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39. Medical and Biochemical Application of Infrared Absorption Spectra. III.[1] Studies on Biological Lipids by Infrared Spectra. (1). Infrared Spectra of Total Lipids in Diabetes Serum.

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Rapid and accurate analysis of various components of serum lipid, i.e. cholesterol, cholesteryl esters, glycerides, phospholipids, and free fatty acids, is most important in the clinics, diagnosis, study of lipid metabolism, and in judging the therapeutic effect of pharmaceutics. It may be said that the process for chemical measurement of these substances is already established. However, process now used for simple measurement and directly useful for daily routine clinical examination is that for total cholesterol and cholesteryl esters. Measurement of glycerides, phospholipids, and free fatty acids is so complicated that its use in its present form is limited as a daily clinical test.

The infrared absorption spectrum would be a valuable measure in this region. There is already the work of Freeman and others in this field, directed toward strict quantitative analysis. They first applied infrared spectrum for the analyses of lipoproteins[2] and related lipids,[3] and more recently established excellent analytical procedures with introduction of chromatography,[4-6] applying this method to atherosclerosis[7] and coronary artery disease.[7,8]

What is required in this field now are first, the establishment of a simplified process which could be used for daily clinical examination and for numerous other studies, and second, to apply such simplified process to the study of serum lipids in various diseases and in a more wider field.

With respect to the first point, it has been found from measurement of infrared spectrum of total lipids in the serum that changes in the spectral form offered sufficient informations that could be used for clinical discrimination. In normal, healthy persons, the ratio of various lipids in the serum is constant and infrared spectrum of such serum shows a constant form. In some diseases, this relative ratio would change and spectral form would also differ from that of normal persons. What is important in

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clinics is not the absolute quantity of various lipid components but their relative ratio. For example, the ratio of cholesterol to cholesteryl esters is said to have a bearing on the liver function and the ratio of cholesterol to phospholipids, to atherosclerosis. For clarification of this point, it is sufficient to examine the infrared spectrum of total lipids in the serum and to classify their spectra. This only requires measurement of infrared spectrum of total lipids extracted from 2 cc. of the serum and the procedure is so simple that several hundred samples could be treated in one week.

As to the second point, several thousands of infrared spectra have been obtained of the sera from various diseases, including diabetes, nephrosis, myxoedema, liver cancer, liver cirrhosis, and leucemia. From these results, studies on the total lipids in diabetes serum are reported herein.

The quantity of total lipids in the serum of diabetic patients is generally higher than the normal value and indicates abnormality of lipid metabolism but their relative ratios and which component has increased are not clear. Infrared spectral examinations showed following facts on serum lipid of diabetes patients.

(1) The shape of infrared spectrum of total lipids in the serum of diabetes patients before treatment can be classified into several types according to differences in the relative intensity of absorptions.

(2) These classified types reflect various changes in the constituent composition of total lipids in the serum.

(3) There is a difinite relationship among these classified types, quantity of total lipids in the serum, and the serum lipoprotein index (ratio of β-lipoprotein/α-lipoprotein) obtained by paper electrophoresis.

(4) Conversion of the abnormal type of spectrum to normal type indicates the possibility of using infrared spectrum for judgement of therapeutic effect of various pharmaceutics.

(5) Frequency of the appearance of various types of spectrum has a definite relationship with progress of diabetic retinosis and nephrosis (proteinuria), and can be utilized as a material for diagnosis.

Experimental

Material—The blood was drawn on empty stomach from 283 patients (183 males, 100 females) who had been diagnosed as diabetes in the Third Internal Clinic of the Tokyo University Hospital. After diagnosis has been established, patients were treated according to their symptoms by diet or by administration of insuline, mesoxalic acid, or tolylsulfonylbutylurea. In order to follow therapeutic effect, serum from each patient was examined several times during the treatment and, therefore, the total number of infrared spectra measured was about 700.

Extraction of Total Lipids and Infrared Spectra—A mixture of 2 cc. of the serum and ca. 50 cc. of 3:1-mixture of EtOH:Et_2O was heated in a liquid extractor, provided with a reflux condenser, for about 3 hr. to extract the lipids from serum. The extract was further extracted with Et_2O, the extract was filtered, and Et_2O was evaporated to obtain total lipids (not containing free fatty acid) of the serum. The residue was dried in a vacuum desiccator over night and weighed. This is the quantity of total lipids in the serum.

In usual cases, the quantity of total lipids extracted was ca. 10~20 mg. This was dissolved in 0.2 cc. of CS_2 and infrared spectrum of this solution was measured in a rock salt cell of 0.5 mm. thickness. When the quantity of total lipids was especially large, the amount of CS_2 used as the solvent was increased.

Measurement of Serum Lipoprotein Index by Paper Electrophoresis—The lipids in serum are not present in a free state but are bound to proteins. The lipoproteins are divided into α-lipoprotein, in which the lipid is bound to α_1-globulin, and β-lipoprotein, in which the lipid is bound to β_1-globulin, by their behavior in electrophoresis. This ratio of β-lipoprotein/α-lipoprotein, called the serum lipoprotein index, is clinically important and comparative examinations were made of this index and

infrared absorption spectrum.

The measurement of lipoprotein index was made by the method of Swahn.[9] The serum was obtained from the blood drawn on empty stomach. Toyo Roshi No. 51 filter paper (2.5 cm. in width) was spotted with 0.03 cc. of the serum and run in a veronal buffer (pH 8.6) at 320 v, 5 mA, for 6 hr. After electrophoretic migration, the filter paper was dried at 60° and the lipids were colored with 5% EtOH solution of Sudan Black B. After coloration, the spots were extracted with 25% AcOH-EtOH solution and the ratio of β-protein to α-lipoprotein, i.e. lipoprotein index, was calculated from the result of photoelectric colorimetry.

The measurement of blood sugar was made by the method of Hagedorn-Jensen[11] and occular fundus test by Dr. H. Tokuda in the Department of Ophthalomology, Tokyo University Hospital.

Results and Discussion

The infrared spectra of total lipids from serum, with special exceptions, generally show four absorptions, a (1052 cm^{-1}), b (1093 cm^{-1}), c (1169 cm^{-1}), and d (1250 cm^{-1}), as shown in Fig. 1. The absorption a is due to cholesterol and serum phospholipids, b to serum phospholipids, c to triglycerides and cholesteryl esters, and d is chiefly due to serum phospholipids, with contribution from a small amount of triglycerides and cholesteryl esters. This is clear from comparison with the spectrum of a standard substance. These four absorptions were chiefly taken up in the present work but, besides these, there is an absorption at around 1750 cm^{-1} which is a total sum of absorption of ester linkages in triglycerides, cholesteryl esters, and phospholipids, and the C-H stretching absorption around 2900 cm^{-1} is approximately in proportion to the total quantity of total lipids.

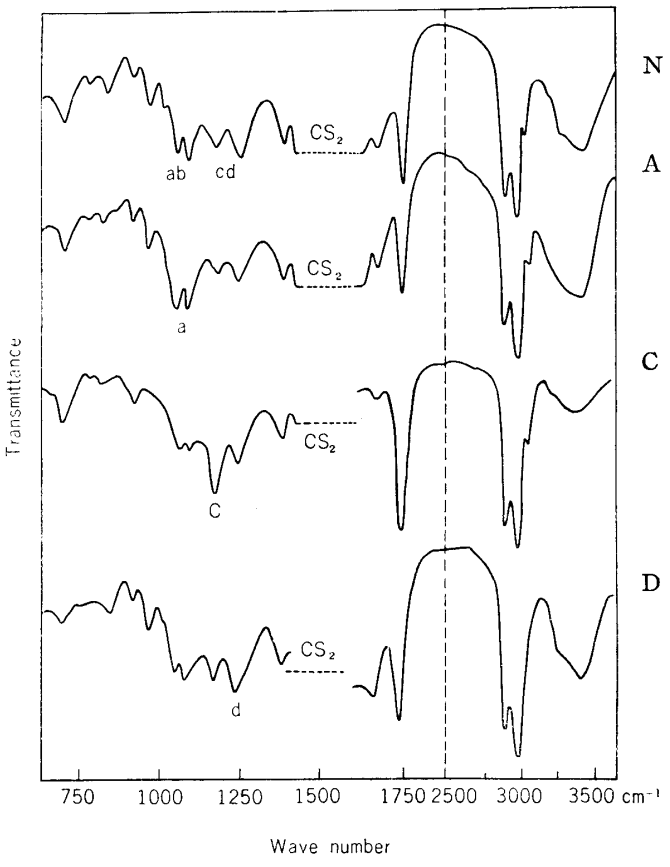

Fig. 1. Four Types of Infrared Spectra of Total Lipids in the Serum of Diabetes Patients

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The infrared spectrum of serum lipids of diabetes patients was classified into N (normal), A, C, and D types by comparison of relative intensity of these four (a, b, c, d) absorptions.

N-Type (Fig. 1-N) is the spectrum of total lipids from normal, healthy human serum and intensity of absorptions is in the order of b-d-c-a or b-d-a-c. This type also occurs, besides the normal serum, in case the quantity of total lipids increases or decreases without variation to relative ratio of the components.

A-Type (Fig. 1-A) is the spectrum in which a and b absorptions are stronger than c and d in intensity. This is mainly due to the increase of cholesterol.

C-Type (Fig. 1-C) is the spectrum in which the absorption c is the strongest or is stronger than that of a or d. This occurs due to increase of triglycerides and cholesteryl esters. In simple diabetes, the ratio of total cholesterol to cholesteryl esters is generally 100:70 and abnormal increase in intensity of c absorption may be considered as increase of triglycerides.

D-Type is the spectrum in which the d absorption is the strongest and this may be considered to be due to a slight increase in relative ratio of phospholipids. This state is comparatively close to the C-type.

The infrared spectra of serum lipids from diabetes patients, numbering about 700, all fell into one of these four types and these four types showed an interesting correlationship with clinical results and biochemical materials, as will be described below.

(I) Relationship between Infrared Spectral Type and Quantity of Total Lipids in Diabetic Serum

The quantity of total lipids in the serum of diabetes patients generally increases and over one-half of the 197 cases of diabetes examined showed values higher than the normal of 700 mg./dl. The infrared spectra in this case are mostly A- and N-types (Table I). Relationship between the quantity of total lipids and infrared spectral type

Table I. Relationship between Infrared Spectral Type and Quantity of Total Lipids in the Serum of Diabetes Patients

IR Spectral type \ Total lipids (mg./dl.)	No. of cases							
	up to 500	500~600	600~700	700~800	800~900	900~1000	over 1000	Total
N	2	16	20	13	11	3	1	66
A	0	4	20	15	11	6	5	61
C	0	2	2	5	11	4	12	36
D	2	5	10	7	6	3	1	34
Total	4	27	52	40	39	16	19	197

in diabetes patients before treatment is shown in Table I and represented by the number of cases out of a total of 197 cases examined, classified by the amount of total lipids in the serum and by infrared spectral types.

According to this result, the increase of total lipids in the serum generally occurs without any great variation in the ratio of componental cholesterol, triglycerides, and phospholipids (N-type), but there are also numerous cases in which marked change occurs in proportional quantity. When a marked increase (over 1000 mg./dl.) in total lipids occurs, the spectrum is the C-type, indicating increase of triglycerides. Infrared spectra of total lipids in the serum of diabetes patients before treatment are mostly A- or N-type. N-Type of spectra appears more frequently when the quantity of total lipids is 500~800 mg./dl., and the quantity of total lipids is still greater in A-type, appearing more frequently at the level of 600~900 mg./dl.

(2) Relationship between Infrared Spectral Type and Serum Lipoprotein Index

The value of serum lipoprotein index was found to be closely related to the type of infrared spectra. This relationship is shown in Fig. 2 in which the values of lipo-

protein index (ratio of β-lipoprotein/α-lipoprotein) are taken on the abscissa and the frequency distribution of N-, A-, C-, and D-types of infrared spectra of total lipids is taken on the ordinate, with the total number of each type taken as 100%.

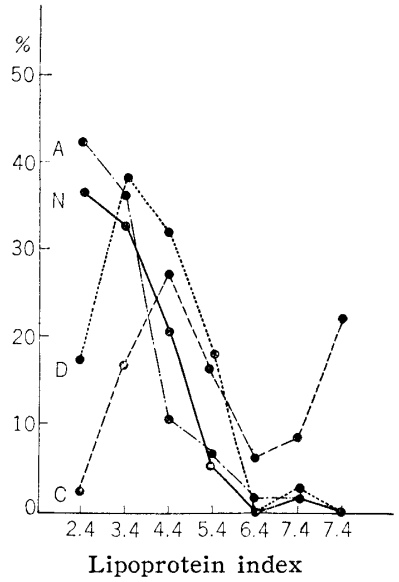

Fig. 2. Relationship between Infrared Spectral Type of Total Lipids and Lipoprotein Index Value in Diabetic Serum

According to this chart, the value of lipoprotein index is extremely high in the serum showing C-type spectrum and none of such cases showed values below the normal of 2.4. On the other hand, about 70% of the serum showing N-type spectrum had values below 3.4 and almost none above 5.4. In the serum showing A-type spectrum, the lipoprotein index value is still lower and 80% showed values below 3.4. The characteristic of C-type spectrum is that it shows two peaks, at around 4.4 and around 7.4.

Composition of lipids in β-lipoprotein has been examined in detail by Chaikoff,[12] Oncley,[13,14] Freeman,[8] and Bragdon.[15] According to these workers, the lipid bound in β-liproprotein contains far greater amount of triglycerides than that in α-lipoprotein and this agrees with the high value of lipoprotein index in the C-type of infrared spectrum.

(3) Comparison of Infrared Spectra of Total Lipids in the Serum of Patients before and after Treatment, and Judgement of Therapeutic Effect

The infrared spectra of total lipids in the serum of diabetes patients before treatment are, as indicated in Table I, N-type (33.51%) and A-type (30.96%) in the majority, and appear twice as much as C-type (18.28%) and D-type (17.26%). The patients were then treated by diet alone or administered insuline, mesoxalic acid, or tolylsulfonylbutylurea, until the blood sugar fell below 170 mg./dl. in the blood drawn on empty

Table II. Comparison of Infrared Spectra in Diabetes Before and After Treatment

Type of IR spectra	Before treatment (%)	After treatment (%)
N	33.51	66.32
A	30.96	1.05
C	18.28	14.74
D	17.26	17.89

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stomach. Infrared spectrum of total lipids in the serum was measured at this period and the results are indicated in Table II.

In this case, distribution of spectral types was 1.05% of A-type, 14.74% of C-type, and 17.89% of D-type to 66.32% of N-type. This result indicates that the A-type serum, which was found in larger number before treatment, decreased gradually during the period of therapeusis and changed into N-type by the end of treatment. On the other hand, the sera showing C- and D-type spectra retained these types throughout the repeated measurements during and after treatment, and the quantity of total proteins in the serum did not show any marked decrease, in spite of lower values of blood sugar. Fig. 3 shows the gradual conversion of A-type spectrum into N-type by treatment.

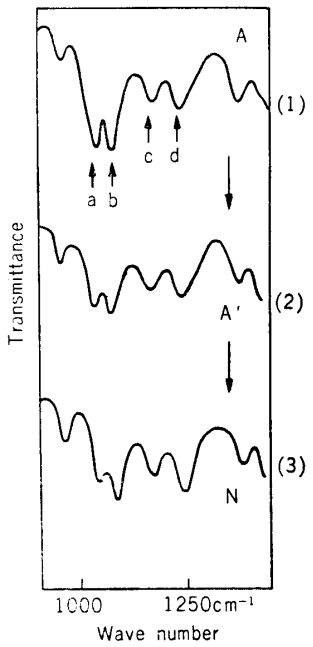

Fig. 3. Changes in Total Lipids of Serum in Diabetes Patients by Treatment (A-Type to N-Type)

(1) Before treatment
(2) During treatment
(3) After treatment

The foregoing shows that there are different types in the change of total lipids in the serum of diabetes patients and that infrared spectrum can be used to advantage in judging therapeutic effect. This mode of use of infrared spectra may also be applied widely in various diseases causing abnormality in lipid metabolism and in judging the therapeutic effect of pharmaceutics.

(4) Relationship between Infrared Spectral Types and Various Complications of Diabetes

Diabetes is often accompanied by complications like diabetic retinosis and nephrosis (proteinuria), and cases with such complications often show A-type spectrum of total lipids in their serum. In order to examine the progress of such complications, infrared spectra were examined in diabetes patients divided into three groups; those within 1 year since appearance of the disease, those suffering for 1~5 years, and those with over 5 years of experience.

Table III. Relationship between Progress in Diabetic Retinosis and the Type of Infrared Spectrum of Total Lipids in the Serum (Frequency of appearance of retinosis)

Spectral type / Diseased period (yr.)	N (%)	A (%)	C (%)	D (%)
Below 1	12.5	16.6	12.5	0
1~5	23.1	45.5	23.1	10
Over 5	20	66.7	33.3	20

Table IV. Relationship between Progress in Nephrosis (Proteinuria) and the Type of Infrared Spectrum of Total Lipids in the Serum
(Frequency of appearance of proteinuria)

Spectral type / Diseased period (yr.)	N (%)	A (%)	C (%)	D (%)
Below 1	31.8	31.3	22.2	35.7
1~5	40	87.5	42.9	35.7
Over 5	62.5	96.3	66.7	68.5

These results show that the frequency of complications increases as the period of the disease prolongs and this tendency was more marked in patients showing A-type spectrum. proteinuria was found in 96.3% of the patients who had the disease for over 5 years and whose serum lipids showed A-type spectrum. This fact suggests that the increase of serum protein which indicates the A-type spectrum or physical conditions closely related to it appears repeatedly during the long period of diabetes and accelerates vascular disturbances characteristic to diabetes. It should especially be noted that a close relationship was observed between the A-type spectrum, in which comparative increase of cholesterol is assumed, and these complications. It is therefore considered that infrared spectrum will be a valuable clinical tool in the diagnosis of diabetes.

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Summary

1) About 700 infrared spectra were examined of total lipids in the sera of diabetes patients, and a new and simple classification of these spectra was devised from the ratio of relative intensities of their absorptions.

2) It was found that there is a close relationship between the quantity of total lipids and their spectral types, and between the spectral types and serum lipoprotein index values.

3) It was found that diabetes is classified into several types from the type of these spectra and that infrared spectra would be valuable means in diagnosis and in judging therapeutic effect of pharmaceutics.

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