DESCRIPTION OF TWO NEW UREOLYTIC *ARTHROBACTER* SPECIES ISOLATED FROM SOIL AND SEWAGE

By M. F. Mullakhanbhai and J. V. Bhat

(Fermentation Technology Laboratory, Indian Institute of Science, Bangalore-12)

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Abstract

The morphological, cultural, physiological and nutritional properties of two new ureolytic species of *Arthrobacter* isolated from soil, sewage and activated sludge were recorded. Both the species showed strong ureolytic activity. A linear relationship was obtained with both when suspensions of cells grown in nutrient broth were tested for release of ammonia against time intervals upto 100 minutes. Optimum pH for the reaction was 7.5 and only very little activity was observed at pH 5.0. Sewage species alone grew luxuriantly in synthetic media containing glycine as a nitrogen source and when grown on different substrates exhibited better capacity than its soil counterpart to oxidize glycine. Ability of the bacteria to oxidize various other substrates was also recorded.

Introduction

The genus *Arthrobacter* (Breed *et al.*, 1957) comprise of 9 species which stain Gram negative or Gram variable and appear as straight, bent, curved, swollen or clubshaped rods in young cultures and as coccoid cells in older cultures. Transference of the coccoidal cells into fresh medium results in the germination of these coccoidal elements to yield long curved rods. In other words the bacteria exhibit a growth cycle in liquid or solid media. True persistent branching has never been observed in typical *Arthrobacter* as in the case of *Nocardia*. However, several authors have described branching on what they call as "rudimentary budding" e.g. in *A. ramosus* by Jensen (1960) and in *A. globiformis* by Mulder (1963).

Since the publication of the Bergey's Manual six more species have been described in the literature. Of these only 3 species are known to be ureolytic. The first ureolytic *Arthrobacter* was described by Jensen (1960) *A. ramosus* n.sp. and subsequently Kuhn and Starr, (1960) and Ensign and Rittenberg, (1963) described *A. atrocyaneus and A. crystallopoietes.* In this paper are described two more ureolytic *Arthrobacter* sp. isolated from glycine enrichments inoculated with soils on one hand and sewage and activated sludge samples on the other.

MATERIALS AND METHODS

Isolation of the bacteria. An enrichment medium containing 0.2% glycine based in the mineral solution used by Khambata *et al.* (1960) was used. The medium was dispensed in 25 ml amounts in 250 ml Erlenmeyer flasks and autoclaved. A gram of soil sample or 1 ml of sewage or activated sludge constituted the inoculum. The flasks were incubated at room temperature RT (20 – 25°C) on a rotary shaker for 36 hours. The contents were examined microscopically. The subsequent two transfers were made in the same medium fortified with 0.05% yeast extract as in its absence the dominant bacteria failed to proliferate rapidly. Inoculum from the third enrichment was plated on the solidified enrichment medium. Colonies were picked up, purified by conventional techniques and the pure cultures made were maintained on two sets of media, *viz.*, on glycine agar and nutrient agar slants.

Characterization of the isolates. Morphology and Gram reaction of agar culteres were studied at intervals of 8, 18 and 36 hours. Changes, if any, in the morphological pattern were also recorded of cultures grown in liquid medium (basal-salts-glucose medium.) Physiological and biochemical properties were studied by adopting the methods described in the "Manual of Microbiological Methods" (1957). Hydrolysis of urea and arginine was tested by the methods of Levine (1954) and Sherris *et al* (1959). Ability to produce acid from carbohydrates was ascertained by growing the cultures in the medium of Hugh and Leifson (1953).

Nutritional Studies. The basal medium used by Khambata *et al.* fortified with 0.001 μ g/ml of biotin was employed for the elucidation of the nutritional requirements of the bacterial cultures. Biotin was subsequently found to be not essential for their growth. Suitability or ortherwise of different sources of carbon was determined by incorporating them individually into the basal medium at the concentration of 1% but the level of oxalate, formate and benzoate was restricted to 0.1%. Various nitrogenous compounds were also tested at the concentration of 0.1%, some of them for their ability to serve as combined source of nitrogen and carbon, in which event the concentrations of the substrates were kept at 0.5%. Inoculum was prepared from cells grown for 48 hours on agar slants after washing them twice with distilled water and suspending them in sterile water in such a manner as to give a turbidity reading of 70 to 75% transmittance. Aliquots of 0.05 ml of this cell suspension was inoculated into each tube containing 10 ml of the nutritional medium. The growth after incubation at R.T. was measured on a Bausch and Lomb "Spectronic 20" Colorimeter at 540 $m\mu$ upto a period of 7 days.

Manometric Studies. Conventional manometric techniques (Umbreit *et al.* (1957) were employed for the determination of oxygen uptake values against certain subtrates. Cultures were first grown for 48 hours in basal media

containing the growth substrates fortified with 0.01% yeast extract on a rotary shaker (250 rpm). The cells were harvested and washed twice with M/30 phosphate buffer of pH 7.0 before their resuspension in M/15 phosphate buffer of pH 7.0. The cell density was adjusted in such a way that 1.5 ml contained 100 mg wet wt. of the cells.

Quantitative Urease activity. For measuring the rate of urea hydrolysis cultures were grown in broth for 36 hours on a rotary shaker. Cells were centrifuged off, washed twice with distilled water and suspended in distilled water in such a manner that 1 ml of cell suspension gave approximately 12 mg dry wt. The enzyme assay was carried out as described by Sumner (1928). The ammonia liberated was measured by Nesslers reagent as described in Manual of microbiological methods (1957). The urease activity was expressed as μg ammonia nitrogen liberated per mg dry wt. of cells at various time intervals.

Results

The morphological, cultural and physiological characteristics itemised below are representative of all the 6 cultures made from sewage and activated sludge on one hand and 8 isolates made from soil on the other.

Morphology of cultures. Morphologically all the *Arthrobacter* cultures were pleomorphic and appeared as long curved rods when young but were coccoidal to small cocci in fairly old cultures. In other words, they were typical species of *Arthrobacter* at all stages of growth and were Gram positive. In liquid media the short rod forms persisted even in the old cultures whereas on the solid media truly coccoidal shapes were observable (occasionally larger Gram positive spherical cells were observed in old liquid cultures). The Gram reaction was somewhat weakly positive when cultures were grown in biotin deficient media. There was no difference between the sewage and the soil strains.

Cultural Characteristics. The soil *Arthrobacter* colonies on nutrient agar appeared moist, lemon yellow, opaque, round, convex with an entire margin and they attained a size of about 3 *mm* on incubation for 5 days at R.T. The yellow pigment was insoluble in water. In nutrient broth they produced a uniform and general turbidity accompanied by a yellow ring and moderate sediment. All the isolates grew luxuriantly on glycine agar and asparagine agar. Aeration of the liquid media had a beneficial effect on the rate of growth of these and the growth was most luxuriant between 20 – 25°C and sparse at 10° and 37°C.

On nutrient agar the sewage *Arthrobacter* colonies were non-chromogenic, pale yellowish, opaque, round, convex, with an entire margin and moist. They also attained a size of 3 *mm* in 5 days. In nutrient broth a whitish ring was visible along with uniform and heavy turbidity and abundant sediment. All the isolates grew luxuriantly on glycine agar and asparagine agar slants.

Physiological attributes. The soil *Arthrobacter* strains gave negative results for indole, V.P. and M.R. tests and were unable to utilize citrate even in the presence of biotin. They, however, hydrolysed arginine, urea, tributyrin, gelatin and starch. They produced catalase and hydrogen sulphide and reduced nitrate to nitrite. Acid was not produced from glucose, maltose, sucrose and lactose, but bromocresol purple milk turned alkaline and a slow clearing thereof was observable. The organism utilized phenol, *m.* cresol and benzoate as sources of carbon.

The sewage strains also gave negative results for indole, V.P. and M.R. They hydrolysed gelatin, urea, arginine and tributyrin but not starch. They produced both catalase and H_2S and reduced nitrate to nitrite. Sugars remained unfermented but bromocresol purple milk turned alkaline. These cultures failed to utilize phenol or any other aromatic compounds unlike their soil counterparts.

Substrate utilization. The soil *Arthrobacter* strains grew poorly in the majority of sugars tested. Of the organic acids only succinate, fumarate and lactate supported a fairly good growth, others being of no use as growth substrates. Benzoate seemed to support growth to a very limited extent.

Except sodium nitrate, urate, methionine, cysteine and tryptophan, all other compounds tested supported good growth. Lysine, aspartic acid, arginine, casein hydrolysate and peptone appeared to be particularly favourable.

Some of the amino acids (β-alanine, leucine, valine, threonine) failed to serve as sources of both carbon and nitrogen. Aspartic acid and glycine, on the other hand, promoted fairly good growth. (Table No. I, II and III).

The sewage ureolytic strains were more versatile in their attack on the carbon compounds. Glucose, fructose, mannose, maltose, sorbitol and glycerol seemed to support their growth well. Surprisingly acetate and propionate also served as carbon source. Tartrate, citrate, fumarate and lactate were suitable as growth substrates.

Ammonium hydrogen phosphate proved to be the best of the inorganic sources of nitrogen tested. Sodium nitrate surprisingly was not utilized. Glycine, alanine, peptone and casein hydrolysate yielded profuse growths. Tryptophan, methionine and urate were unsuitable as growth subtrates. Leucine, valine, hippurate, methionine tryptophan, urate, threonine and cysteine could not serve as sources of both carbon and nitrogen whereas alanine and arginine served very well as combined sources of carbon and nitrogen (Table I, II and III).

Oxygen uptake. The units of oxygen uptake against glycine, formate, glyoxylate, acetate, pyruvate, succinate, citrate and glucose are presented in Table IV and V and they are clearly indicative of the ability of sewage strains to breakdown glycine and acetate irrespective of the nature of the substrates wherefrom the organisms were harvested. None of the growth subtrates,

however, served as a common source for the development of all enzymes required for the various substrate utilization. Glutamate appeared to serve such a purpose in the case of soil strains.

Whereas acetate appeared to lie in the pathway of glycine metabolism, neither formate, glyoxylate, nor pyruvate were involved in the oxidation of glycine by the two groups of bacteria. The sewage strains obviously possessed constitutive enzyme for the utilization of glycine whereas the soil strains were not endowed with the same. In the utilization of the carbon and nitrogen compounds the sewage strains were more versatile than the soil isolates.

TABLE I

Utilization of various carbon compounds by ureolytic *Arthrobacter* species

Carbon Source	Sewage Strains				Soil Strains			
	43	44	45	46	59	60	61	62
	Growth response: 100—% transmittance							
Glucose	31	27	30	31	20	20	40	39
Fructose	30	28	25	28	34	22	30	38
Mannose	45	48	42	46	0	0	0	0
Sucrose	0	0	0	0	20	20	27	30
Maltose	52	48	50	47	0	0	0	0
Sorbitol	23	26	25	27	0	0	0	0
Glycerol	37	29	30	32	0	0	0	0
Acetate	45	47	40	46	0	0	0	0
Propionate	23	28	20	25	0	0	0	0
Lactate	60	76	60	60	40	45	51	40
Tartrate	60	52	60	48	0	0	0	0
Citrate	77	73	70	68	0	0	0	0
Succinate	54	43	50	40	55	50	30	39
Fumarate	73	69	66	59	57	51	40	45
Benzoate	0	0	0	0	20	18	15	20

TABLE II

Utilization of various nitrogen compounds by ureolytic *Arthrobacter* species

Nitrogen Source	Sewage strains				Soil strains			
	43	44	45	46	59	60	61	62
	Growth response 100 - % transmittance							
Ammonium sulphate	33	25	30	28	27	40	36	30
Ammonium nitrate	30	25	35	22	20	25	22	20
Ammonium hydrogen phosphate	60	80	57	55	31	20	28	32
Sodium nitrate	0	0	0	0	21	20	25	20
Urea	32	20	40	30	20	20	37	18
Creatinine	43	35	30	28	31	33	38	39
Aspartic acid	78	62	59	70	36	45	30	42
Asparagine	75	64	60	65	38	50	40	40
Arginine	78	70	65	62	45	33	40	41
Alanine	95	70	77	75	38	30	38	33
βalanine	54	40	39	42	15	33	20	20
Cysteine	62	60	51	63	0	0	0	0
Glycine	88	80	87	76	35	30	44	40
Glutamate	20	32	30	35	31	29	25	28
Histidine	26	30	38	27	21	45	28	36
Lysine	55	71	49	51	40	40	42	40
Leucine	30	20	28	31	15	16	15	15
Proline	67	51	40	46	31	25	40	35
Phenylalanine	42	30	33	40	20	15	20	15
Valine	22	20	18	20	15	15	15	15
Peptone	95	80	87	80	80	72	70	82
Casein-hydrolysate	60	90	91	92	60	55	62	67

TABLE III

Ability to serve as combined nitrogen and carbon source by various compound for ureolytic *Arthrobacter* species

Substrates	Sewage strains				Soil strains			
	43	44	45	46	59	60	61	62
	Growth response : 100—% transmittance							
Aspartic acid	60	60	51	54	50	41	40	46
Asparagine	47	76	48	50	18	31	20	20
Arginine	86	79	80	81	48	25	38	29
Alanine	74	77	70	69	20	25	33	24
βalanine	20	26	28	22	0	0	0	0
Citrulline	31	39	30	35	0	0	0	0
Glycine	56	52	58	53	35	40	35	30
Glutamate	42	48	40	39	39	30	35	32
Histidine	64	70	65	60	34	28	15	20
Lysine	60	60	60	61	35	49	50	42
Proline	40	60	50	55	30	32	20	33
Phenylalanine	31	45	35	40	44	25	41	28
Serine	26	33	28	35	0	0	0	0
Creatinine	17	20	15	15	31	30	20	30
Hippurate	0	0	0	0	35	35	25	30
Peptone	80	78	80	80	45	60	55	60
Caseinhydro-lysate	89	94	90	86	60	58	55	58

TABLE IV

Oxygen uptake by washed cell suspensions of *Arthrobacter* (Sewage strain)

Growth substrates	Respiratory substrates (10 μ moles) ($\mu 10_2$/hr./mg. dry wt.)							
	Glycine	Acetate	For-mate	Glyoxy-late	Pyru-vate	Succi-nate	Citrate	Glucose
Glycine	10.0	8.0	0	0	0	0	0	0
Acetate	16.0	67.6	6.0	3.0	37.6	20	0	0
Glutamate	9.6	14.1	5.2	0	13.5	31.75	0	0
Succinate	9.3	21.3	0	0	18.3	32.6	10.6	0

The values have been corrected for the oxygen uptake in the absence of the subtrates.

Each warburg flasks contained M/15 Phosphate buffer pH 7.0;
cell suspension, 100 mg. wet weight;
10 μ *M* of substances;
Total volume 3.2 ml.

TABLE V

Oxygen uptake by washed cell suspension of *Arthrobacter* (Soil strain)

Growth substrates	Respiratory substrates (10 μmoles) ($\mu 10_2$/hr./mg. dry wt.)							
	Glycine	Acetate	For-mate	Glyoxy-late	Pyru-vate	Succi-nate	Citrate	Glucose
Glycine	12.8	25.0	15.5	0	15.5	0	0	0
Acetate	0	16.0	0	0	15.0	14.0	0	0
Glutamate	13.5	34.8	21.4	6.0	30.0	6.9	4.5	6.0
Succinate	0	29.0	0	0	26.3	13.0	0	0

The values have been corrected for the oxygen uptake in the absence of the subtrates.

Each warburg flask contained M/15 phosphate buffer pH 7.0; cell suspension, 100 mg. wet weight; 10 μ *M* of subtrates; Total volume 3.2 ml.

Ureolytic property. Fig. I represents the linear relationship recorded. It is clear therefrom that increase in ammoniacal nitrogen occured commensurate with the incubation period. Maximum activity was observed at 100th minute, the release of ammonia thereafter being at a uniform level upto a period of 120 minutes to which the test lasted. The results are expressed as per mg. dry wt. bases. The pH optimum for the soil strains tested was close to 7.5 (Fig. II), the enzyme activity at pH 5.0 being little and nil at pH 9.25. Fig. III shows a linear relationship between increase in cell concentration and increase in NH_4 nitrogen released. The sewage strains behaved much the same way as the soil ones, both being of equal vigour in attacking urea.

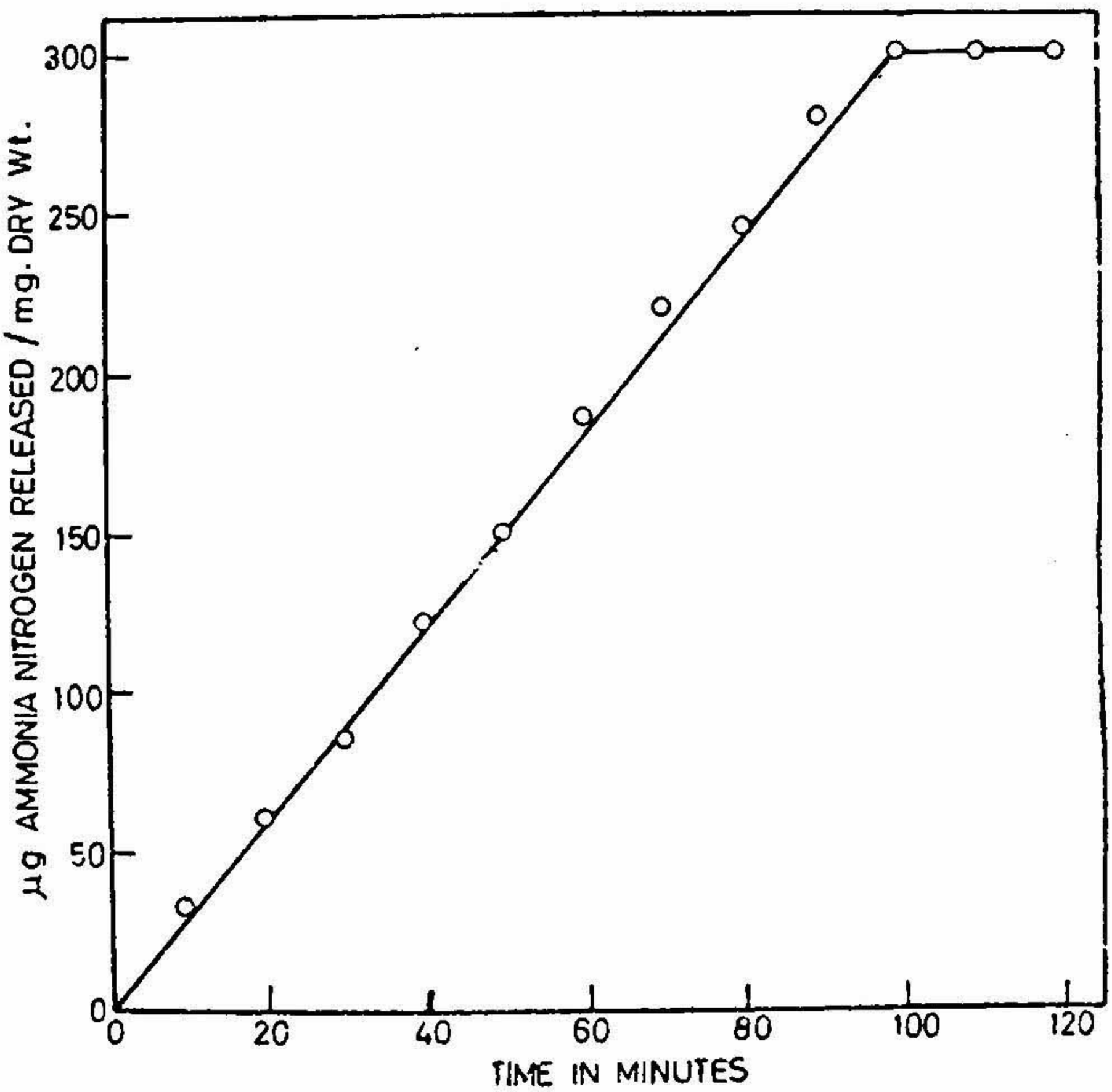

FIG. I

Effect of time on urease activity of *Arthrobacter*, soil strain No. 59

Reaction mixture: Cell suspension, 1,0 ml. (12 mg dry wt.); 3% urea solution, 1.0 ml; 9.6% phosphate buffer pH 7.0 1·0 ml.

Incubated at room temp. for varying period of time and at the end 1.0 ml. of 1N HCl was added to stop the reaction, centrifuged and aliquotes therefrom taken for the estimation of ammonia liberated as the product of the reaction.

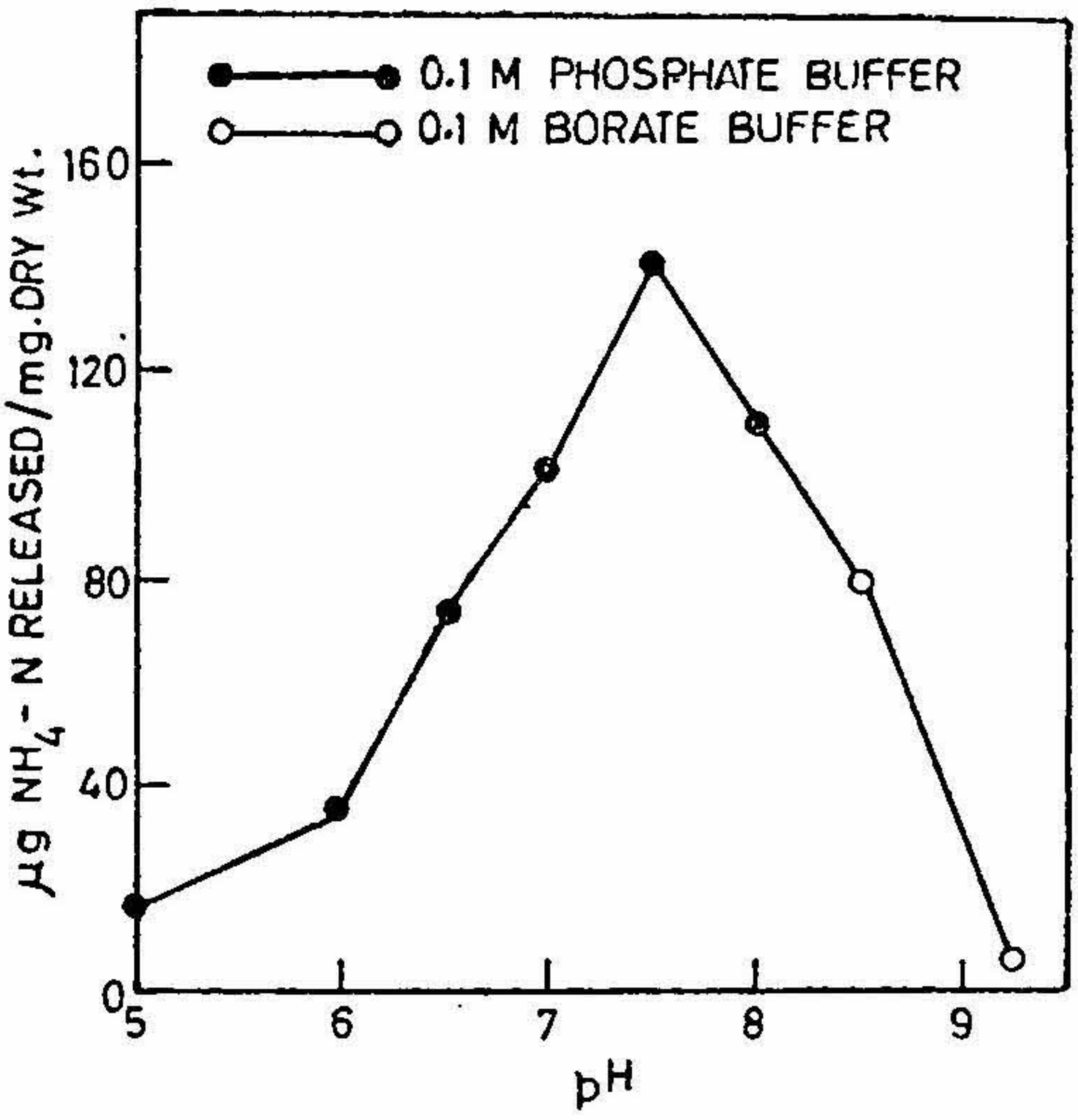

FIG. II

Effect of pH on urease activity of *Arthrobacter*, soil strain No. 59

Reaction mixture: Cell suspension, 1.0 ml. (12 mg dry wt.): 3% urea solution, 1.0 ml; buffers of various pH range (between 5.0 and 9.0) at 0.1 M conc: 1.0 ml.

Rest of the experimental conditions were same as Fig. I except for the period of incubation which lasted for one hour.

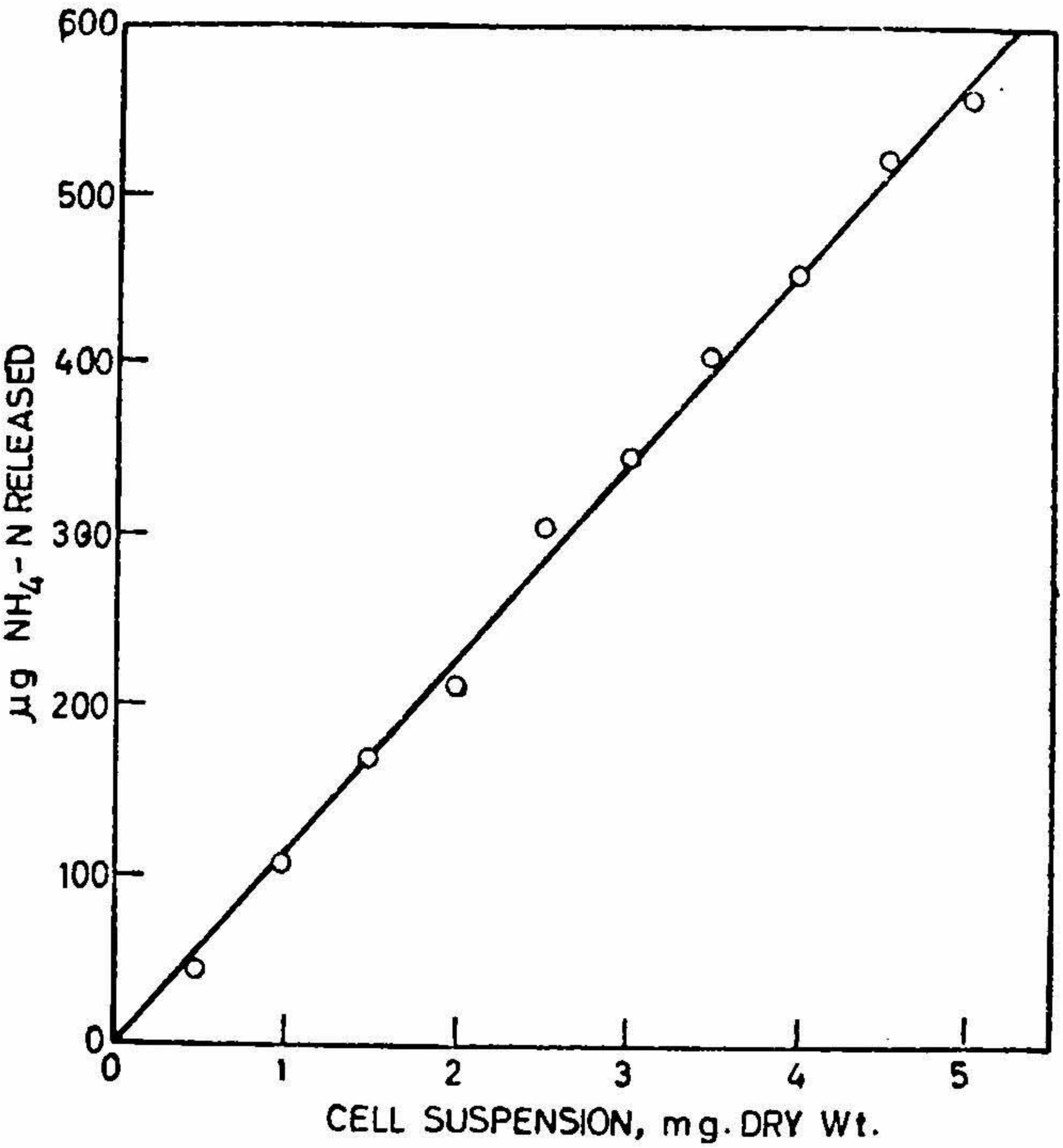

FIG. III

Urease activity of *Arthobacter*, soil strain No. 59 in relation to cell concentration

Reaction mixture: Cell suspension, 1.0 ml. (varying concentration of the resting cells); 3 °/₀ urea solution, 1.0 ml; 9 6 °/₀ phosphate buffer pH 7.0, 1 0 ml. Rest of the experimental conditions were same as Fig. I except for the period of incubation which lasted for one hour.

TAXONOMY AND DISCUSSION

The morphological and physiological attributes of the isolates studied are consistent with those described for the genus *Arthrobacter* and clearly the species belong to this genus. Utilization of citrate has been observed in this laboratory to be an important criterion in the differentiation of the *Arthrobacter* species, and accordingly whereas the soil ureolytic *Arthrobacter* species belong to the citrate non-utilizing group, the sewage species fall into those which utilize it. Both of them, however, possess the ability to utilize ammonium salts as source of nitrogen though the sewage species could not attack nitrates as its source of nitrogen. The soil strains also differ from their sewage counterparts by virtue of their ability to hydrolyse starch and decompose hippurate, benzoate, phenol and other aromatic compounds. Of considerable interest is the fact that the sewage strains are very well equipped with the enzymes necessary for the oxidation of glycine whether or not it was used as a growth substrate, whereas the soil strains can do so only when they are grown on glycine and glutamate. Thus the two groups are clearly distinguishable from each other and appear to be two different species.

The sewage strains may be considered to belong to the non-chromogenic *Arthrobacter globiformis* group. Since they failed to hydrolyze starch they have to be reckoned as strains of *A. simplex* though they differed from *A. simplex* in two important aspects, *viz.*, (i) inability to grow at 37°C, and (ii) ability to decompose urea. They also differed from Jensen's *A. ramosus* in their ability to reduce nitrate not to mention the fact that they were nonmotile in contradistinction to *A. ramosus* described as a flagellated species. Nevertheless, we are inclined to believe that the sewage strains are not dissimilar to *A. simplex* and would therefore prefer to lable them *A. simplex* var. *urealyticus*.

The soil strains resembled *A. citreus* in their pigment and inability to utilize citrate but differed in many other important aspects: (1) soil strains were nonmotile whereas *A. citreus* is described as motile; (2) *A. citreus* fails to hydrolyse starch whereas soil strains could do so; (iii) soil strains were ureolytic whereas *A. citreus* cannot hydrolyse urea; (iv) unlike *A. citreus* soil strains produced H_2S, (v) soil strains produced alkalinity and peptonization of milk whereas *A. citreus* fails to show any reaction in milk, and (vi) soil strains could utilize NH_4 salts and NO_3 as sources of nitrogen without added growth factors whereas *A. citreus* could not do so. The soil strains also differed from the other two ureolytic species *A. atrocyaneus* (Kuhn and Starr, 1960) and *A. crystallopoietes* (Ensign and Rittenberg 1963) either by their virtue of chromogenesis on *n.* agar or ability to utilize inorganic nitrogen compounds or to hydrolyse gelatin.

From all this it would appear that we are dealing with an entirely new species but we do not propose to create any new species as it may result in the

unnecessary additions to the existing number. The need to have a well considered scheme for the identification of species in the genus *Arthrobacter* has however been felt for a long time and one would be proposed by the authors elsewhere for the consideration of workers in the field.

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