AGAR ELECTROPHORESIS

Part III. Animal Hæmoglobins

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Abstract

The blood hæmolysates from some of the common animal species have been studied by the agar electrophoresis method.

Of the 12 species examined, buffalo blood indicated the presence of two components.

There is considerable variation in the electrophoretic mobilities of the hæmoglobins of different animals, the hæmoglobin of the horse having the highest and that of the camel, the lowest mobility.

The results are discussed in detail in the light of the findings of other workers in the field.

Introduction

The studies on hæmoglobins have been mostly concerned with the abnormalities of human hæmoglobins. Recently, however, reports on hæmoglobins of various animal species have appeared. Haurowitz *et al.*[1] reported the presence of two components in rat hæmoglobin on the basis of the discontinuities observed in the alkali denaturation curves. Harris and Warren[2] found two hæmoglobins in sheep and Cabanes and Serain[3] found two hæmoglobins in cattle. Rodnan and Ebaugh[4] who carried out investigations on the electrophoretic heterogeneity of hæmoglobins from a large number of species including chimpanzee, rhesus monkey, pig, cat, hamster, rat, goat, sheep, horse and cow found that except horse and 1 of 5 sheep, which possessed two components all other mammalian hæmoglobins studied were found to have single component. The solubility studies of Roche *et al.*[5] on horse and bovine hæmoglobins and of Karvonen and Leppanen[6] on sheep hæmoglobin showed the presence of more than one component in each. Rabbit blood was reported to contain two hæmoglobins by Schapira, Kruh and co-workers[7-11] on the basis of their isotope incorporation studies. Giri and Pillai[12] employing the agar electrophoretic technique found two hæmoglobins in buffalo blood. Sydenstricker and co-workers[13] have analysed the blood samples of dog, rabbit, rat, chicken, alligator, human and turtle species, using paper electrophoresis, but could demonstrate heterogeneity of hæmoglobins only in turtle and chicken at pH 7·4. Landsteiner and co-workers[14] using the boundary electrophoresis have reported as early as 1938, the electrophoretic mobilities of hæmoglobins from five different animal species. While work was in progress in this laboratory on animal hæmoglobins by the agar electrophoretic technique Fine *et al.*[15] employing the agar electro-

phoresis method have analysed the blood hæmolysates of a number of animals and could not observe any heterogeneity or any appreciable difference in the electrophoretic mobilities in the hæmoglobins of the different animal species. The present communication deals with the results of our investigation on the hæmoglobins of twelve different animal species and their approximate mobilities employing the agar electrophoresis technique.

Experimental

Blood samples were collected fresh into oxalated tubes and centrifuged to remove the plasma. The red cells were washed five times with physiological saline to remove the plasma proteins. To the washed cells, an equal volume of distilled water and a few drops of toluene were added and kept overnight in deep freeze. The cells were force thawed and centrifuged to remove the stroma. The clear hæmolysate obtained was used for electrophoretic analysis.

The agar electrophoresis was carried out as described earlier.[16, 17] For determining the comparative mobilities of the different hæmoglobins, the electrophoresis of each hæmoglobin sample was carried out together with normal human serum on the same agar plate. Suitable amounts of serum and blood hæmolysate were applied one below the other (Fig. 1) by the paper strip application technique on the surface of the agar gel (0·5% B.D.H. agar fine powder) containing barbital buffer of pH 8·6 and ionic strength 0·05. The electrophoresis was carried out at 200 V., 8 m.A for 6 hr. After the run, the agar plate was removed, dried at room temperature in a current of air and stained in a dye-bath of amidoschwarz-10 B (E. Merck). The approximate mobilities of the different hæmoglobins were calculated from the relative positions of the hæmoglobins to that of human serum globulins, whose mobilities are known.[18]

Results and Discussion

The electrophoretic patterns of the hæmoglobins along with that of normal human serum proteins are shown in Fig. 1. The number of components present in the blood hæmolysates of the different species as well as their approximate mobilities are given in Table I.

It could be seen from Table I and Fig. 1 that among the 12 animal species investigated, the horse hæmoglobin has the highest electrophoretic mobility, while the camel has the lowest. Under the conditions of experiments carried out (agar electrophoresis in veronal acetate buffer of pH 8·6), only buffalo blood showed the presence of two hæmoglobins. Rat blood indicated the presence of one hæmoglobin and two minor protein components. Of the two hæmoglobin components in buffalo blood, the minor component (β) has a lower electrophoretic mobility and is about 37% the concentration of the major component (α). In rat blood hæmolysate the major component (α) which has the highest mobility, is present in high concentration. Of the other two minor components, the one having the least mobility (γ) is in a higher concentration than the slightly faster

TABLE I

Electrophoretic heterogeneity and mobility of animal hæmoglobins

Species	No. of components	Approx. mobilities — ($U \times 10^5$ sq. cm./v/sec.)
Horse	1	3·7
Elephant	1	3·4
Buffalo-α	2	3·3
Buffalo-β		2·7
Guinea pig	1	3·1
Man	1	2·9
Rat-α	3*	2·8
Rat-β		1·2
Rat-γ		0·9
Rabbit	1	2·7
Sheep	1	2·6
Cow	1	2·6
Goat	1	2·4
Dog	1	2·3
Camel	1	1·7

* One hæmoglobin and two other protein components.

β-component. The minor components in rat blood hæmolysates are present in such low concentrations compared to the major component, that they are likely to go undetected unless a high concentration of the hæmolysate is used for analysis.

It should be emphasised here that the two minor components present in the rat blood hæmolysate could be detected by the amidoschwarz reagent and not by the benzidine test. It is likely that these two minor components are some other proteins present in the erythrocytes and are characteristic of the rat species. The fact that they are not any other serum proteins, is proved from their electrophoretic behaviour (appearing as well-defined sharp bands in contrast with the diffuse bands of γ-gobulins) as well as their reproducibility even after repeated washings of the cells with physiological saline. Further work on the isolation and characterisation of these two protein components present in rat blood hæmolysates is in progress.

The hæmoglobins of other species, horse, elephant, guinea pig, man, rabbit, sheep, cow, goat, dog and camel showed the presence of only one hæmoglobin. The findings of Roche *et al.*[5] and of Karvonen and Leppanen[6] of different hæmoglobins in horse and sheep were based on the solubility studies. Itano[21] has rightly pointed out that " the discontinuities in salting out curves, on which the conclusions of Roche *et al.* are based, probably indicate changes in the nature of

the solid phase, and such changes may result either from the precipitation of a different form of hæmoglobin or from a change in the type of aggregation of the same hæmoglobin due to interaction with small molecules or ions in the solvent. Hæmoglobin A, S and F as well as the hæmoglobins of other species are known to crystallise in more than one form [19–22, 23] depending on the composition of the solvent. Thus it has not been established unequivocally that discontinuities in the salting out plot always indicate heterogeneity of a protein. The number of discontinuities seems always to exceed the number of components detectable by other methods. The application of this method to the estimation of proportions of components in a hæmoglobin mixture has no theoretical basis ".

Again, the confused situation with respect to the alkali denaturation experiments needs further critical examination of the results.

Schapira *et al.*[7–11] have carried out some isotopic incorporation studies on the rabbit blood hæmoglobin. " As stated by the authors, the fractionations (employing a variety of physical and chemical methods) were performed arbitrarily, so that the fraction obtained could not be identified as homogeneous entities. The proportions of the two fractions obtained and the ratios of their specific activities depended on the methods of fractionation employed. Their data therefore do not permit a choice between their hypothesis and the alternatives given above. In fact the arbitrary limitation of the number of forms present in the rabbit hæmoglobin to two may not be justified " (Itano[24]).

Hæmoglobins are more stable in the alkaline pH rather than in the acid pH, where a certain extent of denaturation of the proteins is likely to take place. As such, the heterogeneity of hæmoglobins in the blood of other species observed by other workers who have used the acid pH may have to be confirmed by experiments conducted under more ideal conditions.

Recently Fine and co-workers[15] have reported the electrophoretic mobility and heterogeneity of the hæmolysates from a number of species including some of the animals investigated in our laboratory, employing the agar electrophoresis technique. They do not find any appreciable variations in the mobilities of the different hæmoglobins, excepting that the hæmoglobin of horse has a higher mobility compared to that of others. The hæmoglobins of all the species without exception migrate towards the cathode in their experiments. But our own observations are to the contrary, as could be clearly seen from Fig. 1. The hæmoglobins of a large number of species move towards the anode, that of horse almost reaching the mobility of α_2-globulin of human serum. There are appreciable variations in the electrophoretic mobilities between the hæmoglobins of the different species, that from camel having the lowest. In the experiments reported by Fine *et al.* the separation of the serum proteins themselves are not very satisfactory. β-globulin is shown to move towards the cathode in their experiments, whereas we could resolve the human serum into six clear components, β_1 and β_2 globulins occupying different positions on either side of the starting

point. The heterogeneity observed in some of the hæmolysates in our experiments have been confirmed by specific colour tests[12] and further characterisation of the individual components present in the buffalo blood has been carried out. The presence of two components in chicken blood hæmolysate reported by Fine *et al.* is in agreement with our earlier observations[17] and that of Johnson and Dunlap.[25] The disagreement between our own observations and those of Fine and co-workers[15] as regards the electrophoretic mobilities and heterogeneity of the different animal hæmoglobins can possibly be attributed to the more unfavourable conditions of experiments they have adopted, *viz.*, a high agar concentration of 1·5%, lower pH 8·2 and the mixing of hæmoglobin solution with agar at 45° C. It has been our experience that a higher concentration of agar as well as incubation of the agar-buffer mixture for prolonged periods before plating for electrophoresis, increases the electro-osmosis of the medium with a resultant shift in the ultimate position of the hæmoglobin bands towards the cathode. Buffalo hæmoglobin which clearly separates into two components (both components moving towards anode) in the veronal acetate buffer of pH 8·6, comes as a single band moving towards the cathode in phosphate buffer of pH 6·8. Thus it is obvious that in the study of the heterogeneity and electrophoretic mobility of hæmoglobins, the most favourable conditions are to be scrupulously observed in the investigations. In the light of these findings the possibility of the agar electrophoresis of hæmoglobins being applied as a tool in medico legal examinations should be explored further.

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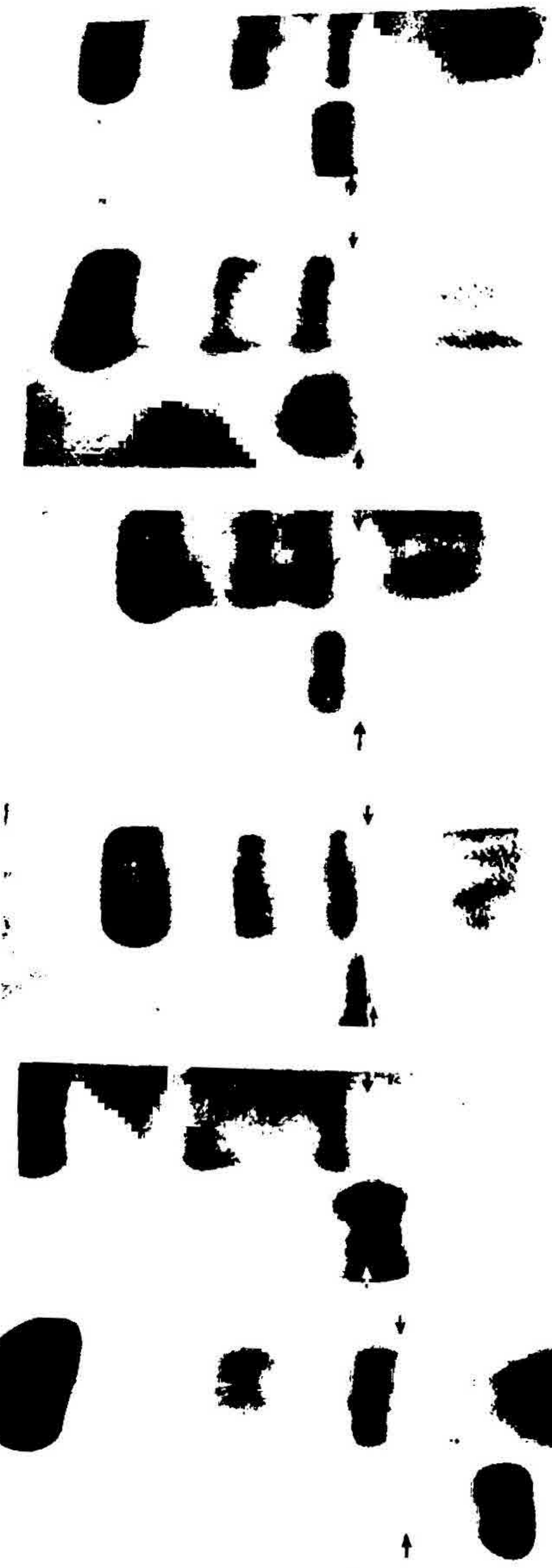


Human serum

Human blood hæmolysate

Human serum

Sheep blood hæmolysate

Human serum

Cow blood hæmolysate

Human serum

Goat blood hæmolysate

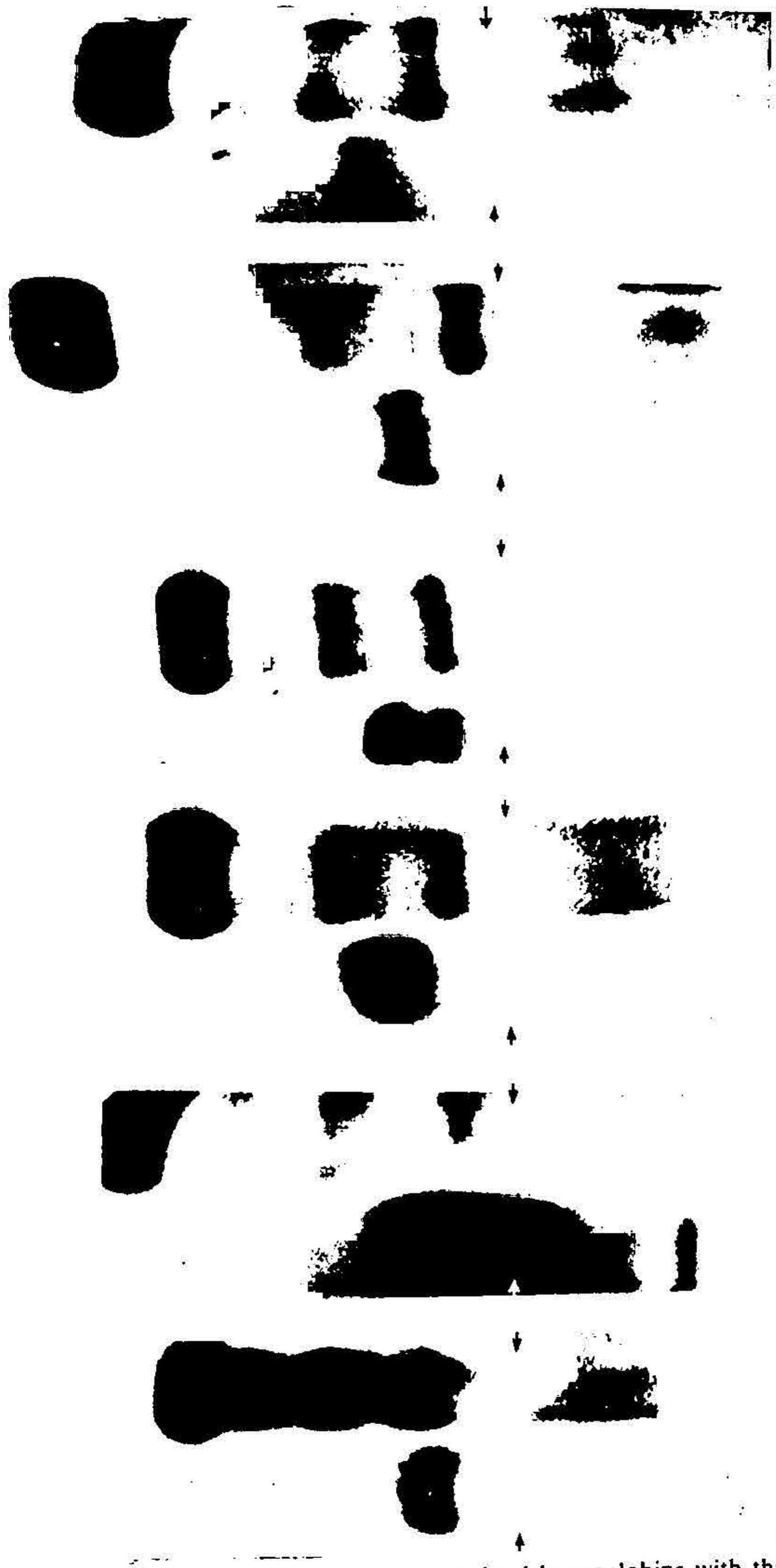
Human serum

Dog blood hæmolysate

Normal human serum

Camel blood hæmolysate

FIG. 1 (*Continued*)



Human serum

Horse blood hæmolysate

Normal human serum

Elephant blood hæmolysate

Human serum

Buffalo blood hæmolysate

Human serum

Guinea pig blood hæmolysate

Human serum

Rat blood hæmolysate

Human serum

Rabbit blood hæmolysate

FIG. 1. Electrophoretic patterns of animal hæmoglobins with that of normal human serum. (Arrow marks indicate the point of application of protein solution.)

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