INFLUENCE OF CARBON:NITROGEN RATIO ON MICROBIAL RESPIRATION BY PURE AND MIXED CULTURES IN SOIL AND SYNTHETIC MEDIA

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INFLUENCE OF CARBON:NITROGEN RATIO ON MICROBIAL RESPIRATION BY PURE AND MIXED CULTURES IN SOIL AND SYNTHETIC MEDIA

Carbon:nitrogen ratio as applied to soils or materials is the ratio of the content of carbon to nitrogen and is customarily designated as the C:N ratio. The C:N ratio of organic matter determines the rate and extent of its decomposition under given environmental conditions. Since this decomposition has a profound influence on soil fertility, directly and indirectly, it is desirable to know how qualitative changes as well as quantitative changes in the C:N ratio may affect activities of the microorganisms involved. Toward this end, soil respiration studies were conducted using different C:N ratios with glucose as a carbon source and ammonium sulfate and sodium nitrate as nitrogen sources. The soil studies were supplemented by experiments with pure cultures of common soil microorganisms in a synthetic medium.

REVIEW OF LITERATURE

Potter and Snyder (8, pp.27-29) found that additions of $(NH_4)_2SO_4$ and $NaNO_3$ to soil depressed the evolution of CO_2 to a level below that evolved from soil containing no additions. Further, they indicated that less CO_2 was produced from limed soil samples containing increased amounts of $(NH_4)_2SO_4$ and $NaNO_3$ than from samples that had only lime additives. They suggested that these salts were

responsible for the decreased CO_2 evolutions from the organic matter of the soil samples. Later Potter and Snyder (7, pp.89-90) reported similar depressions in other experiments and offered the possible but not probable explanation that it was caused by a toxicity of the $NaNO_3$. No explanation was given in regard to $(NH_4)_2SO_4$. A like depression was reported by Merkle (5, p.229) when $(NH_4)_2SO_4$ was added to soybean fodder. Millar, Smith and Brown (6, p.923) added plant materials of a relatively high nitrogen content to soil samples and noted a decrease in total CO_2 evolved compared to additives of a lower nitrogen content. They adduced that a greater amount of carbon is assimulated by microorganisms in the decomposition of plant materials of higher nitrogen content. The chemical composition of plant substances with reference to the form of the contained nitrogen, was considered by Hutchings and Martin (4, p.340) to have as great an influence on the rate of decomposition as the total amount of nitrogen present.

The metabolic activities of soil microorganisms in the decomposition of organic matter result in carbon dioxide as one of the major end products. It is produced in considerable quantity since carbon is the food element utilized in greatest proportion by heterotrophes and sooner or later appears as the dioxide. The value of carbon dioxide evolution as an index of numbers of microorganisms

and their activity, and of the decomposition of organic matter is well established. Russell and Appleyard (10, p.387) avouched the relationship of CO_2 evolution to bacterial numbers to the extent that "This occurs so frequently that it can't be accidental, and we are forced to conclude that the production of CO_2 is definitely connected with the rises and falls in bacterial numbers." Wilson and Wilson (14, p.20) in determining the quantities of CO_2 given off during the decomposition of timothy and clover hay discovered that these quantities were directly proportional to bacterial numbers. Millar, Smith and Brown (6, p.914) state that "practically all of the CO_2 evolved from soils which are not supporting a crop is derived from this source," i.e. from microbial activity. Sievers and Holtz (11, p.20) aver that the rate of decomposition of organic matter may be ascertained accurately by measuring the amount of CO_2 liberated. Starkey (12, pp.293-294) says that the CO_2 released from soils gives a "reliable" indication of the rate of decomposition of organic matter but emphasizes that it is not absolute because some of the CO_2 released is reassimilated by autotrophes and a great deal is assimilated for production of cellular material.

EXPERIMENTAL METHODS

The methods given here will be described only as they are applied to the work as a whole or a major part. If and when changes, elaborations, or refinements are made, they will be cited in detail. The respiration apparatus used was quite similar to that employed by Potter and Snyder (8, pp.3-4) and (7, pp.80-81), Millar, Smith and Brown (6, p.915) and Bollen (10, p.359). The pump and respiration apparatus are pictured in Figure 1. To insure carbon-dioxide-free air, the air, before it reached the respiration apparatus proper, was passed through 35 per cent sodium hydroxide. This removed the greatest portion of the CO_2. Next it was bubbled through a saturated solution of barium hydroxide, which having greater absorption powers for the gas, rendered the air essentially CO_2-free. From there the air was passed to the individual respiration bottles. Here the air was supplied at a relatively constant pressure in an amount required to expell the air through a column of 0.1 N NaOH four inches in height.

Millar, Smith and Brown (6, p.922) considered that aeration by passing air over the soil very probably stimulated the microbiological activity of the soil by the resultant increased aeration of the soil. Potter and Snyder (8, p.35) concluded that by using this method, results produced in the laboratory approximated field

Figure 1. Diagram of Respiration Apparatus

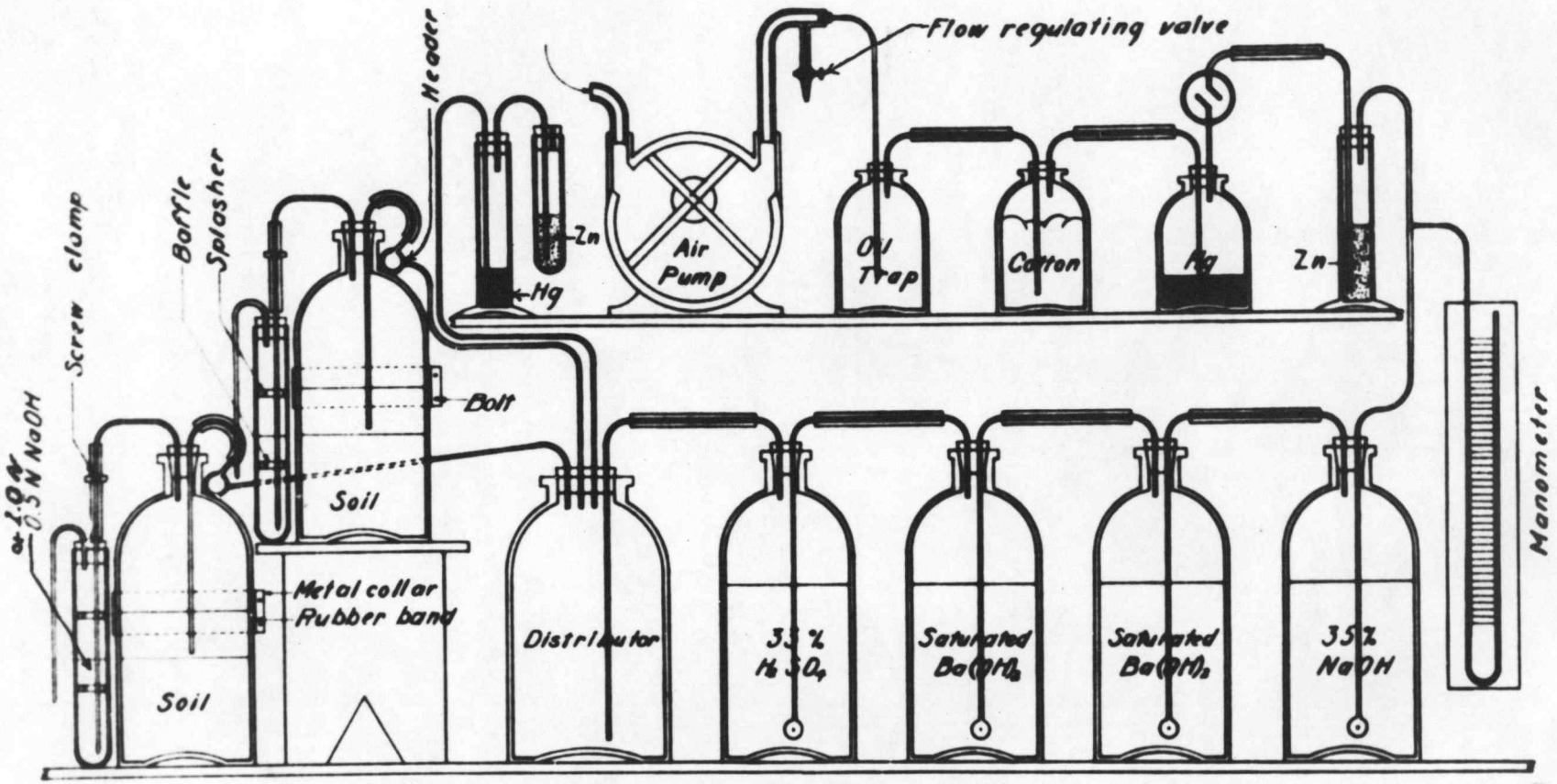

Table 1. Analysis of Chehalis Silty Clay Loam Soil*

Chemical Analysis:	
Moisture, per cent	13.4
Moisture capacity, per cent	57.2
pH	5.7
Lime requirement (Truog)	1 ton per acre
Nitrogen:	
Ammonium	26 ppm
Nitrite	trace
Nitrate	38 ppm
Kjeldahl, per cent	0.130
Total carbon, per cent	1.680
C/N ration	12.9/1
Sulfur as sulfate (water-soluble)	14 ppm
Phosphorus as phosphate (water-soluble)	2.0 ppm
Microorganisms:	
Molds, per gram	24,100
Mucors, per cent	8
Penicillia, per cent	88
Aspergilli, per cent	2
Bacteria, per gram	5,565,000
Streptomyces, per cent	45
Azotobacter	present

*Data expressed on water-free basis.

conditions more closely than by passing air through the soil. In addition, they indicated that if there occurred a slight variation in the volume of air that passed over the soil, the amount of carbon dioxide liberated was not altered to any perceptible degree. Bollen (2, p.359) points out that by using this method approximately normal atmospheric pressure can be maintained over the soil.

Chehalis silty clay loam soil was used in the series of respiration studies involving soil for the following reasons: It was readily available in quantity, its chemical and microbial analysis was known (Table 1), it was a typical Willamette Valley soil, and, having been obtained from experimental mulch plots on the Lewis-Brown Horticultural Farm, had a known cultural history.

Two hundred and fifty grams of previously screened (10-mesh) soil were placed in common, glass milk bottles of pint size. The weight of the soil was always calculated on the water-free basis, the moisture having been determined by drying a sample at 105° C. to a constant weight.

The optimum moisture content was placed at 60 per cent of saturation which was arrived at as follows: A tared gooch crucible was filled with soil and placed in a container of water so that the water came just to the lip of the crucible; in this way the soil was moistened thoroughly but gently from below. After it stood for twenty-four hours in a saturated atmosphere in a bell jar,

the bottom of the gooch was wiped dry. After water failed to appear within two minutes the weight was determined. The saturated soil was then dried at 105° C. to a constant weight. The percent of water retained in the soil was considered the saturation capacity, and was calculated on the water-free basis.

Soil moisture equivalent to 60 per cent saturation is midway in the optimum range cited by Waksman and Starkey (13, p.61) and is generally considered to be favorable for microbial action. An appreciably higher percentage would lead to a less desirable soil structure or induce puddling, while less moisture would tend to reduce microbial activity. The optimum moisture for soil respiration was reported by Bollen (2, p.367) to be approximately 75 per cent of saturation capacity.

Before the soil was placed in the milk bottles, the various additions for the different C:N ratios were thoroughly mixed into the soil. Special care was taken to see that even the smallest lumps were broken up. Similar procedure was employed with the sand used in the pure culture studies.

To encourage a predominance of molds in some of the respiration studies the soil was acidified to pH 4 with sulfuric acid. The required amount of acid was found by preparing a 1:5 suspension of the soil on the water-free basis in tap water, shaking this for one-half hour, and

then titrating to the desired pH with the Fisher Titrimeter.

Organisms used in the pure culture studies were the following: Bacillus subtilis, Aerobacter aerogenes 199M, Penicillium notatum NRRL 1249-B21, and Streptomyces sp. SN10. These were chosen because they were considered relatively representative microorganisms normally found in the soil, and they were readily available. These were grown on slopes of peptone-glucose-acid agar for the P. notatum, and on nutrient agar for B. subtilis, A. aerogenes, and Streptomyces sp. The cultures of P. notatum and Streptomyces sp. were incubated at room temperature (approximately 25° C.) for seven days, while those of the bacteria were incubated at 37° C. for twenty-four hours. At the end of the incubation period a heavy inoculum was prepared from the growth by suspending in normal saline.

To eliminate the influence of the soil organic constituents containing carbon and nitrogen as variable factors, a synthetic liquid medium known to support abundant growth of the test organisms was used in sand cultures. The medium had the following composition:

10.0000 grams glucose
0.5000 grams K_2HPO_4
0.2000 grams $MgSO_4.7H_2O$

*2.8299 grams $(NH_4)_2SO_4$ or 3.6410 grams $NaNO_3$ C:N = 10:1
1.4150 grams $(NH_4)_2SO_4$ or 1.8205 grams $NaNO_3$ C:N = 20:1
0.5660 grams $(NH_4)_2SO_4$ or 0.7282 grams $NaNO_3$ C:N = 50:1
0.2830 grams $(NH_4)_2SO_4$ or 0.3641 grams $NaNO_3$ C:N =100:1

*Nitrogen sources and amounts.

The constituents were dissolved in tap water and made up to a thousand milliliters. Tap water was used in preference to distilled water because it was believed that any trace elements required would thus be supplied. The medium was autoclaved in eight ounce screw-cap bottles, one hundred milliliters per bottle, at 121° C. for fifteen minutes. The bottles were then incubated at 37° C. for twenty-four hours as a test of sterility. To prepare inocula for the sand cultures a bottle of the synthetic medium was inoculated with five milliliters of a specific suspension of microorganism to be studied.

As with the soil respirations, pint milk bottles were used as containers. Three hundred grams of sand were added to each. The sand was used to provide vastly greater surfaces for growth and to accelerate the exchange of oxygen. The efficaciousness of the use of sand in this way was demonstrated by Rahn (9, pp.208-209). After inserting rubber stoppers bearing inlet and outlet tubes closed with screw clamps to prevent contamination, the bottles were autoclaved at 121° C. for thirty minutes. The previously inoculated medium was added aseptically in the amount required to give the optimum moisture content, 60 per cent saturation, determined as before with the soil.

At intervals ranging from twenty-four hours at the beginning to ten to forty days at the end, depending upon the length of the experiment, the tubes of 0.1 N sodium

hydroxide which absorbed the carbon dioxide from the respirations were replaced with tubes of fresh solution and titrated with sulfuric acid to determine the carbon dioxide evolved. The titrations were performed with a Fisher Titrimeter according to these reactions:

$$NaOH + CO_2 \longrightarrow Na_2CO_3 + 2H_2O$$

$$2NaOH + H_2SO_4 \xrightarrow{pH\ 8.35} Na_2SO_4 + 2H_2O$$

$$2Na_2CO_3 + H_2SO_4 \xrightarrow{pH\ 8.35} 2NaHCO_3 + Na_2SO_4$$

$$2NaHCO_3 + N/12\ H_2SO_4 \xrightarrow{pH\ 4.75} Na_2SO_4 + 2CO_2 + 2H_2O$$

Each milliliter of N/12 H_2SO_4 is equivalent to one milligram of carbon as carbon dioxide.

In this whole series of respiration studies all factors, except C:N ratio, that might influence microbial respiration were maintained as constant as possible within each study by using the same soil, screened and well-mixed; the organic matter content, the physical structure, the available nitrogen of the soil itself, the pH, and the microbial population were all kept relatively uniform. Temperature and the moisture were controlled as closely as possible. Aeration was constant by virtue of the same physical structure of the soil and the regulation of the air-volume passed over the soil. These same factors that were pertinent were similarly controlled in the pure cultures.

EXPERIMENTAL RESULTS

Respiration Studies with Soils

The first respiration study of the soil was set up with four treatments in duplicate as follows: (1) soil only, without additions; (2) soil plus 1,250 mg. of glucose to supply 500 mg. of carbon, and (3) and (4) soil plus glucose as in (2) and $(NH_4)_2SO_4$ to provide C:N ratio of 20:1 and 50:1 respectively. For the C:N ratio of 20:1 the ammonium sulfate added was equivalent to 100 ppm of N and for the ratio of 50:1 was equivalent to 40 ppm of N. This respiration was carried out for a period of twenty-nine days and the amount of carbon dioxide evolved was determined six times--after twenty-four hours, on the second day, the fifth, the tenth, the twenty-second, and on the twenty-ninth day. These values are plotted as shown in Figure 2.

A very definite increase in CO_2 evolution was produced by the addition of glucose (2) and glucose plus ammonium sulfate (3) and (4). Further, this stimulation was evident and pronounced throughout the entire period. Also, throughout the entire period of twenty-nine days, (3) leads in total amount of CO_2 evolved, followed by (2) and (4) in order. This would seem to indicate that the addition of material having a C:N ratio of 20:1 is more conducive to the activity of soil microorganisms than the addition of

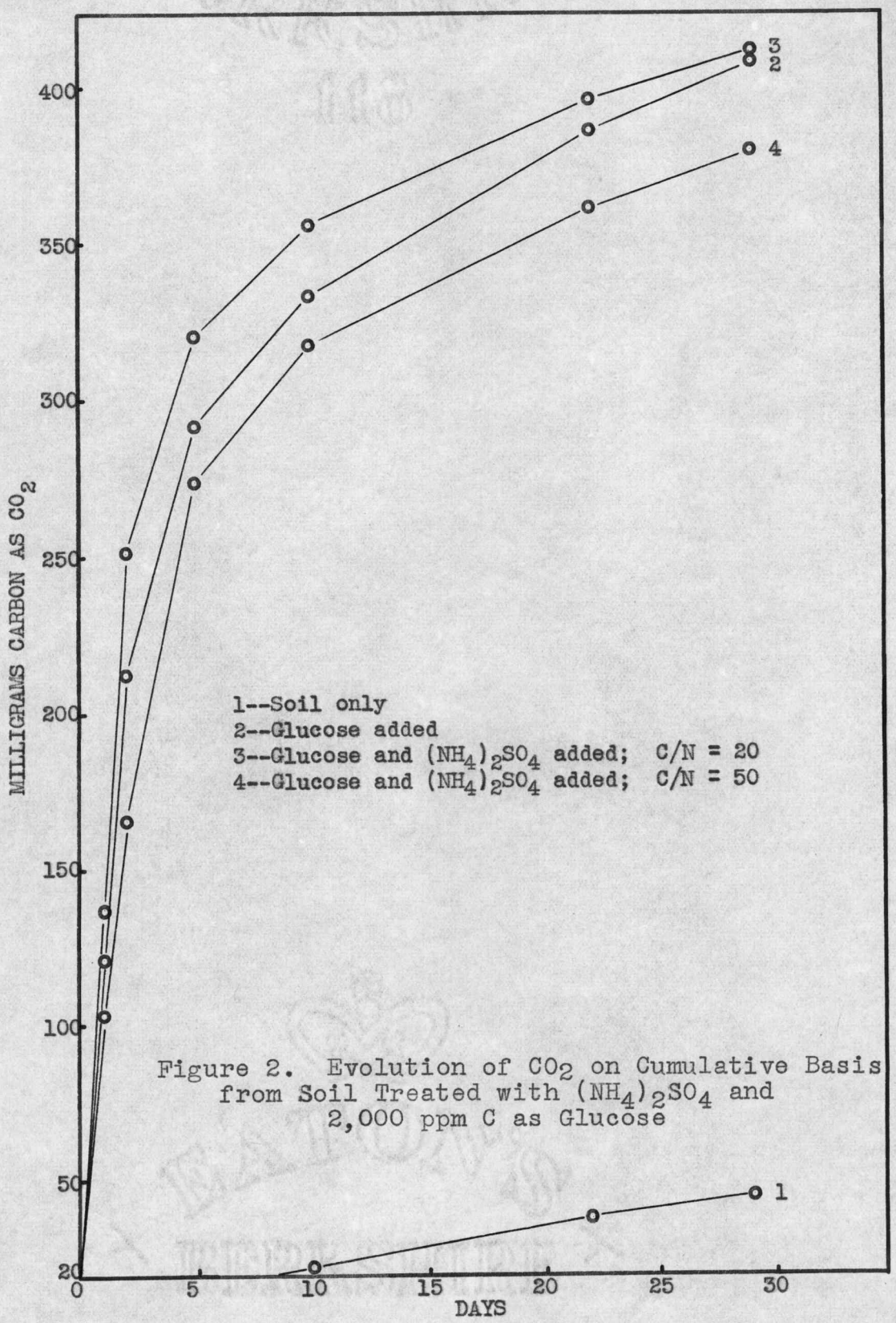

Figure 2. Evolution of CO_2 on Cumulative Basis from Soil Treated with $(NH_4)_2SO_4$ and 2,000 ppm C as Glucose

Table 2. Evolution of CO_2 from Soil Treated with $(NH_4)_2SO_4$ and 2,000 ppm C as Glucose

Treatment				Mg. C as CO_2 from 250 g. soil at intervals indicated												
				Time (days)												
		C	N	0 - 1		1 - 2		2 - 5		5 - 10		10 - 22		22 - 29		
No.	Additions	(ppm)	(ppm)	each	ave	each	ave	each	ave	each	ave	each	ave	each	ave	Total
(1)	Control	0	0	3.5		4.1		4.9		9.7		16.8		7.8		
				3.5		3.9		5.2		8.4		16.9		7.9		
					3.5		4.0		5.1		9.1		16.9		7.9	46.3
(2)	Glucose	2000	0	122.8		89.9		73.9		40.0		51.2		22.0		
				119.9		93.6		84.8		43.4		53.7		21.1		
					121.3		91.8		78.4		41.7		52.5		21.6	408.2
(3)	Glucose + $(NH_4)_2SO_4$ C:N = 20:1	2000	100	128.9		115.2		82.4		35.2		40.9		15.2		
				147.7		112.4		59.2		33.4		38.2		15.6		
					137.3		113.8		70.8		34.3		39.6		15.4	411.3
(4)	Glucose + $(NH_4)_2SO_4$ C:N = 50:1	2000	40	85.3		61.6		125.8		50.0		46.5		18.4		
				123.6		62.9		90.0		35.2		42.9		17.5		
					104.2		62.3		107.9		42.6		44.7		17.5	379.8

glucose alone or with a C:N ratio of 50:1. It should be noted, however, that (2) very closely approaches (3), especially at twenty-nine days, and (4) is also gaining in total output. This is better demonstrated in Table 2. Here it can be seen that in the first two days (3) leads, but after that the output of CO_2 for each period is greater in (4), followed by (2), with a reversal in the last nineteen days. Thus it would seem that the wider the C:N ratio or the lower the amount of ammonium sulfate added, the greater the amount of CO_2 produced. Note also in Table 2 that when the respiration was terminated after twenty-nine days, the level of CO_2 production has not dropped nor returned to that of the control.

In Table 2 it is shown that generally there was good agreement between duplicates. In two cases where there was a pronounced difference after the first twenty-four hours, the discrepancy was quite well compensated at the end of five days. This same compensation is repeated in later respirations to a striking extent.

A second study was conducted with the soil to learn if the previous results could be reproduced and to magnify, if possible, these differences. With these objectives in mind, five different treatments and a control were used: (1) soil only, without additions, (2) soil plus 2,500 mg. of glucose, equivalent to 1,000 mg. carbon, double the quantity added in the earlier experiment, and (3), (4), (5), (6) soil

plus glucose as in (2) with ammonium sulfate in amounts to give C:N ratios of 10:1, 20:1, 50:1 and 100:1 respectively. These ratios correspond to nitrogen additions of 400 ppm, 200 ppm, 80 ppm, and 40 ppm. Respiration was carried out for eighty-three days, a much longer period of time than in the first experiment. Carbon dioxide evolved was determined after two, seven, fourteen, twenty-eight, forty-four, and eighty-three days. Figure 3 shows these values plotted cumulatively.

Again a very explicit stimulation of CO_2 production over the control was demonstrated by all of the treatments (Figure 3); moreover, this was maintained throughout the entire respiration period. The greatest amount of carbon dioxide was produced with the C:N ratio of 50:1. Among the others, though none held a constant relative position, not one lost a superior position after once gaining it. It would appear then that a C:N ratio addition of 50:1 was the most satisfactory for growth or activity of the microbial population. The results of this study at first appear to be contradictory to the first one, but if the quantities of carbon and nitrogen added are compared, a correlation will be exhibited:

Soil Respiration I		Soil Respiration II
0.5 g C	C:N Ratios	1.0 g C
	10:1	400 ppm
100 ppm N	20:1	200 ppm
40 ppm N	50:1	80 ppm
	100:1	40 ppm

The amount of nitrogen added in I for a C:N ratio of 20:1 is nearly the same as in II for a ratio of 50:1, and 50:1 of I is exactly the same as 100:1 of II. Thus it appears that perhaps rather than the C:N ratio as such, it is the quantity of ammonium sulfate added that produced the influence on the respiration of the soil microorganisms. Table 3 indicates that there might be a logical relationship between the C:N ratio or total ammonium sulfate added and respiration. After the first few days, even in this case, which seems to be almost a "shake-down" period, there is a constant inverse ratio between C:N values or total ammonium sulfate added and the respiration intensity.

There was fair conformation between duplicates in Table 3 as in the earlier respiration, and there was some compensation of differences between duplicates as time progressed.

Respiration Study with Soil-Sand Mixture

In order to reduce the influence of the soil constituents, particularly carbon and nitrogen, and perhaps find what this influence is, two hundred and fifty grams on the water-free basis of a soil-sand mixture were used. This mixture was composed of ten parts of soil and ninety parts of sand. In addition, different sources of nitrogen were used: (1) the control, sand and soil only, (2) soil-sand mixture plus 500 mg. of carbon as glucose, (3), (4),

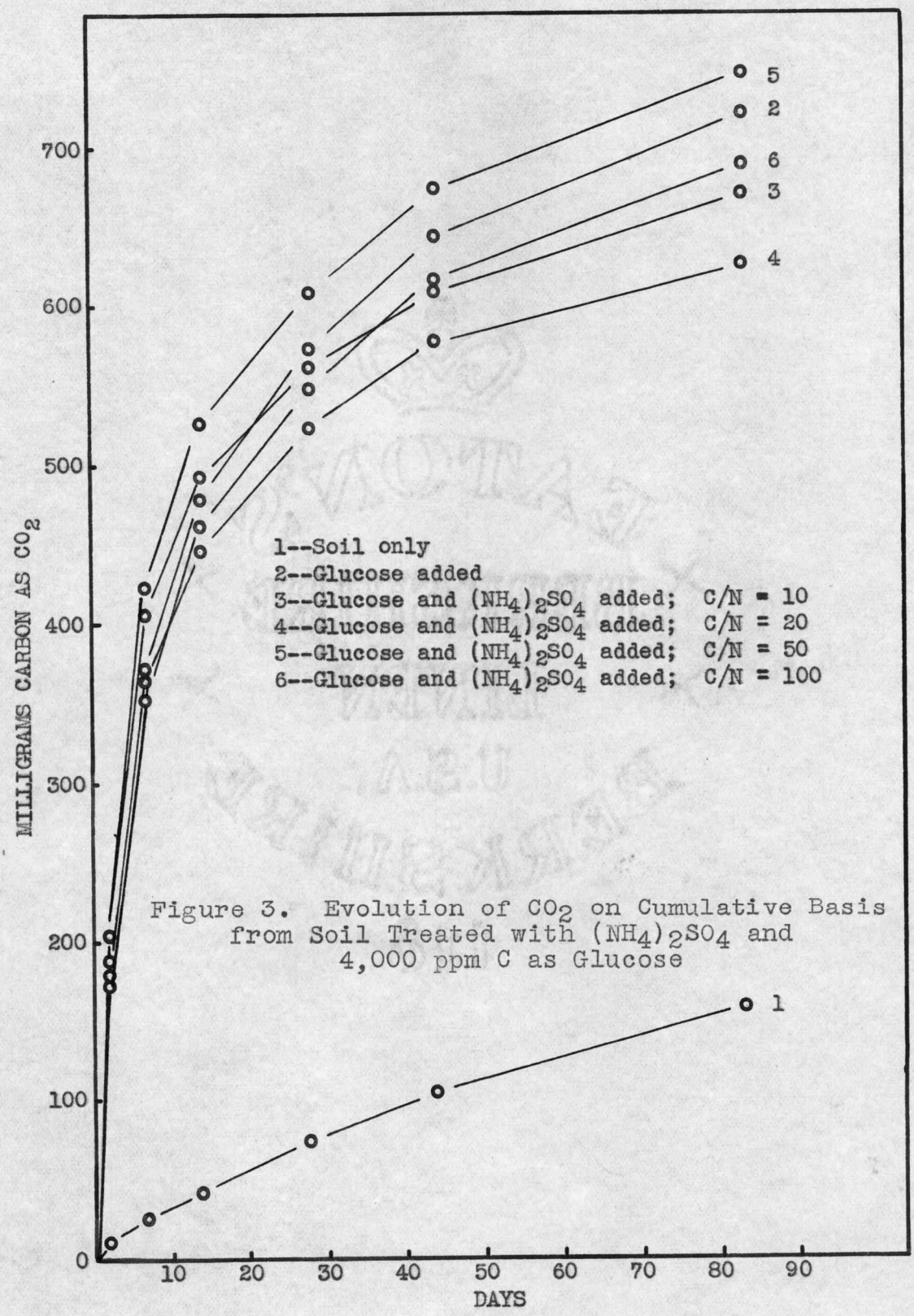

Figure 3. Evolution of CO_2 on Cumulative Basis from Soil Treated with $(NH_4)_2SO_4$ and 4,000 ppm C as Glucose

Table 3. Evolution of CO_2 from Soil Treated with $(NH_4)_2SO_4$ and 4,000 ppm C as Glucose

Treatment				Mg. C as CO_2 from 250 g. soil at intervals indicated												
				Time (days)												
		C	N	0 - 2		2 - 7		7 - 14		14 - 28		28 - 44		44 - 83		
No.	Additions	(ppm)	(ppm)	each	ave	each	ave	each	ave	each	ave	each	ave	each	ave	Total
(1)	Control	0	0	8.4		13.6		15.9		28.3		28.2		52.4		
				11.9		16.8		18.9		32.7		33.4		57.0		
					10.2		15.2		17.4		30.5		30.3		54.7	158.7
(2)	Glucose	4000	0	178.1		187.5		118.6		93.2		76.0		79.3		
				199.2		178.7		94.8		93.0		68.8		71.3		
					188.6		183.1		106.7		93.1		72.4		75.3	719.3
(3)	Glucose + $(NH_4)_2SO_4$ C:N = 10:1	4000	400	190.1		203.9		85.4		72.7		46.2		61.0		
				219.1		196.7		83.6		72.0		44.6		60.5		
					204.6		200.3		84.5		72.4		45.4		60.9	668.0
(4)	Glucose + $(NH_4)_2SO_4$ C:N = 20:1	4000	200	191.0		168.7		75.8		73.0		52.2		71.1		
				151.7		219.5		84.2		83.1		54.6		73.4		
					171.4		194.1		80.0		75.1		53.4		72.3	649.1
(5)	Glucose + $(NH_4)_2SO_4$ C:N = 50:1	4000	80	219.4		210.6		104.5		82.6		64.4		72.2		
				187.7		229.1		97.3		84.0		62.4		72.6		
					203.5		219.9		100.9		83.3		63.4		72.4	743.7
(6)	Glucose + $(NH_4)_2SO_4$ C:N = 100:1	4000	40	189.7		166.5		99.2		85.3		68.5		69.5		
				169.7		181.9		111.9		88.4		70.0		70.8		
					179.7		174.2		105.6		86.9		69.3		70.2	685.6

and (5) soil-sand mixture plus carbon as in (2) with ammonium sulfate, sodium nitrate, and ammonium nitrate, respectively to produce C:N ratio additions of 20:1, and (6) the soil-sand mixture with ammonium nitrate only.

Figure 4 and Table 4 present results in carbon as carbon dioxide determined at the end of each respiration period. Number (1), the control, shows that this mixture is very low in either available carbon or nitrogen or both; however, results for (2) with an addition of only glucose makes it apparent that the deficiency of nitrogen is not great and would seem to indicate that the shortage consists mainly of available carbon. This carbon deficit is further borne out in (6) where the addition of ammonium nitrate results in only slight difference from the control in period-to-period production of carbon dioxide and a very small total difference.

Consideration of the relative amounts of CO_2 produced for each period by the individual respirations shows that with available nitrogen the glucose is decomposed most rapidly during the first five days. Comparison of (2) with (3), (4) and (5) for the first five days shows that, while some soil nitrogen was present, it apparently was much less available during this period than were the added nitrogenous compounds; after five days, however, the nitrogen still available from the soil continued its influence

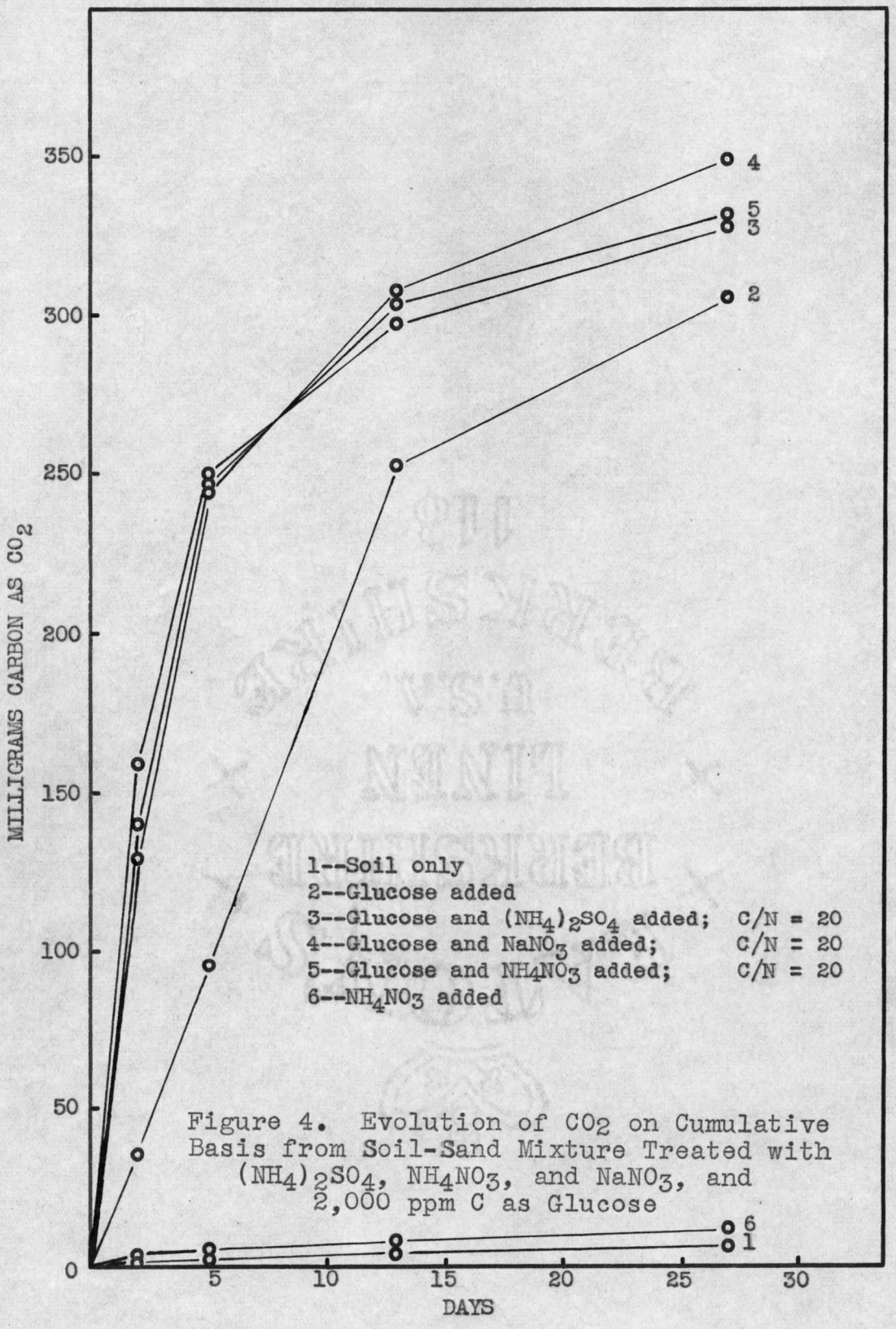

Figure 4. Evolution of CO_2 on Cumulative Basis from Soil-Sand Mixture Treated with $(NH_4)_2SO_4$, NH_4NO_3, and $NaNO_3$, and 2,000 ppm C as Glucose

Table 4. Evolution of CO_2 from Soil-Sand Mixture Treated with $(NH_4)_2SO_4$, NH_4NO_3, and $NaNO_3$, and 2,000 ppm C as Glucose

Treatment				Mg. C as CO_2 from 250 g. soil-sand mixture on cumulative basis and at intervals indicated						
				Time (days)						
No.	Additions	C (ppm)	N (ppm)	0-2	2 - 5		5 - 13		13 - 27	
(1)	Control	0	0	0.8	0.9	1.7	2.4	4.1	3.4	7.5
(2)	Glucose	2000	0	36.8	59.9	96.7	156.9	253.6	53.0	306.6
(3)	Glucose + $(NH_4)_2SO_4$ C:N = 20:1	2000	100	160.3	90.1	250.4	47.2	297.5	29.9	327.4
(4)	Glucose + $NaNO_3$ C:N = 20:1	2000	100	129.7	114.4	244.1	64.7	308.7	40.5	349.2
(5)	Glucose + NH_4NO_3 C:N = 20:1	2000	100	141.3	105.8	247.1	57.8	304.9	26.8	331.6
(6)	NH_4NO_3	0	100	2.6	3.0	5.6	3.2	8.8	4.2	13.0

while the added nitrogen was losing its effectiveness. Further comparison indicates that early in the same first two days, CO_2 evolution was most stimulated by ammonium sulfate followed by ammonium nitrate and sodium nitrate in order; after this, however, the more of the nitrate-nitrogen present the greater the amount of carbon dioxide released. This was further borne out in later pure culture studies with $NaNO_3$ as nitrogen source.

Respiration Studies with Acidified Soil

In order to encourage the growth of a primarily fungus population, the pH was adjusted to a lower level with sulfuric acid. To determine how much acid to add, a one to five dilution of the soil was titrated with 0.88 N sulfuric acid until pH 4.0 was reached, a value considered favorable for the growth of molds to the general exclusion of bacteria and streptomyces. The proper quantity of acid was added to enough water so the solution added to the soil would bring its moisture content up to 60 per cent of saturation.

The treatments included soil only, soil plus only glucose equivalent to 2000 ppm C, and soil with glucose plus nitrogen as ammonium sulfate to give C:N ratios of 10:1, 20:1, 50:1, and 100:1. Respiration was continued for twenty-eight days.

The data are presented in Figure 5 and Table 5. All treatments resulted in an increased evolution of CO_2 over the control. The C:N ratio of 10:1 starts off with the highest CO_2 production and maintains this position throughout the entire respiration. It was slightly lower with the 50:1 ratio, which also keeps its relative position. Next in order are glucose only and the 20:1 ratio, both producing just about equally, with 100:1 producing the least of all consistently. This would indicate that a C:N ratio of 10:1 is most favorable for carbon dioxide production in the acidified soil, and is consistent with the higher nitrogen requirement for mold development.

The smaller additions of ammonium sulfate are stimulative during the first few days only, the apparent depression at the C:N ratio of 100:1 being almost within experimental error. However, a brief stimulative effect could have occurred in the first twenty-four to forty-eight hours; this could then be followed by the typical decrease in CO_2 evolution. This study was repeated using enough sulfuric acid to produce a pH of 3.8, and the quantities of glucose and ammonium sulfate were doubled. The run was continued for eighty-three days.

The results in Figure 6 and Table 6 show a disparity between the control and the five treatments. Among these, No. 5 was constant for the duration of the respiration in the maintenance of its superior position; it was followed,

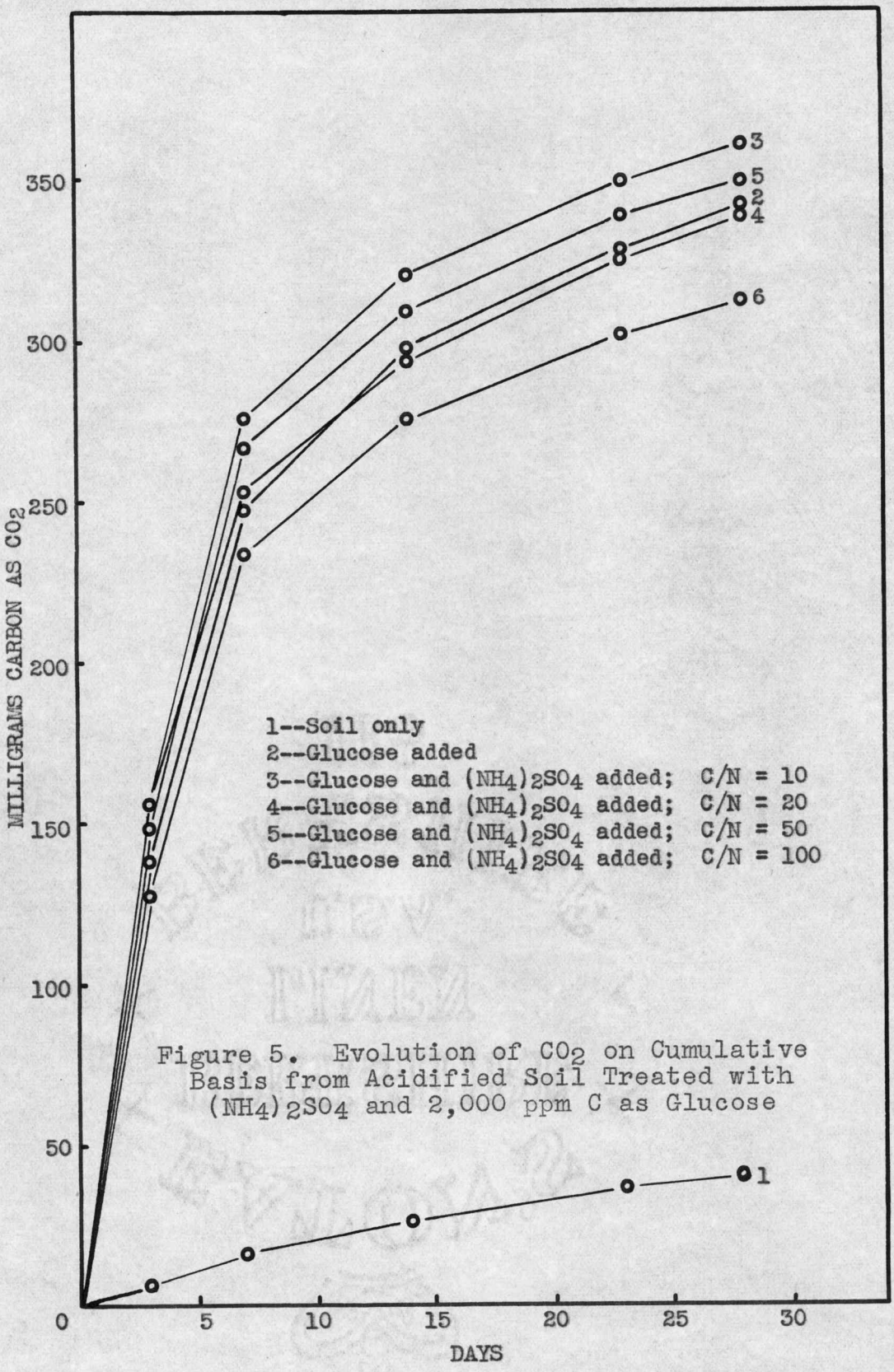

Figure 5. Evolution of CO_2 on Cumulative Basis from Acidified Soil Treated with $(NH_4)_2SO_4$ and 2,000 ppm C as Glucose

Table 5. Evolution of CO_2 from Acidified Soil Treated with $(NH_4)_2SO_4$ and 2,000 ppm C as Glucose

Treatment				Mg. C as CO_2 from 250 g. acidified soil at intervals indicated					
				Time (days)					
No.	Additions	C (ppm)	N (ppm)	0-3	3-7	7-14	14-23	23-28	Total
(1)	Control	0	0	6.8	9.5	8.9	9.9	4.0	39.1
(2)	Glucose	2000	0	138.6	108.4	49.3	32.3	11.6	340.1
(3)	Glucose + $(NH_4)_2SO_4$ C:N = 10:1	2000	200	156.6	119.1	44.6	29.1	11.2	360.7
(4)	Glucose + $(NH_4)_2SO_4$ C:N = 20:1	2000	100	155.0	98.5	40.1	30.5	13.2	337.7
(5)	Glucose + $(NH_4)_2SO_4$ C:N = 50:1	2000	40	149.4	116.6	43.2	29.2	10.2	348.5
(6)	Glucose + $(NH_4)_2SO_4$ C:N = 100:1	2000	20	128.4	105.4	41.2	26.1	10.9	312.1

however, relatively closely by No. 2. The remaining three were far below in the total production of carbon dioxide and generally much lower for each period.

These results would seem to indicate in this case, where at the close of the experiment the pH was found to be 5.3 to 5.5, that a C:N ratio of 50:1 was most favorable for microbial activity as indicated by CO_2 evolution. Less CO_2 was obtained with glucose alone, and with the remaining three C:N ratios the curves were closely grouped rather far below. This seems highly irregular and contradictory when it is considered that all factors of influence are relatively equal except for the different C:N ratios prevailing in the soil and/or the graduated quantities of ammonium sulfate introduced. Table 6 shows that there was fair agreement between duplicate determinations, some differences occurring in the first two days being well compensated by the seventh and not later than the fourteenth day.

A third study was set up with more carefully acidified soil in the hope that more pronounced results would be found. A different approach was used to reveal how much sulfuric acid should be used to achieve and maintain a soil reaction of approximately pH 4.0. It was found that 0.6 ml. of 10.0 N sulfuric acid produced a pH of 1.50 in a 1:5 dilution of this soil or 7.5 ml. for 250 grams of soil on water-free basis. From this, graduated quantities of the acid were added to five 250-gram portions of soil only on the

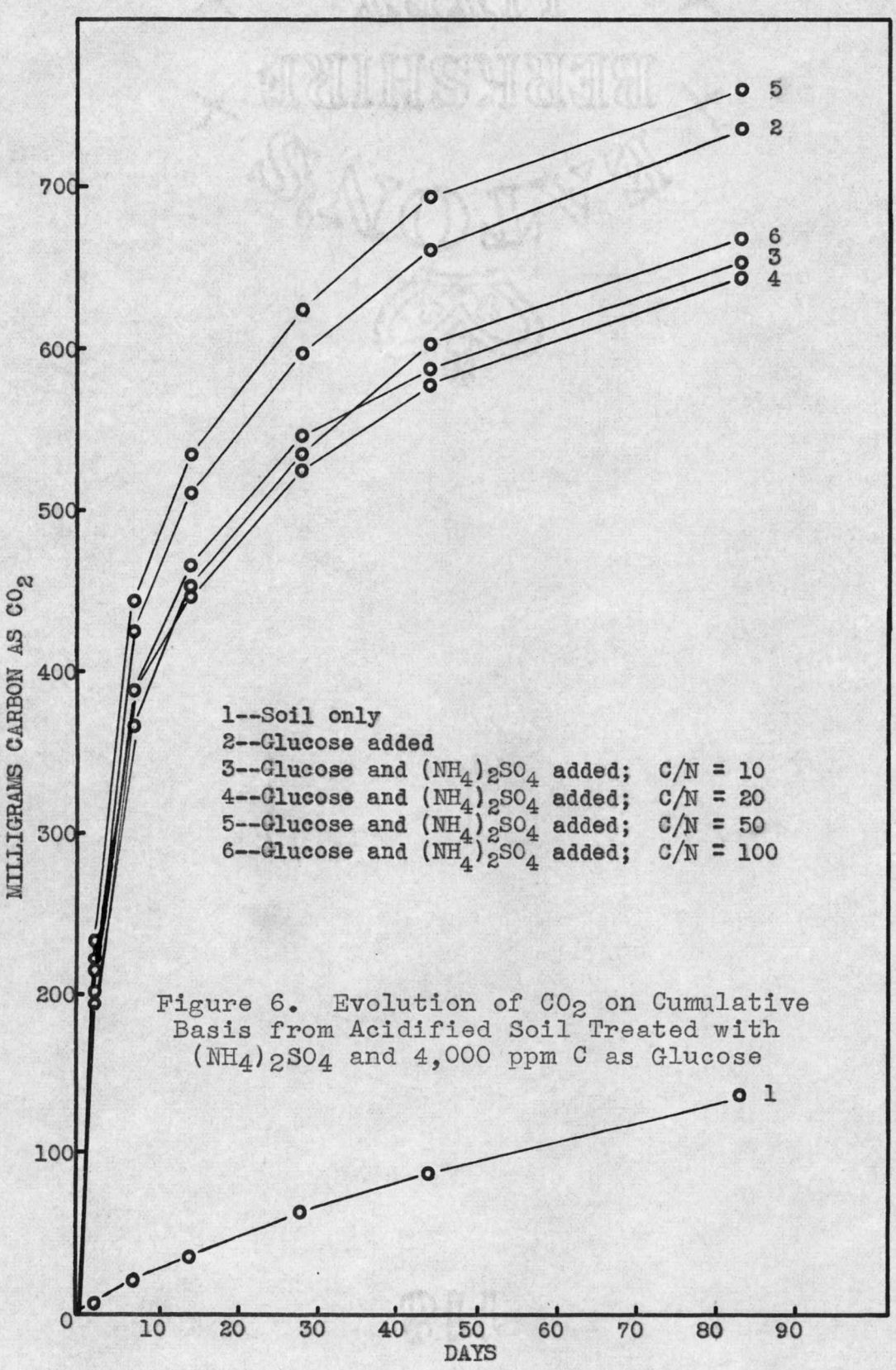

Figure 6. Evolution of CO_2 on Cumulative Basis from Acidified Soil Treated with $(NH_4)_2SO_4$ and 4,000 ppm C as Glucose

Table 6. Evolution of CO_2 from Acidified Soil Treated with $(NH_4)_2SO_4$ and 4,000 ppm C as Glucose

Treatment				Mg. C as CO_2 from 250 g. acidified soil at intervals indic.												
				Time (days)												
		C	N	0 - 2		2 - 7		7 - 14		14 - 28		28 - 44		44 - 83		
No.	Additions	(ppm)	(ppm)	each	ave	each	ave	each	ave	each	ave	each	ave	each	ave	Total
(1)	Control	0	0	7.1		13.2		14.7		24.6		23.5		45.7		
				7.4		15.2		16.7		27.4		26.2		49.3		
					7.2		21.4		37.1		63.1		88.0			135.5
(2)	Glucose	4000	0	200.8		215.5		88.8		89.1		64.4		69.4		
				245.2		183.5		86.7		87.7		63.6		73.0		
					223.0		422.5		510.2		598.6		662.6			733.9
(3)	Glucose + $(NH_4)_2SO_4$ C:N = 10:1	4000	400	193.5		190.6		81.1		78.6		42.7		62.7		
				208.6		183.9		77.5		75.1		46.3		62.4		
					201.0		388.3		467.6		544.5		588.9			651.5
(4)	Glucose + $(NH_4)_2SO_4$ C:N = 20:1	4000	200	175.7		192.7		81.8		79.6		52.4		65.5		
				210.1		194.3		40.4		72.6		55.0		66.4		
					192.9		386.4		447.5		523.6		577.2			643.2
(5)	Glucose + $(NH_4)_2SO_4$ C:N = 50:1	4000	80	*		*		*		*		*		*		
				232.9		209.5		91.1		90.5		68.1		68.6		
					232.9		442.41		533.6		624.1		692.1			760.7
(6)	Glucose + $(NH_4)_2SO_4$ C:N = 100:1	4000	40	218.5		151.3		85.0		84.7		65.5		68.7		
				209.3		158.4		82.4		80.7		66.4		66.7		
					213.8		368.7		452.5		535.2		601.1			668.8

*Leak

water-free basis. After three days aeration the pH was determined on 1:5 dilutions of each soil. The values found were as follows:

7 ml. 10.0 N H_2SO_4	pH 2.60
6 ml. 10.0 N H_2SO_4	pH 2.80
5 ml. 10.0 N H_2SO_4	pH 2.90
4 ml. 10.0 N H_2SO_4	pH 3.20
3 ml. 10.0 N H_2SO_4	pH 3.55

From this it was deduced that 2.50 ml. should yield a reaction of approximately pH 3.7. The previous experiment was repeated using this amount of acid for each 250-gram portion of soil.

From the results in Figure 7, plotted from average values of duplicates, and Table 7 it is seen again that all of the respirations greatly exceed the control, which was a typical result. Glucose only gave the greatest CO_2 production from the second day, gaining over other treatments up to the close of the experiment. The agreement between duplicates in some cases left much to be desired. Considering the results as a whole though, the final cumulative values especially indicate significant differences.

The average amounts of carbon dioxide evolved for each period (Table 7) show that the higher the C:N ratio and/or the lower the amount of ammonium sulfate added, the greater the rate of respiration.

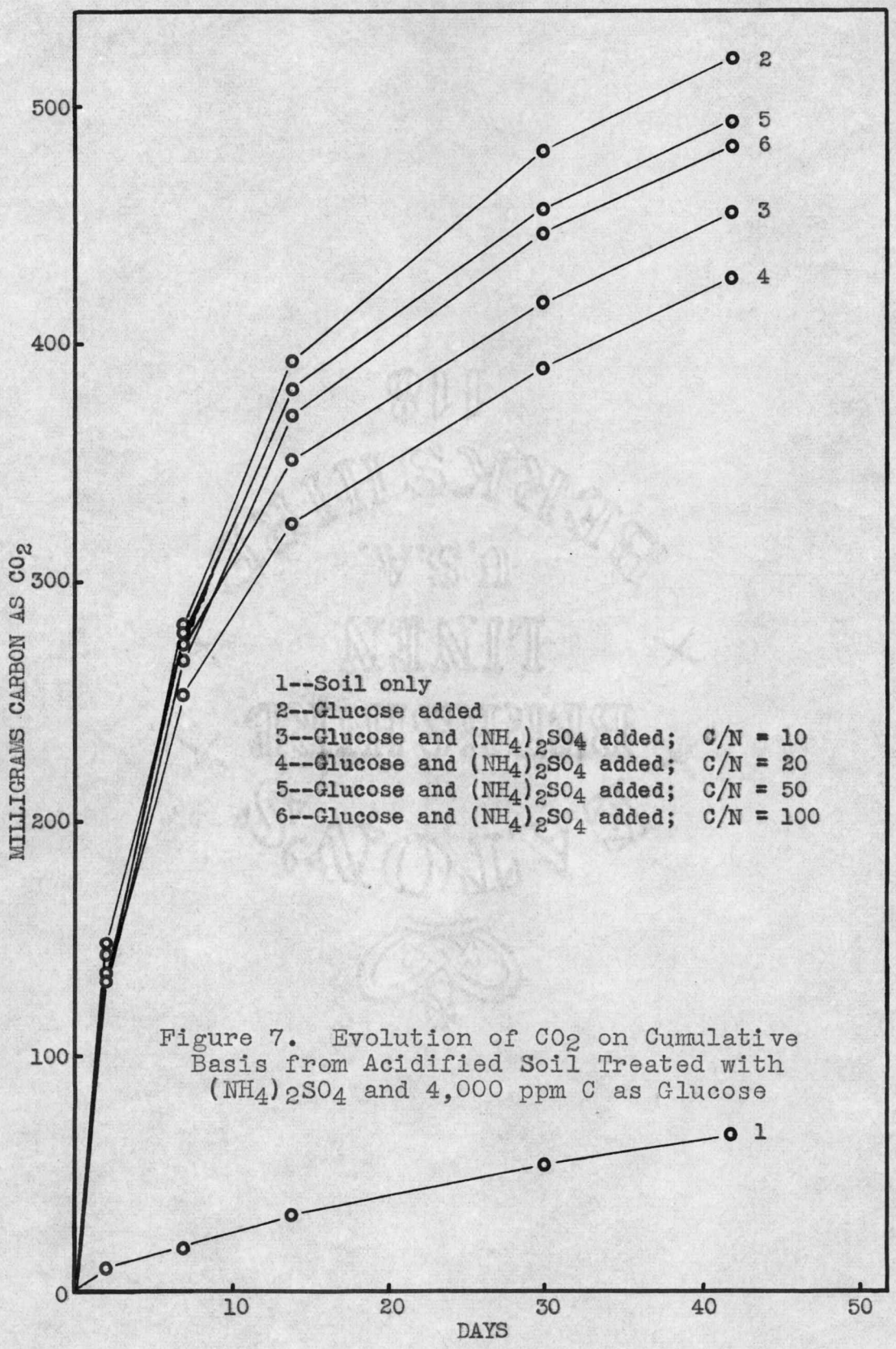

Figure 7. Evolution of CO_2 on Cumulative Basis from Acidified Soil Treated with $(NH_4)_2SO_4$ and 4,000 ppm C as Glucose

Table 7. Evolution of CO_2 from Acidified Soil Treated with $(NH_4)_2SO_4$ and 4,000 ppm C as Glucose

Treatment				Mg. C as CO_2 from 250 g. acidified soil at intervals indicated					
				Time (days)					
No.	Additions	C (ppm)	N (ppm)	0-2	2-7	7-14	14-30	30-42	Total
(1)	Control	0	0	12.0	7.9	13.5	18.8	14.6	66.6
(2)	Glucose	4000	0	133.4	147.8	111.1	88.4	38.6	519.3
(3)	Glucose + $(NH_4)_2SO_4$ C:N = 10:1	4000	400	148.4	126.2	77.4	65.4	37.1	454.5
(4)	Glucose + $(NH_4)_2SO_4$ C:N = 20:1	4000	200	135.2	117.6	72.0	64.2	39.0	427.9
(5)	Glucose + $(NH_4)_2SO_4$ C:N = 50:1	4000	80	143.2	136.2	101.2	75.3	37.2	493.0
(6)	Glucose + $(NH_4)_2SO_4$ C:N = 100:1	4000	40	133.2	134.1	102.6	75.9	37.2	483.0

As pointed out in discussing the results of the third experiment (Table 4), it can be recalled that the Chehalis silty clay loam soil is much more deficient in available carbon than available nitrogen. Therefore the main response is to added carbon. The five curves of respirations resulting from addition of glucose only, shown individually in Figures 2, 3, 4, 5, and 6, are presented for comparison in Figure 8. Calculated decompositions are presented in Table 8.

This shows very well the influence of available carbon on respiration, in the absence of added nitrogeneous compounds. The rate of respiration and total quantity of carbon dioxide evolved is significantly greater when the addition of carbon is increased. It is apparent also that acidification of the soil decreased the CO_2 evolved from equal amounts of added glucose. This difference was probably the result of a change of the flora to one predominately of molds, which would be favored by the lowered pH. As molds are much more economical in their metabolism than bacteria and streptomyces, assimilating on an average of 50 per cent of the carbon metabolized in contrast to an average of 7 per cent for bacteria and 15 to 30 per cent for streptomyces (13, p.94), they release less carbon dioxide per unit of carbon source.

Figure 8. Effect of Glucose Concentration and Soil Acidification on CO_2 Evolved from Soil

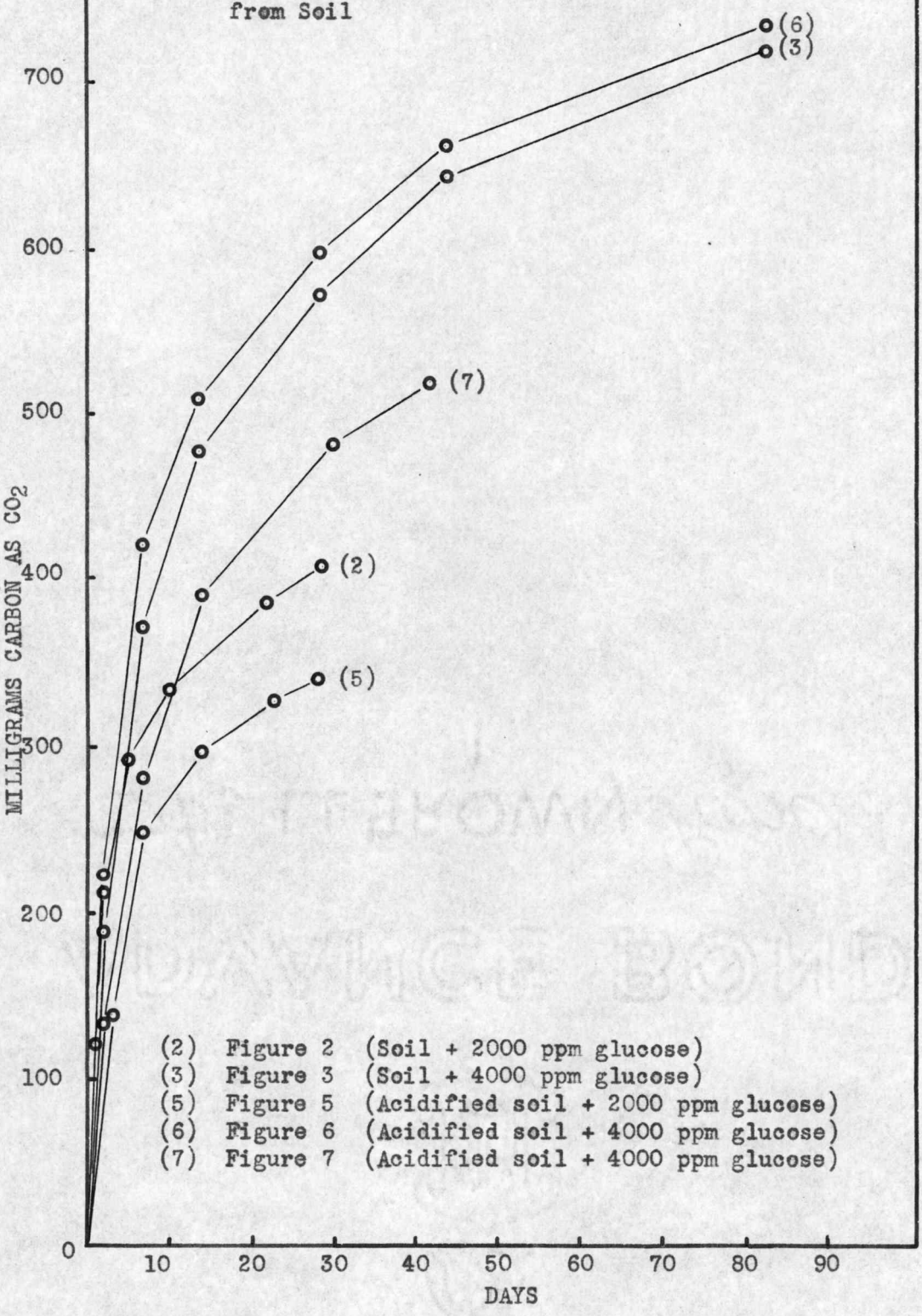

Table 8. Effect of Glucose Concentration and Soil Acidification on CO_2 Evolved from Soil

No.	Treatment	Total C evolved as CO_2 Days	ppm	Apparent Decomposition From Glucose	%
(2)	Soil + glucose at 2000 ppm	29	1633		
	Soil only	29	185	1448	71
(3)	Soil + glucose at 4000 ppm	28	2280		
	Soil only	28	300	1980	50
(5)	Acidified soil + glucose at 2000 ppm	28	1364		
	Acidified soil only	28	156	1208	60
(6)	Acidified soil + glucose at 4000 ppm	29	2392		
	Acidified soil only	29	248	2144	54
(7)	Acidified soil + glucose at 4000 ppm	30	1920		
	Acidified soil only	30	212	1708	43

Respiration Study with Soil Suspension

The study was extended by using synthetic media adjusted to several different C:N ratios. These were inoculated with a one to five dilution of the soil. Five milliliters of this suspension were added in each case to provide the microbial flora. Each respiration bottle contained three hundred grams of sand as outlined under general pure culture procedure. An appropriate amount of the sterile synthetic medium was used to produce 60 per cent of saturation, the optimum moisture content for the soil.

To investigate the possibility of there being any loss of nitrogen from the synthetic medium during autoclaving, either from that containing ammonium sulfate or sodium nitrate, nitrogen determinations were made by a microkjeldahl procedure (2, pp.763-765). Though no loss was detected from the solutions containing nitrate, there was a loss from those to which ammonium sulfate had been added. This decrease was approximately 10 per cent in every case, regardless of the C:N ratio. The C:N ratios as reported in the relevant figures and tables were calculated on the basis of actual loss.

Reference to Figure 9 and Table 9 shows that the C:N ratio of 11:1 produced the greatest total amount of carbon dioxide and also maintained the lead for the duration of

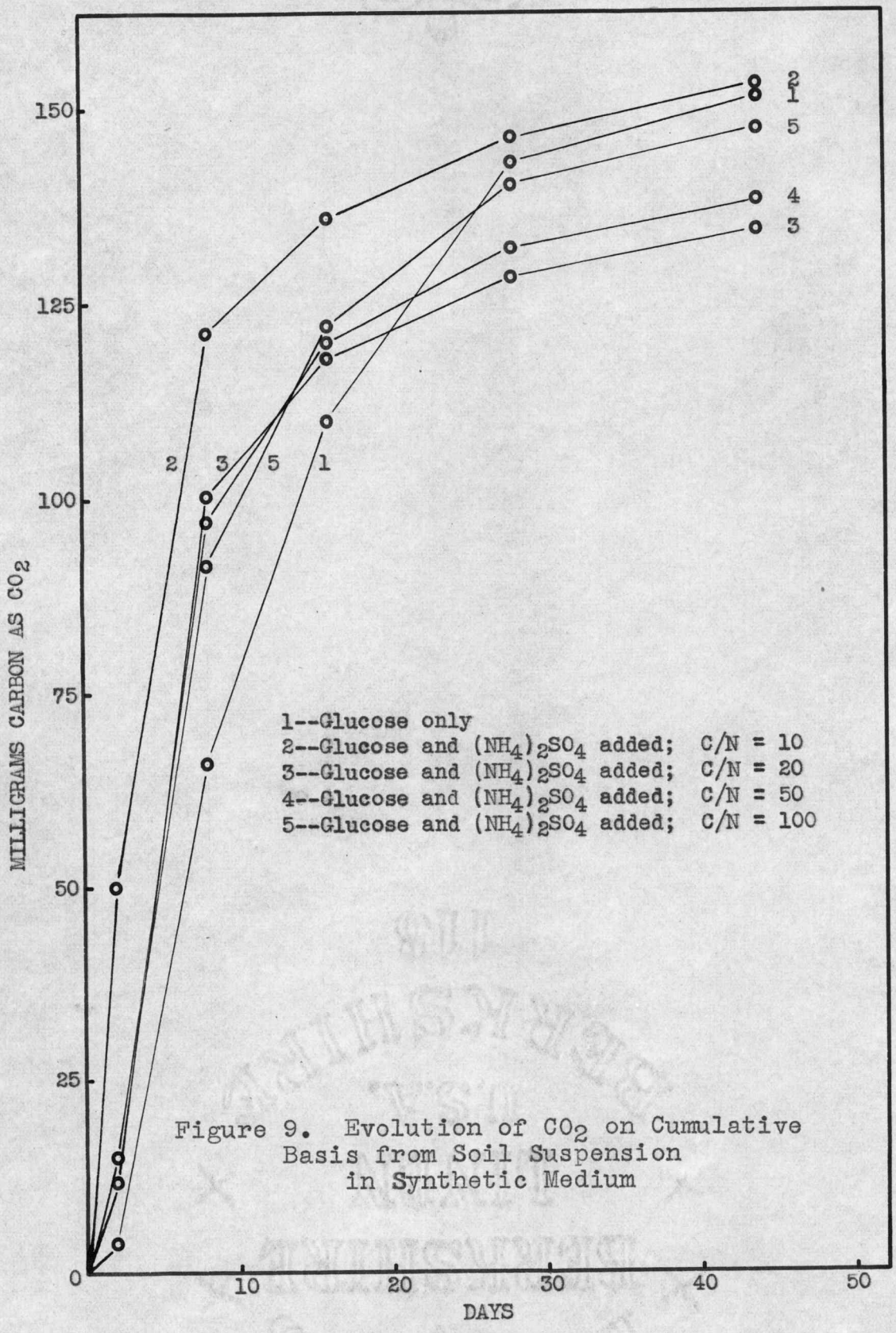

Figure 9. Evolution of CO_2 on Cumulative Basis from Soil Suspension in Synthetic Medium

Table 9. Evolution of CO_2 from Soil Suspension in Synthetic Medium

Treatment		Mg. C as CO_2 from Soil Suspension					
		Time (days)					
No.	Additions	0-2	2-8	8-16	16-28	28-44	Total
(1)	Glucose	4.4	61.8	43.8	33.0	8.4	151.3
(2)	Glucose + $(NH_4)_2SO_4$ 10:1	50.4	71.1	14.3	10.0	7.0	152.6
(3)	ditto 20:1	12.2	88.0	17.8	10.2	6.4	134.4
(4)	ditto 50:1	15.3	81.8	23.0	12.4	6.0	138.3
(5)	ditto 100:1	15.0	76.6	30.3	17.9	6.9	146.6

the incubation period, chiefly by virtue of the relatively large volume evolved in the first two days. At eight days the cumulative CO_2 was in proportion to the amount of nitrogen added. By the end of forty-four days, however, respiration was essentially the same in all cases.

As decomposition proceeded CO_2 evolution fell off more and more in proportion to the amount of nitrogen added, indicating that the more rapid the initial respiration the slower it became thereafter. This is shown to some extent in Figure 9 but is more readily apparent from the data in Table 9. Even in the control, with no added nitrogen, the rate of CO_2 production at the close of the experiment was essentially the same as for the others. Regardless of treatment there is a well defined tendency for all the curves to attain the same slope. It is interesting to note also that the soil nitrogen alone, although sufficient to maintain only the slowest beginning decomposition, was adequate to maintain the process at a later level comparable to levels shown with additional nitrogen.

At the end of seven days and at the end of the period, the heaviest surface growth of mycelium was observed with the 22:1 ratio followed by 56:1 and 11:1; the control and 114:1 had about the same and the least. Bacterial development as indicated by Chlodny slides was apparently equal in

all cases, but the quantity of mycelium of molds and streptomyces was similar in proportion to that found on the surface of the sand.

Pure Culture Respiration Studies

A series of pure culture studies with Bacillus subtilis and synthetic media were undertaken to show the influence of variations in C:N ratio on the respiration of this organism, a typical soil bacterium. Glucose equivalent to 2000 ppm was used throughout while ammonium sulfate additions were varied to give the desired C:N ratios.

The results are shown in Figure 10 and Table 10. They show that without the introduction of nitrogen there is very little growth, which is to be expected. With nitrogen, up to the twelfth day, the narrower the C:N ratio the greater is the production of CO_2; after this, however, with the C:N ratio of 56:1, CO_2 evolution still continues at almost the previous rate while for the other ratios it falls off rather sharply. This greater total production by approximately the same C:N ratio (50:1) was observed in some of the previous studies with the soil (Figures 3, 5, 6, 7).

At the end of the period samples were withdrawn for plate counts with nutrient agar. No contamination was found and there was fair agreement between the total carbon dioxide produced and bacterial numbers as determined on the plates.

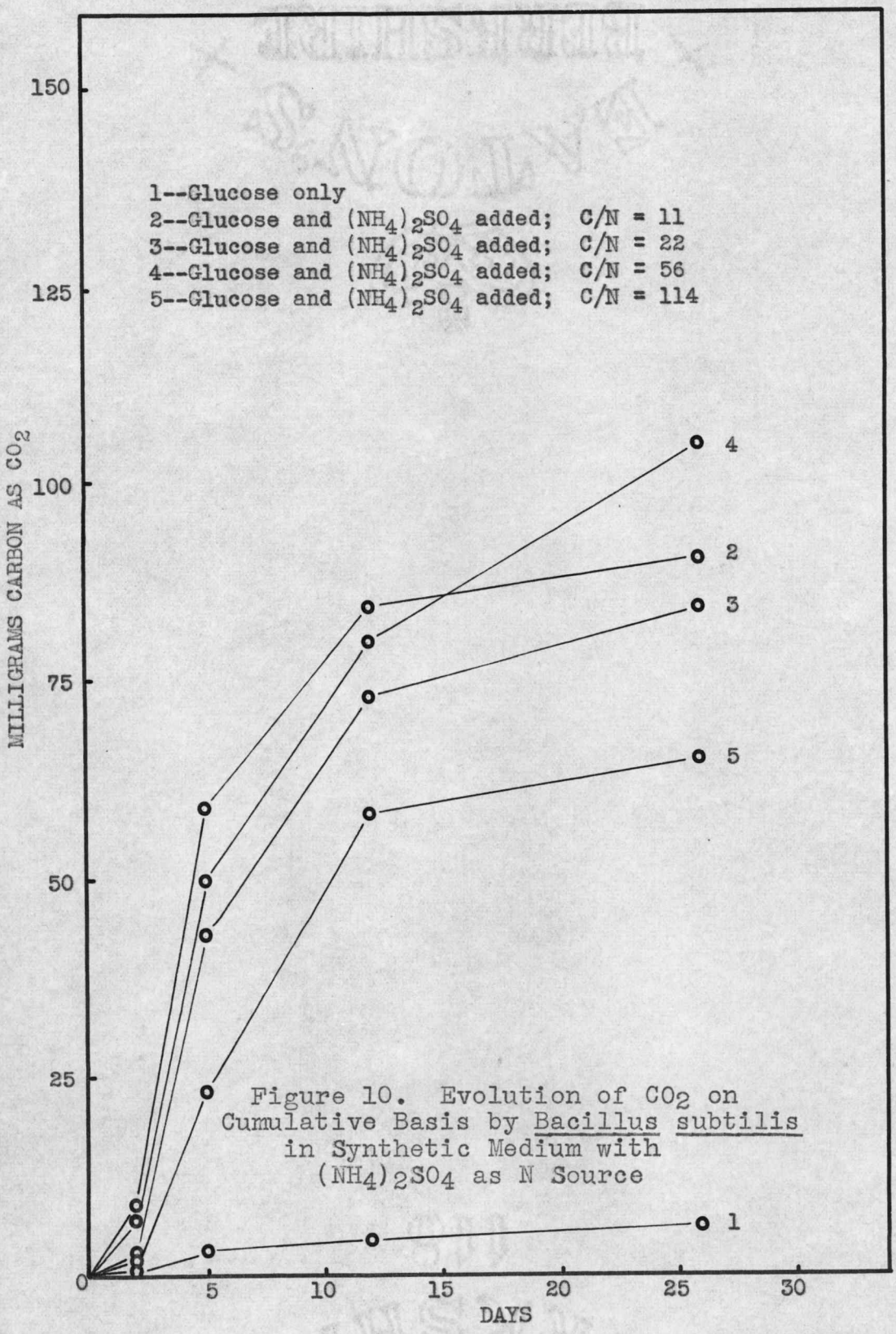

Figure 10. Evolution of CO_2 on Cumulative Basis by Bacillus subtilis in Synthetic Medium with $(NH_4)_2SO_4$ as N Source

Table 10. Evolution of CO_2 by Bacillus subtilis in Synthetic Medium with $(NH_4)_2SO_4$ as N Source

	Treatment		Mg. C as CO_2 at intervals indicated				
			Time (days)				
No.	Additions		0-2	2-5	5-12	12-26	Total
(1)	Glucose		0.5	2.1	1.5	1.3	5.7
(2)	Glucose + $(NH_4)_2SO_4$	11:1	8.6	50.2	25.5	8.2	92.5
(3)	ditto	22:1	3.1	39.9	30.5	10.5	84.0
(4)	ditto	56:1	7.4	42.2	29.2	25.9	104.7
(5)	ditto	114:1	2.1	20.9	34.9	7.5	65.4

The experiment was repeated, again using Bacillus subtilis as the test organism, with sodium nitrate rather than ammonium sulfate as the nitrogen source. Except for this difference and a respiration period longer by several days, this study was the same as the previous one.

Reference to Figure 11 and Table 11 shows a difference from the run with ammonium sulfate as a source of nitrogen. With nitrate, the CO_2 evolved during the most active period bears a direct relationship to the added nitrogen. Later, the rate of respiration falls less rapidly with the lower amounts of nitrogen.

A check for numbers of organisms and purity was made at the end of the study by observing Cholodny slides inserted in each bottle at the beginning. No contamination was indicated. Numbers were relatively low in the control but no outstanding differences were found among the cultures receiving nitrogen.

Aerobacter aerogenes, another typical bacterial inhabitant of the soil, but gram negative and otherwise significantly different from Bacillus subtilis, was similarly used in a pure culture study. The results are given in Figure 12 and Table 12. With ammonium sulfate as the nitrogen source, the highest production of CO_2 in the initial period resulted with the 11:1 ratio. Later, this leveled off very definitely. Though (3) started off slower, it surpassed (2) by the seventh day and did not slow down

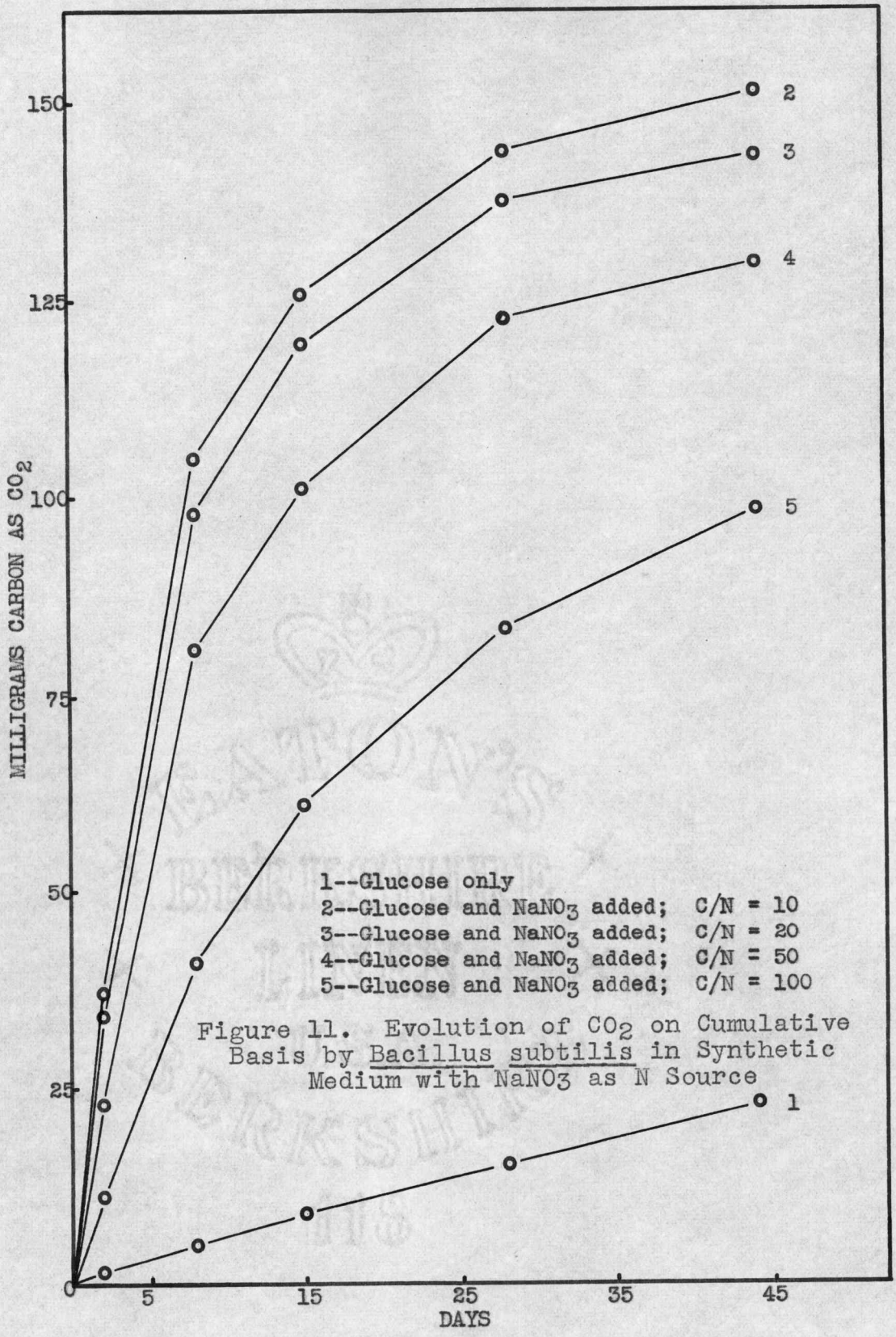

Figure 11. Evolution of CO_2 on Cumulative Basis by Bacillus subtilis in Synthetic Medium with $NaNO_3$ as N Source

Table 11. Evolution of CO_2 by Bacillus subtilis in Synthetic Medium with $NaNO_3$ as N Source

Treatment		Mg. C as CO_2 at intervals indicated					
		Time (days)					
No.	Additions	0-2	2-8	8-15	15-28	28-44	Total
(1)	Glucose	1.6	3.9	3.3	6.6	7.5	22.8
(2)	Glucose + $NaNO_3$ 10:1	37.2	67.5	21.1	17.9	8.0	151.6
(3)	ditto 20:1	34.0	63.9	21.4	18.4	4.9	142.5
(4)	ditto 50:1	22.5	57.8	20.1	21.8	7.5	129.9
(5)	ditto 100:1	10.9	30.2	20.4	22.3	14.6	98.4

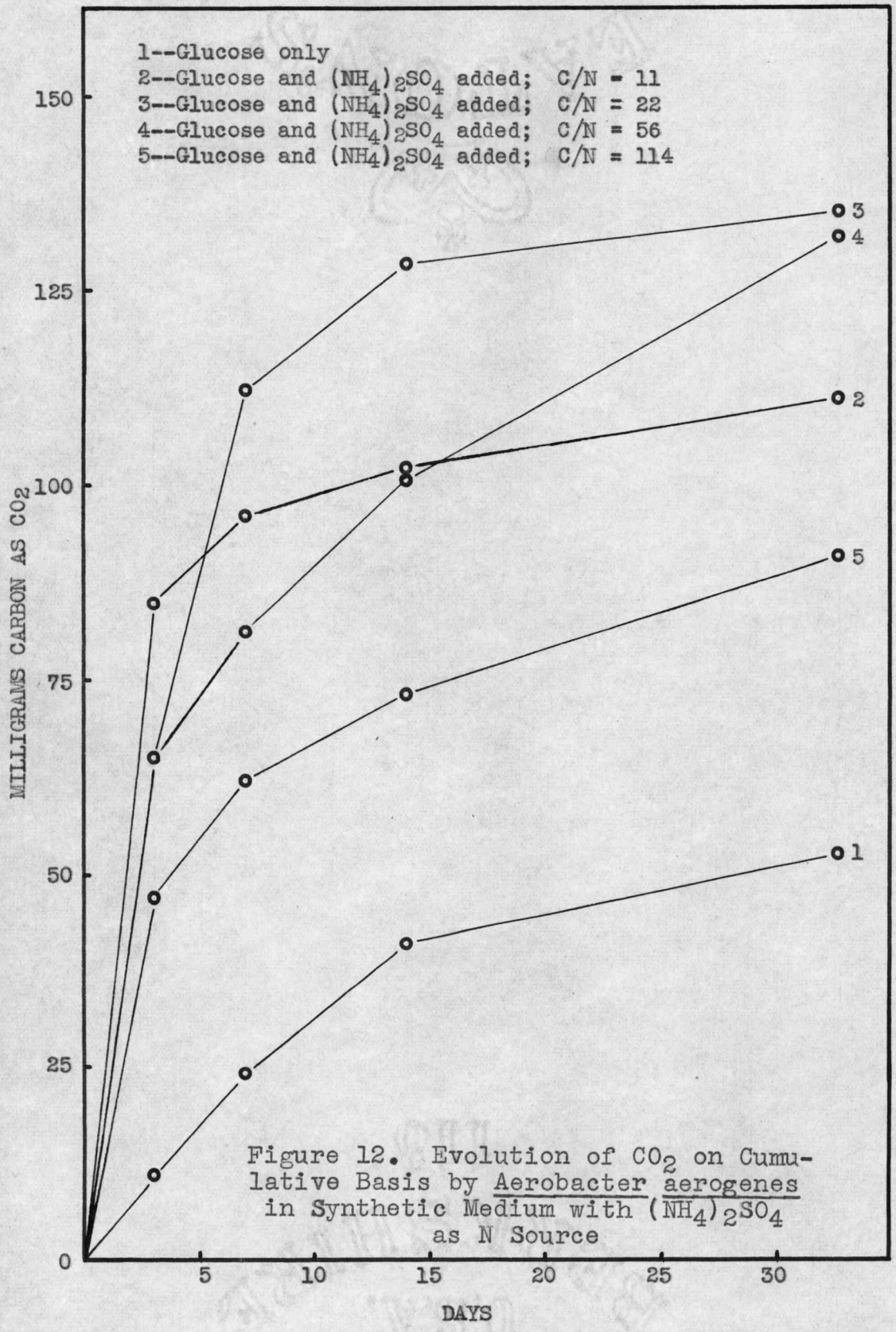

Figure 12. Evolution of CO_2 on Cumulative Basis by Aerobacter aerogenes in Synthetic Medium with $(NH_4)_2SO_4$ as N Source

Table 12. Evolution of CO_2 by *Aerobacter aerogenes* in Synthetic Medium with $(NH_4)_2SO_4$ as N Source

Treatment			Mg. C as CO_2 at intervals indic. Time (days)				
No.	Additions		0-3	3-7	7-14	14-32	Total
(1)	Glucose		10.9	13.4	17.1	11.4	52.8
(2)	Glucose + $(NH_4)_2SO_4$	11:1	84.6	11.1	6.1	9.5	111.3
(3)	ditto	22:1	65.4	47.1	16.0	6.7	135.2
(4)	ditto	56:1	65.4	15.9	19.6	31.5	132.4
(5)	ditto	114:1	46.6	15.5	11.5	17.2	90.8

to marked extent until after the fourteenth day. While (4) had not produced at the rate of the previous two, its rate was retarded least of any and very probably would have evolved the greatest volume of carbon dioxide if the study had been extended for a longer period of time. The amount of CO_2 and its production rate were lowest with the widest C:N ratio; still it showed the same trend for the last period as did (4). No contamination and no appreciable variation in numbers of this organism were shown on the Cholodny slides.

A similar study with an unidentified species of Streptomyces showed that this representative of an important group of soil microorganisms responded much like the bacteria to ammonium sulfate used with glucose in the synthetic medium. Generally the lower the concentration of the ammonium salt the greater the volume of carbon dioxide produced except in the case of the widest C:N ratio. It is very probable that if the respiration had been allowed to continue, this would have surpassed (4) in total volume (Figure 13, Table 13). Cholodny slides showed about equal growth in all cultures at the surface. Beneath the surface (4) had the heaviest growth followed by (3) and (5), (2) and (1). No contamination was discovered.

With sodium nitrate as the nitrogen source this Streptomyces was directly comparable with B. subtilis in

Figure 13. Evolution of CO_2 on Cumulative Basis by Streptomyces species in Synthetic Medium with $(NH_4)_2SO_4$ as N Source

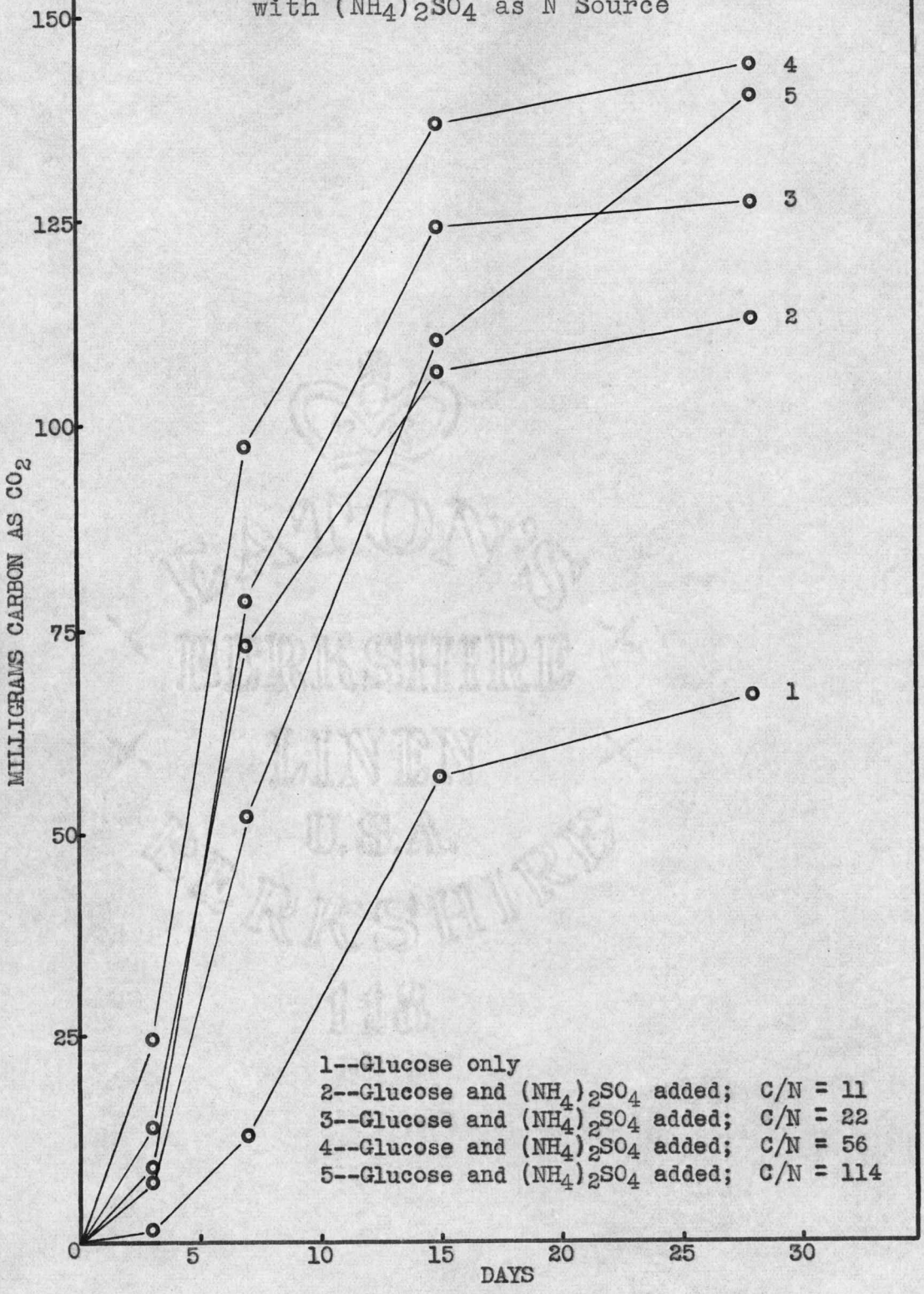

Table 13. Evolution of CO_2 by *Streptomyces* species in Synthetic Medium with $(NH_4)_2SO_4$ as N Source

	Treatment		Mg. C as CO_2 at intervals indic.				
			Time (days)				
No.	Additions		0-3	3-7	7-15	15-28	Total
(1)	Glucose		1.7	11.1	44.0	10.5	67.3
(2)	Glucose + $(NH_4)_2SO_4$	11:1	13.9	58.9	33.6	6.2	112.6
(3)	ditto	22:1	8.6	69.7	45.8	2.6	126.7
(4)	ditto	56:1	24.8	72.6	38.9	7.5	143.8
(5)	ditto	114:1	7.4	44.8	57.9	29.7	139.8

response (Figure 14, Table 14). The total carbon dioxide released was again proportional to the amount of nitrate-nitrogen added, and with the wider C:N ratios started more slowly at first and fell less rapidly toward the end. No significant differences were found in mycelial development, except for a reduction in the control. Cholodny slides again showed no c ontamination.

To study the response of a mold to variations in the nitrogen factor Penicillium notatum was employed. The medium and procedure were the same as with the other pure cultures. Figure 15 and Table 15 show the results obtained with ammonium sulfate as the source of nitrogen. Carbon dioxide production during the first three days was low for all treatments. Except with the widest C:N ratio all the respirations with added nitrogen behaved similarly. Again for the widest ratio, CO_2 evolution while slower at first, was much more rapid than the others during the last period and could have caught up. Thus it would seem that the amount of nitrogen made available at the 11:1, 22:1, and 56:1 ratios was sufficient for the needs of this organism under these conditions, while the 114:1 ratio was inadequate for maximum activity. Cholodny slides exhibited no marked difference in development of mycelium and showed no contamination.

A last experiment, designed to show how this mold was influenced with sodium nitrate as the nitrogen source, was

Figure 14. Evolution of CO_2 on Cumulative Basis by Streptomyces species in Synthetic Medium with $NaNO_3$ as N Source

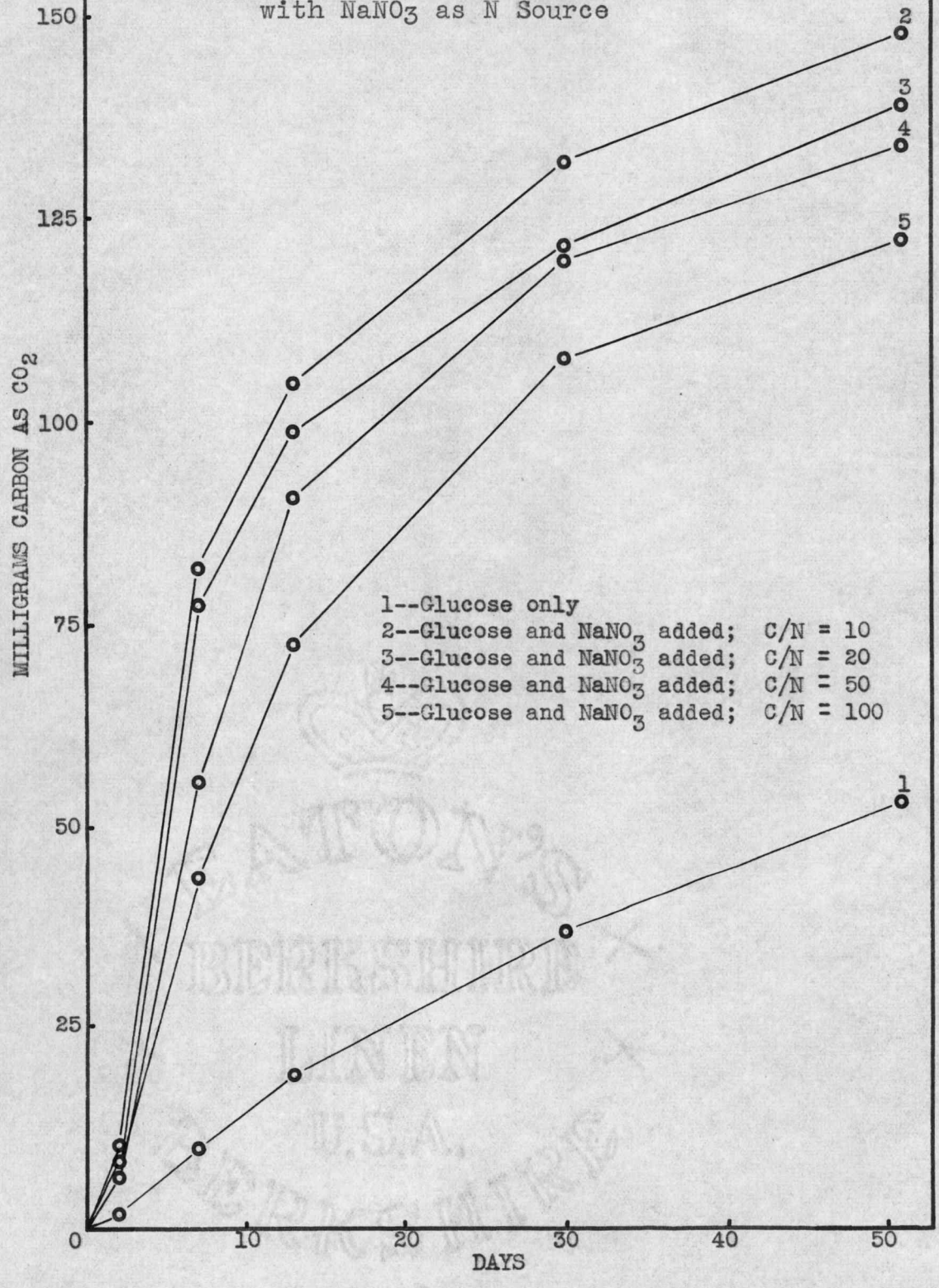

Table 14. Evolution of CO_2 by Streptomyces species in Synthetic Medium with $NaNO_3$ as N Source

Treatment		Mg. C as CO_2 at intervals indicated					
		Time (days)					
No.	Additions	0-2	2-7	7-13	13-30	30-51	Total
(1)	Glucose	1.8	8.1	8.9	18.3	16.7	53.8
(2)	Glucose + $NaNO_3$ 10:1	10.4	71.5	22.8	27.0	15.8	147.5
(3)	ditto 20:1	8.4	69.9	21.5	22.6	17.2	138.6
(4)	ditto 50:1	6.3	49.2	34.7	28.9	14.2	133.3
(5)	ditto 100:1	8.5	35.0	28.7	35.7	14.4	122.3

Figure 15. Evolution of CO_2 on Cumulative Basis by *Penicillium notatum* in Synthetic Medium with $(NH_4)_2SO_4$ as N Source

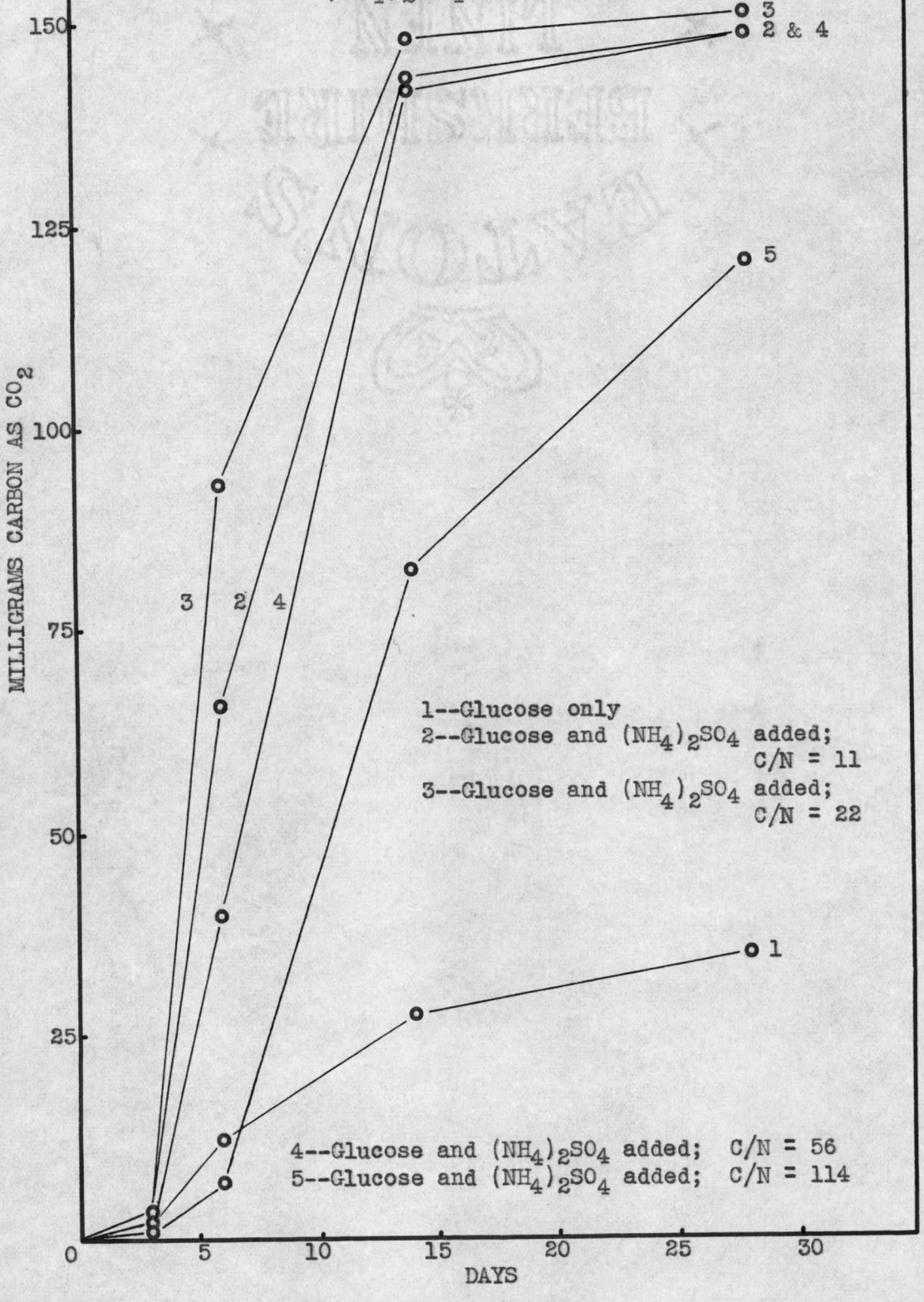

Table 15. Evolution of CO_2 by Penicillium notatum in Synthetic Medium with $(NH_4)_2SO_4$ as N Source

Treatment		Mg. C as CO_2 at intervals indicated				
		Time (days)				
No. Additions	C:N	0-3	3-6	6-14	14-28	Total
(1) Glucose		2.3	10.1	31.8	16.8	61.0
(2) Glucose -	11:1	2.6	63.2	77.1	5.6	148.5
(3) ditto	22:1	3.1	90.3	54.6	3.2	151.2
(4) ditto	56:1	0.6	39.1	102.3	6.1	148.1
(5) ditto	114:1	0.7	5.8	76.0	38.0	120.5

rendered invalid by contamination. Although the data are not presented they indicated in a general way a response similar to those shown by B. subtilis and the Streptomyces.

DISCUSSION AND CONCLUSIONS

In addition to the observations already made in the presentation of results of the various experiments, there are some points worthy of further discussion. The first obvious generalization is that the lesser amounts of nitrogen gave lesser evolution of CO_2 during the earlier periods of respiration. A second general characteristic is that the more rapid the respiration at the beginning, the slower the rate later on.

More CO_2 was not always obtained with more nitrogen except with sodium nitrate. The widest C:N ratios with ammonium sulfate nearly always gave the lowest initial CO_2 evolution but response to narrower ratios was not generally in proportion to the amount of nitrogen added. A certain minimum ratio appeared necessary for rapid respiration. Above this minimum, more nitrogen generally did not greatly alter the rate of CO_2 evolved. Later, glucose without nitrogen ranked close to glucose plus nitrogen. This indicated that the available nitrogen in the soil itself, while not sufficient to appreciably change either the overall C:N ratio or the narrower C:N ratio additions, could

support an active decomposition of added carbon source.

There exists also the possibility of nitrogen fixation by Azotobacter and Clostridium. Available nitrogen in the soil is sufficient to inhibit fixation by Azotobacter. Fixation by Clostridium would be important only where little or no nitrogen was added. In any event the fixation could be significant in amount only during the later period of the respiration studies.

Another point is the possible influence of the contribution to acidity of $(NH_4)_2SO_4$ brought about by the release of sulfate with the utilization of the ammonium ion. The buffer capacity of the soil was considerable as shown by the quantity of sulfuric acid required to lower the pH in the acidified-soil studies. In the synthetic medium employed in the pure culture studies the K_2HPO_4 used as buffer may not have had sufficient capacity to keep the pH up to a desirable level. This might explain the depression of CO_2 evolution shown in Table 8.

SUMMARY

Different amounts of nitrogen source were added to soil and solution cultures with glucose to determine the influence of the C:N ratio on microbial respiration. Ammonium sulfate or sodium nitrate was used as nitrogen source. Respiration studies were conducted on untreated soil, acidified soil, a soil-sand mixture, and with pure cultures in a synthetic medium.

With sodium nitrate the C:N ratio had a direct influence on CO_2 production. The effect of using ammonium sulfate as the nitrogen source in some cases was apparently inhibitory, but often was variable. A possible explanation may be an increased acidity from the residual sulfate ion. Wider C:N ratios often produced initial lower CO_2 evolution but relatively higher evolution rates later.

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