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A Simple Chemically Defined Medium for the Production of Phase I *Bordetella pertussis*

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SUMMARY

A simple, chemically defined medium is described consisting of sodium glutamate, proline, cystine, salts, and growth factors, which is suitable for the large-scale production of phase I *Bordetella pertussis*. More than 30×10^9 organisms/ml. were produced in 48 to 72 h. growth in shake flasks and fermentors. The cultures were detoxified by the addition of 0·14 % formalin to yield vaccines which were non-toxic to mice and guinea pigs and had good mouse-protective antigen levels. The antigenic stability was satisfactory after storage of the final vaccines at elevated temperatures.

INTRODUCTION

Hornibrook (1939) first described the use of a liquid medium for the propagation of phase I *Bordetella pertussis*, and since that time many modifications of his original formulation have been published (Verwey & Sage, 1945; Wilson, 1945; Cohen & Wheeler, 1946; Verwey, Thiele, Sage & Schuchardt, 1949; Sutherland & Wilkinson, 1961). These liquid media have consisted of a casein hydrolysate to which were added various salts, growth factors, and either starch, charcoal, or anionic resins. Studies on the growth requirements of *B. pertussis* have resulted in the publication of several formulae for chemically defined media (Ungar *et al.* 1950; Jebb & Tomlinson, 1955, 1957; Wilson, 1963; Goldner, Jakus, Rhodes & Wilson, 1966), and an excellent review of the problem by Rowatt (1957). In a series of metabolic studies on *B. pertussis* in our laboratories, Wilson (1963), Goldner *et al.* (1966), Vajdic, Goldner & Wilson (1966), used a synthetic medium in rocked L-tubes and obtained good growth of phase I organisms. The composition of their optimal medium is given in Table 1. This is a completely defined medium apart from the liver co-enzyme preparation, but would be unsuitable for large-scale production due to its relative complexity. This paper describes further modifications which have resulted in greatly improved yields of phase I bacteria. The properties of vaccines prepared from these cultures were also examined.

METHODS

Seed culture preparation and growth conditions

The strain of *Bordetella pertussis* used in most of our investigations was one of the Connaught Laboratories' routine vaccine production strains, obtained originally from Dr Pearl L. Kendrick, and designated no. 18334. Two other strains were also used in the large-scale production experiments, Kendrick's no. 10536, and a strain obtained

from Dr R. J. Wilson, no. 1494. The lyophilized cultures were grown on a Bordet–Gengou plate for 4 to 5 days, subcultured to Bordet–Gengou slants in 8 × 1½ in. tubes, and grown for a further 48 h. All Bordet–Gengou media contained 30 % citrated sheep's blood. The growth from one slant was washed off into three 500 ml. Erlenmeyer flasks, containing 100 ml. liquid medium (see below), and grown on a rotary

Table 1. *Composition of chemically defined media for Bordetella pertussis*

Comparison between Goldner's formula and basic 'glutamate + proline' medium ('1 G + 1 P' medium).

Component	Goldner's formula* (mg./l.)	Basic 'glutamate + proline' medium ('1 G + 1 P') (mg./l.)
DL-Alanine	100	—
L-Arginine	40	—
DL-Aspartic acid	130	—
L-Glutamine	400	—
Glycine	50	—
DL-Histidine	50	—
DL-Serine	130	—
L-Proline	240	240
L-Glutamic acid	620	670†
L-Cystine	10	40
NaCl	2500	2500
KH_2PO_4	500	500
KCl	200	200
$MgCl_2.6H_2O$	100	100
$CaCl_2$	20	20
$FeSO_4.7H_2O$	10	10
$CuSO_4.5H_2O$	5	—
Adenine	10	—
ATP (Di-Na salt)	0·3	—
Guanine	0·3	—
Hypoxanthine	0·3	—
Thymine	0·3	—
Uracil	0·3	—
Xanthine	0·3	—
Activated Charcoal (B.D.H.)	15	—
Tris buffer 121	6075	6075
Liver co-enzymes (CoA, DPN, TPN)	100	—
Asorbic acid	5	20
Niacin	1	4
Glutathione	25	100
2-Deoxyribose	0·5	—

* Compiled from Goldner *et al.* (1966).
† Present as sodium glutamate.

action shaker for 48 h. (Model V, New Brunswick Scientific Co., New Jersey, U.S.A.; this model has a 1 in. circular orbit and was used at 350 rev./min). In the early work a casein hydrolysate medium (Wilson, 1945) was used for the growth of seed cultures, but the chemically defined medium was substituted in the shake flask and small fermentor experiments, following certain modifications, which will be discussed later. All growth temperatures were between 35 and 37°. Five ml. inocula were taken from these seed flasks and used to seed each 100 ml. experimental medium in 500 ml. flasks. In the large-scale production work, three sizes of fermentors were used: a Pyrex

jar (18 × 10 in.), containing 9 l. medium, and stainless steel tanks containing either 60 or 140 l. medium. All vessels were aerated by vortex stirring with air blown over the surface at the rate of 0·1 l. of air/l. of medium/min. Approximately 5 % seed was employed for the fermentors, which were grown for 48 to 72 h.

Basic medium preparation

In very early work it was found that the medium described by Goldner *et al.* (1966) could be simplified without interfering with the final yields of bacteria obtained. The nucleic acid derivatives, liver co-enzymes, 2-deoxyribose, $CuSO_4$, and all of the amino acids except glutamic acid, proline, and cystine could be left out. The addition of charcoal, starch or resin was also found to be unnecessary. The formula of the simplified basic medium is given in Table 1, in which it should be noted that the concentration of cystine, ascorbic acid, niacin, and glutathione have each been increased fourfold on those specified in Goldner's formula. Sodium glutamate is used in place of glutamic acid since it is more soluble and less expensive. This basic medium is termed the 'glutamate + proline medium' or '1 G + 1 P medium', and contains 670 mg. sodium glutamate and 240 mg. proline/l. The many other combinations of glutamate and proline used are expressed in terms of the concentrations shown in Table 1, e.g. the medium designated '16 G + 2 P' would contain 10,720 mg. glutamate and 480 mg. proline/l., etc. In the preparation of media for the shake flask experiments, the amino acids, salts and tris buffer were dissolved in the necessary amount of water, the pH adjusted to 7·6 with 2·5 N-HCl, and autoclaved. In the preparation of the large volumes of medium for the fermentors, the amino acids, salts and tris buffer were dissolved in water at 70°, and sterilized by steaming for 40 min. and autoclaving for 60 min. at 260° (17 to 18 p.s.i.). The cystine, glutathione, ascorbic acid, niacin and ferrous sulphate were sterilized by filtration and added to the autoclaved medium. When the ferrous sulphate was added before autoclaving a hazy precipitate of ferrous phosphate appeared and presumably the Fe^{2+} ions were no longer available to the organism since growth was diminished.

Bacterial concentrations

Bacterial concentrations were estimated by photometric comparison with the Opacity Standard for pertussis vaccine and challenge suspension, supplied by the National Institutes of Health, Bethesda, Maryland, U.S.A. Ten opacity units (o.u.) were considered as being equivalent to 10×10^9 organisms/ml.

Toxicity testing of finished vaccines

The cultures were detoxified by the addition of formalin (37 % solution of formaldehyde) at concentrations and for times described below. The treated cultures were centrifuged, the vaccines resuspended in 0·85 % NaCl and tested using the standard N.I.H. mouse toxicity test: ten mice (CMRL strain, random-bred), 14 to 16 g., were injected intraperitoneally with 10 o.u. vaccine and group weights assessed after 7 days. A further test for freedom from toxicity was carried out by injecting three guinea pigs (320 to 340 g.) with 100 o.u. vaccine contained in a volume of 5 ml. Two of the animals were injected intraperitoneally, and one subcutaneously, and observed for 16 days with individual weights being recorded.

Potency assays

The protective potencies of the vaccines were determined by the method set out in *Minimal Requirements for Pertussis Vaccine* (U.S., N.I.H. 1948, revised 1968) and the results calculated by the Worcester–Wilson procedure (Worcester & Wilson, 1943). For the stability studies, samples were made up to contain 10 or 20 o.u./ml., either as straight pertussis vaccine or in combination with 40 Lf Diphtheria toxoid and 8 Lf Tetanus toxoid, and stored for varying periods of time at 4, 25, or 37°. These results were assayed by Probit analysis with 95 % confidence limits. The antigenic potencies of the diphtheria and tetanus toxoids in the Diphtheria–Pertussis–Tetanus preparations were determined as described previously (Stainer, 1968), and the results expressed in International Units (i.u.)/ml. compared to the W.H.O. International Standards for diptheria and tetanus toxoids.

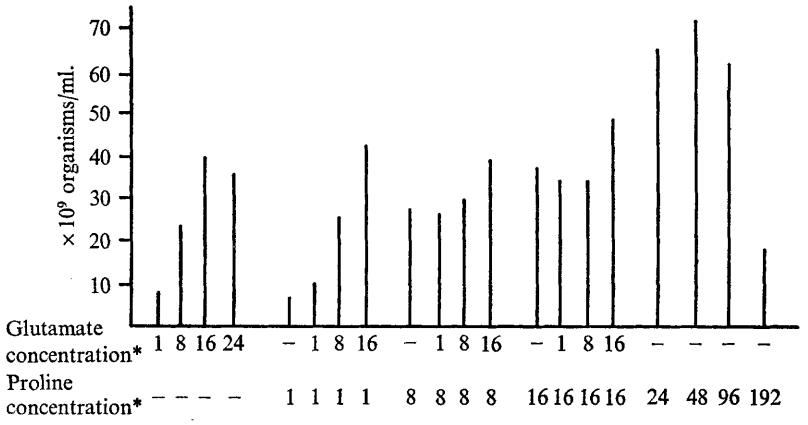

Fig. 1. Effect of various glutamate and proline concentrations on the opacity of *Bordetella pertussis* in shake flasks after 48 h. growth. *Concentrations of the amino acids are expressed relative to the basic 'glutamate+proline' medium ('1 G+1 P') given in Table 1 (1 G, 670 mg. glutamate/l.; 1 P, 240 mg. proline/l.).

RESULTS

Growth in shake flasks

Using the '1G+1P' medium given in Table 1, yields of approximately 10×10^9 organisms/ml. were obtained after 48 h. growth. Fig. 1 illustrates the results of numerous experiments in shake flasks in which an increased concentration of either glutamate or proline in '1G+1P' medium is shown to have a significant effect on the final opacities attained. The seed cultures for these studies were grown in '16G+1P' medium and not in the casein digest medium (Wilson, 1945). This was found to be possible, if as previously mentioned, the ferrous sulphate was added *after* the medium had been autoclaved and also provided that the seed cultures were taken in the early exponential phase of growth. The medium designated '48P' gave the highest opacity values, followed by '24P', '96P', '16G+16P' and '16G+1P'.

Shake flasks containing '12G' medium (no proline) or '12P' (no glutamate) were sampled at 0, 30 and 48 h. after growth, the cultures centrifuged at 10,000 *g* for 1 h. and portions of the resultant supernatants chromatographed on Eastman Cellulose thin-layer chromatography sheets (no. 6065), using *n*-butanol+acetic acid+water

(4+1+1). In view of the high level of seeding employed (4 %) and to avoid the carry-over of extraneous amino acids, the flasks used for the chromatography experiments were seeded in the following way: the flask containing the '12G' medium was seeded with a culture grown in the '12G' medium, and similarly the flask containing the '12P' medium was seeded with a culture grown in the '12P' medium.

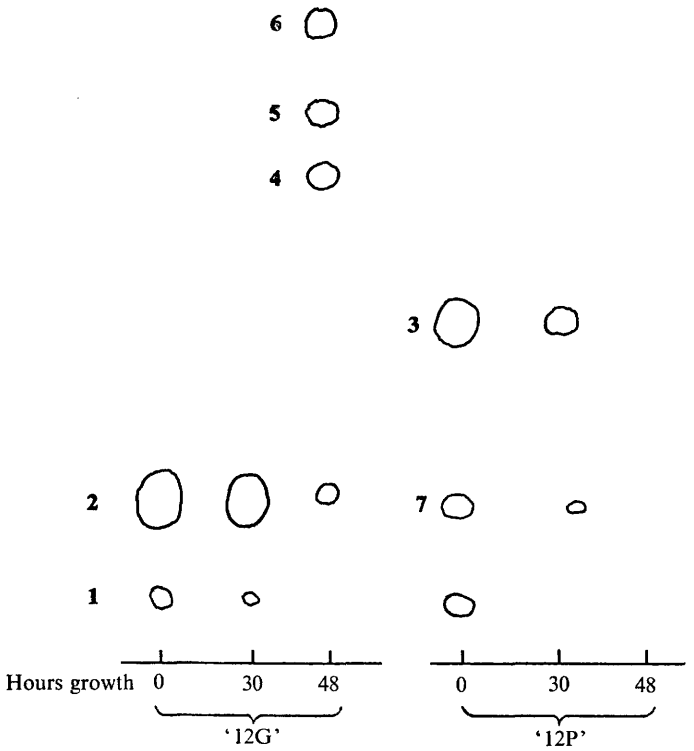

Fig. 2. Thin-layer chromatography of cell-free *Bordetella pertussis* culture supernatants on Eastman Cellulose paper (6065), using *n*-butanol+acetic acid+water (4+1+1).
1, cystine and glutathione; 2, glutamic acid; 3, proline; 4, threonine, 5, alanine; 6, valine; 7, glutamic acid (from glutathione).

Some interesting differences were noted as depicted in Fig. 2. Forty-eight h. after growth in the '12G' medium, three ninhydrin-positive spots were produced which were not present in the original medium. Subsequent analysis with the Spinco automatic amino acid analyser showed them to be threonine, alanine and valine. These amino acids were not present in the culture supernatants from the '12P' medium.

Growth in fermentors

Concurrent with the shake flask experiments, attempts were made to grow *Bordetella pertussis* in various chemically defined media in vessels of large capacity. Seed for the fermentors was prepared by inoculating 100 ml. of a 40 h. culture of *B. pertussis*, grown in a shake flask containing casein digest medium (Wilson, 1945), into 8 l. of the same medium in a 20 l. bottle. Air was bubbled through the bottle of medium at 1 l./min. and the culture grown for 40 h. Approximately 5 % seeding was used in these

studies. Table 2 shows the results obtained in three sizes of fermentors. Four different combinations of glutamate and proline concentrations were compared and gave essentially the same results as those from the shake flask experiments. The medium which gave the highest yields in shake flasks, namely '48P', was not used in large-scale production since proline is an expensive amino acid. The medium designated '16G+1P' gave good yields and was used to prepare numerous lots of culture suitable for conversion to vaccines.

Table 2. *The growth of Bordetella pertussis in chemically defined media in large volumes*

Volume (l.)	No. of experiments	Relative concentrations* G	Relative concentrations* P	Average yield after 48 to 72 h. growth† (× 10^9 org.)
9	2	4	8	29·1
9	6	4	16	43·0
9	3	8	2	28·2
9	10	16	1	42·0
60	6	16	1	38·0
140	5	4	8	24·3
140	15	16	1	36·1

* Compared to basic '1G+1P' medium ('1G', 670 mg. glutamate/l.; '1P', 240 mg. proline/l.).
† The lag phase was extended depending on the age of the seed culture (see text).

Table 3. *Detoxification studies on Bordetella pertussis grown in '16G+1P' chemically defined medium*

No. of tests	Formalin (%)	No. days incubation at 36 to 37°	Av. wt gain per mouse (g.)	Av. no. deaths/test
3	0·07	3	3·8	2
5	0·07	5	4·4	1·4
1	0·07	7	6·8	0
5	0·1	5	5·5	1
3	0·1	6	5·6	0·3
5	0·1	7	7·5	0·5
3	0·12	7	7·7	0
6	0·14	5	7·4	0
		Conventionally-grown vaccines		
12	0·07	3	7·1	0
		Saline		
10	—	—	8·9	0

Detoxification studies

Cultures were detoxified by the addition of formalin as indicated in Table 3. After centrifuging, the vaccines were tested as described previously. Several experiments indicated that the most suitable conditions for producing a nontoxic vaccine were the addition of 0·12 % formalin and incubation for 7 days, or 0·14 % formalin and incubation for 5 days at 36 to 37°. Vaccines prepared in this manner gave satisfactory weight gains in mice. Vaccines produced by growing *Bordetella pertussis* in casein digest

medium (Wilson, 1945) require 0·07 % formalin and incubation for 3 to 4 days. The reason for the extra formalin needed to detoxify the cultures grown in the chemically defined medium could be related to the greater bacterial densities attained in this medium (30 to 40 o.u. compared to 20 to 30 o.u./ml. with conventionally grown cultures). Injection of the finished vaccines into guinea pigs elicited no toxic reactions and good weight gains were noted.

Table 4. *Potency of Bordetella pertussis vaccines prepared from cultures grown in '16G+1P' chemically defined medium*

Lot no.	Potency*	Lot no.	Potency*
P55	4·2	1057	19·4
	4·3	—	10·1
	—	—	15·2
P56	21·4	—	—
	16·2	1059	19·1
	—	—	11·0
P57	8·0	—	18·2
	7·6	—	—
	—	1065	5·3
1005	16·8	—	—
	13·4	1066	5·3
1006	18·2	1068	16·7
1055	10·4	1069	18·5
	13·6	—	—
	13·7	1071	10·4

* i.u./20 o.u. tested against U.S. Pertussis Vaccine Lot 6.

Table 5. *Adjuvant effect of Bordetella pertussis vaccines prepared from cultures grown in '16G+1P' chemically defined medium*

Sample	Time (months)	Temperature (°C)	Diphtheria potency (i.u./ml.)	Tetanus potency (i.u./ml.)
Fluid diphtheria toxoid	7	4	6 (3 to 12)*	—
Fluid tetanus toxoid	7	4	—	22 (11 to 45)
Diptheria toxoid—40 Lf	7	4	20 (10 to 44)	46 (23 to 87)
Tetanus toxoid—8 Lf	7	25	34 (16 to 73)	56 (28 to 108)
Pertussis vaccine—20 o.u.	7	37	29 (14 to 61)	120 (64 to 230)
Diphtheria toxoid—40 Lf	7	4	16 (7 to 39)	69 (26 to 180)
Tetanus toxoid—8 Lf	7	25	53 (22 to 154)	102 (36 to 364)
Pertussis vaccine—10 o.u.				

* 95 % Limits given in parentheses.

Antigenicity studies

Agglutination tests with vaccines prepared by growing *Bordetella pertussis* in the '16G+1P' medium showed that they all agglutinated with phase I antiserum, and further testing proved the presence of appreciable quantities of agglutinogens 1, 2 and 3 (Preston, 1963, 1965).

The mouse-protective potency of numerous lots of vaccine (each diluted to contain 20 o.u./ml.) is shown in Table 4. All lots tested had protective activity of more than 4 i.u./ml.

The adjuvant effect of pertussis vaccine on the response to diphtheria and tetanus toxoids is well known (Fleming, Greenberg & Beith, 1948). Samples of two combined

Table 6. *Antigenic stability of Bordetella pertussis vaccines prepared from organisms grown in '16G+1P' chemically defined medium*

Storage time (weeks)	Temp. (°C)	Pertussis samples at 20 o.u./ml. (i.u./ml.)	Pertussis samples at 10 o.u./ml. (i.u./ml.)
		(A) Pertussis vaccine	
0	—	13·4 (6·6 to 26·1)*	—
4	4	8·6 (4·4 to 16·6)	6·3 (3·3 to 12·3)
26	4	4·6 (2·0 to 10·7)	7·0 (3·1 to 15·7)
44	4	9·4 (4·8 to 18·2)	—
4	25	9·4 (4·7 to 19·6)	5·9 (3·0 to 11·5)
26	25	5·6 (2·4 to 13·0)	4·7 (2·1 to 10·8)
8	37	8·8 (4·6 to 17·0)	6·4 (3·3 to 12·3)
26	37	1·2 (0·5 to 2·8)	2·4 (1·0 to 5·4)
		(B) Pertussis component of D.P.T.	
0	—	13·4† (6·6 to 26·1)	—
4	4	13·2 (6·9 to 25·4)	9·5 (4·9 to 18·2)
26	4	7·8 (3·4 to 18·1)	3·6 (1·5 to 8·3)
4	25	21·9 (11·4 to 42·3)	5·8 (3·0 to 11·2)
26	25	1·4 (0·6 to 3·4)	3·4 (1·4 to 8·2)
8	37	10·0 (5·2 to 19·3)	5·2 (2·7 to 10·0)
26	37	1·3 (0·5 to 3·7)	2·7 (1·2 to 6·2)

Note: vaccines contained either 20 or 10 o.u./ml. and were stored and tested separately against U.S. Pertussis Vaccine Lot 6. * 95 % Limits given in parentheses. † This is a theoretical calculation.

vaccines, containing a mixture of two lots of *Bordetella pertussis* grown in the chemically defined medium ('16G+1P') and diphtheria and tetanus toxoids, were each stored for seven months at three temperatures. The resulting potencies of the tetanus and diphtheria components were compared with the original potencies of the fluid toxoids, and the results are given in Table 5. The combined vaccines contained 40 Lf diphtheria, 8 Lf tetanus toxoids, and either 20 or 10 o.u. pertussis vaccine/ml. The antigenic potencies of the toxoids were significantly increased ($P = 0{\cdot}01$) when combined with the pertussis vaccine. An unexpected finding was the additional adjuvant effect on the potencies of the tetanus component of the combined vaccine when stored at elevated temperatures. This is to be further investigated. The stability of the pertussis component used in the combined vaccines was also determined, and these results are given in Table 6. More information is needed but it appears that the new pertussis vaccine is stable for at least 44 weeks at 4°, and 8 weeks at 37°, when stored as either a plain vaccine or in combination with diphtheria and tetanus toxoids. Three of the four samples stored at 25° were stable for 26 weeks.

DISCUSSION

The use of a chemically defined medium in which all the ingredients essential for the growth of bacteria are known is obviously very desirable, especially in relation to vaccine production. In the case of *Bordetella pertussis* many such media have been described and every author has stressed the importance of certain amino acids, notably glutamic acid, proline and cystine. We have confirmed these findings. Since most of the chemically defined media published were developed for basic metabolic studies, the yields have not been sufficient to warrant their use in the routine production of *B. pertussis* in large volumes. In the present case, however, very good yields were obtained.

In the experiments reported here, the cultures used for seeding the large fermentors were grown in casein digest medium (Wilson, 1945) and could be stored at 4° for 4 weeks. The lag phase became extended, however, as the time of storage of the seed culture progressed. Similar results were obtained using '16G+1P' chemically defined medium for the growth of seed cultures, but the effects of storage of these cultures on the resultant lag phase was much more pronounced unless the seed was taken during the early exponential phase of growth. This could imply that the '16G+1P' chemically defined medium is incomplete and that before growth can start, specific intermediates must be synthesized. We do not feel that this is the case since we have evidence that *Bordetella pertussis* will grow very well in the '16G+1P' medium in shake flasks using a fresh seed culture grown in the same medium, and that the flasks can be serially subcultured for up to 29 transfers. Antigenic analyses of these serially transferred cultures showed no loss in either mouse-protective antigen levels or the ability to agglutinate phase I *B. pertussis* antiserum for up to eight transfers. Beyond this number of transfers, antigen levels declined, although there was still 5 to 10 % of the original mouse-protective activity present after 21 transfers.

The absence of high molecular weight material (e.g. starch), its ease of preparation, and the fact that vaccines produced from it have low toxicity and high antigenicity, make the proposed '16G+1P' chemically defined medium very attractive from the production standpoint. The interesting differences noted when *Bordetella pertussis* was grown in the absence of either glutamate or proline could indicate that different

metabolic pathways exist for these amino acids, and the extreme simplicity of these media makes possible further, more detailed biochemical studies of the organism. Preliminary investigations have already been reported (Stainer & Scholte, 1969), and further work is in progress.

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REFERENCES

COHEN, S. M. & WHEELER, M. W. (1946). Pertussis vaccine prepared with phase I cultures grown in fluid medium. *American Journal of Public Health* **36**, 371–376.

FLEMING, D. S., GREENBERG, L. & BEITH, E. M. (1948). Use of combined antigens in immunization of infants. *Canadian Medical Association Journal* **59**, 101–105.

GOLDNER, M., JAKUS, C. M., RHODES, H. K. & WILSON, R. J. (1966). The amino acid utilization by phase I *Bordetella pertussis* in a chemically defined medium. *Journal of General Microbiology* **44**, 439–444.

HORNIBROOK, J. W. (1939). Cultivation of phase I *H. pertussis* in a semi-synthetic liquid medium. *Public Health Reports, Washington* **54**, 1847–1851.

JEBB, W. H. H. & TOMLINSON, A. H. (1955). The nutritional requirements of *Haemophilus pertussis*. *Journal of General Microbiology* **13**, 1–8.

JEBB, W. H. H. & TOMLINSON, A. H. (1957). The minimal amino acid requirements of *Haemophilus pertussis*. *Journal of General Microbiology* **17**, 59–63.

PRESTON, N. W. (1963). Type-specific immunity against whooping cough. *British Medical Journal* ii, 724–726.

PRESTON, N. W. (1965). Effectiveness of pertussis vaccines. *British Medical Journal* ii, 11–13.

ROWATT, E. (1957). The growth of *Bordetella pertussis*: a review. *Journal of General Microbiology* **17**, 297–326.

STAINER, D. W. (1968). Preparation and properties of diphtheria toxoids in submerged culture. II. Purification, detoxification, antigenicity, and stability. *Canadian Journal of Microbiology* **14**, 327–330.

STAINER, D. W. & SCHOLTE, M. J. (1969). The effect of medium composition on the biological properties of *Bordetella pertussis*. *Bacteriological Proceedings* p. 100.

SUTHERLAND, I. W. & WILKINSON, J. F. (1961). A new growth medium for virulent *Bordetella pertussis*. *Journal of Pathology and Bacteriology* **82**, 431–438.

UNGAR, J., JAMES, A. M., MUGGLETON, P. W., PEGLER, H. F. & TOMICH, E. G. (1950). The cultivation of *Haemophilus pertussis* in partially defined liquid media. *Journal of General Microbiology* **4**, 345–359.

U.S., N.I.H. (1948). *Minimum Requirements for Pertussis Vaccine* (Revised June 1968). Bethesda, Maryland: National Institutes of Health,

VAJDIC, A. H., GOLDNER, M. & WILSON, R. J. (1966). The amino acid use in cultures of phase I *Bordetella pertussis* during growth in chemically defined media. *Journal of General Microbiology* **44**, 445–449.

VERWEY, W. F. & SAGE, D. N. (1945). An improved culture medium for the growth of *Hemophilus pertussis*. *Journal of Bacteriology* **49**, 520–525.

VERWEY, W. F., THIELE, E. H., SAGE, D. N. & SCHUCHARDT, L. F. (1949). A simplified liquid culture medium for the growth of *Hemophilus pertussis*. *Journal of Bacteriology* **58**, 127–134.

WILSON, R. J. (1945). The production of phase I pertussis vaccine in casein hydrolysate broth. *Canadian Journal of Public Health* **36**, 321–326.

WILSON, R. J. (1963). Cultivation of *Bordetella pertussis* (phase I) in a chemically defined medium. *Canadian Journal of Public Health* **54**, 518–523.

WORCESTER, J. & WILSON, E. B. (1943). A table determining LD50 or the fifty percent end-point. *Proceedings of the National Academy of Sciences of the United States of America* **29**, 207–212.