FIGURE 5.

et al. (1981). If the experimental finding of Sutoh is confirmed, Figure 4B in the main text should be replaced by Figure 5, which takes into account the fact that, on dehydration, S1 attaches to two actin globules (Amos et al., 1982).

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Formation of a Supramolecular Complex Is Involved in the Reconstitution of Basement Membrane Components†

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ABSTRACT: Basement membrane macromolecules, including type IV collagen, laminin, and heparan sulfate proteoglycan, do not aggregate when incubated alone. Rather, precipitation occurs in the presence of equimolar amounts of laminin and type IV collagen but variable amounts of heparan sulfate proteoglycan. This interaction requires native laminin and type IV collagen. Heparan sulfate proteoglycan increases the precipitation of laminin particularly in the presence of type IV collagen. Fibronectin does not cause type IV collagen to precipitate. These studies show that the components of basement membrane interact in a highly specific manner and suggest that such interactions may be involved in the deposition of basement membrane in situ.

Basement membranes are thin extracellular matrices which support epithelial and endothelial cells and separate them from the underlying stroma. Progress has been made in defining the structure of basement membrane and its components (Kefalides, 1973; Heathcote & Grant, 1981; Timpl & Martin, 1982). All basement membranes contain type IV collagen (Kefalides, 1973; Yaoita et al., 1978; Timpl et al., 1978); laminin (Timpl et al., 1979; Chung et al., 1979; Foidart et al., 1980), and a heparan sulfate proteoglycan (Hassell et al., 1980) which are unique to basement membranes. Type IV collagen (M_r 540 000) is incorporated directly into the matrix without enzmatic processing to a less soluble form as observed with other collagen types (Minor et al., 1976; Heathcote et al., 1978; Dehm & Kefalides, 1978; Tryggvason et al., 1980;

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Karakashian et al., 1982; Kleinman et al., 1982a). Electron microscopic studies indicate that type IV collagen does not form fibrillar structures in basement membranes but is present in a fine, feltlike meshwork (Leivo et al., 1982). It has been suggested that this meshwork is formed by a continuous network of type IV collagen molecules joined with like ends in apposition (Kühn et al., 1981; Timpl et al., 1981). These interactions are stabilized by lysine-derived cross-links as well as by disulfide bonds (Tanzer & Kefalides, 1973; Karakashian et al., 1982; Kleinman et al., 1982a).

Laminin is a large glycoprotein (M_r 10^6) localized exclusively to basement membranes (Timpl et al., 1979; Chung et al., 1979). It binds to type IV collagen, to heparan sulfate proteoglycan, and to the surface of epithelial and endothelial cells (Terranova et al., 1980, 1983; Kleinman et al., 1982b; Woodley et al., 1983) and possibly of fibroblasts (Couchman et al., 1983). Heparan sulfate proteoglycan (M_r 10^6) is also a component of basement membranes (Kanwar & Farquhar, 1979; Hassell et al., 1980; Kanwar et al., 1981), and it binds to type IV collagen, laminin, and fibronectin (Woodley et al., 1983). Fibronectin (M_r 440 000) is found in serum, in fibrous connective tissues, and in newly deposited basement membranes where it has been implicated in their formation (Mosher, 1980; Ruoslahti et al., 1981; Brownell et al., 1981).

Some studies have been conducted on the reconstitution of type IV collagen extracted from basement membranes (Schwartz & Veis, 1979, 1980; Veis & Schwartz, 1981). For example, Veis & Schwartz (1981) found that type IV collagen obtained by acid extraction of lens underwent self-aggregation and formed ordered structures. However, acid-extracted type IV collagen has smaller chains than those present in the protein in the basement membrane, possibly the result of some proteolytic degradation (Minor et al., 1976; Orkin et al., 1977; Karakashian et al., 1982; Kleinman et al., 1982a). Other studies suggest that type IV procollagen molecules assemble in solution into disulfied-linked aggregates similar to those present in the matrix (Fessler & Fessler, 1982; Bächinger et al., 1982).

Since type IV collagen is secreted in soluble form and does not undergo conversion to a less soluble form (Minor et al., 1976; Heathcote et al., 1978; Dehm & Kefalides, 1978; Crouch & Bornstein, 1979; Tryggvason et al., 1980; Karakashian et al., 1982; Kleinman et al., 1982a), the interaction of type IV collagen with other components of basement membrane may be required for its deposition and stabilization. Here, we studied the precipitation of various purified basement membrane components from solution either by themselves or in combination. These studies suggest that the binding of type IV collagen to laminin may cause its deposition in basement membrane and that the heparan sulfate proteoglycan regulates the amount of laminin that is incorporated into the matrix.

Experimental Procedures

Materials. Type IV collagen, laminin, and heparan sulfate proteolgycan were prepared from EHS tumor (Timpl et al., 1979; Hassell et al., 1980; Kleinman et al., 1982a). After the tumor tissue from lathyritic rats was washed with 0.5 M NaCl in 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, and then with 2.0 M guanidine in 0.05 M Tris-HCl, pH 7.4, type IV collagen was extracted with 2.0 M guanidine in 0.05 M Tris-HCl, pH 7.4, containing 0.005 M dithiothreitol. The collagen was further purified by DEAE-cellulose column chromatography (Kleinman et al., 1982a). The type IV collagen prepared in this manner was essentially homogeneous as judged by sodium dodecyl sulfate (SDS) gel electrophoresis and by amino acid analysis, and the protein contained two chains, pro α1 (IV) and pro α2 (IV) of 185 000 and 170 000 daltons, respectively. The protein prepared in this manner had a native structure based on its appearance in the electron microscope after rotary shadowing and by its resistance to pepsin (Kleinman et al., 1982a). Although the collagen prepared in this manner is as yet indistinguishable from the biosynthetic product, it will be referred to here as type IV collagen since it is the tissue form. Laminin was isolated from the 0.5 M NaCl extract as previously described (Timpl et al., 1979). Heparan sulfate proteoglycan was purified from 4.0 M guanidine extracts of the tumor by cesium chloride density centrifugation followed by ion-exchange and molecular sieve column chromatography (Hassell et al., 1980).

Fibronectin was prepared from freshly drawn human serum by affinity chromatography on a gelatin–Sepharose column (Hopper et al., 1976; Engvall & Ruoslahti, 1977) followed by chromatography on a heparin–Sepharose column (Sakashita et al., 1980). Its purity was confirmed by SDS gel electrophoresis.

Precipitation Assays. In general, 50–125 μg of collagen type IV in 0.5 M acetic acid was neutralized by the addition of concentrated buffer and salt to a final concentration of 0.02 M phosphate, pH 7.4, containing 0.15 M NaCl (PBS), and concentrated NaOH was added until the pH was 7.4. This solution was then centrifuged for 20 min at 7000 rpm to remove insoluble material. The amount of collagen remaining in the supernatant fraction and actually used in the experiment was quantitated after dialyses and lyophilization by weighing. Laminin, fibronectin, or heparan sulfate proteoglycan dissolved in PBS was centrifuged and either added alone or added to collagen and proteoglycan as indicated to a final volume of 1.0 mL. These samples were incubated at various temperatures in either a water bath or a Gilford spectrophotometer with the temperature of the cuvettes maintained by a circulating water bath. In general, samples were incubated at 35 °C. The precipitation of material was followed spectrophotometrically by the increase in the turbidity of the solution with time at 313 nm (Gross & Kirk, 1958) or by sedimentation of precipitated material followed by electrophoresis (Kleinman et al., 1981). Here, after 3 h, the samples were centrifuged at 10 000 rpm for 20 min. The supernatant solutions were decanted, and the pellets were dissolved in sample buffer and electrophoresed in 5% acrylamide under reducing conditions (Laemmli, 1970). Each experiment was carried out a minimum of three times. In all cases where percent is used to present the data, the amount of material precipitated was related to the total amount of material present in the sample. For the calculations, negatives of photographs of the gels were scanned in a Helena densitometer for quantitation of the protein bands. The 200K band of laminin and the 185K band of type IV collagen were used for quantitating these two proteins.

Results

The extent of precipitation of type IV collagen was studied in the presence and absence of laminin, fibronectin, and heparan sulfate proteoglycan. When incubated alone at physiological pH, ionic strength, and temperature, little type IV collagen precipitated as measured by turbidity (Figure 1) or by analysis of the protein in the precipitate by electrophoresis (Figure 2, lane 1). Some laminin did precipitate when it was incubated alone (Figure 1 and Figure 2, lane 3). The amount of laminin that precipitated by itself varied somewhat between different preparations but generally was between 5 and 10% of the total laminin present. However, when both laminin and

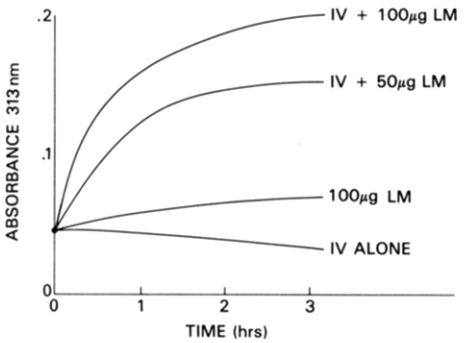

FIGURE 1: Effect of laminin on the rate of precipitation of type IV collagen. Laminin (LM) (100 μg) and type IV collagen (50 μg) were incubated alone or together at 35 °C, and their precipitation was monitored at 313 nm. An increase in absorbance to 0.200 represented the precipitation of over 80% of the protein from solution as determined by electrophoresis of aliquots of the solution and precipitate.

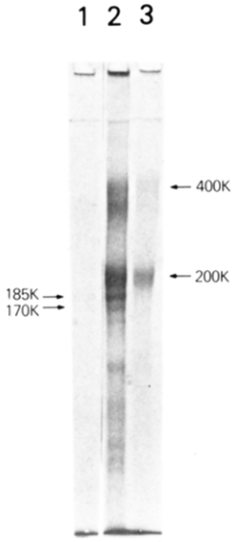

FIGURE 2: Effect of type IV collagen on laminin precipitation. Type IV collagen (50 μg) (lane 1) and laminin (100 μg) (lane 3) were incubated alone as well as together (lane 2), and the aggregated material was collected by centrifugation and electrophoresed after reduction of disulfide bonds. The 200 000- and 400 000-dalton chains of laminin are indicated by arrows as are the 185 000- and 170 000-dalton chains of type IV collagen. Some breakdown products of type IV collagen are more apparent in lane 2 just beneath the 170 000-dalton chain. Also, some contaminants in the laminin preparation (i.e., entactin) are present in lane 2 at ~150 000 daltons and below.

type IV collagen were incubated together, there was a marked increase in material precipitating as detected by an increase in the turbidity of the sample (Figure 1) and by electrophoretic analysis of the material in the precipitate (Figure 2, lane 2, Figure 3, and Table I). Laminin stimulated type IV collagen precipitation in a concentration-dependent manner with maximal precipitation of 50 μg of type IV collagen occurring in the presence of 100 μg of laminin (Figure 3). Type IV collagen also stimulated the precipitation of laminin (Figure 2 and Table I). Type IV collagen did not precipitate in the presence of either low or high concentrations of fibronectin as assessed by changes in the turbidity of the sample (data not shown) or by analysis of precipitates by electrophoresis (Figure 4).

The interaction of type IV collagen and laminin appears to be specific. First, the interaction proceeds better at 35 °C than at 4 or 24 °C (Figure 5). In the presence of laminin, the amount of type IV collagen precipitating is temperature dependent with maximal precipitation occurring at 35 °C (Figure

Table I: Requirement for Native Molecules of Laminin and Type IV Collagen for Precipitation[a]

	% type IV collagen precipitated
(A) type IV	9
type IV boiled	7
type IV + laminin	81
type IV boiled + laminin	20
type IV + laminin boiled	7
type IV boiled + laminin boiled	9
	% laminin precipitated
(B) laminin	4
laminin boiled	2
laminin + type IV	60
laminin + type IV boiled	27
laminin boiled + type IV	7
laminin boiled + type IV boiled	5

[a] Type IV collagen (125 μg) was incubated with laminin (150 μg) in a final volume of 1.0 mL for 3 h at 35 °C. The samples were then centrifuged, and the amount of laminin and type IV collagen in the precipitate was quantitated after SDS gel electrophoresis.

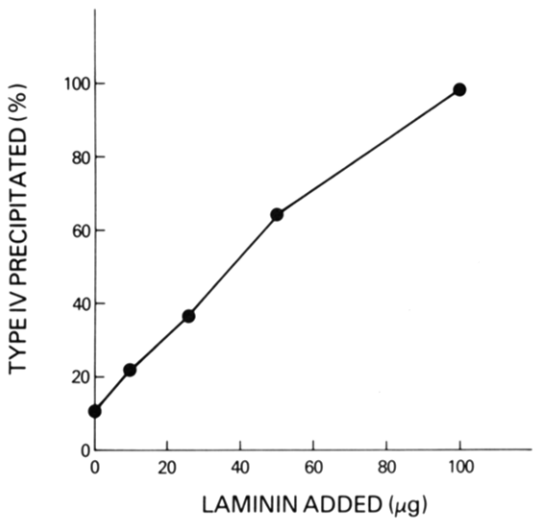

FIGURE 3: Effect of laminin on the extent of type IV collagen precipitation. Type IV collagen (50 μg) and various amounts of laminin were incubated for 3 h at 35 °C and then centrifuged. The pellets were dissolved and electrophoresed. Negatives of the photographs of each lane were scanned with a Helena densitometer, and the amount of type IV collagen precipitated was determined by measuring the areas under the peaks.

5). Little type IV collagen precipitates in the absence of laminin, at any temperature tested (4–35 °C). In the presence of type IV collagen, the amount of laminin precipitating is increased substantially, and the formation of the precipitate occurs at physiological temperature (Figure 5). Little precipitation of laminin occurs when the purified protein is incubated alone (<13% at 35 °C). In the presence of type IV collagen, however, the amount of laminin precipitating is increased with maximal precipitation at 35 °C. The native forms of laminin and type IV collagen are required for maximal interaction (Table I). When laminin is denatured by boiling for 5 min prior to incubation with type IV collagen, it does not enhance type IV collagen precipitation (Table I). Similarly, when type IV collagen is boiled for 5 min prior to incubation with laminin, the amount of type IV collagen precipitated is reduced (Table IA). In the latter case, residual activity could be due to some renaturation of the native col-

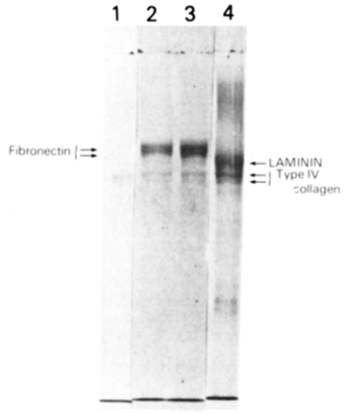

FIGURE 4: Effect of fibronectin on the precipitation of type IV collagen. Type IV collagen (50 μg) was incubated either in buffer alone (lane 1) or with 50 μg of fibronectin (lane 2), 100 μg of fibronectin (lane 3), or 50 μg of laminin (lane 4) as a control as described in Figures 2 and 3, and the pellets were dissolved and electrophoresed in SDS gels. Some minor contaminants present in the laminin preparation are visible in lane 4 near the bottom. Their nature is unknown.

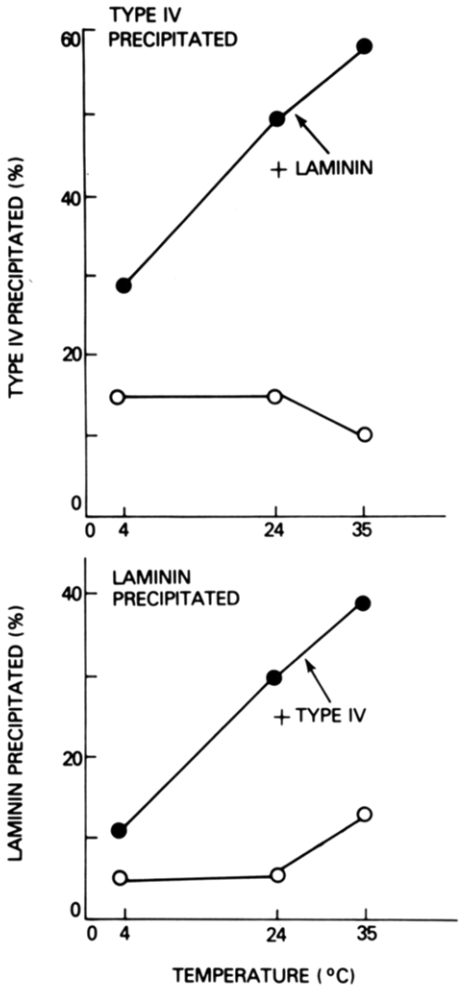

FIGURE 5: Effect of temperature on the amount of laminin and type IV collagen precipitated when incubated alone or in combinations. Type IV collagen (100 μg) or laminin (100 μg) were incubated either alone or together at 4, 24, and 35 °C for 2 h. The samples were then treated as described under Figure 3.

lagen structure, since the chains of type IV collagen are held together by disulfide bonds. The amount of laminin precipitating in the presence of denatured type IV collagen is slightly elevated over that seen when laminin is incubated alone but is not as high as that seen with native type IV collagen. Little or no precipitate formed when denatured laminin and type IV collagen were incubated together. Thus, native laminin and type IV collagen are required for these proteins to interact. In addition, type I collagen had no effect on the precipitation of laminin, confirming the specificity of the interaction. This is expected since laminin does not bind to type I collagen (Terranova et al., 1980; Kleinman et al., 1982b).

Table II: Effect of Heparan Sulfate Proteoglycan on Laminin and Type IV Collagen Precipitation[a]

	% type IV collagen precipitated
(A) type IV	7
type IV + laminin	61
type IV + heparan sulfate proteoglycan	7
type IV + heparan sulfate proteoglycan + laminin	100
	% laminin precipitated
(B) laminin	9
laminin + type IV	31
laminin + heparan sulfate proteoglycan	80
laminin + heparan sulfate proteoglycan + type IV	98

[a] Type IV collagen (50 μg), laminin (35 μg), and heparan sulfate proteoglycan (5 μg) were incubated in the combinations described above and processed as described under Table I. This amount of laminin does not allow complete precipitation of type IV collagen.

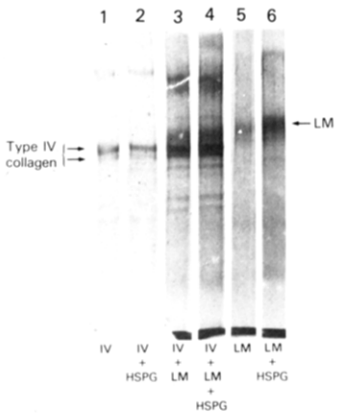

FIGURE 6: Effect of heparan sulfate proteoglycan (HSPG) on type IV collagen (IV) and laminin (LM) precipitation. Type IV collagen (50 μg) was incubated either in buffer alone (lane 1) or with 5 μg of heparan sulfate proteoglycan (lane 2), 35 μg of laminin (lane 3), or 35 μg of laminin plus 5 μg of heparan sulfate proteoglycan (lane 4). Laminin (35 μg) was incubated in either buffer alone (lane 5) or with 5 μg of heparan sulfate proteoglycan (lane 6). All assays were carried out as described in Figures 2 and 3. The additional lower molecular weight bands visible in lanes 3 and 4 originate from the type IV preparation and are breakdown products.

We next tested the effect of the heparan sulfate proteoglycan on type IV collagen and laminin precipitation. Although the heparan sulfate proteoglycan binds to type IV collagen (Woodley et al., 1983), heparan sulfate proteoglycan over a range of 1–100 μg had no effect on type IV collagen precipitation (Figure 6, lanes 1 and 2, and Table IIA). The precipitation of laminin was stimulated 9-fold by heparan sulfate proteoglycan (Figure 6, lanes 5 and 6, and Table IIB) and by heparin (data not shown), both of which bind to laminin (Sakashita et al., 1980; Woodley et al., 1983). Maximal precipitation of laminin (35–50 μg) occurred at 5–10 μg of heparan sulfate proteoglycan, with higher concentrations of proteoglycan resulting in a reduction in the amount of laminin precipitated (data not shown). When heparan sulfate pro-

teoglycan was incubated with laminin and type IV collagen, it increased the amounts of both laminin and type IV collagen which precipitated (Figure 6, lanes 3 and 4, and Table IIA,B). The effect of heparan sulfate proteoglycan on laminin and type IV collagen precipitation was most apparent when suboptimal levels of laminin were used. These data demonstrate that low concentrations of heparan sulfate proteoglycan can stimulate laminin precipitation and that heparan sulfate proteoglycan may have a key role in regulating the proportions of material deposited in the tissue.

Discussion

We have studied the interaction of purified macromolecules isolated from basement membrane by following their coprecipitation. Type IV collagen extracted from the EHS tumor with 2 M guanidine did not precipitate under physiological conditions. However, incubation of type IV collagen with laminin caused both proteins to coprecipitate. Precipitation was maximal at physiological temperatures and exhibited a linear relationship between the amount of laminin present and the amount of type IV collagen precipitated. Based on the ratios of laminin and type IV collagen in the precipitates, it appears that approximately 1 mol of laminin precipitates 1 mol of type IV collagen. Further, it was shown that the interaction between type IV collagen and laminin only occurred when both proteins were native. Heat denaturation of either protein largely abolished their precipitation on subsequent incubation. Previous studies have shown that laminin binds to native but not to denatured type IV collagen (Kleinman et al., 1982b; Woodley et al., 1983) and that the activity of laminin in supporting epithelial attachment was destroyed by boiling (Terranova et al, 1983).

Fibronectin and heparan sulfate proteoglycan, which are also components of basement membranes, do not alter the amount of type IV collagen precipitating from solution. However, heparan sulfate proteoglycan causes laminin to precipitate from solution and increases the coprecipitation of laminin and type IV collagen. Based on the stoichiometry observed here (i.e., 3–10 mol of laminin per mol of heparan sulfate proteoglycan) and the greater amounts of laminin present in the matrix (10 mol of laminin per mol of heparan sulfate proteoglycan; Kleinman et al., 1982a), it is likely that several laminin molecules can bind to heparan sulfate chains in the proteoglycan. Such interactions would result in the formation of a very large macromolecular complex. The amount of heparan sulfate proteoglycan present may regulate the laminin content of basement membranes and vice versa.

The interaction of laminin with type IV collagen and heparan sulfate proteoglycan appears highly specific. The relative amounts of each component in the complex formed are comparable to the amounts found in the EHS tumor (Kleinman et al., 1982a,b). However, the EHS tumor which contains laminin, type IV collagen, and heparan sulfate proteoglycan in the molar ratio 1:1:0.1 may not be representative of normal basement membranes. Similar quantitative studies have not been carried out in other tissues. However, it appears that laminin and type IV collagen are the major components secreted by cultured epithelial cells (Chung et al., 1979; Cooper et al., 1981; Kuhl et al., 1982), suggesing that authentic basement membranes may also contain these components in quantity.

Laminin has several known functions. Of the characterized components of basement membrane, laminin appears first in development (Ekblom et al., 1980). Further, when added to cultured thyroid cells, it initiates the formation of basement membrane (Garbi & Wollman, 1982). In addition to these observations, its role in cell attachment to the basement membrane (Terranova et al., 1980) and the interactions of laminin with type IV collagen and heparan sulfate proteoglycan shown here suggest a key role for laminin in the organization and structure of basement membranes.

It is likely that the interactions studied here are not the sole factors determining the structure of basement membranes as the precipitate which formed here was particulate and not membranous. The importance of other interactions, such as the formation of intermolecular cross-links and disulfide bridges in type IV collagen, was not addressed here. In addition, other components of the basement membrane could play an important role in determining the form and function of basement membranes.

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Natural Abundance Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Liver and Adipose Tissue of the Living Rat†

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ABSTRACT: We have employed the topical magnetic resonance (TMR) technique to obtain natural abundance ^{13}C nuclear magnetic resonance (NMR) spectra from liver and adipose tissue in the living rat. Experiments were performed in a TMR magnet (20-cm diameter) with a two-turn radio-frequency coil ("surface" coil) combined with a focused static magnetic field. The in vivo spectra that were obtained at 20.2 MHz have been assigned by comparison with those from excised organs obtained in a conventional spectrometer operating at 90.5 MHz. Signals in the TMR spectra corresponding to carbons of the carbohydrates, glucose and glycogen, and of the lipids, triglycerides and phospholipids, have been resolved in vivo and assigned. The effects of chronic modification of dietary fat and carbohydrate on the in vivo spectra have been investigated. The levels of carbohydrates and of saturated and unsaturated fats in the liver as measured by ^{13}C TMR reflect the relative amounts of these compounds in the long-term diet.

High-resolution nuclear magnetic resonance (NMR)[1] spectroscopy is presently making significant contributions in physiology, biochemistry, and clinical diagnosis due to its ability to measure noninvasively the concentrations and the dynamics of metabolites in living tissue (Shulman et al., 1979; Edwards et al., 1982; Gordon et al., 1982; Gadian et al., 1979; Ugurbil et al., 1979; Scott et al., 1981; Ross et al., 1982). Many reports have shown that ^{31}P NMR can be used to assay the energetic status of tissue by measuring the concentrations of phosphocreatine, inorganic phosphate, and nucleoside triphosphates. The potential usefulness of ^{13}C NMR has not been explored as extensively partly because the ^{13}C nucleus is only 1% abundant in nature whereas ^{31}P is 100%. However in a number of previous reports (Shulman et al., 1979; Ugurbil et al., 1979; Cohen et al., 1979, 1980, 1981a,b; den Hollander et al., 1981), we have shown that the low natural abundance, in fact, facilitates the ability to observe specific metabolic pathways by using ^{13}C-enriched substrates. Certain molecules have very high intracellular concentrations. For some of these molecules, it has been shown that detectable ^{13}C NMR signals can be obtained without isotopic enrichment (Norton, 1981). For instance, trehalose stored in encysted *Acanthamoeba castellanii* (Deslauriers et al., 1980) and in yeast spores (Barton et al., 1982) has been observed in natural abundance by ^{13}C NMR. Triglycerides and phospholipids in a number

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[1] Abbreviations: NMR, nuclear magnetic resonance; TMR, topical magnetic resonance.

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