl-L-lysine was kindly supplied by *F. Hoffmann-La Roche AG*, Basel. Poly-L-lysine was prepared by polycondensation of ε-carbobenzoxy-L-lysine carboxyanhydride with diethylamine as initiator [8]. Its average chain length was 20 lysine units. ε-Benzylpenicilloyl amidocaproic acid bis-benzylammonium salt or disodium salt [9], benzylpenilloic acid [10] and benzylpenicilloic acid disodium salt were from laboratory stock, the last compound being prepared in high yield by a simplified procedure as follows. 10,8 ml 1N NaOH was added dropwise within 2 h. to 3,33 g sodium benzylpenicillinate in 8 ml water. The solution was kept well stirred and its pH below 11,5. Thirty min. after the last base

addition, 1,4 ml 1 N HCl was added dropwise at 0° with particularly efficient stirring. The solution was lyophylized to give 3,78 g of white fluffy disodium benzylpenicilloate containing 1,4 mmol (81,8 mg) NaCl. $PV_{molar} = 8.0 \cdot 10^3$ l · mol^{-1} · cm^{-1}, $PS_{10} = 31\%$. The IR.-spectrum (KBr) of this preparation was virtually identical to a spectrum of monosodium benzylpenicilloate [10] to which 1 equivalent of NaOH and 2% NaCl had been added after dissolution in water and which had been lyophilized thereafter. The two preparations also showed identical PC.: Rf: 0.33, broadened zone (0,1 M ammoniacal $AgNO_3$), 1-butanol/1-propanol/0,1 M phosphate buffer pH 7,4 2:1:3, paper pretreated with buffer.

1(L),6(D)-Diamino-5,5-dimethyl-3,4-dithiahexane-1,6-dicarbonic acid (D-penicillamine-L-cysteine mixed disulfide, PSSC). To 1,85 g D-penicillamine disulfide [11] in 18 ml methanol containing 12,5 mmol HCl was added dropwise at 0°, 1,85 ml 30% peracetic acid in tert. butylacetate mixed with 11 ml chloroform. After 4 h., 730 mg L-cysteine hydrochloride was added and the reaction mixture was stirred at 0° for another 5 h. After filtration, the solution was mixed with 4 ml pyridine and 15 ml chloroform. The precipitate which formed overnight was collected and extracted with chloroform in a *Soxhlet* apparatus for 20 h. The residue was dissolved in warm water and centrifuged to remove insoluble material. It was precipitated and then crystallized from acetone/water in the presence of 100 mg N-cyclohexylmaleineimide: 527 mg (40%) needles; m.p. (sinters 194°) 206° (dec.) (lit. [4] m.p. 195°, dec.).

$C_8H_{16}N_2O_4S_2$	Calc.	C 35,81	H 6,01	N 10,44	S 23,90%
(286,3)	Found	,, 35,85	,, 6,13	,, 10,31	,, 23,80%

This compound was also prepared similarly by reacting peracetic acid oxydized L-cystine with D-penicillamine. The yield was however less than 20%.

Identification of D-penicillamine-L-cysteine mixed disulfide after reaction of ε-benzylpenicilloyl-amidocaproic acid with L-cystine. A solution of 100 mg ε-benzylpenicilloyl amidocaproic acid disodium salt in 2 ml 0,1 M phosphate buffer pH 7,6 was stirred at 37° with 150 mg L-cystine. After 40 h the suspension was centrifuged and several 50 µl aliquots of the supernatant were chromatographed on cellulose thin layers (Alufolien, *Merck*, Darmstadt, without fluorescence indicator) with 1-butanol/ethanol/water 3:2:2. Similarly, a solution of synthetic PSSC was chromatographed. Narrow longitudinal strips were cut away from each plate and sprayed with ninhydrin. The synthetic sample showed a strong band at Rf 0.17 and a weak cystine band at Rf 0.1. The reaction solution showed the same two bands and two additional ones at Rf 0.35 and Rf 0.40. The unsprayed cellulose containing the Rf 0.17 bands was scraped off, eluted with water and the eluates were lyophilized. In this way 2,4 mg of product from the reaction and 2,5 mg lyophilizate of synthetic PSSC were obtained. The two compounds showed identical behaviour upon PC. with phenol/water (100 g/39 ml): Rf 0.55, with a trace at Rf 0,3 (cystine). Both compounds showed virtually identical IR.-spectra (KBr): 3250, 2800, 1625, 1380, 1160, 895 cm^{-1}.

Rate of production of D-penicillamine-L-cysteine mixed disulfide. Solutions of 100 µmol of penicilloyl compound in 1 ml 0,1 M phosphate buffer pH 7.6 were stirred at 37° with 75 mg L-cystine. During incubation penamaldate values and penamaldate stabilities [7] remained unchanged indicating that no other reactions in addition to the one to be measured consumed penicilloyl compound. Side reactions modifying the penicilloyl structure without affecting penamaldate assays are not expected to take place under present conditions. After 10, 20 and 30 h. aliquots of the reaction mixtures were centrifuged and 60 µl portions of each were subjected to PC. with phenol/water (100 g/39 ml). Usually the 60 µl portions were divided into three equal parts and run separately on the same paper. After drying in air the chromatograms were sprayed with ninhydrin reagent on both sides, left for 30 min. in the hood and were finally dried *in vacuo* in the dark for 20 h. over concentrated H_2SO_4 and solid NaOH. The stained bands from a 60 µl aliquot with the Rf of PSSC were cut out, cut further into small pieces and were shaken for 30 min. with 10 ml methanol. The paper was then filtered off, washed with methanol and the filtrate plus washings were concentrated to 1.0 ml. The concentrate was measured at 505 nm against methanol in 1 cm cells. A piece of unstained paper matched in size to the bands from a 60 µl aliquot was similarly cut out and eluted. The concentrate showed an optical density of 0.09 to be subtracted from the readings of the coloured concentrates. For calibration 60 µl aliquots containing 10 to 50 nmol of synthetic disulfide were chromatographed and the bands were stained and eluted as above. The values obtained on different occasions were quite reproducible (Fig. 2). The rate

constants were calculated from the expression $k = (2.3/tC) \log(P/P - n)$, where P is the initial concentration of penicilloyl compound and n is the decrease after time t. C, the constant concentration of L-cystine in the stirred suspension amounts to $1.37 \cdot 10^{-3}$ M according to a microkjeldahl determination of the supernatant of a L-cystine suspension (100 mg in 1,5 ml 0,1 M phosphate buffer pH 7,6) stirred at 37° for 48 h.

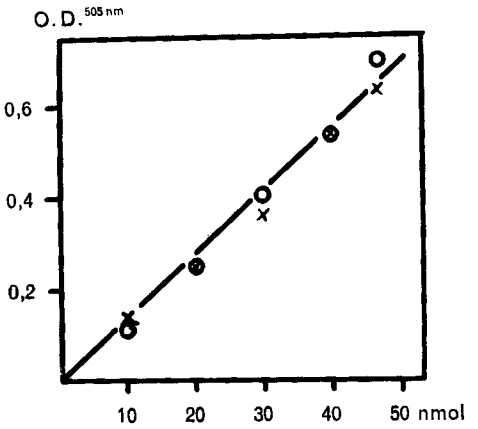


Fig. 2. *Determination of* D-*penicillamine*-L-*cysteine mixed disulfide after elution of chromatographic bands*

Measurements are on two different days: X and 0. Paper background is subtracted

D-*Penicillamine succinylated poly*-L-*lysine conjugate.* To a solution of 164 mg poly-L-lysine in 10 ml dilute NaOH (pH 10.3) 60 mg finely divided succinic anhydride is added at 25° with stirring. The pH is kept at 10.3 by 2N NaOH-additions for 45 min. and then brought to 9.5 with 1N HCl. Iodoacetic anhydride is prepared prior to use by stirring 2.4 g chloroacetic anhydride in 8 ml dry acetone at 0° in the dark with 4.66 g NaJ for 30 min. After centrifugation, $^2/_3$ of the supernatant are added dropwise at 15° to the solution containing the succinylated polylysine. The reaction mixture is kept at pH 9.5 for 2 h. in the dark, centrifuged and then brought into a nitrogen atmosphere. While N_2 is bubbled through the solution 0.48 g D-penicillamine in 6 ml 0.05 M phosphate buffer pH 7.4 (boiled and then cooled under N_2) is added dropwise. The pH is kept at 7.6 with 1N NaOH and 1 h. after the last NaOH addition the solution is concentrated *in vacuo* to 6 ml and passed through a 1.8 × 30 cm Bio-Gel P-4 column with 0.05 M phosphate buffer pH 7.4. The first peak emerging in the eluate is detected by microkjeldahl analysis. It contains 5 to 6 mg N of which about 14% are usually detectable also by *van Slyke* nitrogen determinations [12]. The degree of penicillamine substitution (α), defined as the number of penicillamine groups bound on the average per 100 lysine monomeric units is found from the expression

$$\alpha = \frac{(\text{B mg } N_{van\ Slyke})/14 \text{ mg N mmol}^{-1}}{(\text{A mg } N_{Kjeldahl} - \text{B mg } N_{van\ Slyke})/100 \cdot 28 \text{ mg N mmol}^{-1}}$$

α varies usually between 30 and 35. In one instance a sulfur elementary analysis was obtained showing the expected value for $\alpha = 30$. The conjugate moves towards the anode as a sharp band upon electrophoresis on cellulose polyacetate strips (sepraphore III, *Gelman Inc.*, Ann Arbor, Mich.; 0.07 M phosphate buffer pH 7.4, 10 V/cm, detection with 0.1 M ammoniacal $AgNO_3$). The bulk of the eluted conjugate is usually lyophilized and kept at −20°. In solution (+4°) the compound can be kept for biological tests for about 3 weeks. As a control, a conjugate was prepared following exactly the procedure described except that iodoacetylation was replaced by acetylation with acetic anhydride. After penicillamine treatment and chromatography the conjugate (15.7 mg $N_{Kjeldahl}$) did not contain detectable *van Slyke* nitrogen. This result indicates that the conjugate prepared from the iodoacetylated polylysine carries only covalently bound penicillamine groups and none bound by intermolecular forces.

ε-(N$^{\alpha}$-Formyl benzylpenicilloyl amido)-caproic acid (isomeric mixture). a) *Salt with benzylamine.* ε-Benzylpenicilloyl amidocaproic acid bis-benzylammonium salt (3.40 g) in 20 ml anhydrous formic acid was kept at room temp. until a constant low optical rotation was obtained (90 min.). Acetic anhydride (10 ml) was added and after another 4½ h. the solvent was removed in a cold air stream. The oily residue was dissolved in 50 ml 1-butanol, washed 3 times with a total of 75 ml water and the solvent was removed *in vacuo* after addition of sufficient benzene to give a dry residue. The residue was taken up in 80 ml 1-butanol containing 1.1 ml benzylamine and mixed with 400 ml ethyl ether. The hygroscopic precipitate was reprecipitated 4 times from 1-butanol/ether: 2,0 g; m.p. 85–95°; penamaldate assay [13]: $PV_0 < 0.05$ ($c = 0.69$ g/l). NMR. (D_2O): The signal intensity of the aromatic protons $\delta = 7.55$ ppm (*s*, benzylammonium) was 1.3 times that of $\delta = 7.40$ (*s*, benzyl).

$C_{23}H_3N_3O_7S + 1.3C_7H_9N$	Calc.	C 60.9	H 6.79	N 9.51 S 5.06%
	Found	,, 60.4	,, 6.94	,, 9.4 ,, 4.89%

b) *Free acid.* Compound a) (900 mg) dissolved in 7 ml water was precipitated with 0.24 ml 6N HCl at 0°, taken up in 11 ml 1-butanol and washed with 0.1N HCl (2 times with 10 ml) and water (3 times with 10 ml). The solvent was removed *in vacuo* after addition of sufficient benzene to give a dry residue. The white residue was further dried over P_2O_5 *in vacuo*: 420 mg; PC. (200 μg): Rf 0.62, moderately broad zone (0.1M ammoniacal $AgNO_3$), 1-butanol/ethanol/water 4:1:1, paper pretreated with 0.1M phosphate buffer pH 6.5; NMR. (D_2O, compound neutralized with NaOD): $\delta = 7.40$ (*s*, 5H, aromatic protons); 8.25–8.35 (*d*, 1H, CHO—N=) ppm.

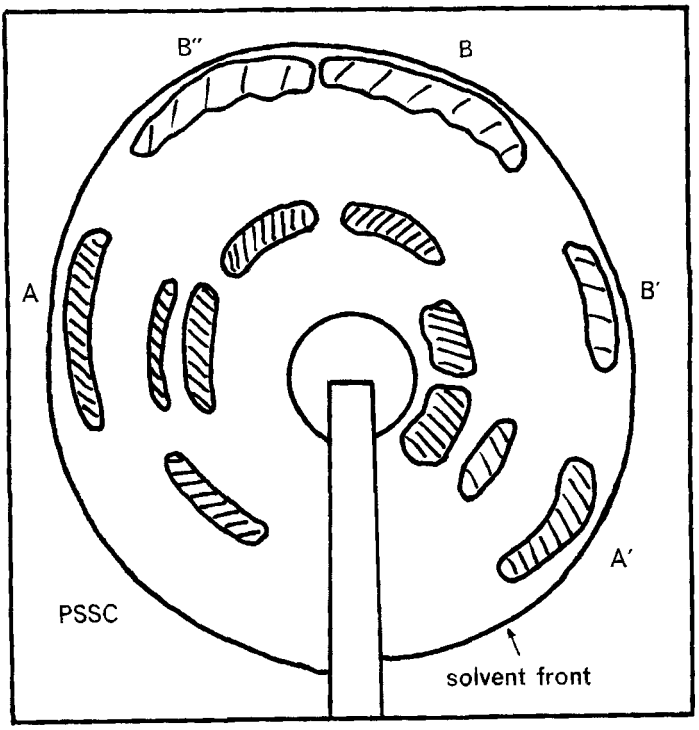


Fig. 3. *Paper chromatography of penicilloyl/cystine reaction mixtures containing and lacking PSSC* Amounts: 10 μl A; 10 μl B; 20 μl A'; 20 μl B'; 10 μl B''; 10 μg PSSC. Detection by ninhydrin reagent

$C_{23}H_{31}N_3O_7S$	Calc.	C 55.97	H 6.33	N 8.51%
(493.58)	Found	,, 56.29	,, 6.25	,, 8.15%

Incubation of ε-(N^4-formyl benzylpenicilloyl amido)-caproic acid with L-*cystine.* The disodium salts of ε-benzylpenicilloyl amidocaproic acid (50 mg) and of the formylated derivative (68 mg) in 1 ml 0.1 M phosphate buffer pH 7.6 each, were stirred at 37° with 75 mg L-cystine each. After 40 h. the suspensions were centrifuged and aliquots of the supernatants (A and B respectively) were used for PC. with phenol/water (100 g/39 ml). A and B (0.2 ml each) were mixed with 0.05 ml 5N HCl, kept 15 min. at ambient temp. and neutralized with 0.05 ml 5N NaOH. These solutions (A′ and B′) were also chromatographed together with PSSC and a B-supernatant (B″) obtained from an unincubated suspension. Fig. 3 shows that B contains no new compound (not present in B″) and B′ contains no PSSC as does A′. In order to establish that the HCl treatment would indeed liberate PSSC from the formyl derivative, 10 mg diformyl-D-penicillamine disulfide [11] were dissolved in 0.5 ml 1N HCl and neutralized after a few min. with 0.5 ml 1N NaOH. 20 μl aliquots as well as D-penicillamine disulfide as a reference were chromatographed with phenol/water on descending paper strips. Densitometry of the ninhydrin (0.3% in acetone) treated strips showed that D-penicillamine disulfide (Rf 0.37) had formed in at least 60% yield.

This work has been supported in part by the *Swiss National Foundation for Scientific Research* and the *Emil Barell Foundation* of *F. Hoffmann-La Roche Ltd.*, Basel.

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125. The Dehydrogenation of 1,4-Cyclohexadienes with 2,3-Dichloro-5,6-dicyanobenzoquinone and Triphenylmethylfluoroborate

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Summary. The dehydrogenation of 1,4-cyclohexadiene (**1**) *cis*-3,6-dimethyl-1,4-cyclohexadiene (**2**) and *trans*-3,6-dimethyl-1,4-cyclohexadiene (**3**) with triphenylmethylfluoroborate in acetonitrile or 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) proceeds in the same reactivity sequence **2** > **1** > **3**. The mechanism of the dehydrogenation of 1,4-cyclohexadienes with tri-