Complete replacement of serum in primary cultures of chick embryo brain cells by growth-promoting alpha-globulin

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Summary. Growth-promoting alpha-globulin (GPAG), a specific serum protein complex which induces mitotic activity in continually replicating metazoan cells in vitro, was shown in this study to support growth of astrocytes and mesenchymal cells as well as process formation of nerve cells isolated from cerebral hemispheres of chick embryos.

Cultivation of neural tissue in vitro in serum-supplemented media has been used in recent years for the study of brain metabolism, and cell culture methods have contributed much in the field of neurological sciences. It has been demonstrated that cells obtained from mechanically dissociated brain tissue can differentiate into neurones and glial cells in medium containing 10–20% serum[1,2]. In this connection, it has previously been reported by Sensenbrenner et al. that the chemically synthesized tripeptide Gly-His-Lys is sufficient for nerve fibre outgrowth of dissociated cells from chick embryo cerebral hemispheres for the first week in culture[3]. Honegger et al. have shown that mechanically dissociated fetal rat brain cells reaggregate, grow and differentiate in a serum-free medium[4]. Little, however, is known about the influence of macromolecular serum substances on the attachment and outgrowth of cellular processes of dissociated chick embryo brain cells in monolayer culture.

Previous work in this laboratory on the growth-promoting alpha-globulin (GPAG) demonstrated the physiological significance of this calf serum protein complex in cell culture. In contrast to hormone-like polypeptides, GPAG supports maximal growth of freshly explanted cells and diploid cell lines in a serum-free medium[5–8]. However, the main concern in our previous work was restricted to cultures of continually replicating cells. For this reason it appears important and desirable to investigate the effect of GPAG on cultured nerve cells.

Material and methods. GPAG was prepared by fractionation with ammonium sulphate and purified by repeated precipitation at pH 5. The biochemical and physiological characteristics of GPAG were described in our previous communications[5,8,13]. Cerebral hemispheres from 7-day-old chick embryos were mechanically dissociated in phosphate-buffered saline (PBS) and the cell suspension was centrifuged at 170× g for 3 min. Cell concentration was adjusted to 1.0×10^6 cells per ml nutrient medium and 5 ml of the above suspension was placed in Müller bottles or Falcon Petri dishes coated with air-dried rat tail collagen[9]. The nutrient medium consisted of Eagle's minimal medium (MEM) supplemented with a) 0.2% GPAG[5] (wt per vol.); b) 20% native fetal calf serum (University School of Veterinary Medicine, Brno, Czechoslovakia). The nutrient medium was changed every 3–5 days depending on its acidity. Cultures were maintained in a humidified atmosphere of 5% CO_2–95% air at 36.5 °C. Phase contrast

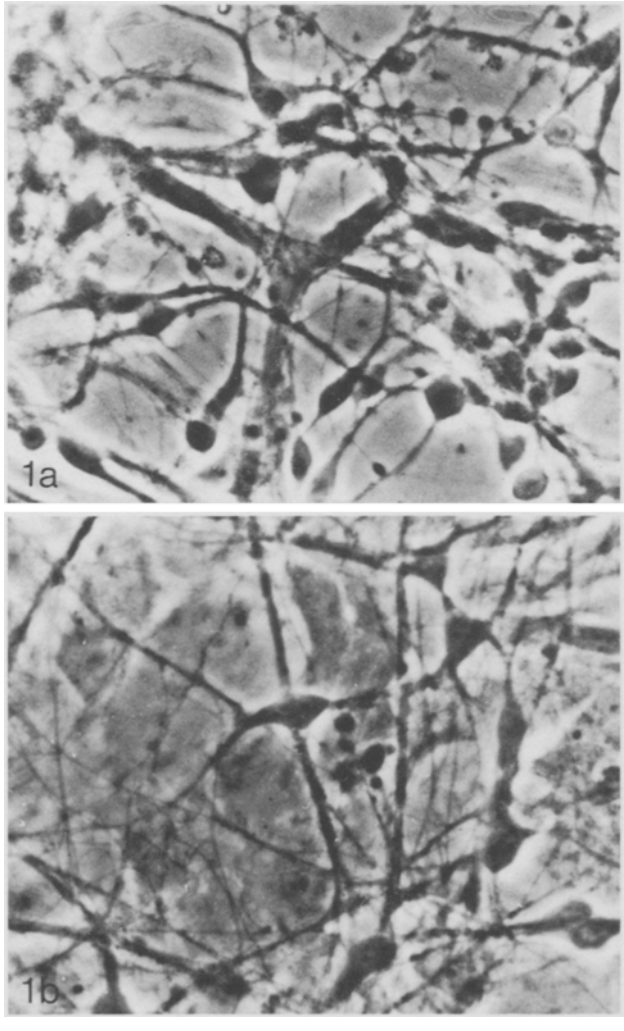

Figure 1. Micrographs of living cells from dissociated cerebral hemispheres obtained by phase contrast microscopy. 7 days in culture. × 560. *a* The cells were cultured in GPAG-supplemented medium. *b* The cells were cultured in serum-supplemented medium.

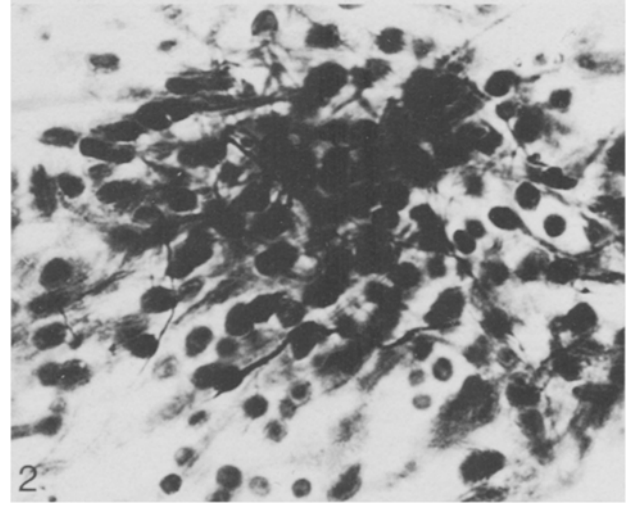

Figure 2. Micrograph of a group of neurons and their fibres from dissociated cerebral hemispheres. 7 days in culture. Silver impregnation. × 320.

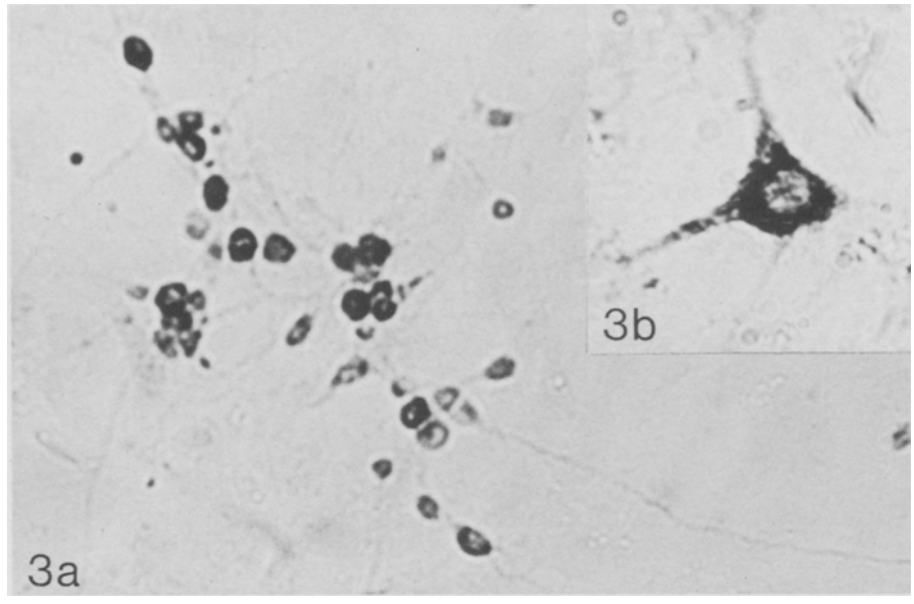

Figure 3. Micrographs demonstrating AChE activity in nerve cells from dissociated cerebral hemispheres. The cells were cultured in GPAG-supplemented medium for 21 days. *a* ×430; *b* ×1040.

microscopy was employed for observation of cultures during their growth period. Some cultures were fixed and impregnated with silver by the Holmes method[10]. The demonstration of acetylcholinesterase activity (AChE) was performed by the method of Karnovsky and Roots[11]; acetylthiocholine iodide was used as substrate and tetraisopropylpyrophosphoramide was used as inhibitor of non-specific cholinesterase activity.

Results and discussion. In GPAG-supplemented medium, dissociated cerebral hemisphere cells from 7-day-old chick embryos attach to the collagen-coated surface as single cells or small aggregates within few hours after explantation. However, during the first 24 h of incubation they exhibit only a little outgrowth of processes. In the following days, the processes elongate progressively and the cell bodies become bipolar or triangular in appearance. After 7 days, a network of fibres interconnects adjacent, single cells as well as aggregates and some neurites are organized in bundles. At the same time only a few glial and mesenchymal cells are present in the culture (fig. 1a). In the serum-supplemented medium differentiation of neurons occurs in a similar way but undifferentiated astrocytes and mesenchymal cells multiply more rapidly and form a bottom cell layer (fig. 1b). The cells with processes were identified as neurons by the Holmes method (fig. 2). In the next 2 weeks the proliferation of astrocyte-like cells increases so that nerve cells are placed on a well-developed monolayer after 3 weeks in GPAG-supplemented medium. In spite of the fact that nerve cell death occurs usually in GPAG-supplemented as well as in serum-supplemented media during the 3rd week of cultivation[12], some well-differentiated multipolar neurons with long and branching neurites are present in both systems. Parallel to the morphological differentiation, a biochemical differentiation occurred and neurons showed high AChE activity (fig. 3a and 3b). The only differences between cells growing in the absence or in the presence of serum are in the density of the glial cell layer and the thickness of the nerve fibre bundles. In comparison with GPAG-supplemented medium the density of the glial cell layer is higher, and the nerve fibre bundles are thicker, in serum-supplemented medium (fig. 4a and 4b). Thus, the GPAG-supplemented medium is sufficient to maintain growth and differentiation of chick embryo brain cells in vitro at least up to 3 weeks.

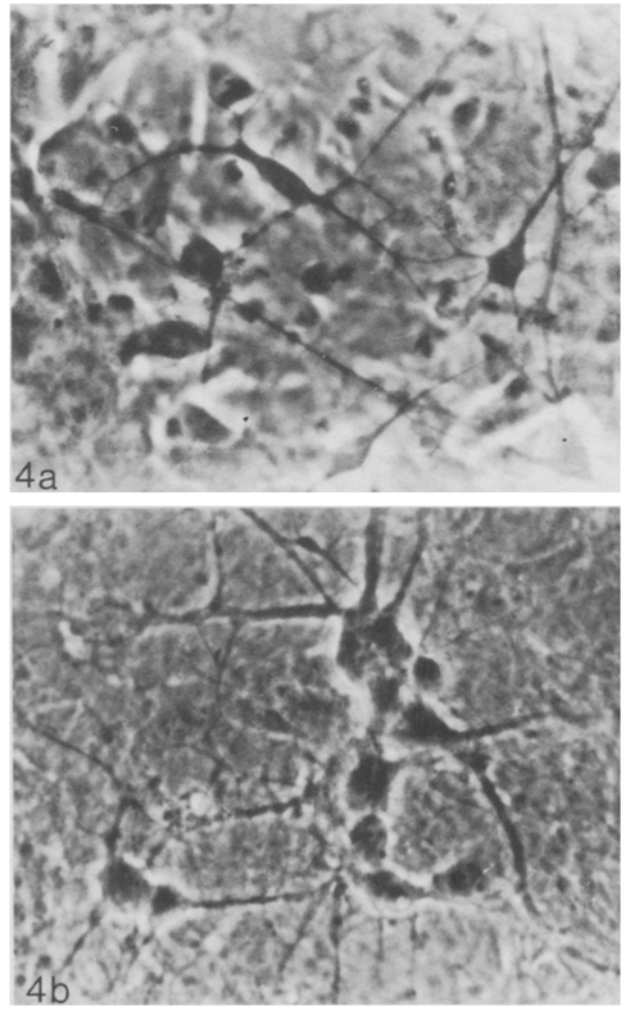

Figure 4. Micrographs of living cells from dissociated cerebral hemispheres obtained by phase contrast microscopy. 21 days in culture. ×560. *a* The cells were cultured in GPAG-supplemented medium. *b* The cells were cultured in serum-supplemented medium.

Our experiments indicate that GPAG is an adequate macromolecular serum factor for attachment of the majority of the brain cells to the collagen-coated surface and for enabling growth and morphological and biochemical differentiation of neural tissue in vitro. The attachment of cells is followed by histiotypic organization and prompt formation of neurites which are characteristic signs of neural tissue differentiation as well as the development of AChE activity. These findings have important implications, because a serum-free medium will facilitate physiological and biochemical studies of freshly explanted neuronal cells, for example neurotransmitter synthesis, synaptogenesis and the development of electrical excitability. Furthermore, in our previous publications we reported that GPAG pinocytosis plays an essential role in transmembrane substrate transport[13] and in the uptake of exogenous DNA by mammalian cells in vitro[14]. It has been demonstrated that GPAG associates with inorganic phosphate, thymidine, uridine, and lysine during incubation at 37 °C and transports the bound precursors into the cells in a compartmentalized manner. The pinocytic carrier function of GPAG is connected with the growth promotion of continually replicating metazoan cells. In this regard, it is interesting to note that since 1953 it is known that the probable cause of the growth of an embryonic neurite is the intake of fluid[15]. Vacuole formation in ganglionic neurites has already been observed by Lewis[16] who first described pinocytosis[17]. For this reason one of the potential uses of GPAG-supplemented medium is to study the mechanisms by which macromolecular serum factor(s) promote the maintenance of neurons in vitro. Since GPAG is present in the blood of metazoan organisms these studies might indicate simultaneously the role of specific serum macromolecules in the maturation of the brain.

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Flow microcalorimetric bioassay of polyene antibiotics: Interaction with growing *Saccharomyces cerevisiae*[1]

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Summary. Microcalorimetric investigation of the interaction of polyene antibiotics with mid-exponential cells of a growing culture of *Saccharomyces cerevisiae* has been used as the basis of a bioassay procedure. The assay is rapid, sensitive and reproducible. The results are compared to classical assays and potency ranking orders.

Calorimetric methods of analysis have been extensively reviewed[3-5]. The application of microcalorimetry to the study of drugs has also been reviewed recently[6]. This review suggests that there are only a few reports of quantitative bioassay procedures for drugs. Given the improvements in sensitivity, reproducibility and speed[7] offered by calorimetric techniques relative to the classical microbiological procedures this is somewhat surprising. The many and varied reports of a qualitative nature on the interaction of drugs with micro-organisms are adequately reviewed by Beezer and Chowdhry[6].

The results of experiments reported previously[7-9] employing isothermal flow microcalorimetry in the bioassay of polyene antibiotics with yeast cells *(Saccharomyces cerevisiae)* used buffered glucose as the only source of nutrient for the organism. Use of this medium produced simple power-time curves (p-t curves; previously termed thermogram, IUPAC, IUB, IUPAB draft recommendations, 1980) which could be analyzed to yield dose/response relationships. The results were used to give an antibiotic biopotency ranking order and this ranking order was compared with the order produced employing minimum inhibitory concentration (MIC) data. The 2 potency rankings were significantly different[10].

The evaluation of the bioassay system depended upon comparison of the appropriate bioassay parameters for the classical and microcalorimetric systems. However, as noted previously there were substantial differences in the experimental conditions. The fundamental difference being that the microcalorimetric system employed nongrowing cells whereas the classical bioassay systems (agar plate diffusion, turbidimetry[11]) employ growing organisms. Thus, the development of a microcalorimetric bioassay involving growing organisms would more nearly mimic the classical technique of bioassay.

Lucensomycin has been shown previously[9] to occupy a borderline position in the response observed with respiring (i.e. nongrowing) cells. Interaction with growing cells may support the view that lucensomycin has 2 modes of action and thus has 2 differing bioassay ranges.

Methods. Frozen inocula of *Saccharomyces cerevisiae* (NCYC 239 Food Research Inst., Colney Lane, Norwich) were prepared, stored and assayed as described previously[12]. The calorimeter (LKB type 10700-1, LKB Produkter AB, Sweden) its design and operation are as described previously[12] except that organism growth regimes rather than respiration regimes were employed. Thus the following variations to the reported[12] procedure were made. Nitrogen