3 ml pooled normal human serum was applied to the column. 81 fractions of 5.5 ml were collected at flow rate of 30–35 ml/h. Chromatography was started with 0.02 *M* phosphate buffer and then a system of stepwise increasing NaCl concentration (0.02, 0.1, 0.2, 0.4, 1 *M*) was applied. Protein content of the effluent was determined by measuring optical density (OD^1_{280}) and by LOWRY method[6].

After chromatography the peaks of each group were pooled and dialyzed. Concentration of the effluent fractions was made by lyofilization after dialysis. Each peak was analyzed by immunoelectrophoresis[7–9], using a horse antiserum, and the individual precipitate arcs were also identified by the method of OSSERMAN[10]. Starch-gel electrophoresis[11] was performed with a discontinuous buffer system.

Results and discussion. The absorption of the effluent (its protein content) separating the serum into 6 fractions (I–VI), is reproduced in Figure 1. The individual fractions analyzed by immunoelectrophoresis are demonstrated in Figure 2.

The bulk of the proteins passes through the liquid phase outside the molecular sieve and is eluted first. The immunoelectrophoretic analysis of the first fraction (I) develops almost a complete protein spectrum. The γ-region – IgA, IgM and IgG appears only in that fraction. The β_1-region seems to be also unvaried, except for hemopexin, which is completely missing. The α_2-region is lacking in many of its components. The α_0, α_1 and prealbumin regions are also very poor. Prealbumin (α_1) is completely retained (Figure 2, I). When the first fraction is adsorbed with antiserum, or when the fractions II–VI are identified by means of direct immunoelectrophoresis[7–9], or using specific method of identification[10], one can see the following: After perfect technical separation the 2nd fraction contains only traces of albumin (Figures 2 and 3). In the 3rd fraction albumin appears again, further α_1-antitrypsin and a precipitation arc in the α_2 region. In the 4th fraction albumin, α_1-acid glycoprotein, α_1-antitrypsin, haptoglobins, ceruloplasmin and hemopexin can by demonstrated. The fraction V contains traces of albumin, α_1-antitrypsin, haptoglobins again and prealbumin. Almost pure prealbumin appears in the fraction VI. By the stepwise increasing NaCl concentration used, it is the protein which is eluted last (Figure 3, VI). The identification of prealbumin by means of direct immunoelectrophoresis is possible only after dilution of antiserum (Figure 2, VI).

The results obtained by starch-gel electrophoresis, are nearly the same as those obtained by immunoelectrophoresis (Figure 4).

Human serum proteins are partly retained on DEAE-Sephadex A-25. The character of the retained proteins shows relationship to the glycoproteins. α_1-acid glycoprotein, α_1-antitrypsin, haptoglobins, ceruloplasmin and hemopexin are retained most of all. The immunoelectrophoretic analysis also revealed the retention of some proteins, which were usually not grouped together with glycoproteins, though albumin or its traces were obtained in the fractions I to V. It is likely that a high degree of heterogeneity, due to complexes of albumin with other small molecules, is responsible for this behaviour, or it is the phenomenon observed at the partial dissolution of these proteins in perchloric acid due to the protective property of the glycoproteins[12–17]. It is of special interest that it was possible to separate almost pure prealbumin in the last fraction, although prealbumin has a carbohydrate composition which is very different from the average of all plasma proteins, with a very low hexosamine to hexose ratio and no sialic acids[18].

Much work has been carried out in recent years in connection the pathological variables with glycoproteins in patients[19–21], nevertheless the pathological significance is not yet exactly understood. The method described may possibly give more significant information about the levels, presence, or absence of individual glycoproteins.

Zusammenfassung. Zur Fraktionierung der Humanplasmaproteine wurde DEAE-Sephadex A-25 verwendet und die Fraktionen I–VI durch Immuno- und Stärkegelelektrophorese charakterisiert. Nach Eluierung der proteinreichen 1. Fraktion wurden zahlreiche Glykoproteine gefunden und die Abtrennung von α_1-Präalbumin erreicht.

M. PODHRADSKÁ and J. ANDRAŠINA

Scientific Laboratory of the Surgical Clinic, University P.J. Šafárik, Rastislavova 53, Košice (Czechoslovakia), 28 December 1973.

[6] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, J. biol. Chem. *193*, 265 (1951).
[7] P. GRABAR and A. C. WILLIAMS, Biochim. biophys. Acta *10*, 193 (1953).
[8] F. ŠKVAŘIL and J. REJNEK, Czech. epid. mikrob. Imun. *7*, 414 (1958).
[9] F. ŠKVAŘIL, Chemické Listy *55*, 1069 (1961).
[10] E. F. OSSERMAN, J. Immun. *84*, 93 (1960).
[11] O. SMITHIES, Biochem. J. *61*, 629 (1955).
[12] A. J. ANDERSON, Nature, Lond. *208*, 491 (1965).
[13] E. R. BERMAN, Biochim. biophys. Acta *58*, 120 (1962).
[14] M. SCHMIDT, Biochim. biophys. *63*, 346 (1962).
[15] G. M. W. COOK and E. H. EYLAR, Biochim. biophys. Acta *101*, 57 (1965).
[16] S. A. BARKER, C. F. HAWKINS and M. HEWINS, Ann. rheum. Dis. *25*, 209 (1966).
[17] R. J. WINZLER, *The Plasma Proteins* (Ed. F. W. PUTMAN; Academic Press, New York, 1960) vol. 1, p. 309.
[18] M. R. SHETLAR, J. C. CAPPS and D. L. HERN, Biochim. biophys. Acta *83*, 93 (1964).
[19] H. W. WALLACE, K. ARAI and W. S. BLAKEMORE, J. appl. Physiol. *28*, 433 (1970).
[20] S. G. MURPHY, A. S. KLAINER and D. F. CLYDE, J. Lab. clin. Med. *79*, 55 (1972).
[21] O. G. EKINDJIAN, J. C. JARDILLIER and J. AGNERAY, Annls. Biol. clin. *30*, 29 (1972).
[22] Acknowledgement. We thank Dr. M. JOUSTRA (Pharmacia, Uppsala) for his constructive criticism.

Histone Synthesis in Amphibian Oocytes and Early Embryos[1]

Amphibian embryos have been demonstrated to synthesize protein at substantial rates during both the pre- and post-fertilization stages of early development[2]. Modulations in the electrophoretic patterns of newly synthesized protein have also been observed during the early cleavage stages of those embryos. Some of those modulations require the presence of functional chromosomes[3], while others are influenced by the composition of the egg cytoplasm[4].

A more complete understanding of the regulation of early protein synthesis in amphibian embryos might be achieved if the identity of some of the newly synthesized

proteins was established. The studies reported in this communication were undertaken as an attempt to fractionate the newly synthesized proteins as a first step towards identifying some of the proteins which are synthesized during early amphibian development. An examination of the size characteristics and electrophoretic mobilities of a substantial proportion of the newly synthesized protein suggested that some of those proteins resembled histones. Appropriate fractionation and characterization methods were therefore employed and the results of these studies, reported here, indicate that both oocytes and early embryos synthesize histones.

Frogs (*Rana pipiens*) were maintained in the laboratory and eggs obtained by standard methods[5]. Embryos which lacked functional chromosomes were produced by mechanical enucleation[6]. [^{3}H]-leucine (36 μCi/mM) was injected in 0.02 μl quantities into either the equator region of oocytes or fertile eggs, the blastocoel of midcleavage stage embryos, both the blastocoel and archenteron of gastrulae, and the area beneath the neural plate of neurulae.

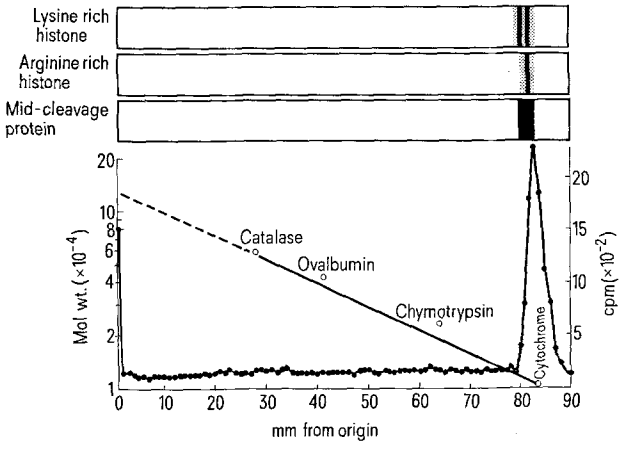

Fig. 1. Molecular weight characterization of acid-soluble proteins from midcleavage stage embryos. 10% acrylamide-SLS gels were calibrated with molecular weight markers. A sample of [^{3}H]-leucine labelled acid-soluble protein was applied to 1 gel and the migration of the radioactivity monitored. A duplicate gel was stained with fast green to reveal the electrophoretic mobility of the bulk of the acid-soluble proteins. Commercially available preparations of lysine rich histones and arginine rich histones were also run on duplicate gels and stained with fast green.

Embryos were incubated for 2 h at 18 °C. During that incubation period approximately 30–35% of the injected isotope was incorporated into cold trichloracetic acid (TCA) precipitated material. Injected embryos were suspended in 0.01 *M Tris* — 0.15 *M* NaCl (pH 7.0) and sonically disrupted for 1.5 min. The method described by Luck et al.[7] was employed for the acid fractionation. Briefly, the embryo homogenate was thrice extracted with 0.4 *N* H_2SO_4 and the acid soluble material precipitated overnight with 95% ethanol at −20 °C. After washing the precipitate 4 times with additional ethanol it was dissolved in an aqueous solution of 6 *M* urea. At each step aliquots of the extraction mixture were precipitated with 15% TCA for monitoring the recovery of incorporated isotope. Special caution was exercised to insure that each extraction was carried out at a temperature of 0–2 °C, and that each extraction step with 0.4 *N* H_2SO_4 was limited to 30 min.

A pH 3.2–8 *M* urea acrylamide (15%) gel system devised by Panyim and Chalkley[8] was used for the characterization of acid-soluble proteins. Fast green was employed for staining protein bands[9]. Commercially available (Sigma Chemical Co., St. Louis) preparations of histones, including arginine rich (catalog number H-9500) and lysine rich (catalog number H-9375) types, were employed as reference markers in the electrophoresis systems. Radioactivity in the gels was monitored by previously described methods[10].

The fraction of the [^{3}H]-leucine which was incorporated into protein and could be extracted from a crude homogenate with 0.4 *N* H_2SO_4 was determined for several developmental stages. A relatively constant proportion of

[1] Paper No. 3 in a series on protein synthesis in amphibian development. Papers 1 and 2 are referenced as 3 and 2, respectively.
[2] R. E. Ecker and L. D. Smith, Devel. Biol. *18*, 232 (1968).
[3] G. M. Malacinski, J. exp. Zool. *181*, 409 (1972).
[4] G. M. Malacinski, Devel. Biol. *26*, 442 (1971).
[5] V. Hamburger, *A Manual of Experimental Embryology* (University Chicago Press, Chicago 1960).
[6] R. Briggs, E. V. Green and T. J. King, J. exp. Zool. *116*, 455 (1951).
[7] J. M. Luck, P. S. Rasmussen, K. Satake and A. N. Tsvetikov, J. biol. Chem. *233*, 1407 (1958).
[8] S. Panyim and R. Chalkley, Arch. Biochem. Biophys. *130*, 337 (1969).
[9] M. A. Gorovsky, K. Carlson and J. L. Rosenbaum, Analyt. Biochem. *35*, 359 (1970).
[10] G. Gordon and G. M. Malacinski, Microchem. J. *15*, 685 (1970).

Table I. Extraction of H^3-leu labelled acid-soluble proteins from various embryonic stages

Step	2-Cell		Mid-cleavage		Early gastrula		Mid-gastrula		Neural plate	
	cpm[a]	Recovery[b] (%)	cpm	Recovery (%)	cpm	Recovery (%)	cpm	Recovery (%)	cpm	Recovery (%)
I. Crude extract	3.7×10^6	100	4.0×10^6	100	4.3×10^6	100	2.5×10^6	100	2.9×10^5	100
II. 0.4 *N* H_2SO_4 extraction	4.9×10^5	13.2 ± 2.1	4.8×10^5	16.6 ± 1.2	1.2×10^6	20.7 ± 1.8	5.6×10^5	18.7 ± 2.0	4.5×10^4	16.4 ± 1.1
III. Ethanol precipitation	4.6×10^5	12.6 ± 1.1	4.4×10^5	11.4 ± 1.5	7.4×10^5	16.0 ± 1.0	3.3×10^5	13.8 ± 2.1	3.4×10^4	10.6 ± 1.8
IV. Electrophoresis[c]	—	93	—	99	—	83	—	97	—	91

[a] TCA precipitation of extracts prepared from 15 embryo samples. Data from 1 experiment. [b] Mean of 3 separate experiments. In addition, occasionally double isotope (eg. H^3-leu-2 cell and C^{14}-leu gastrulae) extractions were performed. The results verified the stage specific differences observed above. [c] Recovery expressed as percentage of cpm of step III material applied to pH 3.2–8 *M* urea gel system which was recovered in the serial sections associated with the fast-green stained bands.

the newly synthesized protein was acid soluble, as the data presented in Table I indicate. For each stage, from 2-cell through neurula, approximately 11–16% of the [^{3}H]-leucine incorporated into trichloracetic acid precipitated material was acid soluble. The apparent increase in proportion of newly synthesized protein which resembles histones may reflect the substantial increase in the number of nuclei in the gastrula stage embryo over the earlier embryonic stages. Recent studies in our laboratory reveal that the presumptive ectoderm-mesoderm display an even greater increase in the relative rate of histone synthesis than the endoderm[11]. Once the embryo has entered into neurulation the fraction of newly synthesized protein which resembles histone appeared to diminish somewhat. Since no determinations of the rates of total protein synthesis were made, it is not possible to speculate as to whether a decrease in the net amount of histone synthesis actually occurred. These newly synthesized acid-soluble proteins were characterized with regard to molecular weight, charge, and staining properties. The data in Figure 1 demonstrate that virtually all of the radioactivity and staining material migrated as a single band to the low molecular weight range of the gel. The low molecular weight and relative homogeneity of the acid-soluble proteins on calibrated SLS gels were very similar to histones. The relative electrophoretic mobilities of commercially available preparations of histones were examined on duplicate 10% acrylamide-SLS gels. The staining patterns displayed in Figure 1 reveal the similarities between the electrophoretic mobilities on SLS gels of lysine rich and arginine rich histones and the acid-soluble proteins.

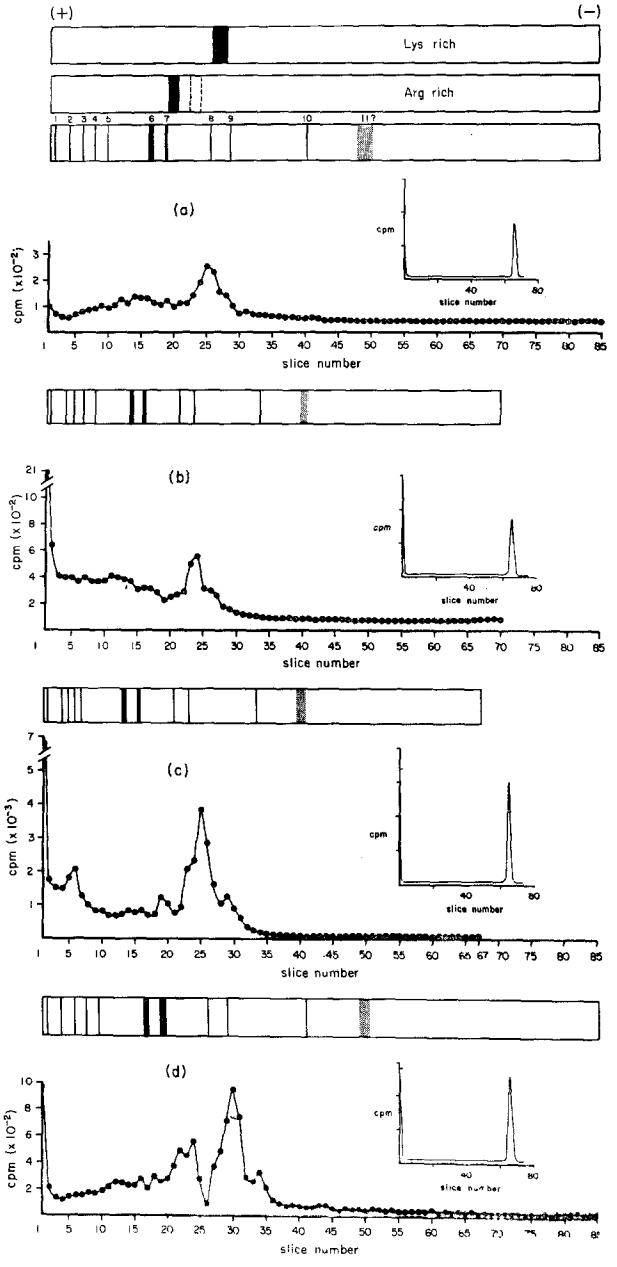

Fig. 2. Electrophoresis of [^{3}H]-leucine labelled acid-soluble protein from various embryonic stages on acid-urea gels. 1 gel was serially sectioned and monitored for radioactivity and a duplicate gel was stained with fast green. a) 2-cell stage; b) midcleavage; c) early gastrula; d) neural plate. The electrophoretic characteristics of lysine rich and arginine rich histones are included in (a) for compariosn. A third sample of each acid-soluble extract was applied to the SLS-gel system described in Figure 2. The cpm profiles are included in the inserts.

Samples of radioactive proteins from the extractions listed in Table I were applied to a pH 3.2–8 *M* urea, 15% acrylamide gel system. The staining patterns and radioactivity profiles for acid-soluble proteins from the 2-cell through neural fold stages are presented in Figure 2. The staining patterns of each gel contained approximately 10 discrete bands. Included in these analyses are the staining patterns of lysine rich and arginine rich histones. No major alterations in the staining patterns of acid-soluble proteins were observed during those developmental stages.

The radioactivity profiles of the acid-soluble proteins from each stage revealed that the relative mobilities of several of the cpm peaks coincided with the stained protein bands. The profiles in Figure 2 also reveal that minor changes in the synthetic pattern accompanied early embryogenesis.

The recovery from these gels of the radioactivity associated with the acid-soluble protein samples is given in Table I. Between 83 and 99% of the cpm in the samples from each developmental stage was recovered in the regions of the gel which contained the fast-green stained bands. A sample of each acid-extract was also applied to a 10% acrylamide-SLS gel system in order to provide a molecular weight characterization of the type described in Figure 1. From the acid-extracts of each developmental stage a single peak of radioactivity in the low molecular weight range was obtained (inserts to Figure 2, a–d).

Table II. Extraction of [^{3}H]-leucine labelled acid-soluble proteins from enucleated *Rana pipiens* embryos and ovarian oocytes

Step	% recovery of [^{3}H]-leucine during acid extraction[a]	
	Enucleated *Rana pipiens* 2-cell	Ovarian oocytes
I. Crude extract	100	100
II. 0.4 *N* H_2SO_4 extraction	18.9 ± 2.1	15.0 ± 1.1
III. Ethanol precipitation	10.7 ± 1.3	7.2 ± 1.2
IV. Electrophoresis	83	85

[a] Mean of two separate experiments.

[11] I. E. LÜTZELER and G. M. MALACINSKI, submitted (1974).

In addition to the above mentioned properties those newly synthesized acid-soluble proteins were discovered to display elution characteristics from Amberlite IRC-50 resin which resembled histones, and when labelled with [^{3}H]-arginine or [^{3}H]-lysine to have high concentrations of those amino acids. These findings are in agreement with previous studies which employed different embryos and developmental stages[12].

Both enucleated eggs and ovarian oocytes are also actively synthesizing histones (Table II). Previous studies on HeLa cells[13,14] emphasized the temporal relationship between DNA synthesis and histone synthesis. In those studies the synthesis of histone was linked to DNA synthesis. The findings described in this report indicate, however, that the amphibian oocyte, dormant in the synthesis of DNA[15,16], synthesized substantial amounts of histones. Indeed, histone synthesis also proceeds in the absence of functional nucleus. The apparent lack of a coordination between DNA and histone synthesis in oocytes may reflect the storage in the oocyte cytoplasm of histones which will be employed during the early cleavage stages when DNA synthesis and nuclear division proceed at exceptionally rapid rates. At 18 °C the number of cells doubles approximately once every 2 h[17].

Zusammenfassung. Proteine, die während verschiedener Entwicklungsstadien der Amphibien-Embryogenese auftreten, wurden isoliert und näher charakterisiert, wobei ein wesentlicher Anteil der mit ^{3}H-Leucin markierten Proteine aus Histonen besteht. In Eiern, deren Nukleus entfernt wurde, sowie in Ovarien-Oocyten, bei denen keine DNA-Synthese stattfindet, wurden jedoch Histone synthetisiert.

G. M. Malacinski

Department of Zoology, Indiana University, Jordan Hall 224, Bloomington (Indiana 47401, USA), 21 November 1973.

[12] E. W. Byrd jr. and H. E. Kusinsky, Biochemistry *12*, 246 (1973).
[13] E. Robbins and T. W. Borun, Proc. natn. Acad. Sci., USA *57*, 409 (1967).
[14] D. Gallwitz and G. Mueller, J. biol. Chem. *244*, 5947 (1969).
[15] M. Izawa, V. G. Allfrey and A. E. Mirsky, Proc. natn. Acad. Sci. USA *50*, 811 (1963).
[16] J. B. Gurdon, Proc. natn. Acad. Sci. USA *58*, 545 (1967).
[17] This investigation was supported in part by N.S.F. Grant No. GB-36973.

Differentiation of Cultured Muscle in the Presence of α-Bungarotoxin

There is evidence that the acetylcholine receptor is present in cultured skeletal muscle at a very early stage of development, even in some mononuclear cells and often before the appearance of organized contractile elements[1,2]. It has been postulated that the cholinoreceptor plays a role in the early events of myogenesis. This possible function of the acetylcholine receptor has been investigated by culturing myoblasts in D-tubocurarine: no effect on development was seen during the first 48 h in vitro[1]. Although D-tubocurarine has a high affinity for the cholinoreceptor, its action is reversible and the receptor-antagonist complex is, therefore, in a

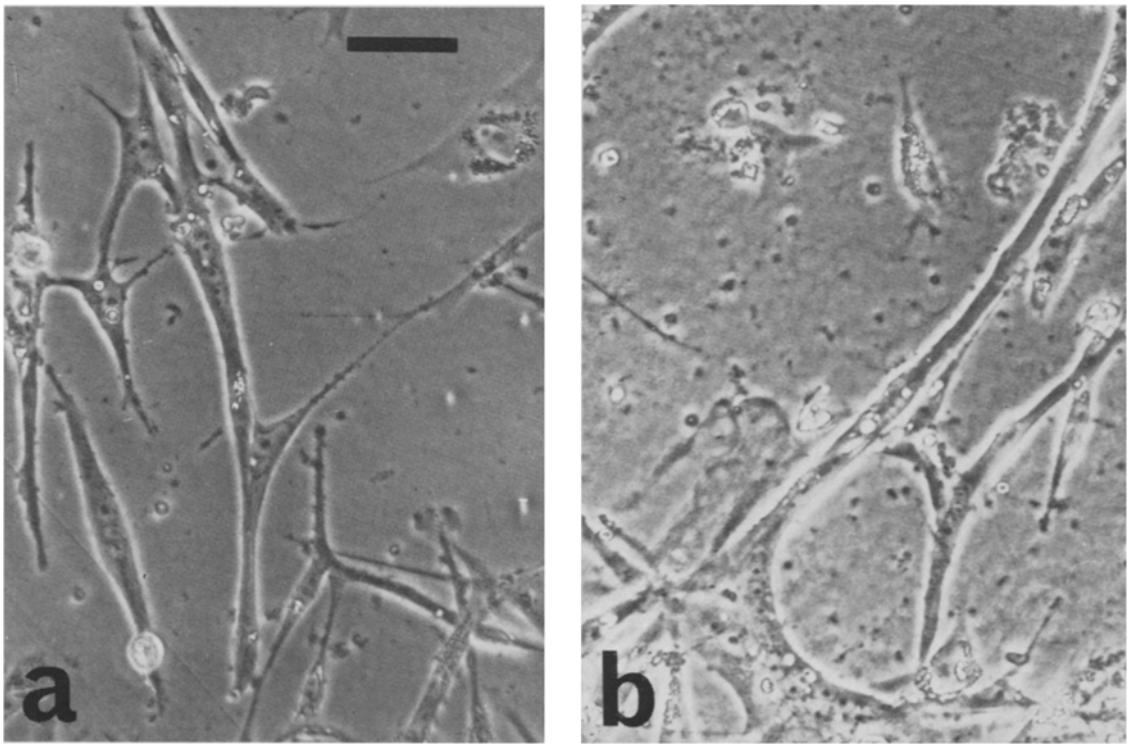

Fig. 1. Morphology of myogenic cells in culture. Phase contrast micrographs. a) 3-day control culture, showing bipolar myoblasts and small myotubes. b) 3-day culture grown in α-bungarotoxin (1 μg/ml). Calibration: 50 μm.