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Physical and Chemical Properties of Polysaccharides and Glycoproteins of the Yeast-Cell Wall

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(*Received* 15 *September* 1959)

The composition of the yeast-cell wall is known to be approximately glucan 29%, mannan 31%, protein 13%, lipid 8·5% and ash 3% (Northcote & Horne, 1952; Roelofsen, 1953). In addition, numerous investigators have reported the presence of 1–3% of glucosamine, which has been attributed to the occurrence of chitin in the wall (Roelofsen & Hoette, 1951; Houwink & Kreger, 1953; Falcone & Nickerson, 1956; Eddy, 1958). The existence of complexes between the polysaccharide and protein has often been inferred, and recently some evidence for a mannan–protein complex has been obtained (Falcone & Nickerson, 1956; Eddy, 1958). Usually strong alkali has been used to isolate the polysaccharide components and this treatment undoubtedly degrades any complexes between these molecules and protein. We therefore decided in these investigations to use milder extraction procedures. This has resulted in the isolation from the cell wall of several fractions which appear to be glycoproteins. As these fractions also contained glucosamine the distribution of this sugar among the cell-wall components was reinvestigated.

EXPERIMENTAL

Materials. The yeast used was a commercial pressed baker's yeast (Ark Yeast, Distillers Co. Ltd.). Cell walls were prepared according to the method of Shockman, Kolb & Toennies (1957). Each stainless-steel cup (internal measurements 12·5 cm. × 2·5 cm.) contained 20 ml. of a 10% (w/v) suspension of yeast and 20 g. of Ballotini no. 12 glass beads (Chance Bros. Ltd., Birmingham). Breakage was continued for 15 min. in an International refrigerated centrifuge at 1400 rev./min. at 4°. The walls were separated from any unbroken cells and cell contents by differential centrifuging according to Northcote & Horne (1952). The yield of freeze-dried walls was 1 g. from 100 g. of pressed yeast.

Analytical procedures

pH *measurements.* These were made with a Pye pH meter and a glass electrode at room temperature and by standardizing the apparatus with standard buffer tablets, pH values 4·01 and 6·99 (Burroughs Wellcome and Co.).

Boundary electrophoresis. The samples were examined as approximately 1·5% solutions in 0·05M-sodium borate buffer, pH 9·2, 0·1M-K_2HPO_4, pH 9·3, or 0·05M-sodium borate–8M-urea, pH 9·2. The Perkin–Elmer model 38 Tiselius electrophoresis apparatus was used.

Diffusion measurements. These measurements were made over a period of 3 days on 0·6% solutions in 0·1M-KH_2PO_4, pH 4·7. The Tiselius electrophoresis apparatus was used.

Sedimentation measurements. The samples were dissolved in 0·1M-KH_2PO_4 (0·05–2·5%) and sedimented at $2{\cdot}69 \times 10^5 g$ for 2·5 hr. in the Spinco Analytical ultracentrifuge model E (Specialized Instruments Co., Belmont, Calif., U.S.A.). Pictures were taken every 16 min.

Total nitrogen and phosphorus. The micro-Kjeldahl digestion method of Chibnall, Rees & Williams (1943) was used, followed by distillation and titration. Phosphorus was determined according to the method of Fiske & Subbarow (1925).

Neutral sugars. Samples were hydrolysed in 2N-H_2SO_4 and the sugars were identified chromatographically (Northcote, Goulding & Horne, 1958).

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Amino acids. Free amino acids were not present before acid hydrolysis. The samples (0·5–3·0 mg.) were hydrolysed in 6N-HCl (0·5 ml.) for 16 hr. at 105° and evaporated to dryness under reduced pressure in a desiccator containing H_2SO_4 and NaOH. The residue was redissolved in a small volume of water and applied as a spot to Whatman no. 52 filter paper. The amino acids were separated by electrophoresis in 2·5% formic acid (pH 2·1) in a field strength of approx. 130 v/cm. for 25 min. The paper was then dried and developed chromatographically in a direction at right angles to the electric field with butanol–acetic acid–water (3:1:1, by vol.). The amino acids were detected by spraying with 0·2% ninhydrin in 96% ethanol. On separate papers, histidine was detected by the Pauly reagent and arginine by the Sakaguchi reagent (Jepson & Smith, 1953). Cysteine was detected as cysteic acid after oxidation of the samples with performic acid (Sanger, 1949). Tryptophan was determined in the unhydrolysed samples by procedure B of Spies & Chambers (1948).

Amino sugar. Glucosamine was detected as a ninhydrin-coloured spot on the papers used for analysis of the amino acids. Its position relative to the amino acids is shown on Fig. 1. It had an electrophoretic mobility corresponding to alanine and moved chromatographically similarly to glycine. This spot also reduced alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950) and gave a positive reaction with the Elson–Morgan reagent (Partridge, 1948). The hexosamine was further identified as glucosamine after separation electrophoretically by chromatography in pyridine–ethyl acetate–water (1:2:2, by vol.) when glucosamine only was detected and after oxidation by ninhydrin (Stoffyn & Jeanloz, 1954) when only arabinose was detected. In addition, the compound was found to have the same mobility as glucosamine upon electrophoresis in pyridine–acetic acid–water buffer, pH 3·5 (1:10:89, by vol.), and pH 6·5 (10:0·3:90, by vol.). The procedures do not distinguish glucosamine from mannosamine.

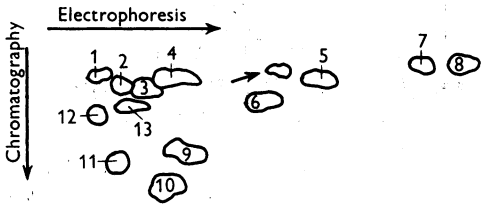

Fig. 1. Separation of amino acids and glucosamine in an acid hydrolysate of fraction *B*. The arrow indicates the glucosamine. Amino acids: 1, aspartic acid; 2, glutamic acid; 3, threonine; 4, serine; 5, glycine; 6, alanine; 7, histidine; 8, lysine; 9, valine; 10, leucine/isoleucine; 11, phenylalanine; 12, tyrosine, 13, methionine.

The glucosamine was quantitatively determined after hydrolysis of the samples in 4N-HCl for 12 hr. at 100° by the Elson–Morgan reaction (Blix, 1948), with both glucosamine and chitin as standards. The chromogen formed had the correct spectrum for glucosamine and no colour developed in the absence of acetylacetone. No free glucosamine was present in any of the samples before acid hydrolysis.

N-*Terminal amino acid.* The samples (50–200 mg.) were dissolved in 8M-urea containing 1% of $NaHCO_3$, treated with fluorodinitrobenzene, and the dinitrophenyl (DNP)-amino acids were determined according to Fraenkel-Conrat, Harris & Levy (1955).

RESULTS

Fractionation of the cell walls

The cell walls were extracted with anhydrous ethylenediamine at 37° (Fig. 2). The mixture was shaken at intervals of 30 min. to resuspend the walls, which after a period of 6 hr. became gelatinous and settled very slowly. During the remainder of the extraction time the mixture was shaken only about three or four times a day and after 3 days it was divided into three fractions as indicated in Fig. 2. The chemical characteristics of these fractions are shown in Table 1.

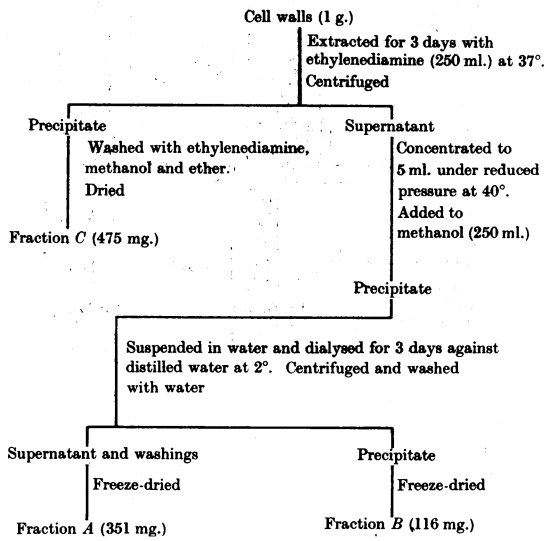

Fig. 2. Fractionation of the yeast-cell wall by ethylenediamine.

Table 1. *Chemical characteristics of the cell-wall fractions*

The protein was calculated from the N content after correcting for the amount of glucosamine present.

Fraction	Neutral sugars	N (%)	Glucosamine (%)	Protein (%)	Phosphorus (%)
A	Mannose	2·0	1·7	11·6	0·30
B	Mannose, glucose	1·0	0·8	5·9	0·12
C	Mannose, glucose	2·7	2·0	15·9	—
Mannan	Mannose	0·35	1·4	1·5	0·30
Glucan	Glucose	0·6	6·0	0·8	0·008

Cell-wall fraction A. This material gave a positive reaction for protein (Lowry, Rosebrough, Farr & Randall, 1951) and contained the following amino acids: aspartic acid, glutamic acid, threonine, serine, glycine, alanine, valine, leucine or isoleucine or both, proline, phenylalanine, tyrosine, tryptophan, cysteine, lysine and histidine. The only DNP-amino acids detectable were alanine and glycine (approx. 0·1 μmole of each/40 mg.).

The sedimentation diagram of fraction *A* shows two components, the relative amounts of which depended upon the time of treatment (24 or 72 hr.) of the cell walls with ethylenediamine (Fig. 3*a*, *b*). After extraction for 3 days the faster-moving component is considerably reduced in amount (Fig. 3*b*). All the data reported refer to the material prepared by extraction for 3 days. The sedimentation and diffusion constants are shown in Table 2. The molecular weight calculated for the slower (major) component is probably lower than its true molecular weight because of the heterogeneity of the material.

When fraction *A* was subjected to electrophoresis in borate buffer only one peak was observed with a slight asymmetry in the trailing edge (Fig. 4, Table 2). Essentially the same picture was obtained in borate–urea buffer. On electrophoresis in phosphate buffer a much smaller mobility was found (Table 2) but again only one peak was evident.

Cell-wall fraction B. This fraction also gave a positive test for protein and contained the same

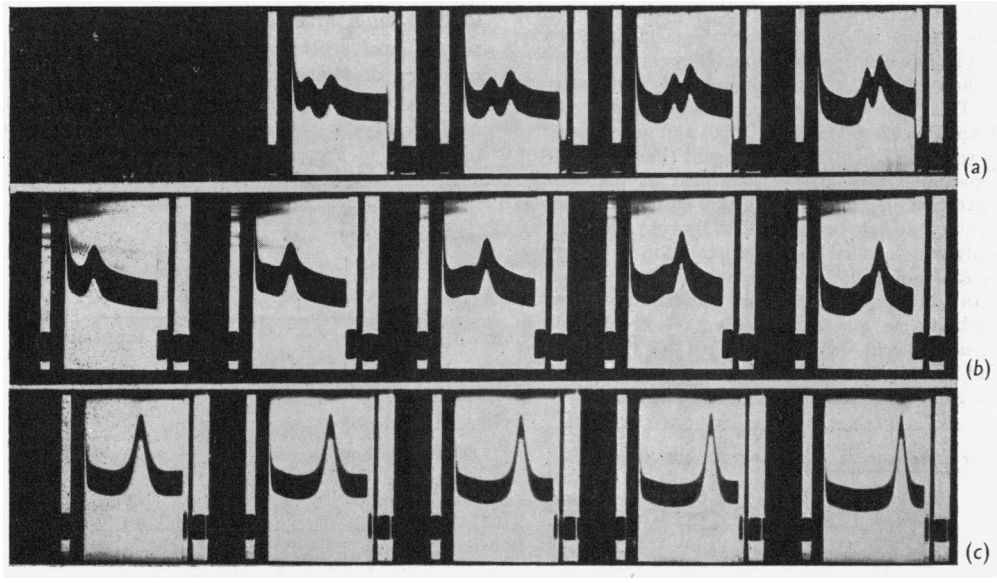

Fig. 3. Sedimentation patterns of yeast-cell-wall fractions dissolved in 0·1 M-KH_2PO_4, pH 4·7. (*a*) Fraction *A* (15 mg./ml.) prepared by extraction of cell walls with ethylenediamine for 24 hr.; (*b*) fraction *A* (15 mg./ml.) prepared by extraction of cell walls with ethylenediamine for 72 hr. (*c*) Mannan (16·4 mg./ml.). Centrifugal force was $2{\cdot}69 \times 10^5$ *g* and the temperature 21°. Photographs were taken at 16 min. intervals. Migration is from right to left.

Table 2. *Physical characteristics of cell-wall fraction A and mannan*

Values for S_{20} and D_{20} were determined at a concentration of 6 mg./ml. in 0·1 M-KH_2PO_4 and the molecular weights were calculated from the equation: mol. wt. $= \dfrac{RTS}{D(1-\bar{v}\rho)}$.

Fraction	$10^{-13} \times$ Sedimentation constant (S_{20}) (cm. g.$^{-1}$ sec.$^{-1}$)	$10^{-7} \times$ Diffusion constant (D_{20}) (cm.^{2} sec.$^{-1}$)	Mol.wt.	$10^{-5} \times$ Electrophoretic mobility (cm.^{2} v^{-1} sec.$^{-1}$)	
				Borate buffer, pH 9·2	Phosphate buffer pH 9·3
Mannan	3·6	5·09	$5{\cdot}9 \times 10^4$	8·8	2·26
A Major peak	4·2	3·50	$9{\cdot}8 \times 10^4$	6·0	1·55
A Minor peak	6·7	—	—	—	—

amino acids as fraction *A*. Again only DNP-alanine and DNP-glycine were obtained and in approximately the same yield as with fraction *A*. Although this fraction was insoluble in water it dissolved in 8 M-urea to give an opalescent solution from which it was quantitatively precipitated upon dialysis against water. The electrophoretic diagram obtained in borate–urea buffer showed the presence of only one component (mobility, $5{\cdot}7 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$).

Cell-wall fraction C. Microscopically this fraction resembled the original form of the cell wall but was greatly swollen. When sectioned and examined with the electron microscope (Northcote *et al.* 1958) two distinct membranes were no longer discernible.

Mannan

This polysaccharide ($[\alpha]_D^{17}$ 88·5° in water (*l*, 2; *c*, 1·1) was prepared from whole yeast by the method of Haworth, Heath & Peat (1941). It gave no colour with iodine. It had a partial specific volume ($\bar{v}$) of 0·65. Although it gave a negative test for protein (Lowry *et al.* 1951) it was found to contain the same amino acids as cell-wall fraction *A*, and again DNP-alanine and -glycine were detected (approx. 0·1 μmole/500 mg.). The electrophoresis diagram of mannan in borate buffer showed only one symmetrical peak (Northcote, 1954). In phosphate buffer again only one peak was observed but it had a much smaller mobility (Table 2). The leading edge of the peak diffused rapidly. This is characteristic of polysaccharides when a buffer not containing borate is used (Northcote, 1954). The sedimentation diagram showed one symmetrical peak (Fig. 3*c*). Other chemical and physical characteristics of this material are shown in Fig. 5 and Tables 1 and 2.

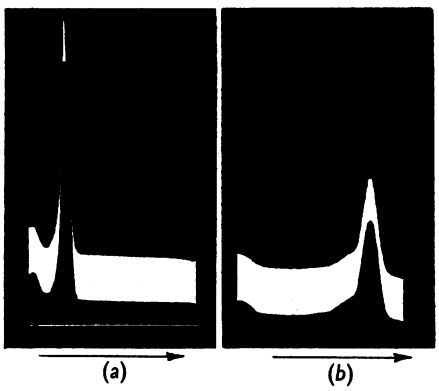

Fig. 4. Boundary electrophoresis of fraction *A* (15 mg./ml.) in 0·05 M-borate buffer, pH 9·2. (*a*) Descending limb, 40 min.; (*b*) descending limb, 150 min.

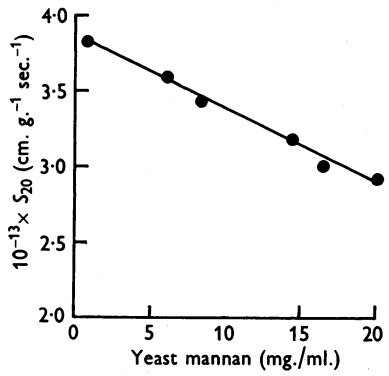

Fig. 5. Variation of sedimentation constant (S_{20}) with concentration of yeast mannan in 0·1 M-KH_2PO_4.

Glucan

This insoluble polysaccharide was prepared from whole yeast by the method of Bell & Northcote (1950). The chemical characteristics of this material are shown in Table 1. It gave a negative test for protein (Lowry *et al.* 1951) but contained all the amino acids found in the other materials, except that tyrosine, phenylalanine and proline were not detected.

Tests for chitin

Glucan (200 mg.) was boiled with 2 % HCl for 1 hr. and then with 3 % NaOH for 15 min. This treatment dissolved 80 % of the glucosamine originally present in the polysaccharide. The residue was heated at 160° for 15 min. in 60 % KOH to prepare chitosan (Roelofsen & Hoette, 1951), but the small residue which remained did not stain with iodine.

Cell-wall fraction *C* (200 mg.) was also treated in the manner described above, when 85 % of the glucosamine originally present dissolved in the dilute acid and alkali. There was no residue left after treatment with 60 % KOH.

Chitin (100 mg.) prepared from crab shells (Purchase & Braun, 1946) was extracted for 3 days at 37° with ethylenediamine (150 ml.). No glucosamine could be detected in the solution and chitin was recovered quantitatively. The chitin (10 mg.) was then treated with dilute acid and alkali in the manner described above. The insoluble residue was found to contain 90 % of the glucosamine of the chitin.

DISCUSSION

Of the total glucosamine in the cell wall, fraction *A* contains 36 %, fraction *B*, 6 % and fraction *C*, 58 %. Fraction *A* is soluble in both ethylenediamine and water and fraction *B* is soluble in ethylenediamine. As chitin is insoluble in both these solvents, the glusamine in fractions *A* and *B*

cannot be present in the form of chitin. When fraction *C* is treated with dilute acid and alkali, 85% of the glucosamine is dissolved and therefore a maximum of 15% of this fraction (9% of the total glucosamine of the cell wall) has the solubility properties of chitin. Similarly, the presence of glucosamine in the soluble mannan and the solubility characteristics of most of the glucosamine in the glucan again indicate that only a small percentage of the total glucosamine in the yeast-cell wall could be chitin. This residual insoluble material could possibly account for the chitin detected by the X-ray-diffraction studies of Houwink & Kreger (1953).

The presence of bound glucosamine in the glucan and mannan which have been prepared by standard procedures raises the question whether the amino sugar occurs in these materials as a separate polysaccharide or as part of the glycosidic chains of the glucan and mannan. In either case it must be considered in chemical structural studies of these compounds and this is especially important with glucan, in which the content of glucosamine is as high as 6%.

The 16 amino acids found in fraction *A* are present in peptide form and as only two *N*-terminal amino acids are found it is probable that the protein portion of the molecule is a specific compound. The low recoveries of DNP-amino acids were probably due to unavoidable losses in the acid hydrolysis because of the large amounts of carbohydrate, and do not indicate the true molecular weight of the protein.

The electrophoretic mobility of fraction *A* in borate buffer is of the same order of magnitude as that found for mannan and must therefore be due to the formation of a complex of the carbohydrate portion with the borate ions. However, the mobility is lower than that of mannan, as would be expected if the protein and polysaccharide are firmly bound. Electrophoresis in the presence of 8M-urea and in phosphate buffer showed no evidence for a separate protein peak.

Fraction *A* would seem to represent a mannan–protein complex similar to those described by Falcone & Nickerson (1956) and Eddy (1958). As the molecular weight of even the slower-sedimenting component of fraction *A* is considerably greater than that of mannan, it may be most accurate to consider the components of fraction *A* and mannan as a sequence of degradation products derived from a much larger mannan–protein complex present in the original cell wall.

Fraction *B* has very similar electrophoretic and chemical characteristics to those of fraction *A* but it contains glucose in addition to mannose and therefore might represent a protein–carbohydrate complex in which glucan and mannan are linked together by protein. As the *N*-terminal amino acids found in this fraction are the same as those found in fraction *A* both may contain the same proteins. If fraction *B* is a mannan–glucan–protein complex, it may be a much closer representation than fraction *A* of the true chemical structure of the yeast-cell wall.

It is noteworthy that all the fractions, including mannan and glucan, contain glucosamine. This sugar might serve as the connecting link between the protein and polysaccharide components of the complexes.

SUMMARY

1. The isolated yeast-cell wall has been separated into three fractions: fraction *A*, soluble in ethylenediamine and water; fraction *B*, soluble in ethylenediamine and insoluble in water; fraction *C*, insoluble in both ethylenediamine and water.

2. The physical and chemical characteristics of these fractions have been determined, from which it is concluded that fraction *A* is a mannan–protein complex and fraction *B* may possibly be a glucan–mannan–protein complex. All three fractions contain glucosamine, and it is suggested that this amino sugar might provide the link between the protein and carbohydrate.

3. It is estimated that no more than approximately 9% of the total glucosamine of the cell wall could be present as chitin.

4. The molecular weight of yeast mannan was found to be $5{\cdot}9 \times 10^4$. It contained 1·4% of glucosamine and 1·5% of protein.

5. Yeast glucan was found to contain 6·0% of glucosamine and 0·8% of protein.

We wish to thank Mr B. R. Slater for assistance with the electrophoretic and diffusion measurements and Mr B. Boon for assistance with the sedimentation measurements. One of us (E.D.K.) is indebted to the National Heart Institute for permission to spend the year 1958–59 at the University of Cambridge and to Professor F. G. Young for providing facilities in the Department of Biochemistry, Cambridge.

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The Frequencies of Certain Sequences of Nucleotides in Deoxyribonucleic Acid

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(*Received* 13 *August* 1959)

The degradation of deoxyribonucleic acid by 66% (v/v) formic acid containing 2% of diphenylamine, at room temperature (Burton, 1956; Burton & Petersen, 1957), appears to be a valuable reaction for the study of certain sequences in the poly-deoxyribonucleotide chains. In this paper we describe the properties of the reaction and a simple chromatographic method by which the frequencies of a considerable number of pyrimidine sequences in deoxyribonucleic acid can be measured on, if necessary, as little as 10–20 mg. of the nucleic acid.

MATERIALS AND METHODS

Deoxyribonucleic acid. This was prepared from calf thymus by extracting the nucleoprotein with 10% (w/v) NaCl and subsequent treatment with sodium dodecyl sulphate (Chargaff, 1955). A sample was freeze-dried to constant weight and found to contain 6·3% of P. Base analysis by hydrolysis in 7·5N-perchloric acid and paper chromatography in propan-2-ol–conc. HCl–water (65:20:10, by vol.; Wyatt, 1955) gave the following molar proportions (mean ± S.E.M.); adenine 28 ± 0·3, guanine 20·9 ± 0·4, cytosine 21·4 ± 0·4, thymine 28·3 ± 0·6, 5-methylcytosine approx. 1. Between 90 and 95 moles of bases were recovered/100 g.atoms of P. These values were obtained from a total of 10 analyses on two hydrolysates.

Apurinic acid. This was prepared from deoxyribonucleic acid (DNA) according to Tamm, Hodes & Chargaff (1952).

Diphenylamine. This was either analytical-reagent grade or a less-pure grade that was recrystallized several times from aqueous ethanol.

Formic acid. Analytical-reagent grade (98–100%) was used.

Calcium bis(p-nitrophenyl)phosphate. This was prepared according to Privat de Garilhe & Laskowski (1955).

Ion-exchange resins. Dowex-1 was nominally 8 or 10% cross-linked and 200–400 mesh. The finer particles were removed by decantation after suspension in water, the resin was packed into a column and washed with 2N-NaOH, water and 3N-HCl until the extinction coefficient of the eluate was less than 0·01 at 260 mμ. Formate and acetate forms were prepared from the chloride form as described by Sinsheimer (1954). Dowex-50 (200–400 mesh) was cleaned according to Moore & Stein (1951). The Dowex-1 was kept clean until required by slowly percolating a solution of the corresponding ammonium salt (0·01M) through the resin.

Paper for two-dimensional chromatography. Sheets of Whatman no. 1 paper for chromatography (47 cm. × 53 cm.) were serrated along the short edge and washed for about 15 hr. with N-HCl by allowing the acid to run along the paper and off the serrated edge in a tank for descending chromatography. They were then similarly washed with water for about 30 hr. and dried in air at room temperature.

Snake-venom phosphodiesterase. This was prepared from *Crotalus adamanteus* venom (Ross Allen's Reptile Institute, Silver Springs, Fla., U.S.A.) by the method of Cohn, Volkin & Khym (1957), including a second acetone fractionation. An increase of 0·13 in the extinction coefficient at 440 mμ was given by 0·1 ml. of the preparation under the conditions of Cohn *et al.* (1957).

Spleen phosphodiesterase. This was prepared according to Heppel & Hilmoe (1955) and dialysed against 0·1M-NaCl and then against water to remove pyrophosphate. The final preparation contained 30 units of activity/ml. (units as defined by Heppel & Hilmoe).

Prostatic phosphomonoesterase. Hypertrophic human prostate glands were dissected free of connective tissue and

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