BIOCHEMISTRY OF BIOTIN DEFICIENCY IN *ARTHROBACTER GLOBIFORMIS*

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Some biochemical aspects of biotin deficiency in *Arthrobacter globiformis*

Biotin deficiency has been shown to cause morphological aberrations in *A. globiformis* 425. Single compounds, such as oleic acid, linoleic acid, linolenic acid, Tween 80, aspartate, and oxalacetate, showed varying degrees of biotin-replacing activity, but none was able to restore the cell to normal growth. Comparative analysis of fatty acid composition indicated that biotin deficiency causes a shift in synthesis from anteiso to normal straight chain fatty acids. RNA, DNA, and protein accumulation were followed throughout the growth cycle. The RNA content per cell was reduced by the vitamin deficiency, however, the DNA per C.F.U. was maintained at a level comparable to that of the normal cell. Protein reached a final level 35% greater than the normal cell protein. It is probable that much of this material is comprised of structural proteins which would account for the morphological abnormalities which accompany biotin-depletion in this organism. Hydrolysates of cell wall material were analysed for amino sugar and amino acid contents. The abnormal cell was found to contain 13.4 times more ninhydrin-reacting material and 19.4 times more hexosamine per mg dry weight over the biotin-sufficient cell.

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SOME BIOCHEMICAL ASPECTS OF BIOTIN DEFICIENCY IN *ARTHROBACTER GLOBIFORMIS*

by

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INTRODUCTION

Biotin is an essential growth factor for many micro-organisms (Briggs, 1961). In 1962 Chan and Stevenson confirmed a previous report by Morris(1960) that Arthrobacter globiformis 425 is also a biotin auxotroph. Two years later Chan (1964) reported that when the organism was grown in medium containing suboptimal concentrations of the vitamin, morphologically abnormal forms appeared. He suggested that cell wall synthesis might be impaired. Examination of the cell using electron microscopy indicated that the abnormal form consists of several membrane-bound bodies surrounded by a thick matrix, giving further support to the view that the cell wall is affected (Robertson and Chan, 1970). The aim of the following work is to relate the morphological aberrations to the biochemical changes which occur as a result of biotin deficiency.

HISTORICAL REVIEW

Vitamin Associated Morphological Aberrations

The involvement of morphological abnormalities with vitamin deficiencies was first reported in a vitamin B_{12}-requiring *Arthrobacter* species by Chaplin and Lochhead in 1956. One year later Holden and Holman described the appearance of an elliptical, swollen form of *Lactobacillus arabinosus* as vitamin B_6 became limiting. They suggested that cell wall composition or structure might be altered, an hypothesis which was proven in later studies (Holden and van Balgooy, 1964,1965).

Shiio, Otsuka, and Takahashi (1962) were the first to involve biotin in a similar context in *Brevibacterium flavum*. Chao and Foster (1959) had previously described a pleiomorphic biotin-requiring *Bacillus* species. However, rather than relating the pleiomorphism to nutritional factors, they felt that this *Bacillus* 14B22 was an intermediate between *B. megaterium* and *B. cereus*. It was later renamed *B. cereus* by Hubbard and Hall (1968) who attributed the morphological changes to vitamin deficiency. Other microorganisms similarly affected are *Arthrobacter globiformis* (Chan, 1964), *Saccharomyces cerevisiae* (Dixon and Rose, 1964), *Escherichia coli* (Gavin

and Umbreit, 1965), Bacillus megaterium (Shirokov, et al, 1965) and Bacillus polymyxa (Summers and Wyss, 1967).

Biotin Involvement in Cell Metabolism

Early evidence for biotin's role in fatty acid biosynthesis was provided by Williams and Feiger in 1946. They reported that oleic acid and elaidic acid both have the ability to substitute for biotin in Lactobacillus casei. This replacement was optimal at a given concentration, time of incubation, pH, and temperature. Despite such strong evidence for an enzyme-linked role, they favoured the view proposed earlier by Kodicek and Worden (1945) that the stimulation was due to physico-chemical properties of the fatty acid. Axelrod, Mitz, and Hofmann (1948) identified as fatty acids the three factors in human plasma which were able to replace biotin for two Lactobacillus species. The carboxyl group of the oleate was an essential factor and a synergistic response was obtained if saturated fatty acids were added as well. They felt that their data suggested an involvement of biotin in lipid metabolism. Similar experiments in which fatty acids enhanced to varying degrees the growth of a biotin-deficient organism gave further support to this hypothesis

(Broquist and Snell, 1951; Hofmann and Panos, 1954; Hubbard and Hall, 1968; Yoshida, 1969). Cheng, et al,(1952) showed that the position of the double bond and the degree of unsaturation were important factors in the biotin-replacing capabilities of certain fatty acids. In vitro studies, using purified extracts of avian liver, finally confirmed suspicions that biotin is involved in fatty acid biosynthesis (Wakil, Titchener , and Gibson, 1958; Wakil and Gibson, 1960).

That biotin is a multifunctional compound became apparent early in the search for its biological role. A variety of compounds including aspartate, oxalacetate and carbon dioxide were able to stimulate growth of biotin-deficient organisms. In Streptococcus faecalis aspartate alone substituted adequately whereas in Lactobacillus arabinosus replacement by the amino acid was only partial (Stokes, Larsen, and Gunness, 1947; Broquist and Snell, 1951). It was incompletely effective in Torula cremoris as well (Koser, Wright, and Dorfman, 1942), indicating that aspartic acid could only compensate for one of biotin's functions. Ahmad and Rose (1962) and Suomalainen and Keranen (1963) independently worked on two different strains of S. cerevisiae. They reported that the biotin requirement could be satisfied by supplying aspartate along

with unsaturated long chain fatty acids. Ahmad and Rose further noted that although the yeast grew well and was less fragile than its biotin-deficient counterpart, restoration to normal cell wall synthesis was not complete. Other studies on this organism revealed that there are multiple restrictions imposed on the cell as a result of decreased biotin. Aside from changes in cell wall composition (Dunwell, Ahmad, and Rose, 1961) there is an increase in acid-soluble U.V. absorbing material within the cell in early growth along with a decrease in RNA, DNA,and protein (Ahmad, Rose, and Garg, 1961). Of the enzymes tested only carbamyl phosphate ornithine carbamoyl transferase activity seemed to be reduced as a direct consequence of the nutritional stress.

Lardy, Potter and Elvejehm(1947) proposed that oxalacetate's ability to stimulate biotin-deficient L. arabinosus can be explained in terms of a metabolic block which prevents its formation via the carboxylation of pyruvate. The oxalacetate provided could then be converted to aspartic acid via the non-biotin requiring transamination reaction. Two years later they reported that there was a decreased fixation of CO_2 in this organism (Lardy, Potter, Burris, 1949). Broquist and Snell found CO_2 stimulatory in 1951.

Biotin was also reported necessary for the carboxylation of pyruvate in E. coli (Shive and Rogers, 1947) and stimulatory in the decarboxylation of oxalacetate in these cells when aged (Lichstein and Umbreit, 1947).

Role of Biotin in Carboxylation Reactions

Despite the accumulation of data involving biotin in a variety of metabolic reactions, there remained much doubt as to its actual role. Failure to detect protein-bound biotin led to the suggestion that the vitamin, rather than being a cofactor, is necessary for the synthesis of certain enzymes (Blanchard, et al, 1950). However this view was discarded when acetyl-CoA carboxylase, an enzyme which participates in the first step of fatty acid biosynthesis, was found to contain bound biotin and to be inhibited by avidin (Wakil, et al, 1958,1960). Other biotin-requiring enzymes include beta-methyl crotonyl-CoA carboxylase (Lynen, 1957), pyruvate carboxylase (Utter and Keech, 1960), propionyl-CoA carboxylase (Kaziro and Ochoa, 1961) and geranoyl-CoA carboxylase (Seubert, Fass, and Remberger, 1963). All are ATP-dependent carboxylating enzymes which require Mg^{++} for their activation. The reaction characteristically occurs in two steps as exemplified by the E. coli acetyl-CoA carboxylase (Alberts and Vagelos, 1968).

1. E-biotin + ATP + HCO_3^- $\xleftarrow{Mg^{++},\ Mn^{++}}$ E-biotin-CO_2 + ADP + Pi

2. E-biotin-CO_2 + RH (acetyl-CoA) $\longleftrightarrow$ E-biotin + R-CO_2 (malonyl-CoA)

This enzyme can be dissociated into two subunits, each one catalyzing one of the above reactions. The enzyme of the first reaction can be further dissociated at alkaline pH into a biotin-containing protein and a larger biotin-free protein which has the ability to catalyse the carboxylation of free biotin in a model reaction (Alberts, Nervi, and Vagelos, 1969).

A transcarboxylase, catalysing the formation of oxalacetate from methylmalonyl-CoA, has been found in a propionic acid-producing bacterium. It differs from the carboxylase enzymes in not requiring magnesium or ATP. It too can be dissociated at alkaline pH into subunits (Gerwin, Jacobson, and Wood, 1969; Northrop and Wood, 1969).

The most recent addition to the magnesium, ATP, biotin-dependent enzymes is a ureaamidolyase found in Candida utilis (Roon and Levenberg, 1970). It requires catalytic amounts of bicarbonate which, with the help of biotin is transferred to the urea to form allophanate. This intermediate is then split to release the end products, ammonia and bicarbonate.

Despite the apparent diversity of functions attributed to biotin, all enzymic reactions that have been closely examined involve carbon dioxide transfer. It is possible then that many reactions known to involve CO_2 will be found to require biotin and, conversely, those suspected of requiring this cofactor will in some way involve CO_2 transfer.

Effect of Biotin on Membrane Composition

Lipid composition of microorganisms have been known to vary widely with age of culture (Kates, Adams, and Martin, 1964; Kanfer and Kennedy, 1963; Law, Zalkin, and Kaneshiro, 1963), temperature of incubation (Marr and Ingraham, 1962; Bishop and Still, 1963; Kates and Hagen, 1964) and composition of the medium (Marr, et al, 1962). The discovery of biotin involvement in fatty acid synthesis (Wakil, et al, 1960) led to a search for changes in the lipid fraction of the cell under conditions where the vitamin was limiting. The observation that splits occur in the lipid layer of S. cerevisiae (Dixon and Rose, 1964) gave morphological evidence in support of a previous report by Suomalainen and Keranen (1963) that there is an increase in C_{16} and a decrease in C_{18} fatty acids. Total lipid content was lower in Aspergillus nidulans (Rao and Modi,

1968), Bacillus subtilis (Kaneda, 1966), and Lactobacillus plantarum (Croom, McNeill, and Tove, 1964; Holden, et al, 1970. The particular changes which have been noted differ in different microorganisms and there is no obvious general trend. Thus B. cereus (Hubbard, et al, 1968) and B. subtilis (Kaneda, 1966) synthesized increased proportions of branched-chain fatty acid, whereas L. plantarum decreased its cis-vaccenic and lactobacillic acids (Croom, et al, 1964). There is also a decrease in C_{18} monoenic acid in Rhizobium japonicum (Bunn, McNeill, and Elkan, 1970) and C_{17} cyclopropane fatty acid in E. coli (Croom, et al, 1964). Increases in non-saponifiable material occurred in E. coli (Gavin and Umbreit, 1965) and L. plantarum (Holden, et al, 1970)

Glutamate Excretion in Biotin-deficient Cells

It is highly likely that these changes, which occur in the membrane as a result of biotin deficiency, are related to other changes that have been reported, such as the high level of glutamate production. It has generally been found that as the level of intracellular biotin drops below that required for optimal growth, glutamic acid excretion increases. This phenomenon has been described in Bacillus 14B22 (Chao and Foster, 1959), Brevibacterium flavum (Shiio, et al, 1962), Micrococcus glutamicus (Kimura, 1963), Microbacterium ammoniaphilum (Miyai et al, 1963) and Brevibacterium lactofermentum (Takinami,

Yamada, and Okada, 1966). Arthrobacter globiformis (Veldkamp and Zevenhuizen, 1963) and E. coli (Gavin and Umbreit, 1965) are also listed among the glutamate excreters. However, this phenomenon has not been as extensively studied in these two organisms.

Most evidence suggests that the increased production of glutamate is due to an increased permeability of the cell. Kimura (1963) found that the level of intracellular free amino acids in M. glutamicus is decreased when biotin is limiting. He suggested that the cells exhibit an increased permeability to amino acids leading to an excretion of glutamate along with a decreased conversion of glutamate to other amino acids. Brevibacterium flavum also has a decreased intracellular amino acid level and an increased uptake of glutamate, aspartate, and alpha-ketoglutarate (Shiio, et al, 1962). They postulated that biotin indirectly regulates cell permeability via the synthesis of unsaturated fatty acids, as evidenced by oleate's ability to substitute for biotin. The increased permeability to glutamate upsets the regulation of its synthesis, causing an overproduction of the amino acid (Shiio, Otsuka, and Katsuya, 1963).

In contrast to this work by Shiio, et al, Hubbard and

Hall (1968) found that the low biotin culture of B. cereus contained twice the intracellular glutamate as that of the normal cell. Furthermore, unlike B. flavum, there was no increased release of amino acids from the vitamin deficient cell by washing. They concluded that in B. cereus an absence of sufficient biotin does not result in an increased permeability. It is possible that B. cereus does not represent a new class of glutamate producers, but rather the lack of permeability observed is due to the choice of citrate as a carbon source for Shiio, et al had reported that the choice of carbon source greatly influences the biotin level needed for optimal glutamate excretion. Furthermore, citrate has been shown to be an activator of liver and yeast acetyl-CoA carboxylase (Knappe, 1970), the enzyme of the first step in fatty acid biosynthesis.

Further evidence in support of the permeability hypothesis comes from the work of Oishi, et al (1970) who found that sucrose or a high concentration of salts are able to restore glutamate uptake to its normal level in B. ammoniagenes. This uptake shows less specificity than the normal cell, since it is not inhibited by aspartic acid. The use of sucrose to raise extracellular osmotic pressure will also increase the accumulation of amino acids in L. plantarum

(Holden and Utech, 1967). Alternatively, stimulation of lipid synthesis by the addition of acetate and biotin will reverse the impaired uptake (Holden, et al, 1970) by restoring the lipid content of the cell to that of the biotin-sufficient organism. S. cerevisiae, though not a glutamate producer, also exhibits increased permeability to chloride, phosphate, and bovine plasma albumin when the concentration of the vitamin is suboptimal (Rose, 1963).

Shibukawa, et al (1965) attempted to relate the shifts in fatty acids of B. ammoniaphilum to the accumulation of extracellular glutamate. The major fatty acids of this organism are a monounsaturated 18-carbon and a saturated 16-carbon fatty acid. When the ratio of saturated to unsaturated fatty acids exceeded one, glutamate excretion began. In high biotin cultures the reverse was true: the synthesis of the monounsaturated fatty acid exceeded that of the saturated, the ratio was less than one and glutamate excretion ceased.

METHODS AND MATERIALS

General:

All chemical materials used were of the highest purity commercially available. Water used was first distilled in a tin-lined still. It was then passed through an ion exchange cartridge and finally glass-distilled.

Organism:

Arthrobacter globiformis 425 was obtained from the stock culture collection of the Cell Biology Research Institute, Canada Department of Agriculture, Ottawa. This strain, also designated NCIB 8602 or ATCC 4336, is identical to the one used by Conn (1928). Stock cultures were maintained on Trypticase Soy Agar slants and were stored at 4 C.

Medium:

a)Stock solutions of inorganic salts were prepared as described by Snell and Strong (1939).

Solution A	K_2HPO_4		12.5	g
	KH_2PO_4		12.5	g
	Distilled water	to	125	ml
Solution B	$FeSO_4 \cdot 7H_2O$		0.25	g
	NaCl		0.25	g
	$MnSO_4 \cdot 4H_2O$		0.25	g
	$MgSO_4 \cdot 7H_2O$		5.0	g
	Distilled water	to	125	ml

b) The basal salt solution (BSS) shown below was supplemented with glucose for use as growth medium (Chan, 1964):

Solution A	5	ml
Solution B	5	ml
KNO_3	5.0	g
Glucose(20%,w/v)	50	ml
Distilled water to	1000	ml

The pH was adjusted to 6.8 using a Beckman Zeromatic pH meter.[1] The medium was autoclaved for 15 minutes at 121 C. Glucose (20%,w/v) which had been autoclaved for ten minutes at 121 C was added to the sterile medium just prior to use. The final concentration of the glucose in the medium was 1% (w/v).

c) Solutions containing 40 ug of biotin per ml distilled water were sterilized by Millipore membrane filtration (0.22 u). Twenty ml aliquots were stored at - 10 C in screw-cap glass tubes. Sufficient biotin to give a final concentration of 0.4 mug/ml was added to the basal medium in order to obtain normal cells. These cells are designated biotin-sufficient. The biotin-deficient culture received no additional vitamin but relied on intracellular carry-over and contaminating traces of the vitamin for its growth. Biotin has been shown to be a contaminant of sucrose (Chan and Stevenson, 1962) and glucose (Robertson and Chan, 1970).

1.Beckman Instruments, Inc., Palo Alto, California

Inoculum:

a) A 300-ml capacity nepheloculture flask[2] containing 50 ml of basal medium and biotin was inoculated with a loopful of cells from a 24-hour Trypticase Soy Agar slant culture. It was incubated at 25 C on a gyratory incubator shaker[3] at 160 rpm.

b) After 19-22 hours of growth, the inoculum cells were centrifuged for 10 minutes at 12,000 x g in a refrigerated centrifuge[4]. They were washed twice in a basal salt solution (BSS), each washing being followed by a 10 minute spin at 12,000 x g. The cells were resuspended in BSS to a turbidity of 100 Klett Units (K.U.). A Klett-Summerson photoelectric colorimeter[5], equipped with a no. 42 blue filter was used for this measurement.

c) Except where otherwise indicated, 0.1 ml of 100 K.U. cell suspension was inoculated into 50 ml of medium. A corresponding increase to 0.8 ml was made when the organism was grown in 2-liter Erlenmeyer flasks, each containing 400 ml of medium. All flasks were grown at 25 C in the incubator, shaking at 160 rpm.

2. Bellco Glass, Inc., Vineland, New Jersey.
3. Psycrotherm, Controlled Environment Incubator Shaker. New Brunswick Scientific Company, New Brunswick, New Jersey.
4. Ivan Sorvall, Inc., Norwalk, Connecticut.
5. Klett Manufacturing Company, New York, New York.

Measurement of Growth:

a) Growth of cultures was measured turbidimetrically using the Klett-Summerson photoelectric colorimeter. All readings were taken using a blue filter (no. 42) and expressed as Klett Units (K.U.).

b) In some studies, viable counts were also used as a measure of cell growth. Serial dilutions were done using 0.5 ml of cell suspension in 4.5 ml BSS diluent. All dilutions were shaken vigorously on a Fisher mini shaker[6] to dissociate aggregated cells as much as possible. One-tenth ml samples of the appropriate dilutions were plated on Trypticase Soy Agar plates using a glass spreader. Plates were allowed to incubate for 2-3 days at 25 C. Colonies were counted using a colony counter [7]. The number obtained was expressed as colony forming units (C.F.U.).

Changes in Macromolecular Levels with Growth

Inoculum

Inoculum used for these determinations deviated from the standard conditions in order to obtain sufficient cell yield from the biotin-deficient culture to permit estimation of the

6. Fisher Scientific Co., Montreal, P.Q.
7. Otto C. Watzka and Co., Ltd., Montreal, P.Q.

protein, DNA, and RNA throughout the growth cycle. Six ml of a 500 K.U. inoculum suspension were added to each of two 2-liter flasks containing 600 ml of the glucose-BSS medium. One of the two flasks was supplemented with the appropriate amount of biotin.

Sampling

The procedure followed was that used by Stevenson (1962). Samples ranging in size from 20-100 ml were taken at 3.5 hour intervals for 21 hours. Each sample was washed twice in BSS and resuspended in 10 ml of 5% trichloroacetic acid (TCA). After centrifuging for 10 minutes at 7,700 x g, the precipitate was suspended in 5 ml of 5% TCA, heated for 30 minutes in boiling water and spun down again under similar conditions. The supernatant contained the RNA and DNA; the pellet contained the protein material.

Growth Measurement

Growth was followed turbidimetrically and by viable counts as described above.

Protein Determination

Protein was assayed according to the method of Oyama and Eagle (1956). The protein-containing pellet was suspended in 1 ml of 1N NaOH and heated for 10 minutes to remove any cloudiness. Dilutions of the sample were done using 0.1 N NaOH as diluent. The reagents were prepared as follows:

Reagent A:	Na_2CO_3		0.2	g
	NaK tartrate		0.2	g
	Distilled water	to	1000	ml
Reagent B:	$CuSO_4.5H_2O$		5.0	g
	Distilled water	to	100	ml

Reagent C: 50 parts of Reagent A plus 1 part of Reagent B. This was prepared just before use.

Folin-Ciocalteau reagent(Fisher Scientific Co.): A dilution of 5:7 was made using distilled water.

Reference standards were prepared using bovine serum albumin.

Five ml of reagent C were added to 1 ml samples and allowed to stand for 10 minutes at room temperature. One-half ml of the Folin-Ciocalteau solution was then added. The contents of each tube were then mixed and allowed to stand for 30 minutes to allow for color development. Optical density readings were measured at 690 mu using a Bausch and Lomb Spectronic 20 spectrophotometer [8].

8. Bausch and Lomb Optical Co., Rochester, New York.

Ribonucleic Acid Determination

RNA was assayed according to the method of Dische (1955). The reagent was prepared as follows:

1 g orcinol
100 ml concentrated HCl
0.5 g $FeCl_3 \cdot 6H_2O$

Reference standards were prepared using yeast RNA. Three ml of the orcinol reagent were added to 1.5 ml samples. After heating for 20 minutes in boiling water, the tubes were cooled and read at 670 mu using the Spectronic 20 spectrophotometer.

Deoxyribonucleic Acid Determination

The diphenylamine method of Dische (1955) was used to determine DNA content. The reagent was prepared as follows:

1 g diphenylamine
100 ml glacial acetic acid
2.75 ml concentrated H_2SO_4

Reference standards were prepared using salmon sperm DNA. 1.5 ml of sample and 3 ml of the diphenylamine reagent were placed in a boiling water bath for 10 minutes. The mixture was then cooled and read at 600 mu using a Spectronic 20 spectrophotometer.

Replacement Studies

Oleic acid, linoleic acid, linolenic acid, Tween 80, aspartic acid and oxalacetic acid were each tested for their biotin-replacing activity in A. globiformis . Stock solutions of oleic, linoleic, and linolenic acids were prepared in ethanol at a concentration of 10 mg/ml. All subsequent dilutions were made in distilled water. The remaining compounds were initially dissolved in distilled water. All solutions were adjusted to pH 7 using 1N NaOH and sterilized by Millipore filtration (o.22 u). The basal medium was supplemented with the desired concentration of the test compound. Growth was expressed in both Klett units and colony forming units. The ranges of each compound tested are given below:

Compound	Ranges of concentrations tested			
Oleic acid	0.1	ug/ml	to	100 ug/ml
Linoleic acid	0.1	ug/ml	to	20 ug/ml
Linolenic acid	0.05	ug/ml	to	1 ug/ml
Tween 80	0.5	ug/ml	to	10 ug/ml
Aspartic acid	13	ug/ml	to	1330 ug/ml
Oxalacetic acid	0.66	ug/ml	to	660 ug/ml

Fatty Acid Analysis

Growth of Cells

Cells were grown in 2-liter Erlenmeyer flasks containing 400 ml of medium. After 18, 29.5, and 51 hours of growth, both the biotin-deficient and biotin-sufficient cells were harvested and washed twice in distilled water. Conditions of centrifugation were 10 minutes at 4 C at 10,000 x g. The ages of the cells collected corresponded to early log, late log, and stationary phases of growth. Comparative analysis of the three sampling times ensured that any difference observed in fatty acid composition was due to the limitations of biotin deficiency and not due to differences in physiological ages of the two cultures.

Extraction of Fatty Acids

Saponification was carried out according to the procedure of Bunn, et al (1970). Cells were suspended in 20 ml of 50% (v/v) methanol containing 15% (w/v) KOH. They were then refluxed at 60-70 C for three hours. Centrifugation for 10 minutes at 12,000 x g sedimented the cell debris which was discarded. The subsequent extraction of the lipid material

followed the method of Walker and Fagerson (1965). The supernatant was extracted twice with ½ volume of diethyl ether. This extracted material contained any non-saponifiable material that might be there and was discarded. The remaining supernatant was acidified to pH 2 with 6N HCl and was re-extracted 4 times with 3/4 volume of diethyl ether. The excess ether was allowed to evaporate to approximately 3 ml.

Formation of Fatty Acid Methyl Esters

Fatty acid methyl esters were prepared using diazomethane according to the method of Schlenk and Gellerman (1960). The apparatus used consisted of three rubber-stoppered tubes interconnected by glass tubing. A stream of nitrogen was introduced into the system, passing first through an ether-containing tube. The gas acted as a carrier of the diazomethane formed in the second tube from Diazald [9] (N-methyl-N-nitroso-p-toluenesulfonamide;0.214 g in 1 ml ether), 0.7 ml Carbitol (2-(2-ethoxy ethoxy)-ethanol), 0.7 ml ether, and 1 ml 60% KOH. This yellow gas was carried to the third tube which contained the extracted fatty acid mixture dissolved in 3 ml ether containing 10% methanol. The reaction was allowed to proceed for 10 minutes.

9. Aldrich Chemical Co., Milwaukee, Wisconsin.

Gas Chromatography

A Hewlett-Packard F and M Gas Liquid Chromatograph[10], equipped with a 3% S.E. 30 methyl silicone rubber gum column on Diatoport S (80-100 mesh) of dimensions 6' x 1/4 ", was used for all the analyses. Injections were made using a microliter syringe[11]. The following conditions were used throughout:

Column temperature	170 C
Hydrogen flame detector temperature	300 C
Flash heater temperature	300 C
Hydrogen flow rate	37 ml/min.
Air flow rate	300 ml/min.
Nitrogen flow rate (carrier gas)	60 ml/min.

Identification of the fatty acid peaks was by comparison of retention times with that of known standards.[12] Analysis of the standards was redone on any given day that samples were chromatographed to check for altered retention times resulting from small changes in any of the conditions of temperature and gas flow. Relative proportions of fatty acids within a given sample were calculated as follows:

$$\% \text{ fatty acid} = \frac{\text{area under peak of fatty acid}}{\text{area of all fatty acids}} \times 100$$

where the area of the peak is proportional to the height X distance from the origin, and is calculated therefrom.

10.Hewlett-Packard, Pasadena, California.
11.Hamilton Co., Whittier, California.
12.Applied Science Laboratories, Inc., State College, Pa.

Cell Wall Analysis

Treatment of Cells

Biotin-deficient and biotin-sufficient cells were grown in two-liter flasks containing 400 ml medium. After 46.5 hours of incubation, the cells were spun at 12,000 x g for 10 minutes, washed twice in distilled water and resuspended in 10 ml distilled water. Twenty grams of glass beads (0.11-0.12 mm in diameter), which had been washed in 1N HCl, rinsed in distilled water and dried, were added to each of the cell suspensions. Disruption of the cells using the Braun Cell Disintegrator[13] was found to be complete by viable counts, after 3.5 minutes of breakage. The cell extracts were decanted, the beads washed three times using 5 ml distilled water for each washing, and the washings were added to the cell extracts.

Isolation of Cell Wall Material

Cell walls were isolated on the basis of differential centrifugation according to the method of Salton (1964). The disintegrated cell suspension was centrifuged for 10 minutes at 2, 445 x g to deposit the intact cells and cell debris. The supernatant was spun at 7,900 x g for 20 minutes to

13. Bronwill Scientific, Inc., Rochester, New York

deposit the crude cell wall material. This pellet was washed once with M NaCl and centrifuged again at 7,900 x g for 15 minutes. The precipitate was resuspended in 1 mg/ml of trypsin dissolved in 0.005 N NH_4OH containing 0.05 M $NaHCO_3$ as directed by Park and Hancock,(1960). After two hours of incubation at 37 C, the mixture was diluted in 10 ml distilled water and centrifuged for 20-30 minutes at 9,250 x g. The pellet was washed again in 5 ml distilled water and centrifuged under the same conditions. The cell wall material was then separated from the debris by resuspending in 5 ml distilled water and spinning at 2,200 x g for 5 minutes. The supernatant contained the cell wall material.

Hydrolysis of Cell Wall Material

The cell wall-containing supernatant was divided into two parts. One was hydrolysed at 100 C for 16 hours in 6N HCl, the other was hydrolysed for 2 hours in 2N HCl at the same temperature. Both were evaporated to dryness, resuspended in distilled water and reevaporated to get rid of the acid. The former was analysed for amino acid-containing material, the latter for amino sugars.

Ninhydrin Reaction

The amino acids were analysed for total ninhydrin-reacting material relative to alanine, which was used as the reference standard. The method was according to the procedure of Rosen as described in "Methods and References in Biochemistry and Biophysics" (1966). The reagents described below were used.

Cyanide-acetate buffer:

A: 4.9 mg sodium cyanide was dissolved in 100 ml distilled water.

B: 27 g sodium acetate trihydrate, 5 ml glacial acetic acid in 75 ml water.

Buffer: 1 ml reagent A in 50 ml reagent B.

3% Ninhydrin dissolved in methyl cellosolve and isopropyl alcohol-water diluent (50% isopropyl alcohol in water).

The analysis was carried out as follows. One ml sample was heated for 15 minutes at 100 C with o.5 ml buffer and 0.5 ml ninhydrin. Five ml isopropyl alcohol-water diluent was added. The mixture was shaken, allowed to cool to room temperature and read at 570 mu in a Spectronic 20 spectrophotometer.

Hexosamine Determination

The procedure followed was the one described by Dische and Borenfreund in 1950. Deamination of the hexosamine was

achieved by allowing 0.5 ml samples to stand at room temperature for ten minutes in 0.5 ml 33% acetic acid and 0.5 ml sodium nitrite (5%, w/v). Excess nitrous acid was removed by adding 0.5 ml 12.5 % (w/v) ammonium sulfamate and shaking at intervals over a period of thirty minutes. Two ml 5% HCl and 0.2 ml 1% (w/v) indole in ethanol were added to each tube. After five minutes of heating in boiling water, two ml ethanol were added to each tube. Optical density readings were taken at 492 mu and 520 mu. Hexosamine concentration was proportional to $O.D._{492} - O.D._{520}$. Undeaminated controls were run for each sample. Glucosamine-HCl served as reference standard.

DNA Determination

DNA was determined according to the method of Dische (1955) as previously described.

Dry Weight

Duplicate samples of 0.5 ml of the washed cell suspension were added to preweighed aluminum pans. The samples were dried by overnight incubation at 100 C. The pans were weighed again and the dry weight of the cells were calculated by difference.

RESULTS

Changes in Macromolecular Levels With Growth

Biotin-requiring enzymes have been implicated in a variety of metabolic pathways. In order to determine whether biotin depletion causes a generalized or specific change in cellular metabolism, a comparison of shifts in DNA, RNA, and protein levels with growth was made between the two cell types. The DNA/ml of the biotin-deficient culture increased at a rate 67% that of the normal culture in log. phase (Figurel). This reflects a generalized decrease in growth rate as measured in Klett units (Figure 2A). The RNA synthesis is impaired to a greater degree, exhibiting an increase of 40% the rate of the biotin-sufficient cell during exponential growth (Figure 3). The protein maintained a level almost exactly that of the normal throughout most of the growth cylcle (Figure 4). Only at the end of log. growth phase (17.5 hr) does it decrease to 67% that of the biotin-supplied cell.

A clearer picture of the situation is obtained when the relative levels are expressed per colony forming unit (Figures 5 and 6). The normal cells followed a pattern similar to the one obtained by Stevenson (1962) in that increases in turbidity, expressed in Klett units, occurred prior to any cell division (Figure2B). In addition during this predivisional lag period, RNA, DNA, and protein have almost reached their maximum level.

The DNA, RNA, and protein have increased by factors of 5.5, 4.7, and 3.5 respectively over the initial value by six hours. Differences in timing can be accounted for by differences in cultural conditions. He grew the cells in a yeast-soil extract medium at 28 C obtaining a generation time of only 83 minutes. When the cells were grown in a glucose-salts medium at 25 C, as in the present work, the generation time was 155 minutes. DNA, RNA, and protein all reached their maxima by 7 hours, followed by a decline. RNA increased first, followed shortly by protein and DNA.

The cells growing in unsupplemented medium exhibited decreased accumulation of RNA, DNA, and protein. RNA increased reaching a maximum level by 12.5 hours. This level was only 70% that reached by the biotin-supplied culture. DNA reached its maximum (0.83 ug/ 10^7 C.F.U.) by 10.5 hours, to a level comparable to the normal peak (0.92 ug/ 10^7 C.F.U.). The subsequent decrease in intracellular DNA and RNA/ C.F.U. reflected the morphological abnormality described by Robertson and Chan (1970). The colony forming unit in the abnormal cells consists of one or more membrane-bound bodies surrounded by a matrix of amorphous material. The degree of biotin depletion determines the degree of abnormality. Therefore, although the

total RNA and DNA per ml of culture was decreased (Figures 1 and 3), the number of membrane-bound bodies per C.F.U. increased (Robertson and Chan, 1970) giving an apparent increase per C.F.U. in RNA and DNA over the normal cell. Protein continued to increase for one hour longer than the RNA (Figure 6), reaching a final level 35% greater than the normal cell protein.

Because of the possible ambiguity in the term colony-forming unit, since each abnormal cell may contain several membrane-bound bodies, DNA concentration was also used as a measure of the cell unit. Throughout the growth cycle the RNA/DNA ratio was much lower in the abnormal cell (Figure 7). Despite the decrease in RNA, there was an increase in protein/DNA in the abnormal cell (Figure 8). The protein content was maintained at a level about 20% higher than the normal cell for most of the growth cycle. By 17.5 hours, this was increased to 35%, followed by a sharp decline.

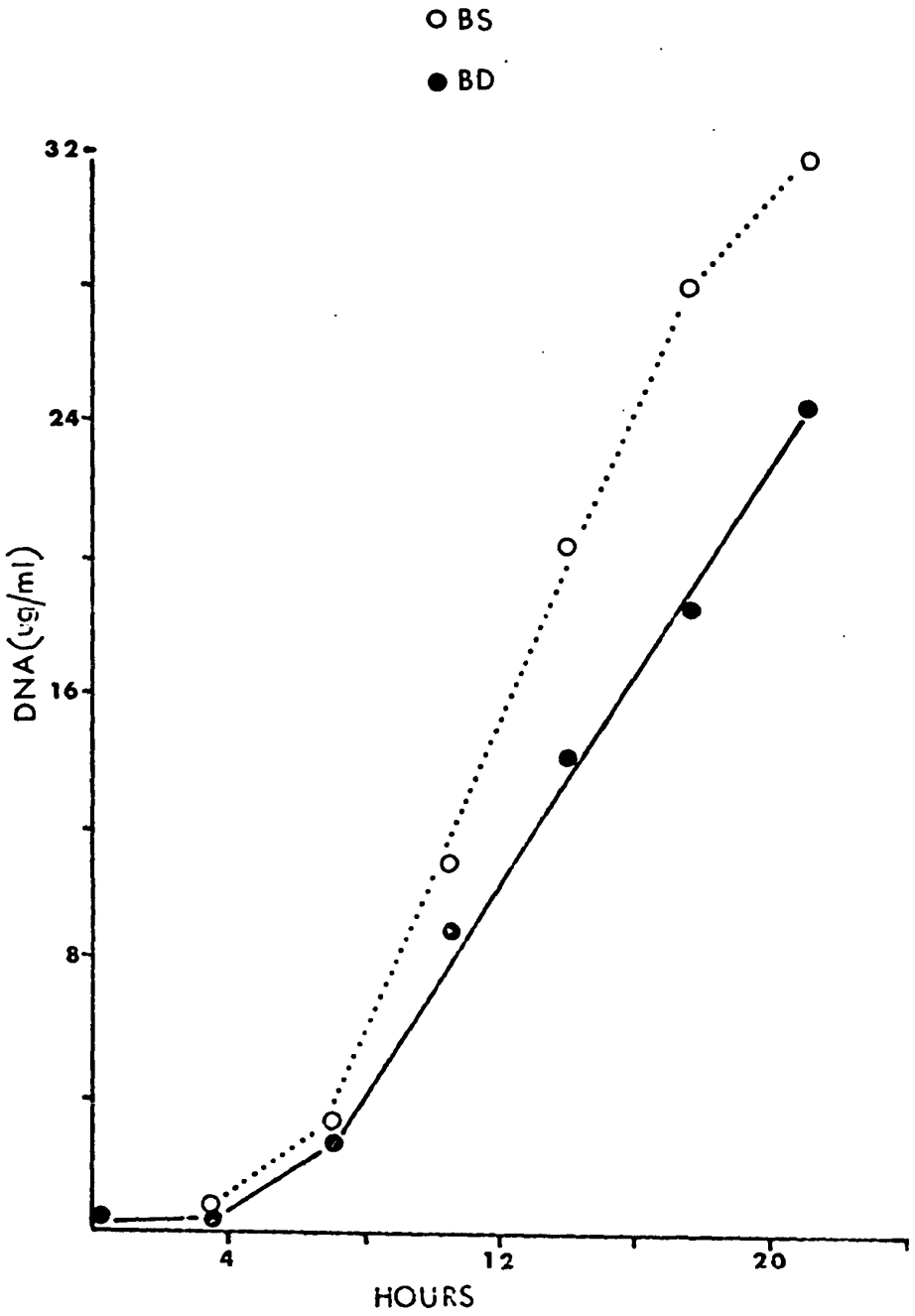


Figure 1. Accumulation of DNA (ug/ml) with Time of Incubation (Hours) in Biotin-sufficient (BS) and Biotin-deficient (BD) Cells.

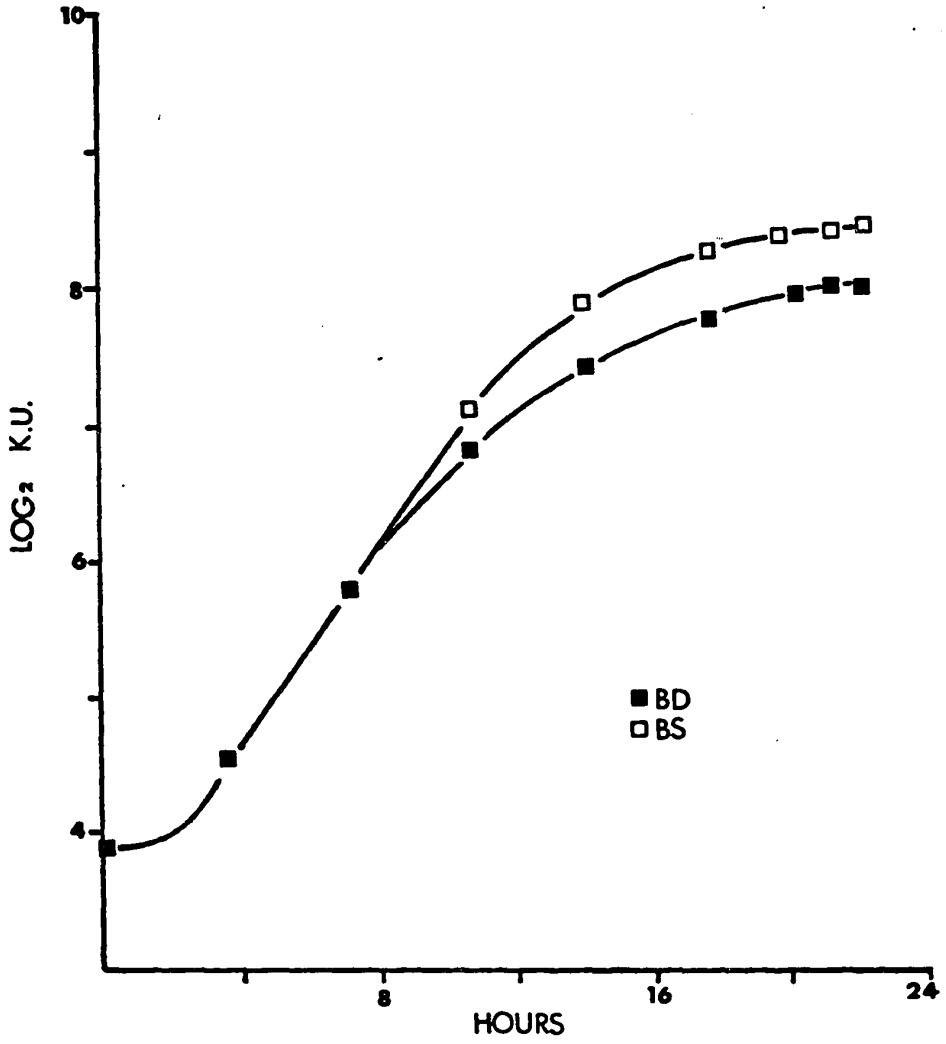


Figure 2A. Growth (Log_2 K.U.) of Biotin-sufficient (BS) and Biotin-deficient (BD) Cells.

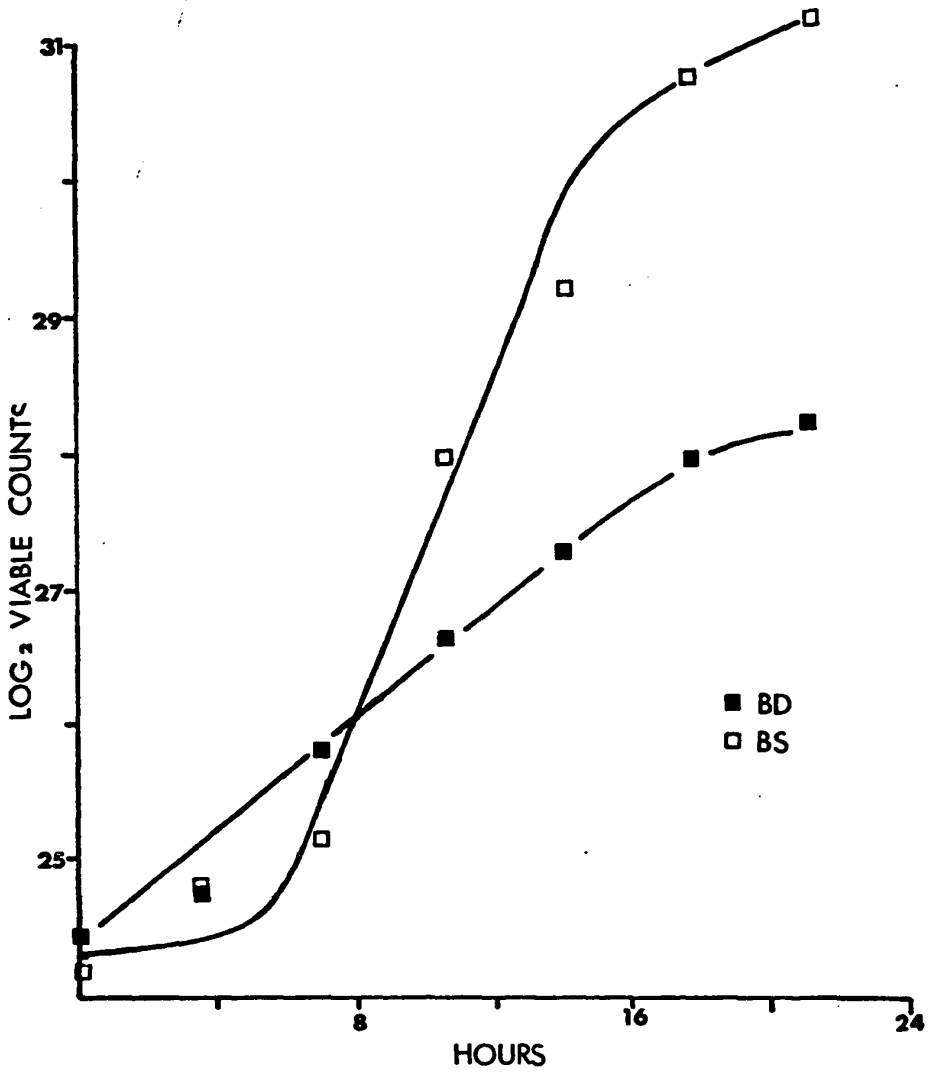
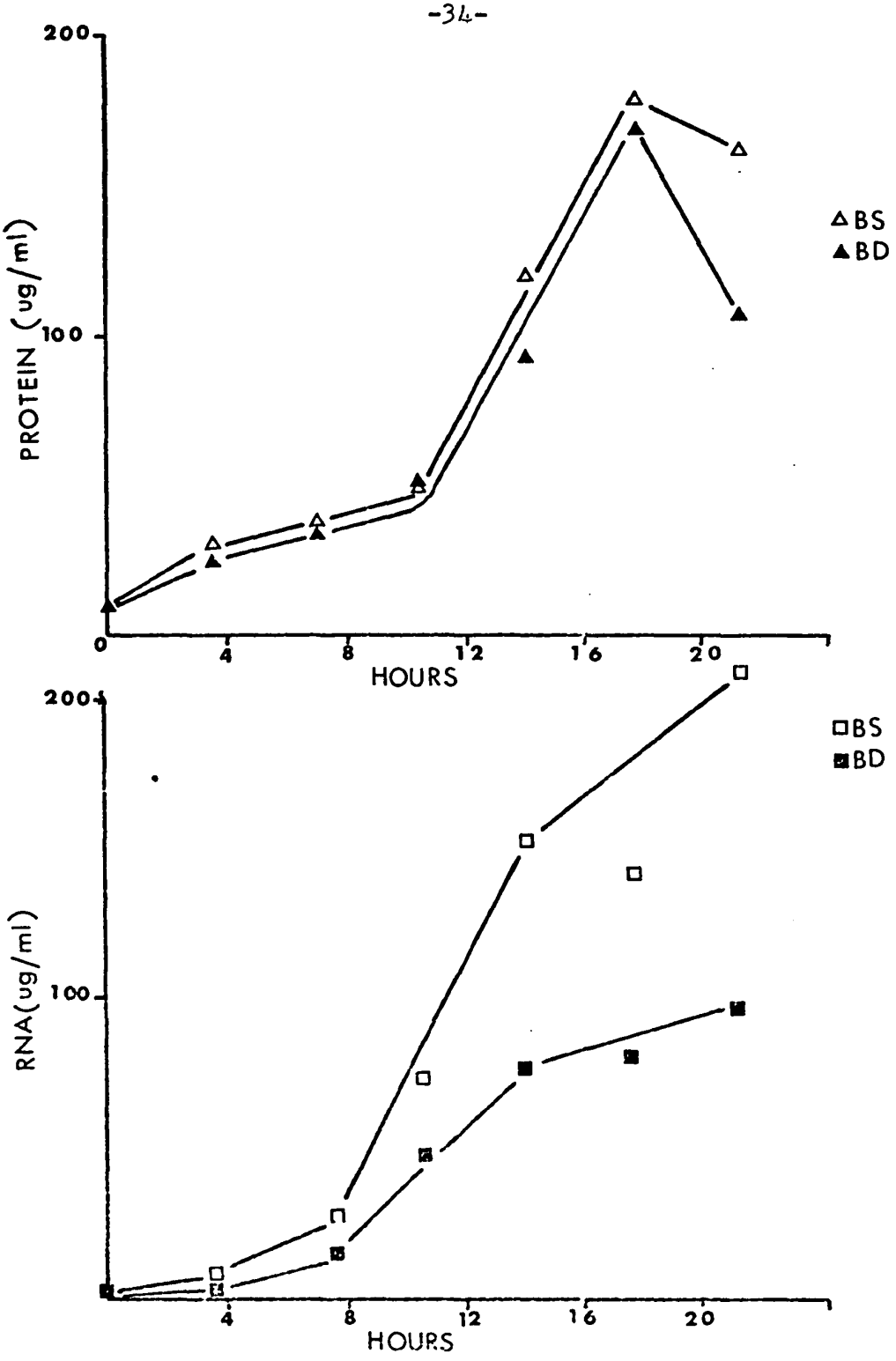


Figure 2B. Growth (Log_2 Viable Cell Counts) of Biotin-sufficient (BS) and Biotin-deficient (BD) Cells.



Figures 3 (Lower) and 4 (Upper). Accumulation of RNA (ug/ml) and Protein (ug/ml) with Time of Incubation (Hours) in Biotin-sufficient (BS) and Biotin-deficient (BD) Cells

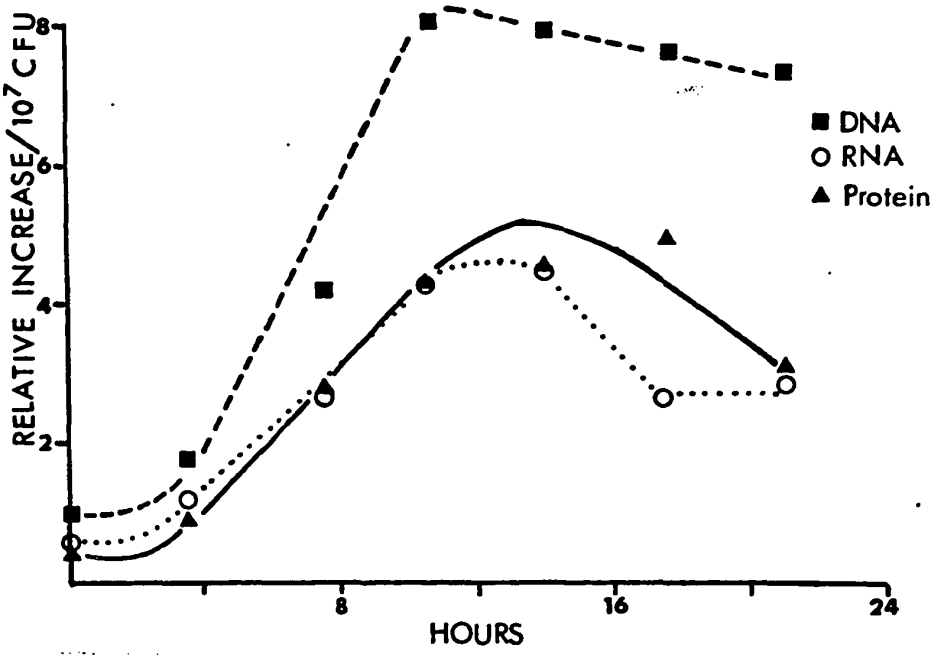


Figure 5. Relative Increase per 10^7 C.F.U. in DNA, RNA, and Protein with Incubation Time in Biotin-deficient Cells.

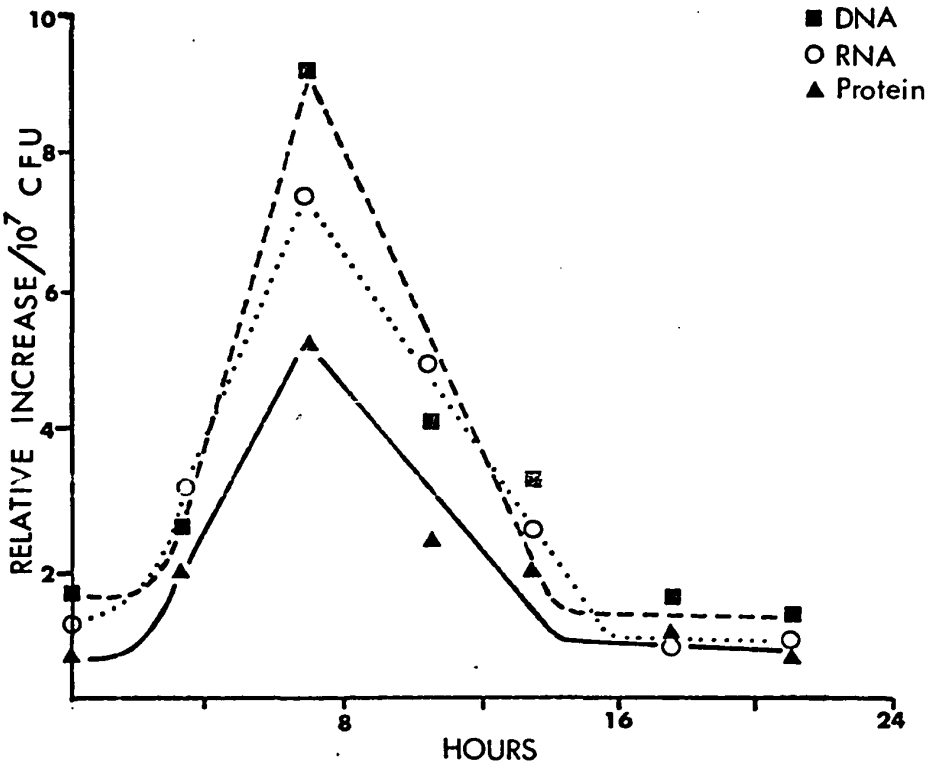


Figure 6. Relative Increase per 10^7 C.F.U. in DNA, RNA, and Protein with Incubation Time in Biotin-sufficient Cells

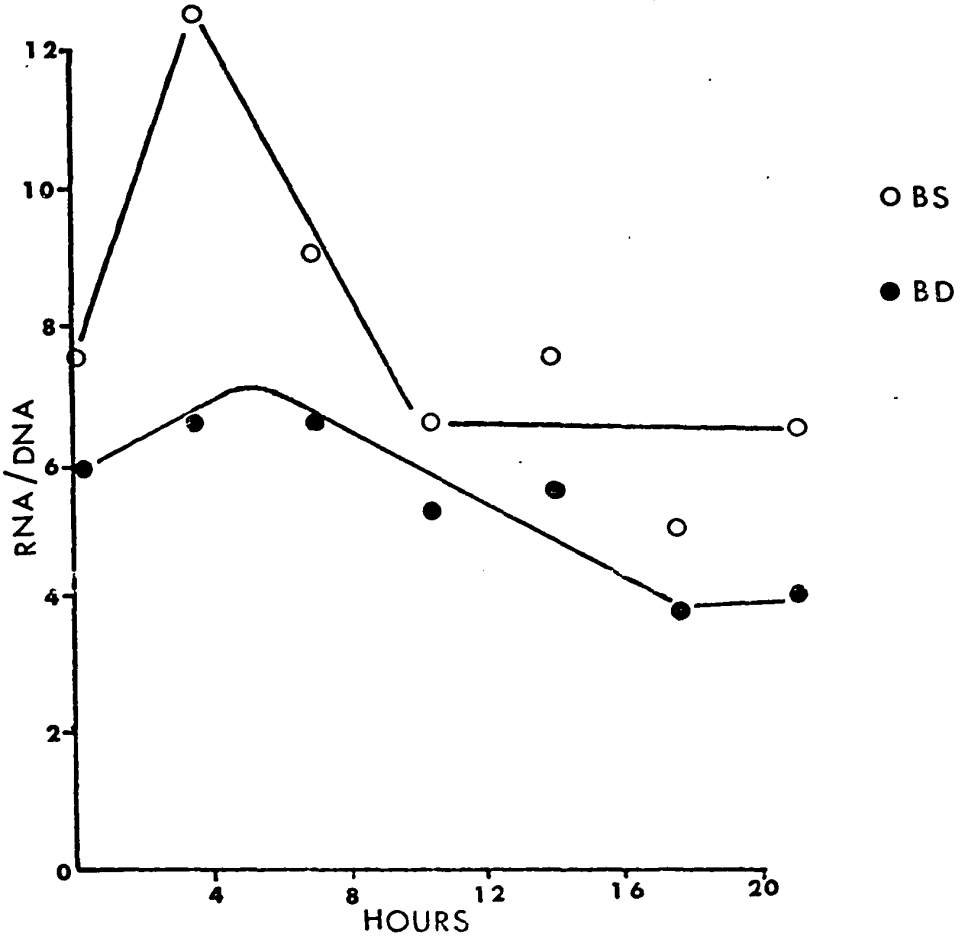


Figure 7. Changes in RNA/DNA Ratio with Time of Incubation in the Biotin-deficient (BD) and Biotin-sufficient (BS) Cells

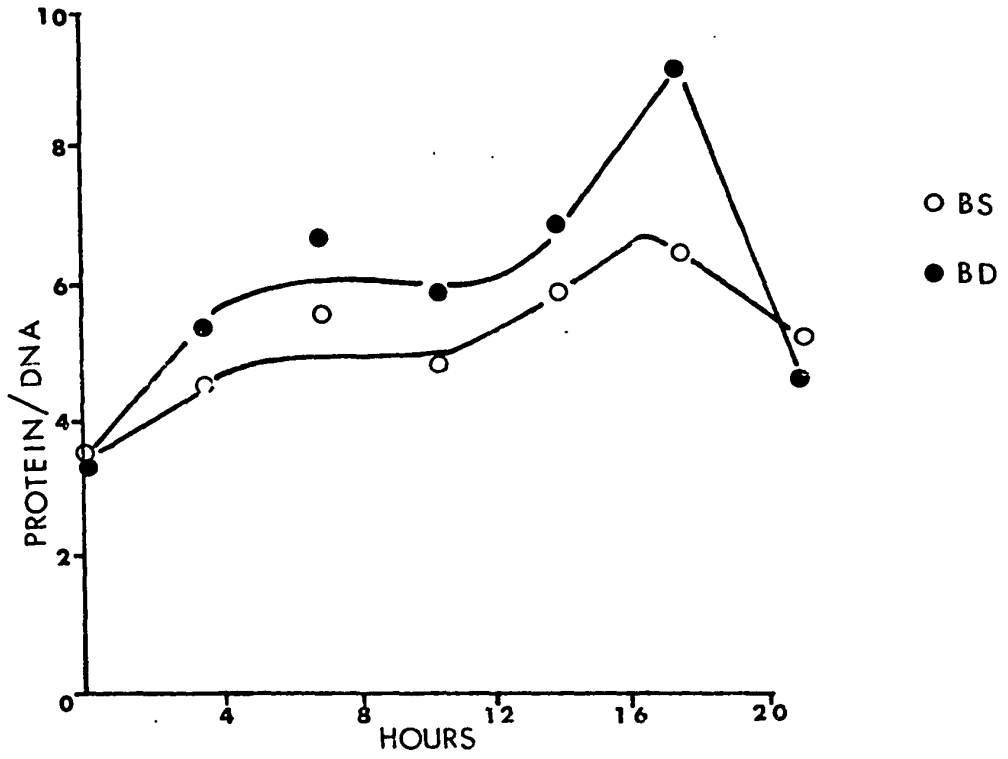


Figure 8. Changes in Protein/DNA Ratio with Time of Incubation in the Biotin-deficient and Biotin-sufficient Cells

Replacement Experiments

In order to determine to what degree various substances are able to replace biotin, it was necessary to assay each compound for that concentration giving optimal growth of the organism. Turbidity readings, as measured in Klett units served as indices of growth for the assay experiments. The concentrations found to give greatest stimulation are listed in Table I.

Of the fatty acids tested, none were found to give significant increases in growth over the control biotin-deficient culture (Table I). Linoleic and linolenic acids gave respective increases of 45 and 6%. Oleic acid was found to be inhibitory, decreasing the net yield of cells by about 50% whereas Tween 80, an alternate source of oleate, caused a stimulation of 27%.

Aspartate more than doubled the growth yield of the culture, whereas oxalacetate gave an increase of only 0.265 generations. Aspartic acid and Tween 80 gave an additive effect. Although there was partial replacement with these compounds, it was only a slight effect when compared with cells grown with an adequate supply of biotin. These showed a 300% increase over the abnormal cell.

Table I. Stimulation of Biotin-deficient Cells by Various Compounds

Compound	Optimal Concentration	Increase in Generations*	% Increase
Tween 80	5 ug/ml	0.273	27.3
Oleic acid	0.5 ug/ml	- 0.927	-46.3
Linoleic acid	1 ug/ml	0.455	45.5
Linolenic acid	0.1 ug/ml	0.059	5.9
Aspartic acid	798 ug/ml	1.37	137
Oxalacetic acid	264 ug/ml	0.265	26.5
Aspartate + Tween 80	798 ug/ml 5 ug/ml	1.60	160

* Calculated as follows:

$$\text{Log}_2 \text{ K.U. (Test Compound)} - \text{Log}_2 \text{ K.U. (Biotin-deficient)}$$

Fatty acid composition

The fatty acid composition of Arthrobacter globiformis 616 has been reported by Walker and Fagerson (1965). Their analysis was done on cells which had grown on a synthetic medium for three days at 27-28 C. For comparative purposes then, the 51-hour or stationary phase sample of A. globiformis 425 would most closely resemble the physiological stage of their cells. It is apparent from Table II that the fatty acid composition of the two A. globiformis strains are similar with a predominance of branched-chain fatty acids in the anteiso series.

The fatty acid composition of A. globiformis remained relatively constant throughout the growth cycle (Table III). Those belonging to the anteiso branched series (a-C_{13}, a-C_{15}, a-C_{17}) comprised 68-84% of the total fatty acids. Normal and even-numbered iso fatty acids were constant at 7-9% and 6-11%, respectively.

When biotin was limiting, however, there was found in abnormal cells higher concentrations of normal fatty acids with a simultaneous decrease in the relative percentage of anteiso fatty acids. At 18 hours, the lipid composition of the biotin-deficient cell was not too different from the

biotin-supplied cell (Table III). There was merely a slight reduction in the relative percentage of anteiso fatty acids. By the end of log. phase, this difference was very marked with 77.2% of the cellular fatty acids being of the normal type whereas only 6% were of the characteristic anteiso type. By late stationary phase, there was a partial return to the composition of the normal cells with only 49.8% normal fatty acids and 32.2% anteiso. The even-numbered iso fatty acids were affected to a lesser degree by biotin-deficiency.

Table II : Comparison of Fatty Acid Composition in Two Strains of *Arthrobacter globiformis*

Fatty acid	*A. globiformis* 425 51 hours % fatty acid	*A. globiformis* 616 3 days % fatty acid *
i C_{10}	0.3	-
n C_{10}	0.7	-
n C_{12}	0.7	-
a C_{13}	0.3	-
i C_{14}	1.0	0.8
n C_{14}	2.1	0.6
a C_{15}	77.9	66.8
i C_{16}	4.9	10.6
n C_{16}	3.6	3.6
a C_{17}	6.4	17.6
Total anteiso(a)	84.6	84.4
Total normal (n)	7.1	4.2
Total iso (i)	6.2	11.4

* Data from Walker and Fagerson (1965)

Table III : Relative Percentages of Fatty Acid Types in Biotin-deficient (BD) and Biotin-sufficient (BS) Cells

Type	Early Log (18 hr)		Late Log (29.5 hr)		Stationary (51 hr)	
	BS	BD	BS	BD	BS	BD
anteiso %	76	60	68.3	6.0	84.6	32.2
normal %	9	11.6	9.3	77.2	7.1	49.8
iso %	7	15.4	11.3	0.4	6.2	7.8

Cell Wall Analysis

The dry weight of material analysed was calculated as 639 mg biotin-sufficient cells and 8.2 mg biotin-deficient cells. Their respective DNA contents were 13.27 and 0.548 mg. Ninhydrin-reacting material was expressed as micromole equivalents of alanine. There was a 4.28-fold increase in ninhydrin-reacting material per mg DNA detected in the abnormal cell, corresponding to a 13.4-fold increase per mg dry weight of cells (TableIV). There was also an increase in hexosamine content in the biotin-deficient cell wall material. When expressed in microgram equivalents of glucosamine, the increase was found to be 5.7 times that of the normal cell per mg DNA and 19.4 times the normal per mg dry weight of cells.

Table IV. Hexosamine and Ninhydrin-reacting Material Contents of Biotin-deficient (BD) and Biotin-sufficient(BS) Cell Walls

	BD Cells		BS Cells	
Total Ninhydrin-reacting Material	0.75	uM	4.35	uM
Ninhydrin/mg DNA	1.37	uM	0.32	uM
Ninhydrin/mg dry weight	0.091	uM	0.0068	uM
Total Hexosamine	21	ug	84	ug
Hexosamine/mg DNA	95.8	ug	16.8	ug
Hexosamine/mg dry weight	6.4	ug	0.33	ug

DISCUSSION

Fatty acid substances have adequately substituted for biotin in several auxotrophs.(see historical review). The role of the vitamin in the formation of malonyl-CoA for fatty acid biosynthesis, as established by Wakil, et al (1958),provided an explanation for such replacing activity. Unsaturated fatty acids were found to give only slight replacement in A. globiformis (Table I), possibly because the normal fatty acid composition of the organism, as determined by Walker and Fagerson(1965) and confirmed by work presented in this thesis (Table II) does not include unsaturated fatty acids.

Aspartic acid, although not found to replace biotin completely, was able to double the growth yield of the biotin-deficient organism (Table I). It is possible that the basis for this replacement is the one proposed by Lardy in 1947. Pyruvate carboxylase is a biotin-requiring enzyme whose end-product from pyruvate is oxalacetate, which in turn can be converted to aspartate via a transamination reaction. That oxalacetate can stimulate the cell slightly (Table I) is also explained by its role in this reaction.

Unlike Streptococcus faecalis (Stokes, et al, 1947) and Lactobacillus casei (Williams, et al, 1946) whose biotin

requirements were adequately satisfied by aspartate and oleate, respectively, A. globiformis was not restored to its normal growth by any single compound tested (Table I). Aspartic acid and Tween 80 were able to work additively, yet complete replacement did not occur. This suggested that although single compounds reduced the requirement for biotin by by-passing single metabolic reactions which involve the vitamin, complete replacement could not occur because the vitamin deficiency evidently affected several metabolic sites in A. globiformis.

Impaired lipid metabolism which occurs as a result of biotin deficiency has been found to cause a shift in fatty acid composition; the direction of this shift differs in different microorganisms (see historical review). In A. globiformis , the predominant series of fatty acids that occur, when biotin is supplied, belong to the anteiso type. Lesser amounts of iso- and normal fatty acids occur as well (Table III). This distribution of fatty acids remains relatively constant throughout the growth cycle. A similar abundance of branched-chain fatty acids has been found in Bacillus species (Kaneda, 1966, 1967). According to Kaneda (1966), in the biosynthesis of branched-chain fatty acids, the appropriate alpha-keto acid must first be converted to its acyl-CoA ester via a

reaction involving the reduction of NAD. Malonyl-CoA units are then added to the acyl-CoA to give the corresponding branched-chain fatty acids of the desired length. In the case of the anteiso series, L-isoleucine was found to be the necessary precursor. Since the relative availability of this precursor determines the relative abundance of anteiso fatty acids formed (Kaneda, 1966), it is likely that under conditions of biotin deficiency A. globiformis does not produce sufficient L-isoleucine, thereby causing a shift in synthesis toward that of normal straight chain fatty acids (Table III).

Of interest is the possible role of aspartate in this biosynthesis. This amino acid which is an endproduct of a biotin-dependent reaction, is also a precursor of L-isoleucine. Therefore any reduction in aspartate, will also influence the amount of amino acid available for biosynthesis of these branched-chain fatty acids. It is reasonable that the shift in abnormal A. globiformis cells would occur in the direction of a "simpler" biosynthetic pathway, using acetyl-CoA rather than an amino acid , such as L-isoleucine, as a precursor. Normal fatty acids make use of acetyl-CoA as their acyl precursor; acetyl-CoA is also the precursor of malonyl-CoA, necessary for chain elongation in fatty acid biosynthesis.

As noted previously, this shift towards synthesis of straight chain fatty acids at the expense of the anteiso series, does occur. By late stationary phase the cells were able to synthesize small amounts of the branched-chain fatty acids typical of the normal cells and a partial reversal to the composition of the biotin-sufficient culture was observed.

When A. globiformis was transferred to medium containing suboptimal concentrations of biotin, DNA, and RNA synthesis decreased to respective levels only 67 and 40% of that achieved by the cells grown with sufficient biotin (Figures 1 and 3). Evidently biotin plays a role in nucleic acid biosynthesis although the actual mechanism of its involvement has not been elucidated. Since aspartic acid, an endproduct of a biotin-dependent reaction, is intimately associated with purine and pyrimidine biosynthesis, a reduction in the level of this amino acid may contribute to this decrease in nucleic acids per cell. The shift in RNA when the organism was first transferred followed the pattern analagous to the "shift-down" experiments (Kjeldgaard, 1967) in that RNA was the first parameter to be affected. A similar decrease in nucleic acids, resulting from biotin-deficiency occurs in S. cerevisiae (Ahmad, et al, 1961). Unlike the yeast, however, whose protein level decreased when biotin was lacking, A. globiformis was

able to maintain protein/cell at a level similar to the one found in the biotin-sufficient cell (Figures 5 and 6). This relative increase in proteins, which are possibly structural in nature, may be related to the changes that have been shown to occur in the membrane (Table III). Increases in protein content have been associated with a 30% reduction in lipid due to biotin-deficiency in A. nidulans (Rao and Modi, 1968), and a complete inhibition of lipid synthesis due to glycerol limitation in B. subtilis (Mindich, 1970). In the latter case the increase in protein was specifically associated with the membrane, indicating that synthesis of the protein and lipid portions of the membrane are under independent control. Although similar measurements were not done on A. globiformis, it is possible that such an effect would partially account for increases in protein/DNA obtained in the biotin-deficient cell (Figure 8). In addition the cell walls of biotin-deficient A. globiformis were found to contain increased hexosamine and ninhydrin-reacting material over the normal cells. This too would account for the high protein levels obtained in studies on macromolecular biosynthesis. Furthermore, since cell wall biosynthesis is membrane dependent, these changes in cell wall may be a result of altered membrane composition, which would

affect the activity of the particulate of membrane-associated cell wall synthesizing or polymerizing enzymes. Alternatively, defective lipid metabolism may alter the formation of lipid intermediates which are essential for cell wall synthesis, and thereby contribute to the aberration of the cell. Altered fatty acid composition in association with defective synthesis of glycoprotein and membrane-associated enzymes has also been found in a nutritionally induced filamentous mutant of E. coli B (Weinbaum, Fischman, and Okuda, 1970).

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