STUDIES IN THE METABOLISM OF ACTINOMYCETES

III. NITROGEN METABOLISM

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The utilization of different nitrogenous compounds by actinomycetes and the transformation of these substances due to the action of the organisms will be taken up in the present paper.

Rullman (1899) stated that *Actinomyces odorifer* cannot nitrify. Beijerinck (1900) observed the reduction of nitrates to nitrites by actinomycetes. Nadson (1903) found that organisms of this type from mineral mud are able to decompose proteins very readily with the liberation of ammonia and hydrogen sulfide. Macé (1905) noted that *Cladothrix chromogena* can split blood serum, with the production of ammonia, propeptone and tyrosin crystals. Fousek (1912) found that the actinomycetes isolated from the soil assimilate nitrate, ammonia and amino-nitrogen and reduce nitrates to nitrites, but cannot assimilate atmospheric nitrogen. Münter (1912) studied seven actinomycetes isolated from the soil; scant growth or no growth at all was obtained on nitrogen free media; all organisms were able to utilize equally well nitrogen in the form of nitrates, ammonia and asparagin; hemialbumin, casein, asparagin and alanin were utilized readily and tyrosin to a smaller extent, both as sources of carbon and nitrogen; urea, thiourea and dicyanamide gave no growth at all when used as a source of both carbon and nitrogen, but were used by some organisms as sources of nitrogen. Münter (1914) has further shown that casein is ammonified well, glue and peptone to a smaller extent, and urea only very slightly; ammonia formed a good source of nitrogen, only small quantities of nitrates being produced from this substance and

no free nitrogen given off; no reduction of nitrates to ammonia could be demonstrated. Krainsky (1914) stated that the actinomycetes assimilate both organic and inorganic nitrogen; of the organic substances, the proteins and amino bodies could serve not only as sources of nitrogen, but also of carbon; casein and peptone were split by all species to ammonia. All species, with one exception, grew readily upon a solution of gelatin in water (with the addition of 0.05 per cent K_2HPO_4), *A. flavochromogenus* forming an insoluble gelatin compound. The actinomycetes assimilated ammonia, nitrite and nitrate nitrogen compounds; NH_4Cl was not always favorable, particularly in the presence of glucose. Nitrates were reduced to nitrites by most actinomycetes, this varying with the composition of the medium; with many species, the nitrite formation was absent, due to the fact that this phenomenon is so slow that all the nitrites formed are at once assimilated by the cells; in no instance could ammonia be demonstrated as a reduction product of nitrates and nitrites; *A. flavus* alone could not reduce nitrates. No urease could be demonstrated, while casein was split by means of a proteolytic enzyme.

Emerson (1917) stated that since Actinomyces colonies were formed on nitrogen free media, these organisms are able to assimilate free nitrogen. This last fact could in no instance be confirmed by the writer. The development of Actinomyces colonies on nitrogen-free agar media is due to the fact that many of these organisms can develop readily with mere traces of nitrogen, which are probably present as impurities in the agar or as traces in the laboratory air.

Waksman and Curtis (1916) found that nearly all the actinomycetes studied could liquefy gelatin, varying in degree of rapidity of liquefaction, but they were found to be rather weak as ammonifying organisms. Lutman and Cunningham (1914) have shown that the ammonia production of *A. scabies* is small compared with that of bacteria under similar circumstances.

EXPERIMENTAL

Since glycerol proved to be a very favorable source of energy (see paper II of series), it was used in the following experiments. To each liter of medium containing the following ingredients: 30 grams glycerol, 1 gram K_2HPO_4, 0.5 gram KCl, 0.5 gram $MgSO_4$, 0.01 gram $FeSO_4$, were added fibrin, casein, powdered egg-albumin, Witte peptone, asparagin, leucin, glycocoll, or urea, 5 grams each; $NaNO_3$, $NaNO_2$, $(NH_4)_2SO_4$ or $(NH_4)_2CO_3$, 2 grams each. The casein and egg-albumin were first dissolved in a dilute NaOH($\frac{N}{10}$) solution, then added to the medium; the fibrin was added in small pieces to the individual tubes. The media were mixed, tubed, 10 to 12 cc. to each tube, and sterilized at 15 pounds pressure for fifteen minutes. Several tubes from each medium were inoculated with each of a series of representative Actinomyces and incubated at 25° for a period of fifteen to sixty days.

Growth is designated by figures as follows: 0—none, 1—scant, 2—fair, 3—good, 4—very good, 5—excellent. In studying the figures for growth one should keep in mind that they are only relative and 2 does not designate twice as much growth as 1 or half as much as 4. The scantest growth, even if only a few tiny flakes at the bottom of the tube or a few minute masses floating on the surface or distributed through the medium, was designated as 1. The most abundant growth was designated by 5, while the other figures fall between. All the cultures were compared on the same basis considering the set as a whole and not each organism separately. In describing the aerial mycelium the signs, + and + + were used, when it was present, the first to designate a thin powdery layer and the second a heavy, usually cottony cover. The ammonia was not determined quantitatively in this experiment, but merely qualitatively, by means of Nessler's reagent. The amino-nitrogen was determined by means of the micro-apparatus of Van Slyke. The hydrogen-ion concentration was obtained by means of the phenol-sulphon-phthalein series of indicators suggested by Clark and Lubs (1917); this was designated by the terminology of Sörensen, using the pH values.

TABLE 1

*The utilization of different nitrogen compounds by actinomycetes (3 per cent glycerol as a source of carbon)**

SUBSTANCE USED	CONTROL	A. VIOLACEUS RUBER	A. GRISEUS	A. AUREUS	A. BOBILI	A. SCABIES	A. ALBUS	A. VIRIDOCHROMOGENUS	ACTINOMYCES 215	A. VERNE	A. BOVIS	A. ASTEROIDES	A. RETICULI	AVERAGE
Fibrin														
Growth†	0	43	44	33(23)	32	23	43	13	43	22	22	31	44(12)	2.75
	0	43	44	44	43	25	43	43	45(13)	22	23	31	44(23)	3.33
Aerial mycelium‡	0	+	+	+	0	0	+	+	+	0	0	+	+	
	0	+	+	+	0	0	+	++	++	0	0	+	+	
Soluble pigment§	0	r	y	0	b	b	0	b	b-sh	0	f-y	0	b-sh	
	0	r	y	y-sh	d-b	d-b	y-sh	b	y-sh	0	f-y	0	b	
NH_2-N, milligrams in 10 cc.	0.15	1.14	1.54	0.09	0.11	0.40	—	0.42	1.14	2.14	4.71	0.18	1.37	
	0.15	—	2.85	0.29	1.22	0.85	0.72	4.20	0.30	4.56	—	0.31	2.28	
NH_3-N¶	0	0	0	1	0	1	0	0	1	1	1	1	0	
	0	0	1	2	0	3	1	0	0	1	2	1	0	
Casein														
Growth	0	43	45	44	23	13	22	34	42(13)	22	43	31(22)	—	3.1
	0	44	45	45	24	14	43	45	42(23)	23	44	31(22)	—	3.7
Aerial mycelium	0	+	++	+	0	+	0	+	+	0	++	+	—	
	0	++	++	++	0	+	0	++	++	0	++	+	—	
Soluble pigment	0	r	g	b	b	b	0	b	b	0	y	0		
	0	r	g	b	b	d-b	y-sh	d-b	b	0	y	0	—	

NH_2-N, milligrams in 10 cc.	0.45	1.37	1.37	0.24	0.12	1.06	0.42	0.56	1.42	2.14	1.57	0.36	–	
		0.60	0.63	0.94	1.10	1.54	0.66	0.97	2.20	3.08	2.85	0.60	–	
NH_3-N	0	0	0	0	0	1	0	0	0	1	1	1		
	0	0	0	1	0	3	0	0	1	2	1	2		
Egg-albumin														
Growth	0	32(22)	44(24)	44(14)	21	22	43	33	42(22)	22	13	23(31)	21	2.5
	0	22	44	45	23	23	44(24)	–	44(22)	22	14	23(31)	–	3.4
Aerial mycelium	0	+	+	++	0	0	+	+	+	0	+	+	0	
	0	+	+	++	0	0	+	–	++	0	+	+	–	
Soluble pigment	0	p-b	0	0	b	b	0	b	b	0	y-sh	0	0	
	0		y-sh	b-sh	d-b	b	0	–	b	0	y-sh	0	–	
NH_2-N, milligrams in 10 cc.	0.15	0.91	0.62	0.22	0.29	0.42	0.45	0.30	0.68	1.23	1.80	0.34	0.38	
		0.85	0.62	0.36	0.72	0.96	0.26		0.68	1.26	2.65	0.62	–	
NH_3-N	0	0	0	0	0	1	0	0	1	0	2	0	0	
	0	0	0	0	0	2	0	–	0	0	1	1	–	
Witte peptone														
Growth	0	33	45	34	42	23	22	34	42(23)	23	22	32(22)	13(44)	3.1
	0	44	45	44(15)	44	24	44(23)	44	42(24)	23	22	32	–	3.7
Aerial mycelium	0	+	++	0	0	0	0	+	+	0	0	+	+	
		++	++	0	0	0	++	++	+	0	0	+	–	
Soluble pigment	0	0	g	b	b	b	0	b	b	0	y-sh	0	b	
	0	0	g	b	d-b	b	0	b	b	f-b	y-sh	0	–	
NH_2-N, milligrams in 10 cc.	0.86	1.34	2.08	1.18	2.11	1.58	2.40	0.90	2.37	2.77	1.90	0.93	1.01	

TABLE 1—*Continued*

SUBSTANCE USED	CONTROL	A. VIOLACEUS RUBER	A. GRISEUS	A. AUREUS	A. BOBILI	A. SCABIES	A. ALBUS	A. VIRIDO-CHROMOGENUS	ACTINOMYCES 215	A. VERNE	A. BOVIS	A. ASTEROIDES	A. RETICULI	AVERAGE
Witte peptone—Continued														
NH_2-N, milligrams in 10 cc.	0	0.60	1.25	0.78	1.62	2.17	1.28	0.96	2.94	2.59	4.02	1.60	–	
NH_3-N..................	0 0	0 0	1 0	1 2	1 0	0 3	1 2	0 1	1 3	1 3	1 2	0 2	0 –	
Asparagin														
Growth.................	0	43(11) 44	33(13) 43	44(13) 44(22)	21 21(41)	22 22	22(42) –	43(11) 43	43 35	22 23	22 23	31(21) 31	33(12) –	2.42 2.90
Aerial mycelium.........	0	+ ++	+ +	+ +	0 0	0 0	0 –	+ +	+ ++	0 0	0 0	+ +	+ –	
Soluble pigment.........	0	r r	g g	b-sh b	0 b-sh	0 y-sh	y-sh	b-sh b	0 y-sh	0 0	0 0	0 0	b –	
NH_2-N, milligrams in 10 cc..................	3.99	1.94 1.65	2.46 2.28	1.30 1.48	2.99 1.90	3.08 2.32	2.56 –	2.91 2.85	2.06 0.63	3.65 3.78	3.34 1.80	3.92 3.86	2.06 –	
NH_3-N..................	0	0 0	2 3	1 0	2 3	3 3	1 –	– –	– –	1 2	1 2	1 2	0 –	
Leucin														
Growth.................	0	33(11) 33(12)	43 43	34(13) 45	22 –	12 23	23 43	13 13(43)	43 44(13)	23 23	22 22	22 22	33(13) –	2.75 3.10
Aerial mycelium.........	0	+ +	+ +	++ ++	0 –	0 0	+ +	0 +	+ +	0 0	0 0	0 0	+ –	

Soluble pigment.........	0	0 r	y-sh g	0 y-sh	b —	b-sh b-sh	0 0	b d-b	f-b f-b	0 0	0 0	0 0	b-sh —	
NH_2-N, milligrams in 10 cc.	3.98	3.00 3.03	2.56 2.56	2.27 0.48	3.12 —	3.09 3.12	2.85 2.82	2.91 2.85	2.85 1.20	3.50 3.38	3.85 3.80	3.83 3.92	2.58 —	
NH_3-N................	0	0 0	0 0	0 0	0 —	0 1	0 0	0 0	0 0	0 0	1 1	1 2	0 —	
Glycocoll														
Growth................	0	33(11) 34	44 43	45(13) 45(13)	22 43	22 13	33 44	33 45	13(32) 45(13)	23 23	12 12	22(31) 22(31)	13 —	2.9 3.5
Aerial mycelium.........	0	+ +	+ +	0 +	0 0	0 0	0 0	+ ++	+ ++	0 0	0 0	+ +	+ —	
Soluble pigment.........	0	0 r	y-sh g	f-b b	0 0	0 0	y-sh y-sh	b d-b	0 gray	0 0	y-sh y-sh	0 0	b-sh —	
NH_2-N, milligrams in 10 cc................	7.40	6.00 5.15	1.50 3.62	2.06 1.18	6.30 6.72	7.13 7.20	3.15 1.25	4.85 2.00	6.30 1.35	— 6.85	6.39 —	6.85 —	5.70 —	
NH_3-N................	0	0 0	3 2	2 2	0 0	0 2	0 1	0 2	1 0	1 2	1 2	1 2	0 —	
Urea														
Growth................	0	31 42	21 21	21 21(0)	21 21	21 22	21 21	43(12) —	0 23	21 21	21 21	21 21	0 0	
Aerial mycelium.........	0	0 0	0 0	0 0	0 0	0 0	0 0	+ —	— 0	0 0	0 0	0 0	— —	

TABLE 1—*Continued*

SUBSTANCE USED	CONTROL	A. VIOLACEUS RUBER	A. GRISEUS	A. AUREUS	A. BOBILI	A. SCABIES	A. ALBUS	A. VIRIDO-CHROMOGENUS	ACTINOMYCES 215	A. VERNE	A. BOVIS	A. ASTEROIDES	A. RETICULI	AVERAGE
Urea—Continued														
Soluble pigment.........	0	0 g	0 0	0 0	0 0	0 0	0 0	green —	— y-sh	0 0	0 0	0 0	— —	
Acetamide														
Growth................	0 0	12 31(12)	31 31	32(22) 42(22)	21 22	21 22	21 21	31 31	21 22	21 —	21 21	21 21	0 —	
Aerial mycelium.........	0	0 +	+ +	+ +	0 0	0 0	0 0	+ +	0 0	0 —	0 0	0 0	0 —	
Soluble pigment.........	0	blue blue	0 0	0 0	0 0	0 0	0 0	0 0	0 y	0 —	0 0	0 0	0 —	
NH_3-N................	0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 —	0 0	0 2(?)	0 —	
$NaNO_3$														
Growth................	0	21 31	21 42	23 45(13)	21 —	21 22	21 25	42 42	22 22	21	21 21	21 21	21	
Aerial mycelium.........		0 +	0 +	0 +	0 —	0 0	0 0	+ +	0 0	0 —	0 0	0 0	0	
Soluble pigment.........	0	0 0	0 g	0 y-sh	0 —	0 0	0 y-sh	green green	0 y-sh	0 —	0 0	0 0	0	

NO_2	0	5	1	1	0	0	2	2	1	1	2	0	
	0	1	2	2	0	1	3	1	1	0	3	4	
NH_3-N	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	—	0	0	0	0	—	0	0	
$NaNO_2$													
Growth	0	33(11)	21	23	0	21	21	31	21	21	21	0	21
		33	41	43(22)	0	33	21	43	21	21	—	—	—
Aerial mycelium	0	+	0	0	0	0	0	+	0	0	0	0	0
		++	+	0	0	+	0	+	0	0	—	—	—
Soluble pigment	0	p	0	0	0	—	0	b-sh	0	0	0	0	0
		p	0	y-sh	0	b-sh	0	b-sh	0	0	—	—	—
NH_3-N	0	0	0	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	—	—
$(NH_4)_2SO_4$													
Growth	0	0	0	21	0	0	0	0	22	0	0	0	0
		0	21	25	0	0	21	0	22	0	0	0	—
Aerial mycelium	0	—	—	0	—	—	—	—	0	—	—	—	—
		—	0	0	—	—	0	—	0	—	—	—	—
Soluble pigment	0	—	—	0	—	—	—	—	0	—	—	—	—
		—	0	0	—	—	0	—	y-sh	—	—	—	—
$(NH_4)_2CO_3$													
Growth	0	0	0	21	0	0	0	0	0	0	0	0	0
		0	0	22(42)	0	0	0	0	41(11)	0	0	0	—

TABLE 1—*Concluded*

SUBSTANCE USED	CONTROL	A. VIOLACEUS RUBER	A. GRISEUS	A. AUREUS	A. BOBILI	A. SCABIES	A. ALBUS	A. VIRIDO-CHROMOGENUS	ACTINOMYCES 215	A. VERNE	A. BOVIS	A. ASTEROIDES	A. RETICULI	AVERAGE
$(NH_4)_2CO_3$—*Continued*														
Aerial mycelium.........	–	–	–	0	–	–	–	–	–	–	–	–	–	
		–	–	0	–	–	–	–	+	–	–	–	–	
Soluble pigment.........	–	–	–	0	–	–	–	–	–	–	–	–	–	
		–	–	0	–	–	–	–	y-sh	–	–	–	–	

* Upper row of records was obtained for the first period of incubation, second row for the later period. The first period was fifteen and the second, thirty days for *A. aureus*, *A. bobili*, *A. scabies*, *A. albus*, *A. reticuli*, *A. viridochromogenus* and *A. verne;* the first thirty and second, sixty days for *A. bovis* and *A. asteroides*.

† 1—designates faint growth, 2—fair, 3—good, 4—very good, 5—excellent; 10 designates masses (colonies) throughout medium; 20 masses or floccules on bottom of tube; 30—masses on surface of liquid; 40—heavy solid surface growth.

‡ 0—absent, + present, scant; ++ abundant.

§ r—red, b—brown, g—golden, y—yellow, db—dark brown, b-sh—brownish, y-sh—yellowish, f-y—faint yellow, p-b—purplish blue.

¶ 0—designates none, 1—traces, 2—good, 3—abundant.

The chemicals used throughout the work were chemically pure, usually Kahlbaum's or Merck's. The casein was purified by the method of Hammarsten.

In glancing through table 1, one can readily see that most of the organic nitrogenous substances both proteins and amino acids, form a readily available source of nitrogen for the actinomycetes; while the amides, namely acetamide and urea used in this investigation and the inorganic nitrogenous substances form a much poorer source of nitrogen for this group of microorganisms. Nearly all the organisms studied, with very few exceptions, notably *A. asteroides*, made a fair to excellent growth upon the proteins, but grew rather poorly on the amides and on the inorganic nitrogenous substances. The quantity of growth is not absolute, but since the record was made by the same investigator under as nearly identical conditions as possible, it lends itself to comparison. The ammonium salts, both the sulfate and carbonate, were found to be the poorest sources of nitrogen under the conditions of the experiment and for the organisms studied; only *A. aureus* and *A. ruber* made a fair growth on these substances, as the only source of nitrogen, while all the others produced no growth or hardly any noticeable growth at all. This held true when these substances were studied in solution; a better growth might have been obtained on solid media. This fact has been brought out by Krainsky (1914), who stated that, with NH_4NO_3 as a source of nitrogen, the nitrate radical was used up in a comparatively short time, while the ammonia persisted for a long period. This relative inability of the actinomycetes to use ammonia compounds as a source of nitrogen may help to explain the fact that in their action upon proteins and amino acids the actinomycetes produce at first very little ammonia, especially when compared in this respect with the bacteria and molds. This brings up the whole question of the relative nitrogen metabolism of these groups of microörganisms, which will be taken up later.

Nitrates are readily used as a source of nitrogen, although the actual amount of growth, particularly on liquid media, is far inferior for most organisms to that produced on the proteins and

amino acids. The particular thing to call attention to in this respect is the reduction of nitrates to nitrites, which is greatly influenced by the source of carbon. Out of 45 species, 35 reduced nitrates in the presence of starch and only 20 in the presence of sucrose. Nitrite reactions were obtained in certain cases even when the proteins and amino acids were used as sources of nitrogen. Notably a strain of *A. californicus*, when freshly isolated from the soil, produced nitrites not only from proteins but also from ammonium sulfate. This fact recalls a paper by Joshi (1915), who reported a bacterium which could transform protein nitrogen directly into nitrites, without the nitrogenous substances undergoing the 3 stages of splitting of proteins to ammonia by one or more groups of organisms, and oxidation of ammonia to nitrites, then to nitrates by Nitrosomonas and Nitrobacter respectively. The photograph of the organism as well as its action strongly resemble that of an Actinomyces. This ability of the actinomycetes to produce nitrites out of nitrates, without any further reduction of the nitrogen compounds to ammonia or elementary nitrogen, was pointed out by Beijerinck (1900), Fousek (1912) and Krainsky (1914). The latter used plate cultures only and found that few species were able to reduce nitrates to nitrites strongly; in many cases the small quantities of nitrites produced were assimilated by the organism as soon as formed. The data on the reduction of nitrates to nitrites, affected by the source of carbon were reported in a previous paper and are summarized in table 2 for the purpose of comparison.

The nitrites were determined by the Griesz (1870) colorimetric method. First 1 cc. of sulfanilic acid solution and 1 cc. of α-Napthylamin solution were mixed in test tubes and allowed to stand a few minutes, so as to insure against the presence of any nitrites in the tubes. Then 1 cc. of the culture was added to the tube, shaken, the mixture allowed to stand in the cold three to four minutes and a reading taken. The active nitrate reducing organisms, such as *A. violaceus-ruber*, reduce with all sources of carbon; some that do not reduce nitrates readily, such as *A. violaceus-caesari*, *A. albus*, and *A. bobili* produce no nitrites

or only traces of nitrites with all sources of carbon studied; while most organisms vary in their power to reduce nitrates to nitrites with the source of carbon in the medium.

Different suggestions have been made concerning the physiological importance of nitrate reduction for the bacterial cell. A complete discussion of this subject can be found in the work of Klaeser (1914). He refuted various theories on this subject

TABLE 2

Reduction of nitrates to nitrites as affected by different sources of carbon

ORGANISMS	A. VIOLACEUS-RUBER	A. VIOLACEUS-CAESARI	A. ALBUS	A. AUREUS	A. EXFOLIATUS	A. GRISEUS	A. SCABIES	A. VIRIDOCHROMOGENUS	A. VERNE	A. DIASTATICUS	A. FRADII	A. ALBOSPOREUS	A. BOBILI	A. POOLENSIS	A. MADURAE	A. HOMINIS	A. ASTEROIDES	A. CHROMOGENUS 205	A. RETICULUS-RUBER	ACTINOMYCES 128	ACTINOMYCES 168	ACTINOMYCES 215
Arabinose	5	1	0	1	0	0	1	2	–	1	2	0	0	0	–	–	0	0	0	0	0	4
Glucose	5	0	0	1	0	2	0	2	1	0	0	1	0	0	–	–	3	0	4	0	2	1
Lactose	5	0	1	1	1	0	1	3	3	1	1	1	1	0	3	5	2	0	1	0	1	3
Maltose	5	0	0	—	3	1	0	2	2	0	0	0	0	0	1	4	4	1	4	3	3	3
Sucrose	3	1	1	0	1	0	0	2	3	0	1	0	1	1	1	0	2	0	1	0	1	1
Mannitol	5	1	1	1	3	3	1	2	4	1	1	3	0	1	–	–	1	0	4	0	2	1
Glycerol	5	0	0	1	0	1	2	3	1	1	1	0	1	1	1	3	4	1	1	0	4	1
Starch	5	1	1	1	1	2	1	2	1	1	2	3	1	3	–	–	0	1	5	0	1	1
Cellulose paper	2	0	0	0	0	0	0	1	1	0	0	0	0	0	–	–	1	1	1	0	0	1
Cellulose precipitated	3	1	0	0	0	1	—	1	1	0	0	0	0	0	1	0	1	0	1	0	0	1
Sodium acetate	5	0	0	0	0	2	0	1	–	0	0	1	0	0	1	0	2	1	2	1	0	2

and concluded that the reduction of nitrate to nitrite and ammonia by bacteria is a process by which these bacteria cover the nitrogen need in their metabolism. This conclusion of Klaeser in regard to bacteria is fully confirmed by the study of the metabolism of actinomycetes. These organisms reduce the nitrates to nitrites, in the process of utilization of this nitrogen compound, but not for any other purpose. This reduction is brought about by hydrogen, as shown by several investigators (Klaeser, 1914). The importance of this phenomenon in relation to the change of reaction of the medium will be discussed in the following paper of this series.

To return back to table 1, we find that when nitrites are present in the medium as the only source of nitrogen, the amount of growth is less than in the case of nitrates, unless the nitrates are present in very small amounts, otherwise (even in the presence of 0.2 per cent $NaNO_2$), they seem to become toxic, so that the amount of growth is very limited. In the presence of 0.2 per cent $NaNO_2$, many organisms made a small growth, limited in most cases to a few floccules on the bottom of the tube and only in the case of the organisms that reduce nitrates to nitrites vigorously (*A. violaceus-ruber*) was the growth more than fair. The question of utilization of nitrites will be discussed later.

The amides, as stated above, form a very poor source of nitrogen in comparison with the proteins and amino acids. Only a few species made more than a very limited growth, the reaction in the case of urea, either remaining unchanged, becoming slightly acid, or, in most cases, turning alkaline; this latter phenomenon is no doubt due to the rapid splitting off of ammonia from urea. In the case of acetamide, the reaction became in nearly all cases more acid, which may be explained by the fact that the NH_2 radical is used up and may be replaced by an OH radical, which may turn the medium more acid.

The utilization of proteins and amino acids can be followed up in two ways, first by observing the actual amount of growth, secondly, by estimating the splitting of the proteins, as measured by the amount of amino nitrogen produced in the case of proteins, or by the decreasing quantities of the amino nitrogen, in the case of the amino acids, using the Van Slyke micro-apparatus.

No sweeping conclusions should be made from these experiments as to the relative utilization of proteins and amino acids by the different species of Actinomyces, because it is quite possible and even probable that different conditions, such as age of culture, time since its isolation from a natural substratum, concentration of nitrogenous substances, nature of carbon compounds, etc., may result in an entirely different range of figures; but, even with all these limitations, certain definite conclusions can be drawn.

A. asteroides splits proteins and uses amino acids only to a very limited extent, although it produces an abundant growth on certain inorganic media (particularly with glucose as a source of carbon). Certain organisms, such as *A. verne,* using only small quantities of amino acids when grown on amino-acid-containing media, allow a large accumulation of amino-nitrogen-rich substances when grown on protein containing media, showing that there is no necessary correlation between the amount of growth and the protein split.

The production of amino acids and other amino-nitrogen-rich substances is not a waste resulting from the growth of the organisms, but is a definite step in the metabolism of the organisms, since in many cases these substances do not accumulate in the medium, but tend to decrease, either due to their transformation into other substances, such as ammonia (which was not the case), or to their assimilation as such or as transformation products, by the organism, as indicated by the increase in growth.

Fibrin, casein, egg-albumin and peptone allow a very good growth of nearly all the actinomycetes studied, the second and the fourth leading, as to the actual amount of growth. Small quantities of amino-nitrogen are present in the proteins; these are equivalent to one-half of the lysin nitrogen, as shown by Van Slyke and Birchard (1914). Some organisms seem to start using this lysin nitrogen at first, particularly the species which are weak proteolytically, such as *A. asteroides* on the casein medium. In most cases, the accumulation of amino-nitrogen becomes prominent only when the organism has made most of its growth. This may be due either to the fact that during the period of its active growth, the organism uses all or most of the amino-nitrogen that it can split, or to the fact that the splitting is accomplished by means of a proteolytic enzyme, which is absent in the early stages of growth or present in only minute quantities.

The amino acids studied, particularly glycocoll, were also well utilized by most organisms. The progress of growth can also be followed by the decrease in the amino-nitrogen present in the medium, either due to its assimilation by the growing organ-

isms or to its transformation into other substances, such as ammonia. It will be noticed that the organisms that made the poorest growth on certain amino acids, used the least nitrogen, while those that made a good growth caused a good deal of the amino-nitrogen to disappear from the medium. For example, in the case of asparagin, the medium containing originally 3.99 mgm. NH_2-N in 10 cc., *A. asteroides* with a faint growth caused a decrease in thirty days to 3.92 and in sixty days to 3.86; while *A. griseus* with a very good growth, changed it in fifteen days to 1.30, which seems to have accompanied the maximum growth, since in thirty days there was a slight increase in the amino nitrogen content, namely 1.48, which may be due either to an individual variability of the cultures or to some autolysis that might have set in.

The production of ammonia does not seem to be a characteristic property of this group of organisms, as was already pointed out elsewhere (Waksman and Curtis, 1916). It is possible that ammonia does not enter as a necessary step in their metabolism, as some investigators claim for other microörganisms and that it is not formed in large quantities as a waste product of metabolism, although we find individual variations between the different species and with different substrata. These organisms seem to assimilate the amino acids directly or indirectly, but not necessarily only after reduction to ammonia. The question of the function of ammonia as a protein split product in the metabolism of molds has been already discussed by the writer elsewhere (Waksman, 1918). It need only be pointed out here that the actinomycetes and molds seem to split the proteins with different results; while most of the latter reduce the proteins to ammonia very quickly and allow its accumulation in the medium, the former seem to split the proteins chiefly to the amino acid stage and only to a limited extent to ammonia. Most ammonia was produced from the peptone and asparagin, least from the egg-albumin and leucin.

The change in the hydrogen-ion concentration of the media are reported in the paper following this one.

Of the different amino acids used, leucin tends to result in a more acid medium, while asparagin and glycocoll lead to a neutral or slightly alkaline reaction. The leucin medium changed on the average for the organisms studied for the first period from pH 7.3 to pH 6.7 and 6.43, while the glycocoll media changed on the average from 7.1 to 7.23 and 7.33, allowing for the variation of the organisms. The protein and peptone media all tended to become more acid, but not to such as extent as the leucin media. It is possible that the reaction of media containing certain proteins may change either to acid or alkaline depending entirely upon whether the organism attacks one amino acid group or another in the protein molecule. The final hydrogen-ion concentration of media, upon which miroörganisms have grown in the presence of the same carbohydrate, is influenced by the nitrogen source and varies greatly, depending upon the source of nitrogen. A more detailed study of this question will be found in the following paper of this series.

The utilization of tyrosin and creatinine by actinomycetes was also studied; the results for the first are reported below in table 3, while creatinine was readily used as a source of nitrogen by all species tested, accompanied by a slightly acid reaction (pH usually changing from 7.0 to 6.4–6.8); the growth was always fair to good and consisted of colonies throughout the medium or as flakes on bottom of tube. No. 205 produced a characteristic soluble yellow pigment, *A. viridochromogenus*, and *A. pheochromogenus* a brownish and *A. violaceus-ruber* a bluish pigment, while *A. scabies*, *A. aureus* and *A. exfoliatus* produced no pigment at all.

A word should be said concerning the pigment production by the actinomycetes upon the media containing proteins and amino acids. These pigments are very characteristic of the species and seem to be stimulated by the organic nitrogen. The chromogenus species, reported in table 1 include *A. scabies*, *A. viridochromogenus*, *A. aureus* and to some extent *A. bobili*, *A. ruber* and *A. reticuli*. These species are characterized by a brown or dark brown (yellowish in few cases, particularly accompanying poor growth) pigment which slowly dissolves into the medium.

The pigment production is usually ascribed to an enzyme tyrosinase, which is able to convert tyrosin into dark colored melanins. Beijerinck (1913) has shown, however, that tyrosin is oxidized into melanin only by a symbiotic action of an *Actinomyces* with a common soil bacterium; neither organism alone can oxidize the tyrosin to the same stage. Attention was called in the previous experiments to the fact that many species of *Actinomyces* produce a yellow brown to dark brown soluble pigment even on media that do not contain any tyrosin. We must therefore conclude that the brown pigment produced by certain species on organic media is due not only to the melanins produced from tyrosin, but also to some other substances produced from amino acids besides tyrosin and from the sources of carbon.

A number of species were inoculated upon an alkaline tyrosin agar (Krainsky, 1914) and only *A. scabies* and no. 205 produced a soluble brown pigment. All the other species made a good growth upon this medium, but produced no soluble pigment. If, as Beijerinck (1913) stated, tyrosinase is a mixture of two oxidizing enzymes, one converting tyrosin into homogentisic acid and the other oxidizing the acid to melanin, then out of all the species studied, only *A. scabies* and no. 205 are able to produce both of these enzymes together. Out of four strains of *A. scabies* obtained from different sources, only two gave the tyrosinase reaction, these two being the more vigorous growers. The experiment on the utilization of tyrosin and on the production of a brown to black soluble pigment was repeated again in solution. A medium was made up containing the same constituents as the synthetic solution used (Czapek's) except that the $NaNO_3$ was replaced by 0.1 per cent of tyrosin and the sucrose by 3 per cent glycerol. The medium was tubed, sterilized, inoculated and incubated at 25° for fifteen days. The results are presented in table 3.

The previous results are confirmed; not all the species that are able to produce a brown to black soluble pigment on gelatin, potato plug and synthetic media containing organic substances, particularly proteins and peptones, are able to produce a soluble

dark pigment on tyrosin. Only some cultures of *A. scabies* and to some extent two to three other chromogenic strains are able to produce this pigment, indicating that only these species are able to form the two enzymes necessary to transform tyrosin into melanin. A detailed study of this question will be taken up in a paper on the "Enzymes of actinomycetes." At present, the fact can only be pointed out that when the actinomycetes were grown on gelatin plus 1 per cent starch, to which HCl and KI had been added only those species that are able to produce a

TABLE 3

The growth of actinomycetes on tyrosin and the production of a soluble pigment

ORGANISM	GROWTH	AERIAL MYCELIUM	SOLUBLE PIGMENT	pH
Control				6.8
A. violaceus-ruber	2*	+	Bluish	6.2–6.6
A. exfoliatus	2	0	0	6.6
A. aureus	3	+	0	6.6
A. viridochromogenus	3	+	Trace of brown	6.6
A. scabies I	2	+	Deep brown	5.4
A. scabies II	3	0	Pinkish	7.0
A. scabies III	2	+	0	6.7
A. 205	3	+	Greenish brown	6.6
A. pheochromogenus	2	+	Brownish	6.7

* The figures have the same designation as in table 1.

brown pigment on the gelatin, produced a purplish color; this indicates that an oxidase is produced by those species that color the cultures containing gelatin and other proteins brown, while they may not be able to convert tyrosin into melanin.

A more detailed study of pigment production by actinomycetes will be published later. Attention may be here called to the fact that *A. violaceus-ruber* which produced a beautiful red brown pigment changing to violet blue is a very active nitrite forming organism; the part played by the nitrites in the synthesis of the pigment may be important.

Attention was called, in connection with table 1, to the fact that ammonium salts and amides form a rather poor source of nitrogen for most actinomycetes. Whether this is true only with glycerol as a source of energy or also with glucose, one can

see from the following experiment, where to the synthetic media previously used 3 per cent of glucose was added in place of glycerol (nitrogen source 0.2 per cent).

Most of the species tested made a better growth with glucose as a source of carbon than with glycerol, using ammonium salts and urea as sources of nitrogen. The poorest growth was obtained with ammonium sulfate, due to the fact that the reaction soon became distinctly acid with this source of nitrogen, the pH values changing from 5.8 to 4.6 and even 4.2 which is a limiting reaction for the growth of actinomycetes, as will be shown in the following paper.

TABLE 4

The utilization of ammonium salts and urea as sources of nitrogen of actinomycetes, with glucose as a source of carbon

ORGANISM	$(NH_4)_2SO_4$		$(NH_4)_2CO_3$		UREA	
	Growth	Aerial mycelium	Growth	Aerial mycelium	Growth	Aerial mycelium
A. violaceus-ruber	1	0	4	–	5	–
A. viridochromogenus	1	0	3	0	4	–
A. aureus	2	–	4	–	4	–
A. reticuli	0	0	1	0	1	0
A. bobili	2	0	2	0	1	0
A. scabies	3	0	3	0	2	0
A. verne	1–2	0	3	0	1	0
A. madurae	Trace	0	0	0	1	0
A. bovis	1–2	0	2	0	2	0
A. asteroides	2	0	3	0	4	0

To test further the utilization of nitrites, different concentrations of this salt were added in place of the nitrate to the synthetic solution containing either glucose or glycerol as a source of carbon. The results are recorded in table 5.

It is obvious that the actinomycetes can assimilate nitrites readily, when these are present in small enough amounts not to exert any toxic effect. In the presence of only 50 mgm. of $NaNO_2$ per 1000 cc. of medium, all the species tested produced a fair to good growth with both glucose and glycerol as sources of carbon. With the increase of the nitrite content, the growth increases, due to the larger amount of available nitrogen, but

TABLE 5

The utilization of nitrites by actinomycetes. ($NaNO_2$ *used*)

ORGANISM	NITRITE ADDED																													
	0.005 per cent						0.01 per cent						0.05 per cent						0.2 per cent						0.5 per cent					
	Glucose			Glycerol			Glucose			Glycerol			Glucose			Glycerol			Glucose			Glycerol			Glucose			Glycerol		
	Growth	pH	NO_2	Growth	pH	NO_2	Growth	pH	NO_2	Growth	pH	NO_2	Growth	pH	NO_2	Growth	pH	NO_2	Growth	pH	NO_2	Growth	pH	NO_2	Growth	pH	NO_2	Growth	pH	NO_2
Control.........		6.4	+		7.3	+		6.4	+		7.1	+		6.4	+		7.3	+		6.4	+		7.3	+		6.4	+		7.3	+
A. violaceus ruber..........	2	5.2–5.8	–	2	6.4–6.7	–	3	5.2–5.4	–	3	5.2	–	3–5	5.0–6.6	+	2–4	6.6–7.0	+	2	5.6	+	1	7.3	+	0	6.4	+	1	6.6	+
A. aureus.......	3	5.0–5.3	–	3	7.1–7.3	±	2	6.0	–	3	7.1–7.2	±	4	5.8	+	4	7.3–7.7	+	0	6.4	+	1–2	6.5	+	1	5.4	+	1–2	6.4	+
A. scabies.......	3	6.4–6.6	+	3	4.6–4.8	–	3	4.6	–	4	4.6–4.8	–	0–1	5.4–6.4	+	4	5.4–6.6	–	0	6.4	+	3	7.5	+	0	6.4	+	0	7.3	+
A. bobili.........	3	5.4	–	2	6.8	–	3	6.4	–	1	6.8	–	1	6–4	+	5	7.7	+	0	6.4	+	1	7.1	+	0	6.4	+	0	7.3	+
A. reticulus ruber..........	3	6.6	±	3	6.4	–	3	6.6	–	3	6.4–7.4	±	1	6.2–6.4	+	5	7.0–7.3	+	0	6.4	+	1	6.8	+	0	6.4	+	0	7.3	+

here the toxic effect of the nitrite may become apparent, particularly with some sources of carbon, so that in the presence of 0.05 per cent of $NaNO_2$, most of the species tested made a very good to excellent growth with glycerol as a source of carbon, while the same species produced only a scant growth in the presence of glucose. When the nitrite content of the medium is still further increased, the growth ceases entirely or is only very limited.

In the previous experiments on the action of the actinomycetes upon different proteins, only purified native substances were used. To study further the action of these organisms upon protein-rich substances, they were grown on gelatin (15 per cent in distilled water), with and without one per cent of starch, on milk and on glucose broth (1 per cent peptone, 0.5 per cent meat extract and 1 per cent glucose). The results are reported in table 6.

Here again no greater importance should be attached to the data presented in table 6 than to any other set of biochemical data with a group of microörganisms, where only one or two tubes for each organism are studied. But, although the results are not absolute, a comparative study of the data will be of interest. These experiments were repeated several times at different temperatures and, although a great deal of variation was obtained, the comparative results hold true as a whole; and it really makes very little difference, when such a variable group of organisms is studied, whether one species accumulates 25 or 35 mgm. of NH_2-N in 10 cc. when grown on a 15 per cent gelatin solution. All the species studied cause a splitting of gelatin, peptone and milk proteins to a greater or less extent, but the organism that produces a maximum splitting of the gelatin does not necessarily split the maximum of peptone, casein and other proteins. As we might expect, however, many species that split one protein actively, also split the others actively. The amount of ammonia accumulated in milk is relatively large, particularly for the organisms which are active proteolytically; this is due to the long incubation period at a rather high temperature; in a shorter period of incubation the

TABLE 6

*The decomposition of organic substances by actinomycetes**

ORGANISM	15 PER CENT GELATIN†	15 PER CENT GELATIN PLUS 1 PER CENT STARCH†	GLUCOSE BROTH‡	MILK §	
	NH_2-N	NH_2-N	NH_2-N	NH_2-N	NH_3-N
Control	6.41	6.35	3.08	2.65	0
A. violaceus-ruber		32.49	5.13	21.38	9.6
A. violaceus-caesari			5.21	20.80	6.5
A. scabies	15.75	15.39	4.85	12.83	9.1
A. pheochromogenus	23.94	22.80	5.70	4.85	2.2
A. viridochromogenus			4.84	17.96	4.3
A. chromogenus 205	34.20	37.05	4.34	13.12	4.3
A. aureus	31.35	23.66	4.87	14.82	7.2
A. exfoliatus			4.57	15.96	9.1
A. albus	10.83	6.56	4.10	23.66	11.0
A. griseus	38.48	23.66	4.56	33.21	13.9
A. fradii	15.96	12.83	5.73	19.95	5.3
Actinomyces (206)	48.74	31.64	5.19	28.32	13.7
A. alboflavus		12.26	6.36	26.32	7.7
A. reticuli		28.79	4.38	14.25	6.5
A. reticulus-ruber	13.68	11.97	3.77	16.18	12.7
A. rutgersensis	48.17	32.21	4.57	22.80	5.8
A. halstedii	20.82		4.52	11.69	1.9
A. albosporeus	25.37	18.53	5.42	9.41	1.9
A. diastaticus	33.06	11.97	6.21	25.94	10.1
A. poolensis	36.48	26.72	4.38	30.21	13.0
A. madurae	27.08	14.82	8.04	35.63	
A. hominis	26.51	13.97	4.52	19.95	
Actinomyces 128	29.92	22.52	3.42	25.65	12.7
Actinomyces 161	45.80	25.65	3.70	21.66	10.8
Actinomyces 96	39.33	27.65	4.16	21.95	11.8
Actinomyces 104	25.65	23.66	4.02	8.55	−2.2
Actinomyces 145	20.52	32.49	3.12	5.70	3.5
Actinomyces 154	10.83	16.53	5.19	23.66	6.7
Actinomyces 168	25.08	21.95	4.14	16.96	13.2
Actinomyces 215			6.05	15.20	
Actinomyces 216	22.80	16.82	3.12	19.95	5.3
Average	27.93	21.72			

* The data present milligrams of nitrogen per 10 cc. of medium.

† Incubated at 16° to 18° for thirty to thirty-five days.

‡ Incubated at 25° for fourteen days.

§ Incubated at 37° for forty days.

quantities of ammonia are much smaller; this again brings out the fact that, while the proteolytic bacteria and molds allow a rapid accumulation, the actinomycetes will produce only small quantities of ammonia in a short period of time, the ammonia tending to increase with the prolongation of the period of incubation.

The presence of starch in gelatin seems to exert in many instances a protective action upon the gelatin, and, though a better growth might have been obtained in the presence of starch, there was less of the gelatin split. This does not hold absolutely true for all species; in a few instances there was a greater splitting of the gelatin in the presence of starch. On the average, however, there was a greater accumulation of amino-nitrogen in the gelatin in the absence of starch than in its presence. The gelatin containing originally 6.35 to 6.41 mgm. amino nitrogen in 10 cc., was found, at the end of the period of incubation, to contain, on an average, 27.93 mgm. of NH_2-N, in the presence of 1 per cent starch, and only 21.72 mgm., in the absence of starch. This is in accord with the investigations of the writer and others which indicate that available carbohydrates exert a protective action upon the proteins.

To get a further insight as to the effect of available carbohydrates upon the splitting of proteins by actinomycetes and compare these organisms in this respect with bacteria and molds, the following experiment was made. One lot of ordinary bouillon was made up and divided into 2 portions; 1 per cent glucose was added to one half and the other half left unchanged. These media were distributed in flasks, sterilized and inoculated, as usual in duplicates, with *B. coli*, *B. proteus*, *Aspergillus niger* and 5 species of *Actinomyces*. The flasks were incubated at 37° and, at the end of seven days, the amino and ammonia-nitrogen were determined. The data are given in table 7.

The addition of 1 per cent glucose causes, in the case of all microörganisms, a lesser splitting of the proteins and peptones accompanied by a smaller ammonia accumulation. But, while the active proteolytic bacterium (*B. proteus*) and mold (*A. niger*) were greatly repressed in their action upon the proteins and in

the production of ammonia, the bacterium, which is recognized as not very active proteolytically (*B. coli*), and the actinomycetes were repressed in their action upon the proteins to a much smaller extent.

It is quite possible that here as well as in the case of higher animals we have two kinds of metabolism: endogenous and exogenous. A definite quantity of ammonia may always be produced out of protein materials, as a waste product, independent of whether an available carbohydrate is present or absent; this

TABLE 7

The action of microorganisms on peptone in presence and absence of available carbohydrates. Period of incubation seven days at 37°*

ORGANISMS	BOUILLON		BOUILLON PLUS 1 PER CENT GLUCOSE	
	NH_2-N	NH_3-N	NH_2-N	NH_3-N
Control	45.73	0	44.25	0
B. coli	50.44	9.45	47.79	7.35
B. proteus	73.75	32.55	48.08	8.40
Aspergillus niger	33.93	32.60	21.54	15.80
Act. diastaticus	89.09	11.55	67.85	9.45
Act. viridochromogenus	68.74	10.50	50.15	10.50
Act. fradii	63.43		56.64	
Act. griseus	97.35	13.65	85.24	11.55
Act. poolensis	80.83	12.60	67.85	12.60

* The data present milligrams of nitrogen per 100 cc. of medium.

ammonia may be reabsorbed, in the presence of available carbohydrates, by organisms that are able to utilize it readily as a source of nitrogen (*A. niger*). In the absence of available carbohydrates, the strongly proteolytic organisms use the proteins readily as sources of carbon, leaving large quantities of ammonia, as waste material, in the medium. The ammonia produced in the first case, will not be appreciably decreased (except when reutilized again), while the production of ammonia in the second case may be entirely prevented by a utilizable carbohydrate.

The milk data in table 5 point clearly to the fact that most of the actinomycetes can attack the milk proteins readily and split them to amino acids and ammonia. This question was dis-

cussed in detail elsewhere (Waksman, 1919). To get an insight into the rapidity of the proteolytic action of some species on the milk proteins, as affected by temperature of incubation and mode of action of the different species, the following experiment was conducted:

A series of tubes, each containing exactly 10 cc. of sterile milk, were inoculated with *A. griseus* and *A. exfoliatus*, inoculating all tubes as nearly alike as possible. Ten tubes for each species were inoculated at 25° and 10 at 37°. Observations were made daily as to the clotting and peptonization. The amino and ammonia nitrogen of the milk were determined at the end of definite intervals and results calculated back to the total amount. This experiment was repeated. The results are given in table 8.

The results reported in the table are not sufficient for plotting an exact curve, because the average error between the duplicates is greater than should be allowed for accurate work. The importance of using a definite period of incubation for all the groups is brought out clearly: while the strongly proteolytic species produce a continuous splitting of the proteins, the weaker forms may, at an early period, allow a splitting, which will not advance much further. It is also interesting to note that when the period of incubation is long enough the difference in the quantities of amino nitrogen content of the milk and ammonia accumulated approaches to zero.

The ammonia accumulation by actinomycetes is slow, but increases steadily. When these organisms are compared with molds and bacteria in their power of producing ammonia, an interesting difference is observed. The rapidly growing bacteria and molds allow a rapid accumulation of ammonia, which soon reaches its maximum as a result of the metabolism of these organisms; the slow growing actinomycetes allow only a slow accumulation of ammonia; if a long enough period of incubation is allowed, the amount of ammonia may even become as large as that produced by the active proteolytic bacteria and molds.

TABLE 8

*The effect of temperature on the action of actinomycetes upon milk**

PERIOD OF INCUBATION	A. GRISEUS						A. EXFOLIATUS					
	25°			37°			25°			37°		
	Action on milk	NH_2	NH_3	Action on milk	NH_2	NH_3	Action on milk	NH_2	NH_3	Action on milk	NH_2	NH_3
days												
3	None clotted	3.9	4.8	Five clotted	5.3	1.6	None	2.8	4.2	Five clotted		0.8
5	Four tubes clotted, digestion definite	3.9	3.4	Nine clotted	5.7	4.8		4.0	3.6	All clotted	6.9	2.4
7	All, but one, clotted digestion begins	6.5	4.8	Two completely digested	10.8	7.0	Thickening of milk in tubes	5.8	4.8	Digestion rapid		4.8
9	All clotted		3.2	Five digested	16.0	13.2	All clotted	7.2	5.0		7.2	5.6
12	Milk half digested	9.5	7.8	All digested	19.7	8.4	Digestion begins	6.9	7.2		7.7	6.4
15		11.7			19.3	12.0		7.4			9.1	
18	One tube all digested		8.5		23.9	8.6		11.4	5.8		12.0	10.6
23		18.2	9.6		23.8	14.9		11.2	6.4			7.6
28		18.2	10.3		24.6	14.5			6.7		11.2	

*NH_2 and NH_3 designate milligrams of amino and ammonia nitrogen in 10 cc.

SUMMARY

1. The actinomycetes do not fix any atmospheric nitrogen, although some colonies will develop on routine nitrogen free media.

2. Most species are able to reduce nitrates to nitrites with the proper source of carbon, a few species are able to reduce nitrates to nitrites actively with nearly all sources of carbon studied, while a few others give no reduction or only traces with nearly all sources of carbon.

3. The proteins and amino acids studied were found to form the best sources of nitrogen for this group of organisms. Amides are used only to a very small extent. Nitrates are used fairly well in the presence of the proper source of carbon. Nitrites present in small quantities in the medium are utilized well by most species, particularly by those that reduce nitrates actively. Ammonium salts form the poorest sources of nitrogen, with glycerol as a source of carbon; with glucose as a source of carbon both amides and ammonium salts are utilized well as sources of nitrogen, if the reaction of the medium does not tend to become too acid.

4. Most actinomycetes split proteins actively as indicated by an increase of the amino-nitrogen content of the medium. The organisms that produce only a small amount of growth split proteins only to a very limited extent and use up only small quantities of the amino acids.

5. The production of ammonia from proteins and amino acids is not characteristic of this group, although, on continued incubation, considerable quantities of ammonia may accumulate in the medium, as indicated by the growth of the organisms in milk or on pure proteins added to sterilized soil.

6. Many species produce soluble yellow, brown to dark brown pigments in media containing proteins and amino acids, the production of a brown pigment being due, in most cases, not to a tyrosinase reaction. Only some strains of *A. scabies* and a few other chromogenus species are able to produce a soluble brown pigment from tyrosin; most of the species that produce

brown pigments on protein media, even if they do not give the tyrosinase reaction, produce an oxidase.

7. For comparative cultural purposes, a definite incubation period is very important, since two organisms will show a different relationship in their metabolism (splitting of milk in this case) at different periods of incubation. With the prolongation of the period of incubation the difference in the quantity of the products obtained from the splitting of milk will greatly decrease and may, in some cases, almost disappear.

REFERENCES

BEIJERINCK, M. W. 1900 Über Chinonbildung durch *Streptothrix chromogena* und Lebensweise dieses Microben. Centrbl. Bakt., *II* Abt., **6**, 2–12.

BEIJERINCK, M. W. 1913 Tyrosinase from two enzymes. Proc. Akad. Wetenscap Amsterdam, **15**, 932–937; Ref-J. Chem. Soc., **104**, *I*, 683.

CLARK, W. M., AND LUBS, H. A. 1917 The colorimetric determination of hydrogen-ion concentration and its application in bacteriology. Jour. Bact., **2**, 134, 109–136, 191–236.

EMERSON, P. 1917 Are all the soil bacteria and streptothrices that develop on dextrose agar azofiers? Soil Sci., **3**, 417–421.

FOUSEK, A. 1912 Über die Rolle der Streptothricheen im Boden. Mitt. Landw. Lehrkanz. K. K. Hochschule f. Bodenkult., Wien, **1**, 217–244.

GRIESZ, P. 1870 Über Diamidobenzoensaure. Ann. Chem. u. Pharm., **154**.

JOSHI, N. V. 1915 A new nitrite forming organism. Memoirs Dept. Agr. India, Bact. Series, **1**, 85–96.

KLAESER, M. 1914 Die Reduktion von Nitraten zu Nitriten und Ammoniak durch Bakterien. Centrbl. Bakt., *II* Abt., **41**, 365–430.

KRAINSKY, A. 1914 Die Aktinomyceten und ihre Bedeutung in der Natur. Centrbl. Bakt., *II* Abt., **41**, 649–688.

LUTMAN, B. F., AND CUNNINGHAM, C. G. 1914 Potato scab. Bull 184, Vt. Agr. Exp. Sta.

MACÉ, E. 1905 De la decomposition des albuminoides par les Cladothrix (Actinomyces). Compt. Rend. Acad. Sci. (Paris), **141**, 147.

MÜNTER, F. 1913 Über Stickstoffumsetzungen einger Aktinomyceten. Centrbl. Bakt., *II* Abt., **39**, 561–583.

NADSON, G. A. 1903 Microorganisms as geological agents (Russian). From the Works of the investigation of the Slavian Mineral Waters. St. Petersburg.

RULLMAN, W. 1899 Der Einfluss des Laboratoriumsluft bei der Zuchtung von Nitrobakterien. Centrbl. Bakt., *II* Abt., **5**, 713–716.

VAN SLYKE, D. D., AND BIRCHARD, F. J. 1914 The nature of the free amino groups in proteins. Jour. Biol. Chem., **16**, 539–547.

WAKSMAN, S. A. 1918 a The importance of mold action in the soil. Soil Sci., **6**, 137–155.

WAKSMAN, S. A. 1918 b Studies in the metabolism of actinomycetes. Jour. Bact., **4**, 189, 307.

WAKSMAN, S. A., AND CURTIS, R. E. 1916 The actinomyces of the soil. Soil. Sci., **1**, 99–134.