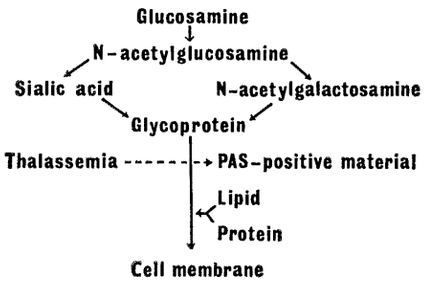

Fig. 2. The biosynthetic pathway of the carbohydrate of the cellular membrane and the proposed block in thalassemia.

the stroma is very likely on the basis of results (*18*) which show an elevation (50 to 80 percent) in the sialic acid content of stroma isolated from the thalassemic erythrocytes. Neither the PAS-positive material nor the radioactivity is identifiable with glycogen, since the former is not destroyed by salivary amylase, and the latter is not in the fraction soluble in trichloroacetic acid. Sometimes PAS-positive material is found in erythroblasts in other anemic conditions.

The reticulocytes of curves *A*, *B*, and *C* (Fig. 1) show an interesting variation that can be correlated with the severity of the thalassemia major. The most severe case clinically, a 2-year-old boy with 95 percent hemoglobin F, is illustrated in curve *A*. The donors of cells represented by curves *B* and *C* had less severe clinical symptoms and had 91 and 85 percent of hemoglobin F, respectively. Curve *A* shows the greatest incorporation of glucosamine; curves *B* and *C* show roughly 40 to 60 percent as much at 1 hour. Each of these curves, however, shows incorporation of much more glucosamine than do curves *D* and *E*.

Thus thalassemia may express itself as a function of several biochemical disorders. In particular, synthesis of the glycoprotein of the cellular membrane, or rather the PAS-positive material, is greatly elevated. Possibly a region of a chromosome is damaged, resulting in the malfunction of several genes associated with hemoglobin, ferritin, and glycoprotein biosynthesis. However, a primary genetic defect in only one of these components may produce abnormal behavior of the other components.

A disturbance in enzyme production along the pathway of membrane synthesis, at the point of association of glycoprotein with lipid and protein, could account for the observed accumulation of PAS-positive material (glycoprotein) in thalassemia (Fig. 2). A block, or shunt, at this point would retard membrane synthesis in general. Thus the regulatory mechanisms would be strongly geared to favor synthesis and thereby the incorporation of glucosamine. In theory the clinical symptoms are understandable in the light of impaired synthesis in the membrane, but, since the cell membrane is a macromolecular, heterogeneous structure composed of carbohydrate, lipid, and protein, it is difficult to evaluate the biosynthesis of any component in relation to the whole without additional data. Knowledge of the chemistry and structure of each component, and of how they are assembled to form a complex unit capable of specific biological activity, is almost totally lacking. More detailed characterization of this specific lesion in thalassemia and its relative importance must therefore await further biochemical studies of the membrane.

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Paraffinic Hydrocarbons in Pasture Plants

Abstract. *The gas chromatographic–mass spectrometric technique recently developed by Ryhage has been applied to the analysis of paraffins extracted from pasture plants, specifically, whole plant and leaves of spotted bur clover* (Medicago arabica). *Normal alkanes from* C_{18} *to* C_{35} *have been found. The* C_{29}, C_{31}, *and* C_{33} *normal saturated hydrocarbons predominate and* n-C_{31} *is the major component. In the range from* C_{24} *to* C_{31} *the ratio of alkanes with an even number of carbon atoms to those with an odd number is approximately 8 for the whole plant and 5 for the leaves. The distribution of paraffins is similar to that reported for cattle manure and also resembles that of some soils and sediments.*

It has been shown (*1*) by a combined gas chromatographic–mass spectrometric technique (*2*) that certain animal excretion products, such as cattle manure, contain a number of paraffinic hydrocarbons, among which *n*-nonacosane ($C_{29}H_{60}$), *n*-hentriacontane ($C_{31}H_{64}$), and *n*-tritriacontane ($C_{33}H_{68}$) predominate.

In an attempt to elucidate the origin of these hydrocarbons we have considered the following three possibilities, that is, the paraffins may be: (i) end products of bovine metabolism, (ii) metabolic end products of the bacteria living in the bovine digestive tract, and (iii) compounds originally present in the pasture plants eaten by the cattle. Since a number of plants are known to produce waxes which contain paraffins (*3*) the third of these possibilities appeared more likely and was investigated first. Spotted bur clover (*Medicago arabica*) was chosen as the plant to analyze because it constituted the bulk of the cattle's diet at the time our other investigation (*1*) was carried out.

Freshly collected spotted bur clover was allowed to dry at room temperature in the shade for a period of 1 week or longer. Two samples of 2 g each (the first one including the whole plant and the second, leaves only) were each extracted with a benzene-methanol mixture (3 : 1), and the extract was fractionated on a silica gel column into four fractions (*1*). The approximate weights, per gram of extracted material, obtained for each of the four fractions of the second sam-

ple (leaves) were: *n*-heptane (second passing), 1.7 mg; carbon tetrachloride, 1 mg; benzene, 4 mg; and methanol, 46 mg.

We analyzed only the *n*-heptane eluates which contain the paraffinic hydrocarbons. These eluates had a green color, which was presumably due to chlorophyll extracted from the plants. The chlorophyll was completely removed by placing the residue of the *n*-heptane eluate on top of a new silica gel column and eluting the column again with the same volume of *n*-heptane. The removal of this pigment was carried out in order to measure accurately the net weight of the fraction, but the presence of chlorophyll does not appear to interfere with the gas chromatographic analysis of the hydrocarbon in this fraction.

The residues from the *n*-heptane eluates were dissolved in 50 μl of benzene, and a 5-μl sample of this solution was analyzed by gas chromatography as described (*1*). Figure 1 illustrates two chromatographic separations performed under these conditions. Chromatogram 1*A* was obtained from the residue of the initial *n*-heptane eluate (chlorophyll present) and chromatogram 1*B*, from the residue of the *n*-heptane eluate after it had been passed a second time through the silica gel column (chlorophyll removed). No essential difference concerning alkane composition can be observed between the two chromatograms, with the possible exception of a small variation of the C_{18} and C_{19} peaks.

Paraffins from C_{18} or C_{19} to C_{35} can be observed in these chromatograms. It can also be seen that the paraffins with an odd number of carbon atoms (C-odd paraffins) are several times more abundant than the alkanes with an even number of carbon atoms (C-even alkanes). In the range from C_{24} to C_{34} the average ratio of peak heights (p.h.) was found to be approximately 8 for the first sample (whole plant) and approximately 5 for the second sample (leaves). By extrapolating the incomplete peaks or bands of chromatograms 1*A* and 1*B* the following ratios were obtained:

$$1A,\ r=\frac{\Sigma \text{ p.h. odd } C_{25}\text{–}C_{33}}{\Sigma \text{ p.h. even } C_{24}\text{–}C_{32}}=5.3$$

and

$$1B,\ r=\frac{\Sigma \text{ p.h. odd } C_{25}\text{–}C_{33}}{\Sigma \text{ p.h. even } C_{24}\text{–}C_{32}}=4.9 \quad (4).$$

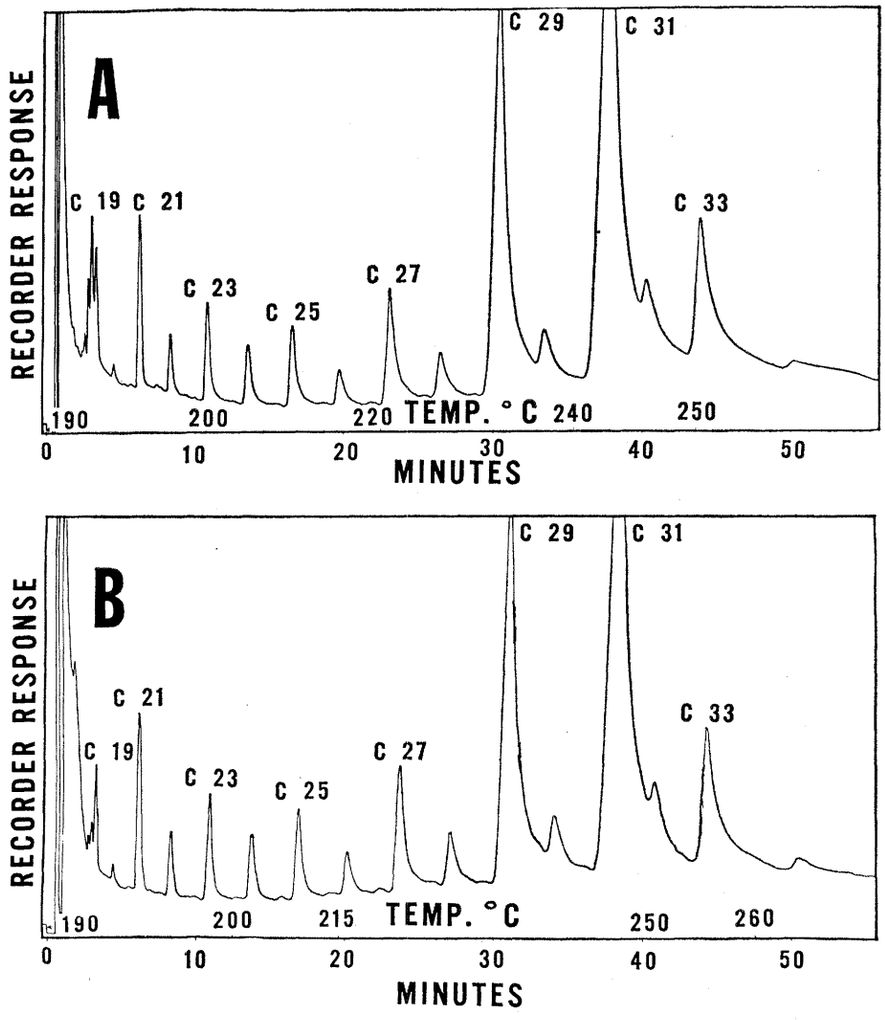

Fig. 1. Chromatographic separation of normal paraffins from spotted bur clover (*Medicago arabica*). Glass column, 1.8 m × 4 mm, containing 0.6 percent thermally stripped silicone (SE-30) and 0.2 percent cyclohexanedimethanol succinate on 80-100 mesh, acid-washed silanized Gas Chrom P. Helium pressure, 1034 mm-Hg.

The three major bands were found to correspond to *n*-nonacosane, *n*-hentriacontane, and *n*-tritriacontane. This identification was made by gas chromatography and was confirmed, according to the method of Ryhage (*1*, *2*), by direct mass spectrometry of the individual bands as they emerged from the gas chromatographic column.

A clear similarity is apparent when the results obtained from spotted bur clover leaves are compared with those obtained from cattle manure (*1*). In both cases we have observed (i) *n*-paraffins mainly in the C_{19}–C_{35} range, (ii) a value of approximately 5 for the ratio of C-odd to C-even alkanes in the C_{24}–C_{34} range, (iii) a predominance of the C_{29}, C_{31}, and C_{33} normal alkanes, and (iv) a major predominance of *n*-hentriacontane. On the basis of these results we conclude that the hydrocarbons with relatively high molecular weights found in cattle manure are derived mainly from the pasture plants which constitute the bulk of the cattle's diet. The leaves of spotted bur clover gave a better correlation (as compared to the whole plant) because the cattle were grazing mainly on the upper parts of this plant which consist essentially of leaves. The relatively larger abundance of *n*-tritriacontane in cattle manure can be attributed to grasses or other pasture plants rich in this hydrocarbon. Indeed, coksfoot grass, rye grass, and other plants are known to have appreciable amounts of *n*-tritriacontane (*5*).

It is surprising that in spite of the large bacterial population in the bovine digestive tract the hydrocarbon composition of the excretion products (*1*) is very similar to that of the diet.

These paraffins are quite inert compounds and can be degraded or absorbed only with difficulty. Thus they tend to accumulate and concentrate in the digestive system and eventually overshadow any hydrocarbons resulting from the animal metabolism or synthesized by the bacteria living in the digestive tract. Our preliminary studies show that some bacteria synthesize a broad range of paraffins with high molecular weights (*6*). However, these paraffins are present in smaller amounts than in plants and show no marked variations in their distribution and no significant predominance of C-odd over C-even alkanes (*6*).

Normal paraffins in the range from C_{25} to C_{35} appear to be widely distributed in the plant kingdom. They have been found in roots, stems, leaves, flowers, fruits, and seeds of a large variety of plants. References to the isolation of individual compounds from plant waxes by classical chemical methods are given in Warth and in Deuel (*3*). A pertinent example is the isolation of *n*-hentriacontane from flowers of common red clover (*Trifolium pratense*), white clover (*Trifolium repens*), and crimson clover (*Trifolium incarnatum*) in 1910 (*7*). The application of modern methods to these studies will lead to a better characterization of the hydrocarbon composition of plants and may correct some unjustified conclusions, such as the absence of C-even alkanes (*8*), made by earlier workers. C-odd as well as C-even *n*-paraffins within the C_{24} to C_{36} range have been detected by modern techniques in pyrethrum wax and string bean wax, sugar-cane cuticle wax, Gramineae, Crassulaceae, tobacco leaves, rose petal wax, and other plants and plant products (*5, 8, 9*). Paraffins with lower molecular weights are also probably common in plants and have been found in cold acetone extracts of red clover leaves, C_{15}–C_{25}, and apple cuticle wax, C_{17}–C_{29} (*10*). Paraffins with higher molecular weights have been observed in cactus leaf, C_{31}–C_{37} (*5*), and in certain microorganisms (*11*).

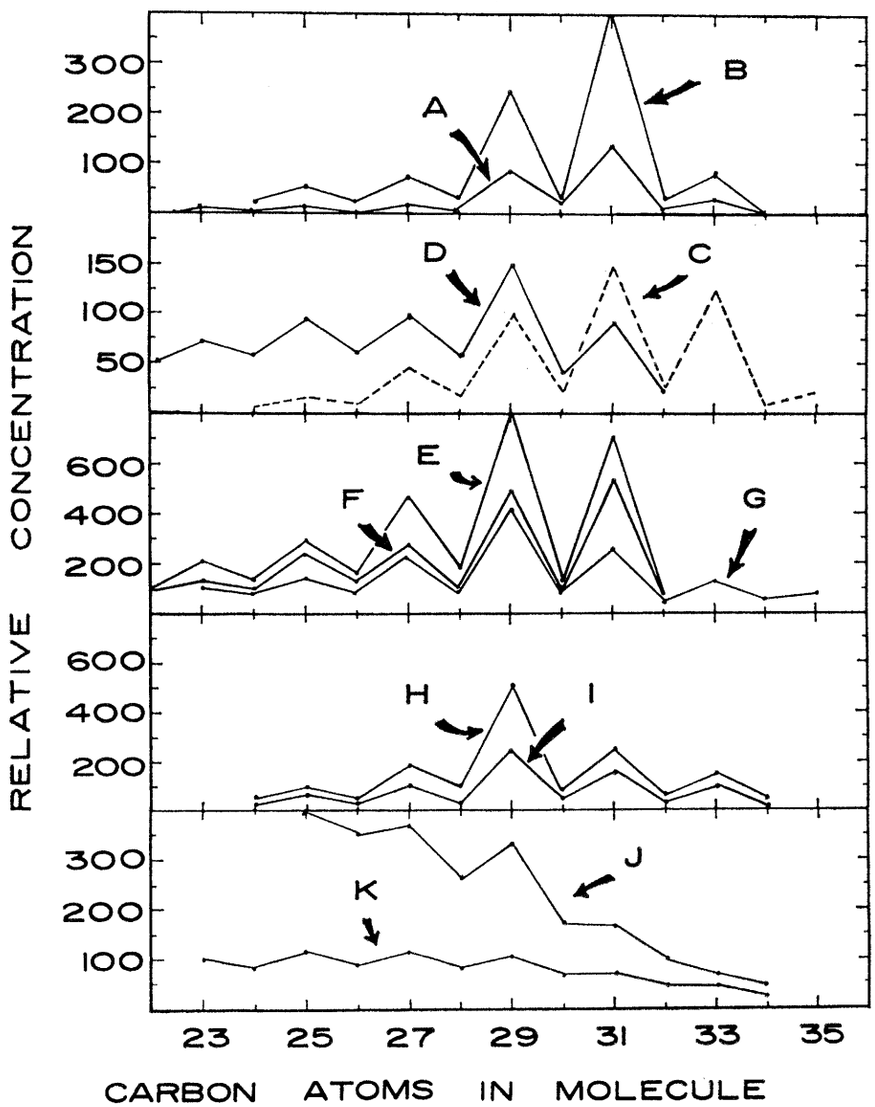

Fig. 2. Distribution of *n*-paraffins in pasture plants, cattle manure, soils and recent sediments, a marine shale, and a petroleum crude. (*A*) Whole plant (*Medicago arabica*), $r = \Sigma \text{ odd}/\Sigma \text{ even} = 8$; (*B*) leaves (*Medicago arabica*), $r = 5$; (*C*) cattle manure, $r = 5$ (*1*); (*D*) recent marine sediment (*12*); (*E*) Gulf of Mexico marine mud (*13*); (*F*) continental soil (*13*); (*G*) San Francisco Bay sediment (*14*); (*H*) West Cortez basin sediment, $r = 3.8$ (*15*); (*I*) Catalina basin sediment, $r = 5$ (*15*); (*J*) Pennsylvanian shale, $r = 1.24$ (*15*); (*K*) Uinta basin petroleum crude (*16*).

It is of interest that the distribution of high molecular weight alkanes in soils and sediments is quite similar to that given in this report and in our other work (*1*). Figure 2 shows a comparison of our results with the analyses of soils and recent sediments (including also a shale and a petroleum crude) carried out by other investigators (*12–16*). The five most common hydrocarbons are the C_{25}, C_{27}, C_{29}, C_{31}, and C_{33} normal alkanes. Normal hentriacontane predominates in spotted bur clover, cattle manure, and continental soil. In most recent sediments from the Gulf of Mexico, San Francisco Bay, and other places normal nonacosane is the predominant paraffin. A predominance of C-odd over C-even alkanes in the C_{24}–C_{34} range is seen throughout these analyses. However, the odd to even ratio decreases from values of 8 and 5 for whole plant and leaves of spotted bur clover, respectively, to values between 5 and 2 for sediments, and to smaller values, 1.24 or less, for Pennsylvanian shale and Uinta basin petroleum crude.

It is possible that the diagenetic reduction of the C-even alcohols normally associated with the above-mentioned paraffins in plant waxes may be responsible for the progressive disappearance of the C-odd predominance during the transformation of recent sediments to old sediments and shales. However, additional work will be necessary to establish firmly a genetic relationship between these high molecular weight hydrocarbons produced by higher plants and the analogous paraffins present in some shales and petroleum crudes of continental formation.

Recent studies on bacteria (*6*), phyto- and zooplankton (*6*, *13*), and "coral" reef organisms (*17*) have shown a ratio of C-odd to C-even *n*-paraffins close to unity, and therefore these organisms appear to be better candidates than higher plants for biological precursors of petroleum of marine origin.

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Contact-Induced Cytotoxicity by Lymphoid Cells Containing Foreign Isoantigens

Abstract. *In tissue culture, immune lymph node cells containing foreign histocompatibility antigens of the H-2 type exert marked cytotoxic effects on tumor cells incompatible with the H-2 antigen. An equally pronounced effect is obtained when normal allogeneic and semi-isologous lymphoid cells of F_1 hybrids are caused to aggregate around the target tumor cells by treating the cultures with either heat-inactivated rabbit antiserum to mouse cells or phytohemagglutinin. Isologous lymph node cells have no effect. Thus, aggregation of lymphoid cells and target cells is a necessary but insufficient requirement for cytotoxicity in vitro; in addition, close contact must be established between histoincompatible cells.*

Immune lymphoid cells have a cytotoxic effect on various normal and neoplastic target cells grown in tissue culture (*1–4*). The effect was presumed to be due to the action of "cell-bound" antibodies, as opposed to humoral antibodies, since it did not require the participation of complement (*2–4*), which is necessary for the cytotoxic action of humoral antibodies. The possibility that the immune lymphoid cells secreted diffusable substances responsible for destroying the target cells was refuted by the demonstration that the cytotoxic effect was prevented by separating the two cell types with a cell-impermeable diffusion membrane (*5*). Also, there are marked differences between the kinetics of the cytotoxic action of immune lymphoid cells, which requires 24 to 48 hours, and the kinetics of the cytotoxic action of humoral antibodies, which requires as little as 1 hour. By the use of heavily x-irradiated target cells incapable of dividing, Wilson demonstrated that immune lymphoid cells actually killed the target cells and did not act by inhibiting their growth (*3*). Different authors have stressed the observation that close cellular contact between the immune lymphoid cells and the target cells always precedes demonstrable cytotoxicity. The importance of close contact is emphasized also by a recent report (*6*) demonstrating that the aggregation in vitro of normal allogeneic lymphoid cells and target kidney cells caused by phytohemagglutinin (PHA) resulted in the destruction of certain target cells. The experiments reported here have led to the conclusion that close contact between lymphoid cells and target tumor cells is a necessary but insufficient requirement for detectable cytotoxic effects in vitro; in addition it seems necessary for contact to be established between cells carrying different sets of H-2 (histocompatibility-2) antigens.

For this investigation tissue-culture systems were used in which cells from sarcomas induced by methylcholanthrene in mice of C57BL and (A × A.CA)F_1 hybrid origin were used as the targets. These cells are designated MC57S and MACD cells, respectively. The tumors were maintained by serial transplantation in isologous recipients. Cellular suspensions were obtained by treating finely minced tumor tissue with 0.25-percent solution of trypsin for 1 hour at room temperature in vitro. The cells were subsequently washed, and 10^6 cells in 1 ml of lactalbumin in Earle's medium supplemented with 10 percent calf serum (or, in later experiments, with the same amount of fetal calf serum) were added to each culture tube. The medium contained mykostatin (30 international units per milliliter) and penicillin (100 IU/ml). After 24 hours the medium was replaced with Parker-199 medium supplemented with 10 percent calf serum. In experiments designed for studying the possible cytotoxic effect of isoimmune and nonimmune lymphoid cells, the tubes were usually treated with 10×10^6 lymphoid cells immediately after the first change of the medium. The cultures were then incubated for 48 hours, after which they were treated with 1 ml of 0.25-percent solution of trypsin for 30 to 60 minutes at 37°C and subjected to repeated shaking. The mixtures were centrifuged, and part of the supernatant was removed. The tumor cells not stained by trypan blue were counted, and the volume of the supernatant was measured. Because of the pronounced differences