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A NEW GROWTH-FACTOR FOR *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT

An active principle has been isolated from the water of tender cocoanuts which has the property of enhancing the growth of *Myco. tuberculosis* in laboratory media. It does not affect the virulence of the bacilli. The growth factor appears to be a low molecular weight polysaccharide.

Myco. tuberculosis is known to be a slow growing organism. To facilitate colony development from small inocula as also quicker growth of the bacilli, numerous attempts have been made by several investigators.[1–5] The growth-promoting and stimulating action observed by these workers has been mainly attributed to the inhibitory effect of these substances on the toxic components customarily present in trace amounts in the culture media. Dubos[6] has also shown that some saturated and unsaturated fatty acids when rendered atoxic exert a favourable growth-promoting action for tubercle bacilli. But none of the standard medias now in use, the egg medias, the Proskeur and Beck type of cultures or the tween-albumin complex of Dubos could be considered as quite satisfactory for the rapid growth of the tubercle bacilli. Though Dubos media slightly facilitates quicker growth, the techniques involved in its preparation and the use of expensive and not easily available albumin factor V, renders this media unsuitable for general use.

In our attempts to shorten the period of incubation and to increase the yield of *Myco. tuberculosis*, it was observed that the addition of the water from the tender

cocoanut could considerably reduce the number of days required for maximum growth of the bacilli on Youmans' media. A detailed study of the factor or factors in the cocoanut water responsible for this stimulation has been undertaken. This paper presents some aspects of this investigation. A preliminary note on this work has already appeared in *Nature*.[7]

MATERIALS AND METHODS

$H_{37}R_v$ strain of *Myco. tuberculosis*, originally obtained from N.C.T.C. (England) and maintained on Petrik's media[8] (Modified Lowenstein) by bimonthly transfers, was the organism used.

Cocoanut water.—A marked area over the tender cocoanut was well sterilized, the rind thinned out and the cocoanut water was withdrawn aseptically by a syringe and kept in cold storage till used.

For the surface culture technique varying concentrations of the cocoanut water taken aseptically was added on to the sterilized Youmans' media. The inoculum was a loopful of the thin spreading growth of *Myco. tuberculosis* from the surface of 14 days growth on the Youmans' media which was then gently floated on to the media containing the cocoanut water. The growth was observed at intervals and the extent was graded as already described.[9]

For the solid media, the required quantities of the water were incorporated into the Petrik's media before inspissation. The inoculum was an uniform suspension of $H_{37}R_v$ grown on the surface of Youmans' media. 0·1 ml. of this suspension was spread over the Petrik's media. The time of appearance of visible colonies and the extent of growth were noted at intervals.

RESULTS

The day of the appearance of serpentine growth and the amount of growth at varying intervals on Youmans' media is shown in Table I.

TABLE I

Effect of cocoanut water on the in vitro *growth of* Myco. tuberculosis $H_{37}R_v$ *in Youmans' media*

Concentration of the cocoanut water	Extent of growth on different days				
	4	7	10	14	21
1/100 ..	+	2+	3+	4+	
1/1,000 ..	Sl.	2+	3+	4+	
1/10,000 ..	Sl.	+	2+	3+	
Control ..	Sl.	Sl.	+	2+	4+

Sl. = Slight; + to 4 + = Various grades of growth.

It was found that the addition of cocoanut water, even in a dilution of 1 in 10,000 to Youmans' medium, facilitated earlier growth. The maximum growth normally attained on the Youmans' medium by about the 21st day was found to have been reached in this modified medium by the 12th day alone.

These macroscopic observations clearly indicate that the initiation of the growth was quicker and the maximum growth attained in the presence of the cocoanut water was much earlier than the controls. Similar trend of results was noticed also in the Petrik's media.

Isolation of the growth factor.—Systematic fractionation was undertaken to isolate this factor. The cocoanut water was devoid of polypeptides or proteins, as indicated by ammonium sulphate fractionation. The ether extract of cocoanut water obtained by shaking the water with an equal volume of ether saturated with NaCl and repeating the process 4 times, was evaporated to dryness, dissolved in a small quantity of ethylene glycol and made up to the original volume. To the water layer was added ethyl alcohol to 75% concentration and the precipitated polysaccharide centrifuged off and dissolved in the original volume of water whose pH had been adjusted to 4·5 by dilute hydrochloric acid. The supernatant was shaken with three times its volume of chloroform and the water layer passed through Dowex-50 resin (200–250 mesh) which had been regenerated in the hydrogen cycle. The eluate containing free sugars was not treated further. The amino-acids from the resin were eluted with 0·15 N sodium hydroxide till the eluate gave no colour with ninhydrin, desalted[10] by evaporation and by the use of 95% alcohol and made up to the original volume. Of the four fractions, *viz.*, lipids, polysaccharide, amino-acids and the residue, only the polysaccharide fraction showed growth-promoting effect on supplementation to Youmans' medium. Fig. 1 shows the growth of *Myco. tuberculosis* on the 5th day in Youmans' medium (control) and in the same medium containing graded concentrations of the polysaccharide fraction. The over-all effect of the cocoanut water and its component fractions is shown in Table II.

Influence of the factor in solid medium.—The factor had similar growth-promoting activity on *Myco. tuberculosis* in Petrik's (solid) medium as in Youmans' medium. Growth was more profuse when the factor was incorporated and could be observable even on the 5th day. The accompanying photograph (Fig. 2) shows more prominently the effect of the factor on the growth of *Myco. tuberculosis* on the 7th day.

Properties of the growth factor.—That the active fraction was a polysaccharide was confirmed by Molisch test. With iodine it gave no colour, nor did it give any test for pectin. The Seliwanoff reagent gave a negative reaction showing that the end-sugar was not a ketose. On hydrolysis with 1 N sulphuric acid for 3 hours it gave tests for glucose and fructose on paper chromatogram developed in pyridine solvent.[11] The fraction does not give test for protein.

TABLE II

Effect of cocoanut water and its fractions on the growth of Myco. tuberculosis *in Youmans' medium*

Fractions	Growth on 4th day
1. Control (Youmans' medium)	Sl.
2. Cocoanut water + Youmans' 1/100 ..	+
3. Ether-soluble fraction ,, ..	Sl.
4. Polysaccharide fraction ,, ..	++
5. Amino-acid fraction ,, ..	Sl.
6. Residue + Youmans' ,, ..	Sl.
7. Cocoanut water alone ,, ..	0

Sl. = slight growth;

+ = growth covering the entire surface;

++ = growth covering the entire surface and extending up the sides of the tube.

The active principle is heat-stable and can be autoclaved at 15 lb. pressure without harm. It is insoluble in water but soluble in dilute acids from which it can be precipitated by alcohol after neutralisation. On paper chromatograms it moves as a single spot showing that it has a low molecular weight. The washed precipitate gives a strong test for phosphate by the Fiske-Subbarao method. It does not have any absorbancy in the ultraviolet region.

Influence of the growth factor on the virulence of Myco. tuberculosis.—Mycobacteria which exhibit rapid growth are usually non-pathogenic or saprophytic in nature. Even amongst the pathogenic mycobacteria the degree of virulence is intimately connected with the rapidity of growth. Since a rapid growth of *Myco. tuberculosis* was observed in presence of the cocoanut factor, the possibility of a change in the virulence had to be considered.

The virulence studies were carried out by the experimentally induced tuberculosis in mice. The standard technique, detailing the inoculum used, method of infection and mode of evaluation in general has been described earlier.[12] In brief, it consisted in the intravenous infection of groups of mice by 0·1 mg. of $H_{37}R_v$ strain of *Myco. tuberculosis* grown in the normal media and in the media incorporating the polysaccharide fraction and observing the weight loss and the survival period.

The gross degree of the extent of pathological involvement of the organs was also noted by autopsy.

TABLE III

The effect of the polysaccharide fraction on the virulence of the tubercle bacilli ($H_{37}R_v$)

No. of animals	Media of growth	Days of death	Average survival period in days
10	Youmans'	21, 23, 21, 24, 21, 19, 20, 20, 22, 24	21·5
10	Youmans' + polysaccharide	19, 20, 20, 21, 23, 24, 22, 21, 19, 20	20·9

No statistically significant difference in the survival rate or the extent of the lesion was noticed in the infection caused by the normal and polysaccharide treated tubercle bacilli.

The effect of the polysaccharide on the course of experimental tuberculosis in mice.—Since the fraction stimulates the growth of the bacilli *in vitro* and does not influence the virulence of the organism when grown in its presence, the possible effect of continuous administration of the material on the course of tuberculosis *in vivo* was next studied.

The experimental design was similar to the ones carried out for chemotherapeutic trials in the experimental tuberculosis in mice. After the *in vivo* infection of the mouse by 0·1 mg. of the organism ($H_{37}R_v$), the fraction, at varying dosages, was injected intraperitoneally once a day, the controls being given only normal saline. The experiment was terminated when all the controls had died. The extent of the weight loss and the survival periods are shown in Fig. 3 and Table IV.

Contrary to expectation, it is noticed that in the dosages tried, no facilitation of growth of tubercle bacilli as judged by the survival period and the extent of lesions is observed, while in higher dosage an actual beneficial effect on the course of the infection is seen. An appreciable gain in the weight during the 1st week and a statistically significant increase in the survival period of the animals treated with 0·5 mg. of the polysaccharide can be observed. It is known that purified polysaccharides from some micro-organisms stimulate the migration of leucocytes[13] while the importance of polysaccharides associated with lipids in the immunological reactions is undoubtedly gaining ground.[14]

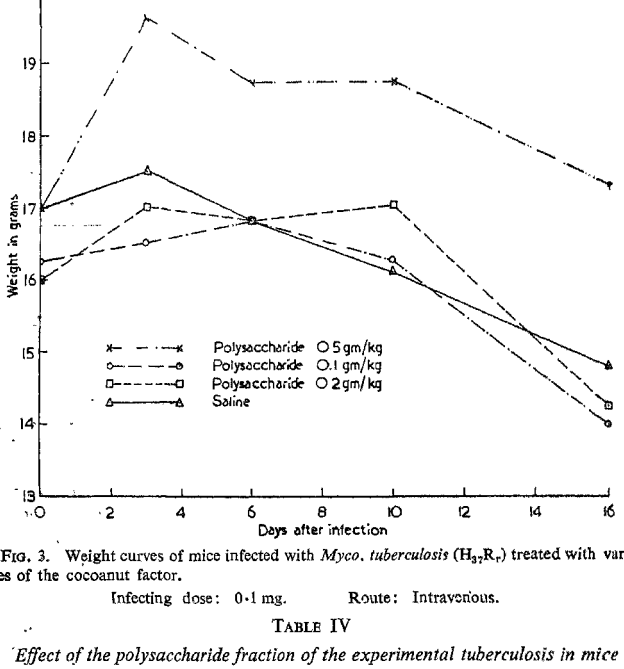

FIG. 3. Weight curves of mice infected with *Myco. tuberculosis* ($H_{37}R_v$) treated with varying doses of the cocoanut factor.

Infecting dose: 0·1 mg. Route: Intravenous.

TABLE IV

Effect of the polysaccharide fraction of the experimental tuberculosis in mice

No. of animals used	Treatment given	Days of death (after infection)	Average survival period	±S.E.	P.
10	Normal saline	20, 19, 17, 20, 20, 18, 19, 20 18, 20	19·1	±0·348	..
10	Polysaccharide 0·5 mg.	21, 24, 18, 20, {21, 22, 20, 23, {20, 21	21·0	±0·542	<0·01
10	,, 0·2 mg.	19, 22, 19, 20, 19, 19·4, 18, 20, 18, 19, 20	19·4	±0·371	>0·5
10	,, 0·1 mg.	20, 17, 22, 19, 18, 20, 18, 17, 19, 19	18·9	±0·482	>0·5

Discussion

It is evident from the foregoing that the active principle from cocoanut water accelerates the growth of *Myco. tuberculosis* in all the media generally used for its propagation but does not modify the virulence of the bacillus.

The fact that this factor acts in concentrations of 1 in 10,000 and facilitates growth even on the most nutritious media used for the growth of tubercle bacilli, *viz.*, the potato egg yolk media, shows that it is not its nutritive value which is responsible for the enhanced growth of the bacilli even in a poor medium like Youmans'. However, it does not seem to be identical with the plant growth factors isolated from cocoanut water by Steward and his co-workers.[15,16] While the latter did not promote the growth of carrot roots to the same extent as the original water itself, the polysaccharide fraction stimulated the growth of *Myco. tuberculosis* even better than the cocoanut water. Steward *et al.* found that the addition of casein hydrolysate improved the activity of the growth-promoting factor for plant tissues, whereas in the case of the polysaccharide fraction, the addition of casein hydrolysate did not further increase its growth-promoting activity for *Myco. tuberculosis.*

The growth factor is not a nucleotide as shown by its lack of absorbance in the ultraviolet region. Since it resists autoclaving, it cannot be one of the usual coenzymes or unstable vitamins.

The insolubility of the material in water permits it to be washed free of nitrogenous materials like amino-acids and soluble inorganic phosphates. The fact that it gives test for phosphate may be due to its admixture with some sugar phosphate or that it exists as a phosphate compound itself. This will be clear only after the structure of the active principle is established, and experiments are under way to elucidate its structure.

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EXPLANATION OF PLATE II

FIG. 1. Influence of cocoanut factor in the growth of *Myco. tuberculosis*—5th day of incubation.

Strain: $H_{37}R_v$. Media: Youmans.

The concentration of the factor in the media from left to right:

1/10, 1/100, 1/1,000, 1/10,000, 1/1 million and control.

The increase in the growth of the surface pellicle can be seen quite prominently in concentrations upto 1/10,000.

FIG. 2. Effect of cocoanut factor on the growth of *Myco. tuberculosis* $H_{37}R_v$ on solid media (Petrik's)—7th day of incubation.

Concentration of the cocoanut factor in the media:

Tube No. 1 .. 1/10.
Tube No. 2 .. 1/100.
Tube No. 3 .. 1/1,000.
Tube No. 4 .. Control.

The increased number of colonies can be seen in all the three concentrations.

FIG 1

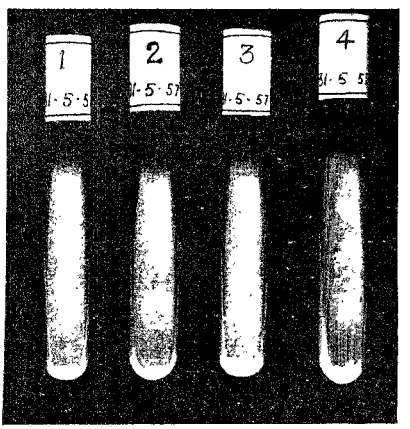

FIG. 2