



FIG. 1. (A) HPLC of naphthol cleavage products from 9.5 mg of CHO protein. The column was eluted with acetonitrile at a flow of 1.5 ml/min. Detection was at 360 nm with a full-scale deflection of 0.01 A unit. The arrow indicates sample injection. In this chromatogram the farnesylcysteine derivative is eluted at 4.3 min and the geranylgeranyl derivative at 7.6 min. (B) HPLC of naphthol cleavage products from 2.4 mg of CHO protein. The column was eluted with a gradient of 95% acetonitrile/water to acetonitrile over 15 min. Detection was at 246 nm with a full-scale deflection of 0.04 A unit. The arrow indicates sample injection. In this chromatogram the farnesylcysteine derivative is eluted at 7.7 min and the geranylgeranyl derivative at 13.2 min. (Inset) Structure of methyl-naphthopyran. For the farnesyl derivative R is geranyl. For the geranylgeranyl derivative R is farnesyl. For the dimethylnaphthopyran R is CH₃.

potassium salt was added in dioxane (0.5 ml). Most dioxane was then removed with warming under a stream of nitrogen and the remainder, by gentle heating under reduced pressure. Dimethylformamide was then added and the sample was treated as described above.

Quantitation. Two methods were used for quantitation of the reaction. The first entailed the addition of a known amount of farnesylcysteine to one of a pair of samples. The amount of the two prenylcysteines present in the sample was calculated by comparison of the peak areas of the naphthopyrans obtained on elution from the column. These results were verified by a second method, in which the response of the detector was calibrated with synthetic dimethyl- and methylisohexylnaphthopyran. Isotope recovery of the naphthopyrans from CHO proteins in conjunction with detector response was then used for calculation of prenylcysteine present in the tissue sample. The two methods were in excellent agreement and indicated a recovery of 22–25% when dioxane was used to add the reagents. When the reagents were added dry, the yield was half. While we have no explanation for this variation, it was consistent and there was no significant difference among five samples of CHO proteins analyzed by the two methods of addition of reagent. All samples were in duplicate and the results were within the standard deviation found for CHO proteins.

Table 1. Prenylcysteine composition in tissue culture cells

Cell line	Prenylcysteine, nmol/mg protein	GGCys/FCys*
CHO (hamster)	0.79	6
3T3 (mouse)	0.83	6
HeLa (human)	0.4	4
Neuroblastoma (human)	0.8	8
Fibrosarcoma (mouse)	0.5	5

*Molar ratio of geranylgeranyl derivative to farnesylcysteine.

Table 2. Prenylcysteine composition in mouse tissues

Tissue	Prenylcysteine, nmol/mg protein	GGCys/FCys*
Liver	0.32	6
Supernatant	0.15	4
Kidney	0.75	8
Supernatant	0.23	7
Brain	1.3	10
Lung	0.64	5

*Molar ratio of geranylgeranyl derivative to farnesylcysteine.

RESULTS

Identification of the Product. When CHO proteins, metabolically labeled with [³H]mevalonate, were treated with 2-naphthol under alkaline conditions about 50% of the radioactivity was released as hexane-soluble material. Most of this product comigrated on HPLC with standard material prepared by allowing geranylgeranyl derivative to react with 2-naphthol under similar conditions. Although this compound is not the naphthyl ether anticipated for a simple S_N2 displacement (9), it is likely a 2-prenyl-substituted 2-methylnaphthopyran (Fig. 1 *Inset*).[‡]

Sensitivity of the Procedure. The experimental procedure was developed with solvent-extracted CHO cellular material. Hexane extracts of the products obtained by treating these proteins with 2-naphthol, when chromatographed on a C₁₈ reversed-phase column, gave the elution profiles shown in Fig. 1. The eluate was monitored at 246 and 360 nm, absorption maxima of the product. Fig. 1A represents an isocratic elution (360 nm), while Fig. 1B is a gradient elution (246 nm). The signal-to-noise ratio found will permit detection of less than 1 pmol of product at 246 nm. In all, 15 different samples of CHO proteins were analyzed, with and without the addition of dioxane. We found 0.79 ± 0.13 nmol of prenyl residue per mg of total cellular protein, which indicates that the method is reproducible. The molar ratio of geranylgeranyl- to farnesylcysteine was 6. The comigration of products as detected by UV absorption and radioactivity has been verified by cochromatography in both reversed-phase and normal-phase systems.

Analysis of Tissues. Samples of other tissue culture cells were examined and found to have a similar content of prenylcysteines. The data are given in Table 1. Table 2 gives the results of the analysis of selected mammalian tissues. The prenyl composition varies significantly from tissue to tissue, as does the ratio of the two different prenyl entities. Sedimentation of membranes by high-speed centrifugation did not remove all of the prenylated proteins, and the cytosolic fraction of both kidney and liver contain substantial quantities of prenylcysteines. The ratio of the two prenylcysteines is the same as is found for whole tissue.

The results from analyses of nonmammalian organisms are shown in Table 3. The prenyl composition is significantly lower and the proportion of the sesquiterpene amino acid is greater. Prenylcysteines were not found in *E. coli*. This determination was on 16 mg of protein and 5 pmol/mg would have been detected.

DISCUSSION

The nucleophilic cleavage of the allylic thiol bond by 2-naphthol provides for qualitative as well as semiquantitative determination of the cysteine-bound prenyl groups of prenyl-

[‡]The basic naphthopyran structure is consistent with IR, UV, NMR, and mass spectral data, a full account of which will be published elsewhere. Although some structures are still tentative, the assignments do not compromise the present work.

Table 3. Prenylcysteine composition in other organisms

Organism	Prenylcysteine, nmol/mg protein	GGCys/FCys*
<i>Escherichia coli</i>	<0.005	—
<i>Methanobacterium</i> <i>thermoautotrophicum</i>	0.04	†
<i>Saccharomyces cerevisiae</i> a factor strain	0.05	3
<i>Phycomyces</i>	0.22	0.7
<i>Peletia fastigiata</i> brown algae	0.06	3
<i>Spinacia oleracea</i>	0.06	‡
<i>Manduca sexta</i> Larvae	0.14	‡
Eggs	0.14	3
		4

*Molar ratio of geranylgeranyl cysteine to farnesyl cysteine.

†This sample had no detectable geranylgeranyl cysteine.

‡These samples had only traces of farnesyl cysteine.

lated proteins. The method has a number of distinct advantages. The naphthylpyrans that are formed are readily isolated and quantified by HPLC. The tissue to be analyzed does not need to be labeled with mevalonate, and this has made it possible to examine organisms, such as bacteria, fungi, and plants, that do not incorporate this metabolite readily. Also, for metabolic labeling, tissue culture cells are frequently treated with inhibitors of mevalonate synthesis, so the observed product distribution may be skewed by this disturbance of isoprenoid synthesis. Another aspect of this method is that the naphthopyrans produced have a UV absorption spectrum that permits verification of the identity of products. This attribute may well be useful in situations where unusual prenylations could occur. For example, insects synthesize homosesquiterpenoids as hormones and similarly modified prenylcysteines would be characterized by altered retention times as well as by absorption spectra. The extinction coefficients of the naphthopyrans are 4450 and 66,070 M⁻¹cm⁻¹ at 360 and 246 nm, respectively.[§] These values are large enough for reasonably sensitive analysis. At 360 nm the maximum sensitivity is about 10 pmol of naphthopyran and at 246 nm the sensitivity is 10-fold greater.

Naphthol cleavage of prenylcysteines has enabled us to examine different tissues for the presence of this modification and to extend the range of organisms in which it is known to occur. Our results clearly indicate that this modification of proteins is very nearly universal. The relative level of modified cysteine is lower in plants and insects than in mammals and lower yet in an archaeobacterium. Prenylcysteines were not found in *E. coli* at a sensitivity that would have detected 5 pmol of prenyl residue per mg of protein. Thus, proteins so modified may not occur in all prokaryotic organisms. The tissue distribution of prenylated proteins that we have found parallels the enzymatic activity reported for protein farnesyltransferase as reported by Manne *et al.* (14). They detected this enzyme in all extracts of mammalian tissue tested but failed to find activity in extracts of *E. coli*.

The subcellular distribution of prenylated proteins in the two tissues that were examined indicates that one-third to one-half of these proteins are in the cytosolic pool. This observation is in agreement with that of Maltese and Sheridan (15), who found significant levels of cytosolic prenylated proteins. While one function of prenylation is to direct cytosolic proteins to membranes, it is apparent that this

modification does not quantitatively partition proteins into membrane compartments.

Our data permit a quantitative comparison of protein prenylation to the other end products of isoprenoid metabolism normally present. Cholesterol, the most abundant polyprenol of animals, occurs in kidney and liver in the range of 40–90 nmol/mg of protein (16, 17). The concentration of ubiquinone in liver is about 0.3 nmol/mg of protein and that of the dolichols is about half this value (17). The levels of prenylated cysteines are in the range of 0.3–1.5 nmol/mg of protein, indicating that these products of mevalonate metabolism are present at levels quantitatively similar to those of the nonsterol lipid products of this pathway.

Prenylation is a surprisingly frequent modification of proteins. Assuming an average molecular weight for proteins (single polypeptide) of 25,000, there would be 40 nmol of protein in each mg of cellular protein. Our finding of about 0.7 nmol of prenylcysteine per mg of protein indicates that about 2% of all proteins are prenylated. Cysteine is one of the less common amino acids, representing approximately 2 mol % of the amino acids of total cellular proteins. One would then anticipate 1 nmol of carboxyl-terminal cysteine in each mg of cellular protein. A search (18) of the amino acid sequences in the Protein Sequencing Library of the National Biomedical Research Foundation (March 31, 1988) indicated that cysteine occurs randomly at this terminus, demonstrating that this is a reasonable assumption. Our results thus indicate that proteins with a carboxyl-terminal cysteine are likely to be prenylated.

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[§]These values were obtained from synthetic 2,2-substituted naphthopyrans (Fig. 1 *Inset*, R = methyl and R = isohexenyl) and literature values for the parent 3H-naphtho[2,1-b]pyran (9), all of which have nearly identical UV spectra.