PREPARATION AND PROPERTIES OF A FREEZE-DRIED B.C.G. VACCINE OF INCREASED STABILITY

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A stable B.C.G. vaccine, prepared and distributed in the freeze-dried form, is now accepted for use in many parts of the world. Recent trials (Medical Research Council, 1958, 1960; Griffiths and Gaisford, 1956) have confirmed that the freeze-dried vaccine induces a high degree of tuberculin sensitivity without untoward local reactions. From its stability the freeze-dried vaccine has undoubted advantages over a liquid vaccine, particularly because it permits standardization and safety-testing to be completed before issue. Nevertheless, since the vaccine contains living cells, of which an optimal number is essential for the efficacy of B.C.G. vaccination, great care has to be taken to ensure that the vaccine is not exposed to elevated temperatures even for relatively short periods. It is therefore obvious that a heat-resistant freeze-dried B.C.G. vaccine that could be kept at temperatures up to 37° C. for a few weeks would have advantages, since it could then be distributed under unrefrigerated conditions and transported in tropical and subtropical countries without special precautions.

Numerous attempts have been made to improve the heat-stability of freeze-dried B.C.G. vaccine. Almost all of them have concentrated on the medium in which the organisms are freeze-dried, since this has usually been regarded as the most important factor affecting loss of viability during both freeze-drying and subsequent storage. Thus, for example, Miller and Goodner (1953) showed that sodium glutamate, as well as sodium aspartate and ascorbic acid, when included in the menstruum, increased the stability of the vaccine on storage. Cho and Obayashi (1956), working with the Japanese strain of B.C.G., concluded that the inclusion of 1% sodium glutamate provided one of the best media for freeze-drying B.C.G. Greaves (1960), in studies with *Bacterium paracoli* and *Neisseria gonorrhoeae*, also found that the presence of sodium glutamate in the drying medium gave subsequent enhanced stability at elevated temperatures. The rationale behind these improvements has been given by Scott (1960). In work on *Salmonella newport* he showed that the sucrose included in the menstruum gave better stability during storage than did glucose or arabinose, both of which contain an available carbonyl group. He advanced the theory that the action of the menstruum is one of inhibiting or preventing damage to the micro-organisms resulting from the reaction between cellular protein and available carbonyl groups.

In our laboratory the actions of many different media for freeze-drying B.C.G. have been investigated: they have included that of sodium glutamate (Muggleton, 1960). Under our conditions and with our substrain of B.C.G., however, no outstanding improvement in survival on storage at elevated temperatures was seen with any of these media, and none showed any great merit, such as had been claimed by the Japanese workers.

It was logical to assume that other factors could influence the stability of the dried vaccine, and it was decided to investigate the culture medium in which the B.C.G. was grown. In previous experimental work (Ungar *et al.*, 1956) Sauton's medium with the addition of 1/4,000 triton WR1339 was used to grow the organisms as a uniform suspension in deep culture. Having then found that the addition of various substances to the freeze-drying menstruum had little effect on the stability of the vaccine, it seemed possible that Sauton's medium either already contained a substance harmful to the cells during and after drying or that such a substance was produced during the growth of the B.C.G. from some precursor in the medium. Heap and Cadness (1924), working with *Salmonella typhimurium*, demonstrated that a fermentable carbohydrate, such as glucose, produced an early enhancement of growth in peptone-water culture but that prolonged incubation caused a rapid fall in the number of viable organisms. A peptone-water culture without glucose showed a fairly constant count for a long time. Dubos and Fenner (1950), searching for a medium for the optimal growth of B.C.G., found that a striking decrease in viability of the culture occurred on prolonged incubation if 0.5% glycerol had been added to the medium instead of glucose.

It was decided to produce a medium without glycerol for the growth of B.C.G. and to study the properties of a freeze-dried B.C.G. vaccine prepared from organisms grown therein.

Experimental

Glycerol-free Medium

After numerous attempts to produce a medium without glycerol in which, with the addition of 1/4,000 w/v triton WR1339, the B.C.G. strain would grow in deep culture, the formula given below was devised—the composition of the modified Sauton's medium used previously (Ungar *et al.*, 1956) being shown for comparison.

Glycerol-free Medium		*Sauton's Medium (Modified)*	
L-Asparagine	4·0 g.	L-Asparagine	4·0 g.
Ferric ammonium citrate	0·1 ,,	Ferric ammonium citrate	0·05 ,,
Monosodium glutamate	4·0 ,,	Citric acid	2·0 ,,
Bacto casitone	1·0 ,,	Glycerol	40·0 ml.
L-Glutamine	4·0 ,,	Dipotassium hydrogen phosphate	0·5 g.
Potassium dihydrogen phosphate	1·0 ,,	Magnesium sulphate	0·5 ,,
Disodium hydrogen phosphate	2·5 ,,	Triton WR1339	0·25 ,,
Calcium chloride (anhyd.)	0·001 g.	Distilled water to	1 litre
Copper sulphate (anhyd.)	0·0005 ,,		
Zinc sulphate	0·0005 ,,		
Triton WR1339	0·25 g.		
Distilled water to	1 litre		

The *p*H value of each medium was adjusted to 7.4, and they were dispensed in 100-ml. amounts into mould culture (" penicillin ") flasks of about 2 litres capacity. The medium was sterilized in the autoclave at 15-lb. (6.8 kg.) pressure for 20 minutes.

Strain of B.C.G.

The Danish (" Copenhagen ") strain of B.C.G. was used in this work. The master cultures were maintained in the freeze-dried state, from which they were recovered on Löwenstein–Jensen medium as required. From these

cultures they were transferred three times through Dubos's liquid medium to produce a "deep-growth" homogeneous inoculum. The third Dubos culture, after seven days' incubation at 37° C., was used as inoculum for the production cultures.

The production cultures were incubated at 37° C. for 12 days. Growth in either the Sauton's medium or the glycerol-free medium was similar, both taking place deep in the medium. The organisms tended to collect at the bottom of the culture, but a gentle swirl produced a uniform turbidity of unclumped cells. The cells were harvested by centrifuging in a refrigerated MSE "Major" centrifuge at approximately 3,000 *g* for 30 minutes.

Preparation of Freeze-dried Vaccine

Dried vaccine was made from the cells grown in the two types of medium by the method we have described previously (Ungar *et al.*, 1956). The freeze-drying menstruum consisted of dextran 8.3%, glucose 7.5%, and triton WR1339 0.025%. The primary freeze-drying and secondary drying (over P_2O_5) conditions were controlled so as to leave a final residual moisture of approximately 1% in the freeze-dried vaccine. The ampoules were sealed *in vacuo* (about 0.05 mm. Hg).

Viability Counts

Viability counts on the reconstituted vaccine suspensions were then carried out. Serial tenfold dilutions (1/10, 1/100, 1/1,000, etc.) were prepared in Sauton's medium with 0.025% triton WR1339 added. The use of a fresh pipette for each dilution-step was found to be important, since the B.C.G. cells, which carry a low negative surface charge, are readily adsorbed on to the glass surface. Drops of 0.02 ml. each from a platinum-tipped pipette were placed on the surface of oleic acid-albumin agar with 10% human blood in petri dishes. About 10 drops were applied to each plate in a manner similar to that described by Miles *et al.* (1938). The colonies from each drop were counted under a stereo-binocular microscope after 21 days' incubation at 37° C. The most reproducible counts were obtained from the dilution giving between 10 and 50 colonies per 0.02 ml. drop. The viability counts of the reconstituted freeze-dried vaccines were obtained on multiplying the average number of colonies counted from each drop by the dilution used and by the number of drops per ml. (theoretically 50) delivered by the dropping-pipette. In practice it was found that different pipettes delivered drops of slightly different sizes, so that it was necessary to calibrate each pipette (numbers of drops per ml.) and apply an appropriate correction factor.

Properties of the Freeze-dried B.C.G. Grown in Glycerol-free Medium

(a) Survival on Freeze-drying

There was a considerable increase in the proportion of organisms from the glycerol-free culture surviving the freeze-drying process when compared with cultures grown in Sauton's medium. Under similar conditions, on the average 57% of the former survived, whereas from the Sauton's medium cultures (with glycerol) survival was 29% (Table I). Thus to achieve the same number of viable organisms in the vaccine after it has been freeze-dried it is necessary to fill into the ampoules only about a half of the number that would be needed had they been grown in Sauton's medium.

TABLE I.—*Survival on Freeze-drying of B.C.G. Grown in Glycerol-free Medium*

Medium in which B.C.G. Grown	Batch	Viability Count/mg. Moist Weight		% Survival
		Before Freeze-drying	After Freeze-drying	
Glycerol-free	A	124×10^6	66×10^6	53
	B	46×10^6	27×10^6	58
	C	97×10^6	58×10^6	60
Control (Sauton/triton)	A	63×10^6	16×10^6	25
	B	57×10^6	21×10^6	36
	C	64×10^6	16×10^6	26

(b) Stability on Storage at Elevated Temperatures

The freeze-dried vaccines prepared from cultures grown in Sauton's medium do not fall significantly in viability over a period of two years at refrigerator (4° C.) temperature. Experiments to compare the keeping qualities of vaccine produced in the glycerol-free medium would therefore be protracted if carried out at this temperature. Comparisons were therefore made of the stabilities of the two types at 37° C. (as representing average tropical ambient temperatures) and 70° C. (as representing temperatures to which the vaccine might be accidentally exposed for a short time—for example, in full tropical sun).

The ampoules were kept in an incubator at 37° C. and in a water bath (totally immersed) at 70° C. Also included in the test at 70° C. were ampoules containing B.C.G. from glycerol-free cultures that had 0.05% glycerol added to the freeze-drying menstruum. The results are summarized in Tables II and III. They show

TABLE II.—*Stability of Freeze-dried B.C.G. Prepared from Glycerol-free Medium Cultures at 37° C.*

Medium in which B.C.G Grown	Viability Count (as Percentage of Starting Value) at				
	0 Months	1 Month	2 Months	4 Months	6 Months
Glycerol-free	100	86	44	23	11
Control (Sauton/triton) ..	100	16	4	0·6	0·01

TABLE III.—*Stability of Freeze-dried B.C.G. Prepared from Glycerol-free Medium Cultures at 70° C.*

Medium in which B.C.G. Grown	Viability Count (as Percentage of Starting Value) at				
	0 Hours	½ Hour	1 Hour	3 Hours	6 Hours
Glycerol-free ..	100	94	64	Not tested	46
Glycerol-free*	100	88	77	70	61
Control (Sauton/triton) ..	100	0·7	0·5	0·1	0·07

* 0·02% glycerol added to the freeze-drying medium.

that the vaccine from glycerol-free cultures withstood a temperature of 37° C. for one month without deterioration below a limit of viability that would preclude their use for vaccination and that a temperature of 70° C. for several hours did not cause any great fall in viable cell content. The addition of glycerol to the freeze-drying menstruum did not affect this increased stability of the vaccine from cultures grown in the glycerol-free medium.

This greater resistance to elevated temperatures occurs only with the sealed ampoules of freeze-dried vaccine. Before freeze drying or after drying and subsequent reconstitution, the B.C.G. cells in suspension were found to be just as readily killed by heating as were those grown in Sauton's medium.

(c) Properties of the Stable Vaccine in Experimental Animals

Ten separate batches of vaccine of the heat-stable type were subjected to the tests for absence of virulence (in guinea-pigs), for local lesion formation (Jensen test), and for skin-sensitizing potency (in guinea-pigs), in accordance with the British Therapeutic Substances Regulations (1952–57). In these tests the batches of heat-stable vaccine were so prepared as to have viability counts falling between the same limits (not less than 4×10^6/ml. and not more than 9×10^6/ml.) as the routine batches of vaccine, prepared from Sauton's medium cultures, under test at the same time. The tests showed that the heat-stable vaccines gave exactly the same results as the control vaccines prepared in Sauton's medium. It was therefore concluded that batches of vaccine could safely be tested in human subjects.

(d) Comparison of Heat-stable and Control Vaccines in Newborn Infants

To make the most valid comparison possible, three pairs of vaccines (heat-stable and control) were compared on separate occasions. The pairs were deliberately chosen so as to have similar viability counts. The viability counts, batch numbers, and optical density equivalents of the batches used are shown in Table IV. Since only minor differences, if any, in the biological properties could be expected between the two types of vaccine, it was decided to carry out the tests on newborn infants, as constituting a more homogeneous group than older children. Moreover, previous experience has shown that differences in the performance of vaccines due to such factors as different viabilities could readily be detected in this age-group (Griffiths and Gaisford, 1956).

TABLE IV.—*Details of Batches of "Heat-stable" and Control Vaccine Tested on Babies*

Batch No.	Type of Vaccine	Optical Density (mg. Equivalent)	Viability Count ($\times 10^6$/ml. of Reconstituted Vaccine)
323B	Heat-stable	0·3 mg./ml.	8·8
325B	Control	0·75 ,,	7·25
406	Heat-stable	0·3 ,,	6·3
407	Control	0·75 ,,	6·7
419	Heat-stable	0·3 ,,	6·0
418	Control	0·75 ,,	5·9

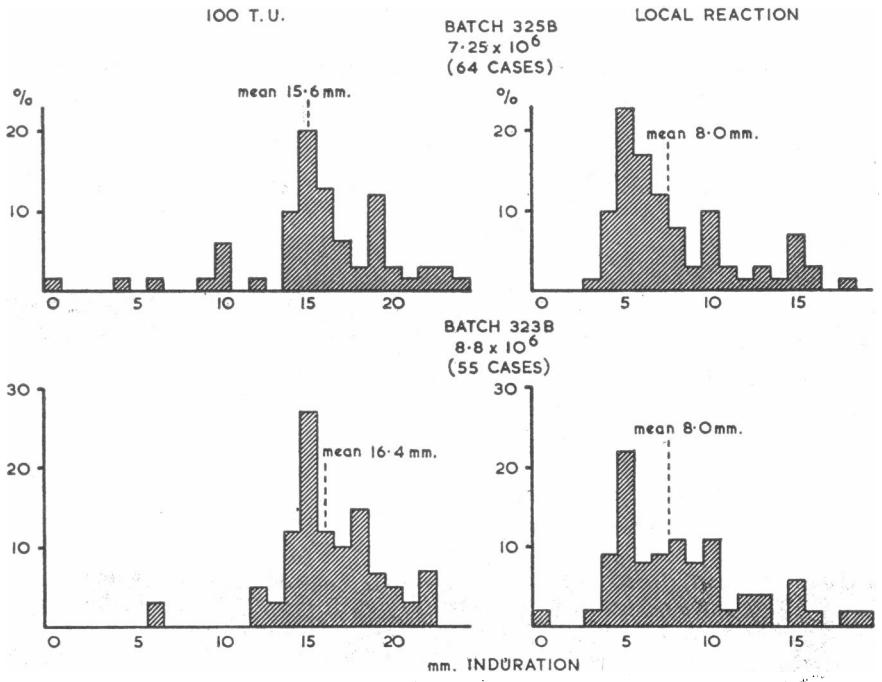
Three batches (323, 406, and 419) of heat-stable vaccine were paired with three batches (325, 407, and 418) of control vaccine of similar viable count. Batches 323 and 325 were given during the period January–March, 1960, batches 406 and 407 in January–February, 1961, and batches 418 and 419 in February–March, 1961. The vaccine was offered to all babies born in St. Mary's Hospital, Manchester, and those whose mothers accepted were vaccinated during the first four weeks of life without preliminary tuberculin-testing. Alternate babies were given 0.1 ml. of heat-stable or control vaccine intradermally into the skin in the region of the insertion of the right deltoid. A separate syringe was used for each vaccine. The infants were seen again four weeks later, the vaccination site was inspected, and a tuberculin test (100 T.U. of O.T.) was performed and read in 48 hours. A test with this dilution of tuberculin and at this interval was chosen for the reasons that (*a*) 4-weeks interval is suitable for examining infants at a routine infant welfare clinic; (*b*) it is a reasonable period for which an infant can be segregated from a source of tuberculous infection if necessary; (*c*) there is sufficient variation in the tuberculin allergy produced to make it a sensitive test for comparing techniques and vaccines; and (*d*) at this short time after vaccination newborn infants have become sensitive only to 100 T.U., but this is a significant indication of tuberculin conversion at this age (Griffiths and Gaisford, 1956).

Those infants in whom a sufficient period had elapsed (batches 323 and 325 given in 1960) were tuberculin-tested (10 T.U. of O.T.) one year after vaccination.

The number of infants examined and the results of tuberculin-testing are shown in Table V and the accompanying Histogram. It is clear from these results that both the mean size of the local lesion and the tuberculin sensitivity to 100 T.U. of O.T. four weeks after vaccination and 10 T.U. of O.T. a year later showed

TABLE V.—*Summary of the Clinical Results (on Babies) with "Heat-stable" and Control Batches of Vaccine*

Batch No.	Type of Vaccine	No. of Cases	Size of Vaccination Lesions (Average mm. Diameter)	Results of Post-vaccinal Tuberculin Test (Average mm. Induration and %)	
				4 Weeks (100 T.U.)	1 Year (10 T.U.)
323B	Heat-stable	55	8·0	16·4 (100%)	16·3 (100%)
325B	Control	64	7·3	15·6 (97%)	16·7 (100%)
406	Heat-stable	55	7·0	14·7 (98%)	Not yet tested
407	Control	52	6·7	15·8 (100%)	,, ,, ,,
419	Heat-stable	59	6·2	16·2 (98·5%)	,, ,, ,,
418	Control	57	7·0	14·8 (98%)	,, ,, ,,



Results of tuberculin-testing. Batch 323B (heat-stable) and batch 325B (control) compared.

no significant difference between the performances of heat-stable and control vaccines of similar viable count.

Discussion

We have shown above that B.C.G. cells grown in a medium in which no glycerol is present survive the process of freeze-drying to a much greater extent, and when so dried have a much greater resistance at elevated temperatures than those grown on media containing glycerol. This finding is in accordance with the hypothesis that some metabolic product of glycerol, at present unidentified but probably an aldehyde or pyruvate, has a toxic action on the B.C.G. cells in the dried state. Glycerol itself would not appear to be incriminated, since the addition of glycerol to the freeze-drying menstruum for cells grown in the absence of glycerol had no adverse effect. It seems possible that the metabolic product of the glycerol contains a carbonyl group capable of reacting to form a toxic "browning reaction" compound in the manner described by Scott (1960).

Laboratory tests and tests on experimental animals have shown that, apart from surviving better during the freeze-drying process and on storage in the dried state, the B.C.G. cells grown in the new glycerol-free medium have the same biological properties as those grown on Sauton's medium containing glycerol. After this finding it seemed justified to compare on babies the relative tuberculin-sensitizing powers of the heat-stable and control vaccines; the results have shown that the effects of the two types of vaccine are identical. Further, the two types of vaccine give the same incidence of local reactions at the vaccination site. We therefore conclude that the heat-stable vaccine would be as acceptable for vaccination as the more thermolabile vaccine it is meant to replace.

The increased resistance to heat of the new vaccine will have obvious advantages in the field. Normal unrefrigerated transportation will generally be permissible, and accidental short exposure to even relatively high temperatures should not prove disastrous to the efficacy of the vaccine. It is not suggested that the vaccine is so stable as to permit abuse of recommended storage conditions; it should, however, enable fully viable B.C.G. vaccine to reach persons living in remote districts in tropical and subtropical countries, where they might otherwise be denied the protection afforded by B.C.G. vaccination.

Summary

A method is described for preparing freeze-dried B.C.G. vaccine with increased stability to heat.

The method involves growing the B.C.G. cells from which the vaccine is prepared in a glycerol-free medium. The hypothesis is advanced that absence of some toxic product of glycerol metabolism permits this stability.

Apart from a greater stability to heat, the new vaccine has the same biological properties as a control vaccine of the same viability prepared in conventional culture media.

Batches of heat-stable and control vaccines of the same viability have the same allergenic properties when used to vaccinate babies and produce similar local reactions at the vaccination site.

The more heat-stable vaccine will have obvious advantages for distribution and use in tropical and subtropical countries.

Our thanks are due to Professor Wilfrid Gaisford, under whose direction the clinical studies were undertaken, for his continuous interest in this work.

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ELECTROENCEPHALOGRAPHIC STUDIES IN TRIPLE-IMMUNIZED INFANTS

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The problem of cerebral complications following triple or pertussis immunization has been very much debated in Sweden since 1959, when Ström at a meeting presented an alarming series of cases, where symptoms of severe brain injury had followed the immunization procedure. The report appeared in this journal (Ström, 1960). In a letter to the Editor shortly afterwards (Malmgren *et al.*, 1960) it was claimed that Ström had shown an uncritical attitude when accepting a connexion between the immunization and the symptoms indicating brain damage in several cases. These objections were partly based on a report of a committee appointed by the Royal Medical Board, in which the incidence of permanent damage to the central nervous system was calculated to be 1 in 50,000 as compared with Ström's figure of 1 in 16,000. A short review of the problem including a preliminary report on some electroencephalographic studies has been published in Swedish (Hellström, 1962). The present paper deals with a more detailed account of these observations.

In a previous investigation by Low (1955) comprising 83 infants, where the E.E.G. was recorded after pertussis or triple immunization, one case was found with a marked slowing in all leads during some days afterwards. In one week the E.E.G. again became normal. In another case less marked and more questionable changes were noted. It was concluded that mild but possibly significant cerebral reactions may occur in connexion with pertussis immunization.

Electroencephalography has also been used as a tool to detect subclinical cerebral reactions in connexion with