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The Minimal Nutritional Requirements of Organisms of the Genus *Bordetella* López

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SUMMARY: Eight strains of bronchiseptica, six strains of parapertussis and fifteen strains of pertussis were examined for their minimal nutritional requirements in defined media. All strains showed an absolute requirement for nicotinic acid and no other vitamin was required for growth. Amino acids were essential for parapertussis and pertussis, but bronchiseptica would grow in either a mixture of amino acids, or lactate or citrate. Two old laboratory strains were exceptional in that they could utilize either glutamic acid, α -ketoglutaric acid, citrate, lactate, succinate or pyruvate. The amino acid requirements of the three species were relatively simple and showed some similarities. Bronchiseptica would grow in a mixture of glutamic acid, proline and leucine, while parapertussis required added cystine and methionine, and pertussis required in addition alanine, asparagine and serine. In simple amino acid mixtures glutamic acid was essential, but was replaceable by α -ketoglutaric acid. Nutritionally the species are very similar, but are quite different from the *Haemophilus influenzae-parainfluenzae-canis*, or the *Brucella abortus-suis-melitensis* groups of organisms. The nutritional evidence supports the already impressive evidence on other grounds that these three groups should be classified separately. If generic status is given to one group it should be given to all three. López (1952) has proposed a new genus *Bordetella*, consisting of the species *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, and this seems a reasonable solution to the present anomalous position. The three species can be differentiated by tests for inhibition. Thus *B. bronchiseptica* is the only species not inhibited by 2.0% citrate and *B. pertussis* the only species inhibited by colloidal copper sulphide.

The Parvobacteriaceae have been subdivided into a multiplicity of genera, primarily on grounds of animal pathogenicity. Many of the genera consist of only a few pathogenic species; in some instances all the species in a genus produce similar diseases in the field or in experimental animals. Since pathogens are highly specialized species it has usually been possible to devise a number of positive or negative diagnostic tests for their identity, tests which are not so readily available for the whole range of bacteria. To some extent these tests have obscured the underlying basis of pathogenicity used for their classification. This classification is, of course, a reflexion of the historical development of bacteriology and has been evolved to satisfy the practical needs of human and veterinary medicine. Even if comparative studies made it possible to present a more aesthetically satisfying classification on general biological lines it would almost certainly be unacceptable at the present time to those workers most interested in this group of organisms. However, the existing system of classification introduces two difficulties that are pertinent here. First, it is difficult to introduce a species into such an arbitrary classification. Thus, when it became apparent that bronchiseptica should be in this group, it had to be classified as either *Brucella bronchiseptica* or *Haemophilus*

bronchisepticus, whereas it was clear that the generic definitions really excluded this species from either genus. The second difficulty is that as further information becomes available the generic definition may exclude established species. Thus Fildes (1923) showed that the generic definition excluded the pertussis organism from the genus *Haemophilus* where it had become well established. In practice any widening of the generic definition introduces chaos into the whole group. For a long time it has been realized that bronchiseptica, parapertussis and pertussis have many common distinctive characters and cannot reasonably be classified in any of the existing genera. López (1952) summarized much of this evidence and proposed a new genus *Bordetella* consisting of these three species. The nutritional studies reported here support this view and this classification has been adopted.

METHODS

Strains. All strains were from the laboratory collection where they had been maintained as freeze-dried cultures. They were all examined for purity and identity by the relevant bacteriological and serological tests.

Nutritional requirements. The methods used were similar to those described by Knight & Proom (1950); for a detailed description reference should be made to that publication, but for convenience the salient points are here given. The usual precautions were taken to exclude contamination by unwanted nutrients. The starting-point for an experiment was a freeze-dried culture from the laboratory collection. At least five subcultures were made on defined media to exclude the possibility of carry-over of essential nutrients. At the conclusion of each experiment the final culture was checked for purity and identity. To exclude the possibility of selection of less exacting nutrients the original strain from the collection was tested for its ability to grow on the minimal medium finally found to be adequate.

Media were dispensed in 5 ml. lots in 6 × 1 in. rimless boiling tubes closed by loosely fitting aluminium caps. Cultures were incubated at 37° in a sloped position to give good aeration. The initial inoculum was taken from an agar slope with a small loop and subsequent inoculations were made with a small loop.

Media

Medium 6 (ammonia basal medium). KH_2PO_4 , 1.5 g.; $(\text{NH}_4)_2\text{HPO}_4$, 7.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3 g.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.04 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025 g.; ammonium molybdate 0.002 g.; dissolved in distilled water to 1 l.; pH adjusted to 7.6 with NaOH. Solution boiled, filtered through paper, and sterilized for 20 min. at 115°.

Medium 7 AA (seven amino acid medium). Medium 6 supplemented with the following amino acids in the concentrations shown (in mg./ml.). L-asparagine, 0.4; L-proline, 0.1; L-leucine, 0.57; DL-alanine, 0.38; L-glutamic acid, 1.4; DL-serine, 0.12; DL-methionine, 0.06. The amino acid mixture was prepared separately by solution in distilled water at 10 times these concentrations. It was gassed with H_2S , filtered through paper and sterilized at 115° for 20 min.

It was then added aseptically to medium 6 to give the required concentration of amino acids.

Medium SC 2 C (complex amino acid medium). Medium 6 supplemented with the following amino acids in the concentrations shown (in mg./ml.). DL-alanine, 0.38; DL-aspartic acid, 0.89; L-arginine HCl, 0.3; L-cystine, 0.02; L-glutamic acid, 1.4; glycine, 0.17; L-histidine HCl, 0.24; DL-isoleucine, 0.76; L-leucine, 0.57; L-lysine HCl, 0.24; DL-methionine, 0.06; L-proline, 0.11; DL-serine, 0.12; DL-threonine, 0.10; L-tyrosine, 0.06; DL-valine, 0.15. The amino acid mixture was prepared separately by solution in distilled water at 10 times the required concentration. It was gassed with H₂S, filtered through paper, sterilized at 115° for 20 min., and added aseptically to medium 6 to give the required concentration.

Medium SC2H. Identical with medium SC2C except that the amino acid mixture was not gassed with H₂S and was sterilized by Seitz filtration.

Medium SC2F. Identical with medium SC2C except that all the amino acids were in the L configuration. Also L-phenylalanine, L-tryptophan were included to give final concentrations in the medium of 0.1 and 0.03 mg./ml. respectively; gassed with H₂S.

Medium SC2G. Identical with medium SC2F except that the amino acid mixture was not gassed with H₂S and was sterilized by Seitz filtration.

Medium 11 (hydrolysed casein medium). Medium 6 supplemented with 'vitamin-free' hydrolysed casein (Allen and Hanburys Ltd.) to give an additional 0.1 % total nitrogen. The hydrolysed casein solution was prepared at 10 times the required concentration, and the pH value adjusted to 7.5 with NaOH. The solution was boiled and filtered through paper, sterilized at 115° for 20 min. and added aseptically to medium 6.

Hornibrook's amino acid mixture. Medium 6 supplemented with the amino acid mixture used by Hornibrook (1940). The amino acid mixture was used in the concentrations shown (in mg./ml.). DL-glutamic acid, 1.8; L-tyrosine, 0.2; glycine, 0.045; L-proline, 0.4; L-histidine, 0.1; DL-arginine, 0.2; L-cystine, 0.01. The amino acid mixture was prepared in distilled water at 10 times the final concentration, sterilized by Seitz filtration and added aseptically to medium 6.

Simple amino acid mixtures. When single amino acids or mixtures of two or three were used, they were added to medium 6 to give the same concentrations as in medium SC2C. The amino acids were dissolved in distilled water at 10 times the final concentration, sterilized for 20 min. at 115° and added aseptically to medium 6.

Medium 14 (citrate + acetate medium). From the medium described by Henderson & Snell (1948): sodium citrate, 2.0 %; sodium acetate, 1.0 %; NH₄Cl, 0.3 %; K₂HPO₄, 0.5 %; 2.0 % (v/v) trace metal solution C from Henderson & Snell (1948).

Soluble starch. A stock 1.5 % solution of Analar grade soluble starch (British Drug Houses Ltd.) dissolved in boiling water and sterilized 20 min. at 115°. The solution was added aseptically to the otherwise complete medium to give a final concentration of 0.15 %. One bottle of a batch was reserved for this

purpose. When tested with *Lactobacillus casei* no appreciable quantities of any of the growth factors required by this species were found in the sample. It was therefore assumed that the addition of starch did not result in the addition of growth factors.

RESULTS

The minimal nutritional requirements of the three species are summarized in Table 1. The detailed results with each species are given below.

Table 1. *The nutritional requirements of Bordetella spp.*

Medium	<i>B. bronchiseptica</i>	<i>B. parapertussis</i>	<i>B. pertussis</i>
1. Ammonia basal salt medium + complex amino acid mixture (SC2H) + nicotinic acid	+	+	+(phase I strains required starch)
2. Medium 1 without nicotinic acid	—	—	—
3. Ammonia basal salt medium + seven amino acid mixture (7AA) + nicotinic acid	+	+	—(+ with added cystine)
4. No. 3 without glutamic acid	—*	—	—(— with added cystine)
5. No. 4 with α -ketoglutaric acid	+	+	+(with added cystine)
6. Ammonia basal salt medium + nicotinic acid + glutamic acid + proline + leucine	+	—(+ with added cystine, methionine)	—
7. Acetate citrate (medium 14) + nicotinic acid	+†	—	—
8. Ammonia basal salt medium + nicotinic acid + either α -ketoglutarate, acetate, lactate, citrate, glucose, glutamic acid or pyruvate	(a) Old laboratory strains + with all except glucose. (b) Freshly isolated animal strains + with lactate or citrate only		—

* One animal strain grew.

† One animal strain would not grow.

Bordetella bronchiseptica

Eight strains of *B. bronchiseptica* were studied: four strains isolated from the lungs of dogs with secondary infection following dog distemper, one strain from the lung of a ferret and one from guinea-pig lung. These strains had been freeze-dried shortly after isolation. The remaining two were very old laboratory strains of unknown origin which had been subcultivated on laboratory media for many years before freeze-drying.

The eight strains grew on the complex amino acid medium (SC2C) and in the seven amino acid medium (7AA) with nicotinic acid (0.5 μ g./ml.) as an essential nutrient. The requirement for nicotinic acid was absolute, and this growth factor had to be added to all media to permit growth.

The growth requirements of the two old laboratory strains were simpler. They would grow in the ammonia basal medium (6) plus nicotinic acid, with the addition of either glutamic acid, lactate, pyruvate, citrate or α -ketoglutarate. Glucose would not support growth; this was to be expected as the species does not ferment glucose. In simple mixtures of amino acids glutamic acid was essential; for example, the strains would grow in a mixture of glutamic acid, proline and leucine but would not grow with proline and leucine alone.

The growth requirements of the remaining six strains were more complex and rather variable. With the complex amino acid medium (SC2C) minus glutamic acid, five strains grew poorly and occasionally failed to grow on serial subculture. In most experiments after growing poorly for a few subcultures the strains improved in growth which eventually became as good, or nearly as good, as in the complete medium. One strain from the start grew equally well in both media. With the seven amino acid medium (7AA) minus glutamic acid two strains failed to grow, while two strains grew poorly for a few subcultures and then died out. One strain grew poorly but growth eventually improved and one strain grew equally well on both media. Three strains grew well in a mixture of glutamic acid, proline and leucine, the other three strains grew poorly and occasionally faded out on serial subcultivation. None of the six strains would grow on proline + leucine, and all six strains gave maximal growth with a mixture of glutamic acid, proline, leucine, cystine and methionine.

Five of the six strains grew in the citrate + acetate medium (14). Growth was at first poor, but after a few subcultures improved and became as good, or nearly as good, as in the amino acid medium. The remaining strain grew in medium 14 after the addition of the seven amino acid mixture. Acetate, α -ketoglutarate, lactate, citrate, pyruvate and succinate in 1.0% concentrations were tested as single carbon and energy sources. The strain that would not grow in medium 14 would not grow in any of the above media. The results with the remaining strains were variable, but followed a definite pattern. When growth occurred it was poor and remained so for one or two subcultures and then either the culture died or growth improved until after five or six subcultures it became as good, or nearly as good, as with the amino acid media. As carbon sources only lactate or citrate supported growth; α -ketoglutarate, acetate, pyruvate and succinate were ineffective. However, since the experiments with lactate and citrate showed clear evidence of selection it is possible that variants could be selected that would grow on all the above substrates. The experiments, however, clearly showed that lactate and citrate were more readily utilized.

α -Ketoglutarate could replace glutamic acid in any of the amino acid media. For example, strains that grew poorly on a mixture of glutamic acid, proline and leucine, grew poorly in a mixture of α -ketoglutarate, proline and leucine, whereas those strains that grew well in the first medium grew well in the second.

Bordetella parapertussis

Six strains of *B. parapertussis* were studied, all having been isolated from cases of whooping cough. Four of the six strains had been freeze-dried within a few subcultures of isolation; the remaining two strains had been subcultured on Bordet-Gengou medium for some years before drying.

The six strains grew in the complex amino acid medium (SC2C) or in the seven amino acid medium (7 AA) with nicotinic acid ($0.5 \mu\text{g./ml.}$) as an essential nutrient. The requirement for nicotinic acid was absolute, and this growth factor had to be added to all media to permit growth. Glutamic acid appeared to be essential, since the six strains would not grow in medium SC2C minus glutamic acid or in medium 7 AA minus glutamic acid. The strains would grow poorly for a few subcultures in glutamic acid + proline + leucine, but they could not be maintained in serial subculture therein. They could be subcultivated indefinitely with added cystine and methionine. Glutamic acid alone would not support growth.

Amino acids were essential for growth and none of the strains would grow on the ammonia + citrate + acetate medium (14). Moreover, the concentration of citrate (2.0 %) in medium 14 inhibited the growth of all strains when added to amino acid media. Acetate, α -ketoglutarate, citrate, lactate, pyruvate and succinate were tested as single sources of carbon and energy but were unable to support growth, although in the 1.0 % concentrations used they were not inhibitory in the seven amino acid medium (7 AA). Citrate and α -ketoglutarate but not lactate, succinate or pyruvate, could replace glutamic acid in the amino acid media.

Bordetella pertussis

Fifteen strains of *B. pertussis* were studied. Thirteen were virulent phase I strains (Leslie & Gardner, 1931) isolated from cases of whooping cough and freeze-dried within a few subcultures of isolation. The remaining two strains were selected avirulent phase IV variants able to grow on nutrient agar.

All thirteen virulent strains would grow and could be maintained in serial subcultivation in some batches of 'vitamin-free' hydrolysed casein (11) with added nicotinic acid ($0.5 \mu\text{g./ml.}$) and with 0.15 % soluble starch to adsorb inhibitory fatty acids present in the medium and produced during growth (Pollock, 1947). The requirement for nicotinic acid was absolute and this growth factor had to be added to all media to allow growth. The two avirulent strains had similar requirements except they would grow in the absence of starch.

With the first inoculum from Bordet-Gengou medium the strains sometimes grew on the above medium when the hydrolysed casein was replaced by the complex amino acid medium (SC2C), but in all cases the cultures died after one or two subcultures. The failure to grow in the absence of 'vitamin-free' casein hydrolysate might have been due to a missing growth factor, to an inhibitor present in medium SC2C or to the balance of amino acid concentrations.

With the first batch of hydrolysed casein used there was no clear evidence that medium SC2C was inhibitory in the presence of hydrolysed casein.

Moreover, medium SC2C was not inhibitory to the related species *Bordetella parapertussis* or *B. bronchiseptica*. A search was therefore made for a missing growth factor. A very wide range of substances was tried, including a preparation of strepogenin made from crystalline insulin by tryptic digestion according to the method described by Sprince & Wooley (1945). None of these substances, individually or in combination, when added to medium SC2C would permit growth. Attempts to isolate a growth-promoting fraction from hydrolysed casein by paper chromatography or by fractionation in an all-glass Craig separator were uniformly unsuccessful.

The unlikely event that failure to grow was due to the relative proportions of amino acids present in the two media was investigated. A batch of medium SC2C was prepared, and by arbitrary addition of various amino acids was matched visually by two-dimensional paper chromatography until it was identical with hydrolysed casein. No growth was obtained with this medium.

Medium SC2G was prepared in which all the amino acids were present in the L-form. Since this medium did not support growth the failure to grow in medium SC2C was therefore not due to an inhibition by D-amino acids.

A number of different batches of hydrolysed casein were used and batch variation was found to be considerable. Some batches supported growth, others failed to support growth or growth died out on the first subculture. Strain variation was also evident. Some batches supported growth of phase IV but not phase I strains, while others supported the growth of phase IV and a limited number of phase I strains. With some batches of hydrolysed casein growth was obtained at the usual concentration equivalent to a total nitrogen (TN) of 0.1 g./l., but was poor at a TN of 0.2 g./l., and there was no growth at 0.4 g./l.; this suggested the presence of an inhibitor and this possibility was reconsidered.

Two recent publications (Schuhardt, Rode, Oglesby & Lankford (1952) and Woiwod (1954)), appeared relevant and suggested that the inhibitor might be colloidal sulphur or colloidal copper sulphide. It was found that media prepared from unsatisfactory batches of hydrolysed casein permitted growth of all strains of *Bordetella pertussis* when sterilized by Seitz filtration instead of by heat. It was also found that the addition of cystine, sterilized by filtration,

Table 2. *The inhibitory effect of citrate and colloidal copper sulphide*

Medium	Strain		
	<i>bronchiseptica</i>	<i>parapertussis</i>	<i>pertussis</i>
Complex amino acid medium (SC2H)*	+	+	+
SC2H + 2.0 % citrate	+	—	—
SC2H + colloidal copper sulphide	+	+	—
Complex amino acid medium (SC2C)†	+	+	—
SC2C filtered through membrane	.	.	+
SC2C + appropriate dilution of β -mercapto- propionic acid	.	.	+

* SC2H sterilized by Seitz filtration and not gassed with H₂S.

† SC2C prepared by gassing with H₂S and sterilized by heat. — = no growth; + = growth; . = not tested.

completely annulled the inhibition by the heat-sterilized medium. These results were consistent with the inhibitor being colloidal sulphur, as Woiwod (1954) showed that inhibition by colloidal copper sulphide was not annulled by cystine.

Chemically defined media SC2H and SC2G, identical with SC2C and SC2F except that they were not gassed with H_2S and were sterilized by Seitz filtration instead of by heat, supported the growth of all strains of *Bordetella pertussis* tested. It was thus clear that failure to grow in the complex amino acid medium SC2C was due to an inhibitor which had been formed during the preparation of the medium. The inhibitory medium SC2C was filtered through membrane filters (Apis Engineering and Research Ltd.) of pore size 200–500 m μ . Two phase IV strains and three of five phase I strains were maintained in serial subculture in the ultrafiltered medium. This was regarded as conclusive evidence that the inhibitor was particulate. Filters of smaller pore size were not used because of the difficulty of filtering sufficient medium for test. Attempts were made to annul the inhibitory effect in medium SC2C with a solution of Seitz-filtered cystine. The results were not so clear-cut as with hydrolysed casein. Phase IV strains and many phase I strains would grow in serial subculture, but a number of phase I strains failed to grow.

Attempts were made to see whether those strains which failed to grow with added cystine would grow after the addition of substances shown by Woiwod (1954) to annul the inhibitory action of colloidal copper sulphide. The results given in Table 3 show that an appropriate concentration of β -mercapto-

Table 3. *Reversal of inhibition present in medium SC2C by β -mercaptopropionic acid (β -m.p.a.)*

Medium	% β -m.p.a. in medium	Growth of <i>B. pertussis</i> after		
		48 hr.	72 hr.	96 hr.
Complex amino acid medium SC2H (non-inhibitory medium)	0.25	—	—	—
	0.125	—	—	—
	0.06	—	—	—
	0.025	—	tr.	±
	0.01	tr.	±	+
	None	+	+	++
Complex amino acid medium SC2C (inhibitory medium)	0.25	—	—	—
	0.125	—	—	—
	0.06	—	—	—
	0.025	tr.	+	++
	0.01	+	++	++
	None	—	—	—

— = no growth; tr., ±, +, ++ = amount of growth.

propionic acid promoted growth. The strain could not be maintained in serial subculture because the toxicity of media containing β -mercaptopropionic acid increased on standing. Bisulphite or β -mercaptopropanol either did not annul the inhibitor or the concentration necessary for annulment was itself toxic. β -Mercaptopropanol was very toxic for *Bordetella pertussis* and inhibited growth in medium SC2H at a concentration of less than 0.01 %. It is possible

that these results could be explained on the basis of variation in sensitivity of different strains of *B. pertussis* to colloidal sulphur. It seems more likely, however, that both colloidal sulphur and colloidal copper sulphide were present but only sufficient colloidal copper sulphide to inhibit the more sensitive strains.

Colloidal copper sulphide (10 mg. Cu/l.), provided by Dr A. J. Woiod, inhibited the growth of *Bordetella pertussis* in medium SC2H at a dilution of 1/64. At a dilution of 1/4 it did not inhibit *B. parapertussis* or *B. bronchiseptica* in any of the media tested including a simple mixture of amino acids. A limited number of experiments showed clearly that the inhibitory effect of colloidal copper sulphide varied both with the strains and the medium to which it was added. Phase IV strains were more resistant than phase I strains, and there was considerable variation in the susceptibility of different phase I strains. The more complex the medium the less was the inhibitory effect of added sol.

The amino acid requirements of *Bordetella pertussis* were examined in some detail, and to avoid inhibitory effects all the amino acids were sterilized by Seitz filtration. All strains grew in serial subculture in the complex amino acid medium (SC2H) or in the seven amino acid medium (7AA) with added cystine. Since both these media contained no utilizable carbohydrate the organism obtained its energy by metabolism of amino acids. Strains of *B. pertussis* grew reasonably well in medium SC2H minus glutamic acid and in this medium glutamic acid was not essential as was the case with *B. parapertussis*. However, glutamic acid was essential with the simpler amino acid medium 7AA plus cystine. With phase I strains the glutamic acid could be replaced by α -ketoglutarate or succinate but not by lactate or pyruvate. Citrate could replace glutamic acid with a minority of phase I strains. With the two phase IV strains glutamic acid could be replaced by citrate, α -ketoglutarate, or succinate but not by lactate or pyruvate. These results are different from those obtained with *B. parapertussis*.

Bordetella pertussis sometimes grew for one or two subcultures in very simple amino acid mixtures, but for serial subcultivation the requirements were more complex. Strains could grow up to five subcultures in Hornibrook's (1940) amino acid mixture but required added alanine for indefinite subcultivation. It was not practicable to determine all the combinations of amino acids that would support growth, but it seemed likely that medium 7AA + cystine or Hornibrook's (1940) amino acid mixture + alanine represented two of the simplest combinations capable of supporting growth. Experiments were performed in which single amino acids were omitted from the above mixtures and from medium SC2H. It was not always easy to interpret the results, and the experiments were very laborious since up to ten serial subcultures had to be made with each combination of amino acids. Cystine appeared essential for growth and could not be replaced by methionine or any other mixture of amino acids. Proline, leucine or alanine, if not essential, could only be replaced by complicated mixtures of amino acids. With simple amino acid mixtures glutamic acid was essential but could be replaced by an alternative source of energy such as α -ketoglutarate. However, a mixture of the above five amino

acids (cystine, proline, leucine, alanine and glutamic acid) was not sufficient to maintain all strains in serial subculture and a mixture of three or more other amino acids had to be added. It seemed that almost any combination of three or four different amino acids would suffice, and the analysis was not further continued.

DISCUSSION

Hornibrook (1939) described a semi-defined medium consisting of inorganic salts, hydrolysed casein, cystine and soluble starch that supported the growth of freshly isolated strains of *Bordetella pertussis*. Yeast extract, though not essential, improved growth. Shortly afterwards Hornibrook (1940) showed that when the inoculum from a culture on Bordet-Gengou medium was small yeast extract became essential, and that *B. pertussis* would grow in a medium consisting of soluble starch, salts, a mixture of seven amino acids, and nicotinic acid. This paper clearly demonstrated the requirement for nicotinic acid but gave no information on the course of events on serial subculture. Later workers have confined their attention to modifications of Hornibrook's medium to give improved growth for the production of pertussis vaccines for active immunization. Such modifications have been in the composition of the salt mixture and in the addition of accessory nutrients such as yeast dialysate or liver extract. With these media it has been common experience that variations in the method of casein digestion, and even batch variations when using the same process, influences considerably the amount of growth obtained. It is possible that these variations might, in part, be due to variations in the amount of inhibitory material present. No reports have been observed in the literature dealing with the exact nutritional requirements of *B. parapertussis*.

While this work was in progress Ulrich & Needham (1953) reported on the nutritional requirements of *Bordetella bronchiseptica* in a paper dealing with the differentiation of this species from *Alcaligenes faecalis*. Sixteen of eighteen strains required nicotinic acid, four strains required in addition pantothenate and two strains did not require any vitamin. In the present study nicotinic acid was required by all the strains of *Bordetella bronchiseptica*, *B. parapertussis* and *B. pertussis*, and was the only vitamin necessary for growth. It is of interest to note that the two very old strains of *B. bronchiseptica* which had been subcultured on artificial media for at least 20 years, as well as the two strains of *B. parapertussis* subcultivated on Bordet-Gengou medium for several years and the two selected variants of *B. pertussis* which grew on nutrient agar, still required nicotinic acid. Knight & Proom (1950) have already commented on the similarity of vitamin requirements of old laboratory strains and fresh isolates in the genus *Bacillus*. Proom & Woiwod (1951) examined a large number of *Proteus* strains, some of which were very old laboratory cultures, and all showed the same basic vitamin requirements. There is cumulative evidence suggesting that the basal vitamin requirements of a bacterial species are among one of its more constant characters. Although variants with more complex vitamin requirements can be produced in the laboratory or may occur in nature, strains less exacting than those of the basal species pattern would seem to be rare.

Bordetella pertussis, *B. parapertussis* and *B. bronchiseptica* do not metabolize glucose and obtain their energy and carbon from either organic acids or amino acids. They do not exhibit the same absolute requirement for specific compounds as is the case with the vitamin requirement for nicotinic acid. Rather they form a graduated series of strains and species having increasing nutritional complexity.

The two old laboratory strains of *Bordetella bronchiseptica* can utilize either glutamic acid, lactate, citrate, pyruvate or α -ketoglutarate. These strains had the most active metabolism of those tested. Chromatograms of cultures grown in hydrolysed casein showed that all the amino acids had been metabolized except for some occupying the position of aspartic acid in the single dimensional chromatogram. They show the typical pattern described by Proom & Woiwod (1949) for biochemically active Gram-negative organisms. With other strains of *B. bronchiseptica* and strains of *B. parapertussis* and *B. pertussis*, the chromatograms showed that cystine, serine, glutamic acid, alanine, proline and leucine had been metabolized. These chromatographic results were a rough guide in the selection of simple amino acid mixtures likely to support growth.

Apart from the two old laboratory strains, five recently isolated strains of *Bordetella bronchiseptica* grew in the absence of amino acids. It was evident that selection occurred before the strains grew readily. There seems no obvious reason why citrate or lactate should be more readily utilized than pyruvate or α -ketoglutarate, particularly since in the amino acid mixture glutamic acid could be replaced by α -ketoglutarate. The remaining strain of *B. bronchiseptica* would not grow in the absence of amino acids. With this strain the high concentration of citrate (2%) in medium 14 was not inhibitory when added to the seven amino acid medium.

The amino acid requirements of the three species show some similarities. *Bordetella bronchiseptica*, in the absence of organic acids, requires glutamic acid, proline and leucine; cystine and methionine are also required to give good growth with all strains. *B. parapertussis* grows poorly with glutamic acid, proline and leucine and for serial subculture requires added cystine and methionine. *B. pertussis* will grow for a few subcultures in Hornibrook's (1940) amino acid mixture but for serial subculture requires added alanine. Alternatively, this species can be maintained in serial subculture in the seven amino acid mixture (7AA) with added cystine or in the complex amino acid medium (SC2H).

Since the three species do not utilize glucose or, with the exception of *Bordetella bronchiseptica*, organic acids, it is not surprising that glutamic acid is specially important, since it is the acid most likely to be used as a source of energy. The chromatographic evidence showed that it was the amino acid most rapidly and completely metabolized in growing cultures. In simple mixtures of amino acids it was found to be essential, but with more complex mixtures the experimental evidence was not so conclusive. Certainly it seems to be preferred, but it was possible here that other amino acids, either alone or in combination, could replace it as an energy source. However, purity is a

relative term and since the actual amounts of amino acid necessary for growth are very small it is unwise to assume that the complex mixtures of amino acids were free from traces of contaminating glutamic acid.

It is usually assumed that when glutamic acid is used as a source of energy it is first converted by the organism to α -ketoglutaric acid. In fact, with all strains of *Bordetella bronchiseptica*, *B. parapertussis* and *B. pertussis*, α -ketoglutarate could replace glutamic acid in simple mixtures of amino acids. However, many strains of *B. bronchiseptica* utilized α -ketoglutarate only in the presence of amino acids but would utilize citrate or lactate in the absence of amino acids. The samples of citrate and lactate used were carefully examined and found free from traces of amino acids.

The investigation of the nutrition of *Bordetella pertussis* was hampered by inhibitory materials formed in the preparation of the defined media. The experiments suggested that the inhibitor present in the heat-sterilized hydrolysed casein was colloidal sulphur. The inhibitor present in the defined amino acid medium was either colloidal sulphur or a mixture of colloidal sulphur and colloidal copper sulphide. The procedures responsible for their production were gassing with H_2S to remove heavy metals and sterilization by heat. The inhibitors were only produced in the presence of cystine, and their production could be eliminated either by sterilization by filtration without gassing with H_2S , or by the addition of cystine as a Seitz-filtered solution to the otherwise complete medium. The observation that inhibitory effects may arise during the preparation of defined media is of course very old, and these particular inhibitors have recently been examined in some detail by Schuhardt *et al.* (1952) and by Woiwod (1954). These results are reported as an example of some of the difficulties that may arise in the preparation of defined media for nutritional studies. It is not always easy to decide whether failure to grow is due to the presence of an inhibitor or the absence of a growth factor. Moreover, the inhibitory effects became progressively greater as the medium became simpler in composition.

It is interesting to note that the three species could be differentiated on the basis of tests for growth inhibition. *Bordetella bronchiseptica* is not inhibited by 2.0% citrate in the presence of amino acids, whereas *B. parapertussis* and *B. pertussis* are inhibited. *B. pertussis* is the only species inhibited by colloidal copper sulphide.

These results show very clearly that nutritionally *Bordetella pertussis*, *B. parapertussis* and *B. bronchiseptica* are similar. All show an absolute requirement for nicotinic acid and require no other vitamin for growth. In simple amino acid mixtures all three species use glutamic acid as a source of energy and this acid can be replaced by α -ketoglutaric acid. The amino acid mixtures necessary for growth are similar, forming a series of slightly increasing complexity. The nutritional requirements of these species are quite different from those of *Haemophilus influenzae-parainfluenzae-canis* group in that they do not require haemin or phosphopyridine nucleotide (X and V factors) and from the *Brucella abortus-suis-melitensis* group in that they do not require thiamine, biotin or pantothenate. These results support the already

impressive evidence on physiological, serological and toxigenic grounds that if generic status is given to one of these groups it should be given to all three. López (1952) has proposed a new genus, *Bordetella*, to include the species *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, and in a footnote stated that this classification will be adopted in the seventh edition of *Bergey's Manual of Determinative Bacteriology*. This seems a reasonable solution to the present anomalous taxonomic position.

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