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Reactions of 6-Aminopenicillanic Acid with Carbohydrates and Related Substances

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The amino group of 6-APA* is involved in many reactions that occur in aqueous solution at room temperature and neutral pH. Grant, Clark & Alburn (1962) reported the formation of large polymers in very concentrated solutions of 6-APA, whereas Batchelor, Cole, Gazzard & Rolinson (1962), working with less concentrated solutions, showed the presence of several compounds of smaller molecular weight. 6-APA undergoes a ready reaction with carbon dioxide through its amino group followed by a molecular rearrangement to 8-hydroxypenicillic acid (Batchelor, Gazzard & Nayler, 1961; Johnson & Hardcastle, 1961). The product of this reaction was identified in, and isolated from, the fermentation liquors of *Penicillium chrysogenum* by Ballio *et al.* (1961). It is known that 6-APA forms anil-like compounds with aromatic aldehydes, and the preparation of such an anil with 2-hydroxynaphthaldehyde has been reported by Wolfe, Godfrey, Holdrege & Perron (1963).

During experiments involving the addition of 6-APA to growing cultures of fungi the present authors observed a number of phenomena that were found to involve the reaction of the primary amino group of 6-APA with mould metabolites and components of the fermentation media. These reactions can be broadly classified into two groups, one in-

volving reducing sugars, the other involving complex carbonyl compounds produced by some fungi. The present paper is concerned with the first group of compounds. The preparation and isolation of compounds thought to be *N*-glucosyl, *N*-maltoysl and *N*-lactosyl derivatives of 6-APA are also described.

METHODS

Assay of glycosyl-6-aminopenicillanic acid, 'total nucleus' and free 6-aminopenicillanic acid. Because most of the derivatives of sugars and 6-APA were found to be stable to penicillinase they could be assayed as the difference between the assay for free 6-APA (penicillinase-labile material) and total 6-APA (NaOH-labile), referred to below as 'total nucleus'. The total nucleus was assayed in reaction mixtures by the hydroxylamine method described by Batchelor, Chain, Hardy, Mansford & Rolinson (1961). Free 6-APA was assayed by the same procedure by using 0.5 ml. of a 20% solution of *Bacillus cereus* penicillinase instead of NaOH for the blank (Batchelor, Cameron-Wood, Chain & Rolinson, 1961). Penicillinase was prepared by the method of Pollock (1957) with the organism *Bacillus cereus* N.C.T.C. 9946.

Chromatography. Descending chromatography was carried out overnight at 5° on sheets and also on 1 cm.-wide tapes of Whatman no. 1 paper with the solvent system butan-1-ol-pyridine-water (1:1:1, by vol.). Reducing sugars, 6-APA and the compounds of the two were detected by dipping chromatograms in an acetone solution of AgNO₃, drying and spraying with ethanolic NaOH. After

* Abbreviation: 6-APA, 6-aminopenicillanic acid.

a few minutes the chromatograms were washed in aqueous ammonia and thoroughly rinsed in water (Trevelyan, Procter & Harrison, 1950).

Penicilloic acid derivatives were detected by spraying with the starch-iodine reagent described by Thomas (1961). 6-APA was also detected by plating chromatograms on agar seeded with *Bacillus subtilis* after first spraying them with NaHCO_3 and phenylacetyl chloride (Batchelor, Doyle, Nayler & Rolinson, 1959).

The carbohydrate derivatives of 6-APA do not react with phenylacetyl chloride on bicarbonate-buffered tapes and are without biological activity. On chromatograms they could, however, be detected biologically by treating first with acid to liberate free 6-APA and then with phenylacetyl chloride to give penicillin G. Chromatograms were sprayed liberally with *N*-acetic acid and left for 30 min. in a humidifying tank, and, after drying thoroughly, they were sprayed with NaHCO_3 and phenylacetyl chloride in the usual manner.

Electrophoresis. By using a pyridine-acetate buffer, pH 4.3, prepared by adding pyridine to 0.05 *N*-acetic acid, paper electrophoresis was carried out for 4 hr. on 1 cm.-wide tapes of Whatman no. 1 paper at 18 v/cm. Under these conditions 6-APA remains on the origin.

Optimum pH for the formation of glycosyl-6-aminopenicillanic acid derivatives. Solutions containing 2% (w/v) of glucose and 0.1% of 6-APA were adjusted to various pH values from 3.5 to 8.5. After 16 hr. at 26° they were all adjusted to pH 6.5 and assayed for both total β -lactam nucleus and residual 6-APA.

Preparation of sugar derivatives of 6-aminopenicillanic acid. Reaction mixtures containing 0.5% of 6-APA and 4% (w/v) of sugar in deionized water were adjusted to pH 6.0 with NaOH and incubated at 26° for 48 hr. During the reaction the pH fell to about 5.7. In a typical experiment 600 ml. of a glucose-6-APA reaction mixture was treated in the following manner.

The residual 6-APA was removed by adsorption on a column (13 cm. $\times$ 2.5 cm.) of Amberlite IR-120 (H^+ form). The effluent was collected at 10 ml./min. and readjusted continuously and immediately to pH 6.5. The residual sugar was then removed from the effluent by adsorbing the 6-APA derivative on to a column (15 cm. $\times$ 2 cm.) of De-Acidite FF resin (acetate form) and washing the column thoroughly with deionized water until no sugar could be detected in the washings. Material was eluted from the column with 0.5 *M*-sodium salicylate at the rate of 5 ml./min., the eluate being collected in 50 ml fractions until salicylate could be detected with FeCl_3 . These fractions were assayed and the bulk of penicillinase-stable β -lactam material was found to be in the fourth fraction. This fraction was stirred slowly into a 20-fold excess of acetone cooled to 5° to give a white emulsion. This was centrifuged and the resulting colourless gum was treated with ethanol until it solidified. After filtration the material was washed with acetone and dried thoroughly in an evacuated desiccator over anhydrous CaCl_2 to give an amorphous white powder.

Determination of the rate of acid hydrolysis of glycosyl-6-aminopenicillanic acid. Solutions of glycosyl-6-APA at a concentration of 1 mg./ml. were adjusted to a series of pH values from 1.5 to 3.5 with HCl. At various time-intervals 0.1 ml. samples were removed and added immediately to 0.4 ml. of 0.05 *M*-phosphate buffer, pH 7.0. The liberated

6-APA was assayed biologically by spotting tapes of Whatman paper with 10 μ l. of solution, spraying with NaHCO_3 and phenylacetyl chloride, plating on agar seeded with *B. subtilis* and comparing the zone diameter with standards of 6-APA treated in the same way.

Analysis of the sugar component of glycosyl-6-aminopenicillanic acid. A 6 mg. sample was completely hydrolysed in 5 ml. of 0.1 *N*-HCl at 37° for 20 min. After adjusting the pH to 6.5, free 6-APA was removed from the reaction mixture by passing the solution through a small column of Amberlite IR-120 (H^+ form). The effluent and washings were combined, adjusted to 50 ml. and assayed for reducing sugar by the method of Somogyi (1926).

Determination of equivalent weight. The glycosyl derivatives were converted into the free acid by passing a solution of the sodium salt through an Amberlite IR-120 (H^+ form) column. The effluent and washings were titrated directly against dilute NaOH.

Fermentation methods. A penicillin-producing strain of *P. chrysogenum* was grown in 500 ml. conical flasks, baffled with internal indentations, closed with cotton-gauze tops and containing 100 ml. of medium. The flasks were incubated at 26° on a rotary shaker having a speed of 250 rev./min. and a throw of 2 in. Fermentations were carried out in two media. Medium 1 contained: corn-steep liquor, 6.0% (v/v); lactose, 5.0% (w/v); glucose, 0.5%; NaNO_3 , 0.5%; KH_2PO_4 , 0.1%; Na_2SO_4 , 0.1%; CaCO_3 , 1.0% (w/v); 3 drops of an antifoam agent, 2% (v/v) octadecanol in cottonseed oil; the pH was adjusted to 5.1 before autoclaving. Medium 2 contained Pharmamedia (Traders Protein Division of Traders Oil Mill Co., Fort Worth 1, Tex., U.S.A.), 2% (w/v); peanut meal, 2% (w/v); lactose, 7.5% (w/v); CaCO_3 , 1.0% (w/v); CaSO_4 , 0.5%; Na_2SO_4 , 0.4%; ground-nut oil, 0.25%; the pH was adjusted to 6.9 before autoclaving. The fermentation media were inoculated with approx. 4% of a 2-day vegetative growth prepared as follows: 500 ml. plain conical flasks, closed with cottonwool plugs and containing 100 ml. of a medium consisting of corn-steep liquor (7%, v/v), raw sugar (2.0%, w/v), CaCO_3 (1.0%, w/v), maize oil (0.25%), with pH 5.8-6.0 before autoclaving, were inoculated with a spore suspension prepared from an agar slope and incubated on a rotary shaker at 26°.

After 5 days the fermentation liquors were harvested and assayed for total nucleus, for free 6-APA and for penicillins.

Acetone extracts of washed mycelium. Mycelium was centrifuged from the fermentation medium and resuspended in a large volume of deionized water. The small pellets of mycelium were allowed to settle and the supernatant material was decanted off. This process was repeated until the supernatant was colourless and fairly clear. The mycelium was collected on a Buchner funnel, pressed free of excess of water and slurried with its own weight of aq. 70% (v/v) acetone. The slurry was stirred for 5 min. before filtering.

RESULTS AND DISCUSSION

When 6-APA was added to solutions rich in sugars, part of the β -lactam nucleus was found to become stable to penicillinase. The effect of pH on this reaction is fairly marked, there being a sharp optimum at about pH 6.0 (see Table 1).

The maltose, glucose and lactose derivatives of 6-APA were isolated as amorphous white powders from reaction mixtures containing only the sugar and 6-APA. Table 2 lists some of the properties of these preparations, and it can be seen that analysis of the components gives a stoichiometry of 1 molecule of 6-APA combined with 1 molecule of sugar.

After conversion into the free acid with Amberlite IR-120 (H⁺ form) resin, 13.9 mg. of the glucose derivative took up 7.5 ml. of 3.9 mN-sodium hydroxide, giving an equiv. wt. of 477. This result and the behaviour of these compounds on electrophoresis (see Table 2) indicate that they are monobasic acids.

That the amino group of 6-APA and the reducing group of the sugar are involved in the formation of these compounds is indicated by the observations that there is neither any interaction between benzylpenicillin and reducing sugars, nor any reaction between 6-APA and any sugar derivatives in which the reducing moiety is protected, such as sucrose, α -methyl glucopyranoside and glucose 1-

phosphate. It was confirmed by chromatography that acid hydrolysis of glycosyl-6-APA liberates only the original sugar and 6-APA. The chemical properties outlined above and the mode of formation all suggest these new compounds are *N*-glycosyl derivatives of 6-APA, e.g. β -*N*-glucosyl-6-APA (I). As would be expected of such a structure these compounds are all extremely soluble in water and insoluble in ordinary organic solvents.

(I)

The rate of acid hydrolysis of the glucosyl derivative was examined at room temperature and the results are given in Table 3. At 37° it was found that a solution of 1 mg. of glycosyl-6-APA/ml. in 0.1N-hydrochloric acid was completely hydrolysed in 20 min.

The glucosyl, maltosyl and lactosyl compounds are all highly stable to staphylococcal penicillinase, and decomposition in the presence of *B. cereus* penicillinase can only be demonstrated with difficulty. Thus, after incubation for 4 hr. at 37° and pH 7.0, a solution of 1 mg. of glucosyl-6-APA/ml. lost 28.5 % of its penicillin nucleus in the presence of *B. cereus* penicillinase compared with a loss of 15 % in its absence. Under the same conditions 6-APA itself was completely destroyed in 15 min.

The presence of the glycosyl side chain not only protects the β -lactam ring from hydrolysis by penicillinase but also decreases markedly the antibacterial activity that 6-APA exhibits. A comparative assay against *B. subtilis* gave the ratio of the activities of glucosyl-6-APA and 6-APA as 1:230. By using both Gram-negative and Gram-positive bacteria it was found that activity was

Table 1. *Effect of pH on a reaction mixture containing 2 % of glucose and 0.1 % of 6-aminopenicillanic acid*

Assays were carried out by the hydroxylamine method after incubating for 16 hr. at 26°, and all results are expressed as mg. of free 6-APA/ml. Details are given in the text.

pH	Total nucleus	Free 6-APA	Glucosyl-APA
3.5	0.7	0.58	0.12
4.0	0.65	0.60	0.05
4.5	0.68	0.52	0.16
5.0	0.68	0.46	0.22
5.5	0.68	0.40	0.28
6.0	0.75	0.40	0.35
6.5	0.74	0.62	0.12
7.0	0.72	0.62	0.10
7.5	0.69	0.66	0.03
8.0	0.74	0.65	0.09
8.5	0.74	0.68	0.06
No glucose	0.94	0.94	0.00

Table 2. *Analysis and properties of three sugar-6-aminopenicillanic acid compounds*

Details are given in the text.

	Glucosyl-6-APA	Maltosyl-6-APA	Lactosyl-6-APA
6-APA (%)*	52	30	27
Sugar (%)†	40	53	49
Sugar:6-APA molar ratio	0.92:1	1.1:1	1.14:1
Purity (%)‡	96	79	70
Decomposition temperature	144–146°	128–140°	142–150°
Mobility toward anode (cm.)	9	—	6
R _F in butan-1-ol-pyridine-water	0.4	0.33	0.30

* Based on direct hydroxylamine assay, with 6-APA as a standard.

† Assayed by Somogyi method after acid hydrolysis and removal of free 6-APA with Amberlite IR-120 (H⁺ form) resin. Standards of the respective sugars were used.

‡ Based on direct hydroxylamine assay of the penicillin nucleus and molecular weights calculated for the sodium salts of *N*-glycosyl-6-APA.

always less than that of 6-APA, values of the minimum inhibitory concentration being generally greater than 1 mg./ml.

The reactions with 6-APA were found to be of a complex nature when studied in closer detail. Apart from rearrangements after the initial reaction and the formation of secondary products, there was usually a definite loss of penicillin nucleus in the presence of sugars (see Table 4). This effect was particularly marked with mannose, but it is not known by what mechanism this sugar causes the non-enzymic destruction of the β -lactam ring at neutral pH. It is possible that the configuration of the compound is such that interaction of the hydroxyl groups with the β -lactam function is particularly easy. There is a direct correlation of the destruction of the β -lactam ring with the configurational instability of the sugars, as reflected in the reaction of the various sugars with hydroxylamine and subsequent time-dependent production of colour on the addition of the ferric chloride reagent (see Table 5).

Because of the interactions of some sugars with the reagents of the hydroxylamine assay additional care was required in performing and interpreting the hydroxylamine assays of penicillin nucleus. In particular it is essential to read the blank and the sample at the same time after the addition of the ferric chloride reagent.

It was shown that 6-APA reacted with several glucose derivatives. With glucosamine it reacted

slowly, there being two new zones on chromatograms of the reaction mixture (see Table 6). The multiplicity of new zones is not unique to glucosamine, for more than one product has been observed in reactions with simple sugars after long reaction times. Glucuronic acid and galacturonic acid react readily with 6-APA to give derivatives that are much less stable both to penicillinase and to phenylacetylation on bicarbonate-buffered tapes. This instability is probably a reflexion of the ease with which they break down to 6-APA in solution and may be due to a ready interaction of the 6-carboxyl group with C-1 of the sugar molecule. The derivatives of glycuronic acids and 6-APA probably exist in equilibrium with the parent compounds, as all attempts to free them from 6-APA have failed.

If compounds of the type described above do occur in fermentation media containing 6-APA, they may not have been previously observed because they are not rendered biologically active by phenylacetylation unless first hydrolysed. Thus, if conditions should become acidic during the spraying of chromatograms with phenylacetyl chloride, or if compounds behaving like the glucuronic acid-6-APA derivative should be present, then phenylacetylation would convert these compounds into penicillin G. It is possible that some of the phenylacetyl chloride-reacting compounds reported by Wolff & Arnstein (1960) to be present in mycelial extracts may have been glycosyl derivatives of 6-APA. Assays of 6-APA in fermentation media are usually carried out after removing natural penicillins either by extraction or by destruction (Batchelor, Chain & Rolinson 1961). As both of these methods involve acid treatment most of the glycosyl derivatives would be hydrolysed to free 6-APA and the assay results would be a measure of the free and bound 6-APA.

The culture filtrate of a *P. chrysogenum* fermentation has been shown to contain a significant amount of 6-APA bound to sugars or sugar derivatives (see Table 7 and Fig. 1).

The results obtained from the chromatography of the acetone extract of the washed mycelium differed markedly from those of the fermented

Table 3. *Acid stability of a solution of glucosyl-6-aminopenicillanic acid (1 mg./ml.) at room temperature*

Results are expressed in terms of liberated 6-APA which was assayed by bioassay after phenylacetylation. Details are given in the text.

pH	Half-life (min.)	Initial rate of reaction (μ g./ml./min.)
1.5	14	23.0
2.0	39	8.5
2.5	92	4.5
3.0	128	2.5
3.5	208	1.2

Table 4. *Stability of 6-aminopenicillanic acid in the presence of sugars*

6-APA (10 mg./ml.) was incubated at 26° in the presence of 4% (w/v) sugar. All results are expressed as mg. of 6-APA/ml. Details are given in the text.

Time (hr.)	Mannose			Galactose			Glucose			No sugar
	Total nucleus	Free 6-APA	Bound 6-APA	Total nucleus	Free 6-APA	Bound 6-APA	Total nucleus	Free 6-APA	Bound 6-APA	Total nucleus
16	5.15	2.3	2.85	8.8	5.7	3.1	9.5	7.6	1.9	10.2
24	4.4	1.7	2.7	8.3	5.2	3.1	9.1	6.6	2.5	9.4
42	3.5	1.1	2.4	7.3	4.4	2.9	8.0	4.5	3.5	8.8
66	2.0	0.5	1.5	5.8	3.5	2.3	6.6	3.8	2.8	7.2

Table 5. *Production of colour by sugars during the hydroxylamine assay*

The reaction mixture contained 2 ml. of sugar (1%) plus 6 ml. of hydroxylamine reagent. After 10 min. 2 ml. of FeCl_3 reagent was added and the colour read on the EEL colorimeter immediately the mixture had cleared. Details are given in the text.

Time after addition of FeCl_3 (min.)	Colorimeter reading		
	Glucose	Galactose	Mannose
0	17	22	23.5
1	17	26.5	38.5
2	17	29.0	48.0
3	17	30.0	53.0
4	17	31.0	57.0
5	17	32.0	60.0
6	17	33.0	61.5
9	17	34.0	64.0

Table 6. *R_F values of compounds of 6-aminopenicillanic acid and glucose derivatives*

The solvent used was butan-1-ol-pyridine-water (1:1:1, by vol.). Details are given in the text.

Sugar used as side chain	R_F of new zones
Glucose	0.4
Glucosamine	0.25, 0.34
Glucuronic acid	0.14
Glucose 6-phosphate	0.17
[6-APA	0.45]

Table 7. *Assay of the culture filtrate of a Penicillium chrysogenum fermentation at 5 days*

The extractable penicillins are expressed as benzylpenicillin. Details are given in the text.

	Concn. ($\mu\text{g./ml.}$)
Extractable penicillins	255
6-APA (free)	680
6-APA (bound)	230

Table 8. *R_F values of new zones reacting with phenylacetyl chloride that appear after acid treatment of chromatograms of culture filtrates and mycelial extracts of Penicillium chrysogenum*

The solvent used was butan-1-ol-pyridine-water (1:1:1, by vol.). These R_F values have been tentatively ascribed to zones observed in several experiments. Details are given in the text.

Medium 1		Medium 2	
Culture filtrate	Mycelium	Culture filtrate	Mycelium
0.29	0.22	0.31	0.16
0.22	0.14	0.24	—
0.17	—	0.20	—
0.00	—	0.15	—
—	—	0.085	—
—	—	0.00	—

Fig. 1. Chromatography of a 5-day fermentation of *P. chrysogenum* in medium 2. The solvent used was butan-1-ol-pyridine-water (1:1:1, by vol.). A, B and C, Acetone extract of washed mycelium; D, E and F, culture filtrate; A and D, untreated tapes; B and E, tapes sprayed with phenylacetyl chloride; C and F, tapes acid-treated and sprayed with phenylacetyl chloride. Details are given in the text. * Cole & Batchelor (1963). † Flynn, McCormick, Stamper, De Valeria & Godzeski (1962).

medium (see Fig. 1 and Table 8). In the latter a series of new zones, usually streaking one into another, appeared on phenylacetylation after acid treatment. The major zone was at R_F 0.3 and corresponded to lactosyl-6-APA, lactose being a major constituent of the media used. In the mycelial extracts, however, a simpler pattern of more discrete zones appeared, and with mycelium grown in medium 2 only a single new zone at R_F 0.17 was detected. Although it behaves like the glycosyl derivatives, nothing is known about this mycelial compound except that it probably contains 6-APA. This zone was also found in extracts of mycelium obtained by using the X-press (Edebo, 1960). That only 6-APA is present in all of these compounds was confirmed by two-dimensional chromatography, running the second dimension, both after acid treatment, with a 6-APA marker, and after acid treatment and phenylacetylation, with a benzylpenicillin marker. Although one cannot assess the significance of these compounds in fermentation media or mycelium it is possible that the formation of glycosyl-6-APA would protect 6-APA from destruction by respiratory carbon dioxide (Ballio *et al.* 1961).

SUMMARY

1. 6-Aminopenicillanic acid (6-APA) reacts with reducing sugars at room temperature and neutral pH to produce derivatives that are probably the *N*-glycosyl compounds of 6-APA.

2. The glucosyl, maltosyl and lactosyl derivatives of 6-APA were prepared as their sodium salts and some of their properties examined.

3. These derivatives of 6-APA are stable to both staphylococcal and *Bacillus cereus* penicillinase, but are readily hydrolysed to the parent compounds by acid.

4. Compounds behaving like those prepared from 6-APA and sugars were identified in both the culture filtrates and the mycelium of a culture of *Penicillium chrysogenum* grown under conditions in which it produced 6-APA.

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The Function of High-Molecular-Weight Ribonucleic Acid from Rabbit Reticulocytes in Haemoglobin Biosynthesis

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There is now considerable evidence for the existence, in bacteria, of a fraction of RNA which is specifically involved in determining the amino acid sequence in protein biosynthesis. This so-called messenger RNA (m-RNA*) appears to have a DNA-like base composition (Volkin & Astrachan, 1956; Volkin, Astrachan & Countryman, 1956; Hayashi & Spiegelman, 1961; Nomura, Hall & Spiegelman, 1962) and is synthesized at a faster rate than either the ribosomal RNA or the s-RNA (Brenner, Jacob & Meselson, 1961; Gros *et al.* 1961). The m-RNA appears to be heterogeneous, as indicated by sedimentation coefficients ranging from about 8 to 30s (Hayashi & Spiegelman, 1961; Ishihama, Mizuno, Takai, Ohtaka & Osawa, 1962; Monier, Naono, Hayes, Hayes & Gros, 1962).

* Abbreviations: m-RNA, messenger RNA; s-RNA, transfer RNA.

In cell-free preparations from *Escherichia coli*, m-RNA isolated by fractionation on sucrose density gradients has been shown to stimulate the incorporation of amino acids into protein (Monier *et al.* 1962), but evidence for the RNA-directed synthesis of specific protein has so far been obtained only with RNA from the f2 coliphage and related viruses. On the addition of such RNA preparations to a cell-free system consisting of ribosomes and amino acid-activating enzymes from *E. coli*, amino acid incorporation into virus proteins has been demonstrated (Nathans, Notani, Schwartz & Zinder, 1962; Ohtaka & Spiegelman, 1963). Tobacco-mosaic-virus RNA also stimulates amino acid incorporation by cell-free preparations from *E. coli*, but protein resembling tobacco-mosaic-virus protein is at most only 10% of the total synthesized