The method described in the present work allows the purity of the solution under test for tocopherols to be assessed and also enables the separation of the commonly occurring α-, γ- and δ-tocopherols to be achieved. Applications of the method to the examination of several oils indicate the usefulness of the method. For example, the presence of δ-tocopherol in soya-bean oil is readily ascertained, whereas its existence escaped detection by conventional methods for many years. In addition, the substance in cocksfoot grass which gives a colour with diazotized *o*-dianisidine is shown to be different from any of the known tocopherols in its behaviour on the chromatogram. Similarly, a non-tocopherol ferric-reducing substance which is not removed by the saponification, adsorption and crystallization processes is present in wheat-germ oil.

Although the method will probably not be readily applicable to routine analysis, its value for reliably detecting the presence of naturally occurring tocopherols is apparent. Application of the method to the quantitative analysis of tocopherol mixtures is being undertaken and will be the subject of a further communication.

SUMMARY

1. A method is described for the detection of α-, β-, γ- and δ-tocopherols by filter-paper chromatography.

2. The method has been used for study of the tocopherols in various vegetable oils and grasses and also in blood and milk.

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REFERENCES

Baxter, J. G., Lehman, R. W., Hove, E. L., Quaife, M. L., Weisler, L. & Stern, M. H. (1947). *Biol. Symp.* **12**, 484.
Cabell, C. A. & Ellis, N. R. (1942). *J. Nutrit.* **23**, 633.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Emmerie, A. (1949). *Ann. N.Y. Acad. Sci.* **52**, 309.
Emmerie, A. & Engel, C. (1938). *Rec. Trav. chim. Pays-Bas*, **57**, 1351.
Emmerie, A. & Engel, C. (1939). *Rec. Trav. chim. Pays-Bas*, **58**, 283.
Glavind, J., Kjølhede, K. T. & Prange, I. (1942). *Kem. Mannedsblad.* **23**, 43.
Quaife, M. L. & Dju, M. Y. (1949). *J. biol. Chem.* **180**, 263.
Quaife, M. L. & Harris, P. L. (1948). *Anal. Chem.* **20**, 1221,
Tošić, J. & Moore, T. (1945). *Biochem. J.* **39**, 498.
Weisler, L., Robeson, C. D. & Baxter, J. G. (1947). *Anal. Chem.* **19**, 906.
Williams, R. J. & Kirby, H. M. (1948). *Science*, **107**, 481.
Winteringham, F. P. W., Harrison, A. & Bridges, R. G., (1950). *Nature, Lond.*, **166**, 999.

Flavin-adenine Dinucleotide and Diaphorase in Resting and Germinated Spores, and Vegetative Cells of *Bacillus subtilis* and *Bacillus megatherium*

By ROSEMARY E. J. SPENCER and JOAN F. POWELL
Microbiological Research Department (Ministry of Supply), Porton, Wiltshire

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There are conflicting reports on the respiration rate of resting bacterial spores, but it is generally agreed that it is lower and less sensitive to cyanide than that of vegetative cells (Tarr, 1933; Keilin & Hartree, 1947). Keilin & Hartree (1949) have also found that although the spores of *Bacillus subtilis* National Collection of Type Cultures 85 (N.C.T.C.) contain considerable amounts of unidentified haematin compounds, their cytochrome content is only 6% of that of vegetative cells, and more recently Chaix & Roncoli (1950) have observed development from an atypical to a classical cytochrome spectrum during the growth of several strains of *B. subtilis*. These findings suggest that bacterial spores differ from vegetative cells in possessing an alternative to the cytochrome-cytochrome oxidase system, possibly in the form of a flavoprotein reacting with oxygen, either directly or through an unidentified haematin catalyst (Slater, 1949*a*, *b*) and that this flavoprotein system may be replaced by the cytochrome system as growth proceeds.

It is interesting to note that respiration, relatively insensitive to cyanide, has also been demonstrated in resting spores of *Neurospora crassa* (Goddard & Smith, 1938) and in unfertilized *Arbacia* eggs (Korr, 1939). The latter were shown to contain haematin compounds but no cytochrome (Ball & Meyerhof,

1940), flavin-adenine dinucleotide (Krahl, Keltch & Clowes, 1940), and a substance resembling cytochrome oxidase (Krahl, Keltch, Neubeck & Clowes, 1941). A direct connexion between decreased cyanide-sensitivity and flavoprotein synthesis in yeast was reported by Pett (1935) who found that the flavoprotein content of this organism was doubled during growth in a medium containing cyanide. In this medium respiration was reduced, although fermentation remained at a normal level.

It seemed of interest, therefore, to determine the relative amounts of flavin-adenine dinucleotide in resting spores, germinated spores (Hills, 1950; Powell, 1950) and fully developed vegetative cells of *B. subtilis* and *Bacillus megatherium*, and to attempt to demonstrate the presence of enzymes containing flavin-adenine dinucleotide as their prosthetic group.

ORGANISMS AND METHODS

Organisms

Spores of the laboratory strain of *B. subtilis* used by Hills (1950) and of *B. subtilis* N.C.T.C. 85 were grown on CCY agar (Gladstone & Fildes, 1940) at 37°. They were reaped after a minimum of 3 days, washed five times with distilled water and stored at room temperature. Vegetative cells of the laboratory strain were grown on meat extract-peptone agar (Tarr, 1933) containing 1% Lab-Lemco, 1% peptone, and 0·5% NaCl, for 16 hr. at 28°. The cells were reaped and washed twice with large volumes of saline. No spores were visible in a stained film.

Spores of the laboratory strain require L-alanine specifically for optimal germination (Hills, 1950). During 30–60 min. incubation at 37° in a medium containing 5 mM-L-alanine and 50 mM-glucose buffered at pH 7·3 with 33 mM-phosphate, 90% of the spores in a 10^9/ml. suspension lose their heat resistance, becoming at the same time less refractile and more permeable to stains (Powell, 1950). Germinated forms for the present investigation were obtained in this way and were centrifuged and made up to a suitable concentration with distilled water.

A chemically defined medium in which spores of *B. subtilis* N.C.T.C. 85 will germinate has not yet been found, so that comparable results are only available for the resting spores of this organism.

Spores of a freshly isolated strain of *B. megatherium* were grown on meat extract-peptone agar (Tarr, 1933) containing 1% peptone, 0·2% Lab-Lemco, 0·5% NaCl and 0·2% glucose. They were reaped after 2 days at 37°, washed three times with distilled water, heated to 60° for 30 min. and finally washed twice more. It was found necessary to ice-cool suspensions of these spores until they had been washed several times, as inadequately washed suspensions germinated completely at room temperature (Powell, 1951). Vegetative cells were grown on CCY agar, reaped after 16 hr. at 28° and washed twice with saline. No spores were detectable in a stained film.

Germinated spores were obtained by incubating 10^9/ml. suspensions at 37° for 60 min. in 50 mM-glucose and 33 mM-phosphate buffer at pH 7·3. During this time about 90% of the spores showed the changes associated with germination (Powell, 1951) described above.

Methods

Determination of flavin-adenine dinucleotide by combination with the protein of D-*amino-acid oxidase.* The method described by Warburg & Christian (1938) was used. To facilitate extraction of flavin-adenine dinucleotide (FAD) the spores, germinated spores or vegetative cells were broken in a Mickle (1948) tissue disintegrator. A maximum of 12 ml. of a suspension containing 10–15 mg./ml. dry weight were shaken with 8 g. Ballotini beads, size 12, for 45–50 min., a drop of tributyl citrate being added to prevent foaming. After this treatment, very few intact cells could be seen in a stained film. Oxygen uptake was measured at 37° in Warburg vessels containing the broken cell material, an appropriate D-amino-acid, and an excess of D-amino-acid oxidase protein, in an atmosphere of oxygen. The system was buffered at pH 7·3 rather than at the optimum pH 8·8, since Krebs (1935) and Ochoa & Rossiter (1939) report a rapid fall in activity of the enzyme under alkaline conditions. Controls were set up in which the cell material was replaced by a crude preparation of FAD. Total FAD was determined on a portion of the broken cell suspension which had been heated to 100° and held at 75–80° for 10 min. in order to split the FAD from its enzyme proteins. Free FAD was determined on unheated samples.

Preparation of D-*amino-acid oxidase protein.* The apoenzyme of D-amino-acid oxidase was prepared from pig kidneys by a modification of the method of Negelein & Brömel (1939). Kidney cortex was minced and immediately freeze-dried. The freeze-dried material was then finely powdered and treated twice with large volumes of acetone. The acetone was removed by straining the tissue in a muslin bag, and then passing a stream of dry air through the powder for several hours. Although the freeze-dried material had quite high D-amino-acid oxidase activity before the acetone treatment, further purification omitting this step resulted in an entirely inactive preparation. The enzyme was extracted from the dried powder with 10 mM-phosphate buffer at pH 7·3 instead of the pyrophosphate used by Negelein & Brömel (1939), and the extract was treated as described in steps 1–5 of the above method. The final precipitate was freeze-dried and kept in a desiccator.

Preparation of flavin-adenine dinucleotide. The prosthetic group of D-amino-acid oxidase was prepared from baker's yeast (United Yeast Co.) according to the method of Warburg & Christian (1938) with a modification in the final stages suggested by the work of Straub (1938). The method was followed as far as the first precipitation of FAD with barium and the removal of the barium as barium sulphate. At this stage, the FAD was precipitated with a large volume of acetone (Straub, 1938), centrifuged, redissolved in a small volume of water and freeze-dried.

Absorption maxima for this preparation occurred at 260, 375 and 450 mμ. (Unicam spectrophotometer). Molecular extinctions at 375 and 450 mμ. were compared with those of the pure barium salt (Warburg & Christian, 1938) and indicated that the crude preparation contained not more than 10% (Table 1) of flavin-adenine dinucleotide, and probably rather less, since absorption by any free riboflavin present as an impurity was not taken into account.

An accurate estimate of the purity of this FAD preparation was obtained by comparison of its activity as the prosthetic group of D-amino-acid oxidase, with that of a sample of the barium salt of FAD (Warburg & Christian, 1938) containing only 13% of water. The conditions were the same as those used in the estimation of FAD in cell material. The weights of the two preparations were accurately determined on a microbalance and checked by measuring the extinctions of the solutions in the Unicam spectrophotometer at 450 mμ.

The molecular extinction coefficient ϵ is the optical density of a solution of concentration 1 g.mol./l. and path length 1 cm. Warburg & Christian (1938) measure the absorption coefficient, μ, which is the natural logarithm of the reciprocal transmission

$$\epsilon = \mu/(2{\cdot}3026 \times 10^3).$$

was transferred to a water bath maintained at 38°. The time needed for 90% reduction of the methylene blue was noted. Broken suspensions of resting and germinated spores and of vegetative cells, previously analysed for FAD were substituted for diaphorase and their activities compared. Some cell suspensions were also tested in the presence of added diaphorase, and of larger amounts of CoI (1 mg.). In a few experiments with broken spores the location of the enzyme activity was determined by testing the supernatant liquid and the cell debris separately after centrifuging.

Preparation of lactic dehydrogenase. The enzyme was prepared from ox heart by a modification of Straub's (1940) method. The heart muscle was minced, and the enzyme extracted with ice-cold water and precipitated by 60% saturation with ammonium sulphate. After centrifuging in the cold, the precipitate was dissolved in 10 mM-phosphate buffer, pH 7·3, and recentrifuged to remove insoluble material. A second precipitation at 60% saturation with ammonium sulphate gave a preparation which, when dissolved in a small volume of phosphate buffer, retained its activity during 2 months' storage at 4°. The enzyme solution was subsequently freeze-dried without loss of activity.

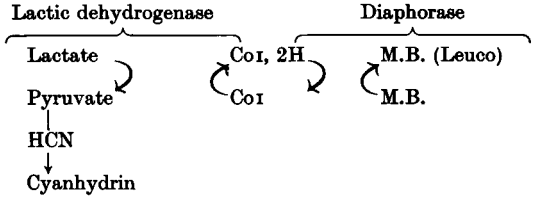
Table 1. *Spectrophotometric absorption data for flavin-adenine dinucleotide preparation*

Wavelength (mμ.)	260	375	450
Concn. of solution of crude FAD in μg. total solid/ml.	89	445	445
Extinction coefficient of crude FAD solution	1·3	0·50	0·535
Molecular extinction of crude FAD solution	$1{\cdot}34 \times 10^4$	$0{\cdot}103 \times 10^4$	$0{\cdot}111 \times 10^4$
Molecular extinction of pure barium salt (Warburg & Christian, 1938)	$3{\cdot}64 \times 10^4$	$0{\cdot}895 \times 10^4$	$1{\cdot}13 \times 10^4$
Percentage FAD in crude preparation	36·8	11·5	9·85

Detection of flavoproteins. L-Amino-acid oxidase, D-amino-acid oxidase and xanthine oxidase were assayed by measuring O_2 uptake, in an atmosphere of O_2, in the presence of the specific substrate.

For the detection of amino-acid oxidases, the system contained 16 mM-amino-acid, 40 mM-phosphate buffer at pH 7·3 and 10–20 mg. dry weight of cell material. Synthetic DL-alanine, valine, phenylalanine, norleucine and methionine were used. L-Leucine and L-proline were also tested at a concentration of 8 mM. To detect xanthine oxidase 3·3 mM-xanthine was substituted for the amino-acid.

The system used to detect diaphorase was that of Straub (1939; 1940) and Sumner & Krishnan (1948). It contained 0·2 ml. 10% sodium lactate, 0·5 ml. lactic dehydrogenase solution, 1 ml. 2% (w/v) HCN, 20–40 μg. coenzyme I (CoI), 1 ml. diaphorase solution, 0·1 ml. 0·05% (w/v) methylene blue, and 0·5 ml. 200 mM-phosphate buffer, pH 7·3. The reaction proceeds anaerobically thus:



diaphorase activity being indicated by decolorization of the methylene blue.

The method of Friedemann & Hollander (1942) was used in this test, because a qualitative result only was required. The results obtained cannot be regarded as quantitative owing to the presence of dissolved oxygen in the medium. An equal volume of 2% (w/v) melted agar, cooled to 45°, was added to the complete system contained in an ice-cooled test-tube. The contents of the tube were thoroughly mixed and when the agar had solidified (1 min.) the tube

Spore respiration. Oxygen uptake of spores was measured in Warburg manometers in 33 mM-phosphate buffer pH 7·3 at 37°, with and without the addition of 50 mM-glucose. In determinations of the cyanide-sensitivity of the respiration, steady overall concentrations of cyanide were maintained by the use of KOH-KCN mixtures in the centre well (Potter, 1948).

Chemicals. Coenzyme I (cozymase) was a commercial preparation supplied by L. Light and Co. Ltd., who also supplied DL-alanine, DL-valine, DL-phenylalanine, and DL-norleucine, and L-leucine and L-proline. L-Alanine was supplied by Roche Products Ltd. HCN 2% (w/v) was prepared freshly by neutralizing a KCN solution with H_2SO_4.

RESULTS

Flavin-adenine dinucleotide content of cells

A system containing 3 mg. D-amino-acid oxidase protein, 33 mM-DL-methionine, 2·7 μg. crude FAD and 75 mM-phosphate buffer (pH 7·3) in a total volume of 2·5 ml. gave an oxygen uptake of 53 μl./10 min. The rate of oxygen uptake was directly proportional to the amount of FAD added, provided

that the apoenzyme was in excess. With methionine as substrate the rate was four times as great as with DL-alanine. This activity ratio is twice that found by Krebs (1935) and is probably due to the presence of inhibitory substances in the synthetic DL-alanine, since another batch of this amino-acid inhibited the enzyme completely. Methionine was therefore generally used as substrate.

When disintegrated cells were substituted for crude FAD in the above system an oxygen uptake was recorded proportional to the amount of cell material added. Thus the relative amounts of free and combined FAD in spores, germinated spores and vegetative cells could be estimated. Compared with the pure barium salt of FAD, the preparation used as a standard contained 76·8 ± 3·0 mg. active FAD/g. The values given in Table 3 for the total FAD content of the cells examined are calculated from this estimation of the purity of the standard. The standard error quoted is that of the mean of two experiments from each of which quadruplicate results for both preparations were obtained.

The results of a typical experiment are summarized in Table 2. It was found that, in the absence of the substrate, or FAD, or the apoenzyme, the system was inactive. No oxygen uptake was recorded when cell material was substituted for FAD, unless both substrate and enzyme protein were present.

In a duplicated experiment, 40 μg. of FAD preparation were added to 10 ml. of spore suspension, the total and free dinucleotide activity then being measured in the usual way. The results indicated that none of the added FAD was destroyed during cell breakdown, but that when the total dinucleotide activity was determined after heating, there was a 30% fall in activity of the added material. On the other hand, when a broken spore suspension was heated for 30 min. at 75–80°, no fall in total dinucleotide activity was detected. It therefore seems probable that the partially purified FAD is more thermolabile than the FAD in the spore.

Experiments in which broken spore suspensions were centrifuged and the supernatant liquid substituted in the test system, showed that FAD activity was not associated with the cell debris. The addition of tri-butyl citrate, used to prevent foaming during cell breakdown, was found to have no effect on the oxygen uptake of the D-amino-acid oxidase system.

Table 2. *Protocol of experiment to assay flavin-adenine dinucleotide content of* Bacillus subtilis *spores*

	Controls (ml.)				Unheated spores (ml.)				Spores heated to 100° (ml.)			
Manometer	1	2	3	4	5	6	7	8	9	10	11	12
Water	1·25	1·25	1·5	1·0	0·5	0·75	0·25	0·75	0·5	0·75	0·25	0·75
Phosphate buffer, 200 mM, pH 7·3	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
DL-Methionine, 163 mM (in side arm)	Nil	0·25	0·25	0·25	Nil	0·25	0·25	0·25	Nil	0·25	0·25	0·25
Flavin-adenine dinucleotide, 8 μg. crude preparation/ml.	0·25	Nil	0·25	0·25	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
D-Amino-acid oxidase protein, 6 mg./ml.	0·5	0·5	Nil	0·5	0·5	Nil	0·5	0·5	0·5	Nil	0·5	0·5
Broken suspension of *B. subtilis* spores 5·5 mg./ml. (dry wt.)	Nil	Nil	Nil	Nil	1·0	1·0	1·0	0·5	1·0	1·0	1·0	0·5
20% (w/v) KOH (in centre well)	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2
Oxygen uptake (μl./10 min.)	0	0	0	40	0	0	21	10·5	0	0	41	20

Table 3. *Flavin-adenine dinucleotide content of bacterial cells*

(The content was estimated by measurement of the O_2 uptake of the unheated and heated suspension (= free and bound FAD) in presence of D-amino-acid oxidase apoenzyme + methionine. 1 ml. O_2/10 min. = 3·56 μg. FAD.)

	No. of experiments	Dry wt. of cells (mg.)	Spore count	Mean O_2 uptake of unheated suspension. Free FAD (μl./10 min.)	Mean O_2 uptake of heated suspension. Total FAD (μl./10 min.)	Percentage combined FAD	Total FAD (μg./g. dry wt.)
B. subtilis (lab. strain):							
Spores	5	5·56	10^{10}	22·2	42·2	48	29·4
Germinated spores	4	2·2 (approx.)	10^{10}	20·25	39·6	49	—
Vegetative cells	4	1·8	—	3·0	27·5	80	123·0
B. megatherium:							
Spores	11	7·76	10^{10}	9·3	59·0	84	29·7
Germinated spores	5	—	10^{10}	9·2	59·2	85	—
Vegetative cells	4	2·07	—	2·0	40·0	90	98·0
B. subtilis N.C.T.C. 85:							
Spores	3	7·35	$2·2 \times 10^{10}$	52	54·5	5	28·9

The figures for FAD content of resting spores of two strains of *B. subtilis* and one strain of *B. megatherium* are given in Table 3. Although there were great differences in the ratio of free to combined FAD in the three organisms, the total amounts were remarkably similar.

Vegetative cells contained considerably greater amounts (3–4 times) of FAD. Comparisons were made on a basis of dry weight, which was determined by heating continuously at 104° for at least 48 hr. In germinated spores of the laboratory strain of *B. subtilis* and *B. megatherium*, both the total FAD and the ratio of free to combined FAD were the same as in resting spores on the basis of spore number. In this case, dry weight was not considered a satisfactory basis for comparison since germinated spores of *B. subtilis* were found to have a dry weight less than half that of the same number of resting spores, although no cell division had occurred. An explanation is at present being sought for this result. Slight inaccuracies must arise in this determination since after centrifuging the germinated spore suspension small amounts of medium still remain associated with the residue. Washing would have removed these, but might have introduced further errors, for example, by extracting soluble material from the germinated spore. The dry weight of germinated spores of *B. megatherium* was not determined.

Occurrence of flavoproteins

L-Amino-acid oxidase and D-amino-acid oxidase. No oxygen uptake generally occurred with resting spore suspensions of the laboratory strains of *B. megatherium* and *B. subtilis* in the presence of any of the amino-acids tested. In some cases in which a small oxygen uptake was recorded, it could be attributed to germination during the course of the experiment. Germinated spores possessed a low amino-acid oxidase activity (1 μl./hr./mg. dry weight) which was completely lost when the suspension was broken up.

Xanthine oxidase. Negligible rates of oxygen uptake were observed in the cells tested with xanthine as substrate. Vegetative cells of *B. subtilis* gave the highest oxygen uptake, which was only about 1 μl./hr./mg. dry weight.

Diaphorase. An enzyme similar in its action to the diaphorase of heart muscle was present in all cells of the three organisms studied. The enzyme remained in the supernatant liquid after the broken cell suspension had been centrifuged at $1100 \times g$ for 20 min. and was completely inactivated by heating for 5 min. at 100°. The diaphorase-like enzyme in resting and germinated spores of *B. megatherium* required the addition of much larger amounts of Co I for its demonstration than did that in the two strains of *B. subtilis*. It was later found that the activity of the heart diaphorase preparation was completely inhibited by broken spores of *B. megatherium*. The inhibition could be prevented by the addition of larger amounts (1 mg.) of Co I, but not by excess FAD. It seems that the spores of *B. megatherium* contain a substance which either removes, or competes with, Co I, under the experimental conditions. The inhibitor was associated with the cell debris and was stable during 10 min. at 100°. No such substance was present in the spores of *B. subtilis*, and moreover the action of the diaphorase-like enzyme which they contain was only slightly inhibited by addition of broken *B. megatherium* spores. This effect could not be accounted for by the presence of relatively large amounts of Co I in *B. subtilis* spores, since they were unable to reduce methylene blue when added to the dehydrogenase system in the absence of added Co I.

It may be significant that the times for reduction of methylene blue by the various cells were roughly in inverse proportion to the amount of bound FAD contained in these cells.

Oxygen uptake of spores

We have attempted to measure the oxygen uptake of resting spores of the laboratory strain of *B. subtilis* in phosphate buffer only. None could be detected using a micro-Warburg apparatus (Barker, 1949) containing up to 30 mg. dry weight of a ten-times washed suspension. A measurable, but variable, oxygen uptake occurred in the presence of 50 mM-glucose. This could be reduced by heating at 60° for 15 min., the Q_{O_2} over an experimental period of 2·5 hr. then being 0·42 compared with Q_{O_2} of 2·1 over the same period reported by Keilin & Hartree (1947) for spores of *B. subtilis* N.C.T.C. 85. The oxygen uptake of spores of the laboratory strain in 50 mM-glucose was found to increase 200-fold after 30 min. incubation in 5 mM-L-alanine. This high oxygen uptake, associated with germinated spores, was found to be considerably less sensitive to cyanide than that of vegetative cells. Thus 0·1 mM-HCN caused 54 % inhibition of vegetative cell respiration and 24 % inhibition of germinated spore respiration. Similar results were obtained with germinated spores and vegetative cells of *B. megatherium*. Here, 0·2 mM-HCN gave 70 % inhibition of vegetative cell respiration and 40 % inhibition of germinated spore respiration. Corresponding figures for 0·4 mM-HCN were 79 and 58 % respectively. These results imply that the oxygen uptake of resting spores may be even lower than that hitherto recorded, and that the cyanide-insensitive respiration previously considered to be a property of resting spores may be in fact due to a small proportion of germinated forms present in the suspensions.

DISCUSSION

Flavin-adenine dinucleotide and an enzyme closely resembling diaphorase in function have been found in all the resting spores, germinated spores and vegetative cells tested. Although the total amounts of flavin-adenine dinucleotide in spores of the three organisms investigated are very similar, the ratios of free to combined dinucleotide differ considerably. Diaphorase activity appears to run roughly parallel with the proportion of bound dinucleotide. These findings may not, however, represent the state in the intact spore, since it is possible that flavin-adenine dinucleotide is split from its apoenzyme to varying degrees in different organisms during cell breakdown.

During germination, i.e. the primary change involving loss of heat resistance of spores, no alteration could be detected in the total, or in the ratio of free to combined, flavin-adenine dinucleotide, when the comparison was made on the basis of spore number. Vegetative cells contained three to four times as much flavin-adenine dinucleotide per unit of dry weight and 80–90 % of this was in the combined state. They also had a much higher diaphorase activity than spores. A comparison between spores and vegetative cells based on dry weight must, however, be made with some caution, since the spore coat contributes an unknown proportion of the dry weight. Even with this reservation it does not seem that development from spore to vegetative cells is accompanied by a fall in flavin-adenine dinucleotide content or diaphorase activity. In view of the findings of Curran, Dewar, Gordon and Green (1939), indicating that xanthine oxidase can replace diaphorase in the dehydrogenase-methylene blue system, it is important to note that negligible xanthine oxidase activity was detected in the organisms tested.

It is significant that the respiration rate of spores of *B. subtilis* N.C.T.C. 85 was reported respectively by Cook (1931), Tarr (1933) and Keilin & Hartree (1947) as 90, 40 and 6 % of that of vegetative cells. It seems probable that the spore suspensions used contained germinated forms not easily characterized except by their lack of heat resistance and slight difference in staining properties (Powell, 1950). For instance, in the spore suspensions employed by Tarr (1933) fully developed vegetative cells were observed after 2 hr. incubation, so that, in this case at least, germination must have taken place at a considerably earlier stage in the experiments. Comparative insensitivity to cyanide cannot be taken as a criterion of resting spore respiration, since we have found that the oxygen uptake of germinated spores is also considerably less sensitive to cyanide than that of vegetative cells.

The occurrence of combined flavin-adenine dinucleotide, together with the demonstration of a diaphorase-like enzyme in spores of *B. subtilis* and *B. megatherium*, offers some support for the view that bacterial spores possess a respiratory mechanism involving a flavoprotein. We have found the oxygen uptake of resting spores in buffered glucose to be extremely low. If the uptake measured is, in fact, that of resting spores and not due to a small proportion of germinated forms, its cyanide-insensitive component may possibly be attributed to slow autoxidation of the diaphorase-like enzyme. If this enzyme is indeed diaphorase, it must be coupled with a readily autoxidized catalyst in germinated spores to account for their relatively high respiration rate. This catalyst may well be a haematin compound of comparatively low cyanide sensitivity.

In accounting for the low sensitivity of their respiration to cyanide, the possibility must not be ignored that spores may contain a large excess of heat-stable cytochrome oxidase (Militzer, Sondegger & Tuttle, 1950) over cytochrome *c* or some other intermediate constituent of the system. The amount of cytochrome *c* or other intermediate compound would then be the limiting factor in the oxidation, even when a large part of the cytochrome oxidase was inactivated by an inhibitor. Preliminary experiments to investigate this point have shown that both intact and broken resting spores of the three organisms used in this report will catalyse the oxidation of dimethyl *p*-phenylenediamine and quinol (Powell, 1952). These oxidations do not, however, show the characteristics of a typical cytochrome oxidase catalysis. Moreover, the oxidizing activity of spores disappears during 3–4 months' storage in water at room temperature, although viability and tendency to germinate in the chemically defined media are maintained. It seems possible, therefore, that an atypical cytochrome-cytochrome oxidase system may, at first, exist in resting spores, but that this system is not necessary to maintain viability or to promote the initial chemical changes involved in germination.

SUMMARY

1. Flavin-adenine dinucleotide has been shown to be present in all the cells tested, i.e. resting spores, germinated spores, and vegetative cells of laboratory strains of *Bacillus subtilis* and *B. megatherium*, and resting spores of *B. subtilis* N.C.T.C. 85.

2. During germination of spores of the laboratory strains of *B. subtilis* and *B. megatherium* in chemically defined media, no change in total flavin-adenine dinucleotide content, or in the relative amounts of free and bound dinucleotide occurred.

3. No L-amino-acid oxidase, D-amino-acid oxidase or xanthine oxidase activity could be demonstrated in resting spores of *B. subtilis* and *B. megatherium* or in germinated spores of *B. megatherium* and the laboratory strain of *B. subtilis*.

4. An enzyme capable of oxidizing reduced coenzyme I and reducing methylene blue occurred in all types of cell of *B. subtilis* and *B. megatherium*.

5. Spores of *B. megatherium* contained a heat-stable substance which inhibited reduction of methylene blue by heart diaphorase, the inhibition being prevented by the addition of large amounts (1 mg.) of coenzyme I.

6. No oxygen uptake could be measured with resting spores of the laboratory *B. subtilis* in phosphate buffer. The Q_{O_2} of these spores in buffered glucose, after preliminary heating at 60° for 15 min., was 0·42.

7. Oxygen uptake of *B. subtilis* and *B. megatherium* spores increased approximately 200 times following germination. This oxygen uptake was considerably less sensitive to cyanide than that of fully developed vegetative cells.

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REFERENCES

Ball, E. G. & Meyerhof, B. (1940). *J. biol. Chem.* **134**, 483.
Barker, S. B. (1949). *Proc. Soc. exp. Biol., N.Y.*, **72**, 390.
Chaix, P. & Roncoli, G. (1950). *Biochim. Biophys. Acta*, **6**, 268.
Cook, R. P. (1931). *Zbl. Bakt.* (1 Abt. Orig.), **122**, 329.
Curran, H. S., Dewan, J. G., Gordon, A. H. & Green, D. E. (1939). *Biochem. J.* **33**, 1694.
Friedemann, U. & Hollander, A. (1942). *J. Lab. clin. Med.* **28**, 353.
Gladstone, G. P. & Fildes, P. (1940). *Brit. J. exp. Path.* **21**, 161.
Goddard, D. R. & Smith, P. E. (1938). *Plant Physiol.* **13**, 241.
Hills, G. M. (1950). *J. gen. Microbiol.* **4**, 38.
Keilin, D. & Hartree, E. F. (1947). *Ned. Tijdschr. Hyg.* **12**, 115.
Keilin, D. & Hartree, E. F. (1949). *Nature, Lond.*, **164**, 254.
Korr, I. M. (1939). *Cold Spr. Harb. Sym. exp. Biol.* **7**, 120.
Krahl, M. E., Keltch, A. K. & Clowes, G. H. A. (1940). *Proc. Soc. exp. Biol., N.Y.*, **45**, 719.
Krahl, M. E., Keltch, A. K. Neubeck, C. E. & Clowes, G. H. A. (1941). *J. gen. Physiol.* **24**, 597.
Krebs, H. A. (1935). *Biochem. J.* **29**, 1620.
Mickle, H. (1948). *J. R. micr. Soc.* **68**, 10.
Militzer, W., Sondegger, T. B. & Tuttle, L. C. (1950). *Arch. Biochem.* **26**, 299.
Negelein, E. & Brömel, H. (1939). *Biochem. Z.* **300**, 225.
Ochoa, S. & Rossiter, R. J. (1939). *Biochem. J.* **33**, 2008.
Pett, L. B. (1935). *Biochem. J.* **29**, 927.
Potter, V. R. (1948). *Methods in Medical Research*, vol. 1, p. 308. Chicago: Year Book Inc.
Powell, J. F. (1950). *J. gen. Microbiol.* **4**, 330.
Powell, J. F. (1951). *J. gen. Microbiol.* **5**, 993..
Powell, J. F. (1952). To be published.
Slater, E. C. (1949*a*). *Biochem. J.* **45**, 1.
Slater, E. C. (1949*b*). *Biochem. J.* **45**, 14.
Straub, F. B. (1938). *Nature, Lond.*, **141**, 603.
Straub, F. B. (1939). *Biochem. J.* **33**, 787.
Straub, F. B. (1940). *Biochem. J.* **34**, 483.
Sumner, J. B. & Krishnan, P. S. (1948). *Enzymologia*, **12**, 221.
Tarr, H. L. A. (1933). *Biochem. J.* **27**, 136.
Warburg, O. & Christian, W. (1938). *Biochem. Z.* **298**, 150.

Pseudo-Cholinesterase Activity in the Central Nervous System

BY MARGERY G. ORD* AND R. H. S. THOMPSON
Department of Chemical Pathology, Guy's Hospital Medical School, London

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In an earlier report on the types of cholinesterases present in the tissues of the rat (Ord & Thompson, 1950), it was shown that whereas whole brain, striated muscle and suprarenal gland contain only small amounts of a 'non-specific' (Nachmansohn & Rothenberg, 1945) or 'pseudo'-cholinesterase (Mendel & Rudney, 1943*a*), the other tissues which were studied each showed a considerable level of activity of this type of esterase; stomach, liver, lung and salivary gland were found to hydrolyse benzoylcholine and acetyl-β-methylcholine at approximately equal rates, while heart, intestinal muscle and mucosa, Harderian gland and skin contain predominantly a pseudo-cholinesterase, benzoylcholine being hydrolysed from two to six times more rapidly than acetyl-β-methylcholine.

Although no evidence could at that time be brought forward as to the physiological significance of this widely distributed pseudo-cholinesterase, it was decided to extend this study to human tissues,

* Present address: Department of Biochemistry, University of Oxford.