Muhlrad, A. (1977) *Biochim. Biophys. Acta 493*, 154–166.
Muhlrad, A., & Ferencz, K. (1973) *Physiol. Chem. Phys. 5*, 13–26.
Muhlrad, A., & Hozumi, T. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 958–962.
Muhlrad, A., Lamed, R., & Oplatka, A. (1975) *J. Biol. Chem. 250*, 175–181.
Okuyama, T., & Satake, K. (1960) *J. Biochem. (Tokyo) 47*, 454–462.
Seidel, J. C., Chopek, M., & Gergely, J. (1970) *Biochemistry 9*, 3265–3272.
Sleep, J. A., Trybus, K. M., Johnson, K. A., & Taylor, E. W. (1981) *J. Muscle Res. Cell Motil. 2*, 373–399.
Stone, D. B., & Cheung, P. H. (1976) *Biophys. J. 16*, A46.
Szilagyi, L., Balint, M., Sreter, F. A., & Gergely, J. (1979) *Biochem. Biophys. Res. Commun. 87*, 936–945.
Taylor, E. W. (1979) *CRC Crit. Rev. Biochem. 6*, 103–164.
Tonomura, Y., Appel, P., & Morales, M. F. (1966) *Biochemistry 5*, 515–521.
Trybus, K. M., & Taylor, E. W. (1982) *Biochemistry 21*, 1284–1294.
Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54–56.
Werber, M. M., Szent-Gyorgyi, A. G., & Fasman, A. D. (1972) *Biochemistry 11*, 2872–2883.
Young, M. (1967) *J. Biol. Chem. 242*, 2790–2792.

# Interaction of Apolipoprotein B from Human Serum Low-Density Lipoprotein with Egg Yolk Phosphatidylcholine and Cholesterol†

Subhash Dhawan and Jacqueline A. Reynolds*

ABSTRACT: Binary and ternary complexes of apolipoprotein B (apo B) with egg yolk lecithin and with lecithin plus cholesterol have been formed from detergent–lipid–protein mixed micelles. These particles appear to be spherical by negative-stain electron microscopy and contain 510000 g of protein (2 mol of apo B) complexed with apparent maximum molar ratios of 780:2 (egg yolk lecithin:apo B) and 1300:280:2 (egg yolk lecithin:cholesterol:apo B). The secondary structure as reflected in the circular dichroic spectra is similar to that of holo-$LDL_2$ when only lecithin is bound, but is significantly altered when cholesterol is also present in the complex, suggesting that the molecular organization of the ternary complex formed in the absence of neutral lipids is significantly different from that of the native lipoprotein. A part of the protein (presumably uncharged) has to be incorporated with the lipid acyl chains in a hydrophobic "core" of the spherical particle. However, the relative amounts of apo B exposed to the aqueous solvent and to the hydrophobic interior of the recombined particles depend on the lipid content and also appear to differ from those in $LDL_2$. The results suggest that the manner in which apo B folds is continuously variable, a result consistent with its ability to bind varying amounts of lipid in vivo.

Apolipoprotein B (apo B)[1] is the core protein common to very low density and low-density serum lipoproteins (VLDL and LDL, respectively) and is present in these water-soluble complexes in a constant mass of 510000 g—equivalent to two polypeptide chains per particle (Schumaker, 1973; Fisher et al., 1975; Tanford & Reynolds, 1979). Apo B forms complexes in vivo with 5–20 times its own mass of various lipids and perhaps more importantly can vary its binding capacity as the lipid content is altered during intravascular catabolism of VLDL to the ultimate product, $LDL_2$.

One approach to understanding how the core protein, apo B, and the naturally occurring lipids combine and organize themselves into a water-soluble structure is to investigate systematically the binding of specific lipid moieties to the purified protein and the effect of this interaction on protein structure. We have used this approach previously (Watt & Reynolds, 1981) and in this study have extended our previous investigations of the binary apo B–EYL complex to the ternary apo B–EYL–cholesterol complex.

## Experimental Procedures

### *Materials*

All materials used in this study were standard reagent grade unless otherwise specified. Sodium dodecyl sulfate was BDH Chemical Corp. specially pure grade purchased from Gallard Schlesinger. Sephacryl S500 and Sepharose CL-4B were products of Pharmacia Fine Chemicals. Egg yolk lecithin used in this study was from Lipid Products and contained less than 0.5% contamination by fatty acid or lysolecithin as determined by high-pressure liquid chromatography. Cholesterol was purchased from Applied Sciences, and [$^{14}$C]cholesterol was from Amersham-Searle.

### *Methods*

*Preparation of Holo-$LDL_2$.* Holo-$LDL_2$ ($d$ = 1.02–1.05 g/cm$^3$) was isolated from fasting, normal human volunteers by centrifugal flotation by employing the procedures described in detail by Steele & Reynolds (1979). After isolation, the two free sulfhydryls per 250000 g of protein were alkylated

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[1] Abbreviations: LDL, human serum low-density lipoprotein; VLDL, human serum very low density lipoprotein; apo B, apolipoprotein B; EYL, egg yolk phosphatidylcholine; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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with iodoacetamide, and the reaction was terminated by dialysis against a buffer solution containing 20 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (Tes) adjusted to an ionic strength of 0.3 with NaCl, pH 7.4 (hereafter referred to as standard Tes buffer). Holo-$LDL_2$ was then passed through sterile 0.22-$\mu$m Millipore filters into sterile test tubes for storage at 4 °C.

*Analytical Procedures.* Protein measurements were by the method of Bensdoun & Weinstein (1976), and lipid phosphorus was assayed according to the micromethod of Bartlett (1959). Radioactive ligands were measured in a Beckman LS-100 C liquid scintillation counter by using ACS (Amersham) scintillation fluid.

*Electron Microscopy.* Protein–lipid complexes were negatively stained with 2% uranyl acetate and examined on a Joelco 100-C electron microscope.

*Circular Dichroism.* Circular dichroic spectra of holo-$LDL_2$ and the reconstituted complexes were recorded on a Jobin-Yvon Dichrographe III calibrated with *d*-10-camphorsulfonic acid. One-millimeter cells were used, and a mean residue weight of 112.5 (Smith et al., 1972) was utilized to calculate values of molar ellipticity.

*Molecular weight* of the protein–lipid complex was determined by equilibrium analytical ultracentrifugation in a Beckman Model E ultracentrifuge equipped with a photoelectric scanner. The mass of apo B per particle was determined from the slope of plots of ln OD vs. $r^2$ which yields $M_p(1 - \phi'\rho)$ directly. $M_p$ is the molecular weight of the protein, and $1 - \phi'\rho$ is the buoyant density factor equal to $(1 - \bar{v}_p\rho) + \sum_i \delta_i(1 - \bar{v}_i\rho)$ (Tanford et al., 1974) where $\delta_i$ is the grams of EYL or cholesterol bound per gram of protein, $\bar{v}_p$ is the partical specific volume of apo B (0.725), and $\bar{v}_i$ values are the partial specific volumes of EYL and cholesterol (0.984 and 0.988, respectively).

*Preparation of Delipidated Apo B.* Delipidated apo B was obtained from holo-$LDL_2$ by using sodium dodecyl sulfate as previously described by Steele & Reynolds (1979). Briefly, holo-$LDL_2$ was incubated with the detergent (30 mg of sodium dodecyl sulfate/mg of apo B) for 3 h at room temperature, followed by gel filtration chromatography on a Sepharose CL-4B column equilibrated with standard Tes buffer containing 2.5 mM sodium dodecyl sulfate, pH 7.4. Appropriate fractions were pooled and concentrated on an Amicon XM-100A Diaflo membrane.

*Preparation of Apo B–Lipid Complexes.* The delipidated apo B–detergent complex was concentrated to 1–2 mg/mL and dialyzed against 0.25 mM sodium dodecyl sulfate in standard Tes buffer to reduce the number of free detergent micelles present in the solution. This free concentration of detergent is sufficient to maintain apo B in a soluble and disaggregated state, the circular dichroic spectrum of which is similar to that of holo-$LDL_2$ (Steele & Reynolds, 1979). The apo B–detergent complex was then transferred to a conical test tube containing a thin film of either EYL or EYL + cholesterol. The sample was allowed to stand for 6–8 h at room temperature and then stirred for another 3–4 h. The lipid–protein–detergent solution was dialyzed against 20 mM sodium carbonate–bicarbonate buffer containing 0.28 M NaCl, pH 10, for 24 h with three 2-L changes of buffer to remove the detergent. During the incubation period and subsequent dialysis, the sample is protected from light exposure to minimize photoinduced oxidation of the lipid moiety. Of the resultant solution, 0.2 mL was then applied to a 0.7 × 50 cm gel filtration column containing Sephacryl S500 presaturated with EYL. Recoveries of protein and lipid from column

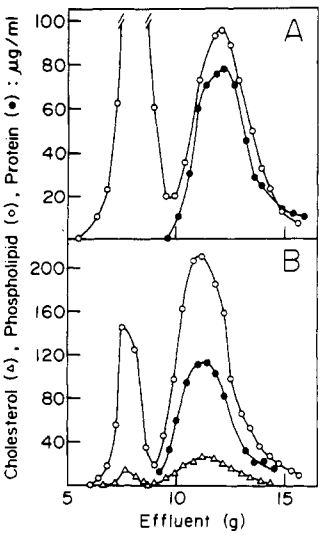


FIGURE 1: Gel filtration of apo B–lipid complexes on Sephacryl S500. (A) Apo B–EYL: $\bar{v}$ = 780 mol of EYL/510 000 g of apo B. (B) Apo B–EYL–cholesterol: $\bar{v}_{EYL}$ = 1258 mol of EYL/510 000 g of apo B, $\bar{v}_{cholesterol}$ = 284 mol of cholesterol/510 000 g of apo B.

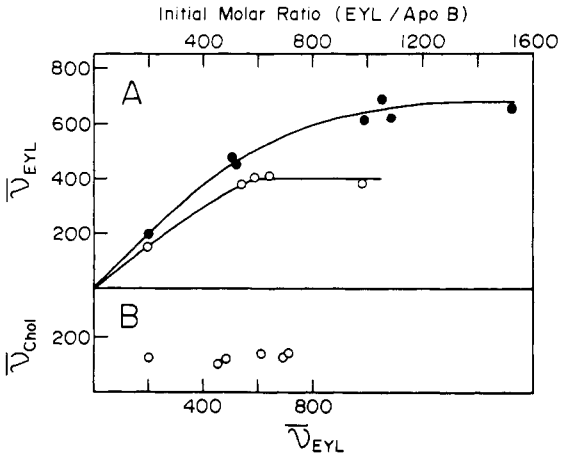


FIGURE 2: Binding of EYL and cholesterol to apo B. (A) Binding of EYL to apo B as a function of increasing initial molar ratio: (O) no cholesterol; (●) cholesterol. (B) Binding of cholesterol to apo B as a function of bound EYL. $\bar{v}$ is expressed as moles of ligand per 255 000 g of apo B.

chromatography were in all cases 90% or greater. Previous studies from this laboratory (Watt & Reynolds, 1981) have demonstrated that dialysis followed by gel filtration chromatography as described removes all detergent from the lipid–protein complex.

This experimental protocol for replacing bound detergent with naturally occurring lipids of low aqueous solubility is highly reproducible. However, it is not possible to demonstrate rigorously that the final lipid–protein complex obtained by detergent removal is the same thermodynamic state that would be reached if it were possible to recombine apo B and lipids by standard binding techniques in the absence of detergent.

## Results

Typical elution profiles of apo B–lipid complexes from an S500 gel filtration column are shown in Figure 1. EYL vesicles containing no protein are eluted in the void volume at 7.5 g of effluent, and the protein–lipid complexes are included with peak elution positions between 11 and 12.5 g of effluent. The binding is in all cases constant across the majority of the included peak. (Errors in determination of the small concentrations of protein and lipid in the extreme leading

Table I: Summary of Apo B Complexes with EYL and Cholesterol[a]

| initial molar ratio | | complex molar ratio | | | |
|---|---|---|---|---|---|
| EYL | cholesterol | EYL | cholesterol | cholesterol/EYL | $[\theta_{208}]/[\theta_{218}]$ |
| 392 | 0 | 350 | 0 | 0 | 0.74 |
| 1078 | 0 | 758 | 0 | 0 | 0.79 |
| 1156 | 0 | 796 | 0 | 0 | nd[c] |
| 1286 | 0 | 824 | 0 | 0 | nd |
| 1956 | 0 | 764 | 0 | 0 | 0.75 |
| 406 | 260 | 392 | 248 | 0.63 | 1.02 |
| 1022 | 418 | 954 | 234 | 0.25 | 1.04 |
| 1046 | 174 | 902 | 212 | 0.24 | 0.96 |
| 1966 | 322 | 1258 | 284 | 0.23 | 0.97 |
| 2106 | 332 | 1378 | 262 | 0.19 | 0.97 |
| 3052 | 328 | 1306 | 278 | 0.21 | 0.99 |
| 2276 | 874 | 1242 | 284 | 0.23 | 0.96 |
| 1286[b] | 199 | 824 | 24 | 0.03 | 0.94 |
| $LDL_2$ | | 621 | 475 | 0.72 | 0.75 |

[a] Molar ratios are expressed as moles of lipid per 510 000 g of apo B. [b] Experiment in which preformed EYL–apo B complexes were added to cholesterol in the absence of detergent. [c] nd, not determined.

and trailing fractions prohibit a meaningful calculation of binding ratios in these regions of the elution pattern.) The molar ratio of bound EYL to 255 000 g of apo B as a function of increasing initial lipid concentration was determined and is shown in Figure 2A as open symbols. The maximum binding observed under these experimental conditions is 390 ± 20 mol of EYL/255 000 g of apo B (780 ± 40 mol/dimer). This apparent saturation value is considerably higher than that for the previously reported EYL–apo B complex of 230–370 mol of lipid/510 000 g of apo B (Watt & Reynolds, 1981). In the earlier studies, two rather than three changes of dialysis buffer were used, and pure phospholipid vesicles containing no associated protein were removed from the mixture by centrifugal flotation in a sucrose solution of density 1.1 g/cm$^3$. The lower value of EYL binding observed in the earlier work may have resulted from separation of uncomplexed lipid prior to total detergent removal or from a perturbation induced by the presence of sucrose.

We have previously reported (Watt & Reynolds, 1980, 1981) a strong tendency for aggregation of neutral amphiphile–apo B complexes at pH 7.4. In the present studies, gross binding heterogeneity occurred when this pH was used for dialysis and column elution. However, transfer of the complex prepared by dialysis at pH 10 to either pH 8.1 or pH 9.2 *after* detergent removal did not alter the binding or homogeneity of the complex. Thus, the critical step in obtaining a homogeneous lipid–apo B complex appears to be the maintenance of significant charge on the protein species during removal of the negatively charged detergent.

The filled symbols in Figure 2A show the results obtained when 130–437 mol of cholesterol/255 000 g of apo B was included with increasing initial amounts of EYL. The interaction with cholesterol significantly increases the amount of EYL associated with the protein (1300 ± 70 mol of EYL/510 00 g of apoB), but as shown in Figure 2B, cholesterol binding is nearly invariant when the initial cholesterol to protein ratio is between 130 and 437. Table I summarizes the binding experiments and shows that the cholesterol to protein ratio is not dependent upon the amount of EYL bound but is constant in all the complexes formed. The maximum cholesterol binding at apparent saturation with EYL is 0.2 mol of steroid/mol of EYL, a value far lower than that found in $LDL_2$ or in pure lipid bilayers saturated with cholesterol.

Negative-stain electron microscopy of the complexes formed at apparent saturation with EYL and with EYL plus cholesterol revealed spherical particles with average diameters of

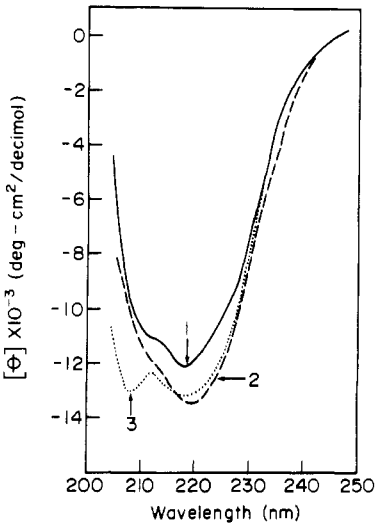


FIGURE 3: Circular dichroic spectra of apo B–lipid complexes. (1) Holo-$LDL_2$. (2) EYL–apo B (780 mol/510 000 g). (3) EYL–cholesterol–apo B (1280 mol of EYL and 280 mol of cholesterol/510 000 g of apo B).

15 and 17 nm, respectively. Hydrodynamic diameters for these two complexes obtained by gel filtration chromatography on a calibrated S500 column are 14.4 and 17.4 nm, respectively. Sedimentation equilibrium of these two complexes demonstrated that 70–75% of the sample was homogeneous with a protein molecular weight of 515 000 (two polypeptide chains per particle). Some aggregation was observed at the bottom of the solution column at protein concentrations greater than 1 mg/mL. The amount of aggregated complex did not vary with time or centrifuge speed. Bound lipid in these systems contributes less than 5% to the buoyant density factor, and thus any experimental uncertainty in the binding ratio, $\delta_i$, has no effect on the determination of the protein molecular weight.

The circular dichroic spectra of all complexes formed were examined and compared with that for holo-$LDL_2$. Figure 3 presents a comparison of the native particle (curve 1) with apo B–EYL at the saturation ratio of 780 mol/510 000 g (curve 2) and apo B–EYL–cholesterol at saturation molar ratios of 1306 and 278 mol/510 000 g, respectively (curve 3). The general shape of the circular dichroic spectrum of apo B–EYL complexes is similar to that of the protein in the native $LDL_2$ particle. The magnitude of the negative ellipticity increases with increasing EYL binding to a maximum at 218 nm that

is 12% larger than that of $LDL_2$. However, binding of cholesterol in the ternary complex EYL–cholesterol–apo B results in a significant alteration in the spectrum characterized by a slight red shift in the 218-nm ellipticity minimum and a second minimum at 208 nm (suggestive of an increase in $\alpha$-helical structure). Table I presents $[\theta_{208}]/[\theta_{218}]$ for the complexes investigated and indicates that the altered shape of the circular dichroic spectrum in the ternary complex is a function only of the presence of cholesterol and not increased binding of EYL.

Apo B–EYL complexes containing 411 and 398 mol of EYL/255 000 g of protein were prepared as described under Methods. These particles were then incubated for 10 h with cholesterol deposited as a thin film on the walls of a conical tube (initial molar ratio of cholesterol to protein, 100 and 200 mol/255 000 g). Nonsolubilized cholesterol crystals were removed by centrifugation, and a 0.2-mL aliquot was chromatographed on the S500 column as described under Methods. Under these conditions, only 24 mol of cholesterol was bound per particle in contrast to the 280 mol bound when the complex was formed from mixed micelles containing apo B, EYL, cholesterol, and sodium dodecyl sulfate. Since the monomer solubility of cholesterol in aqueous medium is 25–40 nM (Haberland & Reynolds, 1973; Gilbert & Reynolds, 1976), the combination of this sterol with a *preformed* apo B–EYL particle must be kinetically limited by the slow rate of transfer of cholesterol from the film to the water-soluble complex. It is significant, however, that the circular dichroic spectrum of this cholesterol–EYL–apo B complex containing only 24 mol of cholesterol displays the same alteration in ellipticity as a function of wavelength as was observed in a complex containing an equivalent amount of EYL and 10 times the amount of bound cholesterol (see Table I).

## Discussion

In vivo, apo B packages in stable, water-soluble form widely varying amounts of lipids. In $VLDL_5$ and $LDL_2$, for example, 510 000 g of apo B bind 39 000 and 2700 mol of total lipid, respectively [e.g., see Shen et al. (1977)]. We have also shown (Watt & Reynolds, 1980; Steele & Reynolds, 1979; Zampighi et al., 1980) that the dimeric form of apo B interacts with detergents, forming a highly asymmetric water-soluble particle quite different from the quasi-spherical VLDL and LDL complexes. Here, we start with an apo B–detergent complex that is asymmetric, and the substitution of lipid for the detergent restores the nativelike spherical shape. This protein, then, can adopt a variety of grossly different morphological structures depending upon the amphiphiles associated with it. Under the experimental conditions employed here, an apparent saturation level of 780 mol of EYL/dimer apo B is observed, and this apparent saturation is increased in the presence of bound cholesterol to 1306 mol of EYL/dimer apo B. The maximum amount of cholesterol that can be incorporated in the complex is 278 mol/dimer apo B, corresponding to 1 mol of cholesterol per 5 mol of EYL. This cholesterol to phospholipid ratio is far lower than those found in VLDL and LDL fractions, which range from 0.8 to 1.1, suggesting that most of the sterol in the latter complex may be incorporated by virtue of the presence of triglycerides and cholesteryl esters, i.e., the presence of a larger hydrophobic core. It is also clear from the circular dichroic spectra that the protein structure in the ternary complex, EYL–cholesterol–apoB, is significantly different from that found in the native particle and that this difference is induced by the presence of bound cholesterol. It is tempting to speculate that in native $LDL_2$ the protein moiety does not "see" the cholesterol in the particle since the circular dichroic spectrum of $LDL_2$ is more nearly akin to that of the binary complex, EYL–apo B.

Structural models of $LDL_2$ have generally been based on the concept of a microemulsion in which triglycerides and cholesteryl esters form a central hydrophobic core surrounded by an outer monolayer of phospholipid and protein [e.g., see Shen et al. (1977)]. There is ample experimental evidence for the sequestered location of triglycerides, cholesteryl ester, and most of the unesterified sterol [e.g., see Deckelbaum et al. (1977), Atkinson et al. (1977), and Yeagle et al. (1978, 1982)] and for the surface location of phospholipid head groups [e.g., see Assmann et al. (1974) and Yeagle et al. (1977, 1978)]. The exact location of apo B in this particle, however, is less easily defined, but recent X-ray and freeze–etch studies (Luzzati et al., 1979; Gulik-Krzywicki et al., 1979) have been interpreted as consistent with a convoluted spherical surface containing approximately 70% of the protein with the remainder of apo B intercalated into the hydrophobic core.

The complexes studied in this work contain no neutral lipids. The experimentally determined radii of the quasi-spherical particles are 75 and 85 Å, respectively, and the maximum extended length of an EYL molecule is 35 Å. Geometrical considerations then lead to the conclusion that protein must penetrate to the center of the particle and that a microemulsion model that locates all of the protein in the surface is not applicable to these complexes.

An alternative way to view the organization of these complexes is to consider the surface area available for each phospholipid head group—183 $Å^2$ for $LDL_2$, 86 $Å^2$ for apo B–EYL (2:780), and 69 $Å^2$ for apo B–cholesterol–EYL (2:280:1300). The minimum surface area occupied by one EYL molecule is approximately 52 $Å^2$ (Luzzati, 1968), leaving the following maximal percentages of surface area available for protein and water in these complexes: 72% ($LDL_2$), 40% (apoB–EYL), and 24% (apo B–cholesterol–EYL). One is forced to the conclusion that apo B is capable of conformational adaptability (Reynolds, 1976) such that varying amounts of the protein are exposed to an aqueous milieu and varying amounts are buried in a hydrophobic medium.

As we have previously noted, it is the protein moiety in all serum lipoproteins that is the guiding factor in the formation of the water-soluble native complexes. Apo B is not associated with high-density serum lipoproteins, and the apoproteins from these latter complexes, AI and AII, are not found in the very low density and low-density serum lipoproteins. Furthermore, the natural association states of pure phospholipids are bilayers (as in cell membranes) or hexagonal phases (e.g., phosphatidylethanolamine). Specific binding proteins such as apo B, AI, and AII are required to form *thermodynamically* stable, water-soluble complexes with phospholipids. In addition, phsyiological function of the circulating serum lipoproteins requires the type of conformational adaptability described herein for apo B (and previously for AI and AII).

## Acknowledgments

We are grateful to Dr. Robert M. Watt for technical advice and continuing interest in the problem of lipid association with apo B and to Dr. Charles Tanford for many helpful discussions.

**Registry No.** Cholesterol, 57-88-5.

## References

Assmann, G., Sokolski, E. A., & Brewer, H. B., Jr. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 549.

Atkinson, D., Deckelbaum, R. J., Small, D. M., & Shipley, G. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1042.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466.

Bensdoun, A., & Weinstein, D. (1976) *Anal. Biochem. 70*, 241.
Deckelbaum, R. J., Shipley, G. G., & Small, D. M. (1977) *J. Biol. Chem. 252*, 744.
Fisher, W. R., Hammond, M. G., Mengel, M. C., & Warmke, G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2347.
Gilbert, D. B., & Reynolds, J. A. (1976) *Biochemistry 15*, 71.
Gulik-Krzywicki, T., Yates, M., & Aggerbeck, L. P. (1979) *J. Mol. Biol. 131*, 475.
Haberland, M. E., & Reynolds, J. A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2313.
Luzzati, V. (1968) in *Biological Membranes* (Chapman, D., Ed.) p 71, Academic Press, London.
Luzzati, V., Tardieu, A., & Aggerbeck, L. P. (1979) *J. Mol. Biol. 131*, 435.
Reynolds, J. A. (1976) *J. Biol. Chem. 251*, 6013.
Schumaker, V. N. (1973) *Acc. Chem. Res. 6*, 398.
Shen, B. W., Scanu, A., & Kezdy, F. J (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 837.
Smith, R., Dawson, J. R., & Tanford, C. (1972) *J. Biol. Chem. 247*, 3376.
Steele, J. C. H., Jr., & Reynolds, J. A. (1979) *J. Biol. Chem. 254*, 1633.
Tanford, C., & Reynolds, J. A. (1979) in *The Chemistry and Physiology of Human Plasma Proteins* (Bing, D. H., Ed.) p 111, Pergamon Press, New York.
Tanford, C., Nozaki, Y., Reynolds, J. A., & Makino, S. (1974) *Biochemistry 13*, 2369.
Watt, R. M., & Reynolds, J. A. (1980) *Biochemistry 19*, 1593.
Watt, R. M., & Reynolds, J. A. (1981) *Biochemistry 20*, 3897.
Yeagle, P. L., Langdon, R. G., & Martin, R. B. (1977) *Biochemistry 16*, 3487.
Yeagle, P. L., Martin, R. B., Pottenger, L., & Langdon, R. J. (1978) *Biochemistry 17*, 2707.
Yeagle, P. L., Bensen, J., Greco, M., & Arena, C. (1982) *Biochemistry 21*, 1249.
Zampighi, G., Reynolds, J. A., & Watt, R. M. (1980) *J. Cell Biol. 87*, 555.

# Microtubule Assembly with the Guanosine 5′-Diphosphate Analogue 2′,3′-Dideoxyguanosine 5′-Diphosphate†

Ernest Hamel,* Anthony A. del Campo, and Chii M. Lin

ABSTRACT: The GDP analogue 2′,3′-dideoxyguanosine 5′-diphosphate (ddGDP) supports efficient tubulin polymerization. Microtubule-associated protein (MAP) dependent microtubule assembly occurs in 0.1 M 2-(*N*-morpholino)-ethanesulfonate, and sheets of parallel protofilaments are formed in 1.0 M glutamate without MAPs. The nucleotide is bound to tubulin in the course of polymerization, presumably in the exchangeable GTP site. The ddGDP is not hydrolyzed, however, and is completely stable in the reaction mixture. Nor was the nonexchangeable GTP bound to tubulin hydrolyzed in ddGDP-supported polymerization: equivalent amounts of GTP remained associated with polymerized tubulin after polymerization with either ddGDP or GTP. Higher concentrations of ddGDP than GTP were required under all conditions. Nevertheless, under optimum conditions for the ddGDP-supported reaction, polymerization began with a shorter lag period and both the rate and extent of polymerization were greater with ddGDP than with GTP. The MAP-dependent reaction with ddGDP is temperature dependent, cold reversible, and inhibited by calcium and antimitotic drugs. It differs from the GTP-supported reaction in being most vigorous at minimal $Mg^{2+}$ concentrations and exquisitely sensitive to GDP inhibition.

Two guanosine nucleotide binding sites are present on tubulin (Weisenberg et al., 1968; Bryan, 1972; Kobayashi, 1974; Hamel & Lin, 1981a), the major protein component of microtubules. One of these is termed the nonexchangeable site, since the GTP which fills it cannot be removed from tubulin without denaturing the protein. The other is termed the exchangeable site, since nucleotide bound in it can be displaced by exogenous GDP or GTP. Although nonhydrolyzable GTP analogues can support tubulin polymerization (Weisenberg et al., 1976; Arai & Kaziro, 1976; Sutherland, 1976; Penningroth & Kirschner, 1977), microtubule assembly generally requires the hydrolysis of exchangeably bound GTP to GDP (Kobayashi, 1975; Weisenberg et al., 1976; Penningroth & Kirschner, 1977; Arai & Kaziro, 1977; David-Pfeuty et al., 1977; MacNeal & Purich, 1978). GDP, particularly if added prior to the onset of polymerization, inhibits the reaction (Arai & Kaziro, 1977; Carlier & Pantaloni, 1978; MacNeal & Purich, 1978; Zackroff et al., 1980; Jameson & Caplow, 1980; Hamel & Lin, 1981a).

We have been examining interactions of ribose-modified analogues of GDP and GTP at the exchangeable site to define structural requirements for the nucleotide in greater detail (Hamel & Lin, 1981b; Lustbader & Hamel, 1982; Hamel et al., 1983). One notable finding has been that while several GTP analogues were more efficient than GTP itself in supporting polymerization, no GDP analogue was as effective an inhibitor as GDP. Perhaps most striking was the contrast between ddGTP[1] and ddGDP.

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[1] Abbreviations: ddGMP, ddGDP, and ddGTP, 2′,3′-dideoxyguanosine 5′-mono-, 5′-di-, and 5′-triphosphates; MAPs, microtubule-associated proteins; Mes, 2-(*N*-morpholino)ethanesulfonate; p($CH_2$)pG, guanosine 5′-($\alpha$,$\beta$-methylenediphosphate).