Isolation of Dipicolinic Acid (Pyridine-2:6-dicarboxylic Acid) from Spores of *Bacillus megatherium*

By JOAN F. POWELL
Microbiological Research Department (Ministry of Supply), Porton, Wiltshire

(*Received* 24 *October* 1952)

In a previous paper (Powell & Strange, 1953) the loss in dry weight of spores of *Bacillus megatherium* and *Bacillus subtilis* during germination was described, and accounted for by the excretion into the medium of amino-acids, peptides, glucosamine, calcium and a compound with a strong and characteristic ultraviolet absorption spectrum. In this paper the isolation and identification of the latter compound is described in detail.

MATERIALS AND METHODS

Lutidines (2:6- and 2:4-dimethylpyridines) were redistilled preparations from the compounds supplied by L. Light and Co.

Dipicolinic acid (pyridine-2:6-dicarboxylic acid) and lutidinic acid (pyridine-2:4-dicarboxylic acid) were prepared from the corresponding lutidines by oxidation with $KMnO_4$ in neutral solution (Hess & Wissing, 1915).

Quinolinic acid was recrystallized from the specimen supplied by L. Light and Co.

Dimethyl ester of dipicolinic acid. Dipicolinic acid (100 mg.) was dissolved in 20 ml. methanol saturated with dry HCl. After refluxing for 24 hr. the ester was precipitated by adding Na_2CO_3 solution. It was recrystallized from water. M.p. 122°.

Ultraviolet absorption spectra were measured with the Unicam spectrophotometer.

EXPERIMENTAL

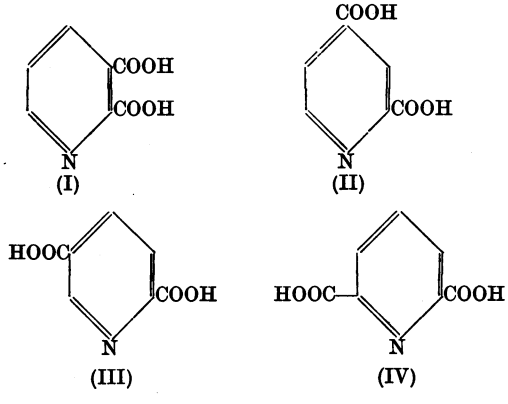
Isolation of the ultraviolet absorbing compound from exudates of germinating Bacillus megatherium *spores*

In preliminary attempts to isolate the ultraviolet-absorbing material, it was precipitated as an impure silver salt from media in which germination had occurred. A much simpler method of isolation was later discovered. The substance is acidic and occurs in exudates as a sparingly soluble calcium salt. Freeze-dried exudates were prepared from *B. megatherium* spores which had germinated spontaneously as a result of heat-activation (Powell & Strange, 1953). The resulting white feathery material was dissolved in the minimum amount of hot water. The calcium salt of the unknown acid crystallized out on cooling. After two further crystallizations from water, this salt was analysed, and found to contain C, 36·3; H, 2·7; N, 5·7; Ca, 15·8%. The free acid was readily obtained by acidifying a hot saturated solution of the calcium salt with HCl, and recrystallized from water. It crystallized in hair-like needles, m.p. 229° with decomposition. Analysis of the free acid gave C, 50·8; H, 3·1; N, 8·3%. On titration with dilute NaOH, one equivalent of alkali neutralized 81·5 g. of acid.

Identification of the ultraviolet-absorbing compound as dipicolinic acid

The first clue as to the nature of the compound appeared when the calcium salt was heated strongly in a tube. It decomposed with a marked smell of pyridine. The distillate was collected, and its ultraviolet absorption spectrum examined. It showed characteristic pyridine absorption, with maxima at 2450, 2500, 2575 and 2625 A.

This production of pyridine as a decomposition product, together with the analytical figures for the free acid and its calcium salt, indicated a pyridinedicarboxylic acid of empirical formula $C_7H_5O_4N$ (C, 50·2; H, 3·0; N, 8·4%) with a calcium salt $C_7H_3O_4NCa\ 2H_2O$ (C, 34·8; H, 2·9; N, 5·8; Ca, 16·6%). This was supported by the alkali-titration figure. The acid from spores gave a strong orange-yellow colour with $FeSO_4$ in aqueous solution, indicating that at least one carboxyl group is in the α position.



Of the four possible pyridine dicarboxylic acids with one carboxyl group in the α position, quinolinic acid (I) was eliminated after examination of its ultraviolet absorption spectrum. Moreover, quinolinic acid readily gave a fluorescein with resorcinol and H_2SO_4 (Ghosh, 1919) while the unknown acid did not. Lutidinic acid (II) gave only a faint colour with $FeSO_4$ and also had an ultraviolet absorption spectrum different from that of the unknown acid. Isocinchomeronic acid (III) was not examined, but seemed unlikely on the grounds of melting point and crystalline appearance. Finally, dipicolinic acid (IV) was prepared from 2:6-lutidine. Its ultraviolet absorption and that of its calcium salt were found to be identical with the corresponding compounds from spores (Figs. 1–3).

The dimethyl esters of dipicolinic acid and of the unknown acid were then prepared. Both substances melted at 122° and there was no depression of melting point on mixing.

It seems reasonably certain, therefore, that the ultraviolet-absorbing material which is present in resting spores of *B. megatherium* and *B. subtilis* and is excreted during germination is dipicolinic acid.

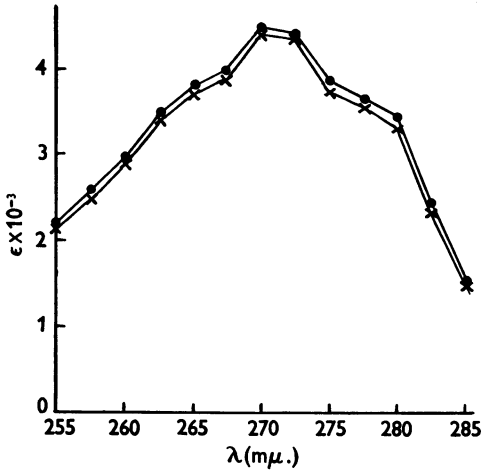


Fig. 1. Molecular extinction coefficients in 0·05 N-HCl. ●—●, dipicolinic acid; ×—×, pyridine dicarboxylic acid from spores.

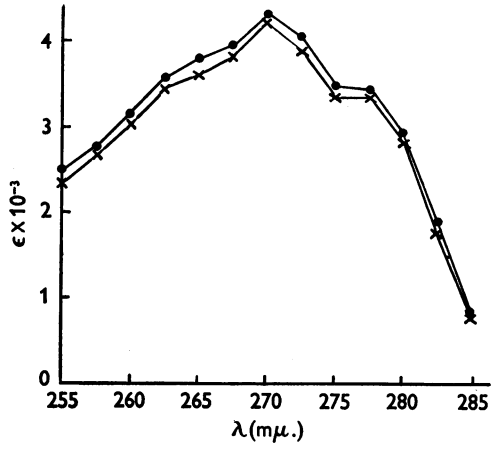


Fig. 2. Molecular extinction coefficients in 0·05 N-NaOH and 30 mM-phosphate, pH 7·3. ●—●, dipicolinic acid; ×—×, pyridine dicarboxylic acid from spores.

DISCUSSION

Dipicolinic acid has not previously been recognized as a constituent of living matter. Calcium dipicolinic constitutes 50 % of the solids excreted by germinating spores of *B. megatherium* and, therefore, approximately 15 % of the spore dry weight (Powell & Strange, 1953). Its physiological properties have yet to be determined. It was interesting to find that the calcium salt of dipicolinic acid is remarkable in its relatively high absorption at 2775 A. (Fig. 3) the ratio $\epsilon_{2700}/\epsilon_{2775}$ being 1:12. The corresponding ratios for the sodium, magnesium, barium, and aluminium salts lie in the range 1·20–1·26. It is possible, therefore, that the calcium salt has some difference in structure which may fit it for a special role in the formation and maintenance of the resting spore.

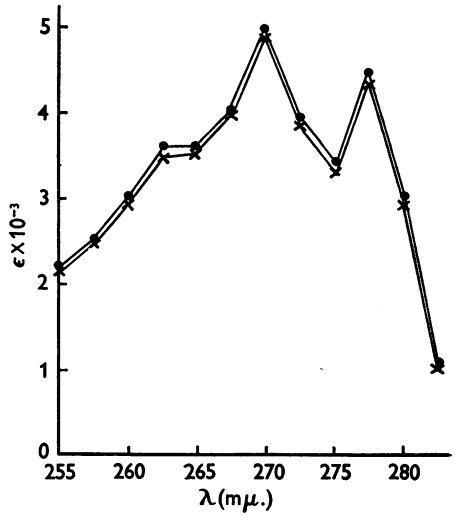


Fig. 3. Molecular extinction coefficients in 30 mM-phosphate, pH 7·3. ●—●, calcium dipicolinate; ×—×, calcium salt of pyridine dicarboxylic acid from spores.

Spore extracts from other members of the *Bacillus* group are now being examined and, so far, the characteristic dipicolinate absorption spectrum has been found in every case. It is hoped to extend these observations to the *Clostridium* group and also to observe the appearance of dipicolinic acid or possible precursors in cells during growth and sporulation.

SUMMARY

1. An acidic compound with strong and characteristic ultraviolet absorption (maxima at 2625, 2700 and 2775 A.) has been isolated, in the form of its calcium salt, from exudates of germinating spores of *Bacillus megatherium*.

2. The compound has been identified as dipicolinic acid.

I am greatly indebted to Mr L. H. Kent for very helpful advice and discussion. I also wish to thank Dr B. R. Record for freeze-drying, Mr F. E. Charlton for micro-analyses, and Mr S. Bailey for technical assistance. Permission to publish has been granted by the Chief Scientist, Ministry of Supply.

REFERENCES

Ghosh, P. C. (1919). *J. chem. Soc.* **115**, 1102.
Hess, K. & Wissing, F. (1915). *Ber. dtsch. chem. Ges.* **48**, 1907.
Powell, J. F. & Strange, R. (1953). *Biochem. J.* **54**, 205.