Journal of Chromatography, 146 (1978) 77—84
Biomedical Applications

CHROMBIO. 171

ELECTROPHORETIC BIMORPHISM OF SERUM ALBUMIN IN THE PRESENCE OF INDOCYANINE GREEN

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(First received December 12th, 1977; revised manuscript received March 1st, 1978)

SUMMARY

The presence of indocyanine green during extended traditional electrophoresis and immunoelectrophoresis of serum is associated with bimorphism of albumin. This occurs over a range of dye—albumin molar ratios an order or more greater than was obtained in similar phenomena described previously. The bimorphism seems not to be dose dependent beyond a certain point, and the two albumins so separated show tinctorial differences. The phenomenon has been observed to apparently the same degree in all normal sera tested, and may represent a means of distinguishing ligand-loaded and ligand-light serum albumin.

INTRODUCTION

Serum albumin generally migrates as a single entity during traditional electrophoresis, but in certain circumstances may separate into two or more components. Two fundamentally different types of such electrophoretic polymorphism are recognized [1] and, in both, demonstration depends to some extent upon the conditions of analysis. The primary variety is based upon hereditary differences in amino acid make-up, includes numerous both unusually fast and unusually slow variants [2] and has been appropriately designated "alloalbuminemia" [3]. The other, apparently secondary, form appears to be attributable to either (a) various ligands including non-esterified fatty acids (NEFAs) [4—9], dyes [10] and penicillin and cephalotin [11]; or (b) partial enzymatic degradation as may occur in acute pancreatitis with hyperamylasemia [12].

In previous reports of the varieties of polymorphism of albumin induced by ligands, the quantity of the abnormal or new component present has varied over a relatively narrow range directly with the concentration of ligand, to the extent that complete conversion to the new form has usually occurred with a ligand—albumin ratio of little more than first order proportions. By contrast,

the anomalous fast albumin component described in this communication is not evident below an indocyanine green (ICG)—albumin molar ratio of 32, and even at a dye—albumin ratio of over 1400:1 has not progressed further than approximately a 50% proportion. The mechanism of this phenomenon has not yet been elucidated, but the relatively high concentrations of inducing agent required for full development suggests that the manifestation may reflect alteration of the electrophoretic medium rather than any straightforward dye-binding artifact.

MATERIALS AND METHODS

Blood was taken into plain tubes from healthy male and female human subjects, and allowed to clot before separation of serum by centrifugation. Sera were analyzed immediately after separation, or after periods of up to 5 days at 4°, or after freezing at —70° and thawing at 37°. These various forms of storage were found not to affect the results of the experiments. The electrophoretic and immunoelectrophoretic analyses were carried out with the serum, undiluted, and at dilutions of 1:2, 1:4, 1:8, 1:16 and 1:32, with immediately-prior additions of either an aliquot of electrophoresis buffer, or of a solution of ICG (K & K Labs., Plainview, N.Y., U.S.A.; M.W. = 774.99) in electrophoresis buffer at a series of concentrations to give dye—albumin molar ratios as shown in Table I (approximated for albumin in serum at G = 4.0%). Exclusion experiments comparing ICG and Ponceau S (C.I. No. 27195; M.W. = 760.598) were carried out by mixing aliquots of different dilutions of serum with one or two aliquots, alternately, of both dyes dissolved in electrophoresis buffer at concentrations of approximately 0.023 *M*, a short time before electrophoretic analysis.

Cellulose acetate electrophoresis was carried out after the technique of

TABLE I

APPROXIMATE RATIOS OF MOLES OF ICG PER MOLE OF ALBUMIN

Calculated in mixtures of dilutions of ICG (vertical) and of serum (horizontal). Thus ICG at 0.9% and serum at 1/32 gives a dye—albumin ratio of approximately 640:1. ND = not done.

ICG solution G(%)	Serum dilution					
	Undiluted	1/2	1/4	1/8	1/16	1/32
0.0028	ND	ND	ND	0.5	1	2
0.0056	ND	ND	ND	1	2	4
0.0112	ND	ND	ND	2	4	8
0.0225	0.5	1	2	4	8	16
0.045	1	2	4	8	16	32
0.09	2	4	8	16	32	64
0.225	5	10	20	40	80	160
0.45	10	20	40	80	160	320
0.90	20	40	80	160	320	640
1.80	40	80	160	320	640	1280
2.0	44	89	178	355	711	1422

Kohn [13] using materials by Helena Labs., Beaumont, Texas, U.S.A., including a "Super-Zee" semi-automatic wire-bridge type serum sample applicator which delivers approximately 0.2 μl of serum sample onto the electrophoresis strip in the form of a narrow band. Specimens were electrophoresed for 15 min at constant 5 mA per strip (6—7 V/cm) in an aqueous barbital buffer of pH 8.6 comprising 0.015 *M* diethyl barbituric acid with 0.075 *M* sodium diethyl barbiturate. Completed preparations were then stained for proteins by immersion in an aqueous solution of Ponceau S at G 0.9/l and sulphosalicylic and trichloracetic acids each at G 13.4/l and afterwards cleared in a solution of glacial acetic acid 20% in methanol.

Micro-immunoelectrophoresis was carried out after the technique of Scheidegger [14], and simple agar electrophoresis was effected by a similar process but omitting the antiserum stage, the preparations being fixed and stained immediately after completion of electrophoresis. In both types of preparation the medium used was "Ionagar No. 2" (Oxoid, London, Great Britain), 1% in aqueous barbital buffer comprising 0.01 *M* diethyl barbituric acid and 0.05 *M* sodium diethyl barbiturate to give a pH 8.5 ± 0.1, with 0.05% (w/v) sodium azide as preservative. Sample volumes of 3 μl of previously mixed aliquots of serum-dilution and either plain buffer or dye-dilution in buffer were subjected to electrophoresis for 60—180 min at constant 10 mA per slide (approximately 20 V/cm). Immunoelectrophoretic preparations were developed with a rabbit antisera either polyvalent to whole human plasma and serum, or monovalent to albumin (Canadian Hoechst, Toronto, Canada) with a diffusion period of 16—18 h at room temperature in a moist chamber. Each slide was then gently overlaid with a 15 cm long strip of 7.5 cm wide Whatman 31ET chromatography paper to allow absorptive removal of fluid and unprecipitated proteins, and afterwards washed for 48 h in changes of stirred normal saline. Completed preparations were simultaneously fixed and stained for protein in a solution of 0.1% (w/v) each of water-soluble Nigrosin (C.I. No. 50420) and Ponceau S for 16 h, and cleared in changes of 2% aqueous acetic acid. All chemicals used were of analytical grade.

RESULTS

The presence of ICG during electrophoresis resulted in anodal extension of the albumin region to the point of bimorphism, and anodal extension of the precipitin arc in immunoelectrophoresis. In runs of ordinary length, these results were very much as have been previously reported [16]. The very extended runs used here, however, directly increased the effect so that bimorphism was electrophoretically much more obvious, and the precipitin arc in immunoelectrophoresis took on a wavilinear* conformation.

*The word "wavilinear" is coined here upon an analogy to curvilinear, to describe a wave form quality of a precipitin arc linear upon a straight axis. The dictionary definition of the word curvilinear is inadequate in this respect since it distinguishes from the conformation here designated wavilinear neither the lesser type of double arc formed as an "S" by two opposite convexities, nor a simple "single-humped" arc. The word was coined by Wendy Somerville, R.T., and the necessary etymological research was carried out by Dr. C. Wood, Department of English, McMaster University, who certified its contemporary absence from the English language, and that its inclusion would fill a need not presently catered for otherwise.

Cellulose acetate electrophoresis

The presence of ICG in sufficient concentration produced two effects. The first was lengthening of the albumin band due to a new fast component which began to appear at a dye—albumin molar ratio of approximately 30:1 and, at a dye—albumin molar ratio of greater than approximately 45:1, what appeared to be a slightly slower than normal small new component in addition. The second alteration was a slight foreshortening of the gamma globulin spread, combined with the greater lateral diffusion of this component previously reported as dye induced diffusion alteration (DIDA) [17]. At the greatest concentrations, some ICG ran free anodally to the electrophoretic spread of the serum proteins, well ahead of the albumin area. This dye disappeared during processing of the cellulose acetate strip but ICG was evident in processed preparations as a dense green band extending from the origin anodally and fading out into the alpha-2 to alpha-1 regions, presumably reflecting especially firm binding by lipoproteins or other proteins [16, 18].

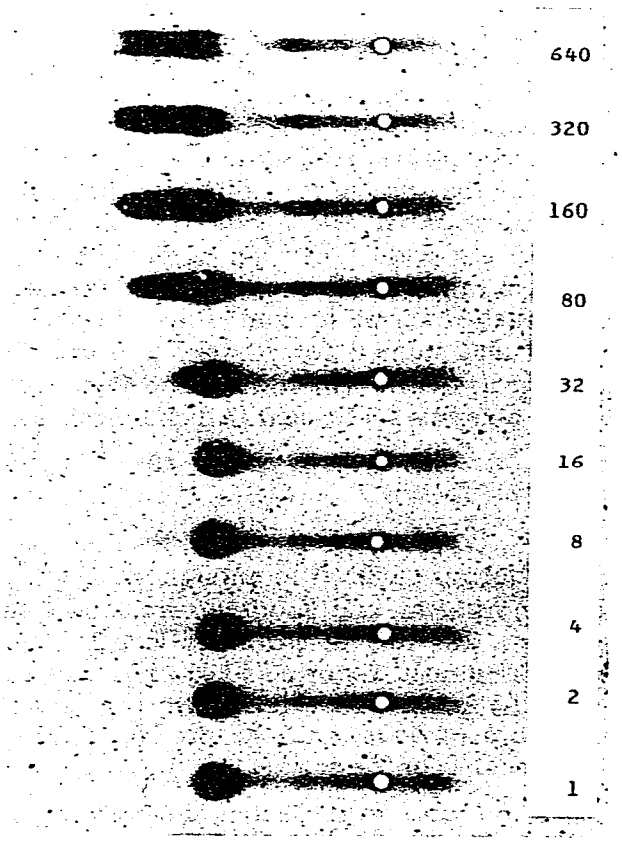

Fig. 1. Simple agar gel electrophoresis of aliquots of normal human serum at a dilution of 1:16 and ICG at the concentrations noted in Table I giving ICG—albumin molar ratios as indicated. Anodes left, cathodes right. The very beginning of the new fast albumin is visible at a molar ratio of 32:1, and is very obvious at all ratios higher than this. At ratios of 160:1, and above, the proportions of the two albumins appear to remain the same.

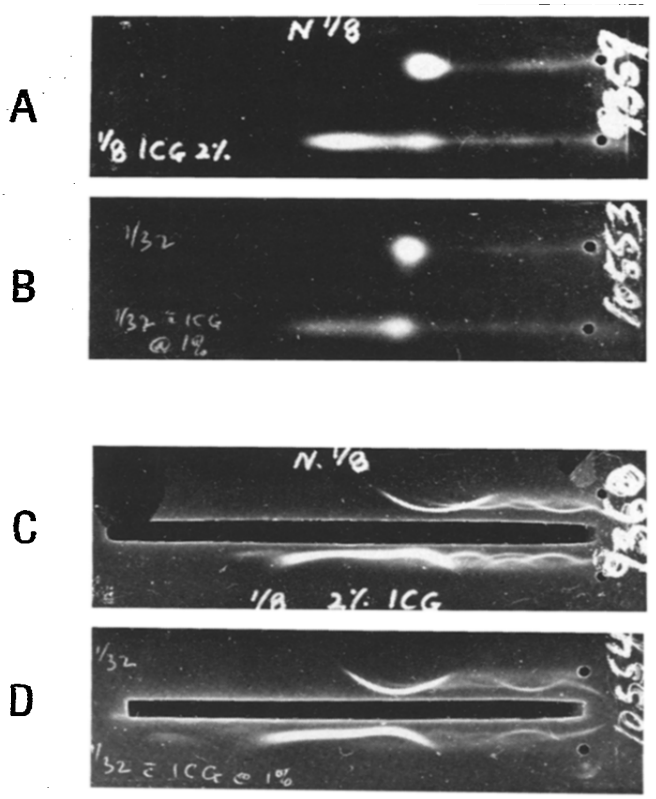

Fig. 2. Anodes left, cathodes right (preparation numbers on right). In each of all four preparations the upper well contains a 1/8 (A and C) or 1/32 (B and D) dilution of native normal human serum while the lower well contains an identical dilution with ICG in a final concentration of 1% (B and D) or 2% (A and C). A and B, simple agar gel electrophoresis. In A the ICG—albumin molar ratio is approximately 355:1 while in B the ratio is approximately 700:1. The electrophoretically fast albumin component is very obvious and, in spite of the molar differences, appears proportionately very much the same in both specimens. Variations in density between the pairs of patterns relates to differences in uptake of stain from the staining solution due to the different final concentrations of albumin in the particular dilutions. C and D, immunoelectrophoretic parallels of A and B respectively, developed with polyvalent rabbit antiserum to whole human serum and illustrating a precipitin line of apparently total identity between the two albumin components. These preparations also show that the ICG—albumin ratio differences between the two specimens do not appear to have affected the quantity or degree of separation of the fast and the slow albumin components. Some other serum proteins are represented by the adjacent faint precipitin arcs developed with this polyvalent antiserum.

Agar gel electrophoresis

In short runs the presence of ICG was associated with short anodal extension of the albumin spot. Extended runs provided greater resolution so that clear bimorphism of the albumin was evident. This bimorphism was first faintly detectable at a dye—albumin molar ratio of approximately 32:1. With further increasing dye—albumin molar ratios the distance between the fast and normal mobility albumin did not increase, but the proportion of fast to slow albumin did increase up to approximately 50:50 proportion at a dye—albumin molar ratio of 160:1. Beyond this ratio no further increase was evident, so that even

at a dye—albumin molar ratio of over 1400:1 the proportion of fast to slow albumin was still no more than approximately 50:50. Dye—albumin ratios greater than this were not explored because (a) solubility of ICG in a buffer at room temperature is little more than 2%, and (b) use of serum at dilutions greater than 1:32 provided albumin spots too faint to be interpreted with certainty. Only minimal cathodal extension of the albumin spot was evident at any dye concentration and however long the electrophoretic run. It became evident at a dye—albumin molar ratio of approximately 40:1, but was undetectable in preparations using high dilutions of serum. An example of the agar gel electrophoretic bimorphism is illustrated in Fig. 1.

Examination of completed agar gel electrophoretic preparations stained in aqueous acetic acid with the Nigrosin—Ponceau S mixture showed that up to an albumin concentration of approximately 0.5% the staining of the fast albumin was different from that of the slow. The slow albumin stained reddish purple with exactly the same tinge as that of albumin in native serum which had been subjected to electrophoretic analysis without ICG prestaining. In contrast, the fast albumin resulting from prestaining with ICG stained almost black, clearly selecting out the Nigrosin from the poststaining mixture. This observation suggested the exclusion experiments referred to previously and indeed it was found that addition of Ponceau S to serum at a final concentration of approximately 0.023 *M* negated almost completely the induction of bimorphism by ICG, whether the Ponceau S was added before or after the ICG at approximately the same concentration. In preparations with albumin at less than 0.5% concentration, the poststaining colours were too faint to be made out with certainty.

Immunoelectrophoresis

Immunoelectrophoretic preparations at all concentrations and at all analytical durations showed no evidence of immunological distinction between the two albumin moieties, nor any difference in apparent precipitin activity. The precipitin arc in immunoelectrophoresis thus took on a wavilinear conformation without any suggestion of crossing or spur formation between its two elements. However, the faster element was less prominent than the slower at the lesser dye—albumin molar concentrations, presumably reflecting less extensive diffusion of the new fast albumin as a result of its relatively lesser proportion at the lower dye concentrations. A typical immunoelectrophoretic result is illustrated in Fig. 2.

DISCUSSION

These results indicate that in the presence of a wide range of concentrations of ICG, two forms of serum albumin can be distinguished by features of electrophoretic mobility at pH 8.4 and relative affinity for Nigrosin and Ponceau S in dilute aqueous acetic acid.

The immunologically indistinguishable reactivity of the two ICG-induced electrophoretic components indicates that both are indeed albumin, and that the new fast moiety is not some other serum protein which has undergone an exaggerated dye-induced electrophoretic mobility alteration (DIMA) as can

occur with alpha-1 lipoprotein and other serum proteins [15—17, 19]. The evidence of immunological identity probably also precludes major but not minor [20] conformational change as a basis for the electrophoretic heterogeneity. It would, however, be consistent with occultation of some of the dye-avid basic groups of arginine and lysine [21] by partial polymerization, or partial saturation of binding sites by natural ligands [22—26] as has been indicated by work with other dyes under other conditions [27—31].

Previously described forms of electrophoretic heterogeneity of serum albumin appear to be distinct from the ICG-associated variety described here. The primary genetic types show close demographic limitations based upon heredity, whereas the ICG phenomenon was present equally in all sera tested from the ethnically very heterogeneous group of normal subjects. Heterogeneity associated with the presence of NEFAs [5—8, 32] which was probably the basis for observations of electrophoretic polymorphism described by Aronsson and Gronwall [33] and McLoughlin [34], or other substances [10, 11], is characterized by progression to complete conversion to the new form across a relatively narrow range of molar ratios commensurate with the recognized [22] number of binding sites on albumin. In sharp contrast, the effect of ICG begins only at a molar ratio at which the effects of other substances are complete, and does not progress beyond a 50% proportion at molar ratios approaching two orders greater. At such levels the dye—albumin molar ratio substantially exceeds that of the buffer molecules, suggesting that the ICG effect may represent no ordinary binding phenomenon but more a modification of the electrophoretic medium. The ICG effect also differs from the transient bisalbuminemia associated with hyperamylasemia in pancreatitis [35, 36] and likely due to enzymatic degradation in vivo [12] in that the dye phenomenon occurs with all normal sera from healthy subjects, is unaffected by incubation, and is not associated with evident antigenic modification.

The observations described here suggest the existence of a new form of dye-induced electrophoretic mobility alteration (DIMA) [15], a phenomenon previously recognized in four different varieties [17]. The phenomenon with ICG and albumin appears, immunoelectrophoretically, to be DIMA-2 (increased electrophoretic spread), but differs from DIMA-2 in that separation of two electrophoretically distinct albumins occurs. Since both of these show immunological identity the dye effect cannot be classified as DIMA-3, which represents separation of different proteins previously appearing as one [19], and thus may represent a fifth form of the phenomenon, i.e. DIMA-5. Definition of this will have to await more extensive characterization of the two ICG—albumins.

ACKNOWLEDGEMENTS

I am most grateful for essential assistance provided in the preparation of this work by J.A. Edwards, A.R.T., A. Eollos, Ll. D., and S. Hendriksen.

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