Bernal, J. D. (1931). *Z. Kristallogr.* **78**, 363.
Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
Craig, L. C., Gregory, J. D. & Hausmann, W. (1950). *Analyt. Chem.* **22**, 1462.
Dent, C. E. (1948). *Biochem. J.* **43**, 169.
Fischer, E. (1906). *Ber. dtsch. chem. Ges.* **39**, 2325.
Fischer, E. & Raske, K. (1907). *Ber. dtsch. chem. Ges.* **40**, 3723.
Hirs, C. H. W., Moore, S. & Stein, W. H. (1954). *J. Amer. chem. Soc.* **76**, 6063.
Kaczka, E. & Folkers, K. (1949). In *The Chemistry of Penicillin.* Princeton University Press.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.
Newton, G. G. F. & Abraham, E. P. (1953). *Biochem. J.* **53**, 604.
Newton, G. G. F. & Abraham, E. P. (1954). *Biochem. J.* **58**, 103.
Newton, G. G. F. & Abraham, E. P. (1956). *Biochem. J.* **62**, 651.
Pavlic, A. A. & Adkins, H. (1946). *J. Amer. chem. Soc.* **68**, 1471.
Synge, R. L. M. (1951). *Biochem. J.* **49**, 642.
Woiwod, A. J. (1949). *J. gen. Microbiol.* **3**, 312.

The Separation of Substances and Estimation of their Relative Molecular Sizes by the use of Columns of Starch in Water

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Starch is frequently used as an inert support in partition chromatography. Departure from ideal behaviour has been interpreted on the basis of adsorption (Moore & Stein, 1948) or the effect of hydrated starch on solubility in the stationary phase (Martin, 1950). It has been assumed that starch grains are uniformly permeable to all solutes. The present study demonstrates that solutes vary greatly in their ability to penetrate hydrated starch grains, and that the extent of penetration is determined primarily by molecular size. Columns of starch in water form a new type of partition system, based on this molecular-sieve effect, and from these columns certain types of substances are eluted in order of decreasing molecular weight. They can be used for separation of mixtures, and under some circumstances the molecular dimensions of solutes may be determined. Some of these findings have been given in a preliminary communication (Lathe & Ruthven, 1955).

METHODS

Starches. Trials were made with potato, wheat, maize and rice starches, as supplied by British Drug Houses Ltd. Because of the ease of washing and the speed of flow, potato starch was used for most columns of unaltered starch.

Swollen maize starch. Because of the relative uniformity of its grains and since they do not burst on heating, maize starch was selected for the preparation of columns of swollen starch. The unwashed, undried maize starch (250 g.) was suspended in 2·5 l. of distilled water, with mechanical stirring which was continued throughout the preparation. The volume of starch was estimated by centrifuging 10 ml. of the mixture for 3 min. at 1250 *g*. The solution was warmed (by a thermomantle) from room temp. to 61° during 75 min., without an appreciable change in the starch volume. During the following 75 min. the temp. was raised to 68° and the procedure was stopped when the starch volume had increased to three times the original. The mixture was poured into 7 l. of distilled water, allowed to stand for 24 hr., and the slightly turbid supernatant was removed. The mixture was kept at 4°, and, in the presence of preservative, did not appear to deteriorate over several months.

A few experiments have been conducted with starch swollen by standing overnight at 4° in the presence of KSCN. 12·9% KSCN resulted in an increase of 0·15 vol., 14·1% KSCN 1·3 vol., and 14·8% KSCN 4·5 vol. The second of these preparations behaved in several experiments much like starch swollen threefold by heat. Although swelling the starch with salt is easy to carry out and to control, the repeated washing to remove the salt is laborious.

Preservative. Early columns had to be discarded in a few days because of gas formation, presumably owing to bacteria. This was avoided in later columns by addition of $CHCl_3$ (about 0·6%) to the water or buffer. Saturation with thymol was also satisfactory for preservation, but it interfered with the anthrone reaction.

Preparation of columns. Untreated potato-starch columns of 20 or 75 g. were set up as follows. The commercial, undried starch (20 g.) was suspended by hand in 100 ml. of water or buffer. After standing for 10 min. the supernatant was drawn off. This was repeated twice more, and the final volume made to about 100 ml. The slurry was delivered in approximately 10 ml. amounts into a 16 mm. (internal diameter) chromatography tube the lower end of which was narrowed and filled with a small cotton plug. Each sample was allowed to stand until it had almost completely settled (5–10 min.) and the supernatant was

sucked off. After all the starch had been added, the column was allowed to stand overnight. Approximately 10 ml. of fluid was then run through the column, which now had a height of 15–16 cm. The material (0·5–5 mg.) to be studied was dissolved in 0·5 ml. of water or buffer and placed on top of the column and was allowed to run into the starch. It was eluted with more water or buffer at the rate of 4 ml./hr.

Columns of swollen starch were prepared from the swollen maize-starch mixture. The starch suspension was remixed to uniformity, and a 65 ml. portion (or sufficient to yield 25–30 ml. of packed starch) was centrifuged for 2 min. at 1500 *g*. The supernatant was poured off. The starch was washed three times with 35 ml. portions of buffer by suspension and centrifuging. Finally a homogeneous slurry was prepared by adding gradually, with stirring, about 30 ml. of buffer. This was pipetted into a glass column of 16 mm. diameter and 70 cm. length; the tip of the pipette was raised during this process so that it was a few mm. above the liquid surface. The sides of the column were washed down with more buffer, which was then allowed to flow through the column overnight or until the height of the column remained constant (about 16 cm.). After addition of the solute in 0·5 ml. of buffer the columns were eluted at about 3 ml./hr.

It was not possible to set up swollen starch columns with constant weights of starch. As a result of this, as well as other factors, there was some variation in the size and behaviour of different columns and a means of comparing different columns was necessary. It was found satifactory to 'calibrate' swollen starch columns on the basis of the elution volume (defined below) of haemoglobin, which was 15 ml. for a 35 ml. column. The elution volumes of other substances reported in this paper have been adjusted proportionately on this basis, except where haemoglobin was not run. In these cases the elution volume has been adjusted on the basis of the volume of the column. After adjustment the error between replicate determinations was less than 0·5 ml. except for smaller molecules. For instance, the elution volumes of cyanocobalamin on six columns of threefold swollen starch, after adjustment to a haemoglobin elution volume of 15 ml., were 28·0, 29·2, 26·7, 26·4, 26·5, 26·7 and 27·3 ml. The error is of a higher order here, but the columns are unsuitable for separating molecules of this size.

Cellulose columns were prepared from 10 g. of Whatman Cellulose Powder, Standard Grade (containing 4·4% w/v water) by a method similar to the one used for unheated starch.

The elution volume is the total volume of liquid which flows from the column between the addition of the solute and the emergence of the maximum concentration of solute.

Determination of 'column water'. The water content of commercial potato starch (about 20% w/v) was determined accurately for each lot by drying a sample to constant weight in a vacuum desiccator over H_2SO_4. The total amount of water held by a column was determined by weighing a loaded column and by subtracting the calculated dry weight of the starch and the weight of the empty column.

Buffers. Phosphate buffer (0·05M) was 0·89% (w/v) $Na_2HPO_4,2H_2O$. Borate and acetate buffers were prepared according to Cole (1933).

Determination of substances. Glucose, streptomycin, sucrose, inulin and glucosamine were estimated by a copper-reduction method (King, 1951). Sucrose and inulin were first hydrolysed with 0·1N-HCl for 10 min. at 100°. Raffinose was estimated by determination of the galactose (King, 1951) present after yeast fermentation of an acid hydrolysate (0·1N-HCl at 100° for 10 min.). Other sugars and polysaccharides were determined by the anthrone method as modified by Loewus (1952), scaled down to 2·25 ml., and carried out in Wasserman tubes. The urea–resorcinol method of Preedy (1954), with the omission of protein precipitation, was also used for inulin when the anthrone method gave excessive blank values.

Haemoglobin was estimated by the extinction at 578 mμ., cyanocobalamin at 362 mμ., bacitracin A at 254 mμ., cytochrome *c* at 520 mμ. and myoglobin at 580 mμ. The extinction at 280 mμ. served for colourless proteins, Borsook's peptide A, benzoic acid, aniline, chloramphenicol and thiamine. Amino acids were determined qualitatively by spotting on filter papers which were stained by dipping in a 0·25% ninhydrin solution in acetone, and were allowed to dry at room temp. Paper chromatography with *n*-butanol–acetic acid–water (40:10:50, by vol.), according to Partridge (1948), distinguished the amino acids, and a solvent mixture of *n*-propanol–ethyl acetate–water (6:1:3, by vol.) was used to characterize sugars (Buchan & Savage, 1952). Urea was determined by nesslerization (King, 1951), and carbonic anhydrase according to Richter & Hullin (1951).

Materials. Crystalline cyanocobalamin was a gift from Dr E. Lester Smith, of Glaxo Laboratories Ltd. Maltotriose, -tetraose, -pentaose, α- and β-Schardinger dextrins (*cyclo*malto-hexaose and -heptaose), soluble laminarin, amylose and amylopectin were provided by Dr W. J. Whelan. Bacitracin A was provided by Dr E. P. Abraham, and polymyxin B sulphate by Dr S. Wilkinson of The Wellcome Research Laboratories. Crude goat β-lactoglobulin was given by Dr B. A. Askonas, and was purified by precipitation at a concentration of $(NH_4)_2SO_4$ between 62 and 72%. Crystalline insulin (Boots), protamine (Light), plasma albumin (Armour), pig pepsin (Armour), ox trypsin (Armour), inulin (Gurr), and most amino acids and peptides were obtained commercially. Haemoglobin was prepared by lysing washed adult red blood cells in distilled water. Cytochrome *c* was prepared from horse heart by the method of Keilin & Hartree (1945). Peptide A of Borsook, Deasy, Haagen-Smit, Keighley & Lowy (1949) was prepared from Witte's peptone. Plasma globulin was prepared from human serum by precipitation with 50 vol. of 42% (w/v) $Na_2SO_3,7H_2O$. Human red blood cells, lysed in 1–2 vol. of water, provided carbonic anhydrase. Myoglobin was extracted from ox-skeletal muscle by an adaptation of the method of Keilin & Schmid (1948).

Molecular weights. These have been calculated for substances of known composition. The values for all other substances have not been determined, but are taken from the current literature.

RESULTS

Composition of potato-starch column

There were minor variations in the water content of potato starch, and in the dimensions of columns. The following is a typical example. A column of

20 g. of potato starch initially containing 19% (w/w) water was set up in water, and after contraction it occupied 25·8 ml., and weighed 32·0 g. Thus for every 10 g. of undried starch in the column there was 7·9 ml. of water. Colloidal carbon (indian ink) was eluted at 4·1 ml. (range 4–4·25 ml.)/10 g. of starch. Under the microscope it was seen that carbon particles did not penetrate starch granules. Thus the elution volume of carbon measured the mobile phase outside the grains. Accordingly, the mobile phase was 4·1 ml., the hydrated grains occupied 8·8 ml. and contained 3·8 ml. of water.

Behaviour of uncharged molecules on untreated starch

A number of uncharged substances, chiefly sugars, were eluted from 75 g. potato-starch columns, in the positions shown in Fig. 1. These are shown in a standardized way, all 'peaks' being recorded at the same height, in order to avoid differences due to the variety of methods of determination, and the varying amounts of material used. Urea, which is not shown in the figure, was eluted at 73·5 ml. Of three monosaccharides, galactose and glucose were eluted together at 49 ml., and fructose was slightly retarded (51 ml.). One curve is given for three disaccharides, since sucrose, maltose and lactose were not separated. Higher sugars of the maltose series showed some unusual behaviour with regard to their positions in a few experiments and, with the exception of maltotriose, there was marked skewing of the curves. They require further study, but are included for completeness. Maltotriose was eluted after raffinose (41 and 37 ml. respectively). Maltotetraose (pH 8·6 in phosphate) spread widely over the region of tri- and di-saccharides (37–49 ml.). Maltopentaose (pH 8·6 in phosphate) had a peak at 37 ml., but trailed badly through the disaccharides region.

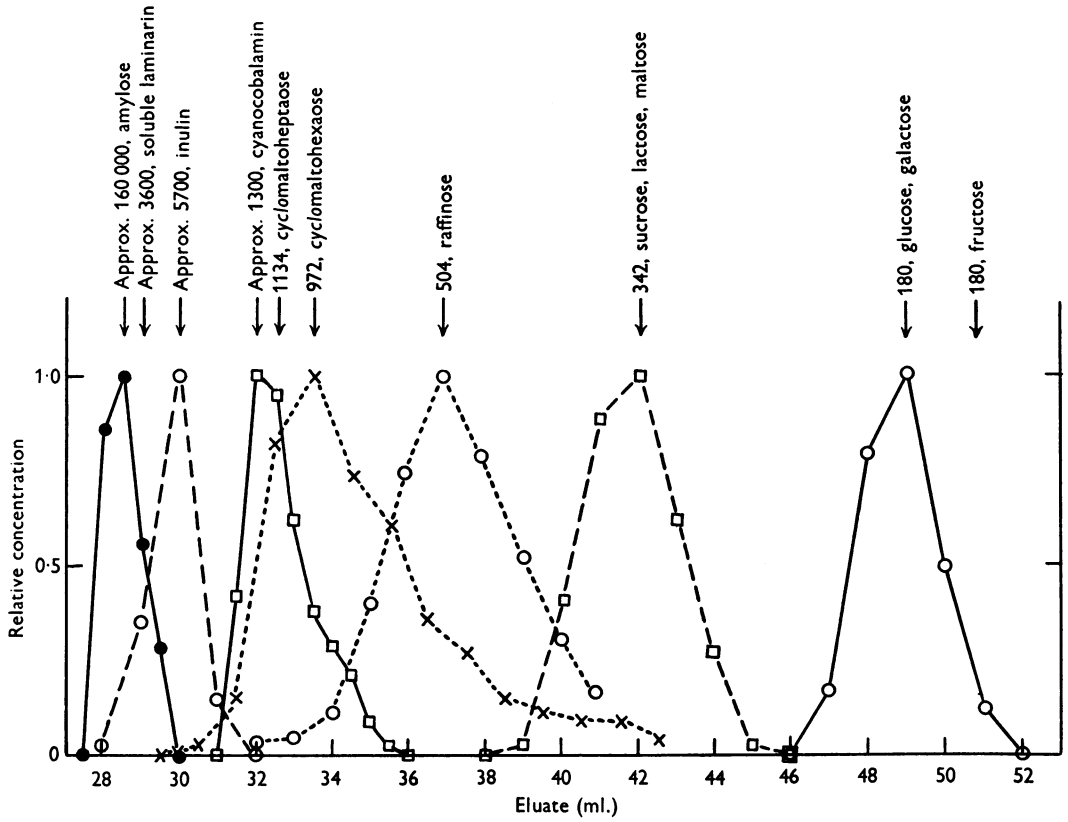

Fig. 1. Elution of neutral molecules in order of descending molecular weights, from columns of 75 g. of untreated potato starch in water. The points of maximum concentration have been adjusted to an arbitrary height. ●——●, Amylose; O— — —O, inulin; □——□, cyanocobalamin; ×- - - - -×, *cyclo*maltohexaose; O- - - -O, raffinose; □— — —□, sucrose; O——O, glucose. The positions of peaks of soluble laminarin, *cyclo*maltoheptaose and fructose are also shown.

Table 1. *Volume of elution of small charged molecules from columns of 20 g. of potato starch in various solutions*

Four uncharged molecules are included as markers. Elution volume is defined in the Methods section.

	Mol. wt.	Elution volume (ml.) pH 8·5, 0·05 M phosphate	pH 8·5, 0·05 M phosphate, 0·25 M-NaCl	pH 10·0, 0·05 M borate	pH 2·2, 0·01 N-HCl	pH 2·2, 0·01 N-HCl, 0·25 M-NaCl
Inulin	5700	9·0	9·0	—	8·0	8·75
Sucrose	342	11·5	12·0	—	12·5	12·0
Glucose	180	14·0	14·0	—	13·0	14·0
Urea	60	19·0	19·0	—	—	—
Galacturonic acid	196	13·0	13·3	—	15·5	14·3
Glucosamine	179	17·0	15·5	—	>22·0	14·8
Benzoic acid	122	18·0	19·3	—	>31·0	>31·0
Aniline	93	28·0	29·0	—	>30·0	17·5
Chloramphenicol	323	25·0	24·0	—	24·0	24·0
Streptomycin	582	>13·0	17·5	—	>16·0	12·0
Thiamine	337	20·0	17·5	—	—	—
Riboflavin	376	>25·0	>25·0	—	—	—
Oxidized glutathione	612	10·5*	—	—	—	—
Reduced glutathione	307	11·0†	—	—	17·0‡	—
Glycylleucine	188	11·5	—	—	—	—
Diglycylglycine	189	12·0	—	—	—	—
Leucylglycine	188	12·5	—	—	—	—
Glutamic acid	163	12·0	13·0	—	25·5	15·5
Aspartic acid	133	13·0	13·5	—	24·0	15·5
Leucine	131	13·5	13·5	—	—	—
Proline	115	13·5	—	—	—	—
Alanine	89	14·0	14·5	—	25·0	14·5
Valine	117	14·7	—	—	—	—
Norleucine	131	15·2	—	—	—	—
Glycine	75	15·5	15·5	—	28·0	16·5
Threonine	119	15·5	—	—	—	—
Serine	105	15·5	—	—	—	—
Cystine	240	16·0	—	—	—	—
Methionine	149	16·5	—	—	—	—
Lysine	146	17·5	15·5	13·5	29·0	—
Folic acid	441	17·5	—	—	—	—
Histidylhistidine	293	18·0	19·0	—	—	—
Histidine	155	18·5	17·0	15·0	—	—
Tyrosine	181	20·5	21·5	—	—	—
Arginine	174	23·5	19·5	20·0	>31·0	18·5
Tryptophan	204	29·0	33·5	—	—	—

* 0·05 M Borate, pH 8·5.
† 0·05 M Borate, pH 8·5, 0·06 M thioglycollate.
‡ Saturated benzoic acid, pH 3·0.

Table 2. *Behaviour of large charged molecules on columns of 20 g. of potato starch in phosphate buffer*, pH 8·5

Sodium chloride when present was 0·25 M.

Protein	Mol.wt.	Elution volume (ml.) Salt present	Salt absent
Haemoglobin	67 000	8·5	8·5
Cytochrome *c*	13 000	8·5	8·8
Insulin*	12 000?	8·5	>19
Peptide A†	?	9·0	9·0
Protamine	3 000	10·0	>29
Polymyxin B	Approx. 1 200	13	>29

* In 0·01 N-HCl.
† Borsook *et al.* (1949).

Behaviour of charged molecules on untreated starch

Preliminary trials of acids and bases of low molecular weight indicated that factors other than molecular weight affected their movement on starch columns. The elution volume of charged molecules, unlike sugars, was altered by change of hydrogen-ion concentration. The effect of variations of pH, with and without additional sodium chloride, were examined for two acid–base pairs (glucosamine–galacturonic acid; aniline–benzoic acid), some naturally occurring acid and basic substances, and a series of amino acids and di- and tri-peptides, on 20 g. starch columns (Table 1). Retardation of charged molecules appeared to be

minimal at pH 8·5 in the presence of salt. The effect of negative charge was examined in 0·05M phosphate buffer at pH 7·0. Glucuronic acid was eluted at 12 ml., galacturonic acid at 12·5 ml., glucose at 14 ml., and glucuronic acid lactone at 15 ml. A few large charged substances were run on columns of 20 g. of untreated potato starch at pH 8·5 in phosphate buffer with 0·25M-NaCl (Table 2). When compared with the 75 g. columns of Fig. 1 the elution volumes of the neutral markers, inulin, sucrose, glucose and urea from 20 g. columns were found to be approximately 1 ml. greater than anticipated. This difference is probably due to closer packing of the starch on the large columns.

Swollen maize starch

To increase the extent to which larger molecules penetrated the starch grains they were swollen by heating in water. Maize starch, granules of which do not burst on heating, were treated until the volume was three times the original. A typical 'standard' column of threefold swollen maize starch, prepared as described in Methods, occupied 35 ml. The mobile phase (measured with colloidal carbon) occupied 11·5 ml., and the volume of stationary hydrated-starch phase was 23·5 ml. The stationary phase contained 5·7 g. of dry starch, and had the following composition: 24·3% (w/v) starch and 93% (w/v) water.

Behaviour of large molecules on swollen starch columns

The positions in which a number of large neutral and charged molecules were eluted from standard columns of this type at pH 8·5 in 0·025M borate buffer containing 0·025M-KCl are shown in Fig. 2. Cytochrome *c*, protamine and polymyxin B could not be eluted from swollen maize-starch columns in this solvent. In view of the basic character of these substances the effect of salt concentration on the

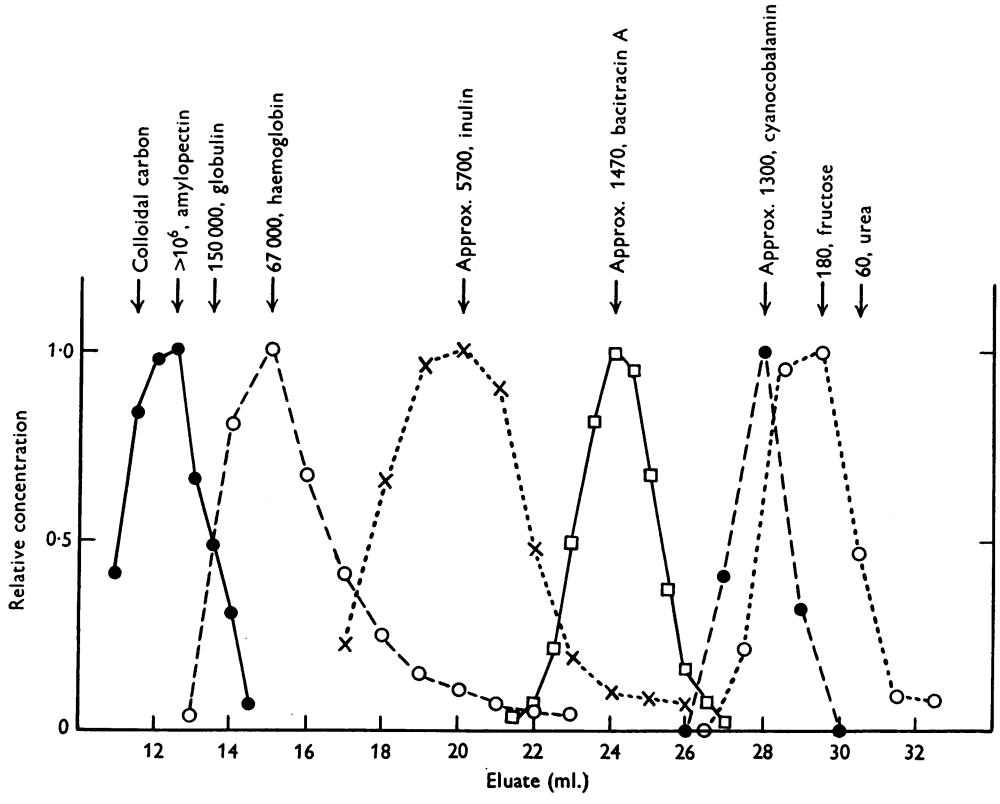

Fig. 2. Elution of large molecules in order of descending molecular weights, from standard columns of heat-swollen maize starch in 25 mM borate and 25 mM-KCl. The points of maximum concentration have been adjusted to an arbitrary height. ●——●, Amylopectin; ○— — —○, haemoglobin; ×- - - - -×, inulin; □——□, bacitracin A; ●— — —●, cyanocobalamin; ○- - - -○, fructose. The positions of peaks of globulin and urea are also shown.

rate of movement of cytochrome was examined (Table 3). As a result, whenever possible proteins were examined in 0·05M phosphate buffer at pH 8·5, containing 0·25M-NaCl. The results from a number of proteins of mol.wt. 150 000 to 12 000 are given in Table 4. Protamine could not be eluted from swollen starch in this solution, and polymyxin B was retarded. For reasons of solubility and stability insulin and trypsin were examined at pH 2·2 in presence of 0·25M-NaCl.

Recoveries

Low recoveries of some sugars were observed on a number of columns of unswollen starch, namely glucose 70 and 86%, raffinose 57%, maltohexaose 60%. Sucrose (114 and 106%), maltotriose (100%), inulin (97%), glucosamine (98%) and galacturonic acid (95–99%) were more satisfactory. Proteins were recovered 80–100% from both unswollen potato starch and swollen maize starch.

Table 3. *Elution volume of cytochrome* c *on standard columns of swollen starch in different solutions*

pH	Solution	Elution volume (ml.)
8·5	25 mM borate, 25 mM-KCl	Approx. 45
8·5	50 mM borate	Held
8·5	50 mM borate, 50 mM-KCl	25
8·5	50 mM borate, 50 mM-KCl, 0·25M-NaCl	18
8·5	12·5 mM phosphate	Held
8·5	25 mM phosphate	26
8·5	50 mM phosphate	18
8·5	50 mM phosphate, 0·25M-NaCl	18
6	Water	Held
6	25 mM-NaCl	18

Table 4. *Volume of elution of proteins from standard columns of threefold swollen maize starch*

Concentrations of buffers and salts were: phosphate, 50 mM; borate, 25 mM; acetate, 50 mM; HCl, 0·01N; KCl, 25 mM; NaCl, 0·25M.

Protein	Solution	Mol.wt.	Elution volume (ml.)
Globulin	pH 8·5, borate; KCl	150 000	14·2
Albumin	pH 8·5, phosphate; NaCl	65 000	14·5
Haemoglobin	pH 8·5, borate; KCl	67 000	15·0
	pH 8·5, phosphate; NaCl	—	15·0
Carbonic anhydrase	pH 8·5, phosphate; NaCl	30 000?	16·5
Lactoglobulin	pH 8·5, phosphate; NaCl	38 000	16·7
Myoglobin	pH 8·5, phosphate; NaCl	17 000?	17·1
Pepsin	pH 5·0, acetate; NaCl	35 000	17·3
Trypsin	pH 3·45, acetate; NaCl	15 000	18·5
	pH 2·2, HCl; NaCl	—	18·1
Cytochrome c	pH 3·45, acetate; NaCl	13 000	18·5
	pH 8·5, phosphate; NaCl	—	18·8
Insulin	pH 2·2, HCl; NaCl	12 000?	20·0
Inulin	pH 8·5, borate; KCl	Approx. 5 700	20·0
Polymyxin B	pH 3·5, acetate; NaCl	Approx. 1 200	30·0

Cellulose columns

Cellulose columns occupied 33 ml. per 10 g. of undried cellulose and contained 29·4 ml. of water. Haemoglobin was eluted at 26·0 ml., glucose and urea at 28·0 ml. Assuming that haemoglobin did not penetrate the cellulose fibres it may be calculated from its elution volume and from the R value of urea, relative to haemoglobin, that urea was distributed in a stationary phase of 1·96 ml. [$R=(M/S)/(1+M/S)$, where M and S are the volumes of mobile and stationary phases respectively].

Role of solubility partition

The possibility that these separations were due to a partition between water and hydrated starch, based on solubility factors (Martin, 1950), has been examined. The stationary phase of unswollen hydrated potato starch has the composition 92% (w/v) starch and 43% (w/v) water. As an approximation to this composition, 87% (w/v) sucrose was prepared, and the ratio of solubilities in water and in this solution were determined for some slow- and fast-moving solutes. In order to calculate the R values which would result from the partitions

indicated by the ratio of solubilities in water and 87% (w/v) sucrose solution, account must be taken of the relative volumes of the mobile and stationary phases. The volume of mobile phase is 4·1 ml./10 g. of starch, while that of stationary phase is intermediate between 3·8 ml. (the volume of water in the phase) and 8·8 ml. (the total volume occupied by the hydrated grains). R values have been calculated for assumed stationary phase volumes of 6·4 and 8·8 ml. and are compared with the observed R values in Table 5.

Smaller molecules, and in particular those of mol.wt. less than about 1000, are retarded to some extent on starch columns. It may also be demonstrated directly that to a varying degree they are removed from solutions in contact with hydrated starch. There are two possible explanations. First, all solutes may be regarded as remaining outside the starch granules, and retardation on the column (or removal from solution) may be attributed to adsorption to the grain surface. Secondly, the retardation on the columns may be due to a

Table 5. *Comparison of observed and theoretical* R *values*

Theoretical R values were calculated from the formula $R=[(M/S)\times\text{solubility quotient}]/\{1+[(M/S)\times\text{solubility quotient}]\}$, where M is the known volume of mobile phase, and S is an arbitrarily assumed volume of stationary phase, in ml., for a column containing 10 g. of undried starch.

	Solubility in water (w/v)	Solubility in 87% sucrose (w/v)	Solubility quotient (water/sucrose)	Calc. R value		Obs. R value
				$S=6·4$	$S=8·8$	
Cyanocobalamin	1·5	0·7	2·1	0·57	0·50	0·91
Raffinose	17	2	8·5	0·85	0·80	0·75
Sucrose	91	4	22·8	0·94	0·91	0·62
Glucose	67	10	6·7	0·81	0·76	0·57
Glycine	23	11	2·1	0·57	0·50	0·52
Urea	51	36	1·4	0·47	0·40	0·42

As an alternative method of investigating the role of solubility partition, we examined the effect on the R values of making the mobile phase more nearly of the same composition as the stationary phase. This was achieved by substituting concentrated sugar solutions for water in the preparation of the columns. There was no change in the R values of haemoglobin, cyanocobalamin and inulin on starch columns when 48% (w/v) glucose was used as mobile phase, and no change in R of cyanocobalamin, glycine and urea on columns in 87% (w/v) sucrose.

DISCUSSION

Starch grains are impermeable to particles of colloidal carbon, which were eluted from a column of 10 g. of potato starch in 4·1 ml. Since the column contained 7·9 ml. of water, it is concluded that during elution water flows about the starch grains, rather than through them. The fact that large molecules [amylose, laminarin, inulin (Fig. 1); haemoglobin, cytochrome (Table 2)] were eluted in a volume that was almost the same as that for carbon indicates that none of these large molecules penetrates untreated starch granules to an appreciable extent. This may also be demonstrated directly by adding a measured amount of solute of high mol.wt. (e.g. inulin) to known amounts of starch and water. The resulting concentration of solute in the water shows that for every 10 g. of starch there is approximately 4 ml. of water in which inulin does not distribute itself.

penetration of the starch granules, the smaller substances being distributed inside as well as outside the granules. The simultaneous occurrence of adsorption and penetration is, of course, not excluded.

Moore & Stein (1952) have stressed the adsorptive properties of cellulose and starch, and they support this view in part by the separation of alanine and glycine on columns of starch in water, without the aid of an immiscible solvent (Moore & Stein, 1948). Adsorption by ionic forces can be excluded as an explanation of the separation of uncharged molecules (Fig. 1), the movement of which is unaffected by changes of pH and salt concentration (Table 1). Hydrogen bonding might play a part. However, hydrogen bonding would be more important for large molecules than small ones, whereas it is the latter that are retarded on columns of starch in water. Thus it is improbable that the retardation of small uncharged molecules can be explained on the basis of adsorption to the granule surface by ionic or secondary linkages. For these molecules penetration of the granules seems more probable. This would have the effect of separating molecules which penetrate the granules from those which do not, the latter being eluted first.

In discussing the factors which may determine the distribution of solutes in partition systems, Martin (1950) suggested that under some circumstances cellulose (or starch) should not be regarded merely as a support for the stationary phase. 'The

stationary phase in a cellulose chromatogram should be compared with, say, a strong solution of glucose, or better some soluble polysaccharide, rather than with water saturated with the organic phase.' According to this argument, solutes could partition themselves on the basis of solubility factors, between water and the hydrated starch grains of starch and water columns. There are two types of evidence in the present study which are inconsistent with this theory.

The first concerns the relation between solubilities and R values. In three cases (Table 5) there is poor agreement between the observed R values and the R values calculated from the relative solubilities in water and in 87% (w/v) sucrose solution. In particular, cyanocobalamin and glycine, which should behave similarly according to solubility-partition theory, have observed R values of 0·91 and 0·52 respectively. Secondly, on the basis of solubility theory, all solutes should move with the same R value, about 0·4, if the ambient and stationary phases had the same composition. Attempts to change the observed R values in this direction, by increasing the carbohydrate content of the moving phase (to 87%, w/v, sucrose), were without effect. Thus solubility-partition theory does not explain the main aspects of starch and water columns, and especially the great differences in behaviour of large and small molecules. Solubility partition may play a part, however, in determining the relative movement of smaller molecules (Table 2). This theory could only be applied to molecules which can move unhindered between mobile and stationary phases.

Cyanocobalamin does not pass into the starch granules, although it is known to be soluble in a medium of similar composition (Table 3). It seems probable that it does not dissolve in the hydrated granules because it cannot penetrate their structure. The behaviour of other large molecules may be explained in the same way, and since substances of intermediate size (e.g. hexaose) separate from both the larger (amylose) and smaller (glucose) molecules it is clear that they must penetrate to an intermediate extent, and that there is no definite pore size which separates materials into those which are larger and those smaller, as considered by Lindqvist & Storgårds (1955). Rather, molecules must be considered as varying in the depth to which they penetrate the starch grains. From the fact that, with few exceptions, molecules are eluted in the descending order of their mol.wt., it is highly probable that the major factor determining the depth to which molecules penetrate the granules is molecular size. In this view substances of the same size penetrate to the same degree, and assuming no solubility-partition effects, they would be eluted in the same volume. This conception is supported by the similar behaviour of substances of identical mol.wt. The three disaccharides which were examined behaved identically (Fig. 1), and the three monosaccharides were almost the same (two at 49 ml., one at 51 ml.). Maltotriose behaved irregularly as compared with raffinose. Solubility factors may play some part in exceptional cases.

The behaviours of small charged molecules (Table 1) are more complex than those of neutral molecules, and the results do not support Lindqvist & Storgårds (1955) who considered that columns of starch in citrate buffer separated peptides and amino acids in the range of mol.wt. 75–500. It is clear from Table 1 that amino acids and peptides of this size vary in their behaviour, and that the mol.wt. is not the primary factor determining the order of elution. The effect of change of pH, and of salt concentration, suggests that the starch is acting as an ion exchanger. From the data of Table 1 it is evident that acidic substances are not greatly retarded in either acidic or alkaline medium. There is some evidence that the addition of a negative charge may slightly accelerate the elution of a molecule [compare: galacturonic acid (13·0 ml.) and glucose (14·0 ml.) at pH 8·5, Table 1; glucuronic acid (12·0 ml.), galacturonic acid (12·5 ml.), glucose (14·0 ml.) and glucuronolactone (15 ml.) in 0·05M phosphate buffer at pH 7·0]. Bases tend to be retarded (see glucose and glucosamine, also streptomycin, thiamine, riboflavin, Table 1) and this effect is greatest in an acidic medium and minimized by the addition of salt. Aromatic character may also produce retardation (see benzoic acid and aniline, tyrosine, tryptophan, folic acid) but, unlike that due to basic character, the aromatic retardation is not reversed by increasing the salt concentration.

Among the amino acids, the dicarboxylic acids move most rapidly, and after them are the 'normal' monocarboxylic mono-amino acids. Cystine, threonine, methionine and serine are all retarded a little, and after them the basic amino acids. The extent of separation of glycine and alanine is of the same order as Moore & Stein (1948) observed on a larger scale. Columns of starch in buffer solutions could be used for the separation of many amino acids, especially the aromatic and basic ones. Their capacity would probably not be high, perhaps only one-tenth of a conventional ion-exchange resin, if judged by the acid-binding capacity of starch (Radley, 1953).

On columns of untreated potato starch most proteins do not penetrate the starch grains and accordingly they move with the leading edge of the solvent (Table 2) when they are not adsorbed. The upper limit of usefulness of such columns is in the region of mol.wt. 1000. When the starch grains are

swollen they are made penetrable to larger molecules and considerable resolution can be obtained, though there is much overlapping among molecules larger than haemoglobin (mol.wt. 67 000). The separation of large molecules can be increased when starch which has been more extensively swollen is used, but the slowness of the columns is a disadvantage. On columns of starch swollen 4·5 times the distance between the peaks of albumin and globulin is increased from 1 ml. (on threefold-swollen starch) to 4·0 ml. The lower range in which threefold-swollen starch is useful appears to be about 1300.

The greater permeability of heat-treated starch is associated with an increased tendency for large bases to be adsorbed, probably because these are adsorbed only after they penetrate the grains. Thus protamin which is not retarded on unswollen starch in the presence of salt (Table 2) cannot be eluted from swollen starch in salt solution. Cytochrome *c* is markedly retarded on swollen starch though salt releases it from adsorption (Table 3). Polymyxin B, which is small enough to penetrate untreated starch to some extent, is considerably retarded by it (Table 2). These observations suggest that swollen starch may be a suitable material to use as an ion exchanger in the separation of basic proteins, and possibly other substances. The adsorption of most proteins appears to be negligible in the presence of 0·25 M sodium chloride. This concentration is ten times that required to free cytochrome *c* from swollen starch (Table 3).

Insulin was eluted in the same position as inulin, which has a mol.wt. of about 5700 (Hirst, McGilvray & Percival, 1950). This value is about half of the usually accepted figure (12 000; Edsall, 1953), although it is in agreement with the value which Harfenist & Craig (1952) obtained by countercurrent distribution of the dinitrophenyl derivatives of insulin.

Soluble laminarin (Fig. 2) behaved as if it were larger than inulin. Connell, Hirst & Percival (1950) estimated its mol.wt. at about 3600, but conceded the possibility of it being larger.

It is desirable that a larger number of proteins should be examined on starch columns to establish the limitations of this method for determining mol.wt. In particular, the effect of hydration of molecules, and of divergence from spherical shape, may alter the behaviour, the former, presumably, affecting a more rapid elution and the latter producing a retardation. These factors may account for some of the discrepancies which have been noted, e.g. carbonic anhydrase. Mol.wt. has been considered here merely as a convenient approximation of molecular size, for substances composed largely of carbon, oxygen and nitrogen.

For general use in the laboratory it would be valuable to have a number of 'markers' of known molecular size, shape and weight, for comparison with substances of unknown dimensions on starch columns. Various polysaccharides might serve this purpose, or possibly synthetic polymers. Should these become available, chromatography on starch may provide a simple method of determining the molecular size of a wide range of proteins and polysaccharides. The method has the advantage that measurements can be made in mixtures, provided that the substance under examination can be estimated by some specific method, such as enzyme activity.

Table 6. *Comparison of starch and cellulose columns*

The data have been calculated per 10 ml. of mobile phase, as indicated by colloidal carbon on starch columns, and haemoglobin on cellulose. The volume of stationary phase (S) for urea, in column 5, was calculated from the observed R value of urea (relative to carbon or haemoglobin) and the volume of mobile phase (M), using the formula $R=(M/S)/(1+M/S)$. It was assumed that the concentrations of urea in the mobile and stationary phases were equal.

Column	Wt. of dry support (g.)	Vol. of hydrated support (ml.)	Water in hydrated support (ml.)	Calc. stationary phase for urea (ml.)
Untreated potato starch	19·8	21·5	9·3	13·0
Swollen maize starch	4·95	20·4	19·0	15·6
Cellulose	3·7	2·7	1·31	0·75

Three proteins were eluted in volumes which were inconsistent with the reported mol.wt. Carbonic anhydrase appeared in a volume appropriate to a mol.wt. of about 40 000 rather than 30 000 (Edsall, 1953). Further study with larger columns is necessary, since the differences were not large.

The elution of ox myoglobin in a position between β-lactoglobulin (mol.wt. 38 000) and pepsin (35 000) suggested that its mol.wt. is double the usually accepted value of 17 000 (Edsall, 1953) for horse myoglobin.

Cellulose columns showed little capacity to separate substances of different mol.wt. Haemoglobin and urea were eluted almost together. The differences between the two types of starch

columns and the cellulose column are clearly seen when the weight of dry supporting material, the volumes of stationary hydrated support and the amount of the water in it are compared, a standard volume (10 ml.) of mobile phase being taken as a basis (Table 6). The volume of hydrated support in the starch columns was twice that of the mobile phase, but with cellulose the hydrated support was one-third of the mobile phase. The proportion of the hydrated support, and of the contained water, which was available for solution of urea was much smaller in cellulose columns than in starch columns.

Thus the capacity to separate materials on a column, by a partition based on varying degrees of penetration, appears to be unique for starch, and probably depends on the structure of the hydrated granules. A possible explanation, for which there is some microscopical evidence (Frey-Wyssling, 1953), is that the amylopectin chains radiate from the centre of the starch granule, and the distance between neighbouring chains is very small at the centre and increases toward the periphery. Solute molecules may penetrate only to the depth at which their diameters are equal to the distance between amylopectin chains. As a result the volume of stationary phase in which solutes become distributed is determined by their molecular size.

SUMMARY

1. From columns of potato starch in buffer neutral molecules were eluted in the descending order of their molecular weights. There was good resolution of substances in the molecular weight range of 100 to 1000.

2. The behaviour of small charged molecules was complicated by ion-exchange effects, which could be minimized by the addition of salt.

3. Large molecules remained outside untreated starch grains. Starch which has been swollen by warming in water was permeable to larger molecules, and in columns separated them in the range of molecular weights 150 000 to 1300.

4. The possibility of determining molecular weights with these columns is discussed. Values of 6000 for insulin and 35 000 for ox myoglobin were obtained.

5. It is suggested that columns of starch in water form a new type of partition system in which the volume of the stationary phase is determined for each substance by the depth to which it can penetrate the starch granules.

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REFERENCES

Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G. & Lowy, P. H. (1949). *J. biol. Chem.* **179**, 705.

Buchan, J. L. & Savage, R. I. (1952). *Analyst*, **77**, 401.

Cole, S. W. (1933). *Practical Physological Chemistry*, 9th ed. Cambridge: Heffer.

Connell, J. J., Hirst, E. L. & Percival, E. G. V. (1950). *J. chem. Soc.* p. 3494.

Edsall, J. T. (1953). In *The Proteins*, vol. 1B, chap. 7. By Neurath, H. & Bailey, K. New York: Academic Press.

Frey-Wyssling, A. (1953). *Submicroscopic Morphology of Protoplasm*, 2nd English ed. Amsterdam: Elsevier.

Harfenist, E. J. & Craig, L. C. (1952). *J. Amer. chem. Soc.* **74**, 3087.

Hirst, E. L., McGilvray, D. I. & Percival, E. G. V. (1950). *J. chem. Soc.* p. 1297.

Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 289.

Keilin, J. & Schmid, K. (1948). *Nature, Lond.*, **162**, 496.

King, E. J. (1951). *Micro-Analysis in Medical Biochemistry*, 2nd ed. London: Churchill.

Lathe, G. H. & Ruthven, C. R. J. (1955). *Biochem. J.* **60**, xxxiv.

Lindqvist, B. & Storgårds, T. (1955). *Nature, Lond.*, **175**, 511.

Loewus, F. A. (1952). *Analyt. Chem.* **24**, 219.

Martin, A. J. P. (1950). *Annu. Rev. Biochem.* **19**, 519.

Moore, S. & Stein, W. H. (1948). *Ann. N.Y. Acad. Sci.* **49**, 265.

Moore, S. & Stein, W. H. (1952). *Annu. Rev. Biochem.* **21**, 521.

Partridge, S. M. (1948). *Biochem. J.* **42**, 238.

Preedy, J. R. K. (1954). *J. biol. Chem.* **210**, 651.

Radley, J. A. (1953). *Starch and its Derivatives*, 3rd ed. London: Chapman and Hall.

Richter, D. & Hullin, R. P. (1951). *Biochem. J.* **48**, 406.