Hexosamine-containing Peptides in Spores of *Bacillus subtilis*, *B. megatherium* and *B. cereus*

By R. E. STRANGE AND JOAN F. POWELL
Microbiological Research Department, Porton, Wiltshire

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In a previous paper (Powell & Strange, 1953) the isolation of non-dialysable peptides containing hexosamine from exudates produced by germinating spores of *Bacillus subtilis* and *B. megatherium* was described. More precise chemical definition can now be given to these peptides. In the following report, a comparison is made between their constitution and that of the peptides present in extracts from disintegrated resting spores, and from vegetative cells. A strain of *B. cereus* isolated from air is also included in the investigation.

ORGANISMS AND METHODS

Preparation of germination exudates and cell extracts

The non-dialysable peptide produced by germinating spores of *B. megatherium* was studied in greatest detail. Well-washed resting spore suspensions of this organism were prepared and allowed to germinate spontaneously after heat activation as previously described. The peptide represented 10–15% of the solid exudate (Powell & Strange, 1953). Germination exudates from *B. subtilis* were obtained by incubating resting spores with L-alanine and glucose (Powell & Strange, 1953). Spores of a laboratory strain of *B. cereus* were obtained after growth for 48 hr. at 37° in potato-extract medium (Robinson, 1951), enriched with 1/10 vol. of casein hydrolysate and yeast extract (Gladstone & Fildes, 1940). The cultures were grown in shaken 1 l. flasks containing 200 ml. of medium. The spores of this strain of *B. cereus*, like *B. anthracis* (Hills, 1949), needed L-alanine, tyrosine, adenosine and glucose for germination. This requirement could be simplified by heating the resting suspension for 1 hr. at 60°, after which, adenosine and glucose were adequate for germination (Powell & Hunter, unpublished). Exudates were therefore prepared by incubating heat activated suspensions containing 2×10^9 spores/ml. in 1 mM adenosine and 5 mM glucose buffered with 30 mM phosphate pH 7.3. Germination exudates were filtered through sintered glass and freeze-dried before extraction and dialysis of the peptide fraction.

Vegetative cells of *B. megatherium* and of *B. cereus* were obtained by growth for 12 hr. on the sporulation media. They were washed 3 times with 0.9% NaCl and suspended in water.

Extracts from resting spores and from vegetative cells were obtained by disintegrating aqueous suspensions containing about 2×10^{10} cells/ml. in the Mickle (1948) tissue disintegrator at 4°, with Ballotini beads, size 12. The disintegrated suspensions were centrifuged at high speed, and the clear extracts freeze-dried.

Paper electrophoresis. The technique of Flynn & de Mayo (1951) was used in the buffer system described by Holt, Voigt & Gaede (1952).

Total nitrogen. This was determined by the micro-Kjeldahl method using the catalyst suggested by Chibnall, Rees & Williams (1943).

α -Amino nitrogen. This was estimated by the technique of Van Slyke, Dillon, MacFadyen & Hamilton (1941).

Paper chromatography. For the detection of amino acids and amino sugars, the technique of Consden, Gordon & Martin (1944) was used, followed by the method of Housewright & Thorne (1950) for quantitative determination of each amino acid.

Hexosamine. This was determined by the methods of Elson & Morgan (1933) with the modification suggested by Immers & Vasseur (1950), of Dische & Borenfreund (1950), and of Tracey (1952).

Acetyl. The *p*-toluenesulphonic acid method was used, hydrolysing at 110° for 18 hr. in sealed tubes, followed by steam distillation.

Carbohydrate. Total carbohydrate (excluding hexosamine) was determined by the method of Sørensen & Haugaard (1933).

Phosphorus, uronic acids and pentose were determined by the methods of King (1932), Dische (1947) and Meijbaum (1939).

Isolation of non-dialysable peptides from germination exudates and cell extracts

The freeze-dried germination exudate from *B. megatherium* which represented about 30% of the spore dry weight was extracted repeatedly with small volumes of water. A solution containing amino acids, peptide, small amounts of protein and calcium dipicolinate was thus obtained the relatively insoluble residue being practically pure calcium dipicolinate (Powell, 1953). The solution was dialysed at 4° for 48 hr., with continuous flow. Batches (1 g.) of germination exudate derived from 3.5×10^{12} spores yielded 100–150 mg. of non-dialysable solid consisting almost entirely of the hexosamine-containing peptide. On one occasion during dialysis in two separate sacs the peptide was almost completely lost (see below). Some loss into the diffusate occurred when the dialysis time was extended to 4–5 days. It seemed, therefore, that the molecules might be barely of sufficient size to be retained by cellophan and, assuming a straight chain configuration, that the molecular weight might lie in the region of 10000 (see Addendum). On treatment of the solution of non-dialysable material with 2.5% trichloroacetic acid (TCA), a protein fraction was precipitated constituting about 4% of the non-dialysable solids. It gave a positive biuret reaction, and paper chromatography after acid hydrolysis showed most of the

common amino acids to be present. Ultraviolet absorption measurements indicated the absence of nucleic acids.

The solid material extracted from disintegrated resting *B. megatherium* spores represented about 50%, and from vegetative cells 45% of the cell dry wt. Freeze-dried spore and vegetative cell extracts were extracted with water and dialysed at 4°. The non-diffusible material and diffusate were concentrated *in vacuo*, and the diffusate was freeze-dried. The non-diffusible material was treated with 1/10 vol. of 25% TCA and centrifuged after standing for 30 min. The precipitated protein was washed with 2.5% TCA, dissolved in 0.1M-NaHCO₃, dialysed and freeze-dried. The TCA solution and washings were combined and neutralized with solid NaHCO₃ then dialysed and freeze-dried. In these extracts, the TCA precipitate was found to be mainly nucleoprotein. The TCA-soluble fraction from resting spores contained a peptide very similar to that found in the germination exudate and constituting about 20% of the total extracted solids. From the acid-soluble fraction of *B. megatherium* vegetative cell extracts, a small amount of material was isolated, representing about 4% of the total extracted solids, and very different from the spore peptides in composition (see below).

Spore extracts and germination exudates from *B. cereus* and *B. subtilis* were treated in the same way, and TCA-soluble, non-dialysable fractions isolated. A 12 hr. culture of *B. cereus* which was already showing signs of sporulation, but contained no free spores was also examined.

Ethanol fractionation of non-dialysable peptides from Bacillus megatherium

The non-dialysable peptide fraction from germination exudates of *B. megatherium* could be partly precipitated from aqueous solution with 2 vol. of ethanol (Powell & Strange, 1953) but it was found, as is usual with this method of fractionation, that the results depended on the concentrations of peptide, ethanol and added electrolyte.

193 mg. of exudate peptide were dissolved in 5 ml. of water. Four drops of potassium acetate solution saturated at 20°, followed by 10 ml. (2 vol.) ethanol were added. A precipitate appeared, and the mixture was left to stand at 2° for 16 hr. The precipitate was centrifuged, dissolved in water, freed from salt by dialysis at 2°, and freeze-dried. A further 5 ml. of ethanol (3 vol.) was added to the ethanol-water mixture and the precipitate thus obtained, after standing, was treated as before. The material remaining in solution was freed from ethanol *in vacuo*, dialysed, and

freeze-dried. Three fractions were thus obtained, the total yield being 171.2 mg. The fractions precipitated by 2 and 3 vol. of ethanol represented 41.3 and 35.0% respectively of the total. The fraction remaining in solution accounted for 23.7%.

An aqueous solution of *B. megatherium* spore extract peptide of the same strength (4%, w/v) was similarly treated with ethanol. It was found that 45% of the preparation was precipitated by the addition of 1 vol., and a further 45% by 2 vol. of ethanol. The remaining 10% was recovered from solution.

RESULTS

Bacillus megatherium peptides

The unfractionated non-dialysable peptides isolated from spore extracts and germination exudates of *B. megatherium* were found to be practically identical in composition (Table 1). After acid hydrolysis, followed by paper chromatography (Consden *et al.* 1944) each was shown to contain glutamic acid, alanine, α -diaminopimelic acid, glucosamine, and an unidentified compound reacting with ninhydrin, the Elson & Morgan reagent (Partridge, 1948) and ammoniacal silver nitrate. These constituents were identified by comparing their positions and ninhydrin colour reactions with those of authentic compounds. Diaminopimelic acid was distinguished from cystine by its stability to hydrogen peroxide (Work, 1951) and its characteristic greyish purple colour with ninhydrin in chloroform-containing collidine (Woiwood, 1949). Eluted from a collidine chromatogram, it contained no phosphorus, and on paper ionophoresis (Wade & Morgan, 1954) it was distinguishable from ethanolamine phosphate. Using as solvent *tert*-butanol, 6N-HCl and water in the volume ratio 70:1:29, it was unequivocally separated on paper from both cystine and ethanolamine phosphate and identified with α -diaminopimelic acid.

It seems likely that the unidentified compound is a sugar amine. On two-dimensional chromatograms, using phenol-ammonia followed by collidine-lutidine, its position varied but was always different

Table 1. *Composition of non-dialysable peptides isolated from B. megatherium (g./100 g.)*

Source of peptide	Sulphated ash	Total nitrogen	α -Amino nitrogen	Glutamic acid	Alanine	α -Diaminopimelic acid
Germination exudate	5.8	7.37	2.91	5.3	9.0	5.1
Spore extract	7.0	7.35	2.75	5.1	8.1	5.6
Vegetative cell extract	—	7.7	—	*	—	—
Source of peptide	α -Amino nitrogen calculated from amino acid analyses	Hexosamine			Acetyl	Total carbohydrate as glucose
		Elson & Morgan	Dische & Borenfreund	Tracey		
Germination exude	2.72	40.3	42.3	56.2	10.9	1.24
Spore extract	2.69	43.2	43.5	57.2	12.0	5.0
Vegetative cell extract	—	6.58	—	—	—	16.3

* Chromatograms showed the presence of most of the common amino acids.

from that of glucosamine and chondrosamine. Using *tert*-butanol-HCl as solvent (Smith & Markham, 1950) batches of 30 mg. of hydrolysed peptide were chromatographed on Whatman no. 3 paper, and the unknown substance was eluted. On steam distillation in phosphate-borate buffer (Tracey, 1952) it yielded ammonia. It gave a reaction with Ehrlich reagent only after previous treatment with alkaline acetyl acetone. The resulting pigment showed maximum absorption between 510 and 520 $m\mu$., the corresponding glucosamine derivative absorbing most strongly at 530–535 $m\mu$. Although, at first, the colour given by the unknown closely resembled that given by glucosamine, it was less stable, and changed slowly to a salmon tint. At pH 9.5, the change of colour intensity produced with increasing concentrations of acetyl acetone (Immers & Vasseur, 1950) was very similar to that observed in the case of glucosamine (Fig. 1). The unknown substance also reacted with indole after deamination with nitrous acid (Dische & Borenfreund, 1950) to give a colour similar to that given by glucosamine but slightly more pink. The ratio of light absorption at 492 and 520 $m\mu$. was approximately the same as with the glucosamine derivative. To simplify the presentation of data, the compound will be referred to as a hexosamine. A diagram of a typical two-dimensional chromatogram from hydrolysed peptide is shown in Fig. 2. The peptide had been freed from protein by TCA precipitation, which accounts for the absence of other amino acids found in the impure preparations of exudate peptide previously described (Powell & Strange, 1953).

The sugar amines were present as their acetyl derivatives and consisted mainly of glucosamine, together with smaller amounts of the unidentified amine sugar (see above). Hydrolysis of the peptides at several acid concentrations for varying periods followed by paper chromatography suggested that hydrolysis was complete after heating at 108° with 6N-HCl for 20 hr., but incomplete at lower acid concentrations and that in concentrated HCl, some destruction of the liberated hexosamine occurred. These indications were well supported by analytical results. Hydrolysis for 20 hr. at 108° with 0.5, 1, 3, 6 and 8N-HCl liberated respectively 24.4, 26.6, 33.1, 42.6 and 40.6 % of hexosamine. Hydrolysis with 6N-HCl for 20 hr. was therefore adopted as standard procedure, although it seemed likely that even at this acid concentration, some decomposition of hexosamine occurred. Thus, glucosamine determinations on a standard solution of the hydrochloride using both the Elson & Morgan and the Van Slyke amino-nitrogen methods indicated a 11 % destruction after heating for 20 hr. with 6N-HCl. The presence of the unidentified amino sugar giving a pigment with a different absorption maximum from that given by glucosamine in the Elson &

Morgan reaction also reduced the accuracy of hexosamine determinations. Measurements were made of the colour with Ehrlich reagent at 520 and at 530 $m\mu$. At 520 $m\mu$., the values for hexosamine concentrations agreed well with those obtained by the Dische & Borenfreund method, while at 530 $m\mu$. lower values were obtained, although this wavelength is nearer to the region of maximum ab-

Fig. 1. The change of colour intensity with increasing concentrations of acetyl acetone in the Elson & Morgan reaction with A 40 μ g. glucosamine and B unknown compound (suspected hexosamine).

Fig. 2. Diagram of two-dimensional chromatogram of hydrolysed non-dialysable peptide from *B. megatherium* spores, developed with ninhydrin. 1, α -diaminopimelic acid; 2, glutamic acid; 3, alanine; 4, glucosamine; 5, unidentified amine sugar.

sorption for glucosamine. It is therefore clear that the figures presented for hexosamine content of the peptides can be only approximate. Using the Tracey alkaline distillation technique, considerably higher values for hexosamine content were obtained. A typical set of results is given in Table 1. It may be significant that if the value for hexosamine nitrogen obtained by the Tracey method is added to that for α -amino nitrogen, all the nitrogen of the peptide is accounted for. If, on the other hand, the value obtained by the Elson & Morgan method is taken, there remains about 15% of the nitrogen unaccounted for.

The three amino acids present were estimated and found to occur in the ratio alanine (3), α -diaminopimelic acid (1) and glutamic acid (1) to 8 of acetyl hexosamine.

The peptides from *B. megatherium* spores contained 0.03% of phosphorus and 0.2% of pentose. No uronic acid, purines or pyrimidines could be detected. The ash content, determined as sulphate, was 5–7% and consisted mainly of calcium.

The non-dialysable, TCA-soluble extract from vegetative *B. megatherium* cells contained only 6.58% of hexosamine (Table 1). Hydrolysis followed by paper chromatography showed that most of the common amino acids were present. The material was obviously different from the spore peptides and was obtained in very much smaller yield.

The peptides isolated from resting spores and from germination exudates were examined by paper electrophoresis (Flynn & de Mayo, 1951) with sodium acetate, acetic acid, sodium barbiturate buffer pH 8.5 (Holt *et al.* 1952). Both types of material appeared to contain a single component reacting weakly with naphthalene black and occupying corresponding positions on the paper. When a preparation of the exudate peptide which had not been treated with TCA was examined, the small protein fraction appeared as a very slow moving, strongly staining component well separated from the weakly staining peptide.

Analysis of ethanol-fractionated peptides from *B. megatherium* spores showed that, within the limits of the techniques used, the fractions from exudate peptides were identical. Each contained about 1% carbohydrate and we have not determined whether this is a constituent of the peptide

molecule or an impurity derived from cell polysaccharides. The unfractionated extract peptide contained 5% carbohydrate. The carbohydrate content of the material precipitated by 1 and 2 vol. of ethanol was 5.4 and 4.2% respectively. The fraction remaining in solution contained 1.7%. All three fractions had very similar nitrogen and hexosamine contents, i.e. $7.0 \pm 2\%$, and $40.7 \pm 2\%$.

Viscosity measurements on aqueous solutions of the *B. megatherium* exudate and extract peptides gave interesting results. A microviscometer of the usual type was used giving a flow time of 141 sec. with water. In 1.33% (w/v) solution, the unfractionated exudate peptide gave a flow time of 190 sec., the fractions precipitated by 2 and 3 vol. of ethanol 204 and 192 sec., and the ethanol-soluble fraction 164 sec. The results on the extract peptide was less complete due to shortage of material. Here, 0.82% (w/v) solutions of the fractions precipitated by 1 and 2 vol. of ethanol gave flow times of 325 and 211 sec.

Bacillus cereus and *Bacillus subtilis* peptides

Very similar results were obtained with *B. cereus* and *B. subtilis*. Paper chromatograms on hydrolysates showed that the spore peptides isolated all contained alanine, α -diaminopimelic acid, glutamic acid, glucosamine and the same unidentified compound suspected to be a sugar amine. Although the hexosamine content varied between 36 and 48% in these preparations, their general composition was remarkably similar. Analyses are summarized in Table 2. The reducing power of the hydrolysate is derived from the hexosamine liberated by 6N-HCl. Under these conditions, sugars are decomposed: hence the low value for reducing power of the *B. cereus* vegetative cell extract which actually contained a high proportion of polysaccharide. The reducing power is expressed in terms of glucose content. It may be significant that these values agree with the sugar amine content expressed as hexosamine, thus supporting though not confirming the suggestion that the unidentified compound is a hexosamine. No systematic study was made on the effect of acid concentration on liberation of hexosamine, but in a single experiment with *B. subtilis* spore extract peptide, 24.0% hexosamine was liberated during 20 hr. heating with 0.5N-HCl and 37.7% with 6N-HCl.

Table 2. *Composition of non-dialysable peptides isolated from B. subtilis and B. cereus (g./100 g.)*

Source of material	Sulphated ash	Total nitrogen	α -Amino nitrogen	Hexosamine (Elson & Morgan)	Acetyl	Reducing power of hydrolysate (as glucose)	Total carbohydrate
<i>B. subtilis</i> spore extract	6.8	7.3	3.1	38.0	9.9	36.1	5.7
<i>B. subtilis</i> spore exudate	8.7	6.6	2.6	37.0	10.4	37.5	4.4
<i>B. cereus</i> spore extract	3.2	7.3	2.5	48.2	10.1	51.7	2.1
<i>B. cereus</i> spore exudate	2.7	7.3	2.9	42.5	9.9	50.0	4.7
<i>B. cereus</i> vegetative extract	3.1	4.9	3.2	8.9	3.9	8.9	51.6

6.2

The *B. cereus* spore peptides appeared also to contain traces of other amino acids. These were reduced, but not entirely eliminated by treating the preparations with chloroform and amyl alcohol, a method used by Sevag (1934) to remove small amounts of protein. All the peptides isolated contained calcium. It is impossible to determine, at this stage, whether this is a constituent of the molecule or a contaminant. The non-dialysable peptide fraction from vegetative cell extracts of *B. cereus* contained most of the common amino acids, less than 10% of hexosamine, and 52% of polysaccharide determined as glucose (Sørensen & Haugaard, 1933). It is interesting that the vegetative culture used was already in the first stages of sporulation, most of the cells showing a round, slightly swollen, non-staining area.

Paper electrophoresis on the peptides from *B. subtilis* spores showed the presence of a single component reacting with naphthalene black. This occupied the same position on the paper as the corresponding derivatives from *B. megatherium* spore peptides. The material isolated from *B. cereus* spore extracts and exudates appeared to contain two components, one identical with that just described, and the other travelling much more slowly. When 10 mg. of peptide was separated into two fractions in this way, and then eluted from the paper, the yield of the slower moving fraction was 1.3 mg. and of the faster component 4.7 mg. The hexosamine content of the two fractions was 31.7 and 35.4% respectively, and each contained glutamic acid, alanine, α -diaminopimelic acid, glucosamine and the unidentified sugar amine.

DISCUSSION

Hexosamine-containing peptides of very similar and characteristic constitution have been isolated from extracts of resting spores and from germination exudates of *B. subtilis*, *B. megatherium*, and *B. cereus*. Peptides of this type were absent or possibly present in only very small amounts in extracts from a young vegetative culture of *B. megatherium* and from vegetative cells of *B. cereus* which were in the first stages of sporulation. It therefore appears that the peptides may be built up at a comparatively late stage of the sporulation process.

All the spore peptide preparations contained alanine, glutamic acid, α -diaminopimelic acid and the acetyl derivatives of glucosamine and of another suspected but unidentified hexosamine. The *B. cereus* spore peptides contained small amounts of the other common amino acids which may have been derived from protein or from other types of non-dialysable peptide. Paper electrophoresis indicated the presence of one component in the peptides from *B. megatherium* and *B. subtilis*, and of two com-

ponents of very similar hexosamine and amino acid content in the peptides from *B. cereus*. Ethanol fractionation of the *B. megatherium* peptides gave products of the same chemical composition, although in the case of the extract peptide, the carbohydrate content of the fraction soluble in 2 vol. of ethanol, was considerably lower than that of the precipitated fraction. It seems possible that the peptide preparations contain molecules of different chain lengths and that during germination some depolymerization of the peptide may occur. This view is supported by the viscosity measurements. In the experiment mentioned early in this paper where there was an almost complete loss of exudate peptide through the dialysis sacs, a greater degree of depolymerization than usual may have occurred.

Hydrolysis of *B. megatherium* exudate peptide showed that approximately two-thirds of the sugar amine molecules were liberated by 0.5N-HCl, while the remaining one-third were much more tightly bound. Since the peptide contained an insignificant amount of phosphorus, this finding does not support the suggestion of Bendich & Chargaff (1946) that ease of liberation of amine sugars is associated with phosphorus-free, and difficulty with phosphorus-containing polymers.

We are unable to attach any biochemical significance to the spore peptides beyond the suggestion previously made (Powell & Strange, 1953) that they may be derived from the spore surface when the permeability of this structure is modified during germination, or damaged mechanically.

SUMMARY

1. Non-dialysable peptides of very similar constitution have been isolated from testing spore extracts and from germination exudates of *B. megatherium*, *B. subtilis* and *B. cereus*. These peptides are not present in appreciable quantities in extracts from vegetative cells.

2. The non-dialysable spore peptides contained α -diaminopimelic acid, glutamic acid, alanine, and the acetyl derivatives of glucosamine and an unidentified sugar amine.

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ADDENDUM

Physico-Chemical Properties and Molecular Weight of Spore Peptides from *Bacillus megatherium*

BY B. R. RECORD AND K. H. GRINSTEAD
Microbiological Research Department, Porton, Wiltshire

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Specimens of the exudate peptide and of the extract peptide prepared from *Bacillus megatherium* according to the above procedure have been examined in the electrophoresis apparatus and the ultracentrifuge. The diffusion constant and partial specific volume have also been determined and these have been combined with the sedimentation constant to give a figure for the molecular weight of the peptide. The questions of homogeneity, monodispersity and molecular shape are also briefly considered.

METHODS

Electrophoresis. This was carried out at 0° in the medium size (10 ml.) U-tube of the Tiselius electrophoresis apparatus and observations were made with the diagonal Schlieren optical system. The solutions were made up to 1% from the freeze-dried sample and were dialysed for 2 days against the buffer used.

Ultracentrifuge. Observations were made in the Spinco analytical ultracentrifuge fitted with the diagonal Schlieren optical system. Solutions were made up directly by dissolving a weighed amount of the freeze-dried material in the buffer used. The rotor speed was 59 780 rev./min. in all experiments and exposures were taken every 64 min.

Diffusion. The diffusion of the samples was followed in the electrophoresis apparatus using the standard 10 ml. U-tube. The solutions, approx. 1%, were dialysed for 2 days against the buffer used. The measurements were made at 0°.

Buffer. All the work was carried out in a sodium acetate (0.1M), NaCl (0.1M) buffer adjusted to pH 4.5 with acetic acid.

RESULTS

Spore exudate peptide

The specimen examined consisted of the 2.5% trichloroacetic acid soluble fraction, which had been dialysed and freeze-dried but had not been subjected to any ethanol fractionation.

Electrophoresis. A current of 9 mA was passed for 4 hr. through a 1.2% solution. The schlieren pattern showed only a single component, migrating anodically. The small stationary boundary clearly evident on the anode limb but hardly perceptible on the cathode side was assumed to be due to the usual buffer gradient. The mobility calculated from the movement of the cathode (descending) boundary was 0.38×10^{-4} cm.²/sec./v.

Sedimentation. The boundary in the ultracentrifuge also showed the presence of only one component having a low rate of sedimentation of about 1 Svedberg. Fig. 1 shows the pattern obtained with a 2% solution. As seen from the results in Table 1 (all the S_{20} values have been reduced to sedimentation in water and are given in Svedberg units) the sedimentation constant is somewhat concentration dependent and the relationship appears to be linear over the range examined.

Determinations of S become unreliable at concentrations much below 1% owing to the rapid spread of the sedimenting boundary and linearity has been assumed in extrapolating the S_{20} values to

Fig. 1. Sedimentation pattern of the exudate peptide. 2% Concentration in sodium acetate (0.1 M) NaCl (0.1 M) buffer pH 4.5. Speed 59780 rev./min. Interval between exposures = 64 min. Sedimentation from right to left.

Table 1. *Sedimentation constants of the exudate peptide*

Concentration, %	2.50	2.00	1.50	1.00
S_{20} (Svedbergs)	0.89	0.98	1.06	1.12

Table 2. *Diffusion constants from sedimentation diagrams*

Concentration, %	2.50	2.00	1.50	1.00
Mean $D_{20} \times 10^7$	3.4	4.3	4.8	5.8

zero concentration. The value so obtained S_{20} ($c \rightarrow 0$) = 1.28 has been used in calculating the molecular weight.

Diffusion. Since a diffusion experiment carried out on a 1% solution showed a very nearly symmetrical concentration distribution, it appeared that diffusion was not appreciably concentration dependent at concentrations below 1%, and the values obtained in this experiment have been used in calculating the molecular weight. The height (H) and area method of calculation was used. Nine exposures were taken over a period of 30 hr. and $1/H^2$ was plotted against time for each of the two boundaries in the U-tube. The points fell very closely on a straight line cutting the time axis at a value of -6×10^8 sec. and this point was taken as zero time in calculating the diffusion constant. The average value obtained for D at 0° was 2.904×10^{-7} and this was corrected to diffusion in water at 20° by the formula

$$D_{20} = D \text{ buffer at } 0^\circ \times \left(\frac{\eta_0}{\eta_{20}} \right)_{\text{H}_2\text{O}} \times \left(\frac{\eta_{\text{buffer}}}{\eta_{\text{H}_2\text{O}}} \right) \frac{293}{273}$$

to give a value of 5.88×10^{-7} for D_{20} .

The partial specific volume of the material was determined in buffer as 0.656 for a 1.3% solution and 0.654 for a concentration of 0.6%. The measurements were made by the pycnometric method at 25° .

Taking a mean value for V of 0.655 together with $S_2 = 1.28$ and $D = 5.88 \times 10^{-7}$ we obtain mol. wt. = 15300.

The frictional ratio, f/f_0 , has a value of 2.4, corresponding to an axial ratio of 30 in the case of a rod-shaped unhydrated molecule. So large a

degree of molecular asymmetry is perhaps surprising in the case of a low molecular weight peptide.

The degree of polydispersity. Both in electrophoresis and in ultracentrifuging the peptide shows only a single moving boundary. By calculating 'diffusion constants' from the sedimentation diagrams and comparing the values obtained with the true diffusion constant we have endeavoured to obtain information on the degree of polydispersity of the material. The problem is complicated by the dependence of sedimentation on concentration which leads to an artificial sharpening of the sedimenting boundary, an effect which becomes more marked the higher the concentration. The sedimentation diagrams were analysed by the height and area method taking times from the point at which the rotor reached full speed. A correction term ($1 - \omega^2 st$) was applied to each exposure to allow for spread due to variation in the centrifugal field with distance from the rotor centre (see Svedberg & Pedersen, 1940, p. 277). No marked drift was found for the D values during a run. The mean values corrected to water at 20° are given in Table 2. By linear extrapolation to zero concentration, the diffusion coefficient calculated from the boundary spread during sedimentation is about 7.1×10^{-7} , a figure not greatly higher than the true diffusion constant. Making allowance for the various errors inherent in the method, especially in the case of a substance with a low sedimentation and a rather high diffusion constant, it may be concluded that the material is either monodisperse or polydisperse only to a very limited extent (compare, for example, the gross polydispersity found in certain polysaccharides, e.g. glycogen (Record, 1948), where spreading coefficients of 30–100 times the true diffusion constant are found).

Spore extract peptide

This material, extracted from freeze-dried *B. megatherium* spores, consisted of the 2.5% trichloroacetic acid soluble fraction, dialysed and freeze-dried as in the case of the exudate peptide.

Table 3. *Sedimentation constants of the extract peptide*

Concentration, %	2.00	1.50	1.00
S_{20} (Svedbergs)	0.93	1.01	1.13

Electrophoresis. The material was examined under precisely the same conditions as the exudate peptide and practically identical behaviour was observed. Electrophoretically it showed only a single component with a mobility, calculated for the cathode limb, of 0.42×10^{-4} cm.²/sec./v in close agreement with that found for the exudate peptide.

Sedimentation. Here also the results show a close parallel with those obtained for the exudate peptide. The corrected S_{20} values are given in Table 3, and the extrapolated value of $S_{20}(c \rightarrow 0) = 1.32$. At the same concentration, the rate of spread of the sedimenting boundary appeared to be slightly less than in the case of the exudate peptide.

Diffusion. An anomaly was observed here in the form of a sharp peak surmounting the main concentration gradient curve and displaced slightly to the buffer side of the main peak. The picture suggests the presence of a small percentage of a high molecular contaminant, though there is no evidence of this in the ultracentrifuge diagrams. The presence of this contaminant makes the analysis of the diffusion curves difficult. The main part of the sample, however, diffuses at a rate very near to that found for the exudate peptide. Without further purification it is doubtful whether a more detailed examination of the extract peptide sample is justified.

DISCUSSION

As seen in the main part of this paper, the crude non-dialysable fraction of the exudate yielded 96 % of a 2.5 % trichloroacetic acid soluble fraction, this

constituting the sample examined here, whereas the crude extract dialysate gave only a 20 % yield when similarly treated. It appears that the germinating spores allow only material of relatively low molecular weight, the exudate peptide, to pass into solution. The extract peptide, on the other hand, has been separated by a single fractionation from the entire spore contents and it is perhaps not surprising that it does not reach the same standard of homogeneity and monodispersity as the exudate peptide.

Evidence of some polydispersity has been adduced in both samples by viscosity measurements on fractions separated by precipitation at various ethanol concentrations. The differences were much more pronounced in the extract than in the exudate peptide, thus supporting the conclusions already reached.

SUMMARY

1. The exudate peptide from germinating spores of *B. megatherium* appears to be homogeneous in electrophoresis and in sedimentation at pH 4.5.

2. At 20°, it has a sedimentation constant at zero concentration of 1.28×10^{-13} , a diffusion coefficient of 5.88×10^{-7} and a partial specific volume of 0.655. These data give a figure of 15 300 for the molecular weight and 2.4 for the frictional ratio, pointing to marked molecular asymmetry.

3. The material is essentially monodisperse.

4. A peptide extracted from resting spores of *B. megatherium* appears to be physico-chemically identical to the exudate peptide except for the presence of a small amount of contaminant.

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The Biological Action of Substances Related to Thyroxine

9. THE EFFECT OF BUTYL 4-HYDROXY-3,5-DIODOBENZOATE ON THE DEIODINATION OF TRIIODOTHYRONINE IN THE RAT

By J. H. WILKINSON AND N. F. MACLAGAN

Department of Chemical Pathology, Westminster Medical School (University of London), London, S.W. 1

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The metabolic interactions of butyl 4-hydroxy-3,5-diiodobenzoate (BHDB) with thyroxine and triiodothyronine in intact rats and mice have been previously reported (Sheahan, Wilkinson & MacLagan, 1951; MacLagan, Sprott & Wilkinson, 1952; MacLagan & Wilkinson, 1952; Sprott, Wilkinson & MacLagan, 1953). These studies have led us to advance the hypothesis that BHDB exerts its

effects by inhibiting the deiodination both of thyroxine and of triiodothyronine *in vivo*. Evidence in support of the first part of this theory has been obtained by studying the effect of BHDB on the urinary iodide of rats treated with thyroxine labelled in the 3' and 5' positions with ¹³¹I (Wilkinson & MacLagan, 1953a; Wilkinson, Sprott, Bowden & MacLagan, 1954). The present communication