Carbamate Formation on Tubulin: CO_2/Bicarbonate Buffers Protect Tubulin from Inactivation by Reductive Methylation and Carbamoylation and Promote Microtubule Assembly at Alkaline pH†

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ABSTRACT: Carbamoylation and reductive methylation of tubulin have been shown previously to inhibit microtubule assembly, probably by attack on essential internal lysine residues [Mellado, W., Slebe, J., & Maccioni, R. B. (1982) *Biochem. J. 203*, 675–681; Szasz, J., Burns, R., & Sternlicht, H. (1982) *J. Biol. Chem. 257*, 3697–3704]. We show first that this inhibition is blocked by the presence of HCO_3^-/CO_2 buffer at physiological concentrations during the carbamoylation or reductive methylation. Under conditions that block assembly, the amount of radiolabeled cyanate or formaldehyde incorporated by these reactions in the absence of HCO_3^-/CO_2 was approximately four carbamoyl or five methyl groups in a ratio of approximately 1.7 α chain/β chain. In the presence of HCO_3^-/CO_2, the formaldehyde incorporation is decreased roughly 0.5 mol in each of the α and β chains, and cyanate incorporation, roughly 1.0 mol/mol of α or β monomer. These results are consistent with the hypothesis that CO_2 competed with formaldehyde or cyanate for uncharged amino groups and led to the reversible formation of carbamates. The complete antagonism of the inhibition of microtubule assembly by reductive methylation by CO_2, even though the number of methyl groups incorporated was reduced by only 0.5 mol/tubulin monomer, was consistent with the possibility that reductive methylation opened up additional residues for attack. Indeed, using an adaptation of the method of Gros et al. for measurement of carbamates [Gros, G., Forster, R. E., & Lin, L. (1976) *J. Biol. Chem. 251*, 4398–4407], we found that reductive methylation with 2 mM formaldehyde (assembly blocked) did not decrease carbamate formation (carbamate formation was inhibited at higher formaldehyde concentrations). Moreover, even after reductive methylation at 2 mM formaldehyde, 50–60% of assembly could be recovered in HCO_3^-/CO_2 buffers. These findings indicate the potential physiological importance of HCO_3^-/CO_2 and carbamate formation in microtubule assembly. We show here that assembly of microtubules above pH 7.0 is enhanced in HCO_3^-/CO_2 buffers.

The mechanisms controlling microtubule assembly are still poorly understood. Several variables such as calcium ion concentration and pH have been identified that potentially regulate assembly, but it has been difficult to demonstrate quantitatively that physiological changes in calcium or pH alone, or in concert with microtubule-associated proteins, can account for the dynamic state of microtubules observed in vivo.

In a prior report, we described the sharp decay in microtubule assembly competence at pHs above neutrality (Regula et al., 1981) and suggested that such effects might be important in regulating assembly in vivo. These and related studies [e.g., see Olmsted and Borisy (1975)], however, utilized unphysiological buffers such as 2-(*N*-morpholino)ethanesulfonic acid (MES),[1] PIPES, or phosphate–glutamate, whereas the physiological buffer system is based upon bicarbonate–CO_2. The special significance of the HCO_3^-/CO_2 system for protein (P) buffering is in the formation of carbamino groups according to the equation:

$$P\text{–}NH_2 + CO_2 \rightleftharpoons PNHCOO^- + H^+ \qquad (1)$$

Note that a proton is generated in the reaction and that the reactant amino group is *un*charged. Consequently, in hemoglobin, for which it has been studied extensively, below pH 7.5 only the terminal α-amino groups with p*K*'s of 6.4–7.3 are involved (Gros et al., 1981; Morrow et al., 1976): the ϵ-amino groups of lysine residues with p*K*'s of 9.8–10.2 contribute to carbamate formation only above pH 8.0. However, the work of Szasz et al. (1982) on the reductive methylation of tubulin and of Mellado et al. (1982) on the carbamoylation of tubulin indicates that tubulin contains one or more highly reactive lysines [the N-termini of both α- and β-tubulins are methionines (Ponstingl, 1981; Krauhs, 1981)]. The generally accepted scheme for reductive methylation is

$$P\text{–}NH_2 + HCHO \rightleftharpoons PN{=}CH_2 \xrightarrow{CNBH_3} PNHCH_3 \qquad (2)$$

and that of carbamoylation is

$$P\text{—}NH_2 + HNCO \longrightarrow PNHC(=O)\text{—}NH_2 \qquad (3)$$

The parallels of eq 2 and 3 to eq 1 are obvious. All reactions require the uncharged amino group. An important difference is that although the initial reactions in eq 1 and 2 are reversible, reduction of the intermediate Schiff base in eq 2 and carbamoylation in eq 3 are irreversible. One can predict, however,

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[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MES buffer, 100 mM MES, 1 mM EGTA, and 0.5 mM $MgCl_2$ (pH as indicated); MAP, microtubule-associated protein; Tris, tris(hydroxymethyl)aminomethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; kDa, kilodalton(s); Rubisco, ribulosebisphosphate carboxylase/oxygenase.

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that CO_2 should be at least partially competitive with formaldehyde and cyanate for the same uncharged amino groups.

We have examined the effects of HCO_3^-/CO_2 on the reductive methylation and carbamoylation of tubulin. We show that the inhibition of assembly by these reactions was completely antagonized by HCO_3^-/CO_2 at physiological concentrations. The nature of this antagonism was explored by measurements of the incorporation of radiolabeled formaldehyde or cyanate in the presence or absence of HCO_3^-/CO_2 and by determination of carbamate formation utilizing a pH stopped-flow method adapted from Gros et al. (1976). These studies establish the occurrence of carbamate formation on tubulin at pHs below 7.0 probably at internal lysine residues as well as the N-terminal α-amino groups. We demonstrate further that microtubule assembly at alkaline pH is enhanced in HCO_3^-/CO_2 buffers, presumably by carbamate formation.

Materials and Methods

Materials. GTP, MES, PIPES, $NaHCO_3$, $Na_2CO_3 \cdot H_2O$, glycylglycine, and $NaCNBH_3$ were obtained from Sigma Chemical Co. $NaCNBH_3$ used in all experiments was periodically recrystallized as described by Jentoft and Dearborn (1979). HCHO was purchased from Aldrich Chemical Co. and KNCO from Fisher Scientific. Radiolabeled $H^{14}CHO$ (53 mCi/mmol) was obtained from New England Nuclear and $KN^{14}CO$ (51 mCi/mol) from Amersham. For preliminary experiments, [^{14}C]formaldehyde was purified by anionic exchange (Jentoft & Dearborn, 1979). However, since 90% of the total radioactivity was recovered after chromatography and 98% precipitable by 5,5-dimethyl-2,3-cyclohexanedione (Lewis et al., 1961), this purification step was later omitted. The fluorescent probe pyranine was purchased from Kodak.

Preparation of Microtubule Protein and Tubulin. Bovine brain microtubule protein was isolated by the procedure of Asnes and Wilson (1979), including three cycles of polymerization–depolymerization in 20 mM sodium phosphate, 100 mM sodium glutamate, 1 mM EGTA, and 0.5 mM $MgCl_2$, pH 6.75. The protein preparation was stored at −80 °C. Except where indicated, on the day of the experiment, aliquots of protein were passed through a final polymerization–depolymerization cycle to yield a preparation of microtubule protein consisting of approximately 80% tubulin and 20% high molecular weight microtubule-associated proteins (MAPs) (4× microtubule protein). When required, purified tubulin (PC-tubulin) was obtained from 3× tubulin by phosphocellulose chromatography. Chromatography was performed in 50 mM PIPES, pH 6.7, using 2.5–3 mg of protein/mL of column bed volume. Both PC-tubulin and 4× microtubule protein were exchanged into the appropriate buffer by serial chromatography using two PD-10 molecular sieve columns (Pharmacia). Removal of free amino groups, i.e., glutamate, was essential since it is a substrate for both reductive methylation and carbamate formation. The final protein concentration was assayed by the biuret method (Gornall, 1949) using bovine serum albumin as standard.

Steady-State Microtubule Assembly. To study the effects of HCO_3^-/CO_2 and reductive methylation or carbamoylation on the assembly of microtubule proteins, two types of experiments were performed. In most experiments, microtubule protein was first incubated with formaldehyde/$NaCNB_3$ or cyanate in the presence or absence of HCO_3^-/CO_2. Free reactants were removed by rapid gel filtration, and the two modified samples and a control were then assembled in identical MES buffer (without HCO_3^-/CO_2) and compared. In some experiments where direct effects of HCO^-/CO_2 on assembly were studied, an aliquot of microtubule protein was chemically modified (MES buffer) in the absence of HCO_3^-/CO_2 and the free reactants were removed as described above. This sample was then divided and was assembled either in MES plus HCO_3^-/CO_2 or in MES buffer alone.

For experiments in which HCO_3^-/CO_2 was employed only during the chemical modification step, subsequent assembly was monitored by using standard 1.0-cm quartz cuvettes. In experiments in which assembly was performed with or without HCO_3^-/CO_2, samples were placed in 2-mL glass vials, adjusted with NaOH or $NaHCO_3$, and the vials sealed with rubber sleeve stoppers. For samples to be exposed to CO_2, the septum was provided with input and exit needles to allow for the continuous delivery of moistened CO_2. After 6–7 min, the samples were removed by syringe and transferred into pregassed cuvettes. These closed cuvettes (Suprasil quartz, micro flow cells, Fisher) were designed with two ports at the top, one of which provided access to the upper surface of the sample, while the other was connected to a tube leading to the cuvette bottom. CO_2 was provided through the former port. The latter was connected to a micro pH flow cell with an electrode (Markson Science, Phoenix, AZ). After temperature reequilibration to 30 °C, GTP–Mg^{2+} (final concentration 2 and 0.5 mM, respectively) was added to initiate assembly which was followed turbidimetrically at 350 nm (Regula et al., 1981). When a steady state had been attained, usually 30–60 min, the sample was withdrawn through the bottom tube and passed (without contact with air) across the pH flow cell.

In general, the numerous column procedures reduced the assembly competence of the microtubule protein, in some cases to nearly half of the unmanipulated starting material. This lability of tubulin was perhaps exaggerated by the selective loss of MAPs: roughly 30% of the high molecular weight MAPs were lost in the usual multicolumn experiments. In all experiments, every sample was subjected to identical incubation times and filtration procedures.

Reductive Methylation. Samples were incubated at 30 or 37 °C, with or without CO_2, as described above. $NaCNBH_3$ was added (final concentration 15 mM), and the reaction was then started by injection of HCHO (0.25–22 mM). For control samples, H_2O was substituted for HCHO. At the designated time, the reaction was partially quenched by immersion in an ice bath, and the unreacted reagents were immediately removed either by repetitive (2 times) rapid gel filtration (Neal & Florini, 1973) for small volumes or by desalting with PD-10 columns for larger volumes. Exchange into the appropriate buffer was achieved simultaneously.

For subsequent assembly studies, the samples were reincubated at 30 °C for 6–7 min with or without CO_2, transferred to cuvettes, and assembled and monitored as described. Since the CO_2 used in all incubations was moistened by bubbling through H_2O, the volume change due to evaporation was in all cases only 0–5%.

In some cases, the degree of reductive methylation was assessed by performing the reaction with [^{14}C]formaldehyde. $H^{14}CHO$ was brought to desired concentration and specific activity by addition of unlabeled HCHO and H_2O.

Carbamoylation. Carbamoylation of tubulin was achieved essentially as was reductive methylation, except $NaCNBH_3$ and HCHO were replaced by KNCO (50 mM final concentration). In some experiments, the number of residues modified by cyanate treatment was quantified by using $KN^{14}CO$.

Analysis of [^{14}C]Formaldehyde- and [^{14}C]Cyanate-Labeled Protein. Microtubule protein was reductively methylated using ^{14}C-labeled reactants and exchanged into 65 mM Tris, pH 6.8. The protein concentration of the samples was assayed, and

aliquots were removed for quantitation of total radioactivity by liquid scintillation. Other aliquots, identical in protein content, were fractionated by SDS–PAGE using 7.5% gels and the Laemmli (1970) buffer system. Gels were stained for 24 h with Fast Green, and the relative protein content of the different bands was estimated by quantitative densitometry (Gorovsky, 1970). The gel was then sectioned and dissolved (Goodman, 1971) and radioactivity counted. Label incorporation was also analyzed by densitometry of autoradiograms.

Identification of the Cyanate-Modified Residues. Carbamoylation has been exploited [e.g., see Manning et al. (1973) and Hershko et al. (1984)] to differentiate reactions at α-amino termini from internal lysines: on mild acid hydrolysis, the carbamoylated α-NH_2 terminus is liberated as the hydantoin; carbamoylated internal lysines are not. The hydantoate can be then extracted into ethyl acetate. Microtubule protein (3.5 mg/mL) was reacted with $KN^{14}CO$ for 15 min at 30 °C as described; 0.5 mL of sample was acidified with 0.5 mL of acetic acid and 1.0 mL of concentrated HCl, heated for 1 h at 100 °C, and then cooled on ice; 2.0 mL of 10 N NaOH and 1.0 mL of saturated NaCl were added to yield a pH of 3–5. Ethyl acetate (10 mL) was then added to the hydrolysate at room temperature and the methionine hydantoin extracted by vortexing for 1 min. The two phases were separated by centrifugation, and the upper organic layer containing the hydantoin was transferred to a fresh tube. The aqueous layer was reextracted with ethyl acetate and the resultant organic phase combined with the previous organics. Radioactivity in both organic and aqueous phases was quantified by liquid scintillation.

Carbamate Measurement. (*A*) *Principle.* When a solution containing neutral amino groups is mixed with an unbuffered CO_2 solution, two reactions occur: carbamate formation (according to eq 1) and the hydration of CO_2. The latter is much slower than the former. Consequently, if the change in $[CO_2]$ can be followed with time, the two reactions can be differentiated and quantified. Earlier methods relied on direct measurements of CO_2 and required voluminous quantities of materal (and effort). Gros et al. (1976) substituted measurement of pH which is altered during the reaction as discussed below. They utilized a stopped-flow apparatus containing a specially designed pH electrode. We have simplified the measurement further by utilizing a fluorescent pH indicator, following the reaction optically, and thus avoiding flow artifacts, electrical grounding problems, and electrode instability. The measured pH change must then be converted to the protons actually released from the reactions. This is done by separate determination of the buffer capacity of the solution. Gros et al. designated this the buffer factor, BF. The amount of H^+ produced by carbamate formation per liter is $Q_{H^+} = pH_{extrap}(BF)$.

The relationship between protons produced and carbamate formation is governed by the reaction of CO_2 with the unprotonated amine (eq 1) and the equilibrium between protonated and unprotonated forms of the amine:

$$P\text{–}NH_3^+ \rightleftharpoons P\text{–}NH_2 + H^+ \qquad (4)$$

Since this reaction is pulled to the right by carbamate formation, between 1 and 2 mol of H^+ is generated for each mole of carbamate. Determination of carbamate and the pK of the amine can be made from measurements of carbamate formation at different pHs and CO_2 tensions. However, in proteins where there are potentially multiple groups involved, this can only be approximated. We aimed here simply to establish that carbamate formation occurred on tubulin and to test the possibility that more than the α-amino termini were involved. More importantly for the purposes of this report, we have used the method to determine if the extent of carbamate formation was altered by reductive methylation.

(*B*) *Apparatus.* The output from a xenon lamp was directed through an interference filter (maximum transmission at 410 or 450 nm) and focused onto the optical window of an Aminco stopped-flow device (SLM-AMINCO, Urbana, IL). The emitted light was directed through a 505-nm cutoff filter perpendicular to the exciting beam and measured with an attached photomultiplier. The chamber was kept at room temperature.

For each experiment, two solutions were prepared. The first contained the protein or peptide to be examined for carbamate formation. In initial control experiments, 4 mM glycylglycine and 150 mM NaCl, pH 7.65, was used. For studies of microtubule carbamate formation, a protein solution of 10–15 mg/mL in 0.5 mM PIPES and 150 mM NaCl, pH 7.05–7.10, was prepared. At this low buffer concentration, the protein accounted for 80–90% of the buffer capacity. The second solution was unbuffered 150 mM NaCl either with or without CO_2. It was delivered to the appropriate reservoir, without contact with air, via a syringe equipped with a three-way valve. Each time the stopped-flow trigger mechanism was activated, equal volumes (usually 0.25 mL) of protein and CO_2 solutions were mixed. The dead time required for the reacting solution to flow from the point of mixing to the point of observation was 4 ms. The intensity of fluorescence obtained was monitored with an Apple IIe computer at a sampling rate of 1000 points/s using the Fast A/D converter option for the input-plotting software, Vidichart (Interactive Microware, Inc.).

The pH of the solutions was measured as the emission of the fluorescent indicator pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid) (Clement & Gould, 1981). The effects of pH on the fluorescence intensity of pyranine vary with the wavelength of excitation: with excitation at 450 nm, increased acidity depresses emission; with excitation at 400 nm, acidity increases emission. Consequently, the ratio of emissions can be correlated with pH independently of the total fluorescence and can be used to obviate variations in signal due to dye concentration or lamp instability. In practice, we extrapolated the emission curves obtained at the two wavelengths back to zero time and calculated the ratio. From the ratios, pH values were determined by reference to a standard curve of fluorescence ratio versus pH. The initial stopped-flow runs for each experiment were performed without CO_2 to establish the fluorescence ratio at a known pH. Once this reference point was established, the reaction with CO_2 was performed.

Effluent from the stopped-flow apparatus was collected and thoroughly degassed. The protein concentration, as expected, was exactly half that of the original protein solution due to the stopped-flow mixing with an equal volume of unbuffered salt solution. The degassed protein solution was titrated with a standard HCl solution. After the contribution of non-protein buffer determined separately was subtracted, the mean buffer value, BF, was calculated to be 19 μmol (0.1 pH unit)$^{-1}$ (mg of microtubule protein)$^{-1}$ over the experimental range of pH.

Results

HCO_3^-/CO_2 Antagonizes the Inhibition of Microtubule Assembly by Reductive Methylation. As reported by Szasz et al. (1982), reductive methylation at relatively low concentrations of formaldehyde inhibited microtubule assembly (Figure 1a, curve A versus curve C). Under our assembly conditions, a 26% inhibition was attained with 0.1 mM formaldehyde, 80% at 0.25 mM, and complete inhibition at 0.5 mM formaldehyde. The latter is somewhat less than the 2.0 mM

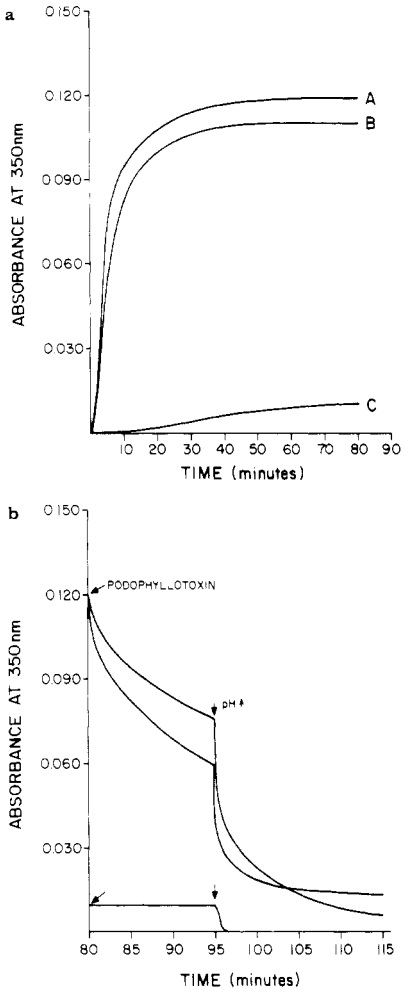

FIGURE 1: Assembly of microtubule protein following reductive methylation in the presence or absence of HCO_3^-/CO_2. Microtubule protein (4×, 3–3.5 mg/mL) in MES buffer, pH 6.35, was adjusted to pH 6.75 by addition of either NaOH or 5% CO_2–$NaHCO_3$ ([HCO_3^-] added = 26–28 mM) and subjected to reductive methylation as described under Materials and Methods. Briefly, one aliquot of NaOH-adjusted sample served as an unmethylated control. Another NaOH-adjusted sample, as well as the HCO_3^-/CO_2 sample, was reacted with 0.5 mM HCHO and 15 mM $NaCNBH_3$ for 15 min, at 30 °C. The $+CO_2$ sample was maintained under a 5% CO_2 atmosphere during the reaction. The three samples were then quenched on ice and unincorporated reactants removed by rapid gel filtration, the sample buffer being exchanged for MES, pH 6.75. (a) The samples (2 mg/mL) were placed in cuvettes, assembly was initiated with GTP, and microtubule assembly at 30 °C was followed spectrophotometrically. Curve A, *un*methylated control; curve B, methylated in the presence of HCO_3^-/CO_2; curve c, methylated in the absence of HCO_3^-/CO_2. (b) Disassembly by podophyllotoxin. Once a steady-state level of assembly for all samples had been reached, podophyllotoxin (final concentration 50 μM) was added, and its effects on assembled microtubules were monitored. After 15 min, NaOH was added to raise the pH of each sample from 6.75 to 7.1 and the absorbance followed for an additional 20 min. All curves represent averages of five experiments. Curves labeled as for (a).

required by Szasz et al., possibly reflecting their use of 2.5 M glycerol to promote assembly.

To determine the influence of HCO_3^-/CO_2 on the effects of reductive methylation, the latter was performed in buffer supplemented with $NaHCO_3$; 26–28 mM $NaHCO_3$ was added to the pH 6.35 MES buffer which was gassed with 5% CO_2, yielding a final pH of 6.75. Controls were adjusted to the same pH with NaOH. Reductive methylation was performed as before. The buffers and reagents were then rapidly exchanged by gel filtration as described under Materials and Methods, and assembly was measured for all samples in the same MES buffer (containing no HCO_3^-/CO_2). The result showed almost complete protection by HCO_3^-/CO_2, with assembly equal to 90–100% of control (Figure 1a, curve B). That the turbidity corresponded to microtubule formation was shown by the loss of absorbance following incubation at 4 °C (not shown) or with the antimicrotubule drug podophyllotoxin, particularly under alkaline conditions (Regula et al., 1981) (Figure 1b). Moreover, electron microscopy of thin-sectioned pellets formed by ultracentrifugation of the reaction mixtures at $10^5 g$ corresponding to curves A and B was indistinguishable and showed abundant microtubules (Figure 2). As predicted from the ready reversibility of carbamate formation, exposure of microtubule protein to HCO_3^-/CO_2 alone did not affect its subsequent assembly in MES buffer. The extent of assembly was unaltered by exchange of microtubule protein into HCO_3^-/CO_2 buffer and back into MES buffer through parallel gel filtrations and incubations. It was also shown, utilizing ^{14}C-labeled HCO_3^-, that the amount of CO_2 remaining with the protein following gel filtration was <0.01 μmol/μmol of tubulin (data not shown).

Table I: Incorporation of ^{14}C-Labeled Formaldehyde into Bovine Tubulin by Reductive Methylation in the Absence or Presence of HCO_3^-/CO_2[a]

	mol of [^{14}C]formaldehyde/ mol of α-tubulin	mol of [^{14}C]formaldehyde/ mol of β-tubulin	α/β
+NaOH	3.12	1.86	1.68
$+HCO_3^-/CO_2$	2.69	1.31	2.05

[a] Microtubule protein (4×) was prepared and reductively methylated as described in Figure 1, except $H^{14}CHO$ (0.59 μCi/μmol) was used in the methylation reaction. After removal of free label by gel filtration, aliquots of microtubule protein were removed for protein assay and quantitation of total dpm per milligram of protein. Other aliquots were examined by SDS–PAGE, both by dissolution and counting of gel slices and by autoradiography (see Materials and Methods). Indicated values are the mean for two experiments and had a maximum range of ±6.4% for $+CO_2$ samples and ±4.3% for +NaOH samples.

This result is consistent with the hypothesis that CO_2 was competing with HCHO for the same uncharged amino groups. To examine this possibility further, we measured the incorporation of labeled formaldehyde into α- and β-tubulins in the presence or absence of CO_2. Figure 3 compares the Fast Green stained gel (Figure 3, lanes 1 and 2) and autoradiograph (Figure 3, lanes 1′ and 2′) of $H^{14}CHO$-labeled microtubule protein. Lanes 1 and 1′ were labeled in the absence of HCO_3^-/CO_2 and lanes 2 and 2′ in their presence. The stained patterns are identical with the usual 80% of protein in the 55-kilodalton tubulin doublet. The autoradiographs show that with or without CO_2, labeling of the α-tubulin is considerably more intense than β. Labeling of the β-tubulin is clearly reduced in the presence of HCO_3^-/CO_2 (lane 2′).

These results were quantified by liquid scintillation counting of the extracted labeled tubulin bands. The extent of methylation measured by $H^{14}CHO$ incorporation showed that the assembly-incompetent tubulin (Figure 3, lanes 1 and 1′) was labeled to the extent of 5 methyl groups per dimer or, if there were dimethylation, a minimum of 2.5 amino sites per tubulin dimer (Table I). This degree of modification associated with complete inhibition of assembly is distinctly less than the 13 methyl groups incorporated per dimer found by Szasz et al. (1982) using formaldehyde at 2.0 mM. However, in agreement with Szasz et al., we found a consistent preponderance of labeling in α- versus β-tubulin in a ratio of approximately 1.7 to 1.

In the presence of HCO_3^-/CO_2, the incorporation of $H^{14}CHO$ was inhibited roughly 20% (Table I). The number

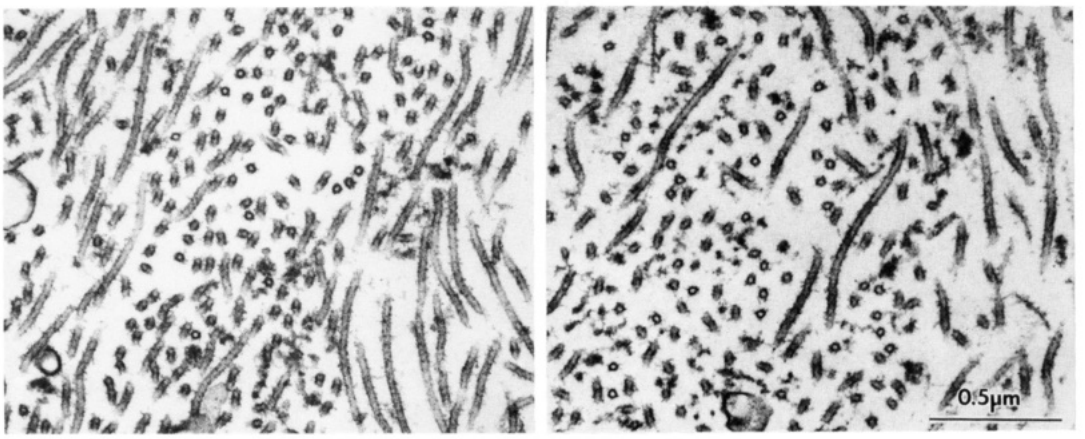

FIGURE 2: Formation of microtubules from control versus microtubule protein reductively methylated in the presence of HCO_3^-/CO_2. Microtubule protein assembled as in Figure 1, curves A and B. Aliquots (2 mg/mL) of assembled protein were fixed in 2% glutaraldehyde for 30 min, and microtubules were pelleted by centrifugation. The pellets were embedded and thin sectioned for electron microscopy. Left, control; right, methylated in the presence of HCO_3^-/CO_2.

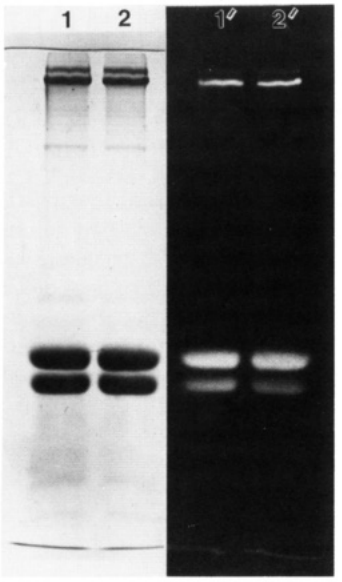

FIGURE 3: Autoradiography of SDS–PAGE-separated microtubule protein methylated in the absence or presence of HCO_3^-/CO_2. In two of the experiments described in Figure 1, $H^{14}CHO$ (0.59 μCi/μmol) was used in the reductive methylation reaction. The microtubule protein was separated by SDS–PAGE on 7.5% acrylamide gels. The stained gel is illustrated in the lanes labeled 1 and 2; the corresponding autoradiography, lanes 1′ and 2′. The protein in lanes 1 and 1′ was reductively methylated in the absence of HCO_3^-/CO_2; lanes 2 and 2′, in the presence of HCO_3^-/CO_2.

of methyl groups per α-tubulin was reduced from 3.1 to 2.7 and the number per β-tubulin from 1.9 to 1.30. Thus, paradoxically, the assembly competence of tubulin was completely restored by inhibiting the incorporation of *less* than one methyl group per monomer. The simplest rationalization of this result was that methylation of certain critical amino groups was prevented by CO_2 in virtually all tubulin dimers but that methylation now occurred in other residues; i.e., carbamate formation altered the reactivity of amino groups on other residues. Analogously, it might be predicted that reductive methylation of certain amino groups would affect the reactivity of others.

Carbamate Formation on Tubulin. To determine directly the formation of carbamates, the stopped-flow technique described under Materials and Methods was used with microtubule protein before and after reductive methylation. We first validated the technique by measurements of carbamate formation on glycylglycine (Gros et al., 1976). Figure 4a illustrates the results of a stopped-flow experiment in which the mixed solutions were 2 mM glycylglycine and 0.15 M NaCl in equilibrium with 20% CO_2. The fluorescence intensity measurements were for an excitation wavelength of 450 nm (Figure 4a, top) and 400 nm (Figure 4a, bottom). It was convenient to invert the electrical polarity of the larger signal at 450 nm which was thus proportional to the hydrogen ion concentration. As described by Gros et al. (1976), there was an initial rapid rise in acidity due to the proton release of carbamate formation, followed by a relatively slow increase corresponding to the hydration of CO_2 and proton release from carbonic acid. Q_{H^+} (the release of protons per mole of glycylglycine) was calculated to be 0.10 mol of H^+/mol of glycylglycine. This is in essential agreement with the data of Gros et al.

Carbamate formation on PC-tubulin was also readily demonstrable, but concentrated solutions were difficult to prepare without some protein denaturation and aggregation. The data were therefore quite noisy. Moreover, since Szasz et al. (1982) have reported the denaturation of tubulin by reductive methylation, we repeated the stopped-flow experiments with the more stable microtubule protein containing approximately 80% tubulin by mass. The microtubule-associated proteins (MAPs) of these preparations are principally MAP 1 and 2 of molecular mass 250–280 kDa as compared to the 55-kDa tubulin. Consequently, the tubulin which accounts for 80% of a microtubule protein preparation will contain approximately 95% of the carbamate-forming α-amino termini. Since our stopped-flow apparatus could not be regulated above room temperature, and since the pK of carbamate-forming amino groups can be expected to decrease roughly 0.03 unit per degree increase in temperature (Gros et al., 1976), we conducted the stopped-flow experiments at pH 7.0 to make them comparable to chemical modifications performed at 30 °C at pH 6.75. Figure 4b illustrates a stopped-flow experiment with 4× microtubule protein. The solutions mixed were 20% CO_2 and 65 μM microtubule protein. The curves were somewhat more noisy than those obtained with the simple dipeptide glycylglycine, but the two phases of proton release were readily discriminated.

From such experiments, a release of protons, Q_{H^+}, of 1.90 protons per tubulin dimer was calculated. Similar experiments with microtubule protein following reductive methylation utilizing 0.5 or 2.0 mM formaldehyde (Figure 4c), i.e., conditions under which CO_2 antagonized the inhibition of assembly by reductive methylation, gave virtually the identical

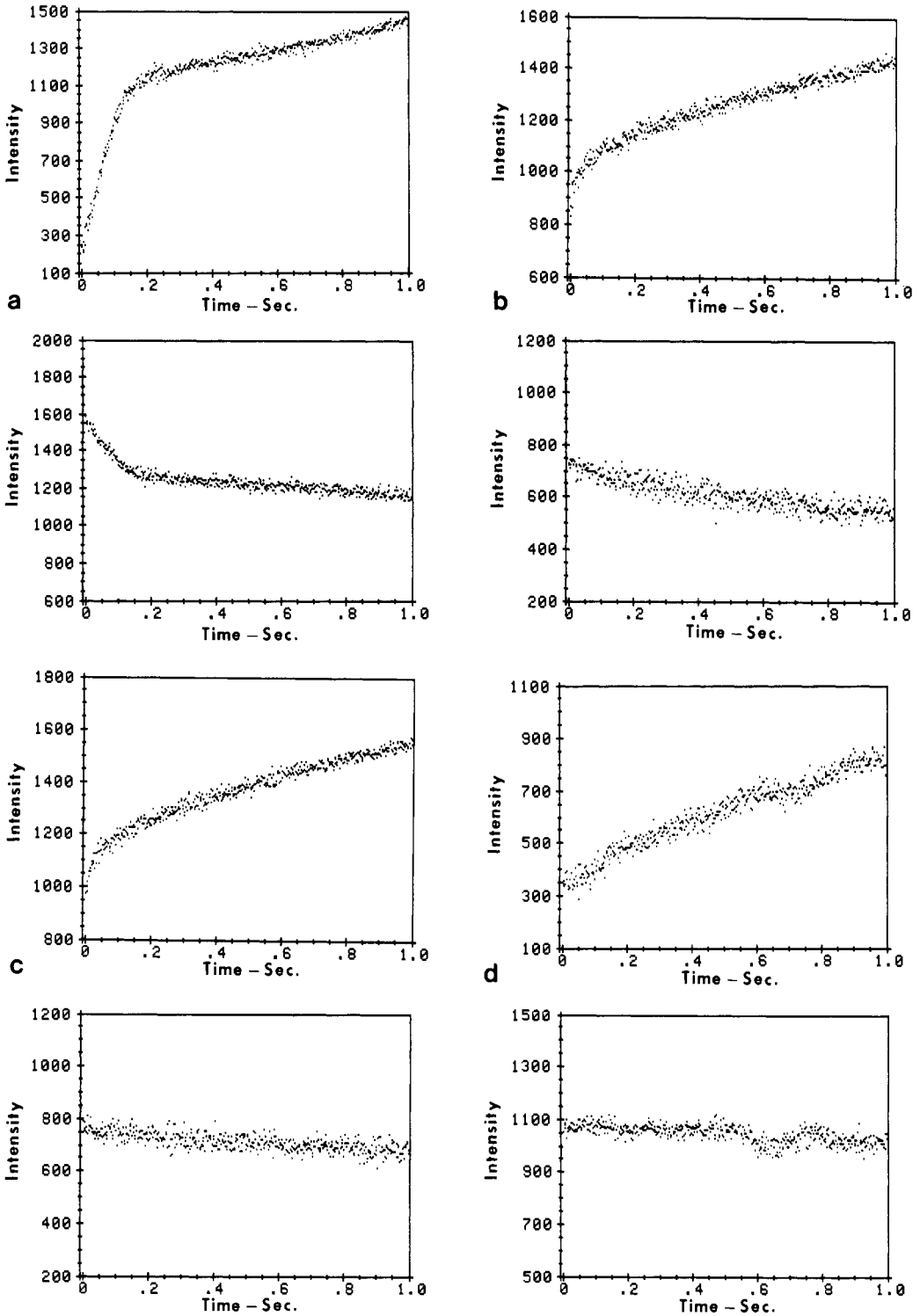

FIGURE 4: Stopped-flow measurement of carbamate formation on glycylglycine and microtubule protein. A solution containing neutral amino groups was mixed with an unbuffered NaCl solution equilibrated with 20% CO_2 (10% CO_2 after mixing). The change in pH due to carbamate formation (rapid) and CO_2 hydration (slow) was monitored by using the pH-sensitive dye pyranine. The fluorescence emission signal was recorded at intervals of 1.0 ms after excitation at 450 nm (top tracing) and 400 nm (bottom tracing). The four representative experiments (a–d) are displayed in arbitrary units of fluorescence intensity. The final concentration of amino group component, the pH change due to carbamate formation, the final pH after mixing, and the number of protons released per molecule, respectively, were the following [the paper of Gros et al. (1976) may be consulted for details of the calculations]: (a) 2 mM glycylglycine, ΔpH 0.54, final pH 6.96, 0.100 H^+ molecule; (b) 65 μM microtubule protein (4×) (assumes a mean molecular mass of 10^5 daltons), ΔpH 0.100, final pH 6.90, 1.90 H^+/tubulin dimer; (c) 44 μM microtubule protein (assumes a mean molecular mass of 10^5 daltons) after reaction with 2 mM $HCHO_2$–15 mM $NaCNBH_3$, ΔpH 0.091, final pH 6.91, 1.90 H^+/tubulin dimer; (d) 51 μM microtubule protein (assumes a mean molecules mass of 10^5 daltons) after reaction with 22 mM HCHO–100 mM $NaCNBH_3$, ΔpH 0.012 (max), final pH 6.99, less than 0.3 H^+/tubulin dimer.

Q_{H^+} (three experiments) (1.90 mol/mol of protein). This is consistent with the hypothesis that reductive methylation enhances the reactivity at nonmethylated residues so that net carbamate formation is unaffected. More extensive methylation would be expected to reduce carbamate formation at these residues. Indeed, at 22 mM HCHO (Figure 4d), when 40 methyl groups were introduced, carbamate formation was abolished.

Effect of CO_2/HCO_3^- on Tubulin Carbamoylation. Stark (1965) has shown that carbamoylation proceeds by reaction

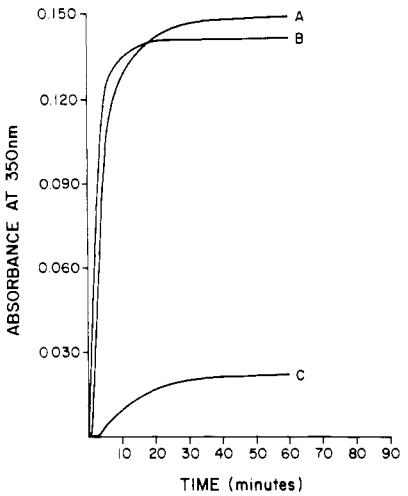

FIGURE 5: Microtubule assembly following carbamoylation in the presence or absence of HCO_3^-/CO_2. Samples were prepared and aliquoted in a manner analogous to that used in the reductive methylation experiments (Figure 1). Microtubule protein (3×) in MES buffer, pH 6.35, was adjusted to pH 6.75 by addition of NaOH or HCO_3^-/CO_2 and carbamoylated as described under Materials and Methods (CO_2, 5%). (A) Noncarbamoylated control; (B) carbamoylated in the presence of HCO_3^-/CO_2; (C) carbamoylated in the absence of HCO_3^-/CO_2.

Table II: Incorporation of ^{14}C-Labeled Cyanate into Bovine Tubulin in the Absence or Presence of HCO_3^-/CO_2[a]

	mol of [^{14}C]cyanate/mol of α-tubulin	mol of [^{14}C]cyanate/mol of β-tubulin	α/β
+NaOH	2.56	1.58	1.62
+CO_2	1.43	0.72	1.99

[a] Microtubule protein (3×) was brought to pH 6.75 and carbamoylated as described in Figure 5, except $KN^{14}CO$ (0.65 μCi/μM) was used in the reaction. After removal of free label, total dpm per milligram of protein was determined, and aliquots of microtubule protein was examined by electrophoresis as described. Indicated values are the mean for three experiments and had a maximum range of ±10.5% for +CO_2 samples and ±8.4% for +NaOH samples.

of isocyanic acid with the unprotonated amine, a mechanism that is formally analogous to carbamate formation (see eq 3).

Mellado et al. (1982) found that carbamoylation inhibited microtubule assembly. As for reductive methylation, we measured assembly following incubation of microtubule protein with cyanate in the presence or absence of HCO_3^-/CO_2. As before, cyanate and HCO_3^-/CO_2 were removed by gel filtration before assembly in MES buffer. In agreement with Mellado et al., we found that prior incubation of microtubule protein with 50 mM KNCO for 15 min almost completely inhibited assembly (Figure 5, curve C). As was the case for reductive methylation, incubation with cyanate in the presence of HCO_3^-/CO_2 blocked the effect of cyanate on microtubule assembly [Figure 5, curves A (control) and B (cyanate with HCO_3^-/CO_2)]. (It should be emphasized that this block occurred with 5% CO_2 or only 40 mmHg partial pressure, essentially physiological conditions.) The corresponding levels of $N^{14}CO$ incorporation were studied, and the results are summarized in Table II. Incorporation of $N^{14}CO^-$ was reduced somewhat more dramatically in HCO_3^-/CO_2 than was incorporation of [^{14}C]formaldehyde: a reduction of over 40% in both α- and β-tubulin was observed or approximately 1 mol/mol of tubulin monomer. The excess label in α versus β was apparent for carbamoylation as it was for reductive methylation.

To determine whether CO_2 affected carbamoylation of the

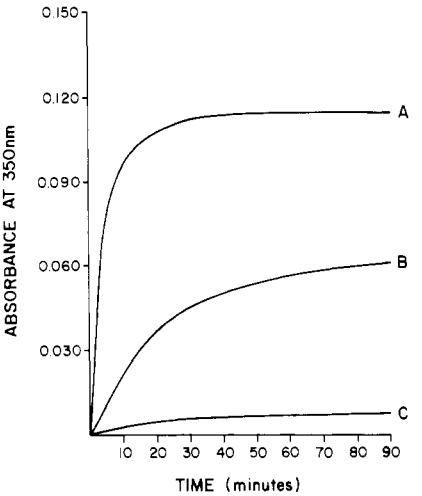

FIGURE 6: Effects of HCO_3^-/CO_2 on the assembly of reductively methylated microtubule protein. Microtubule protein (4′) was exchanged into MES buffer, pH 6.75. One aliquot was reacted with 0.5 mM HCHO and 15 mM $NaCNBH_3$ for 15 min at 30 °C. Another aliquot served as the unmethylated control and received water instead of HCHO. The reaction was quenched and free reactants removed, simultaneously exchanging the sample buffer in all samples for MES, pH 6.35. The unmethylated control and an aliquot of the methylated sample were then adjusted to pH 6.75 by addition of NaOH. Another aliquot of methylated sample was brought to pH 6.75 by addition of 5% HCO_3^-/CO_2. Assembly was initiated and monitored as described. The figure is a composite of four experiments. (A) Nonmethylated control; (B) methylated and then assembled in HCO_3^-/CO_2; (C) methylated and then assembled in MES.

α-NH_2 termini or internal lysines, we subjected protein that had been carbamoylated in the presence or absence of HCO_3^-/CO_2 to mild acid hydrolysis and extracted the released N-terminal hydantoate into ethyl acetate, thereby separating it from the internal carbamoylated residues that are not released by hydrolysis (see Materials and Methods). In two experiments, we determined that virtually all counts were incorporated into internal lysines in agreement with Mellado et al. (1982). The mean values for labeled nonterminal amino acids were 95% for CO_2 treated and 96% for controls. The remaining counts corresponded to less than 0.2 mol of labeled terminal amino acid per mole of dimer. This is ~10% of the total reduction in carbamoylation effected by HCO_3^-/CO_2 (see Table II). Thus, the reduction in labeling must have occurred nearly exclusively on internal lysines.

Some Effects of CO_2 Cannot Be Explained by Action at Residues That Are Reductively Methylated. The inhibition of the effect of reductive methylation and carbamoylation on assembly was consistent with direct competition of CO_2 with formaldehyde or cyanate at critical amino acid residue(s). However, further studies showed that HCO_3^-/CO_2 had noncompetitive effects as well. Thus, we first subjected microtubule protein to reductive methylation (no HCO_3^-/CO_2 present) and then determined its assembly in MES buffer in the presence or absence of HCO_3^-/CO_2 buffer (Figure 6). We found that in MES buffer, assembly was completely inhibited (Figure 6, curve C). In HCO_3^-/CO_2, assembly was 50–60% of control (Figure 6, curve B); i.e., CO_2 still has an effect even if it is not present during methylation. That the turbidity measured corresponded to bona fide microtubule assembly was again shown by its sensitivity to cold and podophyllotoxin; electron microscopy of thin-sectioned pellets (10^5g for 30 min) showed the expected packed microtubule arrays (not shown). This indicates that carbamate formation at nonmethylated residues can compensate at least partially for the effect of reductive methylation and thus demonstrates that carbamate

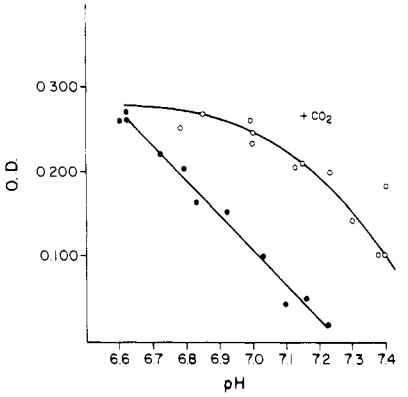

FIGURE 7: Effects of HCO_3^-/CO_2 on steady-state microtubule assembly as a function of pH. Microtubule protein (3×) was exchanged into MES buffer, pH 6.60, and diluted to 3.0 mg/mL. Samples were adjusted to the desired pH by addition of either NaOH or HCO_3^-/CO_2. The final concentration of added $NaHCO_3$ ranged from 0 to 45 mM, and the ionic strength of all samples was held approximately constant by adjustment with NaCl. Assembly was initiated by addition of GTP and monitored at 350 nm until steady-state levels were reached. (○) Samples plus HCO_3^-/CO_2; (●) samples plus NaOH–NaCl.

formation at nonmethylated residues is likely to be important for assembly.

Microtubule Assembly in HCO_3/CO_2 Buffers. The foregoing results establish that carbamate formation occurs on tubulin most probably at internal lysines. Since reductive methylation or carbamoylation at these residues strongly inhibits assembly, it seemed highly likely that assembly would not be indifferent to carbamate formation. As a first step toward exploring the consequences of carbamate formation, we examined the pH dependence of microtubule assembly in HCO_3/CO_2 buffers.

We had reported in detail on the pH dependence of microtubule assembly in phosphate–glutamate buffers (Regula et al., 1981): a sharp decrease above pH 7.0 was noted. A comparison of the pH dependency in HCO_3/CO_2-supplemented MES buffer versus MES supplemented with NaCl to obtain similar ionic strength is shown in Figure 7. Microtubule assembly was considerably enhanced in the CO_2/HCO_3^- buffer at pHs >6.9.

DISCUSSION

The capacity of HCO_3^-/CO_2 to antagonize the inhibition of microtubule assembly by reductive methylation or carbamoylation strongly suggests that there is direct competition of CO_2 with formaldehyde or cyanate for the same critical amino groups. In the introduction, we emphasized that these reactants require uncharged amino groups, and direct measurements of ^{14}C-labeled formaldehyde and cyanate (Tables I and II) showed that their incorporation was reduced in the HCO_3^-/CO_2 buffer.

In general, our measurements of label incorporation in the absence of CO_2 were in agreement with Szasz et al. (1982) and Mellado et al. (1982) with some minor exceptions. Thus, we found that the number of methyl groups introduced that were required for complete inhibition was 5 as compared with 13 found by Szasz et al. However, like them, we consistently found preferential labeling of α- as opposed to β-tubulin. We found also, like Mellado et al., that 50 mM KCNO was required for complete inhibition of assembly. However, these authors found only 1 mol of $KN^{14}CO$ incorporated per dimer which was distributed nearly equally between α and β. Under very similar conditions (but with bovine instead of porcine tubulin), we found 4 mol of $KN^{14}CO$ incorporated per dimer, and this was preferentially distributed to α in a ratio nearly identical with that obtained with labeled formaldehyde by reductive methylation.

In preparations of microtubule protein, reductive methylation (and carbamoylation) occurs on both tubulin and microtubule-associated proteins (MAPs). However, Szasz et al. showed that inhibition of microtubule assembly was the result of tubulin methylation. Our attention has been focused thus on the reactive groups of tubulin. It should be recognized nonetheless that reductive methylation occurred on high molecular weight MAPs and that it was inhibited somewhat in HCO_3^-/CO_2 buffers: in two experiments, the radioactivity incorporated into the combined MAP 1 and 2 bands was decreased 18% in the presence of HCO_3^-/CO_2. Since the quantities of MAP are limited, direct measurements of carbamate formation on them cannot be made at this time. Moreover, since carbamate formation is readily reversible, its separate contribution to tubulin and MAP function in microtubule assembly can also not be made directly. Thus, although we can be reasonably confident that the antagonism of HCO_3^-/CO_2 to inhibition of assembly by reductive methylation or carbamoylation was exerted through tubulin, it is possible that carbamate formation on MAPs may also be important in regulating microtubule assembly. This is currently under investigation.

Sternlicht and his colleagues have developed the hypothesis that microtubule assembly is dependent on a cluster of basic residues in α-tubulin (Szasz et al., 1986). This is based largely on their demonstration that there is a lysine residue (Lys-394) that is highly susceptible to reductive methylation and that is responsible for the excess labeling of α-tubulin (Sherman et al., 1983). Whatever the importance of Lys-394, it should be pointed out that the concentrations of formaldehyde used by Sternlicht and co-workers to demonstrate selective labeling were different from those they used to inhibit microtubule assembly (0.15 or 0.2 mM versus 2 mM). Under the latter conditions, 13 methyl groups were introduced. In our experiments where inhibition of assembly is paralleled by incorporation of fewer methyl groups, the excess of 1.3 methyl groups in α versus β is explicable in terms of a single reactive residue in α (as suggested by Sternlicht). Similarly, carbamoylation of a single α-tubulin residue can explain the excess label of α- versus β-tubulin. However, these results do not establish this residue as critical for assembly.

Except in the unusual case of ribulosebisphosphate carboxylase/oxygenase (Rubisco), whose complex with CO_2 can be enormously stabilized by a high-affinity transition-state analogue and the carbamate then chemically esterified to form a stable methoxycarbonyl (Lorimer & Miziorko, 1980), it has not been possible to identify directly the site of carbamate formation. Cyanate, which has been considered a CO_2 analogue, has been used to specifically block carbamate formation in hemoglobin (Kilmartin & Rossi-Bernardi, 1969) and the site of formation inferred from the known positions of carbamoylation at the N-termini. In our study, carbamoylation occurred almost exclusively at nonterminal sites identified by Mellado et al. as ϵ-amino groups of internal lysine residues. CO_2 did not appreciably affect the small fraction of label at the N-termini but reduced total incorporation of label by roughly 50%. Hence, carbamate formation almost certainly occurs on the ϵ-amino groups of internal lysines. The presence of specific internal lysines reactive to nucleophiles has been observed in other proteins many times [e.g., for tropomyosin, see Hitchcock-DeGregori et al. (1985)]. The structural basis

for this differential reactivity appears diverse. The cluster of basic residues in α-tubulin including the reactive Lys-394 and a neighboring Arg and His analyzed by Szasz et al. is one possible molecular strategy employed. Interestingly, the carbamate-forming N-terminus of the β chain of hemoglobin lies in a cavity that is lined with positively charged groups (Arnone, 1974). On the other hand, carbamate formation on Rubisco occurs within a cluster of negatively charged residues (Lorimer, 1981). In any event, even assuming Lys-394 to be a site of carbamate formation, it cannot be the only site: the presence of HCO_3^-/CO_2 reduces labeling either by reductive methylation or by carbamoylation in *both* α- and β-tubulin. Moreover, assembly in HCO_3^-/CO_2 following reductive methylation at least partially rescues the tubulin from the inhibition of assembly observed in MES buffer. Collectively, the data indicate several sites of carbamate formation occurring in both α- and β-tubulins. Work in progress is aimed at identifying these sites. At this point, however, it may be well to emphasize the likelihood that carbamate formation or reductive methylation at certain residues modifies the reactivity at others. This is indicated most clearly for reductive methylation, after which (at levels that completely block assembly) there is no inhibition of carbamate formation measured directly by the release of protons even though the effect of methylation on assembly is antagonized. In addition, under the same conditions, the HCO_3^-/CO_2 effects a reduction of methylation in both α- and β-tubulins of only 0.5 mol/mol of monomer. This labeling pattern is most consistent with a shift in the reactivities of internal lysines. Moreover, the capacity of HCO_3^-/CO_2 to partially restore assembly competence *after* reductive methylation indicates that functionally significant carbamate formation can occur at nonmethylated sites. Such labeling implies that certain amino groups are less reactive to reductive methylation than to carbamate formation. Carbamates may be stabilized by charge–charge interactions [e.g., see Arnone (1974)].

From our results, obtained at physiological of HCO_3^-/CO_2, it can be inferred that lysine residues in α- and β-tubulins, probably including the reactive α-tubulin Lys-394 identified by Sternlicht and colleagues, are sites of carbamate formation under physiological conditions. Since tubulin is purified in the absence of HCO_3^-/CO_2 and since no tightly bound CO_2 is measurable under these conditions (unpublished observations), it is clear that virtually all reported measurements of microtubule properties and assembly have been made on this "unphysiological" tubulin. Microtubule proteins, of course, can be made to assemble and disassemble in vitro in the absence of HCO_3^-/CO_2 so that one can anticipate that the effects of carbamate will be to modulate basic properties already apparent in nonphysiological buffers. The effects of HCO_3^-/CO_2 on the pH dependence of assembly demonstrated here are perhaps a case in point: a similar optimum pH was apparent in MES with or without supplementation with HCO_3^-/CO_2, but the range of assembly was extended by 0.4 pH unit in HCO_3^-/CO_2. Such modifications may be critical in determining microtubule function in vivo. It is apparent, thus, that a wide range of microtubule properties might be profitably reexamined in the physiological HCO_3^-/CO_2 buffer.

ACKNOWLEDGMENTS

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Registry No. CO_2, 124-38-9; HCO_3^-, 71-52-3; HCHO, 50-00-0; $NCBH_3$, 33195-00-5; OCN^-, 661-20-1.

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