ACID HYDROLYSIS OF THE SPORES OF *B. CEREUS;* A CORRELATION OF CHEMICAL AND CYTOLOGICAL FINDINGS*

by

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Acid hydrolysis, which forms part of the standard procedure for demonstrating the nuclear material of bacteria, produces an odd effect on resting spores. Whereas the Feulgen-positive material (chromatin) of a vegetative cell remains within the cell during acid hydrolysis, that of the resting spore is extruded beyond the cell outline, appearing as an eccentric plate, or side body. (STILLE[1]; DELAPORTE[2]; and ROBINOW[3]). It is now known that, during this hydrolysis, the nuclear structure of the resting spore is markedly rearranged (ROBINOW[4]). In earlier studies of this reaction, ROBINOW[5] observed that if potassium permanganate were added to the acid (hydrochloric or nitric) and the hydrolysis carried out at room temperature, the eccentric basophilic structure of the spore could be selectively removed by extraction with dilute buffers at a pH greater than 6.8. This mild alkaline washing did not remove the side body from spores hydrolyzed without permanganate.

As the side body contains the Feulgen-positive substance of the spore, the fact that it could be selectively dissolved suggested a simple means of separating nuclear from cytoplasmic material. Experiments were therefore designed to study:

a. The effect on the phosphorus fractions of resting spores of hydrolysis with acid mixtures, with and without permanganate.

b. The further effect of mild alkali extraction on the phosphorus fractions of spores which had been hydrolyzed with and without the addition of permanganate.

The effects of hydrolysis on other materials in the spore were also observed.

A preliminary report of this work has already been presented (FITZ-JAMES[6]).

MATERIALS AND METHODS

Spores of *B. cereus* were used exclusively. These were harvested after ten days growth on the agar medium of HOWIE AND CRUIKSHANK[7], supplemented with 1% casamino acids. Spores were washed eight to twelve times with distilled water at room temperature. The washing was extended over one to two days, and after each centrifugation (6000 to 9000 *g*) the upper layer of the pellet was scraped to remove vegetative debris. A spore suspension free of vegetative cells was thus obtained.

One or two ml of the washed spore suspension, containing from 30 to 50 mg of spores, was pipetted to a series of 15 ml centrifuge tubes. Ten to twelve ml of a freshly prepared solution of *N*/3 nitric acid containing 0.1% potassium permanganate ("K" solution) was added to one group of spores which was then allowed to stand for 20 minutes at room temperature. Hydrochloric acid, to a final concentration of 1 *N*, was added to another group and the tubes heated at 60° C for 10

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minutes. The samples were centrifuged and decanted. Following hydrolysis, the residues were washed with distilled water, and the clear hydrolysates and their washes pooled. The phosphorus content and, where possible, the ultraviolet absorption spectra of the hydrolysates were measured.

Those samples of hydrolyzed spores to be further extracted by mild alkali were suspended in 0.5% sodium acetate (ROBINOW[5]) for 5 to 20 minutes at room temperature, centrifuged at 2000 r.p.m. and, depending on the degree of extraction desired, either washed with water or re-extracted with sodium acetate. The cytological effect of these treatments was followed by coverslip smears stained with crystal violet and mounted in water from which photomicrographs were made. The pH of the various spore suspensions was measured with a glass-electrode pH meter.

Duplicate samples of the control spores, as well as the residues following hydrolysis and mild alkali treatment were subjected to the phosphorus fractionation procedures of SCHNEIDER[8] and of SCHMIDT AND THANNHAUSER[9]. Since the DNA of hydrolyzed spores was not precipitated from the alkali extract of SCHMIDT AND THANNHAUSER on acidification, this method of fractionation was of limited use. The individual nucleic acid values in treated spores are therefore based on the MEJBAUM[10] orcinol reaction for RNA and the DISCHE[11] diphenylamine reaction for DNA. The ribonucleic acid standard was a yeast RNA from Schwartz and Company; the desoxyribonucleic acid standard a sample of highly polymerized DNA prepared from calf thymus by the MIRSKY AND POLLISTER method[12] and obtained from Dr. G. C. BUTLER, University of Toronto. At low concentrations of DNA (as in spores free of side body) the absorption spectrum of the colour complex developed in the Dische test was examined in a Beckman (Model DU) spectrophotometer, at wave lengths from 550 to 675 mμ. This helped to decide whether the true colour complex or some interfering substance was responsible for the optical density at 600 mμ.

Total nucleic acid in trichloracetic acid (TCA) extracts was estimated by the ultraviolet absorption procedure described by LOGAN, MANNELL AND ROSSITER[13]. Total P was determined on all samples by the KING[14] method, except that "metol" was used instead of KING's reducing agent (GOMORI[15]). Inorganic phosphorus was measured by the method of ERNSTER, ZETTERSTRÖM AND LINDBERG[16]. All values are expressed as mg phosphorus per 100 g of original spores dried at 80° C and stored *in vacuo* over phosphorus pentoxide to a constant weight.

Optical equipment consisted of a Zeiss achromatic condenser, N.A. 1.4, achromatic oil immersion objective 90×, N.A. 1.3, in conjunction with a compensating eyepiece, 15×. The light source was a tungsten ribbon lamp and the equipment was arranged to give Koehler illumination. The photographs were taken using a Baird Associates interference filter having a transmission peak at 539 mμ. The photographs, with a magnification of 1800, were enlarged twice before mounting.

In one series of studies, the effect of the hydrolysis and acetate treatments was followed by electron microscopy. Small drops of dilute spore suspension were dried on collodion-covered electron microscopy grids, and shadowed with uranium (8 mg, distance 12 cm) at an angle of about 1 to 4 The pictures were taken with an RCA electron microscope, type EMU 2 D.

RESULTS

Hydrolysis with acid permanganate solution

Eight experiments were performed to study the effect on spore phosphorus fractions of acid permanganate hydrolysis ("K" treatment) and subsequent mild alkali extraction. The results of a typical experiment are presented.

Within a few minutes of suspending the spores in the K solution, the permanganate colour was markedly reduced. The spores settled out as a brownish floc, leaving a pale pink supernatant. Fig. 1 illustrates the crystal-violet-stained appearance of spores treated in bulk with the K solution. The refractile, non-staining, resting spores, one of which still persists in the upper left of Fig. 1, are differentiated into two basophilic parts, a deep blue side body and a purple central zone separated from the spore surface by a non-staining layer. The central basophilic zone is connected to the side body by a short neck. The electron micrograph of this change is shown in Figs. 6A and 6B. The exosporium still envelopes the hydrolyzed spore.

Table I records the chemical changes during K treatment. Some 60% of the RNA-P was lost from the protein-bound fraction, while the residue P (Schneider fractionation) was reduced to 40 to 50% of its original whole spore level. Studies on this TCA-residue P of *B. cereus* spores are included in a publication now in preparation. A large part of it

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was found to be an alkali-soluble and acid-labile substance possessing the properties of a polyphosphate. A small, acid-stable part of the TCA-residue P of *B. cereus* spores was both acid and alkali insoluble (equal to the DNA-free residue P of the SCHMIDT AND THANNHAUSER procedure Table III) and is associated with the spore coats.

TABLE I

THE PHOSPHORUS DISTRIBUTION OF ACID-PERMANGANATE HYDROLYZED (K TREATED) SPORES, WATER AND SODIUM ACETATE WASHED

Phosphorus (P) as mg/100 g spores

		Untreated spores	*K-treated spores*		
			water washed	*Sodium acetate washed*	
				Once	*Twice*
Cytological effects			*side body revealed*	*side body partly removed*	*side body completely removed*
K Extract	Total P	—	90	90	90
1st Acetate wash	Total P	—		291	291
2nd Acetate wash	Total P	—			100
Acid-soluble P		8	160	29	14
Lipid-soluble P		3	10	10	8
TCA Extract					
(SCHNEIDER[8])	Total P	499	270	189	112
RNA P (orcinol)		372	145	100	72
DNA P (diphenylamine)		90	84	37	0
TCA					
Residue	Total P	148	63	58	26

From Table I it can be seen that the loss of RNA and residue P from the bounds forms is largely balanced by an increase in acid-soluble P and by the phosphorus in the K extract. Labile phosphorus studies showed that part of the drop in TCA-residue P in the K-treated, water-washed spores of Table I may be accounted for by an increased acid-labile P in the TCA extracts (when appears as KING-GOMORI phosphorus). The DNA-P, based on DISCHE's test for desoxyribose, was never reduced by more than 10% after K treatment and was usually within 2% of the untreated spores.

Surprisingly high estimates for total nucleic acid P were encountered on applying the ultraviolet absorption procedure to the TCA (SCHNEIDER[8]) extracts of untreated spores. The ultraviolet absorption, expressed as nucleic acid phosphorus, could be as much as twice the true phosphorus content of the extract. An example of this interference is shown in Table II. A separate study revealed that a non-phosphorus substance with a peculiar ultraviolet absorption spectrum was extracted by hot TCA from whole resting spores and completely masked the purine-pyrimidine spectrum. This interfering substance could be readily removed or extracted into cold TCA if the spores were first disrupted, heat killed, germinated or (as indicated in Table II) acid hydrolysed. A substance with a similar absorption spectrum to that encountered here has been reported by POWELL AND STRANGE[17] in the spores of a number of bacteria. POWELL[18] has identified this material as the calcium salt of dipicolinic acid. The spectrum of this material is also evident in ultraviolet studies of spore suspensions (MOROWITZ[19]).

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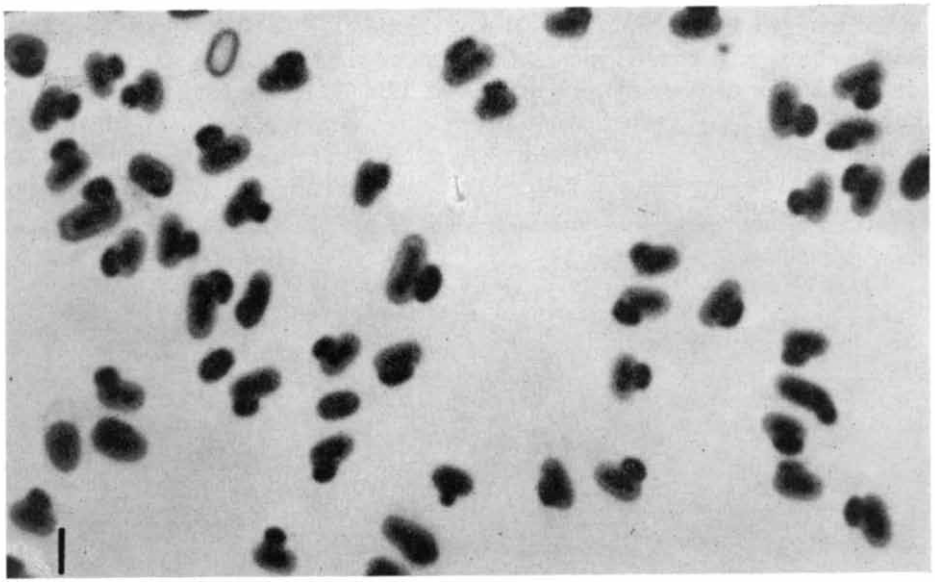

Fig. 1. Resting spores hydrolyzed in bulk for 20 minutes with permanganate-nitric solution (K treated) washed twice with water and stained with crystal violet. All but one spore in the upper left corner have differentiated into a structure with a central core and side body.

TABLE II

THE BOUND PHOSPHORUS OF ACID-PERMANGANATE HYDROLYZED (K-TREATED) SPORES, WATER AND SODIUM ACETATE WASHED (SHOWING THE EFFECT OF THE TREATMENTS ON THE ESTIMATION OF NUCLEIC ACID P BY ULTRAVIOLET ABSORPTION)

mg P/100 g spores

	Untreated spores	*K treated spores*	
		Water washed	*Sodium acetate washed*
TCA Extract (SCHNEIDER[8])			
Total P	476	380	250
u-v absorption as nucleotide P	1020	144	76
RNA-P (orcinol)	400	260	140
DNA-P (diphenylamine)	90	95	46
TCA Residue-P	150	75	45

After hydrolysis ("K" or HCl treatment) the ultraviolet spectrum of the TCA extracts was predominantly that of purines and pyrimidines. But the ultraviolet absorption expressed as nucleic acid P (Table II) gave values below the total P of the extracts or the sum of the nucleic acid P based on the sugar tests. These discrepancies may be readily explained by the degraded state of the nucleic acid residues left by the hydrolysis, as well as by the shift of labile residue P mentioned above.

Mild-alkali washing of acid-permanganate hydrolyzed spores

When the K-treated spores were suspended in 0.5% sodium acetate solution sufficient in quantity (5–10 ml) to elevate the pH of the suspension above 6.5 (the level

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after K treatment and washing was pH 2.0) the side bodies could be removed. Fig. 2 shows the effect on K-treated spores of a 5 minute rinse in sodium acetate. The phosphorus distribution of these spores is recorded in Table I "Sodium acetate washed once". Some 56% of the DNA (DISCHE[11]) of the spores has been removed, along with a further 12% of the original spore RNA. Fig. 3 shows that two rinses in sodium acetate completely dissolved the side bodies, leaving a spore residue containing a basophilic core. The ultraviolet absorption spectrum of the acetate extracts indicated the presence of a nucleoprotein.

The phosphorus distribution of the K-treated spores after two washes with sodium acetate is also shown in Table I. Besides removing the entire spore DNA, the two

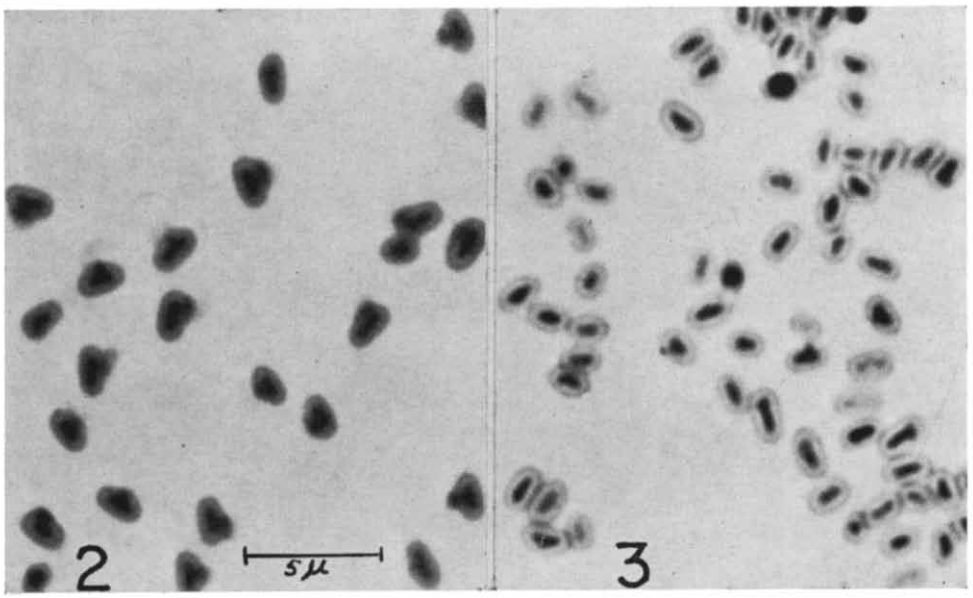

Fig. 2. K-treated spores after a short rinse (5 minutes) in 0.5% sodium acetate. The side bodies are partly dissolved (stained with crystal violet).
Fig. 3. K-treated spores after two rinses with 0.5% sodium acetate. The side bodies are completely dissolved leaving a basophilic central core (stained with crystal violet).

acetate extractions removed a further 19% of the spore RNA-P and a portion of the TCA-residue phosphorus. This left behind, then, a structure devoid of DNA but still containing some 20% of the original RNA and a small amount of residue P. Further RNA could be removed by extending the acetate extraction beyond the time necessary for the complete removal of the side body. This was associated with some shrinkage of the basophilic central cores shown in Fig. 3.

Occasionally, the acetate washing was of insufficient alkalinity to raise the pH above 6.5. In these cases, although the side body did not appear to be altered in basophilia or size, some 50% of the DNA was extracted. Little or no drop in the acid-labile TCA-residue P occurred with such partial treatment. On being rewashed with fresh acetate, however, the spores lost their side bodies together with the remaining DNA and the remaining alkali-soluble part of the residue P.

These chemical and cytological effects of K treatment and acetate extraction on

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the bound phosphorus fractions of *B. cereus* spores are summarized graphically in Fig. 5. The acid-labile part of the non-nucleic-acid P (non NA-P) includes the acid-labile P present both in the TCA extracts and the TCA residue. The acid-stable P is acid and alkali-insoluble material associated with the spore coats. The changes recorded in Fig. 5 are the averages of several studies.

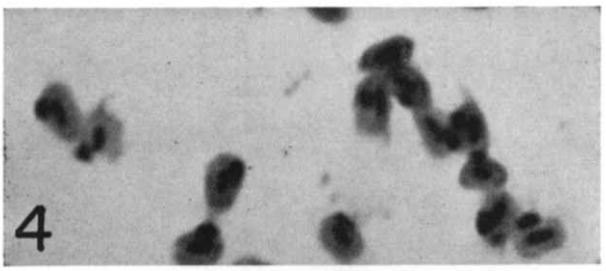

Fig. 4. K-treated spores after extraction of nucleic acids with 5% TCA (90° C, 15 min), water washed and stained with crystal violet. The side body and central core, both slightly contracted, are still stainable.

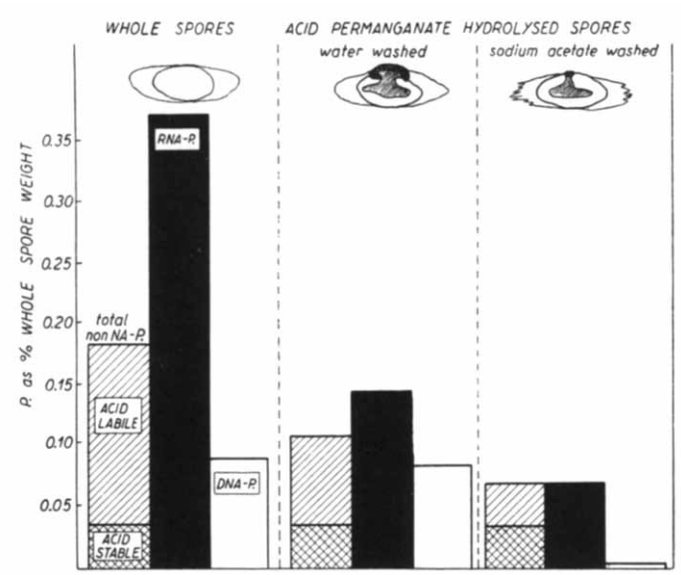

Fig. 5. A summary of the effect of nitric acid-permanganate hydrolysis (K treatment) and subsequent washing with 0.5% sodium acetate on the bound phosphorus of *B. cereus* spores.

To investigate further the dissolution process of the side body, some electron microscopic studies were made. Fig. 6 ,"C" to "G" shows the changes in electron density as the side body is dissolved. These include a mushroom-like swelling and the gradual disappearance of the side body. The stump, or neck, on the central core is indicated in Fig. 6G.

Hydrolysis with hydrochloric acid

The effect of the familiar Feulgen hydrolysis on the phosphorus fractions of resting spores and the further effect of 0.5% sodium acetate extraction on Feulgen hydrolyzed

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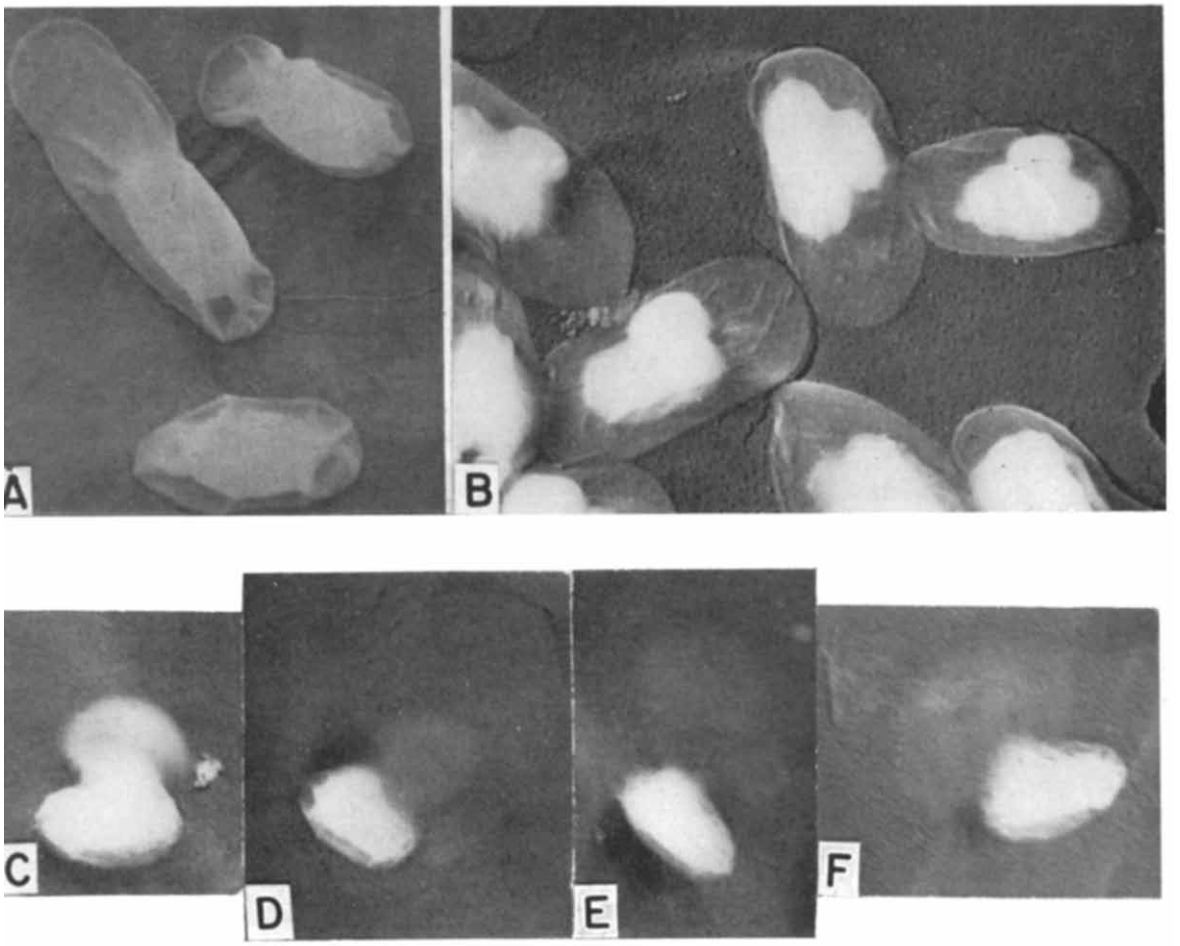

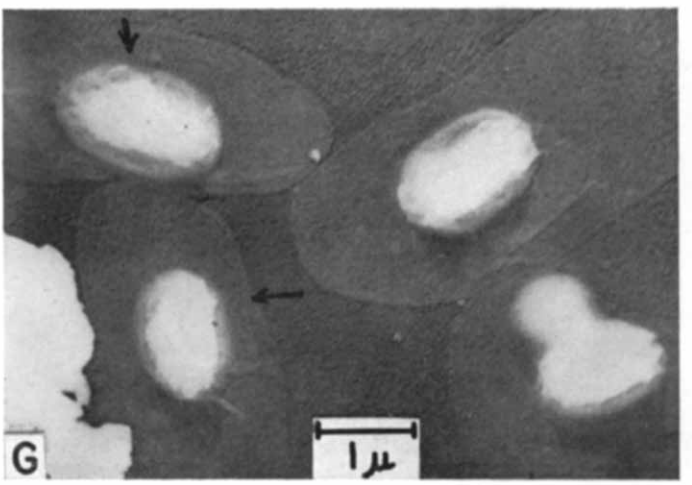

Fig. 6. Electron micrographs showing the effect of acid-permanganate hydrolysis (K treatment) and subsequent extraction with 0.5 % sodium acetate on the resting spores of *B. cereus* (magnification as indicated in G).

A. untreated resting spores.

B. K-treated resting spores (from the same lot as Fig. 1).

C to G. K-treated spores extracted with sodium acetate showing the dissolving side body. The arrows in G point to the necks left on the central core.

TABLE III

THE PHOSPHORUS DISTRIBUTION OF FEULGEN HYDROLYZED SPORES, WATER AND SODIUM ACETATE WASHED (pH 7.8)

Phosphorus as mg/100 g spores

	Untreated spores	*HCl hydrolysed spores*	
		Water washed	*Acetate washed (2 ×)*
Total P in HCl	—	567	565
Total P in Acetate washes	—	—	11
Method of SCHNEIDER[8]			
Fractionation of residues			
Acid-soluble P	39	15	10
Lipid P	12	30	30
TCA *Extracts*			
Total P	645	141	135
u.v. absorption as P	non-nucleotide interference	59	56
RNA-P (orcinol)	560	57	57
DNA-P (diphenylamine)	77	72	66
Residue P	143	110	112
Method of SCHMIDT AND THANNHAUSER[9]			
Supernatant of "S and T"			
Total P	653	217	207
Inorganic P (phosphoprotein)	44	44	44
*RNA-P + Excess P**	609	173	163
TCA Extract of *DNA precipitate*			
Total P	80	3	2
Diphenylamine-colour P	78	0	0
"S and T" Residue P	30	27	26

* *Excess P*, largely present as 7′ acid-labile P. Studies describing this material are being published separately.

"S and T", by the method of SCHMIDT AND THANNHAUSER.

spores (side bodies not removed) were compared with the above permanganate-nitric studies. Cytologically, the spores hydrolyzed in bulk with HCl were all differentiated and similar in appearance to the K-treated spores (Fig. 1).

The chemical effects of Feulgen hydrolysis on the phosphorus fractions of *B. cereus* spores are shown in Table III. The HCl treatment had altered the RNA so that 90% of it was no longer protein-bound. Some 25% of the TCA-residue P had also been removed from its normal position. The DNA-P, however, although non-recoverable as a polynucleotide in the SCHMIDT-THANNHAUSER fractionation, was not significantly altered when estimated by the diphenylamine reaction (method of SCHNEIDER[8]).

Unexpectedly, the general appearance of the crystal-violet-stained K-treated and HCl hydrolyzed spores was not markedly altered when the nucleic acids were removed by hot 5% TCA. Fig. 4 shows a group of K (or HCl) treated spores extracted with hot TCA, washed and stained with crystal violet. A large part of the side body remains, but it now stains a reddish colour similar to the central core. Stains more specific than crystal

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violet for DNA (SO_2-azure A and SO_2-thionine, after DELAMATER[20]), although effective in staining the side body before, were not taken up after hot TCA extraction by these hydrolyzed preparations. The acid dye Ponceau de xylidene RR (0.1% in 0.1 *N* HCl for 1 hour) only faintly stained the side bodies of TCA extracted, K-treated spores but stained deep red those of TCA extracted Feulgen hydrolyzed spores.

DISCUSSION

Acid hydrolysis of resting spores was accompanied by extraction and degradation of phosphorus compounds. On studying the effect of acid hydrolysis (*N* HCl, 60° C) on vegetative cells, VENDRELY AND LIPARDY[2] found that, in addition to completely extracting the RNA, some purines were split from the DNA. The results recorded here indicate that similar changes in the nucleic acids of spores occur on acid hydrolysis. Thus, there is no reason to suppose that spore nucleic acids are more resistant to hydrolysis than those of vegetative cells.

Two different hydrolysis systems were studied. One of these, a *N*/3 nitric acid solution with added potassium permanganate (0.1%) and carried out at room temperature, produces a basophilic side body which can be selectively removed by adjusting the pH to neutrality. The other, the usual Feulgen hydrolysis (*N* HCl at 60° C), produces a side body resistant to mild alkali extraction. These differences were found to be related to the presence or absence of permanganate in the acids used (ROBINOW[5]). Although the hot HCl hydrolysis did remove more RNA and less of the residual labile phosphorus than the room temperature nitric-permanganate hydrolysis, there is no evidence that these chemical effects are related to the difference in solubility of the side bodies (and DNA remnant) in mild alkali solutions. It would be more reasonable to assume that the protein of the side body, possibly the spore histone, had been more markedly damaged by the permanganate hydrolysis. This assumption is supported by the observation that side bodies of permanganate hydrolyzed spores do not stain with an acid dye, while those of HCl-treated spores do stain.

When the pH was raised to above 6.5 the complete removal of the side body was accompanied by a total extraction of desoxyribose. This suggests that the "peripheral nucleus" (ROBINOW[5]) contains all the degraded DNA. The electron micrographic appearance of the side body, the ultraviolet absorption spectrum of the acetate extract and the stained appearance of the TCA residue of K-treated spores all indicate that this side body also contains protein. The extraction by acetate of an acid-labile residue P coincidental with the removal of the side body suggests this substance as another possible component of spore chromatin.

Recent studies applying the techniques of thin sectioning (ROBINOW[4]) and disruption in fixatives (FITZ-JAMES[22]) have revealed the chromatin of the resting spore as a band or layer of variable thickness surrounding an RNA-containing central mass. When these sectioned or disrupted spores are acid hydrolyzed, the chromatin, like that of the vegetative cell, is not apparently shifted. Thus, the extrusion of the spore chromatin as a side body during hydrolysis depends, at least, upon the presence of an intact spore coat.

SUMMARY

Combined chemical (phosphorus) and cytological studies on spores hydrolyzed at room temperature with a nitric acid-permanganate solution followed by dilute alkali washing (pH 7.8) have supported the suggestion (ROBINOW[5]; BISSET AND HALE, 1951) that the "peripheral body" of

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hydrolyzed spores is nuclear in origin. The selective dissolution of the basophilic side body of such hydrolyzed spores was accompanied by the removal of all the spore DNA (as measured by the DISCHE diphenylamine reaction) along with 15 to 20% of the RNA. Combined cytological and chemical evidence indicate that protein and possibly a labile phosphorus material also constitute spore chromatin.

Both the DNA remnant and the peripheral nucleus of Feulgen hydrolyzed spores remained with the spore on washing at pH 7.8.

Both methods of hydrolysis degrade and extract phosphorus compounds, but to different degrees.

RÉSUMÉ

Des études chimiques (phosphore) et cytologiques simultanées sur des spores hydrolysées à la température ordinaire par une solution acide nitrique-permanganate puis lavées par un alcali dilué (pH 7.8) corroborent l'hypothèse (ROBINOW[5]; BISSET ET HALE, 1951) selon laquelle le "corps périphérique" des spores hydrolysées serait d'origine nucléaire. La dissolution sélective du corps basophile de spores ainsi hydrolysées s'accompagne de l'élimination de la totalité du DNA des spores (déterminé par la réaction de DISCHE à la diphénylamine) et de 15 à 20% du RNA. Des indications cytologiques et chimiques associées montrent qu'une protéine et peut-être également un produit phosphoré labile constituent la chromatine des spores.

Le reste du DNA et le noyau périphérique de spores hydrolysées au Feulgen restent dans la spore après lavage à pH 7.8.

Les deux méthodes d'hydrolyse dégradent et extraient des corps phosphorés mais à des degrés différents.

ZUSAMMENFASSUNG

Gleichzeitige chemische und cytologische Studien an Sporen von *B. cereus*, die bei Raumtemperatur mit einer Lösung von Salpetersäure-Permanganat hydrolysiert und anschliessend mit verdünnter alkalischer Lösung (pH 7.8) gewaschen wurden, unterstützen die Annahme (ROBINOW[5]; BISSET UND HALE, 1951), dass der "periphere Körper" der hydrolysierten Sporen vom Kern herstammt. Gleichzeitig mit der selektiven Auflösung der basophilen Seitenkörper solch hydrolysierter Sporen verschwindet alles DNA der Sporen (gemessen durch die DISCHE Diphenylamin Reaktion) zugleich mit 15–20% der RNA. Ferner finden sich chemische und cytologische Beweise dafür, dass auch das Chromatin der Sporen aus einem Protein und möglicherweise einer labilen Phosphorverbindung aufgebaut ist.

Nach Feulgen hydrolysierte Sporen enthalten nach Waschen bei pH 7.8 sowohl einen Rest an DNA wie auch den peripheren Kern.

Beide Hydrolysierungsmethoden erniedrigen und extrahieren Phosphorverbindungen, jedoch in verschiedenem Ausmass.

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