

# Morphological and Chemical Studies of the Spores and Parasporal Bodies of *Bacillus laterosporus*\*

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## ABSTRACT

Spores of *Bacillus laterosporus* were studied to determine the chemical and morphological nature of their basophilic canoe-shaped parasporal bodies. An unusually high phosphorus content of these spores compared to other *Bacillus* species appeared to be associated with the parasporal body. Preparations of these "canoes" still attached to the spore coats were indeed high in phosphorus, but also in nitrogen. They were free of lipide-soluble and nucleic acid phosphorus and stained for protein. Some 50 per cent of the total nitrogen, but only 6 to 10 per cent of the total P were liberated by extraction with alkali-thioglycollate (pH 11.5) or alkali alone (pH 12.2–12.5). Proteinaceous material was recovered from these alkaline extracts and electron microscopy indicated that there had been a marked loss of "canoe" substance. Extraction with acid, removed some 80 per cent of the phosphorus associated with the "canoes" as orthophosphate. Chromatographic analyses for amino acids indicated some 14 ninhydrin-positive spots in the canoe-coat preparations whereas the whole spores contained at least 16.

As part of a wider study of the process of spore formation, the mature spores of a number of *Bacillus* species have been compared from both a structural and chemical viewpoint. The spore of one organism, *Bacillus laterosporus*, Laubach (1), possesses a unique, metachromatic, canoe-shaped body adjacent to the spore body proper, and it has been studied in greater detail to determine the nature and composition of this parasporal structure. It was observed that when these spores were stored as water suspensions the parasporal bodies showed a progressive decrease in size. Furthermore, spores formed on certain media possessed more plump and more deeply stained parasporal parts. These observations prompted some studies of the effects of ageing and of the growth medium on the composition of the spores, and from these initial results it appeared that a high phosphorus content was associated with the

spore-coat residue containing the "canoes." Methods were then developed to separate the canoe-spore-coat structures from the protoplast of the spore. A comparison of the structure with the chemical composition of these isolated forms is presented.

Since this work was begun, Hannay (2) has independently emphasized the intimacy of the canoe and the spore and more recently has shown by electron microscopy of ultra-thin sections that there is a laminated structure within the canoe (3).

## Materials and Methods

The strain of *Bacillus laterosporus* used in this study was obtained from Dr. C. F. Robinow and was maintained on potato agar slopes. Bulk lots of spores were produced in Povitsky bottles containing either the agar medium of Howie and Cruickshank (4) supplemented with 0.5 per cent casamino acids or potato agar (5) or nutrient agar (Difco). Water suspensions of these clean spores were relatively easy to obtain (6) and could be readily pipetted for counting, dry weight determinations, and analysis.

The phosphorus (P) compounds of these spores were

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fractionated by the Schneider (7) and Schmidt and Thannhauser (8) methods as previously modified (6). Disruption of the spores in the Mickle (9) tissue disintegrator with Ballotini beads (Nos. 12 or 14) preceded these fractionations.

Nitrogen (N) was determined by a micro Kjeldahl procedure employing a micro adaptation of the ashing method of Beet (10).

The standard procedures of bright field photomicrography used, have already been described (11). In addition phase-contrast microscopy was applied to wet mounts using a Zeiss dark phase-contrast attachment. Also, material applied in micro drops to formvar-coated grids was examined, both shadowed and unshadowed, in the Philips model 100A electron microscope at 40 or 60 kv. Unless indicated otherwise, the shadowing material was tungsten oxide and was applied at an angle of 16 degrees. Magnifications are approximately as indicated by the markers.

The parasporal structures were stained for bright field microscopy with either toluidine blue (1 per cent for  $\frac{1}{2}$  to 1 minute) or crystal violet (0.2 per cent for 2 minutes).

Counts were made with the Petroff-Hauser counting chamber.

*Preparation of Parasporal Bodies (Canoe-Coats).—*The parasporal canoe and attached spore-coat were separated from the spore body by two methods—germination and disruption.

*Germination.*—Some 400 mg. of heat activated (85°C. for 5 minutes) spores (Lot 8) suspended in 5 ml. of water were mixed and shaken with 750 ml. of a previously warmed germination medium (medium SSB, (11)). After 5 minutes a smear stained with crystal violet indicated that all of the spores had passed into the initial stage of germination, for they now possessed in addition to the stained parasporal body, a deeply stained core (see also reference 3).

Outgrowth of the germinated spores was much slower than expected. By 15 minutes only a slight increase in size was noted. Consequently the culture was diluted with 400 ml. of heart infusion broth (1.5 per cent Difco) and aeration continued. Two hours after inoculation, when 90 per cent of the cells had been liberated from the spore-coats and canoes, aeration was stopped and the culture was chilled. Subsequent procedures were carried out in the cold room at 2–4°C. Following differential centrifugations of the washed suspension in 0.9 per cent NaCl (saline) a layer of liberated vegetative cells could be gradually removed from the top of the pellet. A preparation of canoe-coats slightly contaminated by incompletely germinated spores (Fig. 2) was thus eventually obtained.

It should be noted that heat activation did not appear to alter the size or metachromasia of the parasporal structures. Moreover, during germination and outgrowth the stainability of the canoes not only persisted, but seemed even more intense. In a culture of dividing vegetative cells they soon lost their basophilia.

*Disruption.*—The protoplast of the spore could be readily disrupted in the Mickle disintegrator, while the canoe with attached spore-coat was left intact. The concentration of spores in suspension and the time of vibration in the disintegrator were important factors determining the degree of disruption. Spores (Lot 7) were suspended in saline (170 mg. in 6 ml.) containing one drop of tri-*n*-butyl citrate and vibrated with 3 gm. of Ballotini beads (size 14). After 5 minutes of vibration, although all spores showed some damage, only 20 per cent of the canoe-coats were liberated, whereas after 10 minutes over 90 per cent were free. Another preparation (Lot 8) (200 mg./6 ml.) which was vibrated for 20 minutes, suffered not only disruption of all the spores but also considerable damage of the canoe-coats. In many cases there was marked removal of the coats from the parasporal structures.

A four layered pellet was formed when each of these disrupted preparations was centrifuged (Servall SS-1; 10,000 *g*). The top white layer of non-basophilic fibrous material was relatively free of the canoe-coats but the second layer of turbid slime contained many trapped parasporal bodies. The third white layer, firmly packed on top of the fourth, was composed almost entirely of shattered spore-coats with attached or free canoes. The brownish bottom layer appeared to be composed of the canoe-spore-coat structures mixed with spore cytoplasmic debris. The two top lighter layers were separated and discarded. The two bottom layers were resuspended in saline or water and centrifuged repeatedly. After each centrifugation the white upper layer was removed and collected in a separate tube for recentrifugation. In this manner the free canoes with bits of attached spore-coats could be separated from other spore debris. The final preparations of the parasporal structures of *B. laterosporus* formed homogeneous water suspensions and, providing the disruption had not been too severe, could be readily counted. Both light and electron microscopy were employed to ascertain the degree of "purity" of the preparations and the damage to the structures incurred during preparation (Figs. 3, 4, and 22).

## RESULTS

*Effect of Storage on Spores of B. laterosporus.*—The parasporal body of spores freshly formed on potato agar is highly basophilic and stains metachromatically with toluidine blue. After prolonged storage (270 days) however, the canoe is only lightly stained and thus appears smaller and less plump, although it can still be seen to extend down one side of the spore. Concurrently the light brown colour of a fresh clean spore suspension becomes almost white.

Electron micrographs of these spores before and after ageing indicated that the density of the spore proper was unchanged but that of the canoe was slightly decreased. The canoes of the aged spores

showed a more marked flattening onto the grid surface upon drying.

The effects of such storage on the volume (exclusive of the parasporal part) and the weight of spores harvested from nutrient agar and the amounts of some phosphorus constituents are shown in Table I. It can be seen that a loss of approximately 35 per cent of the dry weight of these spores was not accompanied by any decrease in the volume of the spore body nor significant change in the nucleic acid ratios. This, coupled with the unaltered appearance of the spore body and the changes in the canoe suggests that the weight loss was entirely of the parasporal substance.

A decline in the phosphoprotein or alkali-labile fraction (9) of the spores, paralleled the loss in weight. This fall in bound phosphorus was usually accounted for by a rise in the acid-soluble P of the washed stored spores. A variable amount of phosphorus did appear (as ortho-P) in the storage extracts, particularly after prolonged storage. Much of the fall in weight could be accounted for by the nitrogen content of the storage extracts expressed as protein ( $N \times 6.25$ ). Moreover the addition of trichloroacetic acid to a clarified storage extract produced a turbidity. These studies with aged spores however were not only time-consuming but their results were often obscured by contamination. The subsequent preparation of large samples of isolated canoes (Methods) permitted more direct analyses of these interesting parasporal bodies.

*The Effect of the Growth Medium upon the Weight and Composition of the Spores.*—Preliminary observations had indicated that there was a considerable variability in the size and stainability of the parasporal part of spores when grown on different media. For instance, only 30 per cent of the spores formed on nutrient agar possessed

prominent, well stained canoes 5 days following their formation. On most, the parasporal structures were faintly stained, of variable size, and often showing metachromasia only at the ends. Although both potato agar and the medium of Howie and Cruickshank supported the production of spores which, when fresh, had well stained canoes (Fig. 1) growth on the latter medium produced a more uniform population. The weight, size, total N, and total P of these various spore lots are compared in Table II *a*, while some of the phosphorus fractions are presented in Table II *b*. Compared to the spores of other *Bacillus* species, particularly those of *Bacillus cereus* (6, 12) these were found to be unusually rich in phosphorus. These findings prompted the preparation of isolated canoes, the analyses of which will be compared with those of the intact spores.

*Analysis of Separated Canoe-Coats.*—From the data in Table II *a* it can be seen that the canoe-coat structure separated by disruption represents 65 to 70 per cent of the dry weight of the spore. This proportion of the spore occupied by spore-coats and canoes is much higher than the yield of spore-coats alone obtained by Strange and Dark (13) from spores which do not possess attached parasporal structures (22 to 35 per cent). A comparison of their spore-coat yields with those of canoe-coats indicates that the canoe alone may account for 40 to 50 per cent of the total spore weight—a range that is in keeping with both the appearance and the maximum weight loss found on prolonged storage.

Thus the large proportion of the spore weight represented by the parasporal material and its susceptibility to change with growth media and ageing, emphasize the fallacy of expressing analyses of spore components on a dry weight basis. For this reason, when possible, the results of Tables II *a* and II *b* have in addition been ex-

TABLE I  
*Effect of Storage on the weight, volume and phosphorus content of Bacillus laterosporus spores*

Lot No.	Storage time (at 4°C.)	Average weight /spore	Average volume of spore* $\mu^3 \pm$ s.d. ( $\times 10^3$ )	Total bound phosphorus		RNA DNA	Alkali-labile (phosphoprotein) P‡	
				Per cent dry weight	$g \times 10^{-16}$ /spore		Per cent dry weight	$g \times 10^{-16}$ /spore
	<i>days</i>	$g \times 10^{-12}$						
3	5	1.35	12.0 $\pm$ 0.8	0.838	113.0	4.1	0.181	24.5
2	120	0.96	14.3 $\pm$ 2.9	0.920	88.2	3.6	0.162	15.5
1	270	0.85	15.2 $\pm$ 3.2	0.691	58.6	3.7	0.140	11.9

\* Exclusive of the parasporal part.

‡ As determined by the Schmidt and Thannhauser (1945) fractionation.

TABLE II a  
A Comparison of the Weight, Nitrogen, and Phosphorus Content of Spores and Spore-Coat Canoes of *Bacillus laterosporus*

Lot No.	Agar medium used	Age of growth when harvested	Storage after harvested	Structure	Average weight per structure	Average volume of spore† $\mu^3 \pm$ s.d. ( $\times 10^3$ )	Nitrogen content		Total P		N/P
							a	b	a	b	
4	Nut.*	8	5	Spores	1.03	—	—	—	1.41	111	—
6	Nut.	12	461	Spores	0.95	8.9 $\pm$ 3.0	9.0	864	1.48	142	6.1
7	Potato	8	25	Spores	1.31	16.8 $\pm$ 2.7	8.4	1110	2.16	284	3.9
				Canoe-coats§	0.93	—	8.7	810	1.34	124	6.5
8	H&C	8	17	Spores	1.20	13.1 $\pm$ 1.8	10.1	1210	2.28	274	4.4
				Canoe-coats§	—¶	—	9.4	—	1.65	—	5.7

a, as per cent dry weight; b, as  $10^{-16}$  g/spore or canoe coat.

\* Nutrient agar (Difco).

† Exclusive of the parasporal part.

|| Agar medium of Howie and Cruickshank supplemented with 0.5 per cent casamino acids (Difco).

§ Prepared by the method of disruption.

¶ Too severely disrupted to count.

TABLE II b  
Fractionation of the Phosphorus of Spores and Separated Canoe-Coats of *Bacillus laterosporus*

Lot No.	Structure	Acid-soluble P		Lipide P	RNA-P*		DNA-P†		RNA DNA	Phosphoprotein P§		Residue-P		TCA insoluble residue P¶		
		Total	Inorganic		a	b	a	b		a	b	a	b	a	b	
		a	b	a	a	a	b	a	b	a	b	a	b	a	b	
4	Spores	0.737	76.0	—	0.333	0.220	22.6	0.066	6.8	3.3	—	—	—	—	0.332	34.2
6	Spores	0.857	81.7	0.760	0.034	0.171	16.3	0.064	6.1	2.6	0.126	12.0	0.170	16.2	0.453	43.4
7	Spores	1.19	154	—	0.022	0.148	19.4	0.051	6.6	2.9	0.146	19.0	0.312	40.9	0.288	37.7
	Canoe-coats**	0.300	27.9	0.300	0.011	0.060	5.6	0.000	0.0	$\alpha$	0.213	19.8	0.318	29.6	—	—
8	Spores	0.935	112	0.930	0.187	0.155	18.6	0.051	6.1	3.0	0.175	22.8	0.111	13.3	0.506	60.8
	Canoe-coats**	0.895	—	0.890	0.014	0.033	—	0.000	—	$\alpha$	0.222	—	0.308	—	0.468	—

\*\* Prepared by the method of disruption. a, as per cent dry weight; b, as  $10^{-16}$  g/per spore or canoe-coat.

\* By the orcinol reaction on hot TCA extracts.

† By the Dische reaction on hot TCA.

§ Method of Schmidt and Thannhauser, 1945.

|| Alkali- and acid-insoluble residue of Schmidt and Thannhauser, 1945.

¶ Method of Schneider, 1945.

pressed as a function of the number of spores or canoes.

Fractionation of the phosphorus of the canoe-coat preparations indicated that most of the phosphorus is present in three fractions: the acid-soluble (as inorganic orthophosphate), the

phosphoprotein, and the residual or spore-coat fraction (Table II b). Although some phosphorus was found in the lipide, RNA, and DNA extracts of canoe-coats, the negligible amounts could conceivably be derived from contaminating spore debris. However although the DNA extract

(method of Schmidt and Thannhauser) of the canoe-coat preparations contained 8 to 12 per cent of the total P, this extract showed neither the ultraviolet absorption nor the positive test for desoxy sugar characteristic of DNA.

Similarly it had been observed that the total P of the DNA extract of these spores was 1.5 to 2 times greater than would have been expected from the ultraviolet absorptions and desoxy sugar content of the extract. It is therefore presumed that the high P content of the DNA extract is due to contamination by phosphorus derived from the canoes and does not represent an abnormal composition of the DNA of these spores.

Thus the peculiar distribution of phosphorus (Table II *b*) in these spores compared to other species (6, 12) can be traced to the phosphorus associated with the canoe-coat. The remarkably high inorganic acid-soluble P, phosphoprotein, and TCA residue P can be largely accounted for by the composition of the canoe-coat structures. Although the nucleic acid content of these spores is less than that found in the spores of other *Bacillus* species (12) it is in keeping with the smaller volume of the spore protoplast phosphorus. The various growth media influenced the average nitrogen and phosphorus content of the spores but did not greatly influence the RNA and ANA content (Table II *b*). In fact, the average spore content of the latter is remarkably constant regardless of the medium. Spores formed on potato agar or the medium of Howie and Cruickshank and which bore more plump and deeply metachromatic canoes, possessed twice as much phosphorus, and more nitrogen than the spores formed on nutrient agar.

Storage of intact spores was accompanied mainly by a shift in the state of the phosphorus, rather than by a loss of phosphorus (see above). However when canoe-coats isolated by disruption were stored as water suspensions (0–2°C.) they released a variable amount of phosphorus (as orthophosphate) into the medium. One preparation (No. 7) which contained 50 per cent of its phosphorus in an ultimately "extractable" form, lost 42 per cent of this P during the first 8 days following isolation and 21 per cent during the subsequent 20 days. Another preparation, in which 85 to 95 per cent of the total P was "extractable" lost 21 per cent during the first 8 days and a further 11 per cent in the next 20 days. The subsequent fractionation of these stored canoe-coats indicates that the preparation (No. 8) which had lost less P prior to frac-

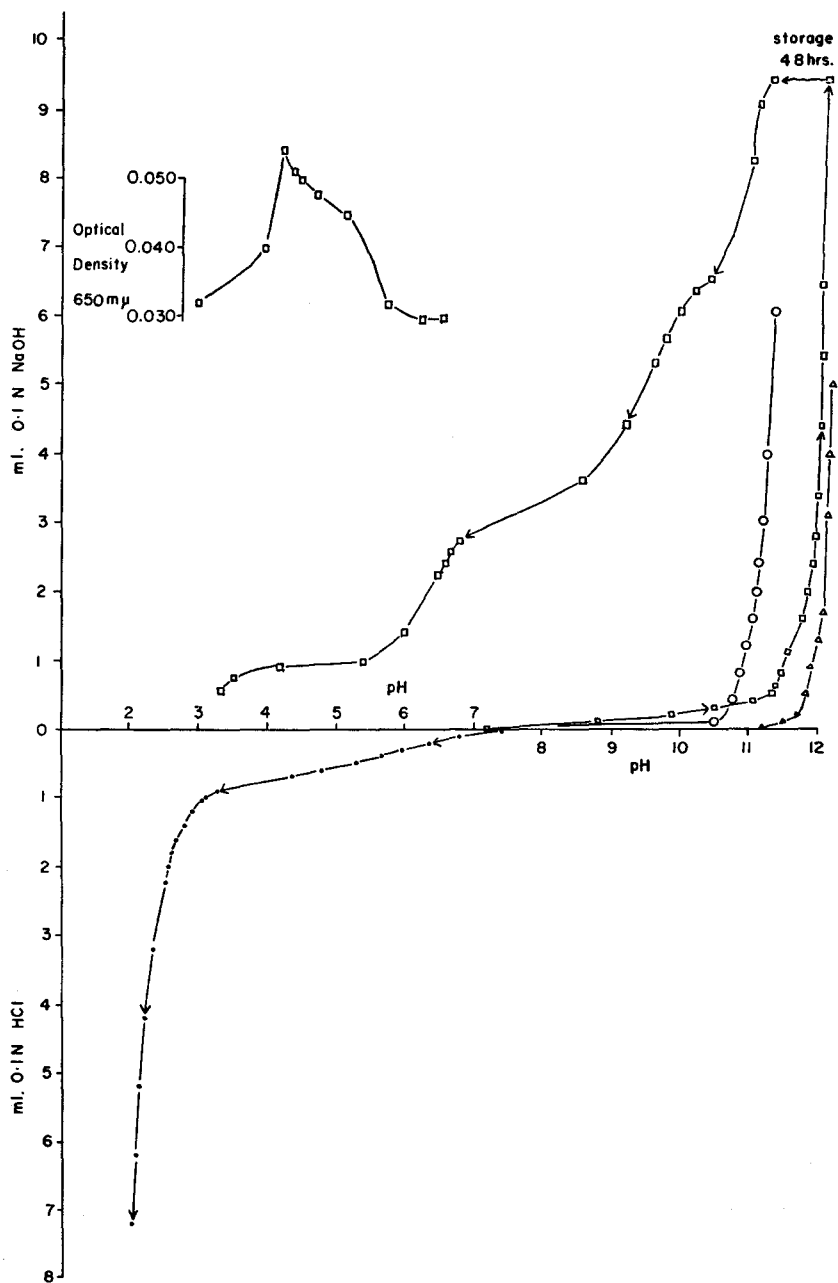
tionation, liberated a greater proportion of P to the acid-soluble fraction (Table II *b*).

Canoe-coats analyzed immediately after preparation can account for 80 per cent of the total spore phosphorus. If the assumption is made that the residual phosphorus of these spores and canoe-coats is located in the spore-coats, then it can be calculated that the canoes alone possess some 64 per cent of the phosphorus of the spores of *B. laterosporus*.

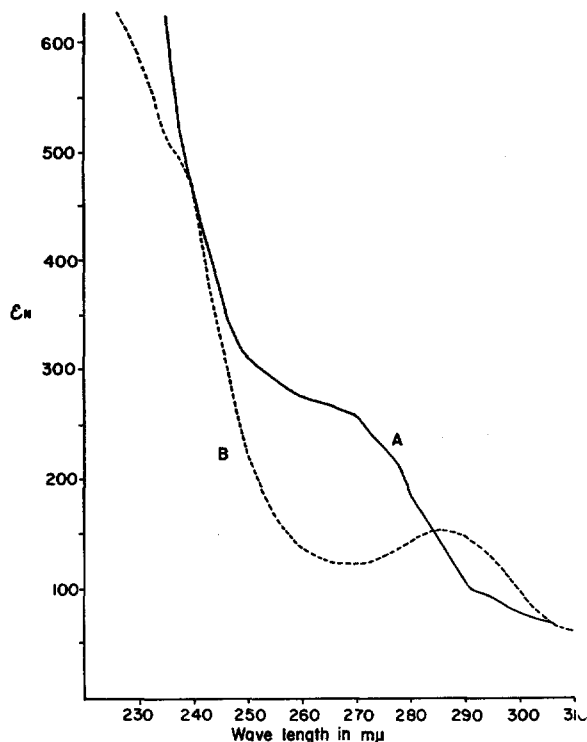
The spore and canoe-coat residues from both the Schneider and the Schmidt and Thannhauser extraction procedures were examined in the electron microscope. The residues of both preparations were composed of two structures, spore-coats and canoe remnants, that could be readily distinguished in spite of the rather drastic pretreatment. Although the canoe parts in these residues from both whole spores (Fig. 17) and isolated canoe-coats (Fig. 16) showed marked alteration, the coats were better preserved and not unlike untreated coats of both this (Fig. 22) and other species (6, 13). The granular structure of the canoes remaining after Schneider's (hot TCA) extraction, distinguished them from the more membranous spore-coats (Fig. 16). However, those residues remaining after the Schmidt and Thannhauser fractionation (Table II *b*) contained canoe remnants which appeared more completely extracted and differed only slightly from the spore-coats (Fig. 17). These findings support the earlier observations with other spores that the residue P left by a modification of the Schmidt and Thannhauser fractionation is located in the spore-coats.

*Extraction of Parasporal Structures.*—The canoe structure, either *in situ* or separated from the spore proper stains with bromphenol blue in mercuric chloride (15) (Fig. 5). This suggestive evidence that there is protein in the canoes was supported by the finding of amino acids upon acid hydrolysis, and prompted the application of extraction procedures at both high and low pH. The effects of alkali and acid extraction were followed by both phase contrast and electron microscopy in addition to nitrogen, phosphorus, and dry weight analyses.

*Alkaline extraction:* When a clean suspension of spores was "titrated" with 0.1 N NaOH, buffering began at pH 10.5 and became marked at pH 11.5 (Text-fig. 1). The pH 11.5 extract contained only a small amount of nitrogen and showed a



TEXT-FIG. 1. Alkali and acid titration curves of spores and separated canoe-coats of *Bacillus laterosporus* (25°C.). o—o titration of whole spores (lot No. 8) with 0.1 N NaOH. □—□ titration of canoe-coats separated by germination (8G) with 0.1 N and 0.5 N NaOH and back titration curve (starting at pH 11.4) of the clarified supernatant with HCl. Drop in pH during storage prior to back titration is indicated. Insert shows change in optical density at 650 m $\mu$ . during this back titration.  $\Delta$ — $\Delta$  titration curve with 0.1, 0.5, and 1.0 N NaOH of spores at same concentration as curve o—o after prior extraction with 1 per cent thioglycolic acid at pH 11.5. •—• titration of intact spores with 0.1 and 0.5 N HCl. Arrows indicate direction of titration.



TEXT-FIG. 2. Ultraviolet absorption curves of material extracted from spores of *Bacillus laterosporus* by alkali-thioglycollate reagent. The nitrogenous residue remaining after dialysis and lyophilization of the alkali thioglycollate extract was partly soluble in 0.16 M sodium acetate (Curve A). The remnant was taken up in 0.05 N NaOH (Curve B). Extinction is expressed as a function of the nitrogen concentration.

low ultraviolet absorption characteristic of protein. However, subsequent extractions at pH 12.2–12.5 removed a considerable, though variable, amount of substance with an absorption spectrum similar to Curve A of Text-fig. 2. Since such a high pH could cause damage to protein structures a more efficient extractant than dilute NaOH was sought. Goddard and Michaelis (16) demonstrated that keratins in alkaline solutions can be rendered soluble by reduction of S-S bonds with thioglycollic acid. More recently, Mazia (17) employing a similar reagent (1 per cent thioglycollic acid, pH 11.5) was able to dissolve the mitotic apparatus of dividing sea urchin eggs. Similarly, this reagent has been used by us to dissolve the parasporal crystals of *Bacillus cereus* variants *sotto*, *thuringiensis*, and *alesti* without affecting the toxicity of the crystal protein to silkworm larvae or the viability of the spores. These observations prompted studies of the effect of this reagent on the solubility of the canoes of *B. laterosporus*.

By the careful addition of 0.2 N NaOH to a

spore suspension, the pH was raised to 11.5 (Text-fig. 1, Curve o-o). An equal volume of 2 per cent thioglycollic acid at pH 11.5 was then added and following centrifugation, the residue was washed with water. The spore preparations by this treatment lost from 7 to 10 per cent of their dry weight and showed a loss of the buffering at pH 10.5–11.5 when retitrated with dilute alkali (Curve  $\Delta$ — $\Delta$ , Text-fig. 1).

There was no noticeable change in the appearance of the spores in wet mounts (dark phase contrast) as the pH was raised to 11.2 but at pH 11.5 there was a slight softening of the parasporal outline (compare Figs. 6 and 7). With the thioglycollate treatment however, there was both an increase in the outline and a reduction of the phase density of the canoes. The spores appeared unaffected (Fig. 8). Subsequent extraction at pH 12.2 caused only a further slight expansion of the canoes (Fig. 9). The nigrosin smear preparations showed more strikingly, the reduction of the

turgidity of the canoes upon treatment with alkali-thioglycollate (compare Figs. 10 and 11).

The alkali-thioglycollate extract was dialysed against water. A flocculant precipitate formed in the bag. The bag contents were carefully collected, lyophilized, and weighed. Seventy per cent of the weight loss of the spores was recovered as a white powder containing some 10 per cent nitrogen but no detectable phosphorus. Two-thirds of this powder readily dissolved in 0.15 M sodium acetate (pH 6.7) and the remainder in 0.05 N NaOH. Ultraviolet absorption spectra of these two solutions are shown in Text-fig. 2, with the extinction expressed as a function of the nitrogen content. The residue in alkali showed an absorption spectrum typical of proteins so dissolved and had  $\epsilon$  N values remarkably close to those already reported for the alkali dissolved protein of the parasporal crystal of *Bacillus thuringiensis* (18).

The spectral data of Text-fig. 2 could be interpreted as evidence for the existence of two protein components in the canoe. However, it is equally possible that the difference in spectra is due to the effect of alkali on the absorption spectrum of a protein containing tyrosine and/or tryptophane (19). The occurrence of the more insoluble component after dialysis and lyophilization may well be due to partial denaturation.

A typical experiment showing the effect of these various alkali extractions on the weight loss and removal of nitrogen and phosphorus are summarized in Table III (Experiment 1).

Separated canoe-coats showed strong buffering at pH 11.5 and 12 (Curve  $\square$ — $\square$ ; Text-fig. 1) when titrated with alkali and underwent a loss of density in phase contrast.

The 0.1 N NaOH extract of one preparation of separated canoe-coats was centrifuged free of undissolved material following 48 hours storage and then back-titrated with 0.1 N HCl (Text-fig. 1). The considerable drop in pH with storage indicated a continued neutralization of alkali by the partially dissolved canoe-coats. The shape of the return curve was markedly different from the alkali titration and indicated the existence of some three buffer plateaus. Precipitation occurred below pH 6.0 and reached a maximum at pH 4.5. The washed precipitate was rich in nitrogen but devoid of phosphorus.

#### *Electron Microscopy Following Extraction.*—

*Alkali:* Extraction of spores of this species with

alkali up to pH 11.5 caused only a slight alteration in the electron density of the canoe edge. However, extraction at the same pH in the presence of the disulfide bond-reducing agent resulted in a marked loss of electron density and expansion of the canoe structures (compare Figs. 14 and 15). The unshadowed extracted canoes looked like a fibrous mat. In the shadow-cast micrographs, the flattening of the canoe remnants onto the grids indicated the extent of the extraction (compare Figs. 18 and 19). Spores, mounted on grids which were then floated on alkaline-thioglycollate, washed and shadowed were occasionally seen enmeshed in masses of fibrous material (Fig. 20).

The canoe parts of the isolated canoe-coats showed similar extraction effects following treatment with dilute NaOH to pH 12.2 or with the thioglycollate-pH 11.5 reagent. The previously plump, dense canoes (Fig. 12) became thin and partly transparent while the coats were unaffected. Phosphotungstic acid readily "stained" the remaining canoes and in Fig. 13 masks the effect of alkaline extraction.

Shadowed electron micrographs of canoes partly dissolved, revealed fibrils of material radiating onto the supporting film (Fig. 23). A similar array was observed at the edge of the partially dissolved parasporal crystal of *Bacillus thuringiensis* (18).

Canoes which had been well separated by disruption (Fig. 22), then repeatedly extracted with alkali still possessed an insoluble component. This appeared to be a membrane-like skeleton (Fig. 24), which was embedded lengthwise in the canoe.

*Acid extraction:* When spores of this species were extracted with dilute HCl, a titration curve (Text-fig. 1) indicated, that buffering began near pH 3.0 and became marked between pH 2.5 and 2.0. However, when the pH of such an acid extract was raised with alkali to pH 4.5, no turbidity was formed suggesting that little parasporal protein had been extracted. Such acid extraction moreover, caused no marked effect on the phase-contrast appearance of the canoes. However, in air-mounted nigrosin films, the canoes, although not swollen, showed a slight softening of their edges.

In the electron microscope, the acid-treated spores showed a variable degree of alteration in the spread and density of the canoes. Unlike the alkali-extracted canoes which showed an extensive flattening onto the grids, in these, the flattening



TABLE III

The Effect of Alkaline and Acid Extraction on the Weight, Nitrogen, and Phosphorus of Spores of *Bacillus laterosporus*

Experiment No.	Extraction procedure	Per cent loss of dry weight	N extracted	P extracted	N/P	Approximate composition of material extracted	
						Per cent N	Per cent P
			$\mu\text{g}/100 \text{ mg. spores}$				
1	pH to 11.5	0.6	76.5	—	—	12	—
	pH to 12.2	10.6	174	67	2.6	2.0	0.7
	pH to 11.5 thioglycollic acid (1%)	7.1	331	30	10.9	5.0	0.4
2	Acid to pH 2.2	8	108	1130	0.095	1.3	14.0
3	Alkali to pH 12.2	11	192	72	2.7	2.0	0.7
	Acid to pH 2.2	7	91	1190	0.076	1.3	16.8-1.70

was largely confined to the outside edge of the canoe (fig. 21). Examination of shadows indicated that the bulk of the parasporal substance was still present. Subsequent extraction of acid-washed spores with dilute alkali now removed much of the canoe substance but left a granular remnant attached to the spore (Fig. 25).

The acid extracts were, in contrast to the alkali extracts rich in phosphorus, but relatively low in nitrogen (Table III, Experiment 2). This prompted a comparison of acid and alkali extraction on the same batch of freshly water-washed spores. One lot of spores was washed twice with 0.1 N HCl (pH to 2.2) and another with 0.1 N NaOH (pH to 12.2). The essential data shown in Table III (Experiment 3) confirm the previous findings.

The results of the last column of Table III are only approximate and are based on the dry weight difference before and after extraction as well as on the weight of the dried HCl extracts. The high percentage dry weight of phosphorus in the substance extracted by acid indicates the inorganic nature of the phosphorus. Thus 0.1 N NaOH (pH 12.2) was found to extract only 3½ per cent of the total spore P (or 6 per cent of the canoe P) while 0.1 N HCl removed 50 per cent of the total P of the spores or some 80 per cent of that in the canoes. In both instances the P as removed was inorganic ortho P. Moreover, direct treatment of

TABLE IV

Amino Acid Analysis of Spores and "Canoe-Coats" of *B. laterosporus*

	Spores lot 8	Canoe-coats		
		Lot 8A*	Lot 8G*	Lot 7A*
Alanine	+	+	+	+
Arginine	+	+	+	+
Aspartic acid	+	+	+	+
Glutamic acid	+	+	+	+
Glycine	+	+	+	+
Lysine	+	+	+	+
Serine	+	+	+	+
Proline	+	+	+	+
Threonine	+	+	+	+
Tyrosine	+			
Valine	+	+	+	+
Phenylalanine	+	+	+	+
Leucine	+	+	+	+
Iso-leucine	+			
Histidine	+	+	+	+
Cystine-cysteine	+	?	?	?
Methionine	+	+	+	+
Unidentified‡	+	+	+	+

? presence suspected.

\* A, by method of disruption; G, by method of germination.

‡ approximate  $R_f$ . 1st solvent (methanol-pyridine- $\text{H}_2\text{O}$ ) 0.5; 2nd solvent (tert. butanol-methylethyl ketone- $\text{H}_2\text{O}$ ) < 0.05.

the spores of this species by the acid-molybdate procedure for ortho P (Ernster *et al.* (14)) removed an amount of P comparable to that removed by HCl extraction. Alkali-extracted spores still readily liberated phosphorus to HCl or to the acid-molybdate reagent but HCl-washed spores gave up no further ortho P. Freshly separated canoes likewise showed the ability to withhold P from water and alkaline washing but released their copious P content to dilute acid or to acid-molybdate.

The canoe nitrogen on the other hand was more readily removed by alkali than by acid. The pH 11.5 thioglycollate reagent was capable of extracting some 50 per cent of the nitrogen of the canoe-coats. Since these structures were 8 to 9 per cent N (Table II a), it can be calculated that approximately 25 per cent of the weight of the canoe-coat structure was extractable protein. The dry weight of a canoe-coat preparation likewise dropped by *ca.* 26 per cent after thorough extraction. If we assume that these spores possess

25 per cent of their weight as insoluble spore-coat material (13) than *ca.* 40 per cent of the weight of the canoe itself was extracted.

Spores which had been subjected to extractions with dilute alkali, acid, or both showed the same ability to form colonies as did untreated spores when plated onto nutrient agar.

The canoes of acid-extracted spores took up both crystal violet and toluidine blue as readily as did those of untreated spores but held these dyes less strongly against water washes. The canoes remaining after alkali extraction held both stains in a narrow band near the spore, the extracted keel being unstained. These observations indicate that the alkali-extractable protein is mainly responsible for the stainability of the canoes. However, the phosphorus may also be partly responsible for the staining characteristics. More quantitative staining procedures are required to study the effects of both ageing and extraction.

*Preliminary Qualitative Amino Acid Analyses of Acid Hydrolysates Using the Chromatographic Technique and the Solvent Systems of Redfield (20).*—The results of these studies are summarized in Table IV. The parasporal structures and adherent coats showed some 14 separate ninhydrin-reacting substances and appeared to lack two amino acids, tyrosine and isoleucine, that were present in the whole spores. Analysis for tryptophane was not made but the absorption spectrum in dilute alkali (Text-fig. 2) suggests its presence. It is remarkable that the protein of these canoes which does not contain tyrosine, should have an absorption spectrum similar to that of the crystal of *Bacillus thuringiensis*, which did contain this aromatic amino acid. More definitive quantitative analyses of these components are planned as well as amino acid analyses of the substances extracted by acid and alkali.

#### DISCUSSION

The nature and location of the abundant phosphorus of these canoes are a matter of considerable interest. Its behavior towards water, alkali, and acid indicates that it all exists in a highly acid-labile monophosphate linkage to some component of the canoe. The presence of protein material and the metachromatic stainability of the canoe suggest that the phosphorus may render this canoe substance polyanionic. That the phosphorus may be protein-bound was suggested by a recent observation that after formaldehyde fixation of sepa-

rated canoes much of the phosphorus was no longer extracted by acid. Also, in the fractionation procedures described, damage to the protein structures by trichloroacetic acid and hot lipid solvents left part of the phosphorus bound in a phospho-protein fraction, whereas direct treatment with acid or acid-molybdate removed it entirely.

The type of linkage holding the phosphorus in the canoe remains to be established. It was not liberated by hydrogen bond disruption (alkali) nor by disruption of S-S bonds (alkali-thioglycollate). The latter treatment however, enhances the solubility of the canoe protein towards alkali indicating that such linkages play a role in the maintenance of its structure. The solubility behavior suggests that the S-S bonds form strong cross-linkages between relatively large units. However, the existence of one dimensional S-S bonding has not been ruled out by these preliminary observations.

The tendency for the canoe phosphorus to remain behind during the extraction of the protein suggests that phosphorus may be part of the membranous residue also left by alkali. In thin sections of developing spores, Hannay (3) observed lamellae which could correspond to the membranous component of the canoes of these ripe spores. Such reasoning would then indicate that the extractable protein corresponds to a matrix of the canoe and that the phosphorus component is located in the lamellae. However a proper evaluation of such a conclusion cannot be made until thin sections of ripe spores as well as acid- and alkali-extracted spores have been observed.

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## EXPLANATION OF PLATES

## PLATE 315

FIG. 1. Intact spores of *Bacillus laterosporus* stained with crystal violet, showing the deeply stained canoes and unstained spore body.

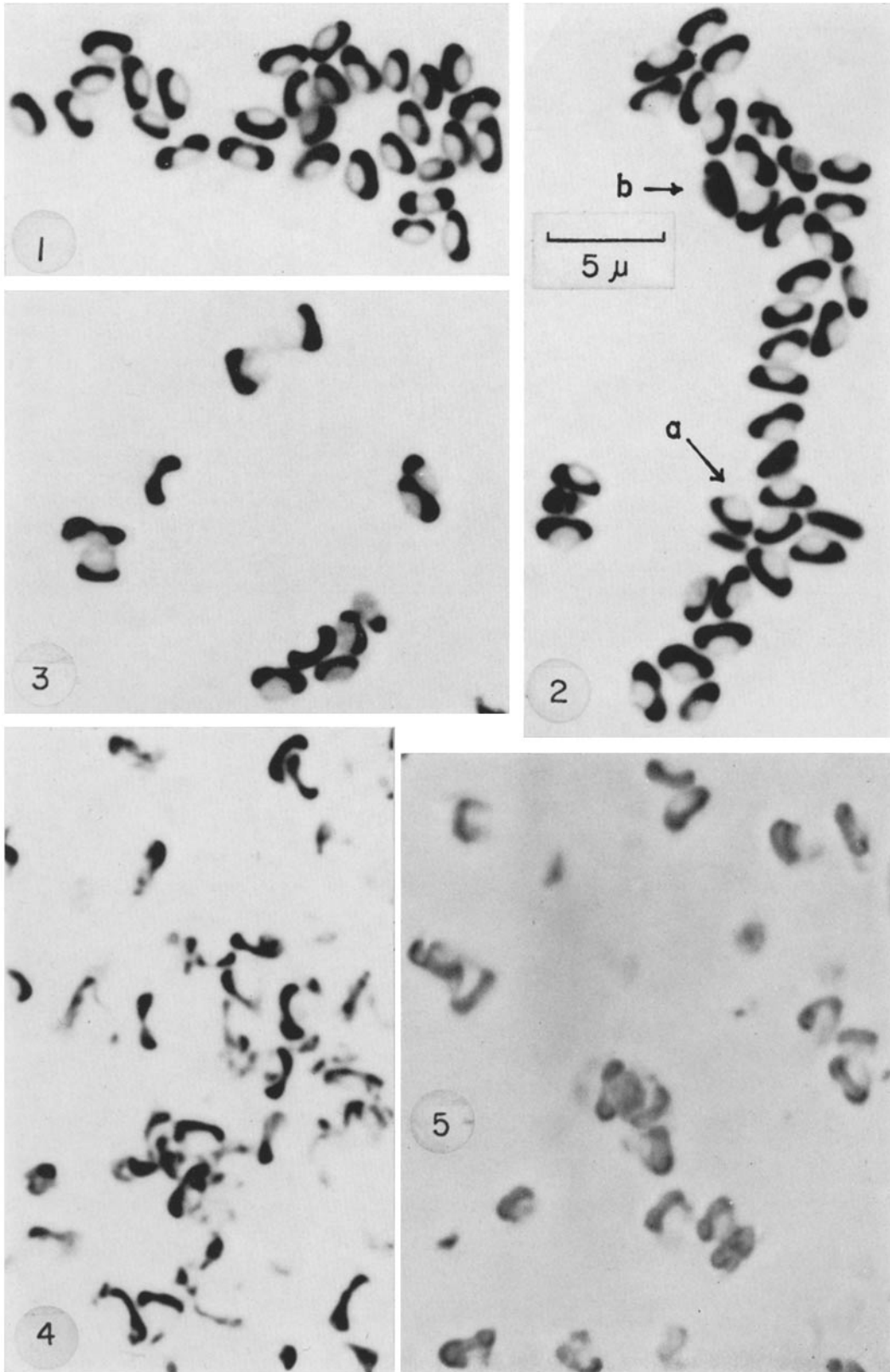
FIG. 2. Canoe-coats of spores of *Bacillus laterosporus* separated by germination (lot No. 8G); crystal violet-stained. The empty spore-coats are, except for the rents (*a*), similar to the intact spore bodies of Fig. 1. An incompletely separated spore is shown at *b*.

FIG. 3. Spore-coat canoes separated by disruption (lot No. 7); crystal violet-stained. A few of the canoes have been separated from the coats.

FIG. 4. A preparation (lot No. 8A) of canoe-coats subjected to excess disruption; stained with crystal violet.

FIG. 5. Material from the same preparation shown in Fig. 3 stained for protein with mercuric chloride-bromophenol blue.

Magnification is indicated by the 5 micron marker.



(Fitz-James and Young: Parasporal bodies of *Bacillus laterosporus*)

PLATE 316

FIGS. 6 to 11. The effect of alkali and alkali-thioglycollate treatment of intact spores of *Bacillus laterosporus*.  
Figs. 6 to 9 dark phase contrast.

FIG. 6. Untreated spores of *Bacillus laterosporus*, water mount.

FIG. 7. Spores of the same preparation raised to pH 11.5 with dilute (0.1 N) NaOH—slight softening and swelling of canoes.

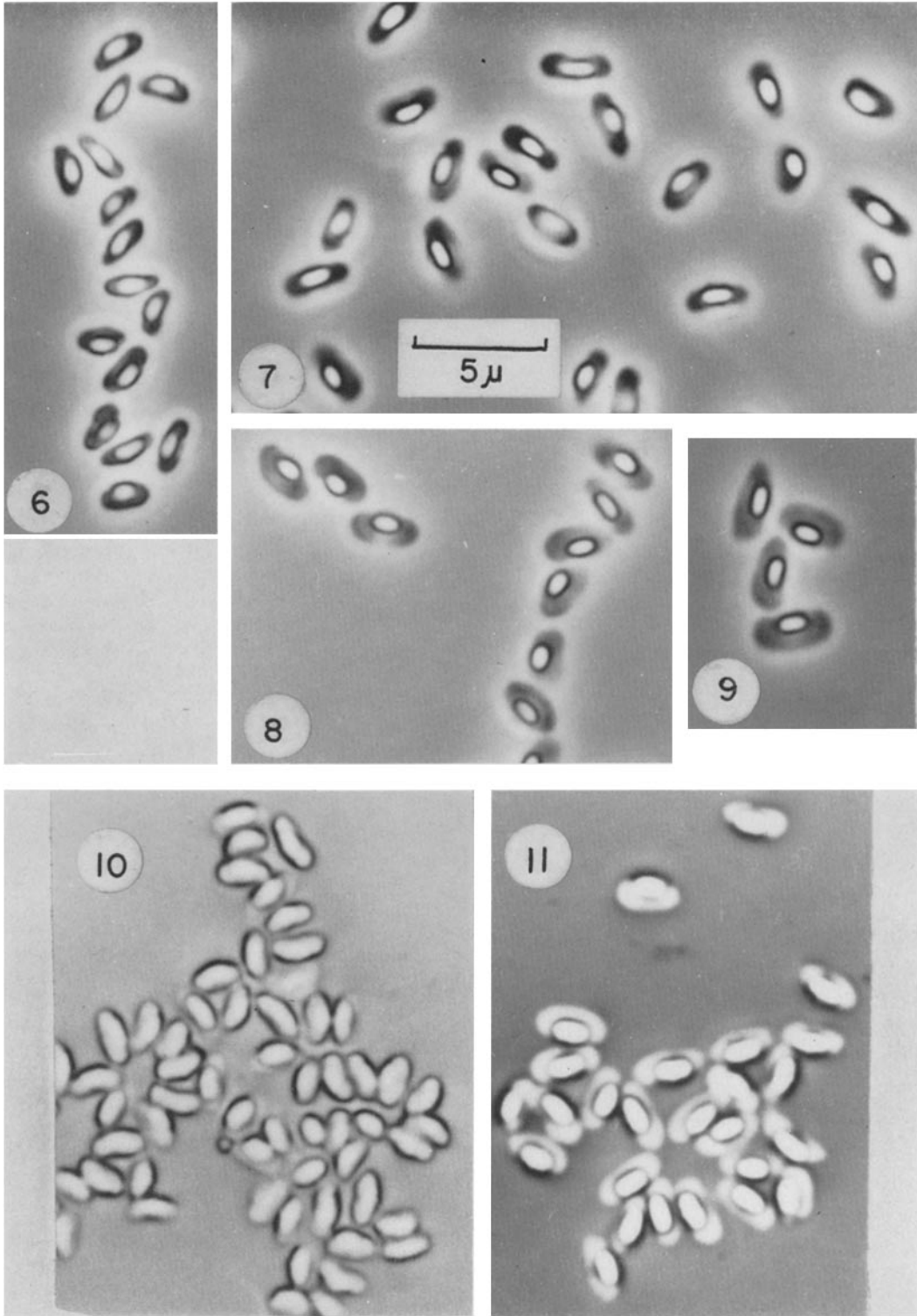
FIG. 8. Spores treated with 1 per cent thioglycollic acid at pH 11.5—more marked swelling of canoes.

FIG. 9. Alkali-thioglycollate treated spores subjected to further extraction with dilute NaOH to pH 12.2—slightly further swelling has occurred.

FIG. 10. Nigrosin smear (dried and mounted in air) of untreated spores showing the sharp outline of the canoe.

FIG. 11. Air-mounted nigrosin smear of alkali-thioglycollate-treated spores. The expanded, extracted canoes no longer blanket the spore refractility.

Magnification is indicated by the 5 micron marker.



(Fitz-James and Young: Parasporal bodies of *Bacillus laterosporus*)

PLATE 317

FIG. 12. Unshadowed electron micrograph of canoe-coats prepared by germination (No. 8G). Not stained with phosphotungstic acid.

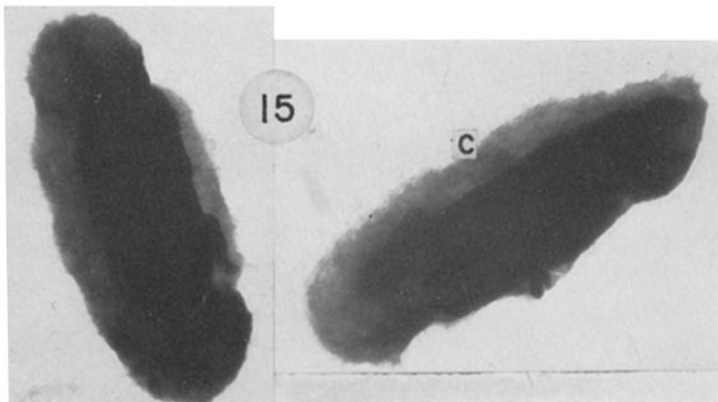
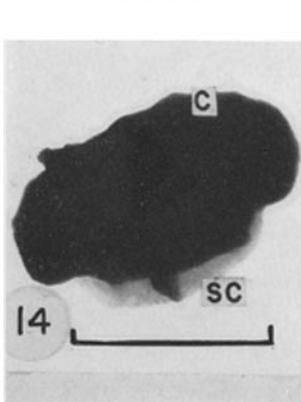
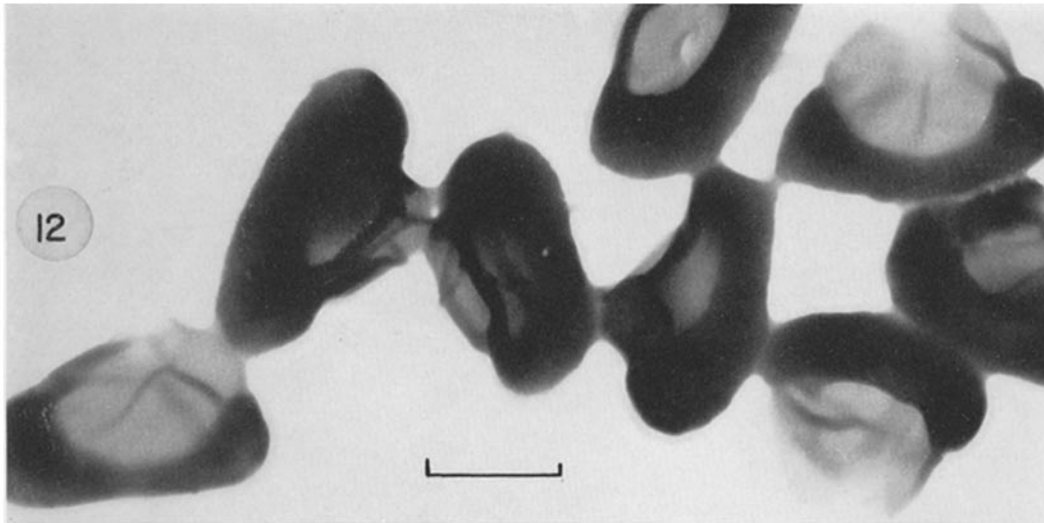
FIG. 13. The same preparation after extraction with dilute NaOH at pH 12.2. Micrograph shows the extraction of the canoes but not the coats. The improved contrast of the spore coats and part of the density of the canoes is the result of staining with phosphotungstic acid. Magnification is as indicated in Fig. 12.

FIG. 14. Untreated spore showing the dense canoe (*c*) and a fold in the spore coat (*sc*) (stained with phosphotungstic acid).

FIG. 15. Spores (of the same lot as shown in Fig. 14) extracted on the grids by floating on alkali-thioglycollate reagent; water-washed, phosphotungstic acid-stained. Magnification is the same as Fig. 14. The canoe is now an expanded matted body, still enclosing the dense spore.

Magnifications are indicated by the 1 micron markers.





(Fitz-James and Young: Parasporal bodies of *Bacillus laterosporus*)

PLATE 318

FIG. 16. Canoe-coats (prepared by disruption, No. 8A) after extraction with 5 per cent trichloroacetic acid, 90°C.; (the phosphoprotein fraction of Schneider (7))

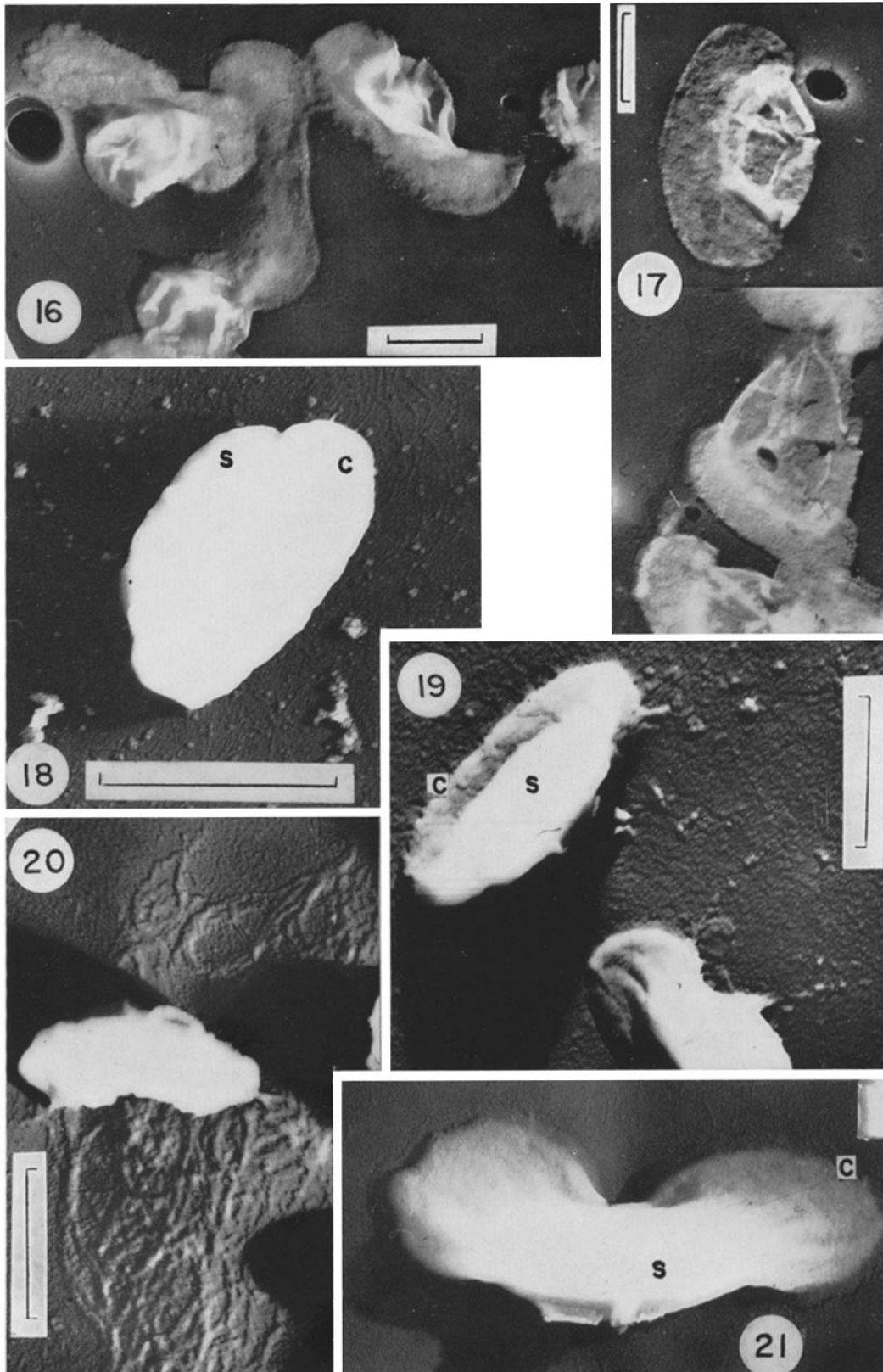
FIG. 17. Top, a typical spore remnant after extraction by both the Schmidt and Thannhauser (N KOH) procedure and hot acid. Bottom, a similar residue of a separated canoe-coat preparation (No. 8A).

FIG. 18. Shadowed electron micrograph of intact spore of *Bacillus laterosporus*. The spore body (*s*) and canoe (*c*) are equally dense.

FIGS. 19 and 20. Spores mounted on grids and subjected to alkali-thioglycollate extraction as were those in Fig. 15. Fig. 19 shows marked extraction of the canoes (*c*) but not of the spores (*s*). Masses of fibrous material, Fig. 20, were occasionally seen about partly extracted canoes.

FIG. 21. A spore from the same preparation shown in Fig. 18 after extraction with 0.1 N HCl. Unlike the flattened extracted canoes after alkali (Fig. 19), these (*c*) had given up much phosphorus but little protein and appeared more expanded than depleted. Same magnification as Fig. 18.

Magnifications are indicated by the 1 micron markers.



(Fitz-James and Young: Parasporal bodies of *Bacillus laterosporus*)

PLATE 319

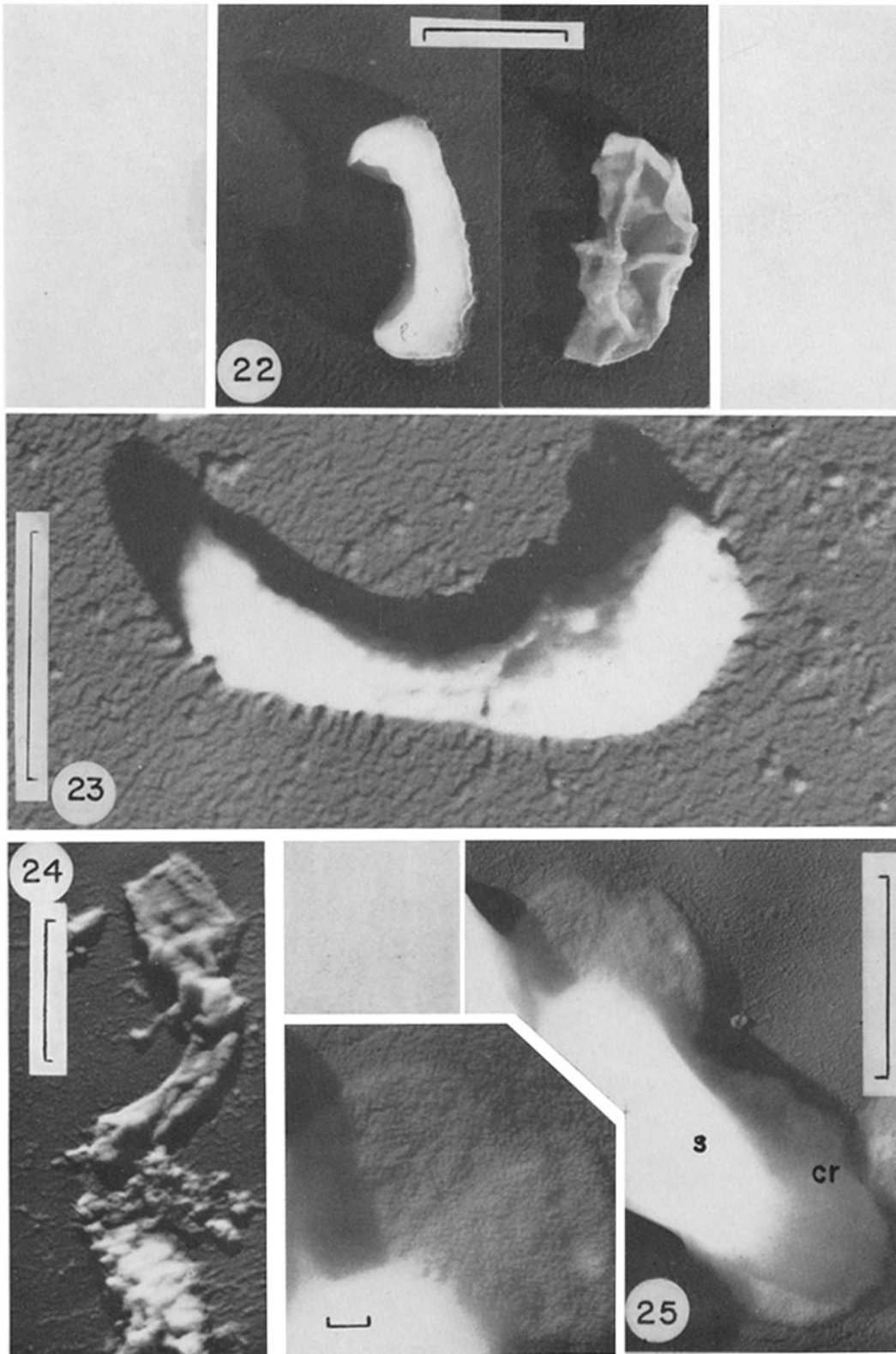
FIG. 22. The two parts of the canoe-coat preparations, frequently separated by the disruption procedure (Preparation No. 7A). Palladium-shadowed.

FIG. 23. A canoe partly dissolved by the pH 11.5 thioglycollate reagent.

FIG. 24. A completely extracted remnant of a canoe separated by disruption.

FIG. 25. An acid-extracted preparation which has been subsequently extracted with dilute (0.05 N) NaOH on the grid. The appearance before alkali is shown in Fig. 21. The canoe remnant (*cr*) is now less elevated and composed of an array of uniformly sized and closely packed granules. In the insert at higher magnification, the scale is 0.1 micron.

Magnification is indicated by the 1 micron markers.



(Fitz-James and Young: Parasporal bodies of *Bacillus laterosporus*)