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The Paraffin Hydrocarbons of Wool Wax. Homologous Series of Methyl Alkanes*

James D. Mold, Richard E. Means, Robert K. Stevens, and John M. Ruth

ABSTRACT: Several families of homologous paraffins with single methyl branching have been identified in wool wax. They were found to the extent of about 4.5% of the total saturated hydrocarbons and included compounds with 17–44 carbon atoms. For compounds of 30–44 carbon atoms with even numbers of carbon atoms, the methyl branch occurred mainly on carbon 13. Lesser amounts of the isomers branched at the 11 and 15 positions were found, with minor amounts of isomers branched at odd carbon atoms of higher number for the longer chain homologs. The general formula may be written: $C_nH_{2n+1}CH(CH_3)C_mH_{2m+1}$, where n is any even number from 10 to 20 and m is greater than n.

The homologs of 31–43 carbon atoms with odd numbers of carbon atoms consisted of mixtures of isomers branched on either even or odd carbon atoms, with the methyl branch occurring mainly on carbon 12. The general formula for these homologs may be written: $C_nH_{2n+1}CH(CH_3)C_mH_{2m+1}$ where n is any number from 11 to 17 and m is greater than n. The hydrocarbons with 17–29 carbon atoms were present in considerably smaller amounts and were more difficult to resolve from other classes in the gas–liquid partition chromatogram. These compounds appeared to be mixtures of isomers with single methyl branches at nearly every position but with the predominant isomers branched on carbon atoms 8–11.

The relative abundance of homologs of the series of normal, 2-methyl, and 3-methyl paraffin hydrocarbons for a sample of wool wax was reported previously (Mold *et al.*, 1964). In that report, the presence of homologous cyclic substituted paraffins and at least two other series of branched paraffins was noted. One of these groups of homologs (designated Series II in the earlier report) has now been separated and characterized by gas-liquid partition chromatography and mass spectrometry.

Results

The procedures used for the preliminary separation of the wool wax were the same as described in our earlier report (Mold *et al.*, 1964). Briefly, this involved chromatographic fractionation of the hexane extract on alumina to separate the saturated hydrocarbons from

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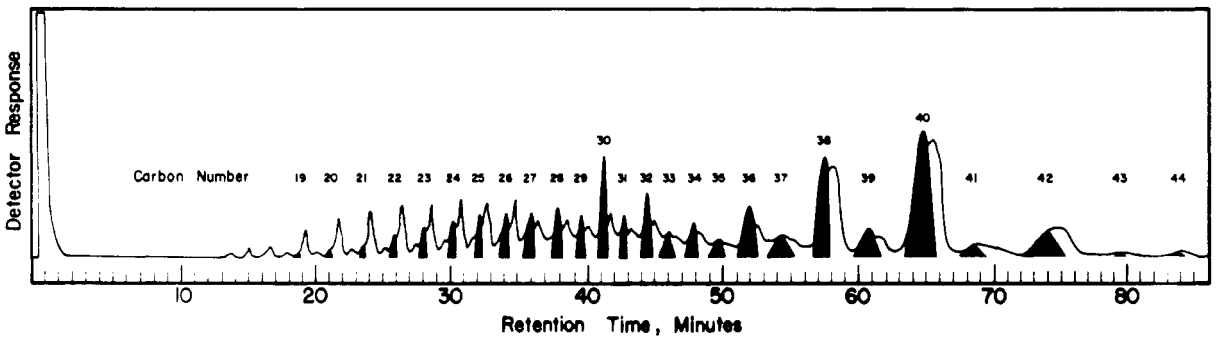

FIGURE 1: Gas–liquid partition chromatogram of the branched paraffins from wool wax which formed urea complexes. The shaded areas indicate fractions representing the branched paraffins of Series II which were collected for further purification and structural identification. Conditions used: sample size, 50 μg; $^3/_{16}$ in. i.d. $\times$ 6 ft dual stainless steel columns containing 80–100 mesh Gas Chrom P coated with 6% silicone rubber (SE-30); helium pressure 38 psi; flow rate 80 ml/min.; column temperature initially 125° for 5.0 min, then programmed to 280° at 4.0°/min.; detector temperature 260°; inlet and exit temperature 300°.

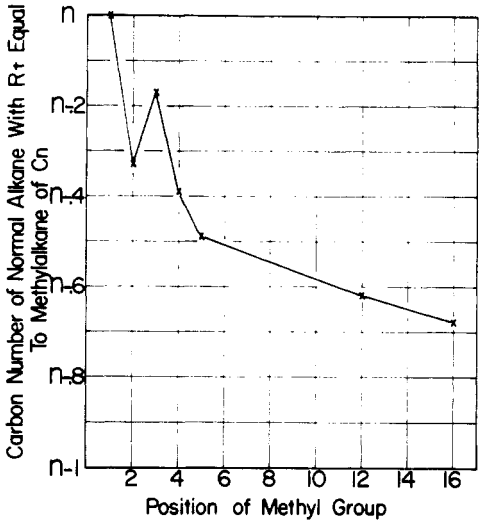

FIGURE 2: Relationship between the position of the methyl group and the gas–liquid chromatographic retention time for monomethyl alkanes.

other wax components, crystallization as a urea–hydrocarbon complex to remove normal and less highly branched compounds from the more highly branched materials, and separation of the normal hydrocarbons from the less highly branched compounds by complexing with a molecular sieve. The further separation of compounds which were not complexed by the molecular sieve was accomplished by gas–liquid partition chromatography on a silicone rubber (SE-30) coated column (Figure 1).

These branched paraffin hydrocarbons of wool wax represented about 9% of the total hydrocarbons. Only about one-fourth of this amount was accounted for by the 2-methyl-, 3-methyl-, and cyclic-substituted paraffins reported in the previous paper. It was speculated that members of Series II (*cf.* Figure 1) appeared to be singly branched paraffins with a methyl group further down the chain. These compounds were estimated from the gas–liquid chromatograph to comprise about 4.5% of the total wool wax hydrocarbons

TABLE I: Amounts of Homologous Branched Paraffins of Series II in Wool Wax.

Carbon No.	Total Paraffins (%)
17	0.005
18	0.007
19	0.014
20	0.023
21	0.025
22	0.057
23	0.084
24	0.12
25	0.085
26	0.11
27	0.098
28	0.12
29	0.086
30	0.24
31	0.089
32	0.18
33	0.048
34	0.094
35	0.050
36	0.25
37	0.12
38	0.66
39	0.23
40	1.2
41	0.18
42	0.27
43	0.014
44	0.050

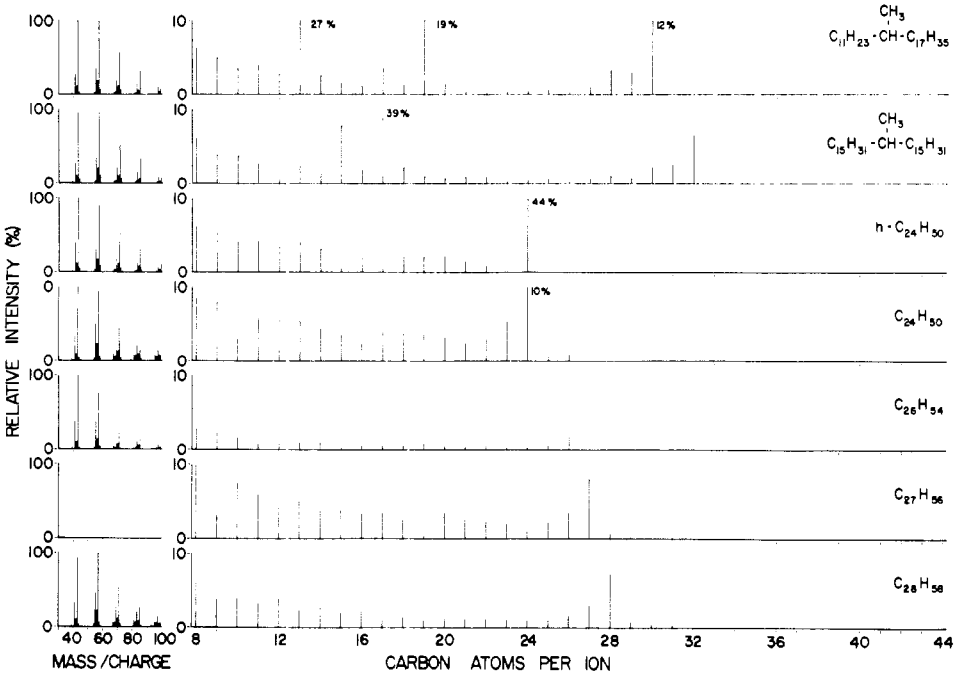

FIGURE 3: Mass spectra of several known compounds and of the methyl alkanes of Series II. The spectra for the authentic reference materials, 12-methylnonacosane, 16-methylhentriacontane, and *n*-tetracosane are given at the top of the figure for reference purposes. Because of the intensity problems associated with the direct inlet probe, the intensities in the spectra of the wool wax fractions are not expected to have the precision of standard reference spectra.

or half of the branched paraffin hydrocarbons which formed urea adducts (Table I). There was an alternating pattern of relative amounts for successive homologs of the higher members of the Series II hydrocarbons while the lower members did not show this. A similar pattern was noted for the normal paraffins of wool wax, but the latter were of lower carbon numbers than were the present series.

Individual homologs of this series of wool wax hydrocarbons were purified by repeated gas–liquid partition chromatography on SE-30 coated columns. The collected individual homologs were finally chromatographed in hexane on small columns of alumina preceding their mass spectroscopic examination. The molecular weights found for these homologs were identical with the 2-methyl and 3-methyl compounds which were eluted from the gas–liquid chromatogram immediately following the Series II hydrocarbons.

Retention times for several position isomers of methyl alkanes were measured relative to normal hydrocarbons of the same molecular weight. These values are expressed in Figure 2 in terms of the equivalent normal hydrocarbons which would be expected to elute at the same time. The decreased retention times observed as a methyl group is removed farther from the end of a paraffin chain has been noted for methyl-substituted fatty acid esters (Abrahamsson *et al.*, 1963). The discontinuity in this curve caused by the 2-methyl isomer was also noted for the methyl esters and is consistent with the boiling points reported for position isomers of methyl alkanes (Rossini, 1960). The retention times for the Series II methyl alkanes were equivalent to normal hydrocarbons of 0.6–0.7 fewer carbon atoms, indicating the position of branching was, on the average, eleven or more carbons from the end of the chain.

Mass spectra, which were obtained for those samples available in sufficient amounts, are given in Figures 3–5. Reference spectra for compounds of known structure are included for comparison. Molecular weights were established by using admixtures with standard compounds. In several of the samples small amounts of impurities of higher molecular weight contaminated the samples. Each of these purified homologs proved to be a mixture of a number of isomeric monomethyl alkanes. For example, the mass spectrum of the $C_{30}H_{62}$ homolog shown in Figure 4 indicates one major component and one other isomer of lower concentration. In addition to the molecular ion peak at *m/e* 422 and the typical alkane spectrum in the low mass range, there are strong peaks at the C-14 and C-18 positions, representing the $C_{12}H_{25}CH(CH_3)^+$ and $C_{16}H_{33}CH(CH_3)^+$ ions expected for 13-methylnonacosane. This isomer makes relatively smaller contributions to the heights of the C-12 and C-16 peaks. The presence of a smaller concentration of 11-methylnonacosane is indicated by the C-20 peak, the other major fragment peak of this isomer being the C-12 peak, which it shares with a minor fragment of the more abundant 13-methyl isomer, as noted above. One of the minor peaks of the 11-methyl isomer also stands

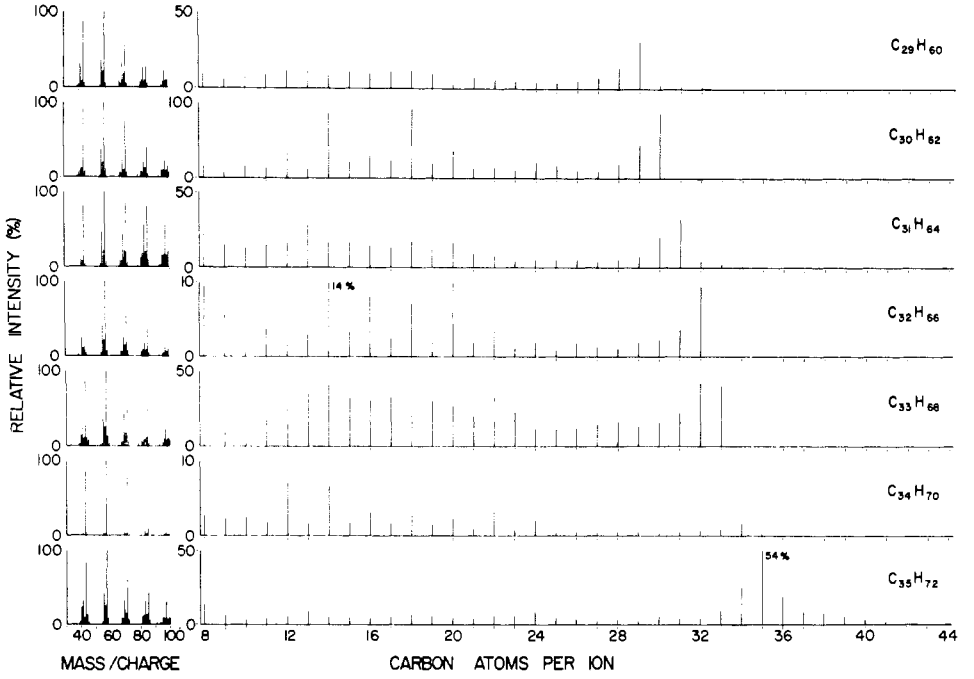

FIGURE 4: Mass Spectra of methyl alkanes of Series II.

out somewhat above its neighbors at the C-10 position, while the other, the C-18 ion, coincides with the strong peak of the more abundant 13-methyl isomer. The spectrum of the $C_{40}H_{82}$ fraction, which is shown in Figure 5, indicates a more complex mixture with one major component, one of intermediate concentration, and probably three others of lower abundance. The C-14 and C-28 ions belong to the 13-methylnonatriacontane, which is the most abundant isomer. The C-12 and C-30 ions are the most important large fragment ions of the 11-methylnonatriacontane, present in somewhat lower abundance. The C-16 and C-26 ions are derived from the 15-methyl isomer, the C-18 and C-24 ions from the 17-methyl isomer, and the C-20 and C-22 ions from the 19-methyl isomer. These ions are the ones formed by cleavage at a branch-bearing carbon atom with retention of the charge on the branched fragment. The peaks representing the less abundant ions in which the charge appears on the unbranched fragment coincide in most cases, in such a complex mixture, with those representing the branched-fragment ions of other isomers. Similarly, the spectra of the other fractions of even carbon numbers from 30 to 44 represent in each case one or two principal isomers with one or more less abundant isomers.

Due to the size of the samples and the necessity for measurement of the spectra using a heated sample probe inserted in close proximity to the ionizing electron beam, it was not feasible to measure precisely the concentrations of each of the components. However, semiquantitative estimation indicated that for the homologs with 30–44 carbon atoms the methyl branch occurred mainly at the 13 position for the even-carbon-number series. Lesser amounts of the isomers with methyl branches at the 11 and 15 position were noted with minor amounts at higher odd-carbon-number positions for the longer chain homologs. In no case was there any appreciable amount of isomer with branching at less than the 11 carbon atom, nor was there any evidence for the methyl branch occurring at less than 17 carbon atoms from the other end of the chain. The branch always occurred on an odd carbon number. The general formula for the even number homologs may be written $C_nH_{2n+1}CH(CH_3)C_mH_{2m+1}$, where n is an even number varying from 10 to 20 and m is greater than n.

The homologs of odd carbon numbers with 31–43 carbon atoms were present in lesser amount than those with even numbers of carbon atoms. For these compounds the methyl branch occurred mainly at the 12 position. Lesser amounts of isomers were present with methyl branches at nearly every higher position. In general, none was found in which the methyl branch was closer to the end of the chain than the 12 carbon position or farther than the 18 carbon position. For this series of compounds branching occurred on either odd or even carbon atoms. In the spectrum of the $C_{39}H_{80}$ fraction, for example, the strong peaks for the C-13 and C-28 ions show that the major component of the mixture is 12-methyloctatriacontane. Ions containing 14, 15, 16, 17, 18, 23, 24, 25, 26, and 27 carbon atoms are more abundant than those containing 19, 20, 21, and 22 carbon atoms, suggesting small concentrations of the isomers branched at carbon atoms 13, 14, 15, 16, and 17. In the even-carbon homologs, branching at alternate positions leads to an alternating pattern of intensities among the

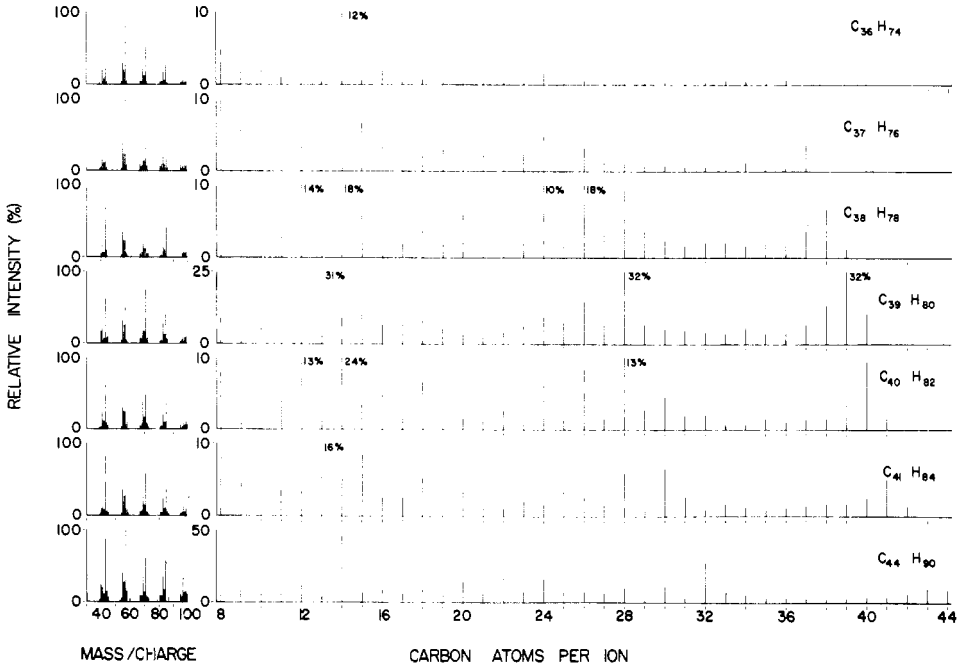

FIGURE 5: Mass spectra of methyl alkanes of Series II.

heavy fragment peaks in the mass spectrum. This pattern is not very evident in the spectra of the odd-carbon homologs, where the branching occurs at every position within the indicated limits. The general formula for these odd carbon number homologs may be written C_nH_{2n+1}-$CH(CH_3)C_mH_{2m+1}$, where n may vary from 11 to 17 and m is greater than n.

The methyl-branched homologs of Series II with 17–29 carbon atoms were present in considerably smaller amounts and were more difficult to purify due to the proximity of the 2-methyl and 3-methyl alkanes as well as the unidentified Series I compounds. The gas–liquid chromatographic retention times for these compounds suggested that they could be monomethyl alkanes with an average position of branching at 8–11 carbon atoms. The mass spectra indicate branched paraffin mixtures which, in this range of molecular weights, are sufficiently complex that the characteristic peaks produced by isomers having a sequence of different branching positions are of comparable intensity. All of the ion peaks associated with this range of branch positions are strong peaks, with no particular one appearing more important than its neighbors. All will be of somewhat lower intensity as compared with those representing small fragments containing three–five carbon atoms because the latter are common to all of the isomers and therefore are reinforced in such mixtures. As was noted above, these homologs did not form an alternating series with odd or even carbon numbers predominant, but rather seemed to increase in quantity to about the 24-carbon homolog and then to decrease as they overlapped the family of higher carbon numbers.

Discussion

The homologous families of alkanes from wool wax described in this report have not previously been noted in any natural waxes. Fatty acids with the methyl branch located 10 carbon atoms from the carboxyl group have been isolated from tubercle bacilli. Tuberculostearic acid, first noted by Anderson and Chargaff (1929), was later shown to be (−)-10-*d*-methyloctadecanoic acid (Spielman, 1934; Prout *et al.*, 1947, 1948; Ställberg-Stenhagen, 1948). The presence of 10-methylheptadecanoic acid and other methyl-branched acids of 15, 16, 17, and 18 carbon atoms were reported in lipids from tubercle bacilli by Agre and Cason (1959). Their degradative studies indicated the presence of mixtures of isomers with branches principally at carbon atoms 8 and 10. While other reports have indicated the possible presence of similar compounds in animal fats, these have not been firmly established. Possible precursors of the branched fatty acids of this type have been shown to be the corresponding unsaturated acids, with methionine serving as the methyl donor for introduction of the methyl group (Lennarz *et al.*, 1962).

Cyclopropane fatty acids have been reported in various bacterial lipids (O'Leary, 1962) and in seed oils (Nunn, 1952). The position of the methylene branch in the compounds for which structures have been established is 9, 10, or 11, 12. It has been proposed that these compounds may also be formed by transmethylation of olefinic acids with methionine.

The predominance of compounds branched at least 11 carbons and predominantly 12 or 13 carbons from the

end of the alkane chain in the homologous series of wool wax paraffins suggests that the above acids are not likely to be directly involved as precursor materials since in every case these methyl or methylene fatty acids have the position of branching at the 8–12 position from the carboxyl group which locates the branch 6–9 carbon atoms from the alkane end of the chain.

It should be pointed out that, due to the proximity of isomeric homologs from the 2-methyl and 3-methyl series as well as from a series of homologous dimethyl alkanes, it was necessary to sacrifice material which was present in the overlapping regions of the chromatogram in the interest of purity of the collected fractions. This may have resulted in a disproportionate loss of certain of the isomers with branching at carbons 4–10. There is no question, however, that they could be major isomers since if this had been the case there would have been no resolution of the series from the 2-methyl and 3-methyl series on the gas–liquid chromatogram.

Experimental Section

Separation of the Methyl-Branched Paraffin Hydrocarbons from Wool Wax. The methyl-branched paraffin hydrocarbons were separated from a sample of centrifugal wool grease (Wellman Combing Co., Johnsonville, S. C.) by the procedures previously described (Mold *et al.*, 1964). A hexane extract of the wax was washed with a methanol–water (2:1) solution, washed with water, and then dried over anhydrous Na_2SO_4. The wax hydrocarbons were separated by chromatography on acid-washed alumina (100–200 mesh, Merck chromatographic grade alumina, which was thoroughly washed with several solvents and reactivated by heating at 110° for 16 hr). The purified fraction, 0.53% of the total wax, gave an infrared absorption spectrum indicating it to be free of carbonyl or olefinic functions.

The more highly branched hydrocarbons were separated into the filtrate by formation of a crystalline urea complex from a *n*-propyl alcohol solution. Twenty-four per cent of the wax hydrocarbons was recovered from the urea crystals. This fraction was dissolved in isooctane and shaken with $^1/_{16}$-in. pellets of 5-A molecular sieve (Linde Co., Division of Union Carbide) to permit absorption of the normal hydrocarbons. Repeated treatment of the filtrate allowed complete removal of the normal compounds, leaving branched compounds in the filtrate to the extent of 34% of the materials which had formed the urea complex. A portion of the branched paraffins which formed urea adducts was analyzed by gas–liquid chromatography as described in Figure 1.

The remainder of this fraction was then fractionated by gas–liquid chromatography on 80–100 mesh Gas Chrom P coated with 6% SE-30 in a $^3/_{16}$ in. (i.d.) × 6 ft stainless steel column using a MicroTek 2500 instrument equipped with dual flame ionization detectors. A flow rate of 80 ml/min with a helium pressure of 38 psi was used. The temperature was programmed at 2°/min from 250 to 285°. The fractions, collected by means of a cold-finger trap (Stevens and Mold, 1963) from a considerable number of individual chromatograms, were combined and rechromatographed under appropriate conditions for optimum separation of each mixture of isomers. A third, and in some cases a fourth, isothermal gas–liquid chromatographic separation was required to purify further the Series II hydrocarbons from materials present in adjacent chromatographic fractions. The samples were finally chromatographed in hexane on small columns of activated acid-washed alumina to remove contaminants introduced from the gas–liquid chromatographic substrate. Examination of these purified homologs was then performed by mass spectrometry.

Determination of Mass Spectra. Mass spectra were obtained with a Bendix Model 14-101 Time-of-Flight mass spectrometer, equipped with an S-14-105 ion source. Each of these samples, contained in a silica capillary, was introduced into the ion source just below the ionizing electron beam by means of a modified Bendix probe (Mold *et al.*, 1963). This was passed through a vacuum lock, after which the capillary was heated by a filament surrounding it. The spectrometer used has no slit or other constriction between the evaporating sample and the pumping system. When the sample is large enough to permit several scans of the spectrum, it is usually possible to adjust the probe filament temperature until a steady evaporation rate is obtained, yielding consecutive scans in which the intensities are reproduced. When the sample is too small to permit such treatment, but large enough for the recording of at least two scans, the intensities may frequently be improved by correcting for the observed rate of change of sample concentration in the ion source. When the sample is so small that only one scan is obtained, this correction cannot be made. The sample concentration will almost surely vary during the recording of the spectrum, and its manner of variation will not be known. Peaks near each other will then show approximately the correct intensity ratios, but those more widely separated will not. In the interpretation of the spectra, allowance must be made for such intensity variations.

Reference Compounds. Compounds which were used as reference materials included: 1-cyclopentylheneicosane, 1-cyclohexyleicosane, *n*-hexacosane, *n*-tetratetracontane, and 2-methyltricosane, supplied by Dr. Joseph A. Dixon (API Research Project 42 at Pennsylvania State University); 2-methyl,-, 3-methyl-, 4-methyl- and 5-methyltritriacontane supplied by Professor E. Stenhagen of Goteborg University; *n*-nonadecane (Humphrey-Wilkinson, Inc., North Haven, Conn.); *n*-hexatriacontane (Distillation Products Industries, Rochester 3, N. Y.); *n*-tetratriacontane (Applied Science Laboratories, Inc., State College, Pa.); and *n*-pentacosane, 2-methyltetracosane, 3-methyltetracosane, 2-methylhexacosane, 2-methyloctacosane, 2-methyltriacontane, 12-methylnonacosane, and 16-methylhentriacontane, synthesized by Mr. T. P. Chen and Mr. T. B. Walker of the Research Department, Liggett and Myers Tobacco Co.

The syntheses were achieved by (a) reaction of a

long-chain bromide with an alkynyllithium or (b) reaction of a ketone with a Grignard complex or an alkyllithium. Dehydration and hydrogenation steps were applied to yield the saturated alkanes.

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Inhibition of Insulin Activity in Mitochondrial Systems and in Normal Rats by Reduced Insulin B Chain–Albumin Complex*

Richard L. Fenichel, William H. Bechmann, and Harvey E. Alburn

ABSTRACT: A complex consisting of reduced bovine insulin B chain with crystalline bovine albumin inhibits the swelling effect of insulin on rat liver mitochondria. The inhibition is concentration dependent and is not seen with S-sulfo B chain or with reduced insulin A chain. The mitochondrial swelling effect of bovine growth hormone is not inhibited by reduced B chain–albumin, and pretreatment of mitochondria with reduced B chain–albumin does not result in subsequent inhibition of insulin activity. These two findings suggest that the B chain may be acting directly on insulin rather than on its site of action. Intraperitoneal injection of the reduced B chain–albumin complex into fasted normal rats significantly increases blood glucose levels, presumably by inhibiting endogenous insulin. Blood glucose is not increased by S-sulfo B chain or when reduced B chain is not complexed with albumin prior to injection. These results obtained by different experimental procedures further confirm the demonstration by J. W. Ensinck, R. J. Mahler, and J. Vallance-Owen (1965, *Biochem. J. 94*, 150) of a reduced insulin B chain insulin-inhibition mechanism.

The observation of inhibition of insulin activity associated with the albumin fraction of diabetic plasma (Vallance-Owen *et al.*, 1958a,b) and the identification of this factor as a complex of insulin B chain with albumin (Ensinck *et al.*, 1965) have presented an attractive new approach to the problem of diabetes.

In the work reported here, we complexed the reduced B chain of purified bovine insulin with crystalline bovine albumin and determined the effect of the complex under various conditions on the swelling of rat liver mitochondria. In addition, we determined the extent of the *in vivo* inhibition of endogenous insulin activity by the reduced B chain–albumin complex in fasted normal rats by measurement of blood sugar levels.

Materials

Crystalline Bovine Insulin (approximately 24 IU/mg). Insulin concentrations were calculated on the basis of a molecular weight of 36,000; thus a 5×10^{-6} M concentration of insulin contained 0.9 mg of insulin in a 5-ml test system. *Insulin, crystalline* L-*cysteine*, and *Trizma*, pH 7.3 [tris(hydroxymethyl)aminomethane hydrochloride, reagent grade] were obtained from the Sigma Chemical Co.

S-Sulfo A and B chains of insulin were prepared from

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