Paper Electrophoresis of Serum Proteins in Diabetic Patients

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The serum protein patterns of diabetic individuals have been investigated intensively in the past few years. Interest in such studies began in 1949 with the histological demonstration of mucopolysaccharides in the vascular lesions of diabetic patients,[1, 2] and with the finding that changes in serum lipoprotein patterns may be associated with the lesions of atherosclerosis.[3] The development of the technic of paper electrophoresis has made it feasible to carry out detailed studies on serum protein patterns in a wide range of clinical disorders.

Although a number of studies have been made of the electrophoretic pattern of the serum proteins in diabetics,[4–15] these earlier investigations were made on small groups of patients and yielded conflicting data. Furthermore, in certain of these studies the clinical status of the patient was not clearly defined, particularly with regard to vascular complications. It seemed that a study of a large group of diabetic patients, with careful evaluation of clinical status, might resolve some of the disparate results reported. Therefore, investigation of such a group, with and without vascular complications, was undertaken in an attempt to provide further correlation of changes occurring in serum protein fractions with those occurring in the vascular system.

MATERIALS AND METHODS

The paper electrophoresis studies were done using, in slightly modified form, the technic of Kunkel and Tiselius.[16] The electrophoretic procedure was carried out on 0.005 ml. serum applied to Whatman 3MM filter paper and used with a sodium veronal-sodium acetate buffer of ionic strength 0.1, *p*H of 8.6.[17] This buffer has given the best separations of the α-globulins in this laboratory and in at least one other.[17] The current was approximately 0.4 to 0.5 ma. per cm. width of paper and electrophoresis was carried out for about fourteen hours at room temperature (20 to 22° C.).* Ninety-five per cent of the strips were stained with Wool Black (Harleco†). The strips were rendered translucent in a mixture of α-bromonaphthalene and white mineral oil.[17] They were then sealed between cellophane tapes and electrophoretic curves determined using a photovolt densitometer (Model No. 525, 610 mμ). The relative percentages of the individual serum protein fractions were calculated from the areas measured with a planimeter. Total serum protein was determined by the Biuret method, and absolute values for each fraction were obtained by multiplying the relative percentages by the total protein content of the serum.

Initially, determinations were made using a buffer of ionic strength 0.05 and staining the paper strips with bromphenol blue. The ionic strength of the buffer was later changed to 0.1 in order to separate better the α-globulins from each other and from albumin.[17] The change to the dye Wool Black was made for two reasons: 1) Wool Black appears to be more sensitive to small changes in protein concentration, and 2) if the serum albumin content of the sample exceeded 3.6 gm. per cent, strips stained with bromphenol blue and read on this densitometer may not yield accurate values for either albumin or γ-globulin. The use of very small amounts of sera would eliminate this, but would introduce a greater sampling error. A detailed discussion of the comparison and validity of the two technics and of other aspects of the method used is reported by Tuller.[19]

Thus it was decided early in the study to use Wool

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*The voltage was not recorded or used as a means of regulation in the method given here, since the procedure had been arrived at empirically and found to be controllable by achieving the current density described. Subsequent work with the horizontal open strip method, however, has been based on voltage per cm. length of free paper.[18]

†Although this is now no longer available, Amido Black 10B (A. H. Thomas) has been shown to be equivalent in purity and results.[19]

Black instead of bromphenol blue. In those instances in which the original determinations were made with bromphenol blue, new separations of the serum proteins were prepared, the strips stained with Wool Black, and compared to the original strips. In about 5 per cent of the cases this comparison was not possible. However, all of these latter individuals had low values for both total protein and serum albumin, and it is probable that the initial staining gave accurate results.

Most studies, including this one, are done on single samples taken from individuals. Because serum protein patterns of a disturbed metabolic state might vary more with time than would those of normal individuals, the serum protein distribution was determined at intervals covering a period of several months in three healthy nondiabetic individuals and in three hospitalized diabetic patients. There were no important variations in the values for any fraction with the passage of time, even in one of the diabetic subjects who had an extremely poor prognosis. Patients with keto-acidosis were excluded, and in a majority of cases the blood samples were taken after control of the diabetes had been established under hospital conditions. Therefore, any changes from normal which appear in the pattern of the serum proteins of any diabetic individual should be due first to the metabolic disorder of long-term diabetes, and secondly might be connected with the presence of the vascular complications.

There is, at present, no evidence that there is any difference in dye uptake between any given fraction of normal sera and the corresponding fraction of pathologic sera. However, it is known that a variety of diseases affect the serum protein patterns.[20] It is, therefore, imperative that a careful clinical evaluation of all patients studied be made, since conflicting results may be obtained, if complications of a nondiabetic nature are present in the diabetic individual.

Clinical Material. The sera of 120 diabetic patients were examined. All patients were eliminated from this report if they had complicating conditions such as multiple myeloma, infectious diseases, hemochromatosis, keto-acidosis or diabetic coma, gangrene, or osteomyelitis. Of the seventy-nine remaining cases, sixty-four had developed diabetes prior to the age of thirty. All were in good nutritional status. The seventy-nine individuals were grouped as follows:

A. No clinical evidence of diabetic complications.

B. Neuropathic changes only.

C. Retinopathy but no clinical evidence of renal involvement.

D. Retinopathy and nephropathy without neuropathy.

E. Retinopathy, nephropathy and neuropathy (diabetic "triopathy"[21]). Retinopathy and nephropathy were diagnosed according to the criteria used by the Joslin Clinic.[22] A diagnosis of neuropathy was based on a clear history or finding of involvement of the central nervous system. The neuropathies included peripheral neuritis, "neuropathic foot," diabetic diarrhea, paresis of the urinary bladder, and postural hypotension.

RESULTS

Table 1 presents the clinical data for the five groups of patients. The age at onset was comparable in the five groups with the exception of Group B (neuropathic changes only), where the age at onset was somewhat later. The duration of diabetes was less in those patients with no complications, as would be expected, and also in the patients with neuropathy. However, 60 per cent of the individuals in both these groups (A and B) had had diabetes for more than ten years. The daily insulin requirement was somewhat less in Groups D and E, patients with the most severe complications.

The data obtained from paper electrophoretic studies of the sera of the five groups of patients and from six normal controls are given in table 2. The data for the six controls are closely comparable to those obtained on twenty nondiabetic controls studied in this laboratory with the horizontal open-strip method,[23] and also agree well with the values obtained by Bogdanowicz, Osinski and Stein[12] in twenty-five nondiabetic subjects.

The most accurate way of presenting values for each protein fraction is in grams per cent (absolute), but in order to compare the results with those of other investigators it is also necessary to present relative percentages; therefore, both types of data have been given in tables 2 and 3.

Statistical analysis of the data in table 2 shows that the α_1- and γ-globulin values are not significantly altered in any of the diabetic groups when compared to those for the normal controls.* Since no significant difference was found in the distribution of the protein fractions between diabetic patients without complications (A) and diabetics with only neuropathy (B), they were combined as Group AB. Similarly, the data from Group D (retinopathy and nephropathy) were compared with the data from Group E ("triopathy": retinopathy, nephropathy and neuropathy), and as there was no statistically significant difference in the serum protein fractions between these groups, they were combined as Group DE.

*Unless otherwise indicated, p is less than or equals 0.01 whenever the term "significant" is used. The t-test was used in calculating comparisons between groups.

TABLE 1

Clinical data on diabetic subjects

		Diabetic groups*				
		A	B	C	D	E
Number in group		28	7	7	11	26
Age at time of study, years	Average	28	38	36	38	40
	Range	(12-55)	(24-51)	(26-42)	(25-57)	(23-75)
Age at onset of diabetes, years	Average	18	27	18	19	20
	Range	(1-55)	(18-36)	(3-32)	(1-45)	(2-64)
Duration of diabetes, years	Average	10	10	18	19	19
	Range	(0.5-29)	(0.5-27)	(10-30)	(12-24)	(11-30)
Daily insulin dose, units	Average	56	63	55	47	42
	Range	(12-112)	(14-100)	(34-72)	(20-80)	(20-80)

*A=Without complications B=With neuropathy only C=With retinopathy only
D=With retinopathy and nephropathy E=With "triopathy" (neuropathy, retinopathy and nephropathy)

TABLE 2

The serum proteins of nondiabetic and diabetic individuals

				Protein fractions				
					Globulins			
Group*		No. of subjects	Total protein per cent S.D.	Albumin per cent S.D.	α_1 per cent S.D.	α_2 per cent S.D.	β per cent S.D.	γ per cent S.D.
				Components expressed as percentages of total planimeter areas				
Nondiabetic		6		56.9±2.2	4.9±0.9	8.7±2.0	13.3±1.8	16.2±2.2
Diabetic:	A	28		53.4±6.0	4.4±1.7	11.5±2.2	14.9±2.5	16.1±3.7
Diabetic:	B	7		50.4±4.0	5.1±1.3	11.3±2.4	16.8±1.4	16.4±2.2
Diabetic:	C	7		50.7±2.7	5.1±0.9	12.4±1.8	15.2±2.9	16.6±2.3
Diabetic:	D	11		43.3±7.4	5.8±1.6	16.7±4.4	16.7±2.5	18.1±3.0
Diabetic:	E	26		42.5±6.6	6.0±1.7	16.8±3.6	17.3±2.6	17.5±4.6
				Components expressed in grams per cent				
Nondiabetic		6	6.9±0.5	3.9±0.24	0.34±0.05	0.60±0.12	0.92±0.15	1.1±0.23
Diabetic:	A	22	6.4±0.8	3.4±0.57	0.29±0.09	0.71±0.14	0.96±0.17	1.0±0.25
Diabetic:	B	5	6.3±0.2	3.1±0.23	0.33±0.09	0.76±0.14	1.04±0.08	1.1±0.15
Diabetic:	C	5	6.9±0.7	3.4±0.46	0.33±0.06	0.82±0.14	1.05±0.18	1.1±0.13
Diabetic:	D	11	6.3±1.0	2.7±0.69	0.36±0.11	1.04±0.28	1.04±0.19	1.1±0.24
Diabetic:	E	21	5.7±0.9	2.8±0.62	0.33±0.08	0.92±0.19	0.98±0.15	1.0±0.29

*For criteria of classification see footnote of table 1.

Table 3 presents the mean ratios and standard deviations for the nondiabetic group and the regrouped diabetics. The data show significantly decreasing values for both relative and absolute amounts of serum albumin and significantly increasing values for the α_2-globulin fraction. However, the only significant change in the values for the β-globulin fraction was the increased per cent of this component in the serum of individuals in Group DE as compared to the nondiabetic group. Table 4 shows the statistical analysis of the comparison of the data for regrouped diabetics and that for the nondiabetic groups.

Relationship of Serum Proteins and Retinitis Proliferans. Since there has been some interest in the possibility of a difference in the serum protein patterns in individuals with diabetes and nephropathy with and without retinitis proliferans, Group DE was subdivided into twenty-three patients with and fourteen without this latter complication. The only difference found in the serum protein patterns of the two groups was an increased value for α_2-globulin in patients with retinitis proliferans. The *p*-value for the increase in both relative and absolute values for α_2-globulin was 0.08, which approaches borderline significance. Thus, this increase in

TABLE 3

The serum proteins of nondiabetic and diabetic individuals*

Group	Total protein per cent	S.D.	Protein fractions: Albumin per cent	S.D.	Globulins: α_1 per cent	S.D.	α_2 per cent	S.D.	β per cent	S.D.	γ per cent	S.D.
			Components expressed as percentages of total planimeter areas									
Nondiabetic			56.9	±2.2	4.9	±0.9	8.7	±2.0	13.3	±1.8	16.2	±2.2
Diabetic: AB			52.8	±5.6	4.5	±1.3	11.5	±2.3	15.3	±2.4	16.1	±3.3
Diabetic: C			50.7	±2.7	5.1	±0.9	12.4	±1.8	15.2	±2.9	16.6	±2.3
Diabetic: DE			42.8	±5.9	6.0	±1.5	16.8	±3.9	17.1	±2.7	17.7	±4.0
			Components expressed in grams per cent									
Nondiabetic	6.9	±0.5	3.9	±0.24	0.34	±0.05	0.60	±0.12	0.92	±0.15	1.1	±0.23
Diabetic: AB	6.4	±0.6	3.3	±0.50	0.30	±0.09	0.72	±0.14	0.98	±0.14	1.0	±0.23
Diabetic: C	6.9	±0.7	3.3	±0.46	0.33	±0.06	0.82	±0.14	1.05	±0.18	1.1	±0.13
Diabetic: DE	5.9	±0.9	2.5	±0.70	0.34	±0.10	0.96	±0.24	1.00	±0.15	1.0	±0.28

*Compilation of data showing means determined by combining the original data of Groups AB and that of Groups DE. Designation of original groups is given in footnote of table 1.

TABLE 4

Statistical analysis of the data from nondiabetic and diabetic groups (p values)

Groups compared	Serum protein fractions: Albumin	α_2	β
	Relative per cent		
Nondiabetic–AB*	N.S.†	<0.01	N.S.
Nondiabetic–C	<0.01	<0.01	N.S.
Nondiabetic–DE	<0.001	<0.01	<0.01
	Grams per cent		
Nondiabetic–AB	<0.02	N.S.	N.S.
Nondiabetic–C	<0.05	<0.02	N.S.
Nondiabetic–DE	<0.001	<0.001	N.S.

*Designation of original diabetic groups is given in footnote of table 1.
†N.S.=Not significant

the values might prove to be significant in larger groups.

The age at onset of diabetes may have influenced these findings. The average age at onset of the disease was fifteen years in the patients with retinitis proliferans, but was twenty-seven years in the other patients. The duration of disease was the same in both groups (approximately nineteen years). No comparative estimate of the severity of the renal disease was made in any of these individuals, but it is possible that it was more advanced in those with retinitis proliferans.

Relationship Between Elevated α_2-Globulin and Prognosis. Immediate examination of the individual values for serum protein fractions showed that five patients in Group DE had extremely high relative and absolute amounts of α_2-globulin. Also, the relative percentages of the β-globulin fractions for these five patients were somewhat elevated. Investigation of these patients revealed that all were comparatively young and had had their diabetes for from seventeen to twenty-nine years. Clinically, their prognosis was poor, and follow-up studies confirmed this since all died within forty-eight hours to one year after the analysis of serum protein had been made. Five additional patients, all in Group DE, were found also to have died within one year of the study. The clinical data and the distribution of the plasma proteins in these ten patients are presented in table 5.

Of the above ten patients, the immediate cause of death was not known in two. One patient who showed a very high α_2-globulin content died of a myocardial infarction and the other seven died in uremia. Although an extremely high relative and absolute value for the α_2-globulin fraction appears to correlate with a poor prognosis, the converse is not necessarily true, since an equal number of individuals with lower values succumbed within the same period of time (table 5).

DISCUSSION

These results confirm the consistent findings of others[10–13, 15] on small or specialized groups of diabetic patients and show values which were decreased for the albumin fraction and elevated for the α_2-globulin in the presence of vascular complications. This study also has demonstrated a tendency for these serum protein changes to be present *before* there is clinical evidence of vascular damage. There were no changes in the α_1- and γ-globu-

TABLE 5

Serum protein patterns on patients dying within two years of protein analysis

Case no.	Age	Duration of diabetes	Total protein grams per cent	Protein fractions: Albumin Per cent of total area	Albumin Grams per cent	Globulins: α_1 Per cent of total area	α_1 Grams per cent	α_2 Per cent of total area	α_2 Grams per cent	β Per cent of total area	β Grams per cent	γ Per cent of total area	γ Grams per cent
1	39	26	3.5	32.0	1.1	8.9	0.31	27.2	0.95	19.0	0.67	12.7	0.45
2	41	17	4.8	31.3	1.8	6.9	0.33	21.3	1.02	19.7	0.95	20.8	1.00
3	34	33	5.1	33.1	1.7	5.4	0.27	26.8	1.37	18.5	0.94	16.2	0.83
4	36	20	5.5	36.3	2.6	5.0	0.28	23.0	1.26	17.5	0.96	18.2	1.00
5	47	29	5.7	29.0	1.6	6.3	0.35	20.4	1.16	14.8	0.85	29.6	1.70
6	33	23	—	33.2	—	6.6	—	20.5	—	18.0	—	21.7	—
7	23	20	5.2	43.4	2.4	10.4	0.52	18.6	0.96	18.3	0.94	9.3	0.48
9	53	15	5.3	41.6	2.2	6.0	0.32	18.7	0.99	15.0	0.79	18.7	0.99
11	75	11	—	41.5	—	7.8	—	15.6	—	15.9	—	15.3	—
12	40	12	5.4	42.3	2.3	7.4	0.40	11.8	0.84	16.6	0.90	21.8	1.18

lin fractions, although other workers have reported changes in the latter component.[8, 13–15] The results reported here are suggestive of an increase in the relative percentages for the β-globulin fraction, although this increase is not statistically significant except in those patients with well advanced vascular complications (Group DE). There was no suggestion of this change in the absolute values.

The data presented here do not, unfortunately, resolve the controversy concerning the values for the β- and γ-globulin fractions in diabetic individuals with retinopathy and nephropathy. Much of this controversy may be due to differences in technics. Reports based on measurements made by free electrophoresis indicate an elevation of the relative percentages of the β-globulin fraction.[4–8] These increased values are probably due to refractive index variations because of the large amounts of lipoproteins present in the sera of diabetic patients with vascular complications.[24] On the other hand, investigators who used the technic of paper electrophoresis have reported an increase in the percentages for the β-globulins, while reporting no change[12] or a decrease[15] in the γ-globulin fraction. Since Amido Black 10B and bromphenol blue only stain the protein moiety, and since the amount of protein material in the lipoproteins is about 25 to 35 per cent of the total molecule, any increases in lipoproteins (such as up to 300 mg. per cent) in the sera of diabetic individuals probably will not produce detectable increases in the protein moiety of the β-globulin fraction. However, large amounts of lipoproteins tend to make it more difficult to resolve the β- and γ-globulin fractions. Therefore, it is possible that the disparity in results concerning these fractions is due to problems of interpreting the electrophorerograms.

The present study indicates that there is a decrease in serum albumin in diabetic patients without clinically demonstrable nephropathy, but that it does not clarify the origin or possible significance of such a decrease. The marked fall in the amount of serum albumin in the diabetic with nephropathy is undoubtedly due to impaired renal function. The change in albumin values in the other diabetic subjects may be due to early subclinical vascular change. An elevation of the blood urea nitrogen (one of the criteria of renal damage) does not occur until 75 per cent of the nephrons have been destroyed,[25] although albuminuria may appear earlier. Also, the work of Ditzel[26] demonstrates that changes in the smaller vessels take place early in the diabetic state, accompanied by the exudation of plasma fluid into the perivascular tissue. Therefore, renal vascular changes may exist undetected in those diabetics with clinically demonstrable retinopathy, and this may be primarily responsible for the lowered values of serum albumin seen in these patients. In patients with no clinically detectable retinopathy or nephropathy, however, changes of the type demonstrated by Ditzel may be the paramount reason for the initial decrease in albumin. Also, Young and Webber[9] have suggested that hepatic function should be studied in many pathological states, since the origin of albumin appears to be the liver. Although it is possible that there is some hepatic dysfunction in the patient who is not in obvious poor control as shown by keto-acidosis, clinical studies have shown such dysfunction to be uncommon in diabetic individuals apart

from frank liver diseases.[27] Thus, in diabetic individuals without clinical nephropathy, both the origin and the significance of the decrease in serum albumin values are unknown.

The increased α_2-globulin values are very striking and may be of importance. Although there is a significant increase in the amount of the α_2-globulin fraction in the *presence* of retinopathy and nephropathy, increased values were also observed in patients *without* these complications. This confirms the work of Bogdanowicz, Osinski and Stein,[12] who also studied a comparable group of individuals with early onset of diabetes. Little is known about the origin of the α_2-globulins, but Young and Webber[9] have suggested a possible relationship to disturbances in bone metabolism. The progressive, marked, and selective increase in this fraction of the plasma proteins suggests that it may be of significance in the pathogenesis of the retinal and renal vascular lesions of diabetes. As has been demonstrated histochemically, these lesions contain large amounts of mucopolysaccharides[1, 2] and a large proportion of protein-bound carbohydrates are known to be bound to the α_2- fraction.[28] On the other hand, this increase cannot be regarded as specific for diabetes, as elevations of α_2-globulin have been reported in acute inflammatory diseases, in acute tuberculosis and in certain malignant diseases.[9, 20] There is no evidence as to whether the increase in the different clinical conditions is due to a common stimulus, or occurs in response to different pathological or metabolic stimuli. A study of the subfractions of the α_2-globulin component might clarify this problem by showing whether the increase in amount is due to the same or to different subfractions in the various disease states.

The possible relationship between vascular complications and variations in the serum protein pattern has been considered above. However, other variables may play a part in these changes. For example, although neuropathy appears to have no effect on the serum protein pattern in the presence of the vascular complications, limited ultracentrifugal studies on lipoproteins[29] indicate that some relationship may be present. It is also possible that the increase in the relative percentage of the β-globulin fraction in Group B (neuropathy alone) may be related to changes in lipoproteins. However, in order to study individuals exhibiting symptoms of neuropathy only, it was necessary to select individuals whose average age at onset of diabetes was greater than that of those diabetics without any complications.

Subsequent investigation has tended to confirm the impression that, whatever the cause of the central nervous system disorder in diabetes, there is a concomitant small change in the serum proteins or their conjugates.

In order to consider the duration of diabetes as an independent variable and its possible effect on the serum protein pattern, it is necessary to consider only the data from Group A (no complications). No statistical correlation of protein values with duration could be made, since there was considerable scatter of the data in this group of patients and the durations of the disease were not well distributed. However, the data gave the impression that the α_2-globulin values became progressively elevated with the increase of the duration of diabetes. A subsequent study in this laboratory also confirmed this impression.[23]

SUMMARY

1. The serum protein patterns of seventy-nine diabetic patients in various stages of the disease were determined by use of paper electrophoresis analysis.

2. Previous results on small groups of diabetics with vascular complications were confirmed: namely, a decrease in the serum albumin, no change in the α_1-globulin fraction and an increase in the α_2-globulin fraction. The controversy regarding possible changes in the β- or γ-globulin fractions was not resolved.

3. Decreases in the albumin and increases in the α_2-globulin fractions also were found, though of lesser degree, in diabetic subjects without complications.

4. The duration of diabetes and the progression of the vascular complications are two factors which appear to affect the serum protein pattern.

SUMMARIO IN INTERLINGUA

Electrophorese a Papiro del Proteinas Seral in Patientes Diabetic

1. Le configurationes del proteinas seral de septantanove patientes diabetic in varie stadios del morbo esseva determinate per medio de electrophorese a papiro.

2. Le resultatos previemente obtenite con pauco numerose gruppos de diabeticos con complicationes vascular esseva confirmate. Iste resultatos esseva un reduction del albumina seral, nulle alteration in le fraction globulina alpha-1, e un augmento in le fraction globulina alpha-2. Le controversia relative a possibile alterationes del fractiones globulina beta e gamma non esseva resolvite.

3. Reductiones de albumina e augmentos del fraction globulina alpha-2 esseva constatate etiam in diabeticos sin complicationes, sed a grados minus pronunciate.

4. Le duration del diabete e le progression del complicationes vascular es duo factores que affice apparentemente le configuration del proteinas seral.

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Information from all available sources indicates a rising incidence of acute myocardial infarction among white individuals in the United States and the data from the autopsies studied by the group at Washington University tend to confirm this rise. The over-all incidence of acute myocardial infarction among the Barnes autopsies was twenty-nine times as high in the decade 1945 to 1954 as it was in the decade 1910 to 1919. The rise occurred in all age groups of both sexes and was not simply a result of an aging population. No evidence was found to indicate that the rise in incidence could be accounted for by a rise in the incidence of diabetes mellitus, hypertension, and obesity. It seems likely that some changing factor(s) in our civilization is responsible and it is remarkable that its effect has been almost exclusively confined to members of the white race.

From "Fatal Acute Myocardial Infarction: Sex, Race, Diabetes and Other Factors," by Wilbur A. Thomas, M.D., in *Nutrition Reviews*: Vol. 15, No. 4, April 1957, pp. 97-101.