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Use of Glutamic Acid to Supplement Fluid Medium for Cultivation of *Bordetella pertussis*

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The amino acid consumption by *Bordetella pertussis* growing in broth containing casein hydrolysate was examined. Serine, proline, alanine, glycine, aspartate, and glutamate were rapidly consumed, in a manner which suggested that they supplied the energy requirements of the organism; exhaustion of the energy source appeared to be the main factor limiting the yield of cells. There was no correlation between the utilization of individual amino acids and the phase of growth; uptake appeared to depend only upon relative concentrations. Consumption of threonine, phenylalanine, histidine, leucine, and methionine was slight; consumption of valine and lysine was variable, and isoleucine was excreted. The addition of monosodium L-glutamate (3 mg/ml) to the broth in shaken flasks increased the cell yield by an average of 43.5%. It had no detectable adverse effect upon the agglutin-producing capacity, agglutinability in antisera versus smooth and rough growth phases, mouse-lethal toxicity, histamine-sensitizing factor potency, or intracerebral protective potency of the culture. Broth supplemented with monosodium L-glutamate has been used over a 2-year period to prepare experimental vaccines by both batch and continuous cultivation methods at controlled *p*H; the cell yields obtained from the supplemented broth have been up to 52% higher than those from the basal broth. The use of glutamate to replace a proportion of casein hydrolysate in the broth caused a reduction in the cell yield, an alteration in cell morphology, and reduction in the mouse-lethal toxicity, the histamine-sensitizing factor potency, and the intracerebral protective potency of the cells.

Bordetella pertussis does not utilize any of the usual carbohydrates (1, 7, 16, 17) and a mixture of amino acids is usually the source of energy. Hornibrook was first to use casein hydrolysate as the source of amino acids (5). The hydrolysate was later incorporated by Cohen and Wheeler into their culture formulation (2) which is now commonly used for the cultivation of *B. pertussis*.

A number of workers have investigated the uptake of amino acids by *B. pertussis* from media containing casein hydrolysate (8, 13-16, 21) and from synthetic media (4, 6, 8, 9, 12-14, 22). The capacity of washed cells to oxidize single amino acids has been examined by Abe (1) and by Jebb and Tomlinson (7).

It is apparent that the organism has an absolute requirement for only a limited number of the amino acids. Glutamic acid appears to be of the greatest importance (4, 6-9, 15, 16, 22), but aspartate, proline, serine, glycine, and alanine may enhance growth. Other amino acids, which are probably of minor significance, are norvaline, hydroxyproline and lysine (1), glutamine (4), phenylalanine (13, 14), histidine (4), threonine (6, 21), tyrosine (13, 14), leucine (1, 12), and arginine (1).

There have been very few attempts to increase the yield of cells from fluid media by adding extra amino acids, and those which have been reported met with only limited success (4, 7, 13, 14, 16, 20, 21). In an attempt to improve the economics of vaccine production, it seemed pertinent to determine whether the cessation of cell growth in Cohen and Wheeler broth (2) was due simply to exhaustion of the energy source and, if so, whether the cell yield could be improved by adding extra amino acids to the medium. The most suitable single amino acid to use as a supplement appeared to be glutamate, since it is rapidly utilized, is inexpensive, and is readily available as monosodium L-glutamate.

The effect upon cell yield and upon the biological properties of the cells of supplementing Cohen and Wheeler broth (2) with monosodium

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L-glutamate is reported here. The possibility of replacing a proportion of the casein hydrolysate in Cohen and Wheeler broth (2) by monosodium L-glutamate was also examined.

MATERIALS AND METHODS

Strain. The strain of *B. pertussis* (code BR1) used for this study had the properties of a typical smooth strain. Stock cultures were freeze-dried and were maintained on Cohen and Wheeler blood-agar slopes and on Oxoid charcoal-agar containing 5% defibrinated sheep blood. No culture line was continued beyond the 10th subculture. A rough variant of the BR1 strain was produced by transferring the smooth strain to nutrient 5% chocolate-agar (18, 19).

Smooth-type antiserum preparation and agglutination. For smooth antiserum production, the strain, grown on Oxoid charcoal-agar containing 5% defibrinated sheep blood, was harvested in saline containing 0.2% (v/v) Formalin, adjusted to about 10×10^9 organisms/ml and stored at 2 C for 2 weeks.

A rabbit was hyperimmunized by repeated intravenous (iv) injection and was bled by cardiac puncture 10 days after the final injection. Low-titer agglutins against the rough BR1 strain and *B. parapertussis* were absorbed from the serum. The final titer of the serum against the smooth BR1 strain was 1:2,048.

Smooth-type agglutination tests were performed in plastic agglutination trays, with a final volume of 0.04 ml, using twofold serial dilutions of serum and a final antigen concentration of 15×10^9 organisms/ml. The tray was rotated on a bench shake-table at 200 rev/min for 15 min, allowed to stand for an additional 15 min, and read by using an X10 binocular microscope.

Rough-type antiserum preparation and agglutination. Rough strains of *B. pertussis* are known to be very poor antigens (10, 19). To elicit an antibody response, intramuscular and subcutaneous depot injections were given in conjunction with iv injections of saline vaccine. An oily adjuvant vaccine was used for depot injections and was prepared by emulsifying 5 ml of cell suspension (40×10^9 organisms/ml) with 4.5 ml of Bayol F [Esso Standard Oil (Australia) Pty. Ltd.] and 0.5 ml of Arlacel A (Atlas Powder Company, Wilmington, Del.). Both the saline and adjuvant vaccines were preserved with 0.2% (v/v) Formalin.

A rabbit was hyperimmunized over a period of 1 month and bled by cardiac puncture 10 days after the final injection. Low agglutination titers of the serum against the smooth strain of organism were removed by absorption; the final titer against rough BR1 was 1:256.

To determine the rough agglutination titer, three drops each of test antigen, adjusted to about 30×10^9 organisms/ml, and twofold serial dilutions of serum were placed in agglutination tubes 6 cm long and 9 mm wide with sloping sides. The tubes were shaken at room temperature at 100 rev/min for 15 min and allowed to stand overnight. The end point was taken as the highest dilution of serum at which agglutination was still perceptible with the aid of an X5 microscope eyepiece.

Cultivation. The broth used was based on the formulation of Cohen and Wheeler (2) and contained 1% (w/v) casein hydrolysate (Casamino Acids, Difco); modifications were made to the content of starch (2% w/v), yeast dialysate (10% v/v) and $MgCl_2 \cdot 6H_2O$ (10 mg/100 ml w/v). Starch was sterilized by autoclaving and all other components were Seitz-filtered into the sterile starch. The broth was dispensed in 400-ml volumes into 1-liter Erlenmeyer flasks. Monosodium L-glutamate (G. T. Winter, Ltd., South Yarra, Victoria; 40 mg/ml) was sterilized by Seitz filtration and added to the broth in duplicate flasks to give 0, 1, 2, 3, 4 or 5 mg/ml final concentration.

The inoculum was grown for 48 hr at 35 C on Cohen and Wheeler (2) 15% blood-agar and then harvested in saline. Shaken cultures were incubated for 72 hr at 36 C on an inclined rotary shake-table turning at 144 rev/min. Cell density was measured in a calibrated colorimeter (Evans Electroselenium Ltd., Halstead, Essex, U.K.).

Vortex-aerated cultures were grown at 36 C in a New Brunswick fermentation jar (model F-5) containing 1.5 liters of broth and stirred at 400 rev/min with air passing over the culture at 100 ml/min. No antifoam was used.

To prepare vaccines, cultures were adjusted to *p*H 7.0 to 7.5 with N HCl. Merthiolate was added to 0.01% (w/v), and the vaccines were stored at 2 C for 1 week.

Toxicity tests and detoxification. The mice used throughout this study were of the Commonwealth Serum Laboratories white strain. Toxicity tests were carried out in mice (4 to 6 weeks old), using 10 mice per group. Each group was weighed and each mouse received 10×10^9 organisms by intraperitoneal (ip) injection. A toxicity value was assigned on the 3rd day according to the following scale: 8 to 10 dead, ++++; 4 to 7 dead, +++; 1 to 3 dead, ++; none dead but weight reduced, +; none dead and weight regained, −. Saline-injected controls were always included and invariably gave a negative toxicity value.

After the toxicity tests had been performed, vaccines were detoxified by incubating them at 35 C for 16 hr.

Agglutinin-producing capacity. The capacity of vaccines to stimulate production of agglutinins in mice was measured by a modification of the method of Evans and Perkins (3) in which only a single-dose level was used (total dose of 10×10^9 organisms). The geometric mean titer of each group was computed and the significance of differences between the means was determined at the 95% confidence level by the Student "t" test.

Histamine-sensitizing factor (HSF). The method was based upon the varied dose/constant-challenge technique described by Preston (11) and was carried out in male mice. To eliminate error due to differences in response between younger and older mice (11; Lane, *unpublished data*), only mice from a narrow

TABLE 1. *Residual amino acid composition of supernatant fluids of samples taken at intervals from either shake-flask or vortex-aerated cultures of B. pertussis incubated at 36 C in broth containing casein hydrolysate (1% w/v)*

Cultivation method	Time sampled (hr)	Amino acid concn (mg/ml)														Ammonia concn
		Lysine	Histidine	Argi-nine	Aspar-tate	Threo-nine	Serine	Gluta-mate	Proline	Glycine	Alanine	Cy-steine	Valine	Methio-nine	Isoleu-cine	Leucine	Tyrosine	Phenyl-alanine	
Shake flask	0	0.486	0.216	0.246	0.459	0.279	0.342	1.07	0.774	0.138	0.309	0.021	0.432	0.213	0.309	0.510	0.054	0.249	0.063
	26	0.492	0.204	0.255	0.435	0.274	0.318	1.02	0.690	0.132	0.288	0.015	0.414	0.195	0.300	0.510	0.048	0.255	0.072
	36	0.501	0.207	0.255	0.453	0.282	0.333	0.996	0.696	0.012	0.264	0.015	0.408	0.207	0.312	0.432	0.057	0.246	0.081
	81	0.576	0.198	0.255	0.009	0.138	0	0	0	0	0	0.021	0.429	0.168	0.330	0.477	0.054	0.234	0.333
Vortex-aerated fermenter	0	0.486	0.216	0.246	0.459	0.279	0.342	1.07	0.774	0.138	0.309	0.021	0.432	0.213	0.309	0.510	0.054	0.249	0.063
	11	0.519	0.204	0.246	0.477	0.282	0.342		0.720	0.135	0.297		0.408	0.201	0.300	0.504	0.051	0.246	0.066
	21	0.501	0.201	0.252	0.420	0.279	0.273	0.879	0.615	0.126	0.288	0.012	0.408	0.210	0.309	0.429	0.057	0.246	0.096
	33	0.504	0.207	0.252	0.351	0.207	0.240	0.081	0.393	0.103	0.258	0.012	0.429	0.201	0.336	0.522	0.052	0.228	0.222
	53	0.510	0.207	0.258	0.105	0.261	0.075	0.009	0.081	0.036	0.114	0.009	0.387	0.186	0.361	0.525	0.054	0.228	0.354
	78	0.441	0.201	0.255	0.006	0.165	0.003	0	0	0	0.003	0.021	0.348	0.171	0.336	0.477	0.051	0.219	0.327

weight range (18 to 21 g) were used. The mice were distributed in groups of 20 in such a way that all groups were of identical weight. Each mouse received by ip injection 0.5 ml of vaccine containing 10×10^9, 2.5×10^9, or 0.625×10^9 organisms/ml. Five days later, each mouse was challenged with 6 mg of histamine diphosphate, and the result was read on the following day. The HSD_{50} (the reciprocal of the vaccine dilution which provokes lethal histamine shock in 50% of the mice) was calculated by the method of Worcester and Wilson (23). The significance of difference between groups was determined at the 95% confidence level using the average standard deviation of all the groups in each experiment.

Intracerebral mouse-protective potency. The capacity of vaccines to protect mice against intracerebral (ic) challenge was measured in female mice (4 to 6-week-old). Each mouse was vaccinated by ip injection with 0.5 ml of vaccine containing 10×10^9, 2.5×10^9, or 0.625×10^9 organisms/ml. Fourteen days later, the mice were lightly anesthetized with ether and challenged by ic injection with 100 to 200 LD_{50} of the U.S. National Institutes of Health challenge strain (#18-323) in 0.03 ml. Deaths occurring within 72 hr were disregarded. Efficacy of the vaccine was based on the mortality 14 days after challenging. The ED_{50} (the reciprocal of the vaccine dilution which protects 50% of the challenged mice) and the significance of differences were calculated as for the HSF assay.

Amino acid analyses. Analyses for the amino acids present in culture supernatant fluids were performed in a Beckman/Spinco Amino Acid Analyser.

RESULTS AND DISCUSSION

Amino acid utilization. The amino acid consumption by strain BR1 *B. pertussis* growing in Cohen and Wheeler broth (2) is shown in Table 1. Aspartate, serine, glycine, alanine, glutamate, and proline were exhausted completely during cell growth. At the end of the cultivation period, the amounts of histidine, threonine, phenylalanine, leucine, and methionine were slightly reduced. Utilization of valine and lysine was variable; isoleucine was excreted by both types of culture. These results were in good agreement with the concensus of previous published results (Table 2).

The rate of consumption of the six amino acids most rapidly utilized in the vortex-aerated culture appeared to depend only upon their relative concentrations (Fig. 1). In contrast with previous results, there was no correlation between the utilization of individual amino acids and the phase of cell growth. Previous workers, using paper chromatographic methods, concluded that either glutamate or proline was utilized first (13, 14, 22); our results suggest that the more rapid initial consumption of these amino acids was due to their higher initial concentrations.

These results suggested that aspartate, serine, proline, glycine, alanine, and glutamate were

TABLE 2. *Amino acids utilized by B. pertussis—a comparison of the results of different authors*[a]

Author	Proline	Serine	Alanine	Aspartate	Glutamate	Glycine	Threonine	Tyrosine	Phenylalanine	Leucine	Histidine	Arginine	Glutamine	Norvaline	Hydroxyproline	Lysine	Methionine	Valine	Isoleucine
Ungar et al. (21)	+	+	+	+	+	+	+												
Jebb and Tomlinson (7)	+	+	+	+	+														
Rowatt (15)	+	+		+	+	+													
Imamura (6)	+	+	+	+	+	+	+												
Pusztai et al. (13, 14)	+	+	+	+	+	+		+	+										
Goldner et al. (4)	+	+	+		+	+					+		+						
Abe (1)	+		+	+	+					+		+		+	+	+			
Proom (12)	+	+	+	+	+					+									
Lane	R	R	R	R	R	R	S		S	S	S					V	S	V	E

[a] Key: R, amino acids utilized rapidly and completely; S, amino acids utilized slightly; V, amino acids whose utilization was variable; E, amino acids excreted during growth.

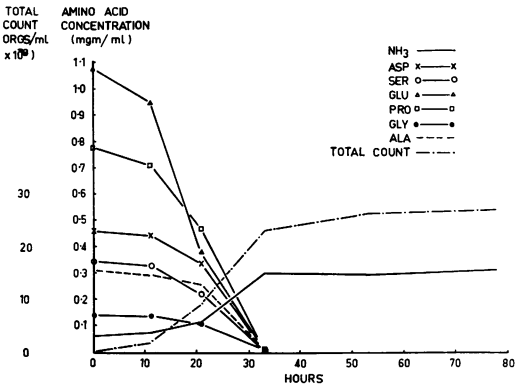

FIG. 1. *Change in concentration of the six rapidly utilized amino acids, of ammonia, and in the total cell count during growth of B. pertussis in broth containing casein hydrolysate (1% w/v); cultivated in vortex-aerated vessel at 36 C.*

used primarily to satisfy the energy requirements of the organism, and indicated that the cell yield might be improved by supplementing the medium with a single amino acid. Glutamate was most suitable, since it was present in the highest initial concentration, was utilized rapidly, and was readily available in the L-form at low cost.

Effect of addition of glutamate: effect upon cell yield. Up to 3 mg/ml, the yield of cells (Fig. 2) increased in proportion to the concentration of the glutamate added; three of the four repeat experiments showed a clear-cut optimum at 3 mg/ml and, in one experiment, there was marked inhibition at concentrations of glutamate above 3 mg/ml. Microscopic appearance of the cultures was normal. It was concluded from these data that the energy requirements

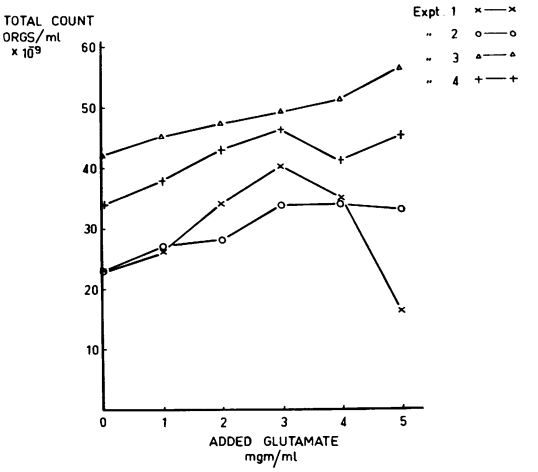

FIG. 2. *Effect of the concentration of added monosodium L-glutamate upon cell yield in shake-flask cultures of B. pertussis. Basal broth containing casein hydrolysate (1% w/v) was supplemented by addition of monosodium L-glutamate (0, 1, 2, 3, 4, or 5 mg/ml) and duplicate shake flasks were incubated at 36 C for 72 hr. The experiment was repeated four times with different batches of broth; each curve represents a separate experiment and each point represents the average total cell count of duplicate flasks.*

TABLE 3. *Addition of monosodium L-glutamate to basal broth containing casein hydrolysate (1% w/v); effect of the concentration of added glutamate upon agglutinability with smooth and rough antisera, mouse-lethal toxicity, agglutin-producing capacity, histamine-sensitizing factor (HSF) potency, and intracerebral protective (IC) potency of B. pertussis cells cultivated in shake flasks at 36 C for 72 hr*

Added glutamate (mg/ml)	Toxicity value	Agglutin-producing capacity (geometric mean titer)	Agglutination titer		IC potency (ED_{50})[a]	HSF potency (HSD_{50})[b]
			Smooth	Rough		
0	++++	441	4,096	32	47	11
1	++++	524	4,096	32	52	12
2	++++	153[c]	4,096	32	24	5
3	++++	371	4,096	32	19	6
4	++++	379	4,096	32	11	8
5	++++	376	2,048	32	11	5[c]

[a] Reciprocal of vaccine dilution which protected 50% of mice against intracerebral challenge.
[b] Reciprocal of vaccine dilution which provoked lethal sensitivity to 6 mg of histamine diphosphate in 50% of the challenged mice.
[c] Significantly different ($D = 0.5$) from the activity of the cells grown in broth containing no added glutamate.

TABLE 4. *Addition of 3 mg of glutamate per ml to different batches of basal broth containing casein hydrolysate (1% w/v); effect upon histamine-sensitizing factor (HSF) potency and intracerebral protective (IC) potency of B. pertussis cells cultivated in shake flasks at 36 C for 72 hr*

Broth	HSF potency (HSD_{50})[a]	IC potency (ED_{50})[b]
1	10.1	
1 + Glutamate	20.6	
2	19.8	
2 + Glutamate	11.7	
3	7.5	317.7
3 + Glutamate	9.0	317.7
4	3.3	
4 + Glutamate	12.1[c]	
5	1.0	
5 + Glutamate	3.3	

[a] Reciprocal of vaccine dilution which provoked lethal sensitivity to 6 mg of histamine diphosphate in 50% of the challenged mice.
[b] Reciprocal of vaccine dilution which protected 50% of mice against intracerebral challenge.
[c] Difference significant ($D = 0.05$).

of the cells growing in shake-flask cultures were satisfied fully by adding 3 mg of glutamate per ml to the broth; at this concentration the cell yield was increased by an average of 43.5%.

Effect upon biological properties. To insure that the addition of glutamate had no adverse effect, vaccines were prepared from the cells and their biological properties were examined (Table 3). The properties of the cells grown in broth containing 3 mg of added glutamate per ml were not significantly different ($D = 0.05$) from the properties of cells grown in unsupplemented broth. However, there appeared to be a downward trend in the HSF and IC protective potencies as the concentration of added glutamate was increased from 1 to 5 mg/ml.

To determine whether this apparent trend was real and reproducible, duplicate flasks of five different batches of broth were prepared by using different lots of Casamino Acids and were supplemented with glutamate (3 mg/ml). The flasks were inoculated and incubated for 72 hr, and the cultures were preserved and detoxified (Table 4). In four of the pairs of cultures, the HSF activity of the cells grown in the supplemented broth was higher than in the cells grown in unsupplemented broth; in one of these the difference was statistically significant ($D = 0.5$). In only one of the pairs of cultures was the activity of the cells grown in supplemented broth lower, and this difference was not statistically significant ($D = 0.05$). In one pair of cultures whose *IC* potency was tested, the addition of 3 mg of glutamate/ml had no effect.

Broth supplemented with 3 mg of monosodium L-glutamate per ml has been used over a 2-year period to prepare experimental *B. pertussis* vaccines by both batch and continuous cultivation methods at controlled *p*H. The cell yields obtained from the supplemented broth have been up to 52% higher than those from the basal

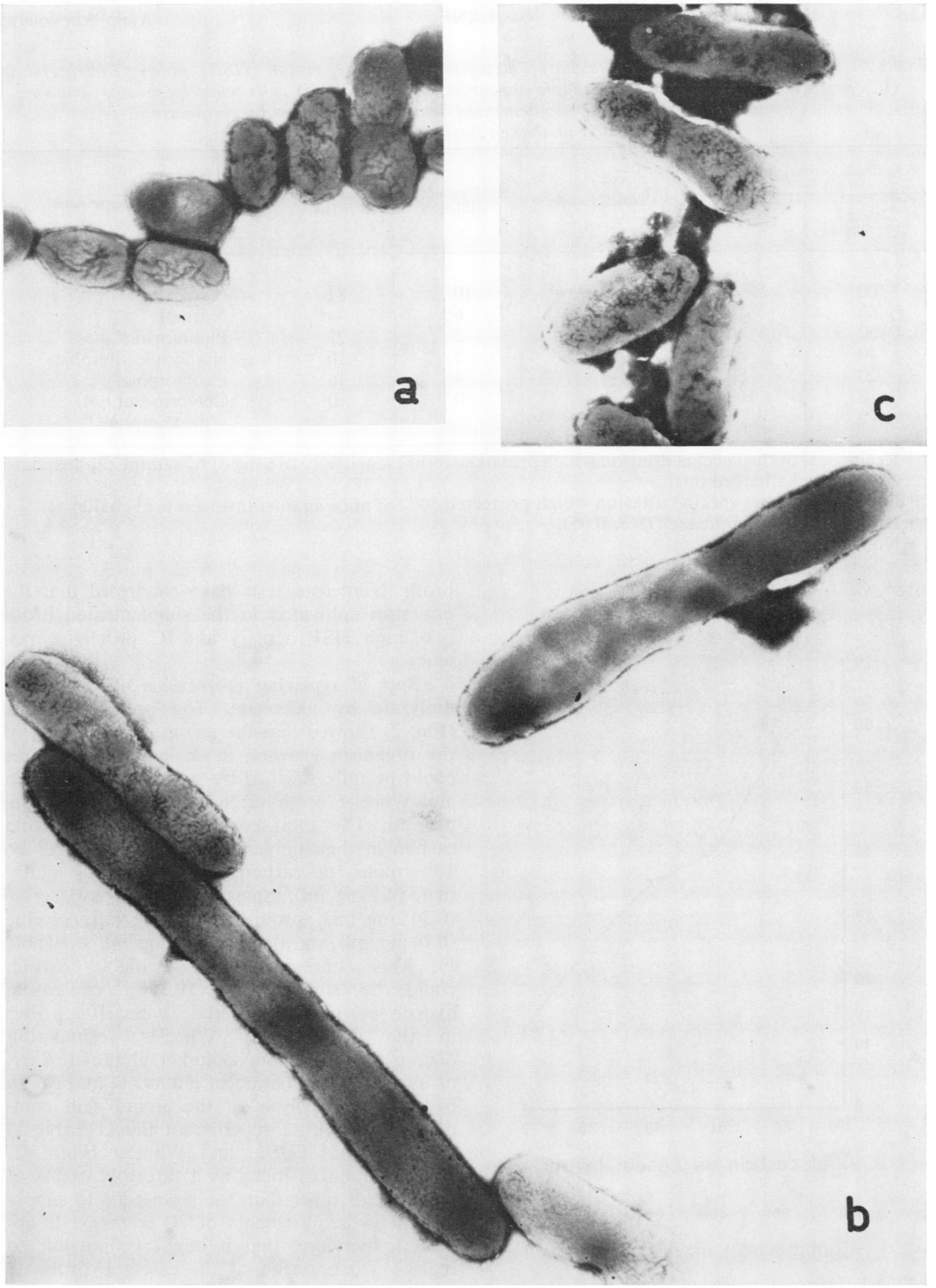

FIG. 3. *Electron micrographs of B. pertussis cells cultivated in shake flasks at 36 C for 72 hr in liquid media in which proportions of the casein hydrolysate had been replaced by glutamate (cells stained by phosphotungstic acid).× 20,000. (a) Normal cells grown in broth in which casein hydrolysate provided 100% of the available carbon. (b) Highly pleomorphic cells grown in broth in which casein hydrolysate provided 40% and glutamate 60% of the available carbon. (c) Cells somewhat coliform but not pleomorphic, grown in broth in which glutamate provided 100% of the available carbon.*

TABLE 5. *Effect upon mouse-lethal toxicity, histamine-sensitizing factor (HSF) potency, intracerebral (IC) protective potency, and microscopic appearance of B. pertussis cells when increasing amount of casein hydrolysate in supplemented broth were replaced by glutamate; cultivated in shaken flasks at 36 C for 72 hr*

Available carbon supplied as		Toxicity value	HSF potency (HSD_{50})[a]	IC potency (ED_{50})[b]	Microscopic appearance
Casein hydrolysate	Glutamate				
%	%				
100	0	++++	10	81	Normal
80	20	++++	6[c]	—[d]	Normal
60	40	++++	5	23	Pleomorhic
40	60	+++	5	10	Highly pleomorphic
20	80	++	4	16	Pleomorphic
0	100	−	0	0	Coliform but not pleomorphic

[a] Reciprocal of the vaccine dilution which provoked lethal sensitivity to 6 mg of histamine diphosphate in 50% of the challenged mice.
[b] Reciprocal of the vaccine dilution which protected 50% of mice against intracerebral challenge.
[c] Difference not significant (D = 0.05).
[d] Not done.

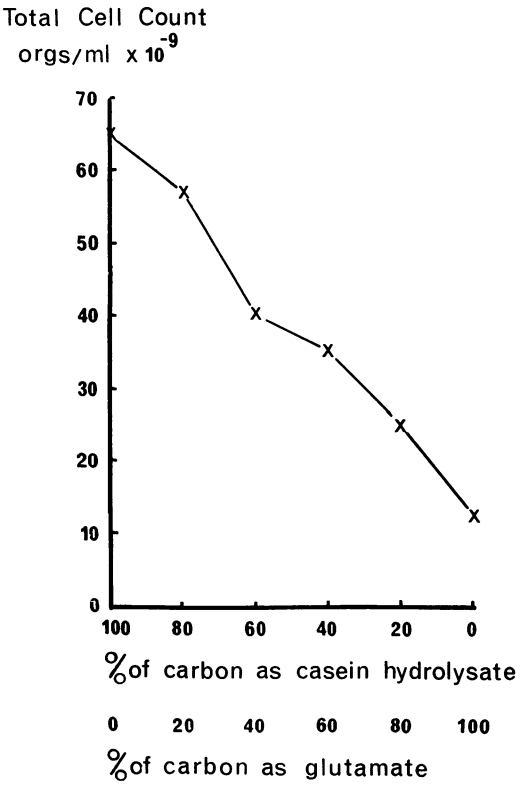

FIG. 4. *Effect of replacing casein hydrolysate in supplemented broth (see text) by glutamate upon the cell yield of B. pertussis in shake-flask cultures incubated at 36 C for 72 hr. The experiment was performed in duplicate flasks and was repeated four times; each point on the graph represents the average total cell count of the eight flasks.*

broth. Numerous tests have confirmed that the organism cultivated in the supplemented broth is of high HSF activity and IC protective potency.

Effect of replacing proportions of casein hydrolysate by glutamate. The previous results (Fig. 2) showed that the energy requirements of the organism growing in shaken flask cultures could be fully satisfied by supplementing Cohen and Wheeler broth (2) with 3 mg of glutamate per ml. The total concentration of available carbon in this supplemented broth was 18.3 × 10^{-5} moles of carbon/ml, supplied by glutamate (4.1 mg/ml), aspartate (0.46 mg/ml), serine (0.34 mg/ml), proline (0.77 mg/ml), glycine (0.14 mg/ml), and alanine (0.31 mg/ml; *see* Table 1). This concentration of available carbon could be supplied as 19.3 g of casein hydrolysate/liter [compared with 10 g/liter in the Cohen and Wheeler formulation (2)], as 6.2 g of monosodium L-glutamate/liter, or as some combination of glutamate and casein hydrolysate. Analysis of the amino acid composition of culture supernatant fluids (Table 1) suggested that Cohen and Wheeler broth (2) might contain as much as a threefold excess of amino acids other than the six needed to supply the energy requirements of *B. pertussis*. It appeared, therefore, that it might be possible to replace a proportion of the casein hydrolysate in the broth by glutamate.

However, when the casein hydrolysate in the supplemented broth in shake flasks was replaced in increasing amounts by glutamate, the cell morphology was altered (Fig. 3, Table 5); there was a dramatic fall in cell yield (Fig. 4), toxicity,

TABLE 6. *Consumption by B. pertussis of amino acids in fluid media in which increasing proportions of casein hydrolysate were replaced by glutamate; incubated at 36 C in shaken flasks for 72 hr*

Amino acid	Amino acid concn (mg/ml) at per cent of available carbon supplied as casein hydrolysate					
	0		40		100	
	Initial	Residual	Initial	Residual	Initial	Residual
Aspartate			0.36	0.10	0.89	0.03
Serine			0.29	0.17	0.66	0.03
Glutamate	6.20	1.58	4.55	0.01	2.07	0.00
Proline			0.60	1.27	1.49	0.00
Glycine			0.12	0.01	0.27	0.05
Alanine			0.24	0.05	0.60	0.09

HSF activity, and IC potency (Table 5). Agglutinability against smooth-type and rough-type antisera was unchanged, this property being obviously independent of the other activities.

Amino acid analyses of the culture supernatant fluids showed that the reduction in cell yield was not due to exhaustion of the media (Table 6), but differences were apparent between the amino acid utilization patterns of the cultures in different media.

The pleomorphism and the reduced cell yield that occurred in broth in which 60% of the carbon was supplied as glutamate were both overcome by the addition of L-alanine to the broth at a concentration of 10^{-5} moles of carbon/ml. However, the addition of alanine had no beneficial effect in correcting the unsatisfactory biological properties of the culture shown in Table 5. None of the other rapidly consumed amino acids (serine, proline, glycine, aspartate) had any useful effect when they were added singly at a concentration of 10^{-5} moles of carbon/ml. Therefore, the attempt to replace some of the casein hydrolysate in the broth by glutamate was abandoned.

Previous workers have shown that *B. pertussis* grows slowly in media containing either glutamate, aspartate, or proline as the sole substrate (6, 8, 9) and that the balance between amino acids in the medium is critical for optimum growth (7, 12, 13, 21). However, little attention has been paid to the effect of the amino acid composition of the broth upon the biological properties of the culture (4, 14, 22). Our results show that both the growth of the organism and its biological properties should be examined when investigating the nutritional requirements of *B. pertussis*.

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