SENSITIVITY OF *CLOSTRIDIUM LACTO-ACETOPHILUM* TO IRON

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Abstract

The feasibility of using *Clostridium lacto-acetophilum* for the microbiological assay of iron in pharmaceutical preparations like *Lohasava* is described. The present method permits estimation of iron in microquantities (1–20 p.p.m.).

The role of iron in the nutrition and metabolism of animals is now well established. Of the most important functions of iron in the body is to act as a catalyst in the oxidation-reduction processes. In iron enzymes this element is much more efficient as a catalyst than in its ionic state. It also acts as an oxygen carrier in the vertebrates as well as some invertebrates, the principal carrier being hæmoglobin. Iron is also stored in the spleen and liver as ferritin. Cytochromes, cytochrome oxidase, catalase and peroxidase are some of the iron containing enzymes. In recent years it has even been concluded that the total iron present in the animal system is greater than the sum of all iron found in other known iron compounds.[13]

Hæmatin, otherwise known as factor X, has for long been recognized as an important growth factor for bacteria of the genus Hemophilus. Lwoff and Lwoff[7] have demonstrated the essentiality of hæmatin in the respiratory activities of *H. influenzæ*. Likewise, the demand for an organo-iron compound, coprogen, for the growth by the coprophylic fungus *Pilobolus*,[4, 5] for ferrichrome (or iron) by the smut fungus *Ustilago sphærogena*,[8, 9] and for "Terregens factor" by *Arthrobacter terregens*[6] has come to be observed in recent years. The ability of *Aspergillus niger* and *Penicillium glaucum* to utilize traces of iron, copper, zinc, manganese and molybdenum has been made use of in the bioassays of these metals, (Nicholas,[11] Donald, Passay and Swaby,[3] Saraswati Devi[12]) but so far as the element iron is concerned the applicability of the assay method under diversity of conditions remains to be established. Some aspects of microbial metabolism of iron have recently been reviewed by Neilands.[10] Literature pertaining to the need for iron in the nutrition and metabolism of bacteria has been pertinently indicated by Bhat[1] whilst demonstrating iron as an essential element for the nutrition as well as metabolism of an anaerobic bacterium *Clostridium lacto-acetophilum* (Strain 3) isolated by him and Barker from soil.[2] It was shown[1] that progressive elimination of iron from the medium by chelation with α-α′-dipyridyl had the effect of rendering an otherwise suitable medium increasingly unsuitable so much so growth could be suppressed with incorporation of 0·0006% of the chelating agent into the medium

containing 0·002% iron in the form of ferrous sulphate. This study gave an indication that the organism could perhaps be used for the microbiological assay of iron contained in certain indigenous pharmaceutical preparations like *Lohasava* (an Ayurvedic preparation containing iron and from which it derives its name) recommended as a tonic and an useful aid in the treatment of tuberculosis, anæmias and jaundice. The attempts made towards this end are presented in this paper.

EXPERIMENTAL

The basal medium employed in all the experiments was the one described previously[2] with this difference that it contained 2% glucose instead of lactate and acetate together as source of carbon and energy, and did not have added $FeSO_4 \cdot 7\,H_2O$ in it. The growth response of the organism to Fe^{++} present in the medium was observed by incorporating graded quantities $FeSO_4 \cdot 7\,H_2O$ solution

TABLE I

Concentration of Fe in 100 ml. mg.	Growth response (Hilger Reading)
Nil	10
0·1	14·5
0·4	15·2
0·6	16·0
0·8	16·5
1·0	17·0
1·4	17·4
2·0	18·5
2·2	18·8
2·8	18·8
3·0	18·9
3·4	19·2
3·8	19·8
4·0	20·0

into the basal medium until the final concentration of iron in the test medium ranged from 0·1 mg./100 ml. to 4·0 mg./100 ml. As the organism is anaerobic the tubes $6'' \times \frac{5}{8}''$ after filling with the medium were plugged with non-absorbent cotton and autoclaved at 15 lb. pressure for 20 minutes. While using the tubes for inoculation they were placed in a boiling water-bath for 10 minutes and then suddenly cooled. They were immediately used for inoculation.

The organism was repeatedly subcultured in the medium containing $FeSO_4 \cdot 7\ H_2O$ in the concentration of 2 mg./100 ml. before a freshly grown (24 hours old) culture was centrifuged and the deposit of cells obtained. The cells were freed from the media, etc., by washing with sterile normal saline and centrifuging again. They were then suspended in 5 ml. sterile saline to be used as the inoculum. Two drops of the inoculum were added to each of the assay tube. The tubes were then incubated at 37° C. for 48 or more hours for maximum growth to appear, which was then estimated in the Hilger-photoelectric colorimeter using filter No. 8 (675 $m\mu$). The basal medium (without iron) which was also inoculated (but which showed no growth) was adjusted to 10.

The growth response of the organism obtained with $FeSO_4 \cdot 7\ H_2O$ added into the basal medium in graded quantities is represented in the accompanying table and figure.

It will be observed from the graph that growth curve is sharp only to concentrations of iron from 0·1 mg./100 ml. to 2·0 mg./100 ml.; also that growth does not take place unless iron is present.

Practical usefulness of the assay method was then studied by recording the growth response of the organism into a medium containing *Lohasava* as the source of iron. The method of inoculation and estimation of the growth in the assay tubes was the same as described before. As the *Lohasava* is a deeply coloured solution the organism was removed by centrifugation from the media after growth had taken place and suspended in distilled water (to volume) for measurement of growth. The growth response obtained with *Lohasava* (Sample No. Z. B. 1028) is recorded in Table II.

TABLE II

Dilution of *Lohasava* in 100 ml. medium	Growth response (Hilger reading)	Corresponding iron concentration per 100 ml. medium (found from graph)	Concentration of iron in 100 ml. *Lohasava* (calculated)	Concentration of iron in 100 ml. *Lohasava* (by chemical analysis)
Nil	10·0	..	..	..
1·22 ml.	16·2	0·7 mg.	57·4 mg.	..
2·45 ml.	17·8	1·5 mg.	61·22 mg.	62·55 mg.
3·30 ml.	18·4	2·0 mg.	60·6 mg.	..

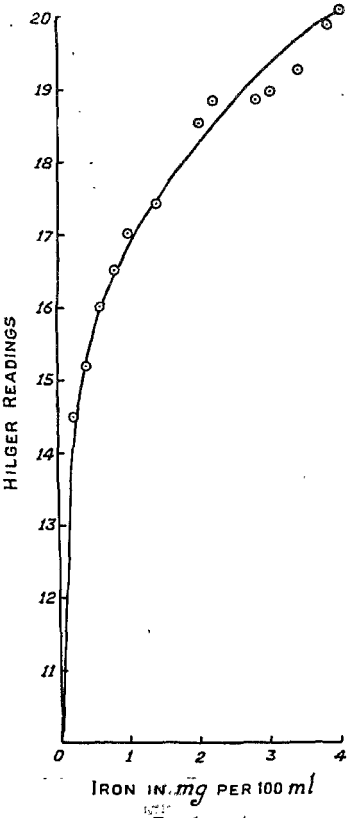

FIG. 1

It will be seen from the table that the values obtained for iron content in the solution agree well with those obtained by the chemical analysis of large amounts of the medicinal preparation.

Having thus observed that the growth response of the organism to iron in the Fe^{++} form (in ferrous sulphate) and *Lohasava* is quantitative, we attempted to study the growth response of the organism to Fe^{++}, Fe^{+++}, iron in both organic and inorganic combinations in order to determine whether the method is applicable in every instance. The following salts were used for the experiment:

Ferrous acetate, ferrous sulphate, ferrous ammonium sulphate, ferric chloride, and ferric ammonium citrate.

The readings obtained with the media containing the above iron salts in graded quantities are recorded in Table III.

TABLE III

Iron salts	Iron in mg./100 ml. medium	Growth response (Hilger reading)
1. Ferrous sulphate	Nil	10
	0·1	14·5
	0·4	15·2
	1·0	17·0
	1·4	17·4
	2·0	18·5
2. Ferrous ammonium sulphate ..	0·1	14·4
	0·4	15·1
	1·0	16·8
	1·4	17·3
3. Ferrous acetate	0·1	14·3
	0·4	15·0
	1·0	16·8
4. Ferric chloride	0·1	14·4
	1·0	16·7
	2·0	18·1
5. Ferric ammonium citrate ..	0·1	14·6
	0·4	15·1
	1·0	16·6

A glance at the above table will clearly indicate that the organism is able to utilise iron in any form—ferrous, ferric, organic or inorganic—and that the growth obtainable is proportional to the total iron present in the media. This permits the organism to be used in the assay or iron.

It may be pointed out here that *Aspergillus niger* and *Penicillium glaucum* have been reported to detect iron in as low a quantity as 0·002 p.p.m.[11] but the ability of the fungi to respond to the element over a period of time is not known.

Clostridium lacto-acetophilum, on the other hand, has now been under critical study for well over a decade. The sensitivity of this bacterium to concentrations of iron lower than 1 p.p.m. has not yet been tried experimentally, though it would appear from the graph presented that it is sensitive to lower concentration of the element. One observation that needs to be emphasised in this connection is that

the organism responds sharply to graded doses of iron so much so that the standard growth curve get overlapped by the experimental readings as the figures presented in Tables I, II and III will clearly indicate.

The practicability of the use of the organism in the assay of iron in the soils, tissue extracts and plants is being further investigated.

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