Conclusions

The Danish B.C.G. vaccine has proved uniformly satisfactory for use, in appropriate dosage, in newborn infants. It suffers, however, from the defects inherent in any living liquid vaccine and it produces complications of a minor, but nevertheless undesirable, nature in a small proportion of cases.

A new British freeze-dried vaccine has been found to be equally effective when given in suitable doses. The clinical effects of such a vaccine have been shown to vary according to the number of viable organisms injected. Since accuracy of bacillary dosage is now a practical possibility, it is probable that satisfactory freeze-dried vaccines with none of the disadvantages of the liquid preparations may soon replace them.

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REFERENCES

- Lorber, J., Hart, P. D'A., Farmer, P., and Muggleton, P. W. (1956). *Tubercle (Lond.)*, 37, 187.
 Medical Research Council (1956). *British Medical Journal*, 1, 413.
 Ungar, J., Farmer, P., and Muggleton, P. W. (1956). *Ibid.*, 2, 568.

FREEZE-DRIED B.C.G. VACCINE

METHODS ADOPTED IN PREPARATION OF A STANDARD PRODUCT

BY

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During the past thirty years B.C.G. vaccine has become established in many countries as an effective agent against tuberculosis. Liquid vaccines, which have been mainly used during this time, have had two major disadvantages—namely, a short life (fourteen days), with its attendant distribution difficulties, and consequent inadequacy of laboratory tests to prove that each batch contains effective antigen and is safe.

With the development of freeze-drying and the discovery that living cultures of bacteria could be freeze-dried without affecting their biological characteristics, including viability for long periods, attempts at producing freeze-dried vaccines from B.C.G. were a logical development. A number of such vaccines have been described (Ungar and Muggleton, 1948; Rosenthal, 1948; Ungar, 1949; van Deinse and Senechal, 1950; Obayashi, 1955).

An effective freeze-dried B.C.G. vaccine needs to satisfy a number of criteria: (1) it must contain a sufficient number of antigenic viable organisms; (2) it must not contain an excessive number of viable organisms producing marked reactions; (3) it must be stable when stored at normal temperatures; (4) it must be produced from a homogeneous suspension of cells containing the minimum of clumps and give an even suspension on reconstitution; (5) it should be pharmaceutically elegant, particularly in ease of reconstitution; and (6) it must be freeze-dried in a medium containing no substances whose subsequent injection would be liable to cause unpleasant reactions.

Almost all B.C.G. vaccines, whether liquid or freeze-dried, are prepared from strains grown as surface cultures; the pellicle collected from such cultures is milled by steel, glass, or agate balls to form a homogeneous suspension. Such suspensions may contain clumps of varying sizes, which can cause reactions to the vaccine injected intracutaneously and, moreover, make difficult the interpretation of viable counts on the vaccine. Further, the process of milling might affect the surface of the cells and render them vulnerable to certain chemical, physical, or thermal insults. In the preparation of freeze-dried B.C.G. vaccine the medium in which the cells are dried is of prime importance. Vaccines have been prepared in which serum, 50% glucose solution, 1% sucrose solution, various concentrations of lactose, sucrose solution with gelatin, and certain other solutions were used. Most of these have disadvantages of one kind or another, such as unsightly appearance, difficulties of reconstitution, unsuitability for injection into human patients, or "fluffy" consistency after freeze-drying so that part of the ampoule content is lost when the vacuum in the ampoule is released.

This paper describes the preparation and properties of a freeze-dried B.C.G. vaccine that has been designed to overcome some of the above-mentioned objections. It represents the result of prolonged and detailed studies for ten years on the numerous factors that had to be separately evaluated before it could reach the production stage.

Technical Details

1. *Strain of B.C.G. Used for Vaccine Production.*—The Copenhagen substrain of the B.C.G. strain has been used throughout our work. When relevant, comparative tests were carried out with the B.C.G. strain from the Pasteur Institute in Paris. Apart from certain minor differences in biological properties, the two strains behave in essentially the same way, such as growth requirements, sensitizing properties for guinea-pigs, and protective action. The strain was freeze-dried at the beginning of this work, regular recoveries from the freeze-dried master cultures being made on Löwenstein-Jensen medium at three-monthly intervals to give submaster cultures; they are subcultivated on the same medium once monthly until a fresh culture is started from the freeze-dried master culture.

2. *Method of Preparation of Bacterial Suspension.*—The submaster culture is carried through three transfers at seven-day intervals in Dubos liquid medium containing "tween 80" and 2% bovine albumin. The third culture invariably shows a homogeneous growth throughout the medium, and this, when seven days old, is used to inoculate the production medium, which consists of Sauton's medium with the addition of 1/4000 w/v of "triton WR1339," a non-ionic polyoxyethylene ether. This wetting agent is stable in the cultures and does not require the addition of albumin to protect the B.C.G. from the bacteriostatic action of hydrolysis products; it was chosen after investigating a large number of wetting agents. The cultures are prepared in "mould culture flasks" containing a shallow layer of medium (8 mm. depth). The depth of the culture medium has a profound effect on the quantity of growth obtained; the highest proportion of growth resulted from shallow cultures. The incubation temperature was also found to be critical, and variations, particularly above 38° C., resulted in a marked growth reduction. These cultures appear as a characteristic thin veil growing at the bottom of the medium on the glass surface. They are left in the incubator undisturbed at 37° C. for nine days. Gentle shaking of the grown culture produces a uniform turbidity in the medium; on microscopical examination this is seen to consist of single cells with a few small aggregates of up to 10 cells. No milling is necessary, and the cells are harvested by centri-

fuging in a refrigerated centrifuge below 15° C. for thirty minutes at a relative centrifugal force of approximately 2,500 g.

3. *Preparation of Vaccine.*—The centrifuged cells are re-suspended in a medium consisting of an 8.3% aqueous solution of dextran with 7.5% glucose. The amount of medium added is sufficient to produce a final concentration of about 2 mg. moist weight of B.C.G. per ml. of vaccine. The B.C.G. content is standardized by opacity measurement in the Spekker photoelectric absorptiometer against a standard formalin-killed suspension. The suspension is then diluted with more dextran-glucose solution to give a concentration of B.C.G. that will contain the required number of viable organisms after freeze-drying. The vaccine is filled into special moulded neutral glass ampoules (0.5 ml. of the suspension into 2-ml. ampoules).

4. *Freeze-drying.*—The ampoules of vaccine are prefrozen in closed aluminium containers on a shelf-freezer at -50° C. and are held there for one hour, though freezing is complete in about five minutes. The lids of the containers are removed and the product is then dried in a specially adapted freeze-drier (W. Edwards & Co., model 31P) for sixteen hours. The temperature of the product during removal of most of the water is below -20° C.; it rises to not more than +25° C. at the end of the drying cycle. After primary drying the necks of the ampoules are constricted in an air/coal-gas blowpipe flame; the ampoules are then put on a secondary drying apparatus over phosphorus pentoxide for four hours and are finally sealed *in vacuo*.

5. *Reconstitution of Freeze-dried Vaccine.*—The ampoule is opened and 1 ml. of sterile physiological saline or sterile distilled water is added to the pad of freeze-dried vaccine. Resuspension of the vaccine is not delayed and aeration is avoided if the ampoule is allowed to stand on the bench for about thirty seconds. An even suspension is then obtained when the vaccine is drawn up into a syringe for injection.

6. *Estimation of Residual Moisture.*—Residual moisture is estimated by the vapour-pressure method (Beckett, 1954) on an Edwards apparatus. With this product it is found to give results agreeing with those obtained by the Carl-Fisher method.

7. *Estimation of Viable Cells.*—Viable cells in the product before and after the freeze-drying are enumerated by using a modification (O'Hea, 1955) of the method of Miles *et al.* (1938), whereby 0.02-ml. drops from a platinum dropping-pipette are applied to the surface of the medium held in small bottles (7-ml. bijou bottles). Blood agar (10% blood in papain digest (horsemeat) agar) is used as the medium for viability counts. The counts are usually made in parallel on Löwenstein-Jensen medium. The two types of medium give similar results, but blood agar is preferable because the colonies are easier to enumerate as they do not become confluent. The vaccine suspensions are diluted in Sauton's medium, with 0.025% triton WR1339 added to inhibit aggregation. Tenfold dilutions from 10⁻³ to 10⁻⁶ are made. After 28 days' incubation at 37° C. the colonies on the surface of the medium are counted by means of a low-power stereoscopic microscope. Duplicate sets of dilutions are prepared from each vaccine suspension and two bottles of medium are inoculated from each dilution. Each result taken is the average of the four counts.

8. *Safety and Potency Tests in Animals.*—The safety of the vaccine and its immunizing power are assessed by the following tests:

Test for Absence of Virulence.—5 mg. of B.C.G. suspension is injected intramuscularly into each of six guinea-pigs. After six weeks the animals are killed for post-mortem examination. The vaccine invariably produces a pus-filled abscess at the injection site and a unilateral enlargement of the inguinal and pre-aortal lymph nodes on the side of the injection; no signs of progressive disease are ever seen. Parallel tests are regularly carried out on guinea-pigs left for six months' observation. No progressive disease has ever been seen up to this time either, and the changes seen at six weeks have never developed further.

Measurement of Degree of Reaction to Vaccine in Guinea-pigs.—Six albino guinea-pigs 250 g. in weight are shaved on the flank, and 0.1 ml. (0.1 mg.) of reconstituted vaccine is injected intracutaneously. The site of the injection is then examined every week for five weeks and the size of the papule measured and recorded, according to a "scoring system" from 0-4 (see Table VI).

Tuberculin Conversion Test in Guinea-pigs.—Six albino guinea-pigs are injected with 0.1 ml. of reconstituted vaccine intradermally into the depilated flank. Twenty and again twenty-eight days later the animals are injected intracutaneously with 10 T.U. of P.P.D. into the other depilated flank. The reaction is read after twenty-four hours by measuring the diameter of the oedema.

Results

Table I shows the results of viability counts carried out on three batches of the vaccine at three-monthly intervals over a period of twelve months. Each figure recorded is the average of the counts from two ampoules carried out at the same time and in duplicate.

The viability counts on different ampoules selected at random from any one batch did not differ by amounts greater than the experimental error of the test (Table II). The results shown are the averages of two counts carried out on each of eight different ampoules of Batch 115 two months after preparation. They show that the batch was uniform and that there was little difference between ampoules.

In Table III are shown moisture contents and the average viable count after drying a B.C.G. suspension in 8.3% dextran solution with 0.025% triton WR1339 containing 0%, 1%, 5%, 7.5%, 10%, and 20% glucose. The results show that with dextran alone virtually all of the water was removed, but that the culture no longer contained any viable cells. The minimum amount of glucose needed to secure maximum retention of viability appears to be 7.5%, and this is the quantity used in the routine production. With this glucose concentration, and under the conditions of freeze-drying normally used, the residual moisture content appears

TABLE I.—Relation Between Storage at 20° C. and the Viability of the Freeze-dried Vaccine

Time After Preparation	Viable-cell Count per ml. Reconstituted Vaccine*		
	Batch A	Batch B	Batch C
0	1.5 × 10 ⁶	115 × 10 ⁶	0.5 × 10 ⁶
1 month	Not tested	Not tested	0.28 × 10 ⁶
3 months	2.6 × 10 ⁶	95 × 10 ⁶	0.2 × 10 ⁶
6 "	1.36 × 10 ⁶	40 × 10 ⁶	0.3 × 10 ⁶
9 "	Not tested	75 × 10 ⁶	0.2 × 10 ⁶
12 "	1.4 × 10 ⁶	Not tested	0.36 × 10 ⁶

* Average of two ampoules enumerated separately, each count duplicated.

TABLE II.—Viable-cell Counts on Eight Ampoules of Vaccine from the Same Batch (Batch 115)

Ampoule	Viable Cell Count per ml. Reconstituted Vaccine
1	1.40 × 10 ⁶
2	1.48 × 10 ⁶
3	2.00 × 10 ⁶
4	2.02 × 10 ⁶
5	1.87 × 10 ⁶
6	1.60 × 10 ⁶
7	1.64 × 10 ⁶
8	2.12 × 10 ⁶

TABLE III.—Relation Between Glucose Concentration in the Medium, Residual Moisture, and Viable-cell Count

Batch	Composition of Drying Medium		Residual Moisture Content	Viable-cell Count per ml. Reconstituted Vaccine
	Dextran	Glucose		
Z1	8.3%	20%	Unsatisfactory product*	
Z2	8.3%	10%	2.07%	12 × 10 ⁶
Z3	8.3%	7.5%	1.26%	10 × 10 ⁶
Z4	8.3%	5%	0.88%	1 × 10 ⁶
Z5	8.3%	1%	0.20%	0.02 × 10 ⁶
Z6	8.3%	0%	0.10%	0

* Medium containing 20% glucose had thawed out during the later stages of freeze-drying to give a "boiled toffee-like" solid of ugly appearance.

to be self-controlled. The moisture has been found by the vapour-pressure method always to fall within the limits 0.8–1.4%. Too low a moisture content was not reached even when secondary freeze-drying was continued for seventy-two hours. Batches of vaccine that had lower concentrations of glucose than 7.5%, and were, therefore, to some extent "over-dried," retained their lower degree of viability well. There has been some indication, in contrast to this finding, that batches containing more than 10% glucose and having more than 2% residual moisture lose their viability more rapidly. Table IV shows the effect of

TABLE IV.—*Relations Between Temperature of Storage and Viable-cell Count*

Time (Weeks)	Viable-cell Count* (per ml. Reconstituted Vaccine) of Ampoules stored at:			
	4° C.	20° C.	27° C.	37° C.
0	15.5×10^6	15.5×10^6	15.5×10^6	15.5×10^6
3	16×10^6	16.5×10^6	14×10^6	9×10^6
4	11×10^6	13×10^6	14×10^6	1×10^6
5	14×10^6	10×10^6	11×10^6	1×10^6
6	7×10^6	15×10^6	12×10^6	$< 0.005 \times 10^6$
10	12×10^6	15×10^6	15×10^6	$< 0.005 \times 10^6$
16	14×10^6	14×10^6	13×10^6	$< 0.005 \times 10^6$

* Average count from two ampoules, each enumerated in duplicate.

storage temperature on the viable-cell counts of a batch of vaccine kept at 4° C. (refrigerator), at room temperature, at 27° C., and at 37° C. The results indicate that at temperatures up to 27° C. the cells retain their viability. At 37° C., however, viability began to fall off in less than three weeks. It is recommended that the vaccine be stored at below 20° C.

By decreasing the glucose content of the drying medium, or by alterations in the freeze-drying procedure, batches of lower remaining viable-cell content can be produced. Some of such batches, together with others of higher viable-cell content, have been tested clinically on children for tuberculin-converting and reaction-provoking properties. The results are reported elsewhere (Griffiths and Gaisford, 1956; Lorber *et al.*, 1956). Table V shows the results of tuber-

TABLE V.—*Tuberculin Conversion in Guinea-pigs After Immunization with Vaccines of Different Viability*

Batch No.	Viable-cell Count per ml.	Tuberculin Test (Time after Vaccination)	Tuberculin Reaction (mm. Induration) 24 hours after 0.1 ml. 1/1,000 P.P.D. Intradermally					
			G. pig 1	G. pig 2	G. pig 3	G. pig 4	G. pig 5	G. pig 6
94b	20.8×10^6	20 days	12	7	8	7	10	8
		28 "	18	16	14	15	17	14
73	10.0×10^6	20 "	10	11	8	8	6	7
		28 "	16	17	14	16	16	14
62	0.7×10^6	20 "	6	9	10	9	8	8
		28 "	7	12	13	13	11	11
50	0.1×10^6	20 "	—	6	—	7	—	—
		28 "	8	8	7	11	9	6
50x	0 Heat killed	20 "	—	—	—	—	—	—
		28 "	—	—	—	—	—	—

Vaccination dose and route—0.1 ml. of vaccine injected intradermally. — Indicates less than 5 mm. (taken as negative).

TABLE VI.—*Relation Between Viable Cell Count of Vaccines and Size of Local Reaction at the Vaccination Site*

Batch No.	Viable-cell Count	Extent of Local Lesion at Vaccination Site at 17 Days after Vaccination					
		G. Pig 1	G. Pig 2	G. Pig 3	G. Pig 4	G. Pig 5	G. Pig 6
78	60.0×10^6	4	4	4	3	4	3
94b	20.8×10^6	4	3	2	2	3	2
73	10.0×10^6	2	2	3	2	2	2
62	0.7×10^6	2	2	2	2	2	1
50	0.1×10^6	0	0	1	1	1	1
50x	0 Heat killed	0	0	0	0	0	0

0=No lesion. 1=Small 4 mm. lesion with small scab. 2=Lesion 4–6 mm. diameter with firm 2–3 mm. scab. 3=Lesion 7–10 mm. diameter with firm 5–6 mm. scab and some ulceration. 4=Lesion >10 mm. diameter with firm >6 mm. scab, ulceration, and deep necrosis.

culin-conversion tests on guinea-pigs carried out with five different batches having different viable-cell contents. They show that a batch having a relatively low viable-cell content was able to accomplish the conversion to the tuberculin-positive state in guinea-pigs in twenty-eight days, although a dead vaccine will not do so. In a similar manner the size of the local lesion produced by the intracutaneous injection of 0.1 ml. of vaccine was related to its viable-cell content (Table VI). Batches having up to 10×10^6 viable cells per ml. of vaccine, which would secure rapid tuberculin conversion in the guinea-pig test and in human children, did not produce severe lesions in the guinea-pigs. On the other hand, some batches having a very high viable-cell content (up to 100×10^6 viable cells per ml.) produced a severe, though rapidly healing (four to five weeks), ulcer of up to 1 cm. diameter.

Discussion

The preparation of a satisfactory viable vaccine from B.C.G. can be accomplished by freeze-drying a suitable suspension of cells in 8.3% dextran with 7.5% glucose. Under conditions of freeze-drying that can be fairly readily standardized, a product is obtained with satisfactory keeping properties over a period of twelve months' storage at below 20° C.

It has been shown that a satisfactory vaccine can be prepared from B.C.G. organisms grown in deep culture. This departure from the traditional practice of using surface cultures has been accomplished by the use of a wetting agent. When this is incorporated in the culture medium a deep growth of the B.C.G. develops as unclumped cells, which disperse well on shaking. It has thus been possible to disperse with the milling procedure necessary to produce a homogeneous suspension from surface growth—a procedure that may result in the retention of small clumps and render bacteria more vulnerable to the stress of freeze-drying.

To produce a B.C.G. vaccine of sufficient viability after freeze-drying, it is essential that a small amount of water be retained in the product, the amount depending on the composition of the freeze-drying medium used. It has been shown that with plain dextran solution as the medium a product can be produced with a very low moisture content and that the bacteria are thereby dried to death. Dextran, therefore, seems to act as a supporting colloidal matrix only. The addition of glucose at the optimal concentration of 7.5% enables enough water to be retained (about 1%) for the viable cells to be preserved and the vaccine to keep well. It has been found desirable to incorporate traces of triton WR1339 in the drying medium to overcome the tendency for the bacteria grown in deep culture to aggregate in dextran solutions and so give a non-homogeneous suspension when the vaccine is reconstituted. The triton has no effect on the survival of the bacteria or on their biological properties.

The freeze-dried vaccine produced in this way is a safe and reasonably constant product. Owing to its long storage life it can be accurately standardized before issue on the basis of its viable-cell content, and each batch can also be checked for safety—that is, absence of virulence. In addition each batch can be tested for its tuberculin-converting power and for its freedom from toxicity as judged by absence of excessive local lesions after injection into guinea-pigs. It is hoped that the elaborations of these procedures will have made available a standardized, effective, and safe B.C.G. vaccine.

Summary

A method is described for the production of a freeze-dried B.C.G. vaccine by using dextran with glucose as a protein-free drying medium.

The B.C.G. organisms for vaccine production can conveniently be grown in deep culture in Sauton's medium with 0.025% triton WR1339—a non-ionic polyoxyethylene ether—without affecting their biological properties.

The bacteria, uniformly dispersed, can be easily harvested in the centrifuge and the deposit resuspended in dextran-glucose solution.

A method is described for enumerating the viable cells with consistent results; the viable-cell content of different batches of vaccine can be accurately standardized.

A study of the keeping properties of the dried vaccine has shown that it has a life of at least twelve months when stored below 20° C.

The relationship between the viable-cell counts of various batches and the tuberculin conversion of guinea-pigs is shown. There is also a direct correlation between the viable-cell count and the size of local lesions in guinea-pigs after intradermal injection.

REFERENCES

- Beckett, L. G. (1954). *Biological Applications of Freezing and Drying*, edited by R. Harris, Chap. 12, p. 285. Academic Press, New York.
Griffiths, M., and Gaisford, W. (1956). *British Medical Journal*, 2, 565.
Lorber, J., Hart, C. B. S., Farmer, Pauline, and Muggleton, P. W. (1956). *Tubercle (Lond.)*, 37, 187.
Miles, A. A., Misra, S. S., and Irwin, J. O. (1938). *J. Hyg. (Camb.)*, 38, 732.
Obayashi, Y. (1955). *Wld Hlth Org. Monogr. Ser.*, No. 28.
O'Hea, A. J. (1955). *J. Path. Bact.*, 69, 169.
Rosenthal, S. R. (1948). *Proc. 1st int. Congr. BCG, Paris*, p. 40.
Ungar, J. (1949). *Tubercle (Lond.)*, 30, 2.
— and Muggleton, P. W. (1948). *Proc. 1st int. Congr. BCG, Paris*, p. 49.
van Deinsse, F., and Senechal, F. (1950). *Tubercle (Lond.)*, 31, 157.

ALDOSTERONE EXCRETION IN NORMAL AND TOXAEMIC PREGNANCIES*

BY

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An increase of sodium-retaining substances in the urine of women with toxæmia of pregnancy, determined by bio-assay, was first reported by Chart *et al.* (1951). Venning, Singer, and Simpson (1954) confirmed this, also using crude urine extracts for bio-assay. Greater release of sodium-retaining substances in urine can be obtained by allowing it to stand at pH 1 for twenty-four hours at room temperature (Axelrad *et al.*, 1954). Using pH 1 and β -glucuronidase hydrolysis prior to extracting the urine, Venning *et al.* (1955) reported an increase of sodium-retaining substances in toxæmia of pregnancy but little increase above normal in normal pregnancy.

Purification of urine extracts by chromatography before bio-assay has an obvious advantage. Such a technique has recently been reported by Barnes and Quilligan (1956), but without stating their evidence that the technique isolates aldosterone. They find a wide range of values in normal pregnancy, but the mean does not exceed the control value. In toxæmia they concluded that there is an increase in sodium-retaining substances, though their figures are all within the range of the non-toxæmic patients, and the mean values have a negative correlation with severity of toxæmia. Using a technique with single chromatography before bio-assay,

* Part of a thesis by I. H. M. for the degree of M.D. in the University of Cambridge.

Venning and Dyrenfurth (1956) reported that the aldosterone in seven normal pregnancies rose steadily from about 4 μ g. per day to about 30 μ g. per day at term, and that in toxæmic patients provisional results suggest that the aldosterone excretion is within the normal pregnancy range.

An alternative method of determining aldosterone by physico-chemical methods was reported by Neher and Wettstein (1955). This technique depends upon the alteration of relative positions of aldosterone and cortisone in two different chromatographic systems, whereby other steroids in the extracts are eliminated. In this way, the capriciousness of bio-assay is circumvented, but a new doubt is introduced about whether the technique is always capable of purifying the aldosterone completely.

Materials and Methods

The method used is a slight modification of that of Neher and Wettstein (1955). A twenty-four hour collection of urine, with chloroform as preservative, was brought to pH 1 and allowed to stand at room temperature for twenty-four hours. It was extracted three times with half volumes of chloroform, with the aid of a continuous stirrer. The chloroform was reduced to a smaller volume on a water-bath at 45° C. under reduced pressure and then washed twice with quarter volume of N/10 sodium hydroxide and twice with quarter volume of water, each wash being backwashed with chloroform. The extract was taken to dryness and then applied to paper which had been dipped in 20% propylene glycol in methanol, for chromatography in the Zafaroni toluene/propylene-glycol system (Burton *et al.*, 1951). The width of paper used was determined by the amount of pigment in the extract and varied between 7.5 and 15 cm. A marker strip with cortisone and hydrocortisone was also run, and after three days it was developed with blue tetrazolium in sodium hydroxide. The cortisone region of the extract strip was eluted with a mixture of chloroform, ethyl acetate, and methanol in equal quantities. Water was added to the eluate to form two phases, the propylene glycol from the paper passing mostly into the aqueous methanol. The latter was extracted a second time with chloroform. The extract was taken to dryness and applied to paper 1.5 cm. wide and run in the Bush C system—toluene:ethyl acetate:methanol:water=9:1:5:5 (Bush, 1952).

The first chromatography separates aldosterone and cortisone (which run together) from hydrocortisone. The second chromatography separates aldosterone (which then runs in the same position as hydrocortisone) from cortisone. The strip is developed with blue tetrazolium in sodium hydroxide so that substances with the Δ^4 -3-ketone configuration, which are also reducing, can be determined. The amount of aldosterone was estimated by comparing the soda fluorescence with that of known amounts of cortisone and hydrocortisone.

Collections of urine were obtained from 55 normal pregnant women and from 20 with toxæmia of late pregnancy; in one normal and one toxæmic patient two determinations were made at different stages of pregnancy. The normal cases were out-patients but they were carefully instructed how to make the collections. The toxæmic patients were all in hospital for the urine collections. When the urine could not be extracted immediately, it was kept frozen at -15° C.

Criteria of Toxæmia of Pregnancy.—These were as follows: the development in a woman 28 weeks pregnant or over of a blood pressure of 140/90 or more, together with albuminuria and oedema. In this series there was only one woman with hypertension but no albuminuria, and she has been grouped among the normal pregnancies.

In the toxæmic group there were 10 primiparae and 10 multiparae; 21 of the normal group were primiparae and 34 were multiparae.