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Attempts at the Cultivation of *M. leprae* in Cell-Free, Semi-Synthetic Soft Agar Media

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Investigations into the effect of ultraviolet irradiation on the acid-fastness of mycobacteria have clearly demonstrated a difference in the degree of acid-fastness among various types of mycobacteria. To illustrate this an acid-fastness map was devised (¹). This map suggested strongly the possibility of cultivating in artificial media the special mycobacterial strains, which have long been believed uncultivable.

Later, attempts were made to cultivate *M. lepraemurium* from nodules of infected mice in a cell-free, semi-synthetic soft agar (0.1%) medium (²). The basic composition of this medium was modified to some extent from that used for the successful cultivation of *M. paratuberculosis Johnei* without addition of mycobactin (³).

Stimulated and encouraged by the results of culture of the above mentioned two mycobacteria, attempts were made, even boldly, to cultivate *M. leprae* in cell-free, semi-synthetic soft agar media, modifying the base compositions from those used for *M. lepraemurium.*

EXPERIMENTS

Chemical compositions of the media used are shown in Table 1. They were the semi-synthetic, semi-liquid (0.1%) agar media with bases containing both glycerol and glucose as carbon sources, and the oxidation-reduction potentials of about −.30 volts as revealed by the dye reduction test. Among them, M-Y 1c, M-Y 4d and M-Y 6f seemed to be approximately adequate at the stage of the preliminary experiment.

Experiment 1. Preliminary experiment. Nodules obtained from four different L-

TABLE 1. *Base compositions of the media.*

Base No.[a]	Asparagine (%)	Glycerol (%)	Glucose (%)	Fe. ammon. citr. (%)	ATP & NAD (ug/ml)
M-Y 1c	0.3	3	1	/	/
3c	0.6	6	1	/	/
3d	0.6	6	2	/	/
4d[a]	0.3	3	1	0.05	/
5e	0.6	3	1	0.05	/
6e	0.6	6	1	0.05	/
6f[a]	0.6	6	1	0.5	/
7a	0.6	/	1	0.05	1
8a	0.6	3	1	0.05	1
8d	0.6	6	1	0.5	1

Note. Composition of M-Y 1c in grams: KH_2PO_4 2.0, $Na_2PO_4\ 12H_2O$ 1.5, Asparagine 3.0, Na. citr. 2.0, $MgSO_4$ 0.02, $CaCl_2$ 0.0025, Glycerol 30 ml, Glucose 10.0, Yeast RNA 100μg, Bov. Serum Alb. Fraction V 5.0, Agar-agar 1.0, Aq. dist. 1,000 ml. (pH: Adjusted to 6.6-6.8, using NaOH).

[a] M-Y 1c, M-Y 4d & M-Y 6f were used most extensively in the studies reported.

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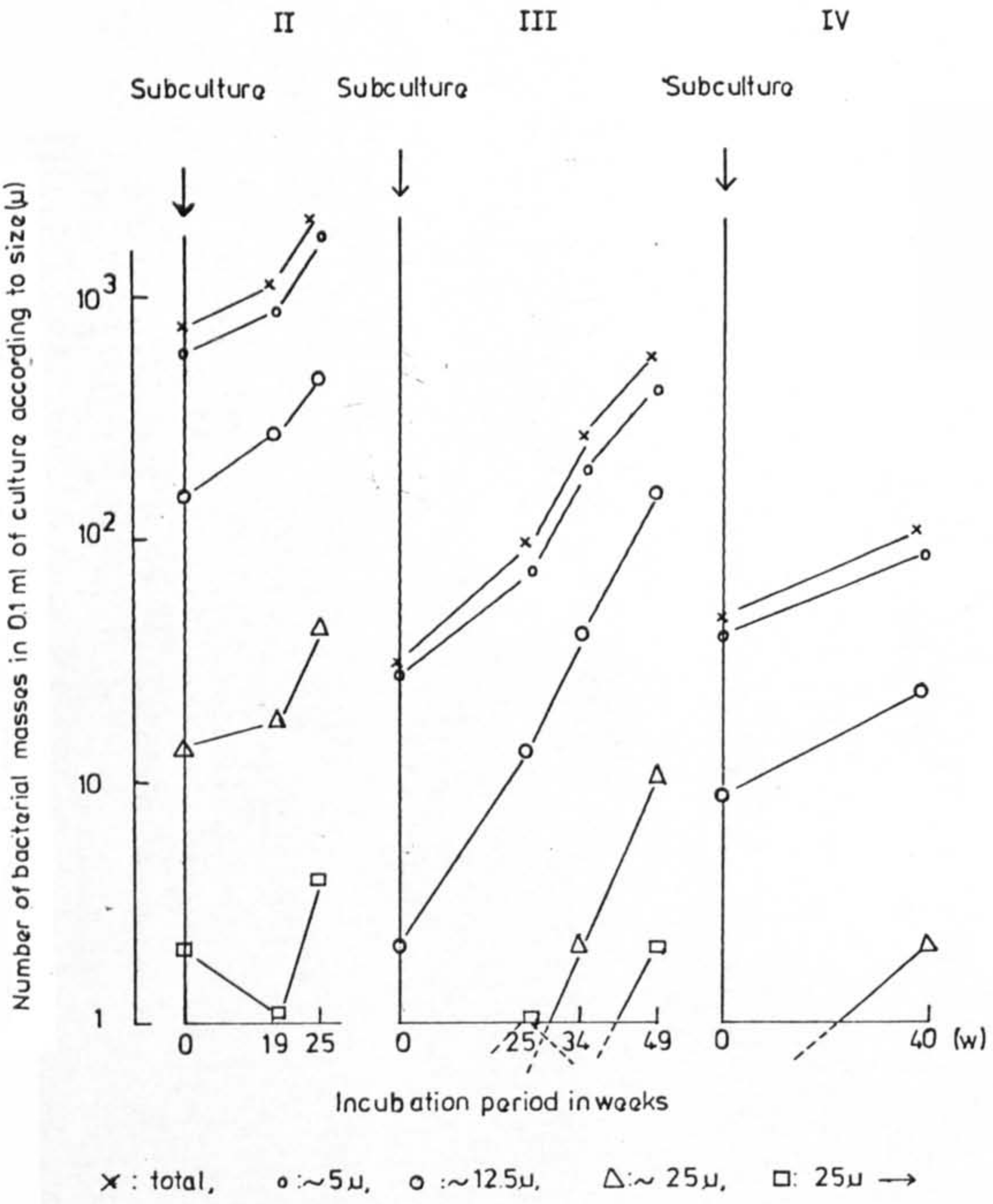


FIG. 1. Increasing trend curves of bacterial masses of L-May-67-1 strain in the 2nd (II), 3rd (III) and 4th (IV) subcultures.

type patients, from May to October of 1967 successively, were homogenized in a mortar at respective times of receipt in the preparation of bacterial suspensions. One-tenth ml. of each bacterial suspension thus prepared was inoculated into 0.1 per cent semiliquid agar media of various base compositions and incubated at 37°C. As no contamination was observed, alkali treatment was omitted. The results obtained were as follows (4, 5):

Rather large, colony-like bacterial masses appeared, in most cases, after about 20 weeks or more of incubation at 37°C, and persisted even after one year of incubation, increasing in number and size extremely slowly. These bacterial masses varied considerably in shape and size. Generally, they were composed of smaller, round bacterial masses 10-20 μ in diameter, adhering with one another. Each mass seemed to have a boundary around it, consisting of strongly acid-fast rods, staining with malachite-green in the malachite-green-fuchsin staining procedure (6), suggesting that the bacterial masses detected in the culture tubes were possibly the colonial growths.

At appropriate times after confirmation of the development of colony-like bacterial masses, a small part (0.5-1.0 ml.) of the parent, primary cultures of the above two strains was transferred to new media in order to learn about adequate conditions for subculturing. The growth trends of the subcultures of the two strains described above are shown in Figs. 1 and 2, in which an increasing trend in number and size of bacterial masses, conforming with the increase of incubation time, is clearly demonstrated. The fourth subcultures of these strains are under observation.

Bacterial suspensions were prepared from the collected bacterial masses derived from the nearly one year-old, primary culture of the above mentioned strains, and injected intradermally into leprous patients for identification. The reactions induced were essentially the same as for Dharmendra's antigen, as shown in Table 2. Therefore, it was revealed clearly that the bacterial masses existing in the culture tubes of the primary isolation at 37°C for about one year were composed just of *M. leprae*.

Experiment 2. Effect of alkali treatment and incubation temperature on the growth. Bacterial suspensions derived from nodules of three different L-type patients were inoculated into semi-liquid agar media of base composition similar to those used in the preceding experiment. Prior to cultivation, homogenates of nodules were mixed with an equal volume of 2 per cent NaOH at 37°C for 60 minutes to eliminate contamination, and then 0.1 ml. of each homogenate was inoculated into media after adjusting the pH to 6.6-6.8 with N-HCl. One half of the culture tubes, taken at random, were incubated at 37°C, and the remaining half at 30°C, to investigate the effect of incubation temperature on the growth. The results obtained were as follows (4, 7):

In all of the media used increase in number and size of bacterial masses were readily observed at the incubation temperature of 37°C, showing fairly strong resistance to alkali treatment, while at 30°C no appreciable increase was observed. Accordingly, it may be said that the adequate incubation temperature for the growth of this organism is 37°C and not 30°C.

At 20 weeks and thereafter of incubation at 37°C, these three strains were transferred to new media for subculturing. The growth trend curves of the subcultures were quite similar to those of the above mentioned two strains, showing clearly the increasing trend in number and size of bacterial masses, even extremely slowly, as shown in Figs. 3 and 4.

It was very interesting to note that fairly large and compact bacterial masses were detected in the primary cultures, which were at first incubated at 30°C for 23 weeks, with no appreciable growth, and then at 37°C for an additional 22 weeks and 43 weeks, respectively. To our great astonishment, further, huge bacterial masses, exceeding 150 μ in diameter were detected in the 50 week-old second subculture, which was derived from the above mentioned primary culture incubated at 37°C for 22 weeks after 23 weeks' incubation at 30°C, and also in the 56 week-old culture, which was derived from that of 43 weeks.

On the basis of the results obtained in the above experiments, a model was de-

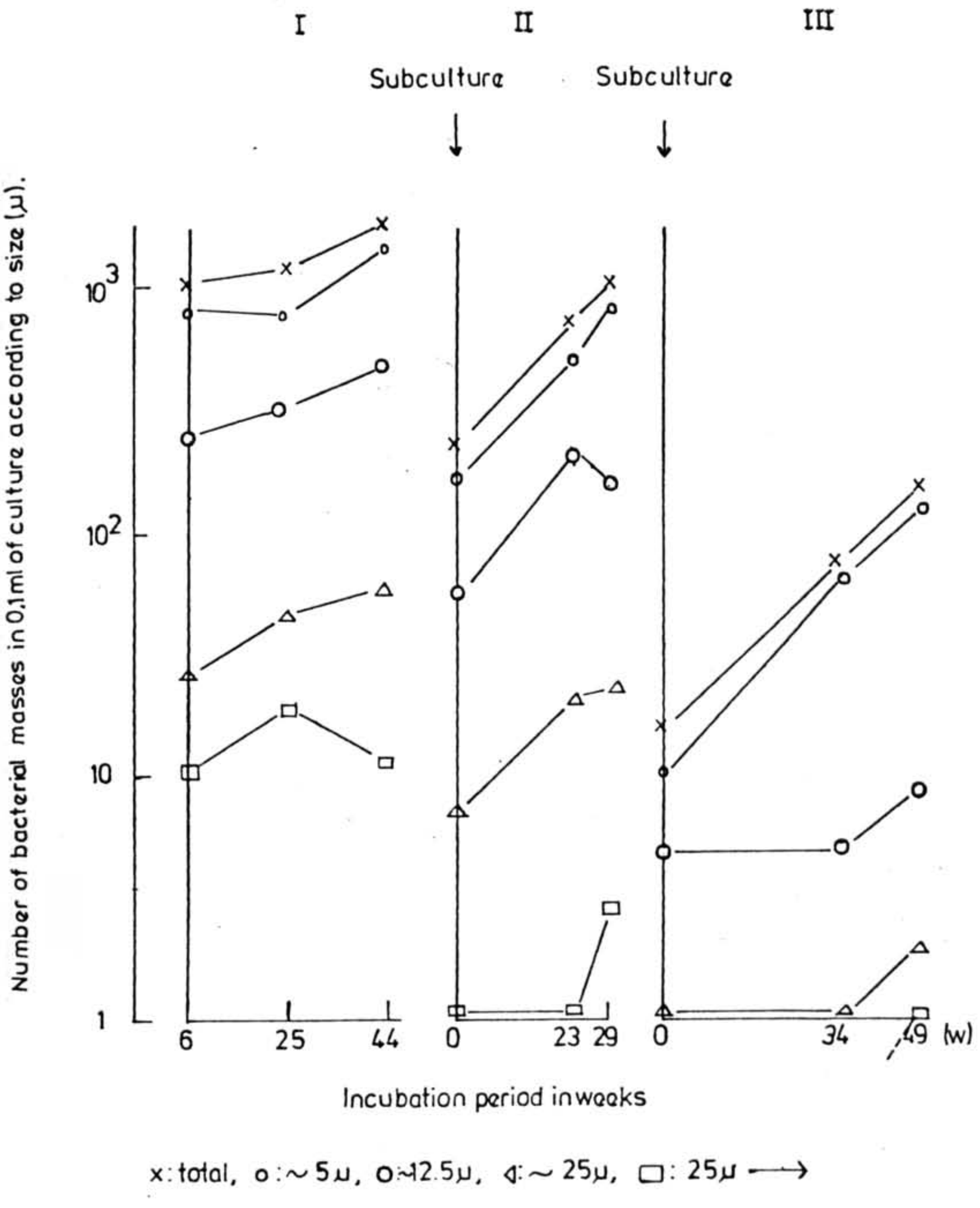


FIG. 2. Increasing trend curves of bacterial masses of L-Jul-67-1 strain in the primary isolation (I), 2nd (II) and 3rd (III) subcultures.

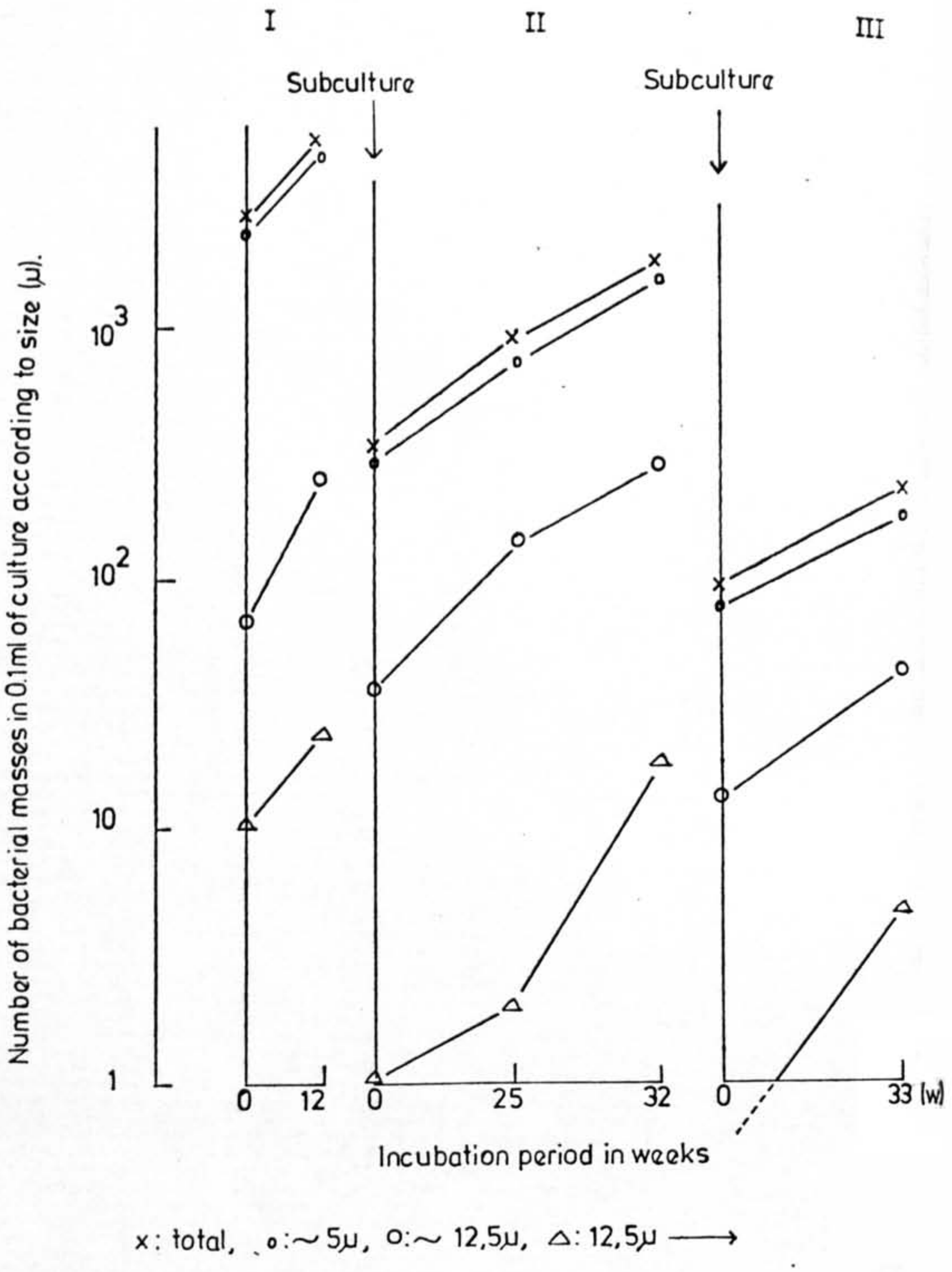


FIG. 3. Increasing trend curves of bacterial masses of L-Feb-68-1 strain in the primary isolation (I), 2nd (II) and 3rd (III) subcultures.

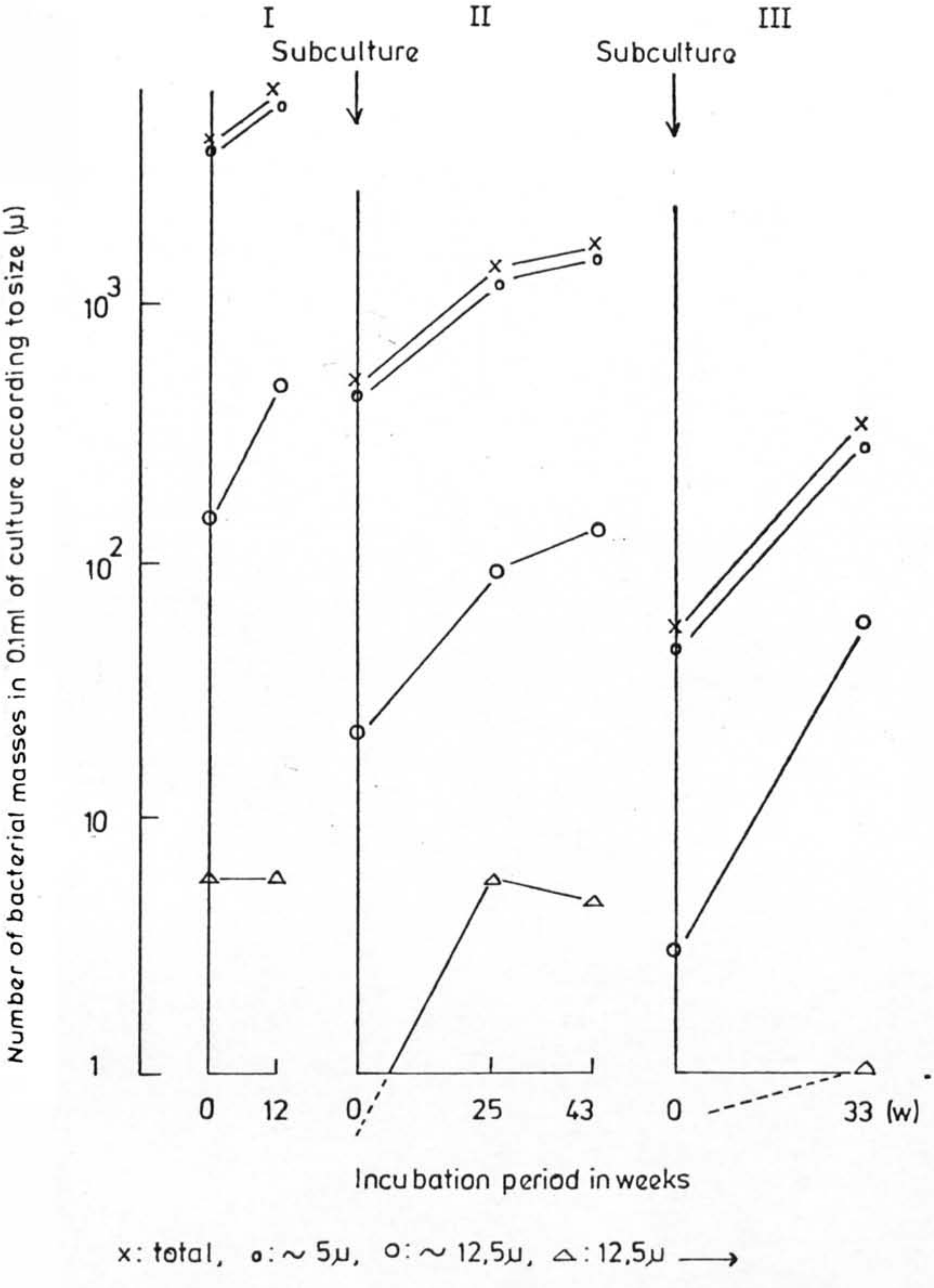


FIG. 4. Increasing trend curves of bacterial masses of L-Feb-68-2 strain in the primary isolation (I), 2nd (II) and 3rd (III) subcultures.

vised (Fig. 5), which enables us to explain the relationship between the incubation period of time and the inoculum size of the parent culture adequate for subculturing, and to estimate the extent of yield of the subcultures to be expected after a certain period of incubation time.

Experiment 3. Objective evaluation of the culture experiment. To examine the objective validity of the above mentioned experiments, investigations were carried out on the same strain (L-Aug-68) independently in two different, remote laboratories, i.e., National Institute of Health and National Suruga Leprosarium. Immediately after inoculation of the alkali-treated and adjusted bacterial suspension derived from a nodule of an L-type patient, several culture tubes at random were brought to the above named leprosarium and incubated at 37°C. At appropriate times after incubation, cultures were examined microscopically in both laboratories independently. The results obtained in both laboratories coincided fairly well, revealing that the incubation at 37°C for about 30 to 50 weeks resulted in an obvious increase in number and size of bacterial masses.

The second subcultures were made in new media prepared in both laboratories by different technicians independently. The results obtained after about 30 weeks and 50 weeks of incubation, respectively, were quite similar, demonstrating that the number and size of bacterial masses increased distinctly in a quite similar trend in the cultures in both laboratories, as shown in Figs. 6 and 7.

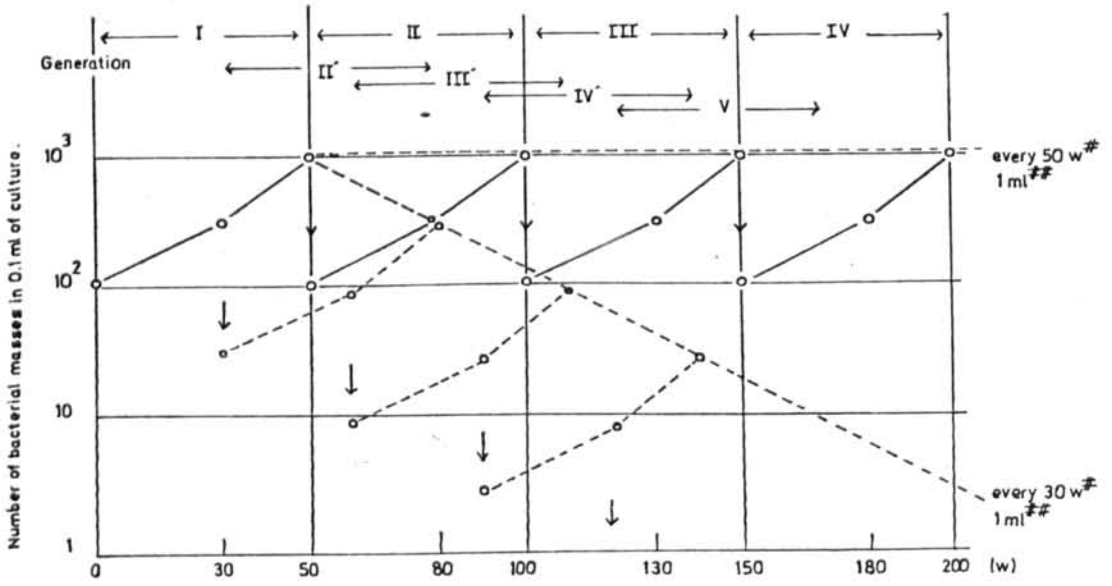
From the nearly one-year-old second subcultures preserved in both laboratories bacterial masses were collected to prepare bacterial suspensions. Intradermal injection of these suspensions into leprous patients induced essentially the same reactions as

TABLE 2. *Intradermal reactions in leprous patients elicited by a 49 week-old primary culture of L-Jul.-67-1 strain.*[a]

Type of disease	Patient	Mean size (mm.) of reactions (erythema)[b]					
		48 hours		15 days		21 days	
		D	M	D	M	D	M
T	N.H.	6	8	6	16	6	7
	S.S.	10	6	9	17	8	9
	O.M.	12	7	14	17	10	11
	O.S.	13	13	13	17	10	12
	K.R.	7	6	6	18	5	7
	K.H.	9	11	10	24	9	10
	O.H.	7	7	6	6	5	6
L	Y.S.	3	6	3	4	3	4
	S.M.	0	0	0	3	0	0
	M.H.	3	4	3	4	0	3
	Y.G.	3	5	3	5	3	4
	S.J.	4	4	0	5	0	3
	E.M.	5	11	5	4	3	4
	T.F.	4	5	3	5	2	3
	S.K.	5	4	0	3	0	3
	Y.S.	5	4	0	6	0	2
	M.Y.	0	4	0	0	0	0

[a] Tests were carried out by Dr. S. Ishihara, National Suruga Leprosarium (July 1968).

[b] D: Dharmendra's antigen M: Murohashi's antigen.



#: Age of parent culture, ##: Inoculum size of new medium to make 10 ml.

FIG. 5. Model suggesting inoculum size and culture age of parent strain adequate for subculturing.

TABLE 3. *Intradermal reactions in leprous patients elicited by a 50 week-old, 2nd subculture of L-Aug.-68 strain.*[a]

Type of disease	Patient	Mean size (mm.) of reactions (erythema)[b]					
		48 hours		15 days		21 days	
		D	M	D	M	D	M
T	O.M.	18	10	11	8	9 u	9 u
	S.R.	18	7	6	7	5	8
	I.F.	23	17	16	9	12	8
	A.H.	18	6	6	6	6	5
	K.H.	26	17	8	8	6 u	6 u
	T.M.	20	10	17	13	8 u	8 u
	U.I.	14	4	6	7	5	5
	S.S.	17	9	6	7	6	7
	T.Y.	15	8	10	9	11 u	10 u
L	N.T.	6	6	4	3	(3)	(2)
	S.T.	0	0	0	0	0	0
	H.N.	5	6	2	3	(3)	3
	I.H.	4	7	0	0	0	0
	K.Y.	4	6	4	4	(4)	(4)
	Y.S.	4	4	4	4	0	(3)

[a] Tests were carried out by Dr. S. Ishihara, National Suruga Leprosarium (July 1970.

[b] D: Dharmendra's antigen, M: Murohashi's antigen, u: ulcer, (   ): pigmentation.

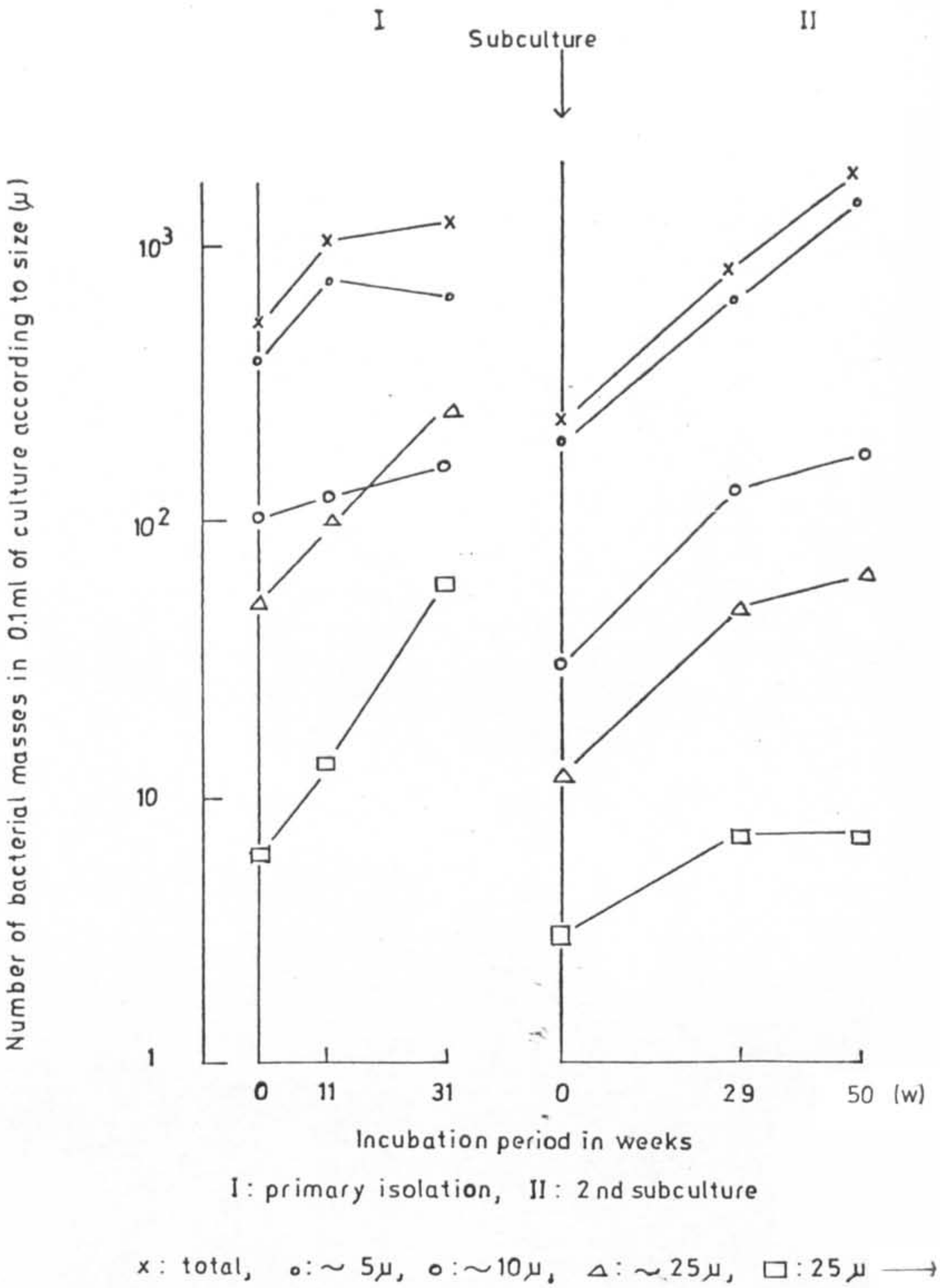


FIG. 6. Increasing trend curves of bacterial masses of L-Aug-68- strain (National Institute of Health).

Dharmendra's antigen. Thus it was proved that the bacterial masses existing in the second subcultures, also, were truly composed of *M. leprae* (Table 3).

Experiment 4. Correlation between colonial growth and differential staining of organisms by malachite-green-fuchsin(6). Between the culture results and the malachite-green stainability of the organisms in the specimens fair parallelism was observed, just as in the case of tubercle bacilli stained by this procedure. In the cases of L-Jun.-68-1, L-Jun.-68-2 and L-Jun.-69 strains, in which almost all of the acid-fast rods detected in smears before cultivation were stainable by

SUMMARY

Attempts were made to cultivate *M. leprae* from leprous nodules employing cell-free, semi-synthetic soft agar (0.1%) media of various base compositions. Many bacterial masses of various shapes and sizes appeared microscopically in the media and were maintained even for more than one year of incubation at 37°C. They could be subcultured successively, with increasing number and size of bacterial masses, under the same *in vitro* conditions as the primary isolation.

The bacterial masses detected were com-

[redacted]