pound, quercetin pentaacetate, is nonmutagenic in the absence of liver microsomes, activity nearly equivalent to quercetin is observed when the compound is metabolically activated, presumably with deacetylation occurring as an initial step. The inactivity of permethylquercetin may be due to the inability of the liver preparation to demethylate the compound.

Quercetin is clearly mutagenic in the absence of liver-mediated metabolism. However, the activity is approximately tripled in the presence of liver microsomes. The hydroxyphenylacetic acids tested, which are known metabolites of quercetin in mammals, were not active. Thus, the proximate mutagen is an intermediary metabolite of unknown nature.

Mutagenic activity of quercetin in bacteria is not proof of genetic hazard to higher organisms. Furthermore, quercetin is from one to three orders of magnitude less active against bacterial mutants than the highly potent mutagens aflatoxin B_1 and 2-aminofluorene. Also, the processes of mammalian absorption, adsorption, tissue distribution, and metabolism, which may have drastic effects on the biological activity of many compounds, are not duplicated in a single nonmammalian mutagen assay. However, evidence that most chemical carcinogens are mutagens in various bioassays (*9*) is increasing. Thus, the carcinogenic potential of a mutagen such as quercetin, which occurs widely in foods and is produced in mammals as a result of the metabolism of various food components, such as rutin in buckwheat (*10*), must not be overlooked.

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3 December 1976; revised 14 March 1977

Quantitation of Cytoplasmic Tubulin by Radioimmunoassay

Abstract. *A radioimmunoassay has been developed for the quantitation of cytoplasmic tubulin. It measures tubulin between 20 and 1500 nanograms and does so independently of decay in colchicine-binding activity. In addition, the state of tubulin as subunit or polymer does not alter the measurement.*

Cytoplasmic microtubules are involved in a variety of cellular activities and differentiative events (*1*). Recently, major advances have been made in understanding the biochemistry of microtubule protein (*2, 3*), but the cellular control of microtubule assembly and disassembly in vivo remains to be elucidated. Ultimate understanding of these regulatory events requires the capability to measure the amount of tubulin, the microtubule subunit protein, present in the cells at various times. However, the only currently available methods for accurately determining the content of cytoplasmic tubulin are the time-decay colchicine-binding assay and the densitometric quantitation of material separated by electrophoresis in polyacrylamide gel systems (*4*), both of which have drawbacks and limitations. Recent reports (*5, 6*) that antibodies to tubulin can be used to localize microtubules in tissue cultured cells suggested to us that such antibodies could form the basis for a radioimmunoassay (RIA) as an alternative method for quantitating cytoplasmic tubulin.

Tubulin used in the RIA was prepared from lamb brain by two cycles of polymerization (*7*) followed by chromatography on a phosphocellulose column (*8*). When prepared by this procedure, tubulin is approximately 99 percent pure, as judged by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (*9*), and is free of microtubule-associated proteins (*8, 10*). Tubulin-specific antiserum was prepared by immunizing rabbits with highly purified tubulin that had been cross-linked with glutaraldehyde (*5*). The globulin fraction of this antiserum produced a faint precipitin line against tubulin in an Ouchterlony double-diffusion assay.

Purified tubulin was labeled with ^{125}I by a lactoperoxidase-catalyzed reaction (*11*), and free ^{125}I was removed by dialysis. Test tubes were coated with 50 μl of 1 mg of rabbit serum albumin (RSA) per milliliter in borate-buffered saline, *p*H 8.0. Radioactively labeled tubulin was added in a volume of 10 μl containing 0.5 ng of total tubulin protein ($\sim 10^5$ count/min). Ten microliters of the antiserum against tubulin was added and the tubes were agitated vigorously. This reaction mixture was incubated at 37°C for 4 hours with occasional agitation, and tubulin-antitubulin complexes were precipitated by adding enough of goat antiserum against rabbit immunoglobulin G (IgG) to precipitate all IgG in 10 μl of the rabbit antiserum. Precipitates and supernatants were separated by centrifugation and counted. Maximum specific binding for different preparations of labeled tubulin ranged from 45 to 70 percent.

Inhibition of a standard indirect RIA is widely used to quantitate numerous proteins, peptides, hormones, and small organic molecules. Unlabeled homologous antigen added to this type of assay competes with the labeled antigen for the limited number of antibody molecules available.

Unlabeled lamb brain tubulin to be used as inhibitor was prepared as described above. It was stored at 4°C in 0.05*M* phosphate buffer (*p*H 7.5) with 0.01 percent sodium azide. For quantitative inhibition studies, tubes were coated with RSA as above and 10 μl of unlabeled tubulin inhibitor was added. Next, 10 μl of rabbit antiserum against tubulin was added and the tubes were incubated at 37°C for 4 hours with occasional agitation; then 10 μl of labeled tubulin was added and the incubation was continued for an additional 4 hours at 37°C. Finally, goat antiserum against rabbit IgG was added and the incubation was continued for 2 hours at 37°C and overnight at 4°C. The precipitates were collected by centrifugation and washed twice with 0.5 ml of borate-buffered saline. Supernatants and precipitates from each tube were counted in a gamma-well scintillation counter. Counts in each precipitate were corrected for (i) background radioactivity, (ii) nonspecific binding (serum obtained prior to inoculation from the same rabbit that synthesized antibody against tubulin), and

(iii) total radioactivity precipitable by trichloroacetic acid. Inhibition results were calculated as percentages of the uninhibited control in which 10 μl of borate-buffered saline was added in place of unlabeled tubulin.

Figure 1 shows a typical standard inhibition curve from two experiments performed 10 days apart, with the same preparation of unlabeled tubulin. The curve decreases as unlabeled tubulin increases from ~20 to ~1500 ng per sample. A standard time-decay colchicine-binding assay requires a number of samples, each containing at least 1000 ng of tubulin. Therefore, the RIA is 10 to 100 times more sensitive than the colchicine-binding assay.

Proteins other than tubulin and its antibody are always present in the RIA. Therefore, we examined the possibility that nontubulin proteins might inhibit the binding of labeled tubulin. Because bacteria do not contain microtubule protein, a soluble extract of *Escherichia coli* was added to the assay. The binding of labeled tubulin was not inhibited by the *E. coli* extract even when 180,000 ng of *E. coli* protein was added. We also examined the effects of certain purified proteins on the RIA. Lactoperoxidase, already present in low concentration in the RIA, bovine serum albumin, and rabbit back

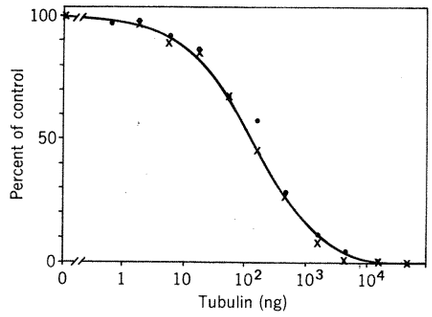

Fig. 1. Standard inhibition curve for cytoplasmic tubulin RIA. The data represented by the (X) and by the (●) are from two experiments performed 10 days apart.

muscle actin were included in the assay. Each of these proteins in the RIA failed to inhibit the binding of [^{125}I]tubulin to its antibody. Thus, these nontubulin proteins do not interfere with the assay.

The colchicine-binding activity of tubulin decays with time. Hence to accurately determine the amount of tubulin present with a colchicine-binding assay, it is necessary to use the time-decay colchicine-binding assay of Bamburg *et al.* (*4*) in which the decay rate is used to obtain the initial colchicine-binding activity. It was, therefore, important to know if a decrease in the colchicine-binding activity of tubulin would affect our RIA. A time-decay colchicine-binding assay (*12*) and an RIA were performed on unlabeled tubulin purified by two cycles of polymerization (*7*) (see Fig. 2). The colchicine-binding activity decayed with a half-life of about 1 hour, in this experiment, while the amount of tubulin measured by the RIA shows no parallel decay. Also, in this experiment, the RIA measured the amount of tubulin more accurately (Fig. 2). Such inaccuracy with the colchicine-binding assay reflects both the time elapsed since the tissue was homogenized and the purification procedures used. In other experiments (data not shown) 14-day chick embryo brain homogenates were shown to contain the same quantity of tubulin when either the time-decay colchicine-binding assay or the RIA, employing labeled chick tubulin and antiserum to chick brain tubulin, was used.

If the RIA is to be used to measure the amount of cytoplasmic tubulin in cells, it must be able to detect tubulin as both dimer and polymer present in the cell. In order to test this capability, we placed samples of tubulin, purified by two cycles of polymerization (*7*), under conditions in which free dimer would be present, rings would be present, or microtubules would polymerize and then measured the amount of tubulin by RIA. The results (Table 1) show that in the RIA the same amount of tubulin is detected regardless of the amount of polymer initially present. Thus the assay is applicable to the quantitation of tubulin independently of its state as free dimer or rings or microtubules.

Since many cells and tissues that we are interested in studying are from either mice or chicks, we determined whether microtubule protein from chick or mouse brain would substitute for lamb brain microtubule protein in the RIA. Samples of unlabeled mouse or chick tubulin, purified by two cycles of polymerization (*7*) followed by chromatography on a phosphocellulose column (*8*), were added to our RIA. Inhibition curves were generated with both tubulins, demonstrating a definite cross-reactivity between microtubule proteins from chick or mouse with antiserum against lamb brain tubulin. However, there are reproducible differences between the standard inhibition curves obtained with lamb, mouse, or chick tubulin, even though they were purified at the same time by identical procedures. The binding of la-

Table 1. The RIA determination of tubulin is independent of state as polymer or dimer.

Incubation prior to RIA*	Tubulin (mg/ml)†	Polymer (%)‡
0°C, 30 minutes	3.1	0
37°C, 30 minutes plus $10^{-4}M$ colchicine	2.9	0
37°C, 30 minutes	3.0	41

*Incubation buffer was 0.1*M* imidazole buffer (*p*H 6.8), 10 percent glycerol, 1 m*M* guanosine triphosphate (GTP), 0.5 m*M* ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA). †Tubulin content determined by RIA after incubation. ‡Percentage of polymer was determined at the completion of incubation by centrifugation (*17*) and protein determination (*18*) in pellet versus supernatant.

Table 2. Quantitation of cross-reactivity of lamb, chick, and mouse brain tubulins to antiserum against lamb brain tubulin.

Source of unlabeled tubulin	Unlabeled tubulin producing half-maximal binding of ^{125}I-labeled lamb tubulin*
Lamb brain	120 ng
Chick brain	70 ng
Mouse brain	45 ng

*The apparent greater ability of chick and mouse tubulin to inhibit the reaction of lamb brain tubulin with its homologous antiserum might be explained by the presence of heteroclitic antibodies (*19*) or by different densities of cross-reactive antigenic determinants shared among the different tubulins.

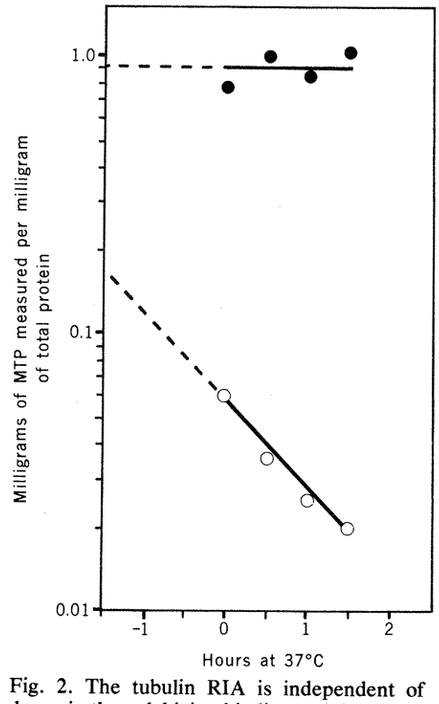

Fig. 2. The tubulin RIA is independent of decay in the colchicine-binding activity. Samples of a single preparation of lamb brain tubulin measured at indicated times by the RIA (●) and the colchicine-binding assay (○). Samples of tubulin for the colchicine-binding assay were mixed with $2.4 \times 10^{-5}M$ [^{3}H]colchicine (0.85 c/mmole), incubated at 37°C for 90 minutes, and the [^{3}H]colchicine-tubulin complexes were collected on Whatman DE81 filter paper disks. Tubulin used in this experiment is approximately 90 percent pure, as judged by SDS polyacrylamide gel electrophoresis. Abbreviation: *MTP*, microtubule protein.

beled lamb tubulin is inhibited by 50 percent with 120 ng of lamb brain tubulin, 70 ng of chick tubulin, or with 45 ng of mouse tubulin (see Table 2). These results suggest that, while there are antigenic differences between brain tubulin molecules from different species that are discernible by these immunological criteria, this system can be used to quantitate tubulins from different sources. However, it is clear that standard inhibition curves should be generated with the use of competitor tubulin from the same species as the tissue being assayed for tubulin content.

The application of immunological methods to the study of microtubule protein has expanded the repertoire of analytical approaches to this ubiquitous protein. Similarities of tubulin in different species have been shown by cross-reactivity to antiserums produced against microtubule-containing structures (*13*). Antibodies produced to outer doublet tubulin of *Naegleria* flagella have been used to quantitate flagellar tubulin in amoeboid and flagellated *Naegleria*, but fail to cross react with cytoplasmic tubulin (*14*). On the other hand, antibodies to both brain (cytoplasmic) and sea urchin outer doublet (flagellar) tubulins have been used to localize cytoplasmic microtubules in cells by immunofluorescence (*5*, *6*). In addition, antiserum against brain tubulin has been studied for its effect on the colchicine-binding activity of the molecule (*15*), and an immunosorbant made with platelet tubulin antibody has been used for rapid isolation of platelet tubulin (*16*). The RIA described here now provides a sensitive tool to quantitate cytoplasmic tubulin which detects amounts ranging from 20 to 1500 ng, and does so independently of colchicine-binding activity and the ratio of tubulin subunits to microtubules in the assay. Radioimmunoassay thus provides a technique for measuring cytoplasmic tubulin in a variety of systems even when only limited amounts of material are available.

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22 November 1976; revised 17 January 1977

Three Hypolipidemic Drugs Increase Hepatic Palmitoyl–Coenzyme A Oxidation in the Rat

Abstract. *Male rats treated with clofibrate, tibric acid, or Wy-14,643 show an 11- to 18-fold increase in the capacity of their livers to oxidize palmitoyl-coenzyme A. This provides a plausible biochemical mechanism for the action of these hypolipidemic drugs in reducing lipid concentrations in the serum.*

Clofibrate is an effective hypolipidemic as well as hypocholesterolemic agent (*1*) and is used extensively in the treatment of human hyperlipidemias. Its mechanism of action is not known in detail, however. Recently, we have found that rat liver peroxisomes oxidize palmitoyl–coenzyme A (palmitoyl-CoA), reducing O_2 to H_2O_2 and nicotinamide-adenine dinucleotide (NAD) to NADH (*2*). This peroxisomal system of fatty acid oxidation was found to be increased approximately one order of magnitude by clofibrate (*2*), suggesting that peroxisomes play a role in lowering serum lipid concentrations during clofibrate therapy.

To test further the possibility that hypolipidemic drugs act by increasing hepatic fatty acid oxidation we have measured the rate of palmitoyl-CoA oxidation in the livers of rats treated with two other hypolipidemic drugs that are structurally unrelated to clofibrate. Like clofibrate (*3*), these drugs have been reported to increase the number of hepatic peroxisomes (*4*).

Four groups of three male F-344 rats were fed ad lib with ground lab chow containing Wy-14,643 (1 g/kg of chow), tibric acid (1 g/kg), clofibrate (5 g/kg), or no drug (*5*). After 6 days, individual liver homogenates were prepared in 0.25*M* su-

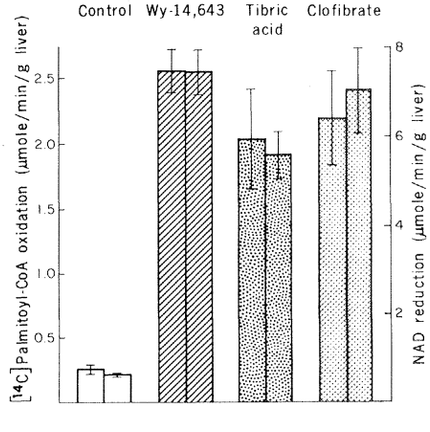

Fig. 1. Palmitoyl-CoA oxidation by liver homogenates of control and drug-treated rats. Assay of the rate of aerobic palmitoyl-CoA–dependent NAD reduction was performed as described (*2*) in the presence of 1 m*M* KCN to prevent reoxidation of the NADH formed (right bar of each pair and right axis). Assay of the oxidation of [1-^{14}C]palmitoyl-CoA was performed under similar conditions except KCN was omitted; the appearance of perchloric acid–soluble products was measured after 10 minutes of incubation at 37°C (left bar and axis). Rates (means and standard deviations) are expressed per gram of liver.