Biosynthesis of the Polyoxins, Nucleoside Peptide Antibiotics: A New Metabolic Role for L-Isoleucine as a Precursor for 3-Ethylidene-L-azetidine-2-carboxylic Acid (Polyoximic acid)†

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ABSTRACT: The biosynthetic origin of the carbon skeleton of 3-ethylidene-L-azetidine-2-carboxylic acid (polyoximic acid) is described. This unique cyclic amino acid is the C terminus of the nucleoside peptide antibiotics, the polyoxins, elaborated by *Streptomyces cacaoi* var. *asoensis*. In vivo experiments show that ^{14}C from [1-^{14}C]isoleucine, [U-^{14}C]isoleucine, [1-^{14}C]methionine, [U-^{14}C]methionine, [U-^{14}C]threonine, and [1-^{14}C]glutamate is incorporated into polyoximic acid; however, ^{14}C from [5-^{14}C]glutamate and [*methyl*-^{14}C]methionine is not incorporated. The distribution of ^{14}C in polyoximic acid clearly shows that the intact carbon skeleton of L-isoleucine is utilized directly. The incorporation of ^{14}C from [U-^{14}C]methionine, [U-^{14}C]threonine, and [1-^{14}C]glutamate into polyoximic acid occurred only after their conversion to isoleucine via 2-ketobutyrate. A scheme is presented in which either of the two β-unsaturated amino acids isolated from *Bankera fuligineoalba*, L-2-amino-3-hydroxymethyl-3-pentenoic acid or L-2-amino-3-formyl-3-pentenoic acid, is regarded as a possible intermediate amino acid between isoleucine and polyoximic acid.

A soil streptomycete, *Streptomyces cacaoi* var. *asoensis* (Isono et al., 1965), elaborates the polyoxins which are nucleoside peptide antibiotics (Isono et al., 1969). The polyoxins are competitive inhibitors of chitin synthetase (Endo and Misato, 1969; Keller and Cabib, 1971; Hori et al., 1971; Jan, 1974) and are used as an agricultural fungicide in Japan. Since the polyoxins contain unique amino acids, the biosynthesis of these di- and tripeptides was studied. The biosynthesis of the 5-substituted uracil base of the polyoxins from uracil and C-3 of serine by a new enzyme system which differs from thymidylate synthetase has been reported (Isono and Suhadolnik, 1973). It was also shown that 5-fluorouracil was able to replace uracil to form the aberrant 5-fluoropolyoxins (Isono et al., 1973).

Polyoximic acid (I, Chart I) is an example of a unique azetidine derivative occurring in nature. It constitutes the C-terminal amino acid of polyoxins A, F, H, and K (Chart II). Only two other naturally occurring azetidine derivatives have been reported. They are L-azetidine-2-carboxylic acid (II) from *Lillyaceae* (Fowden, 1956) and nicotianamine (III) from *Nicotiana* (Noma et al., 1971). Leete (1964) has shown that [1-^{14}C]methionine is a precursor of II and suggested an intramolecular displacement mechanism of *S*-adenosylmethionine for the formation of this amino acid. Later, Su and Levenberg (1967) showed that the intact carbon chain of methionine was incorporated into II by using [2-^{14}C]methionine and [U-^{14}C]methionine.

For the biosynthesis of I (Chart I), two possibilities were considered: first, a pathway analogous to that proposed by Leete for II, i.e., azetidine-1-carboxylic acid followed by addition of either a C-2 unit or two C-1 units to form the ethylidene side chain; or second, a completely new pathway,

Chart I

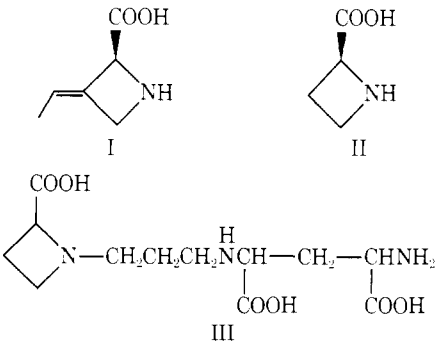
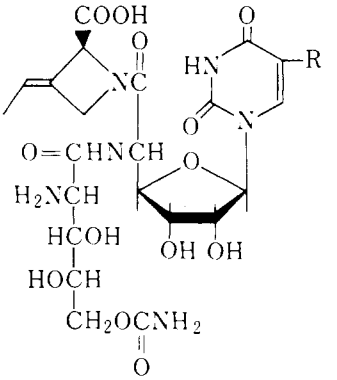


Chart II. Structure of the Polyoxins Containing 3-Ethylidene-L-azetidine-2-carboxylic Acid.



polyoxin A, R = CH_2OH
polyoxin F, R = COOH
polyoxin H, R = CH_3
polyoxin K, R = H

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i.e., formation of the carbon skeleton of I by ring formation involving the methyl and amino groups of isoleucine. Since ^{14}C from methionine and isoleucine was incorporated into I, experiments were designed to prove which of the above two possibilities were operating in the biosynthesis of I. This paper describes the results of ^{14}C incorporation data that indicate a new metabolic role for isoleucine metabolism and favor pathway 2 mentioned above.

Materials and Methods

L-[U-^{14}C]Isoleucine, L-[4,5-^{3}H]isoleucine, DL-[1-^{14}C]methionine, L-[*methyl*-^{14}C]methionine, L-[U-^{14}C]threonine, and DL-[1-^{14}C]glutamic acid were purchased from the Radiochemical Centre, Amersham/Searle. L-[U-^{14}C]Methionine and DL-[5-^{14}C]glutamic acid were purchased from New England Nuclear Corporation; L-[1-^{14}C]isoleucine was purchased from the Calatomic Corporation. Radioactive measurements were made on a Beckman DPM-100 liquid scintillation spectrometer using Bray's dioxane scintillation solution (1960) with the addition of 2% of 0.6 *N* toluene solution of hyamine (NCS tissue solubilizer, Amersham/Searle).

Streptomyces cacaoi var. *asoensis* (Isono et al., 1965) was maintained on agar plugs (Uematsu and Suhadolnik, 1972) which were also used for inoculation. Seed culture medium consisted of soluble starch (1%), glucose (1%), soybean meal (2%), yeast (1%), sodium nitrate (0.2%), and potassium phosphate, dibasic (0.2%). Culture flasks were shaken at 28° on a rotary shaker (210 rpm). Each 500-ml flask contained 60 ml of the medium. After 24-hr incubation of seed culture, 1 ml was used to transfer to each flask containing the following medium: soluble starch (9%), glucose (1%), soybean meal (2%), dry yeast (4%), sodium nitrate (0.2%), and potassium phosphate, dibasic (0.2%). The uptake by cells of radioactive compounds added to the culture was checked by measuring radioactivity remaining in the medium. All the labeled compounds described were taken up by the cells. The culture medium was adjusted to pH 2 with 10% hydrochloric acid and either centrifuged or filtered with the aid of Celite. The polyoxin complex was purified as described (Isono et al., 1967).

Biosynthesis of the Polyoxin Complex from Labeled Compounds. Sterile solutions of radioactive compounds were added to the culture medium 45 hr after inoculation. At this time, the cells had reached the stationary phase and the production of the antibiotics had started. Seventy-two hours later the polyoxin complex was isolated from the culture filtrate. Twenty-five flasks were used for each experiment. The yield of the purified polyoxin complex was 1 g.

Hydrolysis of the Polyoxin Complex and Isolation of Polyoximic Acid. The polyoxin complex (1 g) was hydrolyzed with 40 ml of 0.5 *N* sodium hydroxide for 2 hr on a steam bath. The hydrolysate was passed through a column of Dowex 50W X-8 (H^+) (100–200 mesh) (50 ml). After washing with water, polyoximic acid was eluted with 750 ml of 0.6 *N* ammonia. The eluate was concentrated to a small volume and applied to the column of DE_{52} (DEAE-cellulose) (100 ml equilibrated with 0.2 *M* triethylamine–bicarbonate buffer, pH 7.2).

The first fraction containing polyoximic acid was further purified with Avicel cellulose chromatography using the solvent system, 1-butanol–acetic acid–water (4:1:1, v/v/v). Polyoximic acid was crystallized from aqueous ethanol: yield, 25 mg; recrystallization from the same solvent to constant activity; mp 156–160°.

Table I: Incorporation of ^{14}C-Labeled Amino Acids into Polyoximic Acid (I).

Compd Added[a]	Sp Act. of Polyoximic Acid Isolated (nCi/μmol)	Dilution[b] (×10³)
L-[1-^{14}C] Isoleucine	1.1	0.91
L-[U-^{14}C] Isoleucine	0.89	1.2
DL-[1-^{14}C] Methionine	0.11	9.1
L-[U-^{14}C] Methionine	0.16	6.3
L-[*methyl*-^{14}C] Methionine	0.00	
L-[U-^{14}C] Threonine	0.038	26
DL-[1-^{14}C] Glutamic acid	0.024	42
DL-[5-^{14}C] Glutamic acid	0.00	

[a] Specific activity, 1 μCi/μmol. [b] Specific activity of compound added/specific activity of polyoximic acid isolated.

Ninhydrin Oxidation of Polyoximic Acid. Ten to twenty micromoles of crystalline polyoximic acid (3000–15,000 dpm) was dissolved in 2 ml of water and 2 ml of 5% ninhydrin solution in 2 *M* citrate buffer (pH 2.5) was added. The resulting solution was heated on a steam bath for 45 min. Nitrogen was bubbled through the reaction mixture. The CO_2 was trapped in a solution of NCS (0°). In a control experiment with authentic polyoximic acid, 10 mg was oxidized under identical conditions, and 96% of the CO_2 was recovered and weighed as barium carbonate.

Ozonolysis of Polyoximic Acid. Polyoximic acid (80–100 μmol) was dissolved in 5 ml of methanol. Carrier polyoximic acid was added when needed. The solution was cooled in a Dry Ice–acetone bath and a gentle stream of ozone was introduced for 30 min. At that time, 2 mg of palladium black (prepared by hydrogenation of palladium chloride) was added and the solution was hydrogenated for 1 hr at room temperature. The reaction mixture was added to 5 ml of sodium acetate–hydrochloric acid buffer (equal volumes of 2 *N* sodium acetate and 1 *N* hydrochloric acid), followed by the addition of 10 ml of 0.4% aqueous dimedone. After standing several hours at room temperature, the methanol was removed by distillation. The solution stood overnight at room temperature. A crystalline dimedone derivative of acetaldehyde was filtered off and recrystallized from aqueous ethanol to a constant specific activity and a melting point of 140°. The infrared spectrum was identical with an authentic dimedone derivative of acetaldehyde.

Results

Incorporation of Labeled Compounds into Polyoximic Acid. Table I shows the results of the studies on the incorporation of ^{14}C-labeled compounds into the polyoximic acid moiety of polyoxins by growing cultures of streptomycete. The specific activity of the precursors was 1 μCi/μmol. A total of 12.5 μCi was used for 25 flasks for each experiment. ^{14}C from [1-^{14}C]- and [U-^{14}C]isoleucine was most efficiently incorporated; [1-^{14}C]- and [U-^{14}C]methionine were next, while [*methyl*-^{14}C]methionine and [5-^{14}C]glutamate were not incorporated. If isoleucine is the direct precursor and methionine was incorporated after its conversion into isoleucine via 2-oxobutyrate pathway (vide infra), threonine should also be incorporated because it is a direct precursor of 2-oxobutyrate. Indeed, [U-^{14}C]threonine was incorporated, though less efficiently than isoleucine. Glutamate is known to rearrange to form L-*threo*-β-methylaspartate (Barker et al., 1958), which is then transaminated to yield β-methyloxaloacetate. Decarboxylation of C-5 yields 2-oxo-

Table II: Distribution of ^{14}C in Polyoximic Acid (I).

Compd Added	Sp Act. of I Used for Degradation (nCi/μmol)	Ethylidene Group (Acetal dimedone) nCi/μmol	Ethylidene Group (Acetal dimedone) % ^{14}C Exptl[a]	Ethylidene Group (Acetal dimedone) % ^{14}C Theor.	Carboxyl Group (CO_2) % ^{14}C Exptl[b]	Carboxyl Group (CO_2) % ^{14}C Theor.
L-[1-^{14}C]Isoleucine	0.50	0	0	0	100	100
L-[U-^{14}C]Isoleucine	0.14	0.042	31	33	17	17
DL-[1-^{14}C]Methionine	0.042	0	0	0	100	100
L-[U-^{14}C]Methionine	0.11	0.055	51	50	32	25
L-[U-^{14}C]Threonine	0.042	0.019	45	50	33	25
DL-[1-^{14}C]Glutamate	0.024				109	100

[a] (Specific activity of acetal dimedone/specific activity of compound used) × 100. [b] (Total disintegrations per minute of CO_2/total disintegrations per minute of I used) × 100.

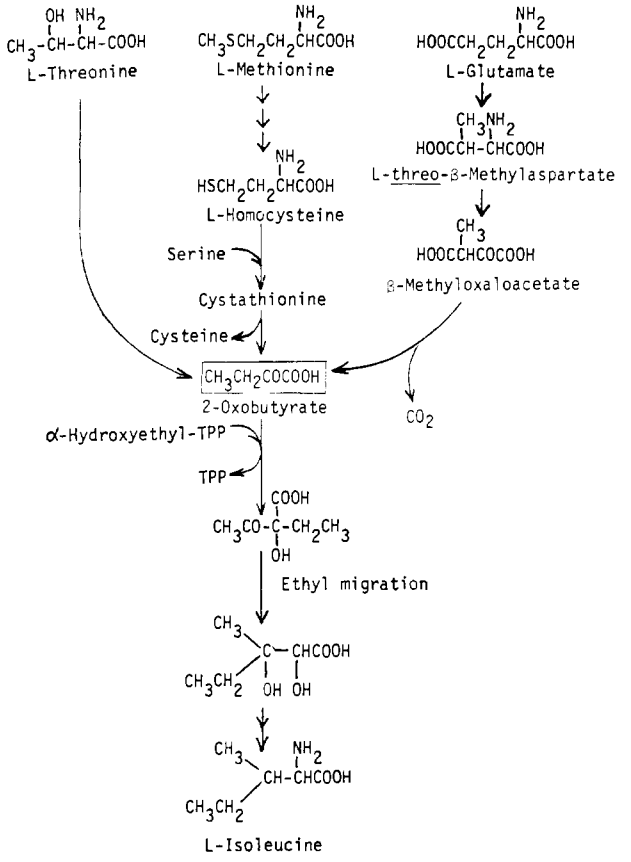
butyrate (Abramsky and Shemin, 1965). Proof that this pathway is operating in *Streptomyces cacaoi* was accomplished by adding [1-^{14}C]glutamate and [5-^{14}C]glutamate to cultures of *S. cacaoi*. ^{14}C from [1-^{14}C]glutamate was incorporated into I, while ^{14}C from [5-^{14}C]glutamate was not incorporated.

Distribution of ^{14}C in Polyoximic Acid from the ^{14}C-Labeled Amino Acids. Four amino acids labeled with ^{14}C and ^{3}H were selected to elucidate the biosynthesis of the carbon skeleton of I. Of the ^{14}C-labeled amino acids studied, L-isoleucine was most efficiently incorporated (Table I). Proof that ^{14}C from the carbon atoms of L-[U-^{14}C]isoleucine was uniformly distributed in I is shown in Table II. If isoleucine constitutes the carbon skeleton of polyoximic acid, 33% (two carbons out of six carbons) of the total radioactivity in I should reside in the ethylidene side chain from the [U-^{14}C]isoleucine experiment and 17% of the ^{14}C should reside in the carboxyl carbon. Experimentally, 31 and 17%, respectively, of the radioactivity was found in these positions. On the basis of these findings, the biological conversion of L-isoleucine to I is proposed.

To determine and explain the incorporation of ^{14}C from [1-^{14}C]- and [U-^{14}C]methionine into I, it was necessary to degrade I. Fifty-one percent of the ^{14}C in I, from the [U-^{14}C]methionine experiment, was found in the ethylidene group and 32% resided in the carboxyl group of I (Table II). All of the ^{14}C incorporated into I from [1-^{14}C]methionine was found in the carboxyl carbon of I. The distribution of ^{14}C in I from [1-^{14}C]- and [U-^{14}C]methionine can be explained from the known metabolic conversion of methionine to isoleucine (Scheme I). From the outline shown in Scheme I, it is predicted that 50 and 25% of the ^{14}C from [U-^{14}C]methionine would reside in the ethylidene and carboxyl carbon of I, respectively. The finding of 51 and 32% of the ^{14}C in the side chain and the carboxyl carbon of I is in close agreement with the theoretical calculations. These data are in agreement with the intramolecular pinacol-type rearrangement in the conversion of methionine to isoleucine to give α,β-dihydroxy-β-methylvalerate which represents the carbon skeleton precursor of isoleucine (Scheme I).

The ninhydrin oxidation data further support the isoleucine pathway as follows. ^{14}C from the amino acids labeled in the carboxyl carbon or uniformly labeled with ^{14}C is incorporated into the carboxyl carbon of I (Table I). With [U-^{14}C]isoleucine, 17% of the ^{14}C resides in the carboxyl carbon of I. This experimental observation is in agreement with the expected distribution of ^{14}C if isoleucine were directly converted to I without loss of any of the ^{14}C carbons in the [U-^{14}C]isoleucine used. Although ^{14}C from [U-^{14}C]methionine and threonine is found in the carboxyl carbon of I, the percent of ^{14}C in C-1 of I suggests that this incorporation occurs only after conversion of these two amino acids to isoleucine (Table II and Scheme I). Since threonine is known to be a direct precursor of 2-oxobutyrate, the incorporation of ^{14}C from L-[U-^{14}C]threonine into I was also studied. Forty-five percent of the ^{14}C resided in the ethylidene side chain and 33% of the ^{14}C was found in the carboxyl carbon of polyoximic acid. Again, the percent distribution of ^{14}C in I from [U-^{14}C]threonine is in agreement with the known distribution of the carbon atoms when threonine is converted to isoleucine (Scheme I).

Scheme I: Metabolic Pathways for the Biosynthesis of L-Isoleucine.



Incorporation of L-[1-^{14}C;4,5-^{3}H]Isoleucine into Polyoximic Acid. To confirm the incorporation of the intact carbon skeleton of L-isoleucine into I, a double-labeled experiment was performed utilizing L-[1-^{14}C;4,5-^{3}H]isoleucine. This experiment would also supply the information

Table III: Incorporation of L-[1-^{14}C;4,5-^{3}H] Isoleucine into Polyoximic Acid (I).[a]

Isoleucine Added			Polyoximic Acid Isolated		
Sp Act. (μCi/μmol)			Sp Act. (nCi/μmol)		
^{14}C	^{3}H	^{3}H/^{14}C	^{14}C	^{3}H	^{3}H/^{14}C
0.74	3.14	4.24	0.59	2.03	3.42

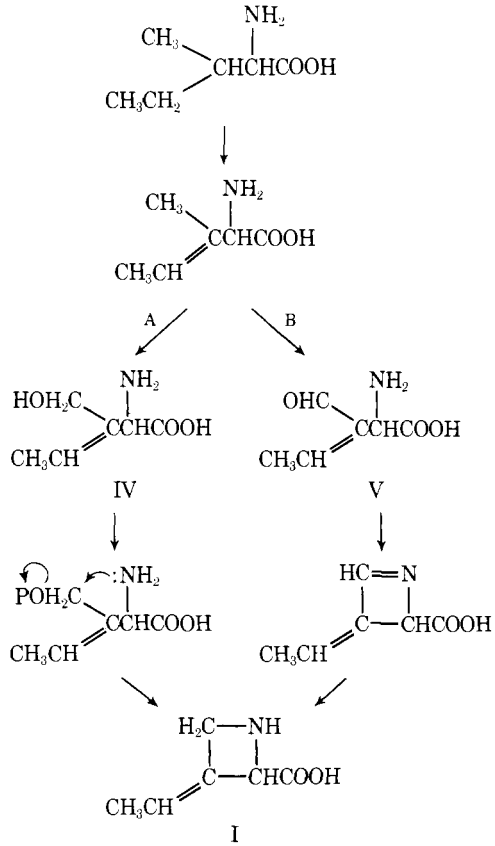
[a] A total 9.25 μCi of L-[1-^{14}C] isoleucine and 39.2 μCi of L-[4,5-^{3}H] isoleucine was added in the given specific activities into 25 flasks of 45-hr culture of *Streptomyces cacaoi*. The other procedure is the same as given in the text.

about the fate of the protons on the ethyl group (C-4 and -5 of isoleucine). The ratio ^{3}H/^{14}C of I isolated was 81% of that of L-isoleucine added (Table III). This shows that four of the five protons in the ethyl group of isoleucine were retained in the formation of the ethylidene group of I. These findings eliminate labilization of the hydrogens in C-4 and -5 of isoleucine.

Discussion

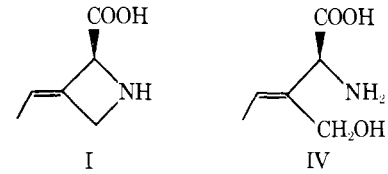
The data presented show that the carbon skeleton of isoleucine is incorporated intact into polyoximic acid. Although ^{14}C from several other amino acids was also found in I, this incorporation can be explained as shown in Scheme I. To accomplish the cyclization between the methyl group and the amino group of isoleucine to form the azetidine ring, the methyl group must be activated. The first event may be dehydrogenation by introduction of the β,γ-double bond (Scheme II). The allylic methyl group could be oxidized either to an alcohol (route A) or an aldehyde (route B). Cyclization may take place either through nucleophilic substitution probably after phosphorylation (route A) or by the direct formation of a Schiff base (route B).

Scheme II: Hypothetical Biosynthetic Pathway of Polyoximic Acid from L-Isoleucine.



It is worth noting that the two hypothetical intermediate amino acids shown in Scheme II, L-2-amino-3-hydroxymethyl-3-pentenoic acid (IV) and L-2-amino-3-formyl-3-pentenoic acid (V), occur naturally in *Bankera fuligineoalba* (Doyle and Levenberg, 1968). Interestingly, the stereochemistry of IV has the L configuration of the α-carbon and the cis relation between the methyl and the hydroxymethyl groups. The stereochemistry of polyoximic acid is the same (Chart III) (Isono et al., 1969).

Chart III: Stereochemical Relation of Polyoximic Acid (I) and L-2-Amino-3-hydroxymethyl-3-pentenoic Acid (IV).



I IV

Because hydroxyethylthiamine pyrophosphate is the two-carbon donor for L-isoleucine biosynthesis, one would expect ^{14}C from [3-^{14}C]pyruvate to be located in C-4 of the azetidine ring of I. Indeed, appreciable incorporation of ^{14}C into I from [3-^{14}C]pyruvate was found. However, 30% of the radioactivity was also found in the ethylidene side chain. This implies that pyruvate was converted to [2-^{14}C]acetate and incorporated into methionine, threonine, or glutamate via the Krebs cycle, which would then lead to the formation of 2-oxobutyrate and isoleucine.

Acknowledgment

The authors gratefully acknowledge Professor E. Leete of the University of Minnesota for his valuable suggestion.

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Ionic and Nucleotide Requirements for Microtubule Polymerization in Vitro†

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ABSTRACT: The ionic and nucleotide requirements for the in vitro polymerization of microtubules from purified brain tubulin have been characterized by viscometry. Protein was purified by successive cycles of a temperature dependent assembly–disassembly scheme. Maximal polymerization occurred at a concentration of 0.1 *M* Pipes (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)); increasing ionic strength by addition of NaCl to samples prepared in lower buffer concentrations did not result in an equivalent level of polymerization. Both Na^+ and K^+ inhibited microtubule formation at levels greater than 240 m*M*, with maximal assembly occurring at physiological concentrations of 150 m*M*. Maximal extent of assembly occurred at pH 6.8 and optimal rate at pH 6.6. Inhibition of polymerization was half-maximal at added calcium concentrations of 1.0 m*M* and magnesium concentrations of 10.0 m*M*. EGTA (ethylene glycol bis(β-aminoethyl ether)tetraacetic acid), which chelates Ca^{2+}, had no effect on polymerization over a concentration range of 0.01–10.0 m*M*. In contrast, EDTA (ethylenediaminetetraacetic acid), which chelates both Mg^{2+} and Ca^{2+}, inhibited assembly half-maximally at 0.25 m*M* and totally at 2.0 m*M*. As determined from experiments using Mg^{2+}–EDTA buffers, magnesium was required for polymerization. Magnesium promoted the maximal extent of assembly at substoichiometric levels relative to tubulin, but was maximal for both rate and extent at stoichiometric concentrations. Elemental analyses indicated that approximately 1 mol of magnesium was tightly bound/mol of tubulin dimer. Viscosity development was dependent upon hydrolyzable nucleoside triphosphate, and stoichiometric levels of GTP were sufficient for maximal polymerization. The effect of magnesium in increasing the rate of GTP-dependent polymerization suggests that a Mg^{2+}–GTP complex is the substrate required for a step in assembly.

Microtubules are abundant in eukaryotic cells and many of the functions with which they are associated, such as cellular shape changes and mitosis, are dependent upon the ordered assembly and disassembly of these structures. In order to analyze these processes, attempts have been made to identify the factors controlling the polymerization of microtubules. In vivo studies on the mitotic apparatus (Inoué and Sato, 1967) and other cellular microtubules (see Tilney (1971) and Margulis (1973) for reviews) indicated that the polymerization reaction was endothermic and sensitive to colchicine. However, the recent development of an in vitro system for the formation of microtubules has facilitated more detailed analyses of the molecular mechanisms governing assembly. The polymerization of microtubules in brain extracts was found to be both temperature and colchicine sensitive (Weisenberg, 1972; Borisy and Olmsted, 1972), and to occur under physiological conditions of neutral pH and moderate ionic strength and be maximal in the presence of GTP (Olmsted and Borisy, 1973). In addition, from centrifugation studies, it was inferred that the formation of tubules was dependent upon a specific particulate structure which might be involved in the initiation of tubule growth (Borisy and Olmsted, 1972; Olmsted et al., 1974). In order to define how solution parameters might affect the overall polymerization process and to attempt to determine the dependence of polymerization on intermediate structures, characterization of purified preparations of tubulin was undertaken. This paper describes the ionic and nucleotide requirements for polymerization, and attempts to identify the mechanisms by which these factors might be involved in the cellular regulation of microtubule assembly.

Experimental Procedure

Preparation of Purified Microtubule Protein. Microtubule protein was purified from porcine brain tissue using two cycles of a reversible, temperature dependent assembly scheme described in detail elsewhere (Borisy et al., 1974, 1975). Microtubules polymerized in the second cycle of purification were pelleted by centrifugation (39,000*g*, 30 min) at 37° for subsequent storage and will be referred to as an H_2P fraction. The pelleted protein was frozen in liquid nitrogen and kept at −80° for periods of up to 1 month with no loss of activity. For the majority of experiments, protein was obtained by resuspension of the frozen pellets in P buffer[1] (0.1 *M* Pipes adjusted to pH 6.9 at 23° with 5 *N*

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[1] Abbreviations used are: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)tetraacetic acid; P buffer, 0.1 *M* Pipes adjusted to pH 6.9 at 23°; EDTA, ethylenediaminetetraacetic acid; H_2P, C_2S, purification fractions (pellet at 37°, 0° supernatant) obtained after two cycles of assembly–disassembly; GMPPCP, β,γ-methyleneguanosine triphosphate; GMPPNP, 5′-guanylyl imidodiphosphate; AMPPCP, β,γ-methyleneadenosine triphosphate; AMPPNP, 5′-adenylyl imidodiphosphate.