Assay of the Mycobactins by Measurement of the Growth of *Mycobacterium johnei*

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A method is described for the assay of mycobactin P and T by turbidimetric measurement of the growth of *Mycobacterium johnei*. The assay agrees satisfactorily with spectroscopic determination of the iron complexes. The growth curves with mycobactins P and T differ somewhat in form. An approximate ratio of activities has been determined.

In the method originally described for the assay of mycobactin (Francis, Macturk, Madinaveitia & Snow, 1953) *Mycobacterium johnei* was grown as a surface culture on solid egg medium. Growth of the organism was slow and the results, based on subjective scoring, were erratic. Replacement of the egg medium by Sutton & Stanfield's (1955) solid medium improved the accuracy and sensitivity of the method, but eventually the use of Hart's (1958) liquid medium allowed the development of a quicker and more reliable assay by turbidimetry.

The use of *M. johnei* ensures a high degree of specificity in measurement of compounds of the mycobactin group, since no other type of substance is known to stimulate the growth of this organism. However, if a less specific method is acceptable, the use of *Arthrobacter terregens* provides a convenient and sensitive assay (Antoine, Morrison & Hanks, 1964; Reich & Hanks, 1964).

ASSAY METHODS

Turbidity measurements. An EEL nephelometer (Evans Electroselenium Ltd., Bishop's Stortford, Herts.) was standardized before each set of readings by adjusting it to give a chosen reading when tested against a selected solid turbidity standard. The setting was arranged so that a culture, showing a well marked turbidity due to completely dispersed growth of *M. johnei* before the onset of flocculation, gave a reading of 80. At this stage the organism would still be in vigorous growth and would give higher readings on further incubation; readings above 80, however, tended to be unreliable because of flocculation.

Test solutions and controls. The samples to be tested were dissolved in propylene glycol, and from the solution successive twofold dilutions were made in water. Care was taken to ensure that the solutions were sufficiently dilute to avoid precipitation on dilution with water. Control solutions were similarly prepared from purified mycobactin P to give concentrations varying from 0.4 to 6.4 $\mu\text{g./ml.}$ Portions (10 ml.) of Hart's (1958) medium (prepared without mycobactin) were dispensed into 20 ml. flat-bottomed

cylindrical ampoules, 2.2 cm. external diam. (Johnson and Jorgenson Ltd.) and each was mixed with 0.05 ml. of test or control solution prepared as above; tubes to be used as blanks received 0.05 ml. of propylene glycol or water; the propylene glycol did not affect the growth of the culture. Replicate tubes were set up for each concentration of each substance to be tested, with a similar replication of the controls and blanks. The number of replicates was never less than three, but where high accuracy was required larger numbers, up to 10, were used.

Because of its greater availability mycobactin P was used throughout as the reference standard. However, the results below show that it would be preferable for accurate results to measure mycobactin T concentrates against standards of the same substance.

Culture of *M. johnei*. In this work aseptic precautions were used throughout. *M. johnei* (A.T.C.C. 9808, received from Dr W. B. Sutton) was maintained on slopes of the medium described by Sutton & Stanfield (1955), to which was added 10 $\mu\text{g.}$ of mycobactin/ml. Good growth occurred within 1 month at 37° and cultures were usually transferred after they had been incubated for 1–2 months.

Assay procedure. Method 1. The organisms from the slopes (about 10 mg. wet wt.) were suspended in 0.85% NaCl soln. (5 ml.) and centrifuged for 10 min. at 1,500 g in an angle rotor. The supernatant was poured off and the residue was homogenized by hand in a ground-glass tissue grinder with 0.85% NaCl soln. (10 ml./10 mg. of organisms originally taken). The suspension was centrifuged for 2 min. at 30 g to remove coarse particles. The ampoules, containing test preparations, controls or blanks, were each inoculated with 0.04 ml. of the suspension; turbidity measurements were made on each, and they were then incubated together at 37°. Any ampoule showing an increase in turbidity during the first 48 hr. was considered to be contaminated and was discarded. From 5 days onwards the ampoules were examined daily, and as soon as an increase in turbidity could be seen in any ampoule nephelometer readings were made on the whole series. Readings were repeated daily for about 6 days until the ampoules containing the higher concentrations of mycobactin gave turbidity readings exceeding 80.

Method 2. Before the organism was required it was temporarily maintained in the liquid medium described by

Hart (1958), containing 20 μg . of mycobactin/ml. Cultures were incubated at 37° and transferred when their turbidities reached 80 on an EEL nephelometer (see above). In assay experiments ampoules containing the test preparations were inoculated with 0.04 ml. of a culture having a turbidity reading between 75 and 85. The ampoules were then incubated at 37° and the development of turbidity was followed as in method 1.

Calculation of results. Each assay experiment included a series of ampoules containing the test samples. The highest concentration of a test sample was chosen to contain the equivalent of more than 20 μg . of mycobactin/ml. of medium, and successive twofold dilutions were made. At the same time control solutions were prepared containing known concentrations of pure mycobactin P. A suitable range was found to be 0, 4, 8 and 16 μg . of mycobactin P/ml. of medium.

Turbidity readings for each ampoule were corrected by subtracting the initial reading; replicates were then averaged. Both methods of inoculation led to a similar type of growth curve with mycobactin P. There was an initial lag of at least 5 days with little change in turbidity; turbidity then rapidly increased in media where the concentration of mycobactin was 20 μg ./ml. or more; concentrations below 4 μg ./ml. permitted very little growth. There was thus an effective range of mycobactin P between 4 and 20 μg ./ml. in which comparative measurements could be made. The increase in turbidity could be usefully measured during

6–10 days. Results of an experiment in which four different concentrations of mycobactin P were used for controls are shown in Fig. 1 (solid lines). For convenience in use, growth curves for intermediate concentrations were interpolated graphically (Fig. 1, broken lines). This was done by plotting the observed turbidities against the logarithm of the concentration of mycobactin for each day on which measurements were made. It was then possible to read off the expected turbidity for any selected mycobactin concentration and to plot the interpolated curves. Growth curves were also plotted for the test solutions and their dilutions. The concentrations of mycobactin P in the test solutions could thus be easily read off by comparing their growth curves with those of the controls. Since twofold dilutions were used there were always two growth curves for each test sample lying in the useful range of measurement, and satisfactory agreement between results derived from these pairs of dilutions provided a check on the reliability of the method.

Most measurements were made with method 1. Its main disadvantage was the uncertainty of the length of the lag period, and experiments in which the lag was unduly prolonged gave unreliable results. These differences from one experiment to another probably reflected differences in the inoculum. There was also probably some variation in the inoculum from one ampoule to another, which caused a slight lack of synchronization of the onset of measurable growth and limited the precision of the assay.

Method 2 was developed to avoid the uncertainties in the length of lag found with method 1. It gave less variation between replicates and greater reproducibility between one experiment and another.

RESULTS

The assay procedure (method 1) was used to follow the purification of ferric mycobactin T (Snow, 1965). Later a spectroscopic method of determination was introduced which was more convenient for fractions containing higher concentrations of the growth factor. Direct comparisons were made between the chemical and the biological assays and the results of one experiment are shown in Table 1. Because pure ferric mycobactin T was not available at the time, mycobactin P was used for the control solutions. The chemical results are calculated from the extinction coefficient of the purest preparation of ferric mycobactin that was subsequently obtained. The chemical method was somewhat unsatisfactory with fractions in which the content of ferric mycobactin T was as low as in stage *F* (Table 1). The biological method was not expected to be closer than $\pm 10\%$. However, comparison of the results in the last two columns of Table 1 show that the numerical values in most cases are reasonably similar. This indicates that the two methods are measuring the same variable, and also that the growth promoting activity of ferric mycobactin T is quantitatively close to that of mycobactin P.

A careful comparison was made between the growth-promoting properties of mycobactins P

Fig. 1. Increase in turbidity of *M. johnei* cultures with time in the presence of varying concentrations of mycobactin P. Figures against the curves show mycobactin P concentrations in μg ./ml. Unbroken lines show experimentally determined curves. Broken lines show curves derived by graphical interpolation. Assay was by method 1.

Table 1. Comparison of biological assay and spectroscopic determination of mycobactin in partly purified preparations of ferric mycobactin T

Sample no.	Purification stage*	Biological assay			Spectroscopic determination (content of solid sample as $\mu\text{g. of ferric mycobactin T/mg.}$)	
		Concn. of sample in medium ($\mu\text{g./ml.}$)†	Dilution	Estimated activity ($\text{m}\mu\text{g. of mycobactin P/ml.}$)‡		Mean content of solid sample ($\mu\text{g. of mycobactin P/mg.}$)
1	<i>F</i>	9.4	$\left\{ \begin{array}{l} 0.5 \\ 0.25 \end{array} \right.$	$\left\{ \begin{array}{l} 12 \\ 6 \end{array} \right.$	2.6	1.7
2	<i>F</i>	13.7	$\left\{ \begin{array}{l} 0.5 \\ 0.25 \end{array} \right.$	$\left\{ \begin{array}{l} (11) \\ 7.5 \end{array} \right.$	2.3	2.8
			0.125	4		
3	<i>H</i>	1.1	$\left\{ \begin{array}{l} 0.5 \\ 0.25 \end{array} \right.$	$\left\{ \begin{array}{l} 9 \\ 5 \end{array} \right.$	17	20
			0.5	11		
4	<i>H</i>	1.0	$\left\{ \begin{array}{l} 0.5 \\ 0.25 \end{array} \right.$	$\left\{ \begin{array}{l} 5 \\ 5 \end{array} \right.$	21	20
			0.5	9.5		
5	$\left. \begin{array}{l} \text{Various} \\ \text{chromato-} \\ \text{graphic} \\ \text{fractions} \end{array} \right\}$	0.049	$\left\{ \begin{array}{l} 0.5 \\ 0.25 \end{array} \right.$	$\left\{ \begin{array}{l} 9.5 \\ (5) \end{array} \right.$	390	490
6		0.062	$\left\{ \begin{array}{l} 0.5 \\ 0.25 \end{array} \right.$	$\left\{ \begin{array}{l} 9.5 \\ 5 \end{array} \right.$	320	340
		7	0.145	$\left\{ \begin{array}{l} 0.5 \\ 0.25 \end{array} \right.$	$\left\{ \begin{array}{l} 9 \\ 4.5 \end{array} \right.$	120

* See mycobactin T purification (Snow, 1965; Table 1). Samples are from two different purification runs.
† Figures show the top concentration of the series. Assay results are given only for dilutions lying in the assay range.
‡ Figures in parentheses have been excluded from the calculation, because of unsatisfactory growth curves.

Fig. 2. Increase of turbidity of *M. johnei* cultures containing differing concentrations of mycobactins P (open symbols) and T (filled-in symbols). Each point is derived from the mean of 10 replicates. Concentrations of mycobactin ($\mu\text{g./ml.}$): 5 (Δ , $\blacktriangle$); 10 ($\circ$, $\bullet$); 15 ($\square$, $\blacksquare$); 20 (∇ , $\blacktriangledown$).

and T by method 2. Growth curves were determined with each compound at several different concentrations. The results (Fig. 2) show that the pattern of growth response was not identical for the two growth factors. An absolute comparison of their activities was therefore not practicable. For instance, at the early stages of growth the turbidity reading with 10 $\text{m}\mu\text{g.}$ of mycobactin T/ml. was greater than that with 20 $\text{m}\mu\text{g.}$ of mycobactin P/ml., and at the time of the final measurement the turbidity was less with the former solution than with 15 $\text{m}\mu\text{g.}$ of mycobactin P/ml. The conclusion is that for most conditions of test mycobactin T is at least 1.5 times as active as mycobactin P in the promotion of growth of *M. johnei*.

If the method is to be used accurately test samples must be measured against standards of the appropriate mycobactin. The sensitive range of measurement for mycobactin T probably lies between 2 and 10 $\text{m}\mu\text{g./ml.}$

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