ELECTROPHORETIC STUDIES OF HUMAN SERUM AT pH 4.5 [1]

By JOHN W. MEHL and FLORENCE GOLDEN

(From the Department of Biochemistry and Nutrition, University of Southern California, School of Medicine, and the Los Angeles County Hospital, Los Angeles, Calif.)

(Submitted for publication April 25, 1950; accepted, June 12, 1950)

During the course of a recent study of certain mucoproteins in serum (1, 2) we became interested in the general properties of the electrophoresis pattern of serum at pH 4.5. It was felt that the electrophoresis pattern at pH 4.5 might be of some value in the study of pathological sera in other respects than as a means of demonstrating changes in mucoprotein. The present paper is concerned primarily with the comparison between the electrophoresis patterns of various samples of pathological sera at pH 8.5 and pH 4.5.

METHODS

The methods were those previously described (1, 2). The pH 8.5 buffer consisted of sodium diethyl barbiturate-diethyl barbituric acid and sodium chloride, and the pH 4.5 buffer of sodium acetate-acetic acid and sodium chloride. The ionic strength was 0.1 in both cases. The temperature of the electrophoresis experiments was 2.0° C., the time three hours, and the voltage gradient 5–6 v./cm. Solutions were dialysed against two changes of buffer for 24 hours at 4–6° C. against each portion of buffer. In some instances the sample was allowed to remain in the second portion of buffer for two to three days. The dilution of the serum was kept uniform by diluting a measured volume of serum with three volumes of buffer before dialysis. After dialysis, the bag was rinsed with buffer and the contents and washings diluted to a volume five (and occasionally six) times that of the original serum.

RESULTS

Choice of pH. Some of the reasons for the choice of pH 4.5 have been noted previously (2). The choice was originally based upon the fact that the study of isolated samples of mucoprotein indicated that it should be largely negatively charged at this pH, whereas the albumin would be essentially immobilized, and the globulins positively charged. In Figure 1 the electrophoresis patterns of a normal serum sample at pH 3.5, 4.5, and 5.1 are shown. It had already been shown by Petermann and Hogness (3) that at pH 4.0 the albumin is not well separated from the globulin. At the still more acid pH of 3.5, the same problem of separation of albumin and globulin fractions is also found. It is also evident that the ascending and descending boundaries show a marked difference at pH 3.5 (as they do at pH 4.0). The same normal serum at pH 4.5 shows a reasonably good separation of albumin from globulin, and three globulin peaks. The ascending and descending boundaries differ, but not quite so markedly as at the lower pH. A pH of 5.1 might offer a somewhat more satisfactory separation of globulin fractions, but the slower components present in the

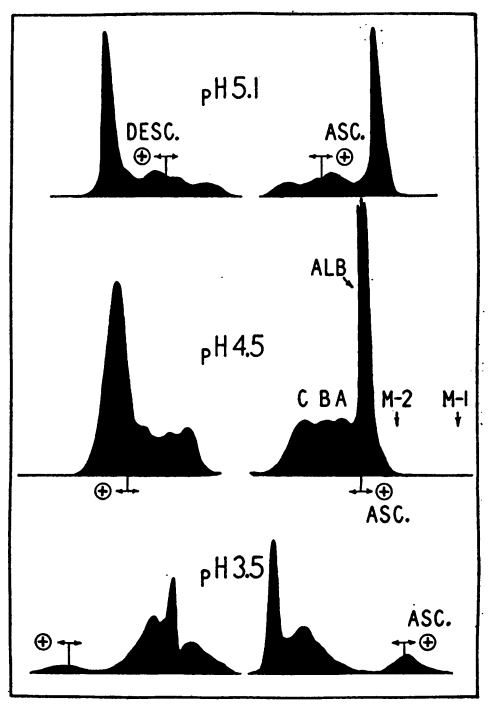

FIG. 1. ELECTROPHORESIS PATTERNS OF A SAMPLE OF SERUM AT pH 5.1, 4.5, AND 3.5

Samples at pH 5.1 and 3.5 were diluted to six volumes and at pH 4.5 to five volumes. Boundaries were photographed after three hours at 5.6 volts/cm.

[1] This investigation was supported by a research grant from the National Cancer Institute, U. S. Public Health Service. Contribution number 258 from the Department of Biochemistry and Nutrition.

TABLE I

	Sample 1		Sample 2		Sample 3	
	Original	After pH 4.5	Original	After pH 4.5	Original	After pH 4.5
Albumin	50	51	55	58	46	53
α_1-globulin	5	5	5	5	5	3
α_2-globulin	9	9	7	9	8	6
β-globulin	15	16	15	12	14	12
γ-globulin	21	20	18	16	20	19

mucoprotein fractions would be obscured by the albumin.

The effect of dialysis at pH 4.5. A small amount of protein has always been found to precipitate during the dialysis of serum at pH 4.5. This is, of course, removed by centrifugation, and although the amount is small, we were concerned that some significant change in the electrophoresis pattern might be produced. Three samples of serum were, therefore, subjected to electrophoresis at pH 8.5. A second sample of each serum was dialysed as though preparing it to be run at pH 4.5, and the precipitate removed. The clear supernatant was then redialyzed and run at pH 8.5. There is no striking difference in the character of the electrophoresis pattern after dialysis at pH 4.5. The comparison of the distribution of areas is given in Table I. Although there is some indication of a decrease in globulin, no fraction is predominantly affected, and this rather superficial investigation satisfied us that no major portion of one component was being removed in the precipitate formed during the dialysis of normal serum at pH 4.5.

Nature of the components observed at pH 4.5. It will be seen from Figure 1 that three components which have a positive charge can be observed at pH 4.5. These have been designated A, B, and C, and must be identified with the globulins. In addition to these and the "albumin" fraction, there are the components M-1 and M-2, the positions of which have been indicated. The M-1 component has been shown to be a mucoprotein (2) and it is possible that M-2 is also a mucoprotein.

It is of some interest to compare the values ob-

TABLE II

Comparison between electrophoretic components at pH 8.5 and pH 4.5

Case	Diagnosis	Per cent of total, pH 8.5						Per cent of total, pH 4.5					
		Alb.	α_1	α_2	β	x*	γ	M-1	M-2	Alb.	A	B	C
1	Multiple myeloma	65	6	13	11		5	<1	3	63	12	9	13
2	Plasmoma	61	5	7	14		15	1	—	57	8	14	21
3	Normal?	53	5	10	16		16	<1	2	54	11	11	21
4	Periarteritis	51	4	13	18		15	1	6	50	12	17	14
5	Malignant thymoma	50	6	15	15		14	<1	5	52	10	12	21
6	Normal ?	49	5	10	16		20	1	—	54	10	11	24
7	?	48	7	25	12		8	3	6	48	15	12	16
8	Rheumatoid arthritis	47	6	14	19		13	1	7	53	11	17	12
9	Rheumatoid arthritis	46	4	25	11		13	1	10	51	11	14	14*
10	Rheumatoid arthritis	42	7	12	23		14	1	8	46	12	14	20
11	?	41	7	14	17		20	1	6	50	12	17	14
12	Pneumonia	39	4	12	15		30	1	3	46	7	9	34
13	Rheumatic fever	38	7	15	19		21	3	4	51	10	10	21
14	Multiple myeloma	36	2	4	7	47	3	1	<1	38	—	26	35
15	Multiple myeloma	34	2	12	46		7	<1	—	44	30	16	9
16	Carcinoma	33	4	17	22		23	3	9	51	12	10	16
17	?	33	12	18	17		19	2	9	43	16	14	16
18	Multiple myeloma	32	5	11	15		37	<1	3	43	9	12	32
19	Rheumatoid arthritis	32	11	16	20		21	2	11	51	6	↔	30
20	Kimmelstiel-Wilson dis.	31	7	18	30		14	1	7	47	10	24	11
21	Reticulum cell carcinoma	23	6	9	21	14	27	1	4	42	15	12	27
22	Multiple myeloma	22	2	5	57		3	<1	<1	29	35	34	3
23	Kimmelstiel-Wilson dis.	21	3	19	39		17	1	3	38	11	31	16
24	?	20	7	11	14		48	2	5	30	5	—	57
25	Multiple myeloma	19	2	9	3		66	<1	4	23	2	3	68
26	Cirrhosis, Cellulitis	16	2	12	7		63	<1	2	26	13	16	43
27	Aleukemic leukemia	15	2	2	4	70	6	<1	<1	23	—	67	9
28	Multiple myeloma	9	3	11	74**		3	<1	<1	20	11	69	—

* Abnormal component between β- and γ-globulin.
** Two components.

tained for the proportion of albumin and globulins A, B, and C at pH 4.5 with those for albumin and α-, β-, and γ-globulin at pH 8.5. For this comparison we have used the descending boundary at pH 8.5 and the boundary descending toward the negative electrode at pH 4.5. The latter choice was determined by the fact that the peaks are sharper in this boundary, so that the albumin does not overlap M-2 and component A as badly. For these, and probably other reasons, the albumin peak is always considerably larger in the boundary ascending to the negative electrode.

The salt boundary introduces certain complications into the comparison at pH 4.5, since it will be included with the albumin. However, the fact that the albumin is near its isoelectric point should reduce the magnitude of the salt boundary, and no attempt has been made to correct for this effect. At pH 8.5, in the buffer which we have employed, the mobility of the γ-globulin is less than it is in the buffer of diethyl-barbiturate and diethyl-barbituric acid only, and the δ and ε boundaries are not separated from the γ-globulin. The values for the components at pH 8.5 have been corrected for the contribution of the salt boundary by attributing 6 per cent of the total area to the salt boundary. This figure was arrived at by measuring the area of the ε boundary in albumin solutions of various concentrations, and by a comparison with some sera between values in the buffer which we have used, and the diethyl-barbiturate buffer without sodium chloride. In Table II we have collected the results obtained with 28 sera covering a wide range of albumin and globulin concentrations. These have been arranged in the order of decreasing relative albumin concentrations as measured at pH 8.5.

Electrophoresis of pathological sera at pH 4.5. In Figure 2 are presented some electrophoresis patterns which illustrate changes which may be seen at pH 4.5 in pathological sera. The patterns at pH 8.5 have been included for comparison. Figures 2A and 2B are illustrative of cases in which the M-2 component is increased, and in which this increase appears to be associated with an increase in α_2-globulin. The patterns at pH 4.5 are like those commonly seen in cases of carcinoma and pneumonia, respectively. In 2C is a case of Kimmelstiel-Wilson disease which illustrates the fact that an increase in the α_2-globulin need not indicate a marked increase in M-2. The patterns 2D and 2E are from patients with rheumatoid arthritis, the former being a very severe case in which inanition may be responsible for the marked depletion of the albumin. Changes seen in rheumatic fever, shown in 2F, are not too dissimilar, except for the increased γ-globulin and component C. In 2G, the increase in γ-globulin appears to

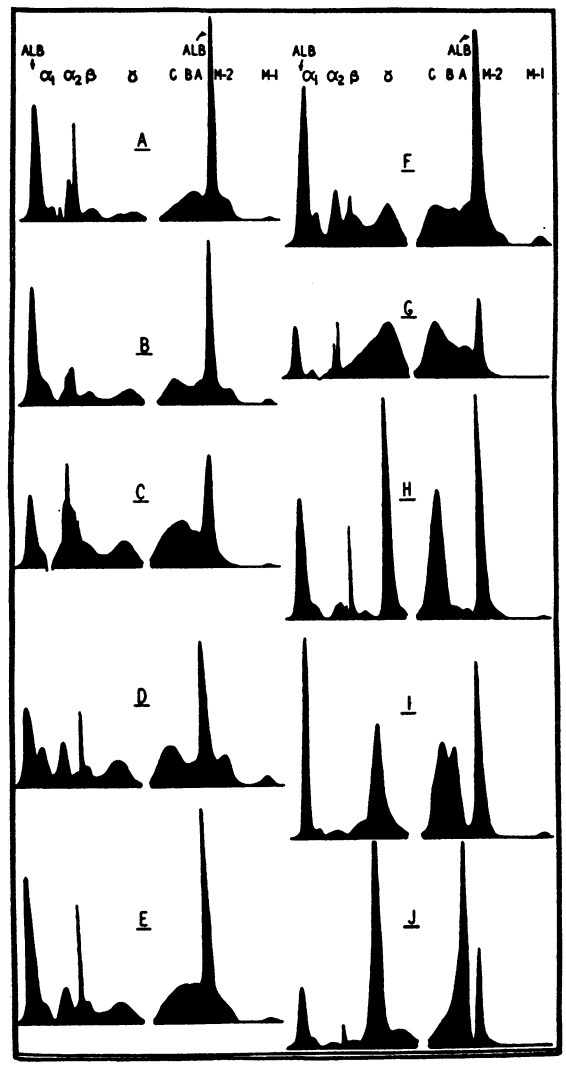

FIG. 2. A COMPARISON BETWEEN SOME ELECTROPHORESIS PATTERNS OF PATHOLOGICAL SERA AT pH 8.5 AND pH 4.5

The descending boundary at pH 8.5 on the left, and the boundary descending toward the negative electrode at pH 4.5 on the right. A, carcinoma; B, pneumonia; C, Kimmelstiel-Wilson disease; D and E, rheumatoid arthritis; F, rheumatic fever; G, cirrhosis with cellulitis (?); H and I, multiple myeloma; J, aleukemic leukemia.

involve the normal γ-globulin fractions, with the shift toward larger proportions of the more rapidly moving γ-fraction that is usually seen when the γ-globulin increases. In this case, the pattern at pH 4.5 shows a general increase in component C, with the same evidence of heterogeneity that is seen in the γ-globulin. A case of multiple myeloma, with an abnormal component in the γ-globulin (2H) which appears to be quite homogeneous at pH 8.5 also shows a large increase in component C. Another case of multiple myeloma in which the abnormal globulin is between the γ- and β-globulins (2I), on the other hand, shows an increase in both components B and C at pH 4.5. Still another case (2J) which also shows an abnormal globulin between the γ- and β-globulin shows an increase at pH 4.5 which is almost entirely in component B.

DISCUSSION

The electrophoresis patterns presented indicate that experiments carried out at pH 4.5 may be of value in supplementing those obtained at pH 8.5. The separation of the globulin components A, B, and C at pH 4.5 is not as satisfactory as the separation of the α-, β-, and γ-globulins is at pH 8.5. Despite this difficulty, it is often possible to separate components at pH 4.5, even though they may appear to be relatively homogeneous at pH 8.5 (Figure 2I).

The relation between components at pH 8.5 and pH 4.5 is not entirely established by the present work, but certain conclusions may be drawn. The nature of M-1, which appears to be a homogeneous mucoprotein, has already been discussed and its relation to the α_1-globulin has been noted (2). It is also evident from the electrophoresis studies presented here that this mucoprotein is not the only component present in the α_1-globulin, which is almost always larger than the M-1 component. We have no further evidence regarding the nature of M-2, but it is again suggested that it is primarily associated with the α_2-globulin at pH 8.5. As a general rule, the sera showing a high M-2 also show a considerable elevation of α_2-globulin. However, M-2 is never as large as α_2, and in one of the cases α_2 is increased markedly without a very notable increase in M–2 (Figure 2C).

The results given in Table II leave little doubt that the albumin is all in the fraction which we have designated as albumin at pH 4.5. The trend of albumin concentration measured at pH 4.5 is generally the same as that at pH 8.5, and the agreement is reasonably good at higher albumin concentrations. At relative albumin concentrations below about 40 per cent, however, the values at pH 4.5 are usually larger by more than 10 per cent of the total area, and in some cases the difference amounts to 20 per cent. Although any effects due to the inclusion of the salt boundary with the albumin at pH 4.5 would be in this direction, it does not seem likely that this can be the major factor. In serum No. 25, for example, with 68 per cent of the area in the most highly charged globulin component, C, the discrepancy in the albumin is only 8 per cent of the total area. It appears that part of the α-globulin may be nearly isoelectric at pH 4.5, for those cases, in this lower range of albumin values, in which the α-globulins are also very low show the best agreement between the relative albumin concentrations at pH 4.5 and pH 8.5 (Sera Nos. 14, 22, 25, and 27). It should also be noted, that these same sera have abnormally low concentrations of β-globulin except for No. 22, in which the β-globulin is probably abnormal.

Nothing very conclusive can be said about components A and B, except that some degree of correspondence with α- and β-globulin, respectively, is suggested by the data of Table II. However, in sera from patients with multiple myeloma in which a large component with the mobiltiy of β-globulin is present, the increase may be largely in component A, in A and B, or almost entirely in B. Component C generally shows a rather close correspondence with the γ-globulin, and in a number of very abnormal sera the agreement is good (Sera Nos. 12, 22, 25, 27, and 28). Certainly, none of the sera in which the γ-globulin is markedly increased have failed to show an increase in component C. In those cases of multiple myeloma in which abnormal amounts of globulin are present in the slower γ-globulin region, the increase at pH 4.5 is confined to component C.

The studies of Cohn and co-workers (4) have made the heterogeneity of the globulin fractions very evident, particularly in the case of α- and β-globulins. It is not surprising, therefore, that the present study indicates that material with the mobility of β-globulin may appear in component A or

B, or that α-globulin may contribute to the "albumin" peak at pH 4.5.

Experience with pathological sera at pH 4.5 is still very limited, and at present it would appear that studies at this pH will be of particular value where changes in M-1 and M-2 are of interest, or in such conditions as multiple myeloma, where further evidence regarding the homogeneity or heterogeneity of an abnormally large globulin component is desired. It is possible, of course, that a further, systematic investigation of pathological sera at pH 4.5 would reveal changes of particular significance for particular diseases.

SUMMARY

In addition to the mucoprotein component, M-1, and another component with a more acid isoelectric point than albumin, M-2, the albumin and three globulin components may be distinguished at pH 4.5. The agreement between the proportion of albumin measured at pH 4.5 and that at pH 8.5 is generally reasonably good. The globulin component C, at pH 4.5, shows the best correspondence with γ-globulin; and components A and B correlate best with α- and β-globulin, respectively.

In addition to making possible the recognition of changes in M-1 and M-2, electrophoresis at pH 4.5 may supplement the information at pH 8.5 by providing further evidence regarding the homogeneity of abnormal components appearing in such conditions as multiple myeloma.

BIBLIOGRAPHY

1. Mehl, J. W., Humphrey, J., and Winzler, R. J., Mucoproteins of human plasma. III. Electrophoretic studies of mucoproteins from perchloric acid filtrates of plasma. Proc. Soc. Exper. Biol. & Med., 1949, **72**, 106.
2. Mehl, J. W., Golden, F., and Winzler, R. J., Mucoproteins of human plasma. IV. Electrophoretic demonstration of mucoproteins in serum at pH 4.5. Proc. Soc. Exper. Biol. & Med., 1949, **72**, 110.
3. Petermann, M. L., and Hogness, K. R., Electrophoretic studies on the plasma proteins of patients with neoplastic disease; acid protein present in plasma. Cancer, 1948, **1**, 104.
4. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K., and Uroma, E., A system for the separation of the components of human blood: quantitative procedures for the separation of the protein components of human plasma. J. Amer. Chem. Soc., 1950, **72**, 465.