Separation of Serum Proteins in Different Amphibian Species by Polyacrylamide Gel Electrophoresis

The electrophoretic separation of serum proteins in various animal groups has been shown to be a useful tool for taxonomic purposes[1–4]. The serum proteins in several European amphibians have previously been analysed by using either paper[5,6] or cellulose acetate[7] electrophoresis. The results of these studies demonstrated the presence of albumin and 3–5 additional fractions which probably correspond to globulins. The more recent technique of zone electrophoresis in polyacrylamide gel has been shown to be superior to previous methods[8]. As will be reported below, by means of this method we were able to resolve at least 15–20 protein fractions in the serum of 11 amphibian species, all of which commonly occur in the vicinity of Zürich, Switzerland.

The animals, which were usually collected during the breeding season between March and May, included 4 species of the order Urodela: *Triturus alpestris*, *T. cristatus*, *T. vulgaris*, *T. helveticus* (Salamandridae), and seven species of the order Anura: *Rana temporaria*, *R. esculenta* (Ranidae); *Bombina variegata*, *Alytes obstetricans* (Discoglossidae); *Bufo bufo*, *B. calamita* (Bufonidae); *Hyla arborea* (Hylidae). Blood samples were obtained by puncturing the heart and serum was separated from the clotted blood by brief centrifugation.

The electrophoretic run was carried out according to procedures described by Davis[9], employing a 2.5% upper polyacrylamide gel and a 7.5% lower gel (pH 8.3). The serum samples usually contained 200–400 µg of proteins. During each run a constant current of 3 mA per tube was maintained. At room temperature and within about 1 h, the tracking dye (bromphenol blue) has an average migration of 64 mm from the origin. After separation the extruded gels were stained with amido black, destained electrophoretically, and preserved in 7% (v/v) acetic acid.

The electrophoretic patterns of 4 urodele species are shown in Figure 1. For the purpose of comparison, the separation of human serum (HS) under the present experimental condition is depicted. As described by Davis[9] and shown in Figure 1, the most concentrated fraction in human serum is albumin (A) which migrates about 40 mm from the origin (O). The second most concentrated fraction is transferrin (T), the iron-binding serum protein, which moves about halfway between the origin and albumin. One fraction migrates ahead of the albumin

[1] H. F. Deutsch and M. B. Goodloe, J. biol Chem. *161*, 1 (1945).
[2] H. F. Deutsch and W. H. McShan, J. biol. Chem. *180*, 219 (1949).
[3] H. C. Dessauer and W. Fox, Nature *126*, 225 (1956).
[4] B. L. Antonaci and G. Macagnino, Monitore zool. ital. *65*, 19 (1957).
[5] B. Lanza and F. M. Antonini, Monitore zool. ital. *63*, 293 (1955).
[6] V. Kiortsis and M. Kiortsis, Revue suisse Zool. *67*, 119 (1960)
[7] F. Gasser, C. r. hebd. Séanc. Acad. Sci., Paris *258*, 457 (1964).
[8] L. Ornstein and B. J. Davis, *Disc Electrophoresis*, Parts I and II (Distillation Products Inc., Rochester, New York 1962).
[9] B. J. Davis, Ann. N.Y. Acad. Sci. *121*, 404 (1964).

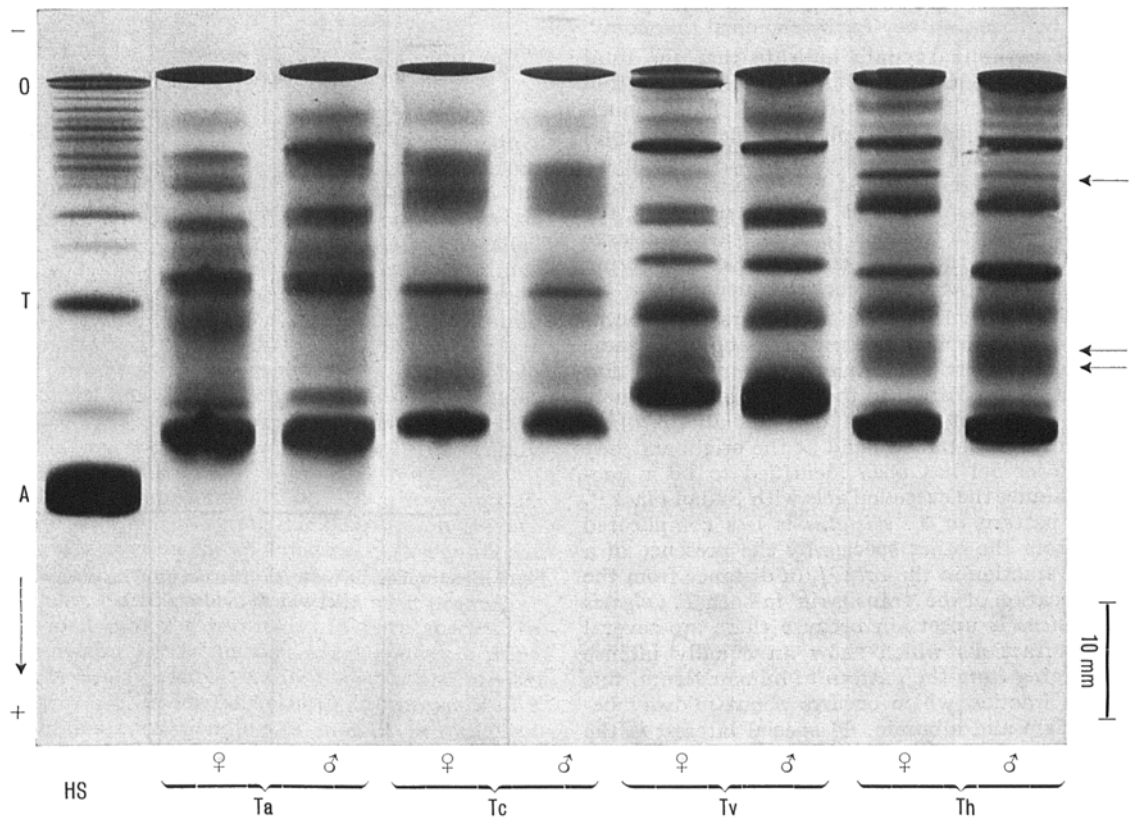

Fig. 1. Electrophoretic separation of human serum (HS) and serum proteins of *Triturus alpestris* (Ta), *T. cristatus* (Tc), *T. vulgaris* (Tv) and *T. helveticus* (Th) in polyacrylamide gels. (O) origin, i.e. junction between upper and lower gel, (T) transferrin, (A) albumin.

(prealbumin) and there are 2 fractions in the gel region between albumin and transferrin (postalbumins). One intensely stained fraction located near the origin is the slow β_1-lipoprotein, and in front of this the $S\alpha_2$-globulin which has an equally heavy staining. Between the $S\alpha_2$-globulin and transferring there are at least 12–15 very thin and sharply demarcated bands, some of which correspond apparently to haptoglobins. This is also the region usually occupied by the so-called 7 Sγ-globulins.

Compared to human serum, one very evident character of the amphibian sera is the absence of the numerous sharply resolved thin bands in the globulin region (see Figure 1). This has been observed repeatedly not only for the genus *Triturus*, but also for the other species so far examined by us. The present observation is in agreement with that noted by FRIEDEN et al.[10] that the phylogenetic complexity of higher animals is associated with the appearance and increase in the number and concentration of bands of the globulin fraction in serum protein components.

A second point of interest is that in urodeles the mobility of serum proteins towards the anodal direction is distinctly slowler than the mobility of human serum proteins. This is visible especially in the albumin fraction which has been identified by its specific binding with Evans blue (also called dye T-1824)[11]. On the other hand, with the exception of the Bufonidae, the protein fractions of the anuran species move at about equal rates as those of the human serum (Figures 2–4). This is also in excellent agreement with the findings of DESSAUER and FOX[12] who, based on paper electrophoresis, reported that the leading anodal peak in Urodela (Caudata) migrates less than 12 cm from the origin, and that in Anura (Salientia) has a migration of 14 cm corresponding to the albumin fraction in human serum. They proposed that this species-specific mobility can be used as a key for biochemical taxonomy.

Finally our preliminary data indicate that the total serum protein concentration of Amphibia varies from 2.1 to 3.6% (based on the biuret reaction). This is much lower than that of human blood plasma which has a total concentration of about 7%. The range of variation for different amphibian genera cited by PROSSER[13] is from 2.16–3.42%. Although there is as yet no strict proof, the present result supports the idea that, at least in a large variety of animals, the total concentration of serum proteins increases in the phylogenetic sequence[10].

As shown in Figure 1, in *T. alpestris* a rather concentrated protein fraction which migrates in about the same position as the transferrin of human serum is divided into 2 subfractions. Immediately to the rear of albumin there is a clear postalbumin band. In this and all the other species, the intensely stained band at the origin and just within the lower gel has been identified to be a lipoprotein by staining the extruded gels with Sudan black[14]. The general pattern of *T. cristatus* is less complicated and differs from the other species by the presence of a broad diffuse fraction in the first $^1/_3$ of distance from the origin. The location of the 'transferrin' in both *T. vulgaris* and *T. helveticus* is uncertain because there are several intermediate fractions which show an equally intense staining. Judging from the pattern of human serum, this could be the fraction which occurrs about midway between the origin and albumin. Of special interest is the distinct difference in the electrophoretic patterns between these 2 closely related species. There is a thin, sharply separated band which occurs in a much higher concentration in *T. helveticus* than in *T. vulgaris* (indicated by single arrow in Figure 1). Behind this a faint narrow band is visible in the former species, but apparently absent in the latter. Furthermore, a broad diffuse fraction situated about 6 mm to the rear of albumin is only present in *T. helveticus* (see double arrow in Figure 1). From the systematic viewpoint such differences are very useful because the morphological distinction of the females between these 2 species is not always easy.

In both *Rana temporaria* and *R. esculenta* the electrophoretic patterns are characterized by a major protein fraction of relatively low mobility (14 mm from the origin) which has a concentration nearly as high as that of the albumin (Figure 2). The intermediate gel regions are occupied by a large number of faint bands which are difficult to resolve. In the electropherogram depicted for the female of *R. esculenta*, the albumin is divided into 2 subfractions (shown by their specific binding with Evans blue). Since all serum samples used for the analyses were prepared from a single female, further work is needed to confirm whether this is really the case or represents artifact due to long storage at low temperature or other factors.

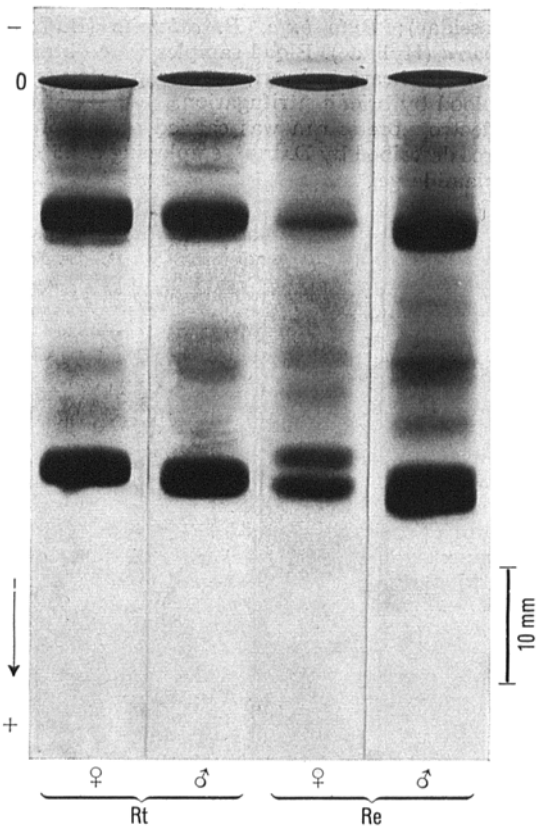

Fig. 2. Electrophoretic patterns of serum proteins of *Rana temporaria* (Rt) and *R. esculenta* (Re).

[10] E. FRIEDEN, A. E. HERNER, L. FISH and E. J. C. LEWIS, Science *126*, 559 (1957).
[11] R. A. RAWSON, Am. J. Physiol. *138*, 708 (1943).
[12] H. C. DESSAUER and W. FOX, Science *124*, 225 (1956).
[13] C. L. PROSSER, *Comparative Animal Physiology* (W. B. Saunders, Philadelphia 1950), p. 104.
[14] J. M. FINE and M. BURSTEIN, Experientia *14*, 411 (1958).

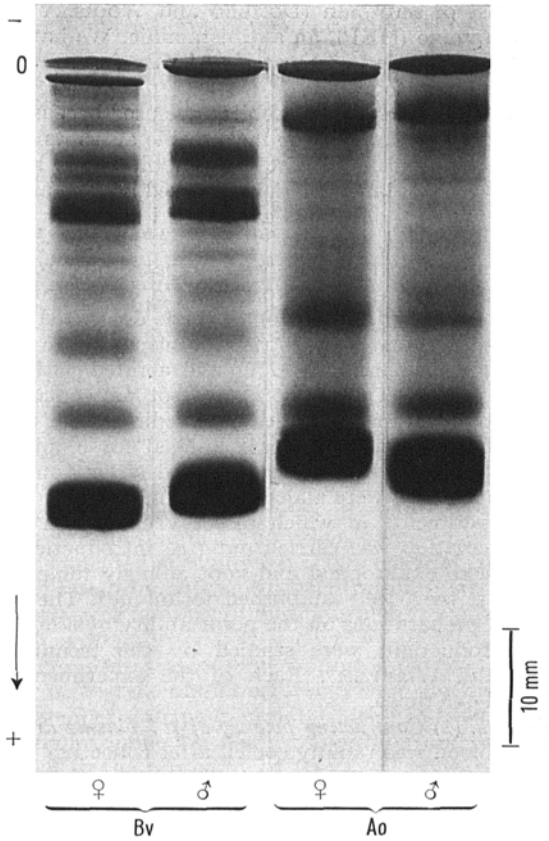

Fig. 3. Electrophoretic patterns of serum proteins of *Bombina variegata* (Bv) and *Alytes obstetricans* (Ao).

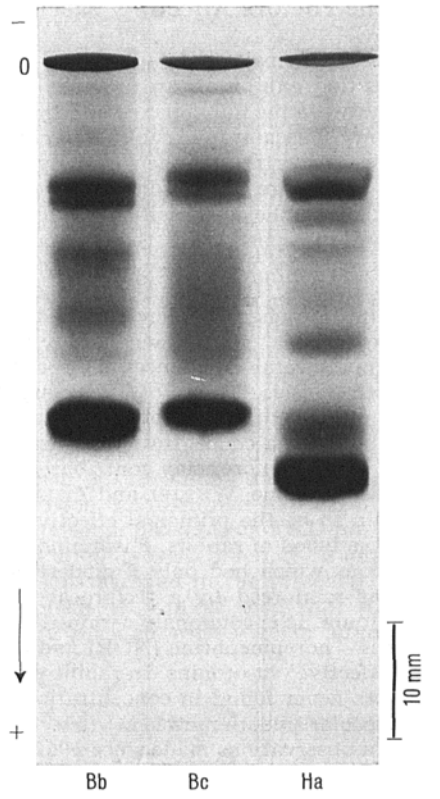

Fig. 4. Electrophoretic patterns of serum proteins of *Bufo bufo* (Bb), *B. calamita* (Bc) and *Hyla arborea* (Ha). The serum samples of all 3 species were obtained from males.

In *Bombina variegata* and *Alytes obstetricans* 2 distinct bands of considerably high concentration can be observed: one is adjacent and the other one about 12 mm to the rear of albumin (Figure 3). From their electrophoretic behaviour, these correspond probably to the so-called postalbumins of the human serum. *A. obstetricans* differs from the other species by the occurrence of an intensely stained and very slow-migrating band only about 5 mm distant from the origin. As can be seen in Figure 3, there is a sexual difference in the serum protein pattern of *B. variegata*. In the female of this species about 7 fractions are detectable in the globulin region, whereas in the male the corresponding region is occupied by only 1 intensely and 2 faintly stained bands.

The major serum proteins of both *Bufo bufo* and *B. calamita* exhibit a markedly similar distribution (Figure 4). The albumin fraction shows a distinctly slower anodal migration, which is comparable to that already described for the urodeles. Furthermore, the second most prominent band is divided into 2 subfractions in both species. In *B. calamita* a faint anodal protein which moves in front of albumin is clearly visible. As in *Bombina* and *Alytes*, the tree frog (*Hyla arborea*) also shows the presence of 2 protein fractions corresponding in mobility to that of human postalbumins. In contrast to the former 2 species, *H. arborea* has only a few very faint bands in the gel section near the origin.

In conclusion, the present survey demonstrated that the patterns of serum proteins are species-specific for the various amphibians examined. Up to now only minor differences between the 2 sexes within one species could be detected. More extensive work is necessary to answer the question as to whether or not sexual polymorphism occurs or is nonexistent in Amphibia. Many interesting problems raised by this survey, such as the functional significance of the individual protein components and the ontogeny of the electrophoretic patterns, must await future investigation[15].

Zusammenfassung. Mittels Polyacrylamid-Gel-Elektrophorese wurden die Serumproteine von 11 Amphibienarten in 15 bis 20 Fraktionen aufgetrennt. Das elektrophoretische Muster wurde mit demjenigen des menschlichen Serums verglichen und erwies sich als artspezifisch.

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