2. The suitability of immobilized bacterial proteases for extended hydrolysis of purified bakers' yeast autolyzate has been studied.

3. The possibility has been established of increasing the concentration of free amino acids in purified bakers' yeast autolyzate as a result of extended hydrolysis with toluene-p-sulfonic acid or bacterial proteolytic enzymes chemically immobilized on to an inert inorganic carrier.

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ENZYMIC HYDROLYSIS OF PHENYLPENICILLINS

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UDC 615.334 (Penicillinum)].074

When investigating structure—activity relationships in a series of physiologically active compounds it is of considerable interest to use correlation equations permitting quantitative assessment of the properties of many drugs in relation to their chemical structure. Correlations are known for antibiotics of the penicillin series. The dependence of the biological activity of penicillins [1] and of their acid stability [2] on the structural elements of the side chain acyl radical has been characterized.

Establishment of quantitative ratios between the structure of penicillins and the rate of their hydrolysis by the specific enzyme penicillinase, as in the case of other enzymic reactions, encountered a series of difficulties explained by the strict specificity of action of the enzymes.

Such a study seemed important since it permitted an opinion on the character of the enzyme—substrate interaction, the properties of the enzyme—substrate complex, and the character of the active center of the enzyme (penicillinase in the present case). It also made it possible to carry out a search for penicillins stable to penicillinase-producing microorganisms.

Previously, we attempted to apply correlation analysis to assess the stability of phenoxymethylpenicillins substituted in the benzene ring [3]. It was discovered that in this case there was a close correlation (correlation coefficient n was 0.94) between the logarithm of the rate constant for enzymic hydrolysis of meta and para substituted phenoxymethylpenicillins and the Hammet σ constant for the substituent, the characteristic reaction constant ρ was equal to -0.12.

Phenylpenicillins in which the benzene ring in the side chain is closest to the bicyclic system are more convenient models for studying structure—activity relationships in the penicillin series. It therefore seemed expedient to obtain phenylpenicillins substituted in the benzene ring to assess their stability towards penicillinase and to compare the results obtained with those for the case of enzymic hydrolysis of phenoxymethylpenicillins.

Phenylpenicillins (I-XII) (see Table 1) were obtained by the acid chloride method [4] in yields of 45-85% by acylation of 6-aminopenicillanic acid with the appropriate benzoyl chlorides and were isolated as sodium salts.

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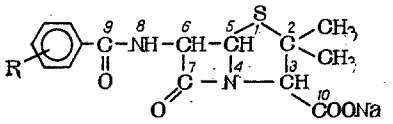
TABLE 1. Chemical Structure and Properties of Phenylpenicillin Sodium Salts



Compound	R	Iodometric activity μg/ml	$[\alpha]_D^{17}$ deg (conc. 0.5, water)	V units (ml·min) at [P] = 67 units/ml	$K_{cat} \cdot 10$ min^{-1}
I	p-N $(CH_3)_2$	920	+210	38,1±0,6	5,6
II	p-OCH_3	740	+216	28,3±0,5	4,2
III	H	970	+250	24,9±0,5	3,8
IV	p-Cl	855	+282	21,7±0,5	3,3
V	p-Br	855	+228	21,6±0,3	3,2
VI	p-NO_2	850	+252	16,0±0,2	2,4
VII	m-NO_2	820	+222	28,2±0,7	4,5
VIII	m,m'-di-NO_2	860	+196	20,7±0,3	3,3
IX	o-CH_3	970	+292	8,0±0,3	1,2
X	o-Cl, p-NO_2	860	+196	3,7±0,1	—
XI	o-Cl	860	+265	3,2±0,4	—
XII	o-Br	870	+228	1,8±0,4	—

Penicillinase from *Bacillus licheniformis* 749/c was used in the experiments to study the enzymic hydrolysis of phenylpenicillins. The kinetics of the hydrolysis was studied by the method in [3] at a temperature of 37°C in buffer solution of pH 6.8 at initial concentrations of substrate 1000-1200 units/ml and of protein 20-67 units/ml. To determine the activation energy for hydrolysis certain derivatives were also investigated at temperatures of 25 and 30°C.

Under these conditions the inactivation of phenylpenicillins by pencillinase is described by a zero-order equation: A linear dependence of the loss in concentration of the antibiotic with time was observed as is seen in Fig. 1.

In addition, it was discovered that the rate V of enzymatic hydrolysis of all the penicillinase [P]. This circumstance made it possible to determine the catalytic enzymic hydrolysis rate constants of the phenylpenicillins K_{cat}.

Some physicochemical characteristics of the synthesized phenylpenicillins, the sizes of the hydrolysis rate of phenylpenicillins at [P] = 67 units/ml, and the catalytic rate constants of the enzymic reactions investigated are presented in Table 1.

From Table 1 it is seen that the enzymatic hydrolysis rate of phenylpenicillins depends to a marked degree on the character and position of the substituent on the benzene ring in the side chain.

However, values of V for ortho-substituted phenylpenicillins were found to be 5-20 times lower than for meta- and para-substituted phenylpenicillins. These data confirm the known fact that the stability of penicillins towards penicillinase is undoubtedly linked with steric factors of the side chain structure.

Both mono- and di-meta-nitro-substituted phenylpenicillins were characterized by large values of V in comparison with para-nitro-phenylpenicillin. This observed phenomenon is seemingly connected wiht the fact that the presence of an electron acceptor nitro group in the meta position on the benzene ring provides additional interaction between substrate and enzyme increasing their mutual affinity. Since other meta-substituted phenylpenicillins were not present in the series studied it is impossible to say whether this is a characteristic only of nitro groups. On investigating the hydrolysis of meta- and para-nitrophenylpenicillins by staphylococcal penicillinase Depue and coauthors [5] determined a lower value (close to 50%) for the Michaelis constant for meta-nitrophenylpenicillin which also indicates

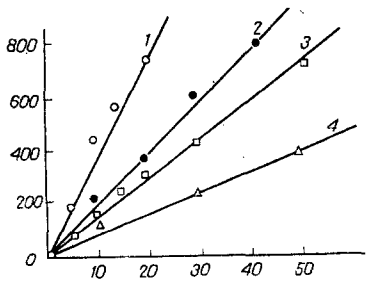


Fig. 1

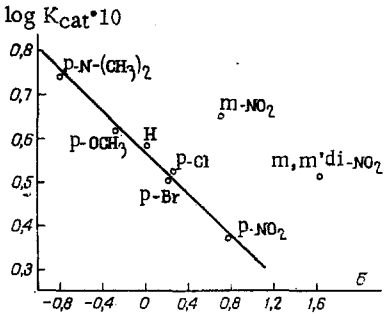


Fig. 2

Fig. 1. Hydrolysis of sodium salts of phenylpenicillins by penicillinase from *Bacillus licheniformis* 749/c at 37°C, pH 6.8, [P] = 67 units/ml. The concentration of hydrolyzed penicillin (units/ml) is shown on the ordinate and time (min) is shown on the abscissa. 1) (I); 2) (V); 3) (VI); 4) (IX).

Fig. 2. Correlation of logarithms of catalytic rate constants for enzymic hydrolysis of phenylpenicillins with Hammet σ constants.

the appreciably larger affinity of this compound towards penicillinase in comparison with para-nitrophenylpenicillin. The low correlation coefficient log K — $\sigma_{m,p}$ in the case of enzymic hydrolysis of phenoxymethylpenicillins is apparently caused by the deviations from the regression line of points for the meta-substituted derivatives which were less marked in the phenoxymethylpenicillin series than in the phenylpenicillin series.

In the series of para-substituted phenylpenicillins the rate of enzymic hydrolysis and the catalytic hydrolysis rate constant increased regularly with the strengthening of the electron-donor properties of the benzene ring substituent. In this way the dependence between log K_{cat} and the Hammet σ constants for para-substituted compounds possessed a linear character (see Fig. 2) and was expressed by the equation:

$$\lg K_{cat} = -0.42 - 0.42\,\delta; \quad \rho = -0.24 \pm 0.03; \quad r = 0.98.$$

The size of the coefficient r was 0.98 which indicates the satisfactory correlation of log K_{cat} values with σ in this equation and testifies to the applicability of the Hammet equation for describing the effect of the nature of the substituent in the para position of the benzene ring in phenylpenicillins on the rate of their hydrolysis by penicillinase.

In its absolute value the size of the constant ρ proved to be close to 2 times greater than the sizes of the ρ constant found for the case of hydrolysis of substituted phenoxymethylpenicillins by *Bacillus licheniformis* 749/c penicillinase under the same conditions [3]. This indicates, as was thought, the high sensitivity of the studied enzymatic reaction towards the influence of electronic effects of the substituents in the benzene ring in comparison with the analogous process using phenoxymethylpenicillins as substrates. In the molecules of the latter the benzene ring is separated from the bicyclic system by the intermediate OCH_2 group.

It is interesting to note that the activation energy of the hydrolysis reactions of compounds (I) and (VI) with strong electron-donor and electron-acceptor para substituents proved to have values of one order: $E_I = 11.2 \pm 0.7$, $E_{VI} = 10.6 \pm 0.4$ kcal/mole. Thus the observed differences in the rates of hydrolysis of para-substituted penicillins are not connected with the value of the activation energies and may be explained only by differences in the values of the probability factor PZ in the Arrhenius equation. In the enzymic reactions this factor finds expression first of all in larger or smaller probabilities of forming an enzyme—substrate complex depending in the present case on the character and position of the substituent on the benzene ring in the side chain.

The negative ρ constant values, disclosed in the two reaction series (phenyl- and phenoxymethylpenicillins), showed that the strengthening of the nucleophilic properties of penicillin aid in increasing its affinity towards the active center of *Bacillus licheniformis* 749/c penicillinase, bearing an apparently electrophilic character. The study [6] must be mentioned in this connection. In this study the conclusion was drawn on the basis of a detected dependence of K_m on pH, as to the existence in the active center of the penicillinase of a grouping of basic character in protonated form. Charged imidazole rings of histidine, for example, may be proposed as such groups bringing about transfer of a proton to the penicillin molecule and thereby catalyzing its rearrangement into a highly reactive penicillenic acid. The latter, as is known [7], is 100% converted under physiological conditions into penicillinic acid, the final product in the hydrolysis of penicillins by penicillinase.

The strong catalytic effect of the histidine imidazole on the process of forming penicillenic acid occurring under physiological conditions was shown in [8]. The hydrolysis of penicillins by a penicillinase-containing histidine in its active center [5, 9] and the process of acid inactivation of penicillins in course of which penicillenic acid is formed as an intermediate are similar in mechanism. The negative values of the ρ constants found both for enzymatic and acidic hydrolysis [2] of phenylpenicillins indicate the general nature of this mechanism.

EXPERIMENTAL

Sodium Salts of Phenylpenicillins. To a solution of 6-aminopenicillanic acid (2.16 g; 0.01 mole) in a mixture of 5% sodium bicarbonate solution (50 ml) and pure acetone (30 ml) cooled to 0°C was added dropwise, with stirring, a cooled solution of benzoyl chloride (1.5 ml; 0.013 mole) in acetone (20 ml). Acylation was carried out for 15-30 min maintaining a neutral reaction medium by the addition of $NaHCO_3$ solution. After completion of the reaction the untreated acid chloride was extracted with ethyl ether. The ether extracts were discarded and the aqueous layer acidified with 6 M sulfuric acid solution to pH 2.0. The acid thus separated was extracted with ether (50 ml). The ether layer was washed with water (two times with 30 ml) and separated. The ether extract was then extracted with 1% $NaHCO_3$ solution (60 ml). The aqueous layer was separated, filtered through filter paper, the residues of ether were evaporated in vacuum, the salt was isolated from the aqueous solution by lyophilization, and purified by reprecipitation from a mixture of acetone–water (9:1) into dry acetone. The yield of phenylpenicillin sodium salt was 2.98 g (87.1%).

Substituted phenylpenicillins were obtained by an analogous method.

IR absorption spectra of phenylpenicillins were taken on a Nippon–Bunko DS-301 spectrometer (Japan) in potassium bromide disks.

The spectrum, cm^{-1}: 1700-1790 ($C_7 = 0$); 1600-1680 ($C_9 = 0$); 1400 ($C_{10} = 0$); 1600 (C = C).

The optical activity of the phenylpenicillins was determined on an EPL-1 electropolarimeter and the iodometric activity was determined by the method in [10].

Bacillus licheniformis 749/c Penicillinase. It was given by the All-Union Scientific-Research Institute for Antibiotics as a dry preparation (25 mg) of activity 1,000,000 units. The enzyme was diluted in pH 6.8 phosphate buffer to low concentration and the solution stabilized by the addition of gelatin.

Catalytic rate constants for the hydrolysis of phenylpenicillins were determined as the slopes of the lines describing the dependence of the reaction rate V on the enzyme concentration [P]. Values of V, ρ, and mean square deviations were determined by the method of least squares starting from the equations: $C_0 - C\tau = V \cdot \tau$; $\log(K_{cat}/K_{cat_0}) = \rho\sigma$, where C_0 and C_τ are the concentrations of unhydrolyzed penicillin at the start of the reaction and at time τ; K_{cat} is the catalytic rate constant for hydrolysis of the phenylpenicillin substituted in the benzene nucleus, K_{cat_0} is the catalytic rate constant for the hydrolysis of phenylpenicillin.

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MICROBIOLOGICAL HYDROXYLATION OF STEROIDS. III.* STUDY OF SIDE REACTIONS OCCURRING IN THE HYDROXYLATION OF CORTEXOLONE IN CULTURES OF *Cunningamella blakesleeana* AND *Curvularia lunata*

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We have previously [1, 2] developed methods for the chromatographic (thin layer) identification of the transformation products of cortexolone (I) brought about by cultures of *T. orchidis* and *T. hyalospora* and have shown that these molds effect not only hydroxylation, but also other conversions of the steroid molecule.

In the present work the transformation has been studied of (I) and its derivatives with an oxygen function at C_{11}, namely cortisol (II), epicortisol (III), and cortisone (IV), by cultures of *C. blakesleena* and *C. lunata*. These cultures have been described in the literature [3], however, chiefly hydroxylation processes of the steroid molecule have been reported for them.

The aim of the investigation was the establishment of the character of the side products on hydroxylation of (I) by the molds *C. blakesleeana* and *C. lunata*. Substrates (I-IV) were placed in contact with the cultures, the transformation products were extracted from the culture mass, and were investigated chromatographically [1, 2]. Results of the experiments are given in Table 1.

As is seen in Table 1, the investigate cultures displayed multiple forms of fermentative activity.

A general property of the cultures was their hydroxylating activity, the introduction of an oxygen function into position 11 of (I). *C. lunata* formed almost exclusively the 11β-hydroxy compound (II), *C. blakesleeana* in contrast did not show any preference for the isomeric 11-alcohols (II) and (III) or for the 11-ketone (IV).

Both molds reduced the carbonyl at C_{20} of the steroid molecule [20β-hydroxysteroid dehydrogenase activity (HSD)] forming the 20β-hydroxy compounds (V) and (VI). Such conversions were known only for (I) and (IV) in the case of cultures of *C. lunta* [4]. We discovered that these cultures also form the 20-hydroxy derivative (V) when transforming compounds (II) and (III). The ability of cultures of *C. blakesleeana* to reduce the carbonyl at C_{20} has been discovered for the first time by us.

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