On the Biosynthesis and Variability of Respiratory Enzymes in *Azotobacter vinelandii*

By

KIMIKO SASAKI

(Formerly KIMIKO MAEDA)

Department of Botany, Faculty of Science
Hokkaido University, Sapporo, Japan

(With 10 text-figures, 43 tables)

CONTENTS

[Journal of the Faculty of Science, Hokkaido University, Ser. V, Vol. VIII, No. 1, 1960]

INTRODUCTION

Metabolic processes concerned with growth, adaptation, mutation, or variation should be studied in detail from the view point of physiology.

In this laboratory, several experiments have been concerned with the problem of the mechanism of growth, enzyme biosynthesis, and of the enzymic variation in *Azotobacter* (1, 2) or other organisms.

Azotobacter has been considered to be an important microorganism in the soil enrichment owing to its active aerobic nitrogen fixation (3–6). This organism has been concerned with the studies of mechanism of nitrogen fixation (7–17) and of the biochemical and biophysical properties of important enzymes because of its activity in attacking numerous substances (18–52). Furthermore, this organism is an interesting microbe owing to the favourable variability (53–55, 57–59). The capacity of nitrogen fixation (25–99) and formation of a nitrogen fixing enzyme system is affected by different conditions (25–29, 33, 34). The morphological (53) and physiological (54) properties of this organism are variable with aging or different cultural conditions. It is interesting that the origin of this organism is discussed in the comparative investigations. For example, THIMANN (60) stated that *Azotobacter* is a "colorless blue-green algae". This statement arose from the morphological and physiological comparison which shows that the morphology of *Azotobacter* resembles that of the small blue-green algae, such as *Chroöcoccus dispersus*, more than any other bacteria and that its nitrogen fixing properties also resemble those of the blue-greens. The respective data indicate the favourable metabolic variability of this organism,

which, probably, controls the morphological and physiological variability. Then, *Azotobacter* offers an unique opportunity for the investigation of processes of formation and variation of enzymes.

The present paper is concerned with detailed investigations of the biosynthesis and variability of respiratory enzymes with *Azotobacter vinelandii*. Particular attention is directed to the essential conditions for enzymic adaptation and its variation, and to the investigations of the nature of the enzyme system involved in the biosynthesis and variation of respiratory enzymes.

EXPERIMENTAL METHODS

*Azotobacter vinelandii**) was used through these studies. The organisms have been maintained in our laboratory on agar slants containing inorganic salts and mannit without any nitrogen compounds.

CULTURE METHOD. Nitrogen compound-free liquid medium containing mannit and inorganic salts was used as a basal medium (61).

A medium modified from one described above was also used, in which mannit was replaced by glucose (2%), succinate (2×10^{-2}M.), or others. In another case, KNO_3 (1×10^{-2}M.), NH_4Cl (1×10^{-2}M.), L-leucine (0.2%), or pepton (1%) respectively was added in the basal medium as the combined nitrogen source. In this case, the media were named respectively: N_2-, KNO_3-, NH_4Cl-, L-, and P-medium, and each of the cells grown in the above listed media were named respectively as N_2-, KNO_3-, NH_4Cl-, L-, and P-cells. The solid medium contained 2% of agar.

Unless otherwise stated, inoculum was incubated in 250 ml. Erlenmeyer flasks containing 25 ml. of liquid medium at 30°C. for one day (NH_4Cl- or P-medium) or three days (N_2-, KNO_3-, or L-medium).

PREPARATION OF CELL SUSPENSION. The organisms grown in the above mentioned media were harvested by centrifugation, washed with deionized water three times, and suspended in the deionized water to yield a suspension containing 1 to 2 mg. dry weight of cells per ml.

PREPARATION OF CELL-FREE EXTRACT. All procedures were carried out at a temperature of 0°C to 4°C. After the disruption of washed cells by grinding with glass powder, quartz sand, or alumina powder, in 2×10^{-2}M.

*) This organism was kindly presented by Prof. Y. SASAKI of the Faculty of Agriculture of this University.

phosphate buffer (pH 7.2), homogenate was centrifuged at 6,000 to 8,000 r. p. m. for 5 to 10 minutes to remove homogenizing powder and intact cells. The supernatant (S 1) was centrifuged again at 10,000 r. p. m. for 5 to 10 minutes to separate the remaining intact cells and cell debris. The resulted supernatant (S 2) was centrifuged at 16,000 r. p. m. for 10 minutes yielding a brown sediment (R 1) and an opalescent supernatant (S3).

MANOMETRIC DETERMINATION. The rate of oxygen uptake in air was determined by the conventional techniques of Warburg respirometer at 30°C. Reaction times were 60 and 20 minutes with the cells and cell-free extract, respectively. The activity of enzymes was expressed as Q_{O_2} or μl oxygen consumed per mg. protein in cell-extract per hour.

For assay of oxidative enzyme, Warburg vessels contained 1 mg. of cells or protein in cell-extract, 0.2 ml. of 5×10^{-1}M. phosphate buffer, pH 7.2, 0.2 ml. of 2×10^{-1}M. substrate, in a total volume of 2.0 ml. Inhibitor was placed in side-arm of Warburg vessel together with substrate.

For assay of dehydrogenase, Warburg vessels contained 2×10^{-4}M. methylene blue or 1×10^{-3}M. 2,6-dichlorophenolindophenol and 1×10^{-2}M. potassium cyanide together with the above listed compounds for assay of oxidative enzyme (63, 66, 67).

For assay of cytochrome c oxidase, Warburg vessels contained 1 mg. of cells or protein in cell-extract, 0.2 ml. of 5×10^{-1}M. phosphate buffer, pH 7.2, 0.2 ml. of 1×10^{-1}M. hydroquinone or *p*-phenylenediamine, and 0.8 to 1.0 mg. of yeast cytochrome c, in a total volume of 2.0 ml.

THUNBERG METHOD FOR DETERMINATION OF DEHYDROGENASE ACTIVITY. Thunberg tubes contained 1 to 2 mg. of resting cells or protein in cell-extract, 0.5 ml. of 2.5×10^{-1}M. phosphate buffer, pH 7.2, 0.25 ml. of 2×10^{-1}M. substrate, and 0.25 ml. of 1×10^{-3}M. methylene blue or 2×10^{-3}M. 2,6-dichlorophenolindophenol, in a total volume of 2.5 ml. After evacuation, the times for complete decoloration were determined at 30°C. The activity of dehydrogenase was expressed as Q_{Dye}, μ moles of dye reduced per mg. of dry cells or protein in cell-extract per hour (62).

SPECTROPHOTOMETRIC DETERMINATIONS. For estimation of cytochrome system of cell suspension or cell-extract, absorption spectra in wave-length range 400 to 700 mμ under reduced and oxidized conditions were observed with SHIMAZU's spectrophotometer Type QB-50. The differences between the optical dencity of the reduced state and that of the oxidized state of cytochromes were determined at 1 to 10 mμ intervals over the desired spectral range. The differences, plotted against wave-length, yield the

difference spectra of cytochrome system. Sodium dithionite as a reductant and air or hydrogen peroxide solution as an oxidant were used conveniently (68).

Quantitative determination of cytochrome c oxidase was made on cell suspension or cell-extract by means of measurement of decreased value of absorption at 550 mμ related to reduced cytochrome c in the presence of air. In this case, the reaction cuvettes contained 1 mg. of resting cells or 1 to 5 mg. of protein in cell-extract, 0.6ml. of 2.5×10^{-1}M. phesphate buffer, pH 7.2, and 0.8 to 1.0 mg. of reduced cytochrome c, in a total volume of 3.0 ml. Reaction time was 3 minutes under room temperature of about 20°C. (40).

For dehydrogenase assay by ferricyanide-method, reaction media contained 1 mg. of resting cells or 1 to 5 mg. of protein in cell-extract, 0.5 ml. of 2.5×10^{-1}M. phosphate buffer, pH 7.2, 0.25 ml. of 2×10^{-1}M. substrate, 0.25 ml. of 1×10^{-2}M. ferricyanide, and 0.25 ml. of 1×10^{-1}M. potassium cyanide, in a total volume of 2.5 ml. These reaction mixtures were incubated at room temerature for 2 to 4 hours. Then, the amounts of non-reduced ferricyanide were determined spectrophotometrically. The amounts of reduced ferricyanide were calculated from the decreased value of absorption at 400 mμ wave-length related with ferricyanide, and then dehydrogenase activity was expressed as Q_{Fe}, μ moles ferricyanide reduced per mg. dry cells or protein in cell-extract per hour (63, 66, 67).

PAPER CHROMATOGRAPHY. For estimation of flavin compounds, *n*- or *iso*- butanol/acetic acid/water (4 : 1 : 5, v/v) was used as solvent. Filter paper, TOYO's No. 2, was plotted with samples or standard solutions, RF, FMN, and FAD water solutions (68).

PREPARATION OF CYTOCHROME **c** FROM BAKER'S YEAST. Yeast cytochrome c was prepared by the method of HAGIHARA *et al.* (69). The crude cytochrome c preparation obtained by this procedure contained 0.8 mg. of cytochrome c per ml.

PROCEDURE FOR INDUCED BIOSYNTHESIS OF RESPIRATORY ENZYMES. Warburg vessels contained 1 to 10 mg. of resting cells, 0.4 ml. of 2.5×10^{-1}M. phosphate buffer, pH 7.2, and 0.2 ml. of 2×10^{-1}M. or 1×10^{-2}M. substrate as a inducer, in a total volume of 2.0 ml. Another vessels contained inhibitor together with substrate. The above reaction mixtures were incubated at 30°C. under continuous aeration or under other conditions for 1 to 4 hours. The cells treated were separated from the reaction mixture by centrifugation, washed with deionized water twice, suspended in the same water, and then employed for the measurement of activity of oxidative enzymes. The cells treated by substrate were named as induced cells. The cells treated by glucose,

succinate, acetate, lactate, or others were named as G-, S-, A-, L-cells and so on, respectively. The cells treated by air alone, with endogenous substances, were named as endogenous cells, E-cells, and the nontreated cells as initial cells, I-cells. When the substrate-treated cells could oxidize the substrate more actively than the nontreated cells, it is probable that the induced synthesis of enzymes concerned with these oxidations could occur during the treatment by substrate in the resting cells.

DETERMINATION OF PHOSPHATE. Phosphate was determined by the methods of ALLEN (70) on growing medium separated from cells.

DETERMINATION OF PROTEIN IN THE CELL-FREE ENZYME SOLUTION. Protein was determined by the method of NEILSON (71).

EXPERIMENTAL RESULTS

I. EFFECTS OF DIFFERENT GROWTH CONDITIONS ON ENZYME CONSTITUTIONS

A. EFFECTS OF DIFFERENT CARBON SOURCES

(1) Extracellular Substance(s) which Stimulate the Biosynthesis of Oxidative Enzymes during Growth.

The earlier observations on *Azotobacter vinelandii* (1) indicated that fundamental differnce of constitutions of main respiratory enzymes does not occur between the cells which were grown in the medium which contained mannit or glucose.

In the present paper, the activities of the oxidation of mannit, glucose, and succinate were observed in cells grown in the medium having mannit, succinate, or mannit plus succinate as the carbon source. As seen in Table 1, high active succinic oxidase was found in the cells grown in the mannit-plus-succinate-containing medium, while active growth took place in the mannit-containing medium. The previous studies on the optimum pH for the formation of succinic oxidase indicated that succinic oxidase was formed favourably at 5.0 to 7.8 of pH. In the medium contained succinate, pH rose to the inhibitory level of 8.0 during the earlier growth stage owing to the consumption of succinic acid. Thus, it was evident that the formation of succinic oxidase during the

The following abreviations are used throught this study: MIA, monoiodoacetic acid; DNP, 2,4-dinitrophenol; Mb, methylene blue; 2,6-DCPIP, 2,6-dichlorophenolindophenol; RF, riboflavin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; RNA, ribonucleic acid; DNA, desoxribonucleic acid.

TABLE 1. Effects of succinate on enzyme synthesis during growth

Warburg evssels contained 0.3 mg. of resting M-, MS-, or S-cells, 0.4 ml. of 2.5×10^{-1}M. phosphate buffer, pH 7.2, 0.2 ml. of 2×10^{-1}M. succinate, glucose, or mannit, in a total volume of 2.0 ml.

	Culture conditions			Qo_2		
	Carbon source	Age (days)	Growth (mg. dry cells/25ml.)	Succinate	Glucose	Mannit
(1)	Mannit	2	1	16 23* 97**	41	57
(2)	"	3	14	64	113	486
(3)	Mannit+ Succinate	2	1	690	297	310
(4)	"	3	2	938	26	
(5)	Succinate	2	1	10		
(6)	"	3	1	92		

* or ** The value was obtained by measurement of O_2 uptake in presence of succinate plus heated--(*) or nonheated--(**) supernatant of MS-cells respectively.

growth was stimulated by succinate. Therefore, it was assumed to be a possibility that succinic oxidase, which is known as a constitutive enzyme, might be an induced enzyme in *Azotobacter vinelandii*.

(2) Intracellular Substance(s) which Stimulate the Enzyme Activity.

After the treatment with succinate at 30°C. for an hour under continuous aeration, the resting MS-cells, which could grow in the mannit-and-succinate-containing medium, were separated from the medium by centrifugation at 10,000 r.p.m. to yield the cell-free supernatant. The supernatant heated at 100°C. or nonheated was added to the reaction medium for the measurement of succinic oxidase activity of M-cells, which were grown in the mannit-containing medium. As seen in Table 1, the nonheated supernatant could stimulate the oxidation of succinate by the M-cells, remarkably. The MS-cells, which were separated from the reaction medium, were resuspended in deionized water, heated at 100°C. for 10 minutes, and then centrifuged at 10,000 r.p.m. for 10 minutes to yield the cell-extract. The clear cell-extract was added to the reaction medium for the measurement of succinic oxidase activity of M-cells. As seen in Fig. 1 c, the M-cells could show active oxidation of succinate in the presence of MS-cell-extract in as great degree as the MS-cells. For example, the former cells after 110 minutes of reaction time and the latter cells after 40 minutes could show the same maximum activity of succinic oxidase, while the M-cells

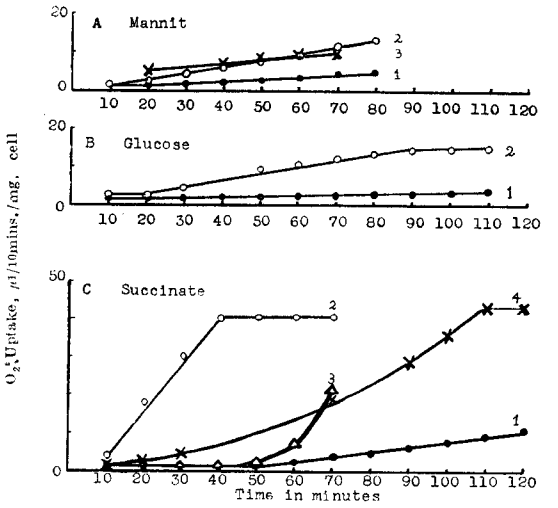

Fig. 1. Time courses of oxidation of mannit (A), glucose (B), and succinate (C) by M-, MS-, and S-cells. Curve 1, M-cells; Curve 2, MS-cells; Curve 3, S-cells; Curve 4, M-cells in the presence of MS-cell-extract. Assay conditions were listed under "Experimental Methods".

in the absence of extract could not show remarkable activity. Therefore, it was assumed to be possible that the cells grown in the succinate-containing medium could form substance(s) which stimulate the succinic oxidase activity or its induced synthesis which was suggested by the increasing oxidation of succinate under the resting state.

B. EFFECTS OF VARIOUS NITROGEN SOURCES

In the previous paper (1), it was shown that the activities of the main respiratory enzymes of *Azotobacter vinelandii* varied with the culture age, for example, the high active oxygen uptake in the presence of various substrates was found at the late lag or initial log phase of growth in the N_2-medium, while such uptake occured at the log phase in the NH_4Cl and pepton media. As seen in Table 2, the constitution of the main respiratory enzymes was not different between the N_2-, KNO_3-, NH_4Cl- and pepton-cells, except that amino acids were not oxidized or were oxidized at a low level by the N_2-cells. As indicated in Fig. 2 and 3, the time courses of oxidation of substrates by the N_2- and KNO_3-cells are represented by increasing curves, while those by the NH_4Cl- and pepton-cells are constant from the start.

TABLE 2. Activities of oxidative enzymes of N_2-, KNO_3-, NH_4Cl-, L-, and P-cells

Conditions of cultivation of N_2-cells or other cells and assay procedures were described under "Experimental Methods.

Culture conditions	Nitrogen source	N_2	KNO_3	NH_4Cl	Leucine	Peptone	
	Age (days)	3	2	2	3	0.5	1
	Growth*	26	41	41	14	25	39
Q_{O_2} with	Glucose	148	41	56	40	32	37
	Succinate	293	36	61	65	140	62
	Acetate	133	60	136		87	38
	Lactate	246	51	127		106	47
	Ascorbate	79				73	72
	L-Leucine	±	±	28	41	90	40
	L-Tyrosine	±	±			118	40
	L-Glutamate	±	±		90		
	L-Aspartate	±	±		91		

* Growth rate, mg. dry cells/25 ml.
± Trace or absence of oxidiative activity.

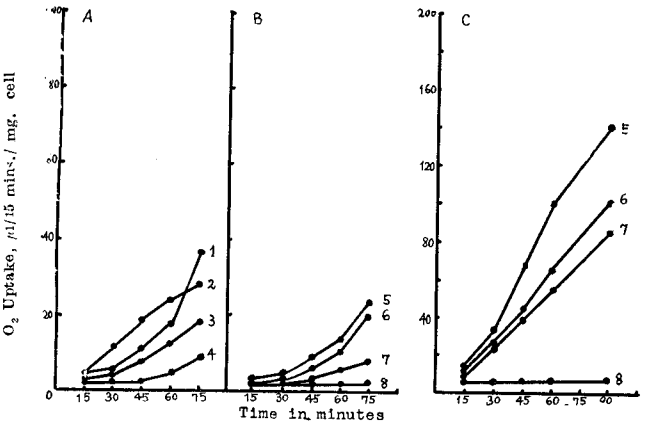

Fig. 2. Time courses of oxidation of various substrates by resting N_2-cells. Assay conditions were already described under "Experimental Methods". A and B, nontreated initial cells; C, succinate-treated cells. Curve 1, acetate; 2, ethyl alcohol; 3, glucose; 4, mannit; 5, malate; 6, succinate; 7, fumarate; 8, citrate.

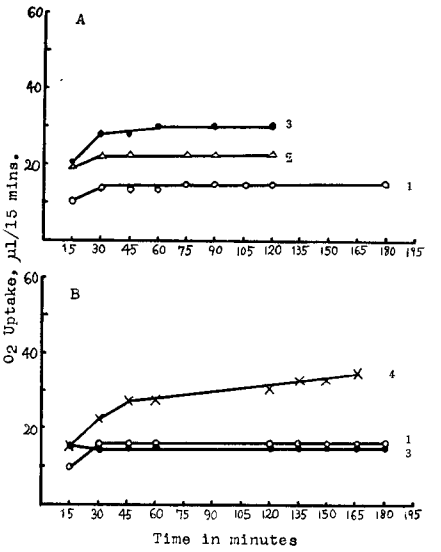

Fig. 3. Time courses of oxidation of succinate, acetate, glucose, or L-leucine by NH_4Cl- and P-cells. Expt. A., resting NH_4Cl-cells, 1.2 mg.; Expt. B., resting P-cells, 6.0 mg. Curve 1, succinate; Curve 2, acetate; Curve 3, glucose; Curve 4, L-leucine.

C. EFFECTS OF DIFFERENT AMOUNTS OF PHOSPHATE

(1) Relationships between Growth, Formation of Respiratory Enzymes, and Assimilation of Phosphate.

N_2-cells were inoculated into N_2-, KNO_3-, NH_4Cl-, and pepton-media, containing phosphate of 1×10^{-6} to 5×10^{-2} M. of final concentrations.

N_2-MEDIUM. As seen in Table 3, the concentration of phosphate lower and higher than the optimum, 1×10^{-4} to 1×10^{-3} M., brought about poor growth. According as the increase of amounts of phosphate in the growing medium the amounts of phosphate assimilated by the cells were increased. The growing medium, glucose, succinate, acetate, or lactate was oxidized rather actively by the cells grown in the phosphate-rich media. In the 1×10^{-6} M. phosphate medium, the uptake of phosphate and the increase of respiratory activity by the growing cells lasted for 1 day of cultivation, while the growth proceeded for 3 days. In the 2×10^{-2} M. phosphate medium., the phosphate uptake by the cells lasted for 2 days of cultivation, whilst the growth rate and the activities of respiratory enzymes increased for 7 days, except for the acetate and lactate oxidizing enzymes. In the medium which contained the suitable amounts of

phosphate, the growth, the formation of respiratory enzymes, and the assimilation of phosphate were increased in parallel with the growth, though these enzyme activities were declined before the stop of growth.

TABLE 3. Effects of different amounts of phosphate on growth, formation of respiratory enzymes, and assimilation of phosphate by N_2-cells

Methods of cultivation of N_2-cells and assay procedures were already described under "Experimental Methods".

No.	Age (days)	Phosphate presented (M.)	Phosphate consumed (μ moles,) (/25 ml.)	Phosphate consumed (μ moles,) (/mg. dry cells)	Growth (mg. dry cells/ 25 ml.)	Q_{O_2} M*.	G.	S.	A.	L.
(1)	2	1×10^{-6}	0.32	0.03	10	47	26	31	51	41
		1×10^{-5}	3.20	0.16	20	214	20	52	32	40
		1×10^{-4}	2.50	0.10	25	193				
		1×10^{-3}	22.50	0.98	23	167				
		1×10^{-2}	43.75	3.12	14	159				
		2×10^{-2}	40.00	4.00	10	135	29	35	103	119
(2)	3	1×10^{-6}	0.32	0.03	13	20	23	1	43	32
		1×10^{-5}	3.20	0.11	24	154	72	64	108	98
		1×10^{-4}	16.38	0.53	33	250	60	32	81	55
		1×10^{-3}	65.00	1.57	35	231	37	30	55	169
		1×10^{-2}	43.50							
		2×10^{-2}	40.00	2.50	16	170	76	122	73	63

* M., medium in which cells were grown for 2 or 3 days (see the text); G., glucose; S., succinate; A., acetate; L., lactate.

Thus, the possibility was suggested that the phosphate uptake during the earlier growth stage was important for the further growth and the synthesis of enzymes, especially under the condition of unsuitable amounts of phosphate.

NH_4Cl-MEDIUM. The optimum concentration of phosphate for growth was 1×10^{-2} to 2×10^{-2} M. In the phosphate-rich medium, 2×10^{-2} M., the growth and medium oxidizing activity were limited on a low level. Therefore, it was indicated that NH_4Cl-cells were more resistant to large amounts of phosphate than N_2-cells (Table 4).

TABLE 4. Effects of different amounts of phosphate on growth and formation of respiratory enzymes of NH_4Cl-cells

Methods of cultivation of NH_4Cl-cells and assay procedures were the same as in Table 3.

Phosphate		Growth	Qo_2 *					
presented (M.)	remained	(mg. cells/ 25 ml.	M.	G.	S.	A.	L.	Leu.
1×10^{-5}	–	10	22	17	19	138	—	59
1×10^{-4}	–	19	30	24	23	27	25	3
1×10^{-3}	+	19	44	—	—	—	—	—
1×10^{-2}	+	34	77	68	23	49	38	14
2×10^{-2}	+	37	87	90	30	66	50	28
5×10^{-2}	+	22	72	56	61	30	—	—

* M., growing medium; G., glucose; S., succinate; A., acetate; L., lactate; leu., L-leucine.

LEUCINE-MEDIUM. The optimum concentration of phosphate for growth was 1×10^{-2} to 5×10^{-2} M. In the concentration of $1\times10^{-4\text{ and }-5}$ M. phosphate, the cells consumed all phosphate in the media within 2 days of cultivation, while the growth rate and activitiy of the oxidation of succinate were increased

TABLE 5. Effects of different amounts of phosphate on growth and formation of respiratory enzymes of L-cells

Methods of cultivation of L-cells and assay procedures were the same as in Table 3.

Age	Phosphate		Growth	Qo_2			
(days)	presented (M.)	remained	(mg. cells/ 25 ml.)	M.*	G.	S.	Leu.
2	1×10^{-5}	–	6	49	11	10	27
	1×10^{-4}	–	6	6	13	12	18
	1×10^{-3}	+	8	10	13	—	7
	1×10^{-2}	+	12	42	3	8	13
	5×10^{-2}	+	17	18	6	6	10
3	1×10^{-5}	–	18	17	0	18	8
	1×10^{-4}	–	18	+ 3	1	18	15
	1×10^{-3}	+	17	4	—	—	—
	1×10^{-2}	+	17	7	12	21	16
	5×10^{-2}	+	21	9	11	15	24

* M., growing medium; G., glucose; S., succinate; Leu., L-leucine.

for 3 days. In accordance with the increase of concentration of phosphate in the growing medium the growth rate increased. The activity of oxidation of glucose, succinate, or L-leucine was increased with the aging of culture in the phosphate-rich medium (Table 5).

The growing medium and L-leucine were oxidized by the cells grown in phosphate-poor medium more rapidly than by the cells grown in phosphate-rich medium at the earlier growth stage. Thus, it was assumed that the deficiency of phosphate in the growing medium could bring about the favourable formation of L-leucine oxidizing enzyme. As described in the previous paper (2), it was assumed that there was some competition with relation to the formation of L-leucine and glucose oxidizing enzymes in phosphate-poor medium.

(2) Effects of Suitable Amounts of Phosphate on the Respiration of Growing Cells.

In the N_2-medium, the addition of phosphate buffer, pH 7.2, at the final concentration of 1×10^{-2}M. to the 1×10^{-5}M. phosphate-medium of 2 or 3 days' age could stimulate the oxygen uptake by the cells. The addition of it to the 1×10^{-2}M. phosphate medium of 3 days' age depressed the oxygen uptake by the cells, so that the higher concentration of phosphate than 1×10^{-2}M. could depress the respiratory activity of growing cells (Table 6).

TABLE 6. Effects of 1×10^{-2}M. phosphate buffer on respiratory activity of growing N_2-cells

Methods of cultivation of N_2-cells and assay procedures were the same as in Table 3.

Culture conditions		Qo_2	
Age (days)	Phosphate (M.)	−	+*
2	1×10^{-5}	47	94
	1×10^{-4}	214	189
	1×10^{-3}	193	184
	1×10^{-2}	87	51
3	1×10^{-5}	20	38
	1×10^{-4}	154	174
	1×10^{-3}	250	335
	1×10^{-2}	235	207

* Presence (+) or absence (−) of 1×10^{-2}M. phosphate buffer, pH 7.2.

(3) Effects of Large Amounts of Phosphate on the Respiration of Growing Cells.

In the N_2-medium, the addition of phosphate buffer, pH 7.2, at the final concentration of 5×10^{-2} M. inhibited the oxygen uptake by the cells in the 5×10^{-4} to 1×10^{-2} M. phosphate media; in the NH_4Cl-medium, the addition of the same concentration of phosphate could stimulate the respiratory activity of the cells (Table 7).

TABLE 7. Effect of 5×10^{-2}M. phosphate buffer on respiratory activity of growing N_2- or NH_4Cl-cells

Methods of cultivation of N_2- or NH_4Cl-cells and assay procedures were the same described in Table 3.

Growing conditions			Qo_2	
Nitrogen	Age (days)	Phosphate (M.)	−	+*
N_2	3	5×10^{-4}	126	107
		1×10^{-3}	137	139
		5×10^{-3}	176	133
		1×10^{-2}	136	122
NH_4Cl	1	1×10^{-4}	30	47
		1×10^{-3}	44	
		1×10^{-2}	77	86
		2×10^{-2}	87	97
		5×10^{-2}	72	106

* Addition of phosphate buffer, at a final concentration of 5×10^{-2} M. pH 7.2, in the media in which cells were grown.

As seen in Table 8, the respiratory activity of young cells in the N_2- or KNO_3-medium contained 1×10^{-3} M. phosphate was inhibited by the addition of large amounts of phosphate, while the older cells were stimulated by it. On the other hand, the respiratory activity of NH_4Cl-cells in the 1×10^{-3} M. phosphate medium was not inhibited by the large amounts of phosphate in any age. Therefore, it was assumed that the NH_4Cl-cells were more resistant to the presence of large amounts of phosphate than the N_2- or KNO_3-cells.

(4) Effects of Large Amounts of Phosphate on the Activities of Oxidative Enzymes of Resting Cells.

As seen in Table 9, the activity of the oxidation of glucose or succinate by the resting N_2-cells was depressed by the 5×10^{-2}M. phosphate buffer in comparison

TABLE 8. Effects of phosphate buffer on respiratory activity of growing cells of varying curture age

Conditions of cultivation of N_2-, KNO_3-, or NH_4Cl-cells were described under "Experimental Methods". Concentration of phosphate in each medium, 1×10^{-3} M. Assay conditions of respiratory activity of growing cells were the same described in Table 3.

Growing conditions			Qo_2	
Nitrogen	Age (days)	Growth (mg. dry cells/25 ml.)	−	+*
N_2	1	20	33	21
	2	23	57	33
	3	45	60	80
	4	83	48	53
	8	120	55	70
KNO_3	1	19	46	38
	2	47	53	38
	3	48	83	85
	4	75	48	54
	8	123	30	35
NH_4Cl	1	32	52	51
	2	47	48	54
	3	47	92	125
	4	137	145	145
	8	105	0.2	0.1

* Presence (+) or absence (−) of 5×10^{-2} M. phosphate buffer, pH 7.2, in the media in which cells were grown.

TABLE 9. Effects of concentrated phosphate buffer on activities of several respiratory enzymes of resting N_2-cells

Warburg vessels contained 1.0 mg. of resting N_2-cells, 0.4 0.16. or 0.08 ml. of 2.5×10^{-1} M. phosphate buffer, pH 7.2, 0.2 ml. of 2×10^{-1} M. glucose, succinate, acetate, or lactate, in a total volume of 2.0 ml.

Phosphate buffer (M.)	Qo_2			
	Glucose	Succinate	Acetate	Lactate
1×10^{-2} M.	23	31	53	58
2×10^{-2} M.	22	—	—	—
5×10^{-2} M.	16	21	59	84

with the control, 1×10^{-2} M., while the acetate and lactate oxidizing activities of these cells were stimulated by it. Then, glucose and succinate oxidizing enzymes were more sensitive to the large amounts of phosphate than are the acetate and lactate oxidizing enzymes.

D. EFFECTS OF VARIATIONS IN GROWTH TEMPERATURE

The effects of temperatures of 30°, 15° amd 3°C. on the growth and enzyme formation were observed on the cells grown in the different nitrogen-source-containing media.

(1) Temperature of the growth.

Azotobacter vinelandii could grow at 30°, 15° and 3°C. in the pepton medium, but not in the N_2-, KNO_3-, and NH_4Cl-media at 15° and 3°C. (54).

(2) Effects of the Low Temperature Treatment.

Each medium supplied with the different nitrogen sources was kept at low temperature (3°C.) for 1 to 30 days after the inoculation of the N_2-cells, before the cultivation at 30 C. for 1 (pepton-medium) or 3 days (N_2-medium), and then the activities of the oxidative enzymes and of the growth of cells were measured, in comparison with corresponding activities of the nontreated control cells.

N_2-MEDIUM. Measurements were made of the activities of growth, com-

TABLE 10. Effects of treatment by low temperature (3°C.) on growth and formation of oxidative enzymes of N_2-cells

Conditions of treatment by 3°C. and of assay procedures were described in the text.

3°C.-treatment (days)		0	10	15	20	25	30
Culture medium	final pH	6.6	5.8	5.8	5.7	7.0	7.0
	growth*	34	30	24	35	34	34
Qo_2 with	Glucose	149	249	150	98	230	149
	Succinate	233	218	68	189	110	128
	Acetate	133	375	310	352	110	38
	Lactate	246	37	120	338	49	224
	Ascorbate	79	15	2	17	63	66
	Oxalate	-	19	37	40	43	41
	Formate	21	228	20	30	130	24
	L-leucine	0	24	28	2	43	120
	L-tyrosine	0	2	5	3	33	27
	None	25	30	17	0	0	3

* Growth rate, mg. dry cells/25 ml.

plete oxidation of 1×10^{-3} M. glucose or succinate, and the oxidation rate of glucose, succinate, ethyl alcohol, acetate, lactate, L-leucine, L-tyrosine, oxalate, or formate by the resting cells, which were treated at 3° C. for 5 to 30 days. The activities of growth and complete oxidation of glucose or succinate by cells which were treated for 5 to 15 or 20 days were lower than those of the nontreated control cells (Tables 10 and 11). On the other hand, these activities of cells which were treated for more than 25 days could be shown to be at as high level as the control cells. L-leucine and L-tyrosine were oxidized actively by the cells treated at 3°C. for 25 to 30 days The formation of succinic oxidase by the treatment with succinate was depressed by the 3 C. treatment for 14 days and restored by the 17 days-treatment (Table 12). The same treatment after the 30°C. cultivation for 3 days brought about remarkable depression in the formation of succinic oxidase.

TABLE 11. Effects of treatment by low temperature (3°C.) on activity of complete oxidation of glucose or succinate by N_2-cells

Conditions of 3°C.-treatment of resting cells were the same as in Table 10.
Conditions of complete oxidation: Resting N_2-cells, 1.0 mg.; 2.5×10^{-1} M. phosphate buffer, pH 7.2, 0.4 ml.; 1×10^{-2} M. glucose or succinate, 0.2 ml.; total volume, 2.0 ml.; temperature, 30°C.

3°C.-treatment (days)	0	10	15	20	25
Growth (mg. cells/25 ml.)	33	30	26	20	42
Complete oxidation ($\mu l.$, total O_2 uptake)					
Succinate	132	70	42	6	205
Glucose	361	129	210	+2	267

TABLE 12. Effects of treatment by low temperature (3°C.) on activity of induced synthesis of succinic oxidase system by N_2-cells

Conditions of enzyme synthesis and assay procedures were described under "Experimental Methods".

	Activity of succinic oxidase (Qo_2)			
3°C.-treatment (days)	0	14	17	17*
Before **	95	80	50	52
After	357	144	227	26

* Cells treated by 3°C. after cultivation for 3 days at 30°C.
** Before or after treatment with succinate.

PEPTON-MEDIUM. The growth rate was depressed by the 3°C. treatment for 15 to 20 days, whilst the activities of the oxidation of L-tyrosine and L-leucine by resting cells were strongly stimulated by the 3°C treatment. The growth could be reactivated by treatment for longer than 25 days and then the cells could grow at 3°C. (Table 13).

TABLE 13. Effects of treatment by low temperature (3°C.) on growth and constitution of enzymes of P-cells

Conditions of treatment by 3°C. and assay procedures were described in the text.

3°C.-treatment (days)		0	5	10	15	20	25	31	41
Growth (mg. cells/25 ml.)		54	53	55	17	15	50	50	50
Q_{O_2} with	Glucose	34	13	23	36	40	26	30	30
	Succinate	41	36	48	61	257	60	44	61
	Acetate	32	41	27	22	104	14	14	108
	Lactate	30	38	58	53	70	67	82	48
	Ascorbate	29	44	11	16	16			48
	Oxalate	16	16	20	15	55		29	35
	Formate	14	33	26	—	72		75	36
	L-leucine	62	10	4	7	18	90	69	120
	L-tyrosine	39	20	54	127	248	174	220	146
	None	5	3	8	9	26		17	

Accordingly, the possibility was assumed that 3°C. treatment for the longer period causes the development of the low-temperature-resistant cells which could oxidize amino acid actively.

II. VARIATION OF GROWING PROPERTY INDUCED BY TREATMENT AT LOW TEMPERATURE

A. CONVERSION OF PEPTONE-CELLS TO N_2-CELLS

(1) Conversion of 30°C.- and 3°C.-grown Peptone- to N_2-cells.

As seen in Table 14, the 3°C.-treated P-cells (for 41 days) could grow in the N_2-medium to the nearly same level as the control N_2-cells, while the nontreated P-cells could not after 35 days of cultivation at 30°C(2).

Therefore, it was suggested that 3°C.-treated P-cells contained some substance(s) concerned with the conversion, and that the favourable growth in the pepton medium at 30°C. brought about the inability of growth in the N_2-medium which might depend on the inability of assimilation of molecular nitrogen.

Thus, the pre-treatment of inoculum P-cells at 3°C. was useful for the growth in N_2-medium.

TABLE 14. Effects of intracellular substances of N_2-cells on conversion of P- to N_2-cells

Assay methods and conditions of 3°C. treatment of P-cells are listed in the text.

Cells inoculated in N_2-medium (ml.)*	Age (days)	Growth (mg. cells/25 ml.)	
		−	+**
3°C. treated P-cells (0.1)	6	28	43
" (")	8	40	46
" (0.5)	6	30	37
" (")	8	39	34
Nontreated P-cells (0.1)	35	Trace	Trace
" (0.5)	35	"	"
Control N_2-cells (0.1)	3	47	

* Cell suspension.
** Addition of extract of heated control N_2-cells.

(2) Conversion of 15°C. grown Peptone- to N_2-Cells, and the Effect of Mannit on this Conversion.

N_2-cells inoculated in the pepton (1%) or pepton (1%) plus mannit (2%) medium (P- or PM-medium) were cultivated at 15° and 30°C. for different number of days as indicated in Fig. 4 and 5, and then the grown cells were re-inoculated in the N_2-media for the observation of growing capacity. The cells grown at 30°C. in the P- and PM-media were named PH- and PMH-cells, and the cells grown at 15°C. in these media, PL- and PML-cells, respectively. Then, the cells which grew as a result of the inoculation of N_2-cells in these media were named 1-P- and 1-PM-cells; the cells

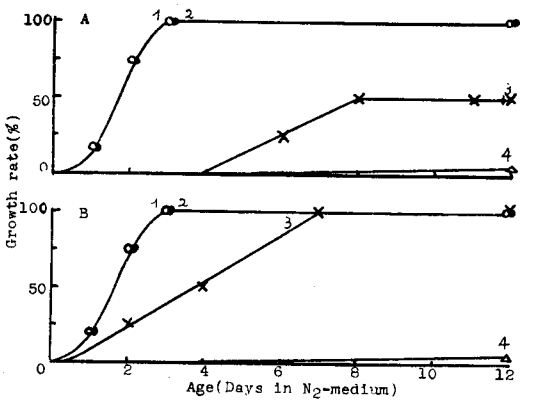

Fig. 4. Growth rate of PH-(A) and PMH-cells (B) in N_2-medium. Assay methods are described in the text. Curve 1, growth by inoculation of control N_2-cells (100%); Curve 2, 1-PH- or 1-PMH-cells; Curve 3, 2-PH- or 2-PML-cells; Curve 4, 3-PH- or 3-PML-cells.

which grew as a result of the inoculation of 1-P- and 1-PM-cells, 2-P- and 2-PM-cells, respectively, and so on.

The growth rate of PL-cells was lower than that of the PH-cells, because 15°C. was not optimum for the growth. The growth rate of 2-PL-cells was higher than that of 1-PL-cells, for example, the same maximum growth occurred in the former cells after 2 days' age and in the latter after 6 days respectively. Therefore, it was assumed that 2-PL-cells could be rendered adaptable to the low temperature.

As seen in Fig. 4, 1 and 2 days' age 1-PH- and 1-PMH-cells could grow in the N-medium as well as the control N_2-cells, while 5 days' age 1-cells could not grow in this medium. Two days' age 2-PH-cells were able to grow poorly in the N_2-medium after the lag time of 4 days, while the 2 days' age 2-PMH-cells could grow at the same maximum level as the control N_2-cells. Two days' age 3- to 6-PH-cells and PMH-cells could not grow in the N_2-medium.

As seen in Fig. 5, 16- days' age 1-PL- and 1-PML-cells or 2-days' age 2-PL- and 2-PML-cells grew at the same level as the control cells, while 21 days' age 1-PL- cells, 25 days' age 1-PML-cells, 2 days' age 5- to 9-PL- and PML-cells could not at all. In this

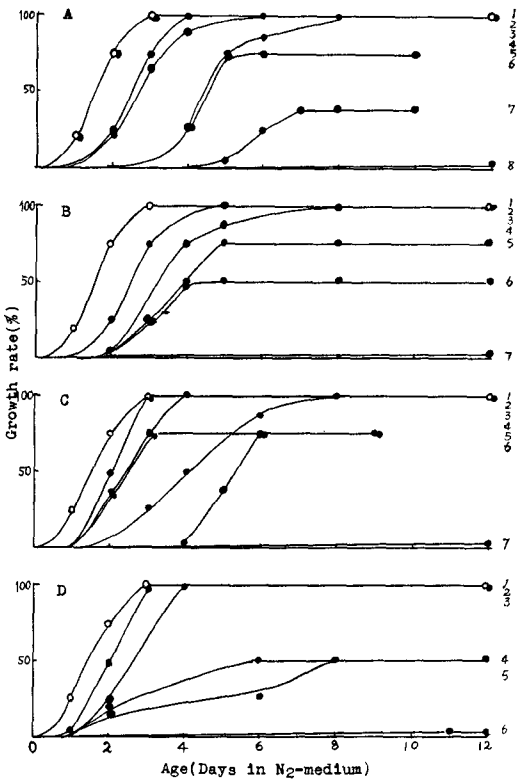

Fig. 5. Growth rate of PL- and PML-cells in N_2-medium. Assay methods are listed in the text. Curves were obtained by inoculation of following cells. Expt. A, 1-PL-cells of varying age (days). Curve 1, control N_2-cells (100%); Curve 2, 3, 4, 5, 6, 7, and 8, P-cells grown at 15°C. for 8, 11, 16, 19, 23 and 25 days, respectively. Expt. B, PL-cells of varying generation number. Curve 1, control N_2-cells; Curve 2, 3, 4, 5, 6, and 7, 1-, 2-, 3-, 4-, 5-, and 6-PL-cells, respectively. Expt. C, 1-PML-cells of varying age (days). Curve 1, control N_2-cells; Curve 2, 3, 4,5, 6, and 7, 1-PML-cells grown at 15°C for 6, 8, 13, 17, 19, and 21 days, respectively. Expt. D, PML-cells of varying generation number. Curve 1, control N_2-cells; Curve 2, 3, 4, 5, and 6, 1-, 2-, 3-, 4-, 5-, and 6-PML-cells, respectively.

case, PML-cells grew more rapidly than the PL-cells in the N_2- medium. Then, it was evident that comparatively lower temperature and the presence of mannit in the pepton medium were useful for the growth of P-cells on molecular nitrogen.

As seen, it was found that according as the increase of generation number of P-cells the more rapid inactivation of growth in the N_2-medium occurred.

(3) Intracellular Substance(s) Concerned with the Conversion of Peptone- to N_2-Cells.

When the extract of heated N_2-cells was added to the N_2-medium inoculated with 3 C.-treated or nontreated P-cells, stimulated or nonstimulated growth was observed, respectively (Table 14). Thus, the extract of N_2-cell might contain substance(s) concerned with the conversion within limits.

When large numbers of P-cells were inoculated in the N_2-medium, the growth was not detectable, so it follows that the P-cells could not contain the substances concerned with the conversion.

(4) Extracellular Substance(s) Concerned with the Conversion of Peptone- to N_2-Cells.

The P-cells were inoculated in the N_2-medium which contained 1 mg. of *p*-aminobenzoic or nicotinic acid, biotin, DL-alanine, FMN, FAD, RF, or all of these together, in 100 ml. volume Erlenmeyer flasks containing 10 ml. of culture medium, and then cultivated at 30°C. There was no growth in these media even after 30 days of cultivation. Therefore, none of these substances mentioned above were concerned with the conversion of P- to N_2-cells.

(5) Conversion of Peptone-Cells to N_2-, KNO_3-, and NH_4Cl-Cells.

It was found previously that the P-cells could not grow in the N_2-medium (2). Now, the aspects of conversion of P-cells to N_2-, KNO_3-, and NH_4Cl-cells were observed to define the fundamental difference between the P-cells and other cells.

At first, the same amounts of N_2-cells were inoculated into each 10 ml. of N_2-, KNO_3-, NH_4Cl-, and pepton-media, in 100 ml. volume Erlenmeyer flasks, cultivated for 3 days, except 1 day of P-medium, and then the respective degree of growth were compared. As seen in Table 15, the cells could grow in these media. After then the P-cells were re-inoculated in the different media containing the different nitrogen sources, and the growth rates in each medium were measured comparatively. As indicated in Table 15, the P-cells could not grow in these media excepting pepton medium. Therefore, it was evident that the P-cells lost growing capacity not only in the N_2-medium but in the other

TABLE 15. Conversion of P- to N_2-, KNO_3-, and NH_4Cl-cells

Control N_2-cells (0.04 mg.) were inoculated in 10 ml. of N_2-, KNO_3-, NH_4Cl,- or P-medium in 100 ml. Erlenmeyer flasks. P-cells (0.19 mg.) were inoculated in these media.

Inoculum Cells	Growth (mg. dry cells/10 ml.)			
	N_2-Medium	KNO_3-medium	NH_4Cl-medium	P-medium
1. N_2-	11	14	2	19*
2. P-*	1	0.6	0.8	12

* Cells were inoculated in the different media.

inorganic nitrogen containing media.

(6) Specific Propeties of N_2-Cells converted from the Peptone-Cells treated at Low Temperature.

As seen in Table 16, amino acids were oxidized actively by the N_2-cells converted from the 3 C. treated P-cells to the same degree as by the parent P-cells, while they were not by the control N_2-cells. And then, the progeny

TABLE 16. Activities of several oxidative enzymes of N_2-cells converted from 3°C.-treated P-cells

Conditions of 3°C.-treatment, conversion of N_2-cells from P-cells, and of assay procedures are listed in the text.

Cells*		Cont. N_2-	Conv. N_2-	Cont. P-		3°C. P-	Prog. 3°C. P-
Culture conditions							
Age (days)		3	6	0.5	1	1	1
Growth (mg. cells/25 ml.)		26	28	25	55	50	80
Q_{O_2} with	Glucose	148	260	3	30	30	42
	Succinate	233	54	140	41	61	34
	Acetate	133	305	89	32	108	32
	Lactate	246	205	106	30	48	0
	Formate	21	31	50	14	36	15
	L-tyrosine	±	29	118	39	146	145
	L-leucine	±	11	90	62	126	30
	None	25	21	17	5	±	16

* Cont. N_2-, control N_2-cells; Conv. N_2-, N_2-cells converted from 3°C.-treated P-cells; Cont. P-, control P-cells; 3°C. P-, 3°C.-treated P-cells; Prog. 3°C. P-, progeny of 3°C.-treated P-cells.

of the converted N_2-cells could oxidize amino acid actively. Thus, the possibility was assumed that the capacity to oxidize amino acids could transfer from the 3°C. treated P- to N_2-cells, and then could be received into the progeny of converted N_2-cells.

III. INDUCED BIOSYNTHESIS OF RESPIRATORY ENZYME

A. TIME COURSE OF OXIDATION OF SUBSTRATES BY N_2-, KNO_3-, NH_4Cl., LEUCIN-, AND PEPTONE-CELLS

The time courses of the oxidation of various substrates by the resting cells grown under various conditions were observed comparatively, because the increasing oxidation of a substrate could indicate the induced formation of respiratory enzyme concerned with the oxidation of the substrate.

(1) N_2-Cells. As seen in Fig. 2, the time courses of the oxidation of various substrates by the resting N_2-cells are shown as increasing curves after the lag time. In this case, the cells could not grow. Thus, the possibility was suggested that the enzymes concerned with the oxidation of these substrates could be formed by induction in the presence of each substrate respectively.

(2) KNO_3-Cells. The time courses of the oxidation of substrates are shown by increasing curves under resting state.

(3) NH_4Cl-Cells. As seen in Fig. 3, the time courses of oxidation of substrates were constant from the start, although an immediate increase occurred as a result of the addition of 1×10^{-2}M. NH_4Cl. When the cells grew in the diluted NH_4Cl- medium, at final concentration lower than the 1×10^{-2} M., time courses of the oxidation of substrates were not constant as seen in N_2-cells. In this case, the possibility was assumed that the NH_4Cl-cells converted to N_2-cells during the growth under the NH_4Cl-poor condition.

(4) Peptone-Cells. The time courses of oxidation of substrates by the resting P-cells were constant from the start as also were the case in NH_4Cl-cells (Fig. 3).

(5) Leucine-Cells. The resting L-cells as well as the P-cells oxidized the substrates as a constant rate from the start.

Then, the possibility was suggested that the increase of oxidation rate of substrates by the resting N_2- (or KNO_3-) cells in the absence of nitrogen compounds are the result of the induced synthesis of respiratory enzymes and that the induced synthesis of respiratory enzymes might depend upon the assimilation of molecular nitrogen.

B. INTRACELLULAR SUBSTANCES CONCERNED WITH INDUCED BIOSYNTHESIS OF RESPIRATORY ENZYMES

In this experiment, resting N_2-cells were used.

(1) Starvation of Resting Cells. As seen in Fig. 6, the starvation of N_2-cells, for several hours at 30°C., caused the longer lag time before the oxidation of substrates with remarkable values. Therefore, it was evident that some unknown intracellular substance (s), which disappeared during the starvation of resting cells, could be concerned with the enzyme formation.

(2) Cell Amounts. As seen in Fig. 7, a small amount of cells, 0.17 mg. of

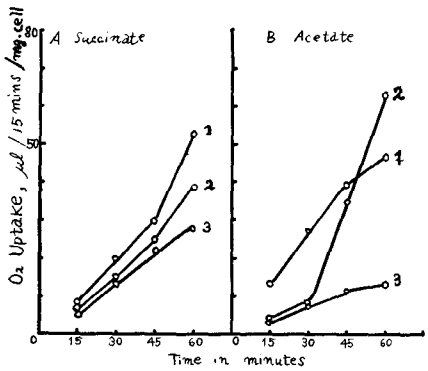

Fig. 6. Effects of starvation of resting cells on activity of oxidation of succinate or acetate. Suspensions of resting N_2-cells were aerated at 30°C., in 5×10^{-2} M. phosphate buffer, pH 7.2. Curve 1, nonaerated cells; Curve 2, 3 hours-aerated cells; Curve 3, 5 hours-aerated cells.

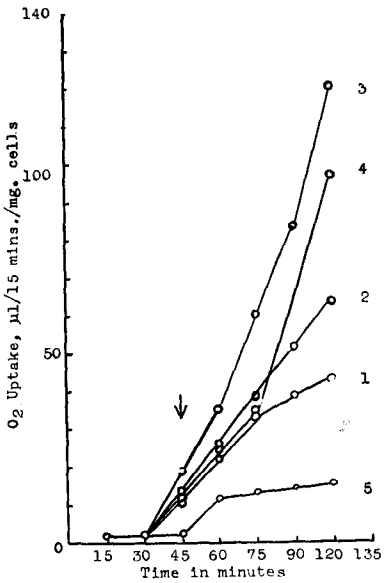

Fig. 7. Effects of different amounts of resting cells on time course of oxidation of succinate. At arrow, succinate was added to respective warburg vessels. Curve 1, 3.4 mg. cells; 2, 2.5 mg.; 3. 1.7 mg.; 4, 0.8 mg.; and 5, 0.17 mg.

dry cells per 2 ml. of reaction medium, could not oxidize succinate actively although 0.34 to 1.7 mg. of cells could oxidize it remarkably with increasing curve. Therefore, there was suggested the presence of unknown intracellular substance(s) involved in the induced synthesis of respiratory enzymes, as seen in the example of succinic oxidase system.

C. CONDITIONS OF INDUCED BIOSYNTHESIS OF RESPIRATORY ENZYMES

(1) Relationships between the Inducer and the Induced Enzyme.

The resting N_2-cells were treated with various substrates for the synthesis of enzymes in the medium having 5×10^{-2}M. phosphate buffer, pH 7.2, under continuous aeration at 30°C. for 1 to 5 hours. After that, the treated cells were harvested, washed, suspended in the deionized water, and measured for activitie of oxidation of various substrates in comparison with the cells treated without external substance and with the nontreated cells. As seen in Tables 17 to 22 and in comparison of Fig. 2B and 2C, the substrate-treated-cells showed the remarkable oxidation of substrate used as the inducer. Then, some specific relations were to be found between the inducer and the induced enzyme as seen in Table 23. In this case, the treated cells were shown to be able to oxidize the assumed intermediate of substance used as the inducer at a higher level than the cells nontreated and treated with oxygen alone. The presence of air alone, was not effective for the formation of respiratory enzymes under the resting state.

TABLE 17. Relationships between inducer and inucedd enzyme

Conditions of induced synthesis of enzymes and assay procedures were those described under "Experimental Methods".

	Inducer	Q_{O_2}				
		Glucose	Succinate	Acetate	Ethanol	Mannit
(1)	None	100	100		43	
	Glucose	200	90		47	
(2)	None	220	90	463	272	131
	Succinate	120	235	474	396	353
	Ethanol	153	77	395	452	340
	Mannit	170	87	353	260	300

The activity of oxidation of citrate by the resting cells was very low before treatment with the inducer, and then it was increased by treatment with citrate or succinate. The activity of oxidation of pyruvate, malate, or pyruvate plus malate was increased by treatment with succinate. However, such activity was not increased by treatment with pyruvate (Tables 21 and 22).

TABLE 18. Relationships between inducer and induced enzyme

Assay conditions were the same described in Table 17.

	Inducer	Q_{O_2}				
		Glucose	Succinate	Acetate	Mannit	Lactate
(1)	None	—	79	95	131	89
	Succinate	—	355	262	145	307
(2)	None	—	77	180	48	140
	Mannit	—	72	150	89	160
(3)	None	—	—	112	—	80
	Acetate	—	—	226	—	190
(4)	None	35	22	42	34	43
	Lactate	41	26	95	25	83

TABLE 19. Relationships between inducer and induced enzyme concerned with oxidation of members of Krebs cycle

Conditions of induced synthesis of enzymes and assay procedures were those described under "Exptl. Methods".

	Inducer	Q_{O_2}			
		Succinate	Fumarate	Malate	Oxalacetate
(1)	None	37	32	16	28
	Succinate	310	307	165	171
(2)	None	80	143	87	60
	Fumarate	450	407	460	215
(3)	None	24	21	53	70
	Malate	293	142	261	225
(4)	None	45	33	145	88
	Oxalacetate	206	264	169	> 88
(5)	None	37	16	32	
	Glucose	18	56	89	
	Mannit	83	13	102	

TABLE 20. Induced synthesis of citrate oxidizing enzyme

Conditions of formation of enzyme and assay procedures were the same described in Table 19.

Inducer	Q_{O_2}	
	Citrate	Succinate
None	7	206
Citrate	64	136
Succinate	39	550

TABLE 21. Induced synthesis of enzyme system concerned with oxidation of pyruvate

Conditions of synthesis of enzyme and assay procedures were the same described in the text except that 1×10^{-2}M. pyruvate as an inducer or substrate.

Inducer	Q_{O_2}	
	Pyruvate	Succinate
None	36	71
Pyruvate	37	33
Succinate	159	114

TABLE 22. Induced synthesis of enzyme system concerned with oxidation of pyruvate plus malate

Methods of synthesis of enzyme system and assay procedures were those described in the text except that 1×10^{-2}M. pyruvate as a substrate.

Inducer	Q_{O_2}			
	Succinate	Malate	Pyruvate	Malate + pyruvate
None	60	26	53	108
Succinate	150	187	171	290

TABLE 23. Relationships between inducer and induced enzyme

Inducer	Probable formation of enzyme systems concerned with oxidation of
Mannit	Mannit, glucose
Glucose	Glucose, fumarate, malate
Succinate	Succinate, fumarate, malate, oxalacetate, citrate, pyruvate, pyruvate plus malate, mannit, acetate, lactate
Fumarate	Succinate, fumarate, malate, oxalacetate
Malate	Succinate, malate, fumarate, oxalacetate
Oxalacetate	Succinate, oxalacetate. fumarate, malate
Citrate	Citrate, succinate
Acetate	Acetate, lactate, succinate
Lactate	Acetate, lactate, succinate
Ethanol	Ethanol, mannit

(2) Mutual Relations between Respective Members of Krebs Cycle on the Induced Biosynthesis of Enzymes which are Concerned with the Oxidation of the Members.

As indicated in Fig. 2B and 2C, and Tables 19 and 20, the activity of oxidation of all members of Krebs cycle of resting cells was increased as a result of treatment with succinate, fumarate, malate or oxalacetate which has inhibitory effect on succinic dehydrogenase. Thus,it was evident that members of Krebs cycle were effective for the induced synthesis of enzymes which are concerned with the oxidation of all these members.

(3) Competitive Relations in the Induced Synthesis of Respiratory Enzymes.

(a) Competitions in the Induced Synthesis of Enzymes Concerned with the Oxidation of Glucose, Succinate, Actate, and Lactate.

The simultaneous synthesis of the enzymes concerned with the oxidations of glucose, succinate, acetate, and lactate was observed by treatment with a mixture of all the listed substances at the same final concentration of 1 or 2×10^{-3}M. As seen in Table 24, the four enzyme systems which oxidize the substrates were synthesized inducibly and simultaneously by this mixture. Lactate, with the diluted mixture, and glucose oxidizing enzymes, with the concentrated mixture, were formed at the highest rate. The mixture was oxidized at a lower level than the sum of each substrate-oxidizing value. Therefore, the possibility was assumed that simultaneous and limited synthesis of respiratory

TABLE 24 Formation of enzyme system concerned with oxidation of glucose, succinate, acetate, or lactate by treatment with a mixture of all the listed substrate

Conditions of synthesis of enzyme and assay procedure are those described in the text.

Inducer	Q_{O_2}				
	Glucose	Succinate	Acetate	Lactate	Mixture
None	33	164	217	46	284
Mixture (2×10^{-3}M.)	298	277	354	112	380
Mixture (1×10^{-3}M.)	65	238	264	219	309

enzymes took place as a result of the presence of the mixed inducer, and that some general mechanism of these oxidations might be induced more poorly than the specific mechanism.

(b) Competition in the Induced Synthesis of Enzymes Concerned with the Oxidation of Members of Krebs Cycle.

As seen in Table 25, the rate of oxidation of mixture by the cells treated with mixture was higher than by the cells treated with any single member, although

TABLE 25. Formation of enzyme system concerned with oxidation of members of Krebs cycle by treatment with a mixed inducer.

Conditions of synthesis of enzyme and assay procedure are those described in the text.

	Inducer	Q_{O_2}					
		Mixture	Succinate	Fumarate	Malate	Citrate	Oxal-acetate
(1)	None	130	37	16	32	6	
	Succinate	157	210	165	307	13	
	Malate	77	83	130	102	—	
	Mixture (1)	317	280	182	110	37	
(2)	None	193	52	23	44	9	75
	Succinate	222	297	236	437	29	245
	Mixture (2)	587	280	181	146	37	74
	Mixture (3)	233					

Mixture (1), succinate plus fumarate and malate.
Mixture (2), Mixture (1) plus citrate.
Mixture (3), Mixture (2) plus oxalacetate.

it was lower than the total value of oxidation of each member. The formation of succinate or malate oxidizing system of resting cells by the treatment with succinate, was stimulated or depressed, respectively, in the presence of fumarate and malate. Oxalacetate inhibited the total activity of formation of enzyme system concerned with the oxidation of all the listed four substances. Therefore, the possibility was assumed that limited and simultaneous synthesis of enzymes concerned with the oxidation of Krebs cycle members took place as a result of the presence of the mixture.

(4) Effects of Inorganic Nitrogen Compounds.

As indicated in Table 26, the resting N_2- and KNO_3-cells could form succinic or acetic acid oxidizing system in the absence of any nitrogen compound under aeration, the former cells more actively than the latter ones. However, the NH_4Cl- and P-cells could not form the respiratory enzymes under the condition of absence of nitrogen compound. The addition of 1×10^{-2}M. NH_4Cl to the enzyme-forming medium could bring about an inhibited synthesis of acetic acid oxidizing enzyme with the N_2- and NH_4Cl-cells, or a stimulated

TABLE 26. Effects of inorganic nitrogen compounds on formation of enzymes by resting cells

Methods of enzyme formation and assay procedures are those described in the text.

Conditions of enzyme synthesis			Q_{O_2}				
Cells	Inducer	Nitrogen	Succ.	Fum.	Mal.	Acet.	Mann.
N_2-	None		37	80	81	110	6
	Malate		248	118	214	184	158
	Malate	NH_4Cl	216	116	230	76	23
	None		82				
	Succinate		182				
	Succinate	NH_4Cl	105				
KNO_3-	None		34			92	
	Succinate		51			102	
	Succinate	KNO_3	48			132	
NH_4Cl-	None		43			120	
	Succinate		25			120	
	Succinate	NH_4Cl	104			28	

Succ., succinate; Fum., fumarate; Mal., malate; Acet., acetate; Mann., mannit.

synthesis of succinic oxidase system with the NH_4Cl-cells. The addition of 1×10^{-2}M. KNO_3 to the enzyme-forming medium could bring about the stimulated synthesis of acetic acid oxidizing enzyme with the KNO_3-cells. Therefore, it was determined that nitrogen compound might be a substance concerned with the variation of activity of enzyme formation.

(5) Effects of Different Gas Conditions.

The N_2- and KNO_3-cells were treated with succinate under various gas conditions, with air, oxygen, nitrogen, or without any one, for 2 hours at 30°C. under continuous shaking. Then the activity of succinic oxidase system of treated and non-treated cells was measured in comparison with each other.

TABLE 27. Effects of different gas conditions on formation of succinic oxidase system

Methods of enzyme formation and assay procedures are those described in the text.

Conditions of enzyme synthesis		Activity of succinic oxidase (Q_{O_2})	
Inducer	Gas phase	N_2-cells	KNO_3-cells
Endogenous substance	Air	0	10
	O_2	6	1
	N_2	1	1
	None	0	1
Succinate	Air	36	34
	O_2	14	54
	N_2	0	7
	None	8	13
None*		8	22

* Nontreated initial cells.

As seen in Table 27, succinic oxidase system was formed largely in the air and in oxygen but not in molecular nitrogen or under gas-less condition. Especially, in the N_2-cells succinic oxidase system was formed in the air more actively than in the oxygen alone, while in the KNO_3-cells more largely in the oxygen than in the air. Therefore, it is suggested that the N_2-cells require molecular nitrogen and oxygen for the induced synthesis of respiratory enzymes, while the NH_4Cl- or P-cells can not form the enzymes unless some nitrogen compound is present in the enzyme forming medium, because of the inability to assimilate molecular nitrogen.

(6) Effects of Inhibitors.

The resting N_2-cells were treated with the inducer, succinate, together with or without various inhibitors under continuous aeration for an hour, washed, and suspended in de-ionized water. The succinic oxidase activity was measured in comparison with the nontreated cells.

(a) Effects of KCN.

KCN was added to the enzyme forming medium together with succinate at final concentrations of 1×10^{-3}M. to 1×10^{-2}M. The presence of 1×10^{-3}M. KCN inhibited the oxidation of inducer during the course of induction, although it did not inhibit the induced synthesis of succinic oxidase system. The presence of 2.5×10^{-3}M. KCN inhibited the induced synthesis of succinic oxidase system incompletely; the presence of 1×10^{-2}M. KCN inhibited it completely. When malate was used as an inducer under the same conditions, the presence of 1×10^{-3}M. KCN inhibited the induced synthesis of succinic oxidase system completely (Table 28) (ref. 72).

Then the possibility was assumed that the induced synthesis of succinic oxidase system depends on the residual respiration in the presence of 1×10^{-3}M. KCN. It follows that the system concerned with the induced synthesis of

TABLE 28. Effects of cyanide on enzyme formation

Methods of synthesis of enzymes and assay procedures are those described in the text.

Conditions of enzyme formation			Q_{O_2}	
Inducer	KCN	Respiratory activity μl., O_2 uptake/ 2 hr./mg. dry cells	Succinate	Malate
(1) None		0	39	
Succinate		1353	62	
Succinate	1×10^{-3}M.	67	67	
(2) None		0	39	
Succinate		333	63	
Succinate	2.5×10^{-3}M.	28	50	
(3) None		0	78	
Succinate		1120	180	
Succinate	1×10^{-2}M.	14	42	
(4) None		0	131	343
Malate		826	380	683
Malate	1×10^{-3}M.	121	45	50

this enzyme was more resistant to the presence of KCN than the action of succinic oxidase itself, and that the sensitivity of the enzyme forming system to KCN could vary with the variation of the inducer at the various levels (see Table 28). Then, it was evident that the oxidative enzyme induced by its direct substrate was more resistant to KCN than the same enzyme induced by the other indirect substrate.

(b) Effects of DNP.

The presence of 1×10^{-3}M. to 2.5×10^{-3}M. DNP inhibited the induced synthesis of acetic or lactic acid oxidizing enzyme system, but not the succinic

TABLE 29 (A). Effects of DNP on formation of succinic oxidase system

Conditions of enzyme formation and assay procedure are those described in the text.

Conditions of enzyme formation			Activity of respiration μl. O_2/2hr./mg. cells	Activity of succinic oxidase (Qo_2)
	Inducer	DNP		
(1)	None		0	161
	Succinate		200	266
	Succinate	1×10^{-3}M.	180	272
(2)	None		0	36
	Succinate		282	450
	Succinate	2.5×10^{-3}M.	322	525
(3)	None		0	248
	Succinate		762	398
	Succinate	5×10^{-3}M.	400	114

TABLE 29 (B). Effects of DNP on formation of oxidative enzymes

Conditions of enzyme synthesis			Qo_2		
	Inducer	DNP	Succinate	Acetate	Lactate
(1)	None		9	82	57
	Succinate		107	176	122
	Succinate	1×10^{-3}M.	107	152	106
(2)	None		64	138	
	Acetate		87	204	
	Acetate	1×10^{-3}M.	86	163	

acid oxidizing system, during the treatment of resting cells with succinate. The presence of 5×10^{-3}M. DNP inhibited remarkably the synthesis of all these enzymes in the succinate-treated cells. Therefore, it was assumed as possible that the mechanism of formation of succinic oxidase system was more resistant to DNP than that of acetic acid oxidizing system (Table 29).

(c) Effects of NaF.

The presence of 1×10^{-2}M. NaF inhibited weakly an induced formation of succinic oxidase system but not the action of this enzyme during the induction (Table 30).

(d) Effects of MIA.

The presence of 1×10^{-3}M. MIA together with the inducer, succinate or malate, inhibited the formation of succinate or malate oxidizing system completely and the oxidation of succinate or malate during the course of induction of enzyme incompletely (Table 30).

TABLE 30. Effects of MIA and NaF on formation of enzymes

Methods of enzyme formation and assay procedures are those described in the text.

	Conditions of enzyme formation			Q_{O_2}	
	Inducer	Inhibitor	O_2 uptake μl. O_2/100 min./mg. cells	Succinate	Malate
(1)	None		0	131	343
	Malate		837	456	745
	Malate	MIA (1×10^{-3}M.)	173	45	56
(2)	None		0	65	
	Succinate		255	406	
	Succinate	MIA (1×10^{-3}M.)	189	43	
	Succinate	NaF (1×10^{-2}M.)	602	361	

(e) Effects of Malonate.

The presence of 3×10^{-2}M. malonate together with 2.5×10^{-2}M. succinate in the inducing medium inhibited the formation of succinic oxidase system but not the oxidation of succinate during the course of induction (Table 31). Therefore, it was possible that the induced formation of succinate- and, especially, malate-oxidizing systems required the reaction of succinic dehydrogenase with succinate which was competitively inhibited by malonate (ref. 75, 76).

TABLE 31. Effects of malonate on formation of enzymes

Methods of enzyme formation and assay procedures are those described in the text.

Conditions of enzyme formation			Q_{O_2}	
Inducer	Malonate	Respiratory activity $\mu l. O_2/1.5$ hr./mg. cells	Succinate	Malate
None		0	131	343
Succinate		525	456	745
Succinate	3×10^{-2}M.	530	62	+ 32

(f) Effects of NaN_3.

The presence of 1×10^{-3}M. NaN_3 together with inducer, succinate, inhibited the induced synthesis of the succinic oxidase system. As seen in Fig. 8, the increasing curve of oxidation of succinate by the resting cells was converted immediately to a non-increasing constant curve by the addition of NaN_3 (72, 73).

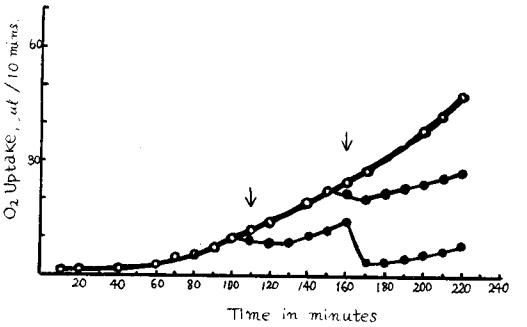

Fig. 8. Effect of NaN_3 on time course of oxidation of succinate by resting N_2-cells. At arrow, NaN_3 (1×10^{-3}M.) was added in reaction mixture. Assay systems have been described under "Experimental Methods".

(g) Effects of Chloramphenicol.

The presence of chloramphenicol, at 100 mg. per ml., together with the inducer, succinate, inhibited the induced synthesis of the succinic oxidase system, completely, and the oxidation of inducer during the course of induction, incompletely (Table 32 and Fig. 9) (77–79).

Then, it was found that: (1) the increasing curve of oxidation of substrates by the resting N_2-cells indicates the induced synthesis of enzymes

TABLE 32. Effects of chloramphenicol on formation of succinic oxidase system

Assay conditions are described in the text.

Conditions of enzyme formation			Activity of succinic oxidase
Inducer	Chloramphenicol	Respiratory activity μl. O_2/3 hr./mg. cells	(Q_{O_2})
None		0	70
Succinate		365	184
Succinate	100 μg./ml.	65	71
Succinate	500 μg./ml.	8	+ 44

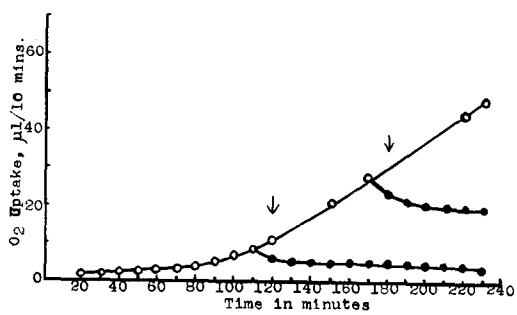

Fig. 9. Effects of chloramphenicol on time course of oxidation of succinate by resting N_2-cells. At arrow, chloramphenicol (100 μg./ml.) was added in reaction medium. Assay procedures have been described under "Experimental Methods".

concerned with the oxidation of these substrates, (2) the induced synthesis of respiratory enzyme, for example, the succinic oxidase system, requires following internal conditions, (a) an energy supply by means of metallo-enzyme, (b) the reaction of dehydrogenase with its substrate, and (c) the free sulfhydryl group of this enzyme. The system of enzyme formation was more resistant to the presence of KCN and more sensitive to malonate or MIA than the succinic oxidase system itself.

(7) Effects of Several External Amino Acids.

L-glutamic or L-aspartic acid, DL-alanine, L-leucine, L-cysteine, L-arginine, or mixture of all the listed amino acids was added in the enzyme inducing medium together with the inducer, succinate or acetate, and aerated for 2 hours in order to observe the necessity of a external amino acid for the inducible synthesis of succinic or acetic acid oxidizing enzyme system.

As indicated in Table 33, the resting cells could show the depressed synthesis of succinic or acetic acid oxidizing enzyme with cysteine and the stimulated synthesis of succinic oxidase system with L-leucine or of acetic acid oxidizing system with L-aspartic acid. The cells treated with the mixture of all the listed amino acids and inducer show a stimulated synthesis of these enzyme systems, as seen in the comparison with the cells treated without any external amino acid.

TABLE 33. Effects of amino acids on synthesis of enzymes

Methods of synthesis of enzyme and assay procedures are those described in the text.

	Conditions of enzym synthesis		Qo_2	
	Inducer	Amino acid	Succinate	Acetate
(1)	None	None	109	
	None	L-Cysteine	159	
	Succinate	None	210	297
	Succinate	L-Cysteine	169	197
(2)	None	None	49	
	None	L-Glutamic acid	75	
	Succinate	None	74	
	Succinate	L-Glutamic acid	82	
(3)	None	None	76	
	None	L-Aspartic acid	71	
	None	DL-Alanine	71	
	None	L-Leucine	71	
	None	Mixture	71	
	Succinate	None	227	128
	Succinate	L-Aspartic acid	256	238
	Succinate	DL-Alanine	290	166
	Succinate	L-Leucine	336	151
	Succinate	Mixture	349	251

Therefore, it was assumed as possible that a specific combination of amino acids was effective for the synthesis of a specific enzyme.

(8) The Stability of the Induced Enzyme.

The stability of an enzyme induced by different inducers to the starvation of cells was observed.

As indicated in Table 34, the succinic oxidase system non-induced or

TABLE 34. Stability of induced enzymes

Conditions of enzyme formation and assay procedures are those described in the text.

Cells		I-cells				S-cells				A-cells	
Time of starvation (hr.)		0	1	2	4	0	1	2	4	0	1
Q_{O_2} with	Succinate	73	49	41	26	120	127	110		90	24
	Acetate	48		28		83	68		61		

induced by means of acetate was more labile to starvation than the same system induced by means of succinate.

Therefore, it was evident that the stability of an induced enzyme, as seen in the example of succinic oxidase system, could vary with the changing of the inducer, as if the enzyme induced by its direct substrate was more resistant to starvation than the same enzyme induced by another indirect substrate.

TABLE 35. Comparison of activity of formation of enzymes by the N_2-, KNO_3-, NH_4Cl-, and P-cells

Conditions of enzyme formation and assay procedures are those described in the text.

Cells		Q_{O_2}	
Grown on	treated with	Succinate	Acetate
N_2	I-*	10	65
	S- (or A-)	148	(96**)
	E-	21	102
KNO_3	I- (or A-)	34	92
	S-	51	(102**)
	E-	12	31
NH_4Cl	I-	190	
	S-	50	
	E-	194	
Pepton	I-	24	
	S-	24	
	E-	24	

* I-cell, nontreated initial cells; S- (or A-), succinate- (or acetate) treated cells; E-, cells treated without any external substrate, with endogenous subtances.

** Activity of oxidation of acetate by A-cells.

(9) Comparison of the Activity of Induced Synthesis of Enzymes by the N_2-Cells and the Other Cells.

As summarized in Table 35, the NH_4Cl- and P-cells could not form the succinic oxidase system in the absence of a nitrogen compound, while the KNO_3-cells could do so more poorly than the N_2-cells. Then, it was evident that the N_2-cells could induce synthesis of the respiratory enzymes and that their ability depended on the capacity of assimilation of molecular nitrogen.

D. DIRECT EVIDENCE OF INDUCED BIOSYNTHESIS OF SUCCINIC OXIDASE SYSTEM

To decide whether increase of activity of succinic oxidase by treatment with succinate of resting cells depends on increase of permeability of cells to succinate or on net synthesis of this enzyme, cell-free enzyme preparations were used in these experiments. After treatment with succinate, the cells were washed, suspended in deionized water, and then the activity of succinic oxidase in the intact and cell-free states was measured in comparison with the nontreated cells.

(1) Succinic Oxidase.

As summarized in Table 36, the succinic oxidase system was more active in the succinate-treated cells (S-cell) than in the air-treated (E-cell) and nontreated cells (I-cell) under the intact and also under the cell-free states. Therefore, it

TABLE 36. Activity of succinic oxidase system of succinate-treated and nontreated cells

Conditions of treatment of resting cells and assay procedures were listed under "Experimental Methods".

Cells	I-	S-	E-
O_2 uptake during treatment (μl./hr./mg. cells)	0	1411	1010
Flavin contents	+	+++	+
Activity of succinic oxidase			
Whole cells (Q_{O_2}) "	92	503	20
Cell-free preparation (S_3) (μl.O_2/hr./mg. protein)	0	42	0
Residue (R_1)	0	0	0
E at 550 mμ (difference spectrum) (S_3)	0	28	0
420 " (") (")	140	190	140
260 " (minus E value at 270 mμ) (")	130	310	150

was found that net synthesis of enzyme took place during the induction process. Flavins and substances which absorbed the wave-length of 550, 420, or 260 mμ were formed in the succinate-treated cells.

(2) Succinic Dehydrogenase.

The value of oxygen uptake in the presence of succinate, 1×10^{-2}M. KCN, and 1×10^{-3}M. Mb was higher in the succinate-treated cells than in the nontreated cells in the intact and cell-free states. The presence of 1×10^{-3}M.

TABLE 37. Activity of succinic dehydrogenase of succinate-treated and nontreated cells

Conditions of succinate-treatment of resting cells and assay procedures are those described under "Experimental Methods".

Reaction medium			Q_{O_2}	
Cells	Substrate	Mb+KCN	Whole cells	Cell-extract (S_2)
I-	None	−	30	4*
	"	+	5	4
	Succinate	−	155	15
	"	+	8	17
S-	None	−	27	0
	"	+	+ 7	0
	Succinate	−	285	27
	"	+	21	25
E-	None	−	13	
	"	+	5	
	Succinate	−	103	
	"	+	17	

* μl. O_2/hr./mg. protein.

TABLE 38. Activity of succinic dehydrogenase of succinate-treated and nontreated cells

Conditions of treatment of resting cells and assay procedures are those described under "Experimental Mehds".

Cells	Whole cells		Cell-extract (S_2)	
	Q_{O_2}	Q_{Fe}	Q_{O_2} *	Q_{Fe}
I-	68	226	36	120
S-	460	830	76	580
E-	73	174	44	120

* μl. O_2/hr./mg. protein.

Mb and 1×10^{-2}M. KCN inhibited the oxidation of succinate with the intact cells (Table 37). Then, the ferricyanide method was applied more conveniently. As indicated in Table 38, the amounts of ferricyanide reduced in the presence of 1×10^{-2}M. KCN and succinate was larger in the succinate-treated cells than in the air-treated and nontreated cells under the intact and also under the cell-free states. Thus, it was evident that synthesis of succinic dehydrogenase system could be induced by the treatment with succinate under aeration, namely, under condition of its "functional state".

(3) Cytochrome c.

Maximum and increased values of oxidation of succinate in the presence of external cytochrome c were measured in the intact and the cell-free states. In this case, the former denoted the maximum activity of succinic oxidase system and the latter the degree of saturation of cytochrome c in the cells and cell-extract. It was seemed to be caused by the external cytochrome c the remarkably stimulated oxidation of succinate in the lower concentration of internal cytochrome c.

TABLE 39. Effects of yeast cytochrome c on oxidation of succinate by succinate-treated and nontreated cells

Conditions of treatment of resting cells and assay procedures are those described under "Experimental Methods".

Reaction medium			Whole cells Qo_2	Cell-extract (S_3) μl. O_2/hr./mg. protein
Cells	Succinate	Cyt. c		
I-	+	−	68	3
	+	+	86	8
S-	+	−	460	14
	+	+	460	14
E-	+	−	73	4
	+	+	141	9

As indicated in Table 39, the maximum value of oxidation of succinate with or without the external cytochrome c was higher in the succinate-treated cells than in the air-treated and nontreated cells, and the stimulation degree of oxidation of succinate by the added cytochrome c was lower in the succinate-treated cells than in the other cells under the intact and also under the cell-free states.

Therefore, it was assumed as possible that the contents of cytochrome c and other components of the succinic oxidase system were greater in the succinate-treated cells than in the nontreated cells. The air-treated cells without succinate could not induce synthesis of cytochrome c and other components of succinic oxidase system remarkably as was also true of the nontreated cells.

On the other hand, as seen in Table 40, the activity of the succinic oxidase system of NH_4Cl-cells was not affected by the added cytochrome c, since the inability of inducible synthesis of succinic oxidase system of NH_4Cl-cells may be depended on the saturation of internal cytochrome c and other components of the succinic oxidase system.

TABLE 40. Effects of yeast cytochrome c on oxidation of succinate by N_2- and NH_4Cl-cells

Assay conditions were listed under "Experimental Methods".

Reaction medium			Q_{O_2} with Succinate
Cells	Succinate	Cytochrome c	
N_2-	−	−	15
	−	+	26
	+	−	94
	+	+	112
NH_4Cl-	−	−	3
	−	+	6
	+	−	107
	+	+	109

(4) Absorption Spectra of Cytochromes.

Spectrophotometric observations of the absorption spectra of cytochromes were made on the succinate-treated and nontreated cells under the intact and cell-free states.

The absorption spectra of intact cells were obscure, except for the absorption bands at 420 and 601 mμ wave-lengths in the oxidized form and at 430 and 600 mμ in the reduced form, which were more remarkable with the succinate-treated cells than with the nontreated cells.

As seen in Fig. 10, the difference spectra of extract of succinate-treated or nontreated cells were remarkable at 520, 550, 563, 620, and 640 mμ or 520, 552, 563, and 610 mμ, respectively, and the extract of air-treated cells could not show remarkable absorption band at 550 and 610–640 mμ. In the extract of succinate-treated cells, the absorption band at 550–551 mμ corre-

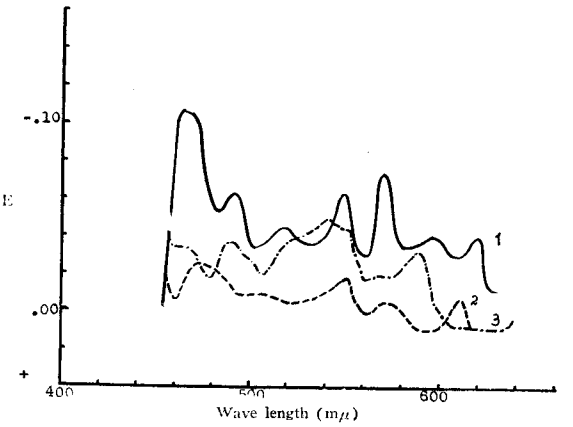

Fig. 10. Difference spectra of sucacinte-treated and nontreated cell-extracts (S_3). Each cuvette contained 0.5 ml. of cell-extract (0.8 mg. protein)and 2.5 ml. of 2×10^{-2}M. phosphate buffer, pH 7.2. Reductant, sodium dithionite; Oxidant, hydrogen peroxide. Curve 1, S-cell-extract; 2, I-cell-extract; 3, E-cell-extract.

sponding to reduced cytochrome c_4 was remarkable, while the band at 552 mμ corresponding to reduced cytochrome c_{4+5} (49–52) and b bands (30, 31) were obscure (32–39, 45). On the cell debris, it appeared that the absorption band at 630 mμ, related to reduced cytochrome a_2, was more remarkable with the S-cells than the othcer cells.

Then, it was possible that the induced formation of succinic oxidase system is involved with the formation of cytochrome c_4, or that the absorption band of 550–551 mμ appears during the succinate-treatment of resting N_2-cells under continuous aeration remarkably.

(5) Cytochrome c Oxidase.

The activity of cytochrome c oxidase of the succinate- or air-treated and nontreated cell-free preparations thereof were compared with each other by the manometric or spectrophotometric method. As indicated in Tables 41 and 42, the activity of cytochrome c oxidase of succinate-treated cells was greater than the air-treated and nontreated cells in the cell-free state, (ref. 51, 52).

Then, it was proven that cytochrome c oxidase is an induced enzyme, and that the induced formation of cytochrome c oxidase requires its own particular "functional state" in the simultaneous presence of an organic substrate and air (ref. 40, 51, 52).

TABLE 41. Activity of cytochrome c oxidase of succinate-treated and nontreated cells

Methods of succinate-treatment and assay procedures are those described under "Experimental Methods".

Reaction mixture			μl., O_2/hr./mg. protein.
Cells	Substrate	Cyt. c	Cell-free prep. (S_3)
(1) I-	Hydroquinone	—	1
	"	+	2
S-	"	—	2
	"	+	5
E-	"	—	3
	"	+	3
(2) I-	Paramine	—	2 (S_2)
	"	+	35
S-	"	—	2
	"	+	86

TABLE 42. Activity of cytochrome c oxidase of succinate-treated and nontreated cell-free extract (S_3)

Conditions of succinate-treatment of resting N_2-cells and assay procedures were described under "Experimental Methods".

Cells	Activity of cytochrome c oxidase mμmoles, cyt. c oxidized/hr./mg. protein
I-	10
S-	18

TABLE 43. Comparative aspects of nitrogen fixers

Organism	CO_2 fix.	O_2 dependence	Nitrogenase						Hydrogenase		
			Pre-sence	Inhibition with H_2	O_2	CO	N_2O	N-*	Pre-sence	Inhib. with N_2	O_2
1. Sulfate-reducing bacteria	+	strict anaerobic	+	?	?	?	?	?	?	?	?
2. Photosynthetic bacteria	+	"	+	+	+				+	+	−
3. Blue-green algae	+	aerobic	+	+	−	+	+	+ (−)	+	+	
4. *Rhizobium*	−	strict aerobic	+	+	−	+	+	+	+	+	
5. *Azotobacter*	−	"	+	+	+	+	+	+	+	+	
6. *Clostridium*	−	strict anaerobic	+	+ (−)	+	− (+)	+	−	+	−	+

1. *Desulfovibrio* (110–112); 2. *Rhodospirillum* (21, 26, 104, 105); 3. *Myxophyceae* (6, 11, 22, 25, 29, 98, 103); 4. *Legume-Rhizobium* combination (2, 11, 22, 25, 29, 34, 98–112); 5. (6, 11, 22, 25–34); 6. (110–112).

* Nitrogen compounds, nitrate, nitrite, ammonium salts, or urea.

DISCUSSION

A. OBSERVATION ON METABOLIC CHANGE UNDER DIFFERENT CONDITIONS OF GROWTH

In the present observations on *Azotobacter vinelandii*, it was found that some respiratory enzymes could change under different environmental conditions, qualitatively and quantitatively. Especially, amino acid oxidizing enzyme could be formed largely during the growth in the NH_4Cl- or organic-nitrogen-medium, but not in the molecular nitrogen-medium. 30°C. grown N_2-cells grew in KNO_3-, NH_4Cl or P-medium, while 30°C. grown P-cells could not grow in N_2-, KNO_3-, or NH_4Cl-medium. On the other hand, 3°C. treated P-cells could grow in the N_2-medium, as well as the 3°C. grown P-cells which were derived by a longer period treatment. The lower growing temperature of 15°C. or 3°C. and the presence of mannit in P-medium stimulated the growth on molecular nitrogen. When the N_2-cells were derived from 3°C. treated P-cells, amino acid oxidizing enzyme could be formed favourably during growth as much as P-cells. Also, the treatment of inoculum N_2-cells in N_2- or P-medium at 3°C. for a longer period could bring about the stimulated formation of L-leucine or L-tyrosine oxidizing enzyme. This stimulated activity was transferred into

progeny of 3°C. treated cells. Thus, it was possible that some enzymic change could be derived by the combinations of different temperatures and different nitrogen sources. MEYERHOF and BURKS (35) reported that the fixation of nitrogen proceeds only in the presence of carbon source and is probably linked to its oxidation, since the fixation is proportional to the oxygen consumption. They, further, reported that there is little growth or fixation on pepton, but very favourable fixation on carbohydrate and sugar alcohol, such as mannit. In the present experiments, it was evident that *Azotobacter vinelandii* grew more rapidly on pepton than on molecular nitrogen, depending, perhaps, on the more rapid assimilation of organic nitrogen, accompanying with the depressed fixation of nitrogen. Then, it is assumed that the nitrogen fixation system could not be formed further in a pepton medium because nitrogen fixation system was not working The presence of mannit may stimulate the nitrogen fixation in the pepton-medium, bringing about the protective effect against the inactivation of nitrogen fixation. Thus, it seems that the nitrogen fixation system is an induced enzyme system which is formed in the functional state of itself under appropriate conditions, such as in the presence of oxidizable carbon source, oxygen, and molecular nitrogen. The ability of formation of respiratory enzyme under the resting state differed between the N_2- and other cells. This ability is the specific property of N_2-cells, while KNO_3-cells have only minimized activity.

All the data support the view that the different conditions for growth could bring about the important changes in metabolic properties.

B. OBSERVATIONS ON THE FORMATION OF RESPIRATORY ENZYMES UNDER THE RESTING STATE

(1) On the Induced Synthesis of Respiratory Enzymes.

MONOD (90) postulated on the induced enzyme formation that; (1) induced enzyme formation involves the complete *de novo* synthesis of the enzyme protein, from its elements or elemental building blocks; (2) the induced synthetic process is virtually irreversible, and the "finished" enzyme molecule is not renewed at a measurable rate within the cells; (3) the process of enzyme induction is independent of enzyme activity; and (4) the inducer is not consumed during induction.

From the present data, it was found: that (1) the specific respiratory enzymes are synthesized by the resting N_2-cells, without growth, in the presence of a specific substrate together with air rather than oxygen alone (but not by the NH_4Cl- and P-cells); (2) there are some specific relations between the substrate, as the inducer, and the induced enzyme. The direct substrate of

specific enzyme is most effective for causing the enzyme synthesis; (3) the respiratory enzyme formation involves the net synthesis of enzyme; (4) the specific substrate effective for the synthesis is consumed oxidatively; (5) the process of enzyme synthesis depends on the enzyme activity; (6) the respiratory enzyme to be formed is contained in the resting cells preliminarily at the lower level; and (7) the process of enzyme synthesis is inhibited by KCN, NaN_3, MIA, malonate, DNP, and chloramphenicol.

The requirement of a specific substrate, as an inducer, for the net synthesis of enzymes suggests that the synthesis of respiratory enzyme can be regarded as a case of an induced enzyme synthesis, namely, that some respiratory enzymes are the induced enzymes in *Azotobacter vinelandii*, which are grown on molecular nitrogen, although there are some differences between the data presented here and the MONOD's postulation. In the case of *Azotobacter vinelandii*, there are interesting points that: (1) the process of enzyme formation is dependent on enzyme activity; (2) the inducible synthesis of respiratory enzyme is specific in the cells assimilated molecular nitrogen; and (3) the synthesis of respiratory enzyme system involves the cosynthesis of each component of this system for example, the synthesis of succinic oxidase system involves the cosynthesis of succinic dehydrogenase, cytochrome c_4, and c oxidase.

From the data on the formation of succinic oxidase system, it is evident that: (1) succinic dehydrogenase or cytochrome c oxidase is not formed by treatment with succinate or air alone; (2) 1×10^{-2}M. KCN inhibits the induced formation of succinic oxidase system, while 1×10^{-3}M. KCN fills up the energy requirement of enzyme synthesis; (3) MIA or malonate inhibits the synthesis of succinic oxidase system, remarkably, inspite of the little inhibition of the succinate-oxidizing activity. Thus, it is assumed that the induced synthesis of succinic oxidase system by the no growing N_2-cells is concerned with the activity of preliminarily included succinic oxidase system, supplying the energy derived from the oxidation of succinate by means of a metallo enzyme, namely that the synthesis of this system requires the "functional state" of preliminary enzyme system in the presence of succinate and air.

(2) On the Nature of Precursor.

From the isotopic examinations, VELICK (90), SPIEGELMAN *et al.* (91), and others (92, 93) are in agreement with the idea that enzyme proteins are synthesized *de novo* from amino acids following the alternative mechanism of enzyme synthesis;

Free amino acids ——→ active enzyme.

The data of specific properties of N_2-cells involved in the synthesis of respiratory enzyme, namely the requirement of air rather than oxygen alone, together with the specific inducer, and the specific effects of external amino acids on the formation, suggest the possibility that the induced synthesis of respiratory enzyme depends on the supply of precursors, probably amino acids, formed by means of the fixation of nitrogen.

(3) On the Mechanism of Enzyme Synthesis from the Precursors.

Cosynthesis of RNA with enzyme protein is reported by GALE (92), PARDEE (94), SPIEGELMAN and co-workers, and others (93). PARDEE (94) concluded that the synthesis of RNA was essential for the enzyme formation but that the nucleic acid formed was inactive, since enzyme formation ceased as soon as the supply of RNA precursor ceased. SPIEGELMAN and co-workers (93) have shown that the rate of synthesis of β-glucosidase in yeast can be affected by the size of "purine pool" in the cells. SPIEGELMAN postulated on the mechanism of the participation of RNA and DNA in the enzyme-formation that DNA and RNA are associated with the enzyme-forming mechanism as the template which serves as a device for specific protein synthesis. The investigations by POLLOCK (95) on the penicillinase-synthesizing system of *Bacillus cereus* suggest that inducer molecule appears to be bound specifically to some site concentrated with enzyme synthesis. Chloramphenicol, at growth-limiting concentration, prevents the synthesis of protein, stimulates the formation of RNA, and inhibits the synthesis of DNA (GALE and FOLKES (96)). In the present investigations, it was found that chloramphenicol inhibited the formation of succinic oxidase system under resting state and that the absorption band at 260 mμ wave-length related with nucleic acids was more remarkable with the succinate-treated cells than with the nontreated cells.

Thus, it is possible that the induced synthesis of the succinic oxidase system depends on the cosynthesis of nucleic acid from the precursors which is, probably, supplied by means of the fixation of nitrogen.

C. ON THE MECHANISM OF NITROGEN FIXATION

The mechanism of nitrogen fixation is still incompletely known. From the investigations on the *Legume-Rhizobium* combination, VIRTANEN (7–10) suggests that hydroxylamine is the first product of nitrogen fixation. However, WILSON *et al.* (6, 11) reported that the first product of fixation is ammonia. BUCH (16, 17) has reported recently the production of hydrazine.

Discussion is offered on the nature of nitrogen fixing system and its variability in *Azotobacter*, in comparison with other nitrogen fixers. The

fixation of nitrogen is specifically inhibited by nitrogen compounds such as nitrate, nitrite, ammonium salt (25), amino acids, or pepton (54), also by hydrogen gas or carbon monoxide in the *Legume-Rhizobium* combination (2, 6), *Azotobacter* (6, 11, 26, 27), and blue-green algae (101), which are aerobic nitrogen fixers. In the case of *Legume-Rhizobium* combination and *Azotobacter*, the fixation is inhibited by N_2O (22, 28, 29). The inhibition by H_2 and N_2O is competitive. In photosynthetic bacteria, the development of H_2 from some organic acids in the light is inhibited by N_2 (21, 26). In *Clostridium*, the fixation is not affected by H_2 (110), carbon monoxide, or nitrogen compounds in contrast with aerobic fixers. A comparison of some important properties of nitrogen fixers is seen in Table 43. *Azotobacter* contains the enzyme hydrogenase and some conditions inhibiting nitrogen fixation depress the activity of hydrogenase and nitrogenase. Hydrogenase or nitrogenase formation in blue-green algae is depressed under some heterotrophic conditions (34). Hydrogenase of *Clostridium* is a molybdo-flavoprotein (116). Because of the special relations between hydrogen gas and nitrogen fixation, it was thought that the reciprocal relationship might be applied here, such as that hydrogen competes with nitrogen and displaces nitrogen from the fixation system nitrogenase. Also, WILSON and BURRIS (6) reported that the affinity of H_2 for nitrogenase is about a fifth of that of N_2. The fixation requires molybdenum in the various nitrogen fixers (61). In the present investigations, the occurrence was found of the secretion of specifically large amounts of flavins in the molybdenum containing N_2-medium during the growth of *Azotobacter vinelandii*. From the data, the possibility was suggested that nitrogenase is a molybdo-flavoprotein similar to hydrogenase.

On the other hand, VIRTANEN points out that the leg-hemoglobin prepared from nodules has a function in nitrogen fixation but has no function in respiration (99, 100, 117). *Azotobacter* does not contain leg-hemoglobin, though it does contain cytochromes (30–40). The nitrogen fixation of *Azotobacter* is linked probably to respiration. There is reported the presence of cytochrome b_1 (30, 31), c_{4+5} (50), and a_2 (30, 31) in *Azotobacter*. Also, recently, there is reported the change of absorption bands of cytochromes during nitrogen fixation (17, 45).

Thus, it is possible that process of nitrogen fixation is linked with the respiration in the presence of nitrogenase, e. g. that nitrogenase may be linked with cytochromes following the production of the oxidized or reduced form of nitrogen.

In *Azotobacter*, the form and function (53, 54) are remarkably affected by the aging or different environmental conditions. On the origin of *Azotobacter*, THIMANN (60) assumed that it may be a "colorless blue-green algae", because

the nitrogen fixation is related to photosynthesis and the form of this organism resembles that of some of blue-green algae, such as a small blue-green algae *Chroöcoccus dispersus*, more than that of any other bacteria.

The problem of the origin of *Azotobacter* should be considered more in detail from the view point of enzymic change and in comparison with other related organisms. Further, the mechanism of nitrogen fixation and of enzymic change, the properties of nitrogenase and the system of its formation, or cytochromes, probably, connected with nitrogen fixation should be studied more definitely.

SUMMARY

The effects of different conditions of growth on the enzyme constitution and the induced synthesis of respiratory enzymes were investigated on *Azotobacter vinelandii*.

1. The presence of succinate in the N_2- medium was effective in the synthesis of succinic oxidase system, predominantly.

2. The extract of cells which grew in the succinate containing medium stimulated the activity of the succinic oxidase system of the cells grown in the succinate absent medium.

3. The different nitrogen sources brought about some enzymic differences during the growth. For example, the cells grown in the nitrogen compound-free medium could actively oxidize succinate or glucose but could not amino acids, while the cells grown in the NH_4Cl-, leucine-, and pepton-media could actively oxidize amino acids.

4. There was an optimum concentration of phosphate for the growth, for the synthesis of respiratory enzymes, or for the phosphate assimilation. In the N_2-medium, the increased uptake of phosphate by the growing cells occurred in accordance with the increase of amounts of phosphate in the growing medium, although the growth and the synthesis of respiratory enzymes were inhibited by the concentrated phosphate. In the leucine-medium, smaller amouts of phosphate brought about more active synthesis of L-leucine oxidizing enzyme in comparison with the large amounts of phosphate. The respiratory activity of growing cells was inhibited by the large amounts of phosphate in the N_2- and the KNO_3-medium, though there was no effect in the NH_4Cl-medium.

5. The treatment of inoculum cells at the comparatively lower temperatures in the various media brought about the change of enzymic or growing properties. For example, the peptone-cells treated or grown at 3°C. could grow in the N_2-medium, while the nontreated cells could not under the same

conditions. The treatment of inoculum cells at 3°C. for 5 to 20 days in the N_2-medium inhibited the growth and synthesis of respiratory enzymes and then the treatment for longer than 25 days was effective for the recovery of growing and enzyme-forming capacities to the same degree as the nontreated control cells. The same treatment of inoculum cells in the pepton-medium for 15 to 20 days inhibited the growth and the synthetic activity of respiratory enzymes except for the L-leucine and L-tyrosine oxidizing enzymes which were stimulated by this treatment at 3°C.; then the treatment for longer than 25 days was effective for the recovery of capacities of growth and enzyme synthesis to the same degree as the nontreated control cells. During the longer, more than 25 days, treatment at 3°C., in the pepton-medium, the inoculum cells could grow at this temperature and their progeny could grow more actively than the parent pepton-cells.

6. The cells grown at 30°C. in the pepton-medium could not grow in the N_2-, KNO_3-, and NH_4Cl-media. The low-temperature treatment of inoculum cells or the presence of mannit in the pepton-medium stimulated the growth in the N_2-medium. In accordance with the increase of generation number of pepton-cells the more rapid inactivation of growth in the N_2-medium occurred. The N_2-cells derived from the 3°C. treated peptone-cells and their progeny could oxidize amino acids to the same degree as the pepton-cells.

7. The induced formation of respiratory enzymes by the resting N_2-cells required the presence of some specific substrate and air. The NH_4Cl-, leucine-, and pepton-cells could not induce the enzymes. Such formation was inhibited in the anaerobic condition and in the presence of KCN, NaN_3, MIA, malonate, DNP, or chloramphenicol at different degrees of inhibition.

8. Mixture of glucose, succinate, acetate, and lactate or members of Krebs cycle, used as an inducer, brought about the simultaneous and limited synthesis of enzymes concerned with the oxidation of each substrate.

9. Succinic oxidase system induced by means of succinate was more resistant to starvation than the same enzyme system induced by means of acetate.

10. The synthesis of the succinic oxidase system in the presence of succinate and air by the resting N_2-cells involved the cosynthesis of succinic dehydrogenase, cytochrome c_4, and cytochrome c oxidase, and also of a substance which showed the absorption band at 260 mμ wave-length.

ACKNOWLEDGEMENT

To Professor S. USAMI, the writer is greatly indebted for his valuable advice during the course of this work.

REFERENCES

1. MAEDA, K., and USAMI, S., Symposia on Enzymology (in Japanese), **10**, 288 (1954).
2. MAEDA, K., and USAMI, S., Symposia on Biological Science (in Japanese), 11 (1955).
3. BEIJERINCK, M. W., Zentr. Bakt., II **7**, 561 (1901).
4. BEIJERINCK, M. W., and VON DELDEN, A., Ibid., II **9**, 3 (1902).
5. FISCHER, W. K., Arch. Mikrobiol., **14**, 353 (1949).
6. WILSON, P. W., and BURRIS, R. H., Ann. Rev. Microbiol., **7**, 415 (1953).
7. VIRTANEN, A. I., and LAINE, T., Biochem. J., **33**, 412 (1939).
8. VIRTANEN, A. I., Biol. Revs., **22**, 239 (1947).
9. SARIS, N., and VIRTANEN, A. I., Acta Chem. Scand., **11**, 1438 (1957).
10. SARIS, N., and VIRTANEN, A. I., Ibid., 1440 (1957).
11. WILSON, P. W., and BURRIS, R. H., Bacteriol. Revs., **11**, 4173 (1957).
12. APRISON, M. H., and BURRIS, R. H., Science, **115**, 264 (1952).
13. ALLISON, R. M., and BURRIS, R. H., J. Biol. Chem., **224**, 351 (1957).
14. BURMA, D. P., and BURRIS, R. H., Ibid., **225**, 287 (1957).
15. MOZEN, M. K., and BURRIS, R. H., Biochim. Biophys. Acta, **14**, 577 (1954).
16. BACH, M. K., Ibid., **26**, 104 (1957).
17. SHUG, A. L., HAMILTON, P. B., and WILSON, P. W., in "Symposium on Inorganic Nitrogen Metabolism", 344 (MCELROY, W. D., and GLASS, B. H., Edt., Johns Hopkins Press, Baltimore, 723 pp., 1956).
18. NICOLAS, D. J. D., and STEVENS, H. M., Ibid., 178 (1956).
19. ROBERTS, E. R., and AJIM, M. J., Ibid., 176 (1956).
20. CHENIAE, G., and EVANS, H. J., Ibid., 184 (1956).
21. GEST, H., JUDIS, J., and PECK, H. D., Ibid., 298 (1956).
22. BURRIS, R. H., Ibid., 316 (1956).
23. ROBERTS, E. R., and RAKESTRAW, J. A., Ibid., 361 (1956).
24. LEE, S. B., and WILSON, P. W., J. Biol. Chem., **151**, 379 (1943).
25. WILSON, P. W., HULL, J. F., and BURRIS, R. H., Proc. Natl. Acad. Sci., **29**, 289 (1943).
26. WILSON, P. W., UMBREIT, W. W., and LEE, S. B., Biochem. J., **32**, 2084 (1938).
27. WILSON, J. B., and WILSON, P. W., J. Gen. Physiol., **26**, 277 (1943).
28. WILSON, P. W., and LIND, C. J., J. Bact., **45**, 219 (1943).
29. GREEN, H., ALEXANDER, M., and WILSON, P. W., Proc. Exp. Biol. Med., **82**, 361 (1953).
30. NEGELEIN, E., and HASS, E., Biochem. Z., **266**, 1 (1934).
31. NEGELEIN, E., and GERISCHER, W., Ibid., **268**, 1 (1934).
32. CHANCE, B., Rev. Sci. Inst., **22**, 619 (1951).

33. REPASKE, R., and WILSON, P. W., J. Am. Chem. Soc., **74**, 3101 (1952).
34. REPASKE, R., J. Bact., **68**, 555 (1954).
35. KAMEN, M. D., Bact. Revs., **18**, 195 (1954).
36. KAMEN, M. D., Ibid., **19**, 350 (1955).
37. SMITH, L., Arch. Biochem. Biophys., **30**, 299 (1954).
38. SMITH, L., Bact. Revs., **18**, 1066 (1954).
39. TISSIERES, A., and SLATER, E., Nature, **176**, 736 (1955).
40. ALLEXANDER, A., and WILSON, P. W., Proc. Natl. Acad. Sci., U.S., **418**, 43 (1955).
41. MAGEE, N. E., and BURRIS, R. H., J. Bacteriol., **71**, 635 (1956).
42. ROSE, I. A., and OCHOA, S. I., J. Biol. Chem., **226**, 307 (1956).
43. BREMMA, T. H., WILSON, P. W., GLENN, J. L., and CRENE, F. L., J. Bacteriol., **73**, 113 (1957).
44. TISSIERES, A., HORENKAMP, H. G., and SLATER, E. C., Biochim. Biophys. Acata, **25**, 336 (1957).
45. CLARK, W. W., ROBERTS, E. R., KAPLAN, N. O., and KAMEN, M. O., Bacteriol. Revs., **19**, 250 (1957).
46. QUINNEL, C. M., J. Bacteriol., **73**, 688 (1955).
47. RAKESTRAW, J. A., and ROBERTS, E. R., Biochim. Biophys. Acta, **24**, 3 (1957).
48. CLARLES, C. R., and KOCHOLATY, W., Arch. Biochem. Biophys., **68**, 30 (1957).
49. WILSON, T. G. G., and WILSON, P. W., J. Bacteriol., **70**, 30 (1956).
50. TISSIERES, A., and BURRIS, R. H., Biochim. Biophys. Acta, **20**, 436 (1956).
51. VENON, L. P., and KAMEN, M. D., J. Biol. Chem., **211**, 643 (1954).
52. LAYNE, E. C., and NASON, A., J. Biol. Chem., **231**, 899 (1958).
53. ROBERG, M. X., Jahrb. Bot., **82**, 1 (1953).
54. JENSEN, H. L., Bacteriol. Revs., **18**, 195 (1955).
55. MONSOUR, V., and WYSS, O., Federation Proc., **14**, 257 (1955).
56. SLONIMSKI, P. P., La formation des enzymes respiratoires chez la levure (Masson et Cie., Paris, 1953).
57. MARCH, M. J., and KOCHOLATY, W., J. Biol. Chem., **224**, 909 (1957).
58. TCAM, M., and DRABKI, D., J. Biol. Chem., **224**, 92 (1957).
59. GOUCHER, C. R., and KOCHOLAY, W., Arch. Biochem. Biophys., **68**, 30 (1957).
60. THIMANN, K. V., in "Life of Bacteria", 337, 343 (McMillan Press, New York, 775 pp., 1955).
61. BORTELS, H., Arch. Mikrobiol., **1**, 333 (1930).
62. BURRIS, R. H., in "Manometric Techniques and Tissue Metabolism", 105 (UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F., Edt., Johns Hopkins Press, Baltimore, 835 pp., 1951).
63. BONNER, W. D., in "Methods in Enzymology", **1**, 722 (COLOWICK, S P., and KAPLAN, N. O., Edt., Johns Hopkins Press, Baltimore, 835 pp., 1957).
64. ESHEL, B., WAINIO, W. W., and COOPERSTEIN, S. J., J. Biol. Chem., **138**, 89 (1950).
65. COOPERSTEIN, S. J., and LAZAROW, A., Ibid., **189**, 665 (1951).
66. GREEN, D. E., MII, S., and KOHOUT, P. M., Ibid., **217**, 567 (1955).
67. BASFORD, R. E., TISIDALE, M. P., and GREEN, D. E., Biochim. Biophys. Acta, **22**, 495 (1956).

68. YAGI, K., in "Standard Biochemical Experiment" (in Japanese), 149 (EGAMI, F. et al., Edt., Bunkodo Press, Tokyo, 625 pp., 1953).
69. HAGIHARA, B., in "Protein, Nucleic Acid, and Enzyme" (in Japanese), **2**, 53 (1957).
70. ALLEN, R. J. L., Biochem. J., **34**, 858 (1940).
71. NEILSON, N. E., J. Bacteriol., **72**, 356 (1956).
72. KEILIN, D., and HARTRY, E. F., Proc. Roy. Soc., **13127**, 206 (1929).
73. RAKESTRAW, J. A., and ROBERTS, E. R., Biochim. Biophys. Acta, **24**, 388 (1957).
74. RAKESTRAW, J. A., and ROBERTS, E. R., Ibid., 555 (1957).
75. QUASTEL, J. H., and WOOLDIDGE, W. R., Biochem. J., **22**, 689 (1928).
76. SINGER, T. P., KEARNEY, E. B., and MASSY, V., in "Enzymes: Units of Biological Structure and Function", 424, 429 (GAEBLER, O. H., Edt., Academic Press, New York, 624 pp., 1956).
77. GALE, E. F., and FOLKES, J. B., Biochem. J., **53**, 483 (1953).
78. YCAS, M., and BRAWERMAN, G., Arch. Biochem. Biophys., **68**, 118 (1957).
79. GALE, E. F., and MCQUILLEN, K., Ann. Rev. Microbiol., **11**, 283 (1957).
80. WAINWRIGHT, S. D., and NEVILL, A., J. Gen. Microbiol., **14**, 47 (1956).
81. NEIDHARDT, F. C., and MAGASANIK, B., Biochim. Biophys. Acta, **21**, 324 (1956).
82. POLLOCK, M. R., TORRIANI, Z. M., and TRIDGELL, E. J., Biochem. J., **62**, 387 (1956).
83. KOGUT, M., POLLOCK, M. R., and TRIDGELL, E. J., Ibid., 391 (1956).
84. LOVTRUP, S., Biochim. Biophys. Acta, **19**, 249, 433 (1956).
85. BORTELS, H., Zentr. Bakt., II **95**, 193 (1936).
86. HORNER, C. K., BURK, D., ALLISON, F. E., and SHERMANN, M. S., J. Agr. Res., **65**, 173 (1942).
87. MOCKERIDGE, E. A., Biochem. J., **9**, 272 (1915).
88. MEYERHOF, O., and BURK. D., Z. physiol. Chem., A **159**, 117 (1928).
89. MONOD, J., in "Enzymes: Units of Biological Structure and Function", 7 (GAEBLER, O. H., Edt., Academic Press, New York, 624 pp., 1956).
90. VELICK, S. F., Ibid., 90 (1956).
91. SPIEGELMAN, S., HALVERSON, H. O., and BEN-ISHAI, R., in "Amino Acid Metabolism", 124 (MCELROY, E. D., and GLASS, B., Edt., Johns Hopkins Press, Baltimore, 1048 pp., 1955).
92. GALE, E. F., in "Enzymes: Units of Biological Structure and Function", 49 (GAEBLER, O. H., Edt., Academic Press, New York, 624 pp., 1956).
93. SPIEGELMAN, S., Ibid., 67 (1956).
94. PARDEE, A. B., Proc. Natl. Acad. Sci., U.S., **40**, 263 (1954).
95. POLLOCK, M. R., Brit. J. Exptl. Pathol., **31**, 739 (1950); **32**, 387 (1951); **33**, 587 (1952).
96. GALE, E. F., and FOLKES, J. P., Biochem. J., **59**, 661, 675 (1955); Nature, **175**, 592 (1955).
97. STANIER, R. Y., Ann. Rev. Microbiol., **5**, 35 (1951).
98. BRENNER, S., and STENT, G. S., Biochim. Biophys. Acta, **17**, 473 (1955).
99. SMITH, J. D., Biochem. J., **44**, 585, 591 (1949).
100. KEILIN, D., and WANGE, J. L., Nature, **155**, 227 (1945).
101. BURRIS, R. H., and WILSON, P. W., Bot. Gaz., **108**, 254 (1946).
102. ROSENBLUM, E. D., and WILSON, P. W., J. Bacteriol., **59**, 83 (1950).

103. DREWES, K., Zentr. Bakt., II **76**, 88 (1928).
104. FOGG, G. E., J. Exptl. Biol., **19**, 78 (1942).
105. WATANABE, A., NISHIGAKI, S., and KONISHI, C., Nature, **168**, 743 (1951).
106. ALLEN, M. B., Sci. Monthly, **83**, 100 (1956).
107. GEST, H., KAMEN, M. D., and BREGOFF, H. M., J. Biol. Chem., **182**, 153 (1950).
108. LINDESTROM, E. S., TOVE, S. R., and WILSON, P. W., Science, **112**, 197 (1950).
109. WINOGRADSKY, S., Zentr. Bakt., II **9**, 43, 107 (1902).
110. ZELITCH, I., Proc. Natl. Acad. Sci., **27**, 559 (1951).
111. HINO, S., J. Biochem. (Tokyo), **42**, 775 (1955).
112. HIAI, S., MORI, T., HINO, S., and MORI, T., J. Biochem. (Tokyo), **44**, 839 (1957).
113. STEPHENSON, M., and STICKLAND, L. H., Biochem. J., **25**, 215 (1931).
114. BUTLIN, K. R., ADAMS, M. E., and THOMAS, M., J. Gen. Microbiol., **3**, 40 (1949).
115. SISLER, F. D., and ZOBELL, C., Science, **113**, 511 (1951).
116. MAPHER, H. R., Adv. Enzymol., **17**, 233 (1956).
117. THOROGOOD, E., Science, **126**, 1011 (1957).