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STUDIES ON HUMAN SALIVA WITH NUCLEAR MAGNETIC RESONANCE

I. PROTON LINE WIDTHS AND SPIN-SPIN RELAXATION TIMES

by

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Numerous investigations have been carried out concerning different properties of the human saliva. The constituents of saliva vary greatly in different individuals and in the same subject under different circumstances. Only a few figures for the average composition are available at present. Many inorganic and organic compounds have been found in the saliva which is, in principle, a dilute solution of electrolytes (0.1—0.2 per cent) in water containing as the most important organic constituents mucins and enzymes (*Hildes & Ferguson,* 1955). The total solid matter of human saliva averages about 0.5 per cent and the protein content about 0.3 per cent or 280 mg per 100 ml saliva, range 180—420 (*Dewar & Parfitt,* 1954). No separate figures are available for mucin. However, most of the protein present in mixed salive exists in so-named mucinous form; it is a protein containing a carbohydrate group. The carbohydrate part of the mucin molecule includes sialic acid $C_{11}H_{19}NO_9$ (*Berggård & Werner,* 1958).

The mucins are largely responsible for the special physical properties of the saliva such as viscosity and "Spinnbarkeit". This in turn plays an important physiological role (*Ericsson*, 1949 and 1961, *Hanson*, 1961, *Schulz*, 1961). Mucin is only slightly soluble in water and is insoluble in weak acids; it can be precipitated from saliva by dilute acetic acid or alcohol, but redissolves in the form of the sodium salt in NaOH (cf. *Jenkins*, 1960). The concentration of mucin in mixed saliva falls slightly with increasing rate of secretion. This is caused by an increased proportion of the protein from the parotid glands which does not secrete mucoprotein.

The way in which water molecules interact with organic macromolecules to bring about the special physical properties of saliva has so far received little attention. One non-destructive method which can give information on molecular interactions is nuclear magnetic resonance (NMR). The present paper is the first report on studies of human saliva with NMR techniques.

THEORY

The physical properties of atomic nuclei play the fundamental role in NMR; some of these properties are that some nuclei have a spin angular momentum (an "intrinsic rotation") greater than zero and a nuclear magnetic dipole moment. As a nucleus with spin >0 can be imagined as a rotating electric charge, we understand that it should give rise to magnetic effects. The generated nuclear magnetism is measured by the nuclear magnetic moment. The unit of nuclear magnetism is the so-named nuclear magneton $= 5.050 \times 10^{-24}$ erg/gauss.

When a sample, containing magnetic atomic nuclei, is introduced in a strong magnetic field of some thousands of gauss, the nuclei will take up certain allowed directions in this field. The directions differ slightly in energy and if quanta of energy exactly fitting the energy differences are supplied, e.g. by radiation, the nuclear axis will jump from lower to higher directions. This phenomenon is called nuclear magnetic resonance.

In a field of 4930 gauss (as in our experiments) the radiation causing nuclear magnetic resonance has a frequency of 16.7

Mc/sec for protons. The exact position of the magnetic resonance lines varies somewhat with the chemical composition (so-named chemical shift). The lower energy states are normally somewhat more populated than the higher energy states so that an additional minute fraction of the nuclei is in each lower state compared with the higher states.

Let us now assume that this small fraction is completely removed by strong absorption of radiation, so that both energy levels become equally occupied. Experiments show that such a so-named "saturated" condition cannot persist for a very long time. Some of the atomic nuclei in the higher energy state lose energy to the surroundings (lattice) and return back to the lower energy state. This is the process of thermal relaxation, which is characterized by a time constant, the so-called *spin-lattice relaxation time,* or longitudinal relaxation time, or T_1. In liquids, the most important mechanism of relaxation is provided by the rotational or translational oscillations of molecules.

Different nuclei can also mutually exchange their spin energy, so that one nucleus flips "up" at the same moment as a neighbouring nucleus flips "down". The time constant describing this process is the *spin-spin relaxation (interaction) time,* T_2.

The magnetic resonance method when applied to protons in biologic samples primarily gives information about the nature of the water in the tissues, just because the protons of the aqeous phase dominate. The water present in tissues has a structure different from that of "free" water because of the presence of electrolytes and macromolecules of various kinds capable of interaction with water molecules. When water is adsorbed to macromolecules the line gets broader and the value of the spin-spin relaxation time is reduced. For detailed information on theory and techniques of NMR the reader is referred to the books by *Andrew* (1955) and *Pople, Schneider, & Bernstein* (1959).

MATERIAL AND METHOD

Mixed saliva samples from eight male students, 19- and 20-year-old, were obtained by allowing the subjects to chew paraffin wax. The collection period for 20 ml mixed saliva varied between 10 and 15 minutes. The saliva samples were collected directly in

Table I

Line widths and spin-spin-relaxation times of solid substance of freeze-dried saliva dissolved in various concentrations in distilled water.

g solid substance per ml of distilled water	g mucoid per ml of distilled water (approximately)	Full line width c/sec.	T_2 from line width sec.	T_2 from wiggle re-growth sec.
0	0	2	—	1.5
0.04	0.02	7.0	—	—
0.09	0.05	13	—	0.3
0.19	0.10	18	0.02	0.04
0.26	1.14	25	0.015	0.03
0.35	0.20	30	0.01	0.01
0.69	0.40	42	0.007	0.008
1.9	1.0	99	0.003	0.002

glass bottles chilled continuously with solid carbon dioxide. After the specimens of 20 ml saliva were collected, the samples were freeze-dried. The solid matter of the saliva was then stored in a refrigerator at —5 °C until required for the NMR studies.

Different concentrations of solid matter of saliva per ml of distilled water were used as shown in Table I and Fig. 2. These solutions were prepared about one hour before analyzing.

Purified mucin was prepared as follows: 50 ml stimulated mixed saliva was freeze-dried as mentioned above. 10 ml distilled water was added, and the mixture was centrifuged at a rate of 2000 r.p.m. for 30 minutes. To the filtrate 40 ml alcohol were added, and two days later the precipitate was centrifuged. The precipitate was washed with 50 % alcohol + 50 % ether, centrifuged twice, washed with ether and again centrifuged. The samples were then stored in a desiccator containing P_2O_5 until required. This purification was *ad modum Högberg* (1960, personal communication). Different concentrations of the purified mucin in distilled water were used as shown in Table II. Samples of unstimulated natural mixed saliva were also analyzed immediately after being obtained.

The equipment used is described in a separate paper (*Odeblad*, 1961). The saliva samples were contained in soda glass tubes of

Table II

Line widths of purified mucin from freeze-dried saliva dissolved in various concentrations in distilled water.

g mucoid per ml of distilled water	Line width
0	2
0.06	8
0.14	13
0.22	15

1.2 mm inside diameter and centrifuged before reading. Sample spinning during recording at about 1,200 r.p.m. was employed. This rotation of the samples served to average small inhomgeneities in the magnetic field (4.930 gauss). The radiofrequency used was 16.7 Mc/sec. The instrumental resolution of the spectrometer was 2 cycles per sec. (0.13 ppm). The measurements were carried out at +20°C. The side-band audiomodulation technique of *Arnold & Packard* (1951) was used for measurement of line width. The wiggle-regrowth method of *Gabillard* (1951) was used for measurement of T_2.

RESULTS

A record is exemplified in Fig. 1. Fig. 2 and Tables I and II show the proton magnetic resonance line widths as a function of the concentration of solid substance and mucin in distilled water (the mucin assumed to be 3/5 of total solids). Except from a weak initial curvature the line width increased about linearly, and even at higher concentrations of solid matter there was an increase in line width; the curve consequently did not level off. Approximate measurements of the spin-spin interaction time, T_2, by the wiggle regrowth method indicated that T_2 decreased from about 1.5 sec. for distilled water to about 0.002 sec. for the most concentrated sample. The rate of decrease of T_2 is shown in Table I.

The line widths for purified mucin are shown in Table II.

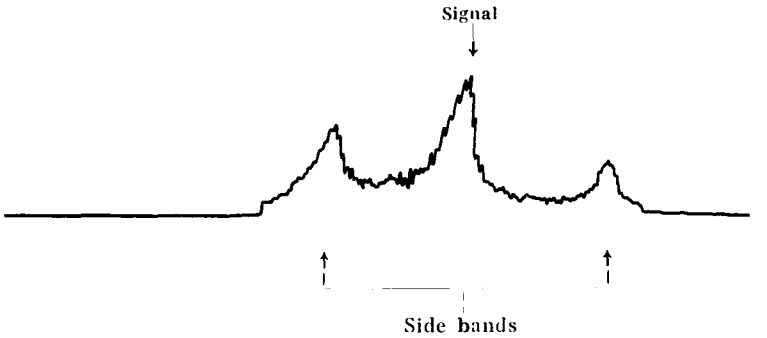

Fig. 1. Proton magnetic resonance signal of the water peak of freeze-dried human saliva material dissolved in distilled water, ratio 0.35. The calibration side bonds were at ± 85 c/sec and the line width was 29 c/sec. The spin-spin interaction time, T_2, measured by wiggle decay and growth was 0.01 sec.

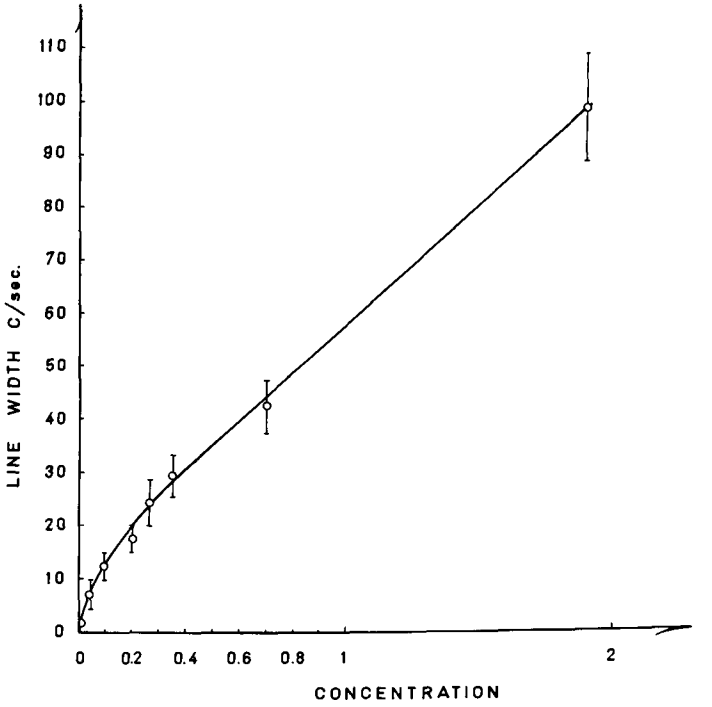

Fig. 2. Proton magnetic resonance line widths as a function of solid matter from freeze-dried stimulated mixed saliva dissolved in various concentrations in distilled water.

In the samples of natural undried saliva which thus contained about 99.5 per cent water the line widths varied between 4 and 6 cycles per second.

DISCUSSION AND CONCLUSIONS

The present investigation is a first attempt to study human saliva with nuclear magnetic resonance methods. A study on this subject can be performed in different ways, e.g. on freeze-dried and redissolved samples and on purified mucin as in the present paper. Any attempt at purification may change the physical-chemical properties of the mucin. Redissolution of freeze-dried saliva at various concentrations may also change the normal interaction between mucin and electrolyte ions and water molecules. The various difficulties of investigating the physical, chemical and physical-chemical properties of the saliva have been discussed by *Jenkins* (1960). Whatever method is chosen genuine "native" conditions cannot be obtained at all concentrations studied. The line widths of comparable concentrations of "native" and "purified" mucin, however, turned out to be about the same.

The normal water molecule has an electric dipole moment due to accumulation of negatively charged electrons around the oxygen atom. Between the water molecules there occur hydrogen bonds which in part form polymerized tetrahedral units in the water. In which way the saliva mucin changes this normal water structure is not clearly understood. The water layers near the mucin molecular chains may have a structure differing in lattice order from the intermolecular water. It can be suspected that the rheologic properties of the saliva mucin could be accounted for by the assumption of intermolecular forces of either the hydrogen bridge type or the electrostatic dipole interaction type. The large increase of signal width and decrease of T_2 with increasing concentration of the saliva mucin would indicate that the water is not free but bound to a considerable degree. However, it cannot be decided what type of bonding mechanism is active. The strength of bonding cannot be calculated from available experimental data. Such interpretation requires additional studies, e.g. measurement of chemical shift, of T_1, of signal areas, as well as studies on effects of different pH and of temperature on reso-

nance lines. Such and other investigations are now under way and will be reported later.

The following conclusions may be drawn from the present investigation:

1. The line-width increases with increasing concentration of dry substance.

2. This increase is largely due to the mucin present.

3. The increase is approximately linear and no levelling of the dilution curve is evident up to the highest concentration studied.

4. The values of T_2 calculated from line widths are identical with the values of T_2 obtained by the wiggle regrowth method within a factor of two (experimental error). This result indicates that line broadening is not due to superposition of unresolved partial resonances but is due to molecular interaction between macromolecules and water.

SUMMARY

The essentials of the nuclear magnetic resonance technique are described.

This method was applied to the study of some properties of human saliva. The line widths of the resonance peaks were measured as well as the spin-spin relaxation times, T_2.

The line width increased about linearly, and even at higher concentrations of solid saliva matter there was an increase in the line width. T_2 decreased from 1.5 sec. for distilled water to about 0.002 for the most concentrated sample. There was agreement between the results obtained from the solid substance and purified mucin of freeze-dried saliva.

The results are discussed in relation to the hydration of water.

RÉSUMÉ

ÉTUDES DE SALIVA D'HOMME PAR RÉSONANCE MAGNÉTIQUE NUCLÉAIRE

I. LARGEURS DE RAIE DE PROTON ET TEMPS DE SPIN-SPIN RELAXATION

L'essentiel de la technique de résonance magnétique nucléaire est décrit.

Cette méthode a été appliquée à l'étude de quelques propriétés de salive d'homme. La largeur de raie de la résonance a été mesurée ainsi que les temps de spin-spin relaxation, T_2.

La largeur de raie a augmenté presque linéairement et même aux concentrations très grandes de substance de salive solide il y avait une augmentation de la largeur de raie. T_2 diminuait de 1,5 sec. en eau distillée à environ 0,002 chez le spécimen le plus concentré. Il existe une conformité entre les résultats obtenus de la substance solide et mucin purifié de salive séchée par froid.

Les résultats sont discuté en relation a la hydration en eau.

ZUSAMMENFASSUNG

STUDIEN AN MENSCHLICHEM SPEICHEL MIT KERN-MAGNETISCHER RESONANZ

I. DIE PROTONLINIENWEITEN UND "SPIN-SPIN" RELAXATIONSZEITEN

Das wesentliche der Technik der kernmagnetischen Resonanz wird beschrieben.

Diese Methode wurde zum Studium einiger Eigenschaften des menschlichen Speichels angewendet. Die Linien-Weite der Resonanzsignalen sowie die "spin-spin" Relaxationszeit, T_2, wurden gemessen.

Die Linienweite wuchs ungefähr liniär. Selbst in grösseren Konzentrationen getrockneten Speichels zeigte sich ein Aufsteigen der Linienweite. T_2 sank von 1,5 sek. für destilliertes Wasser bis ungefähr 0,002 für die am meisten konzentrierte Probe. Es zeigte sich Übereinstimmung zwischen den Resultaten, die von den festen Substanzen und denen, die von gereinigtem Muzin kälte-getrockneten Speichels erhalten worden waren.

Die Ergebnisse wurden diskutiert in Beziehung zur Hydrierung des Wassers.

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