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STUDY ON THE GROWTH PROMOTING CAPACITY OF CALF AND FETAL BOVINE SERUM FOR ANIMAL CELLS "IN VITRO"

II — Electrophoretic study and survey on the antiproteolytic activity of pools of calf and fetal bovine serum

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SUMMARY

Calf serum and fetal bovine serum present great variability as to its growth promoting efficiency (GPE). As supplement of culture media to cultivate cells of animal origin they stimulate the "in vitro" multiplication and maintain cell viability. When fourteen lots of calf sera of variable GPE had the total protein contents as well as the percentages of serum fractions determined, no significant differences that could possibly explain the variability of the GPE were observed. Evaluation of the antiproteolytic activity of nineteen lots of calf serum and eighteen serum lots of younger calves showed that the former exhibited lower antiproteolytic titers (1:40 to 1:80) than the latter (1:80 to 1:160). Twelve lots of fetal bovine serum studied in parallel, showed the highest concentration of antiproteolytic factors, with titers equal to 1:320. Sera of bovine origin, but not fetal sera, are usually heat-inactivated, what was demonstrated to be responsible for the decrease of the antiproteolytic activity of 75% of the lots tested. This could explain the inability of certain heat-inactivated sera in promoting multiplication of some cells "in vitro", as verified with primary monkey kidney cells. The results obtained in this study indicated the convenience of submiting each lot of serum to be introduced in cell culture to previous determination of its characteristics, such as growth promoting efficiency, antiproteolytic activity and also toxicity, absence of extraneous agents, etc., in order to minimize the possibility of using serum lots of questionable quality, thus preventing not only the loss of cell lines, but also undesirable and sometimes expensive delays.

INTRODUCTION

Animal serum is an ordinary component of cell culture systems since it is essential to maintain viability and to stimulate proliferation for most cell types.

The division of mammalian cells "in vitro" is controlled by a variety of factors that reach the cell surface coming from serum present in the nutrient medium [20,21,22]. They may be either macromolecular factors [4,6,8,9], or low molecular weight nutrients [7]. However, other growth factors (GF) must also be considered [10,11,12,13]. They are growth-stimulating substances, stable on heating at 56°C for 30 min [19], which bind to a cell membrane receptor to be then transferred whithin and degraded [17,18]. GF in the bloodstream may be extracted from plasma, serum or platelets. These corpuscles are the source of a potent mitogen that is released during the

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process of blood coagulation, when they aggregate [5,14,15,16]. While sera prepared from cell-free plasma show a considerably lower activity in stimulating growth and do not support growth of cells that require platelet factor [1], blood serum contains molecules present both in plasma and in platelets.

Serum of bovine origin is the most commonly used in cell cultivation. Presenting great variability as to its growth promoting efficiency (GPE), even when the product results from the pooling of sera of large number of animals, any serum should be checked for such efficiency, as well as for the presence of any virus, bacteriophage, mycoplasma and citotoxicity, prior to use.

Considering the widespread use of bovine serum, this paper reports data on the electrophoretic patterns of pools of calf sera of known GPE and of fetal sera. The antiprotease activity of such sera are also evaluated since this characteristic was demonstrated to be of relevance for the multiplication of some cells "in vitro" [24].

MATERIAL AND METHODS

Bovine Serum — Samples of calf serum prepared as described elsewhere [22], as well as samples of fetal serum were studied.

Heat inactivation — Calf sera were inactivated at 56°C in a water-bath for 30 min prior to sterilization. Fetal bovine sera were used "as is".

Electrophoretic assay — Electrophoresis was carried out with a Mikrophor Boskamp equipment. Samples were applied on Boskamp cellulose acetate strips (25.5 x 145 mm) with 2m A per strip for 20 min. Tris-acetate pH 8.6, 0.1 μ ionic strength was used as buffer solution. The strips were stained in Amidoschwarz — 10B, destained in methanol/acetic acid (9/1), and made transparent with dioxan/isobutanol (7/3). They were then scanned in a Zeiss EI-3 desintometer.

Antiproteolytic titer (APT) — Using Tris buffer pH 7.4, serum samples were diluted from 1:10 to 1:2,560, and skin milk to 1:4. From a 10% trypsin stock solution in water (stirred for 5 min and filtered through an AP-20 pad) a 1:1,000 dilution was prepared with the same buffer and used in the test. Aliquots were mixed and homogeneized as follows: 0.5 ml dilutions of the sample in test, 0.5 ml trypsin 1:1,000, 0.5 ml skin milk 1:4. After incubation in a 37°C water-bath for 60 min, the endpoint was considered as the highest dilution of the serum sample which was not digested by trypsin. Appropriate controls containing no sample and no trypsin were ran in parallel.

RESULTS

Fourteen lots of bovine sera assigned for cell cultivation and thus, with pre-determined GPE for a large number of cell lines [22], were submitted to electrophoresis. For all lots, no significant variations were observed among differential protein concentrations of albumin, alpha, beta and gamma globulin, as well as for total proteins (Table I). Three serum lots picked up at random from the same group, lot 75: good; lot 72: fair; lot 69: poor, showed great electrophoretic similarity despite their different GPE, as can be seen in Figure 1.

Figure 2 presents electrophoretic tracings of three fetal bovine serum lots exhibiting albumin, alpha, beta globulin, and the characteristic absence of gamma globulin. Comparable values for total and differential proteins were also found for them.

A group of final serum pools (29 lots of heat inactivated bovine serum: 11 of calf and 18 of young calf; 13 lots of non inactivated fetal bovine serum) processed by different manufacturers had their antiprotease activity assayed. While FBS exhibited the highest APT (usually 1:320), the majority of serum pools obtained from young calves reached titers varying from: 1:40 to 1:160. Meanwhile, calf sera, which are the most used to supplement cell culture media, exhibited the lowest APT with values ranging from 1:40 to 1:80 (Table II).

Since heat inactivation of calf serum prior to sterilization is a routine procedure in sera preparation, the effect of such heating on the APT of eight lots of calf serum was surveyed, when aliquots taken before and after inactivation were titrated. It was found that heating determined a decrease by half, in the APT of 75% of the serum lots tested (Table III).

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T A B L E I

Total and differential serum proteins presented by 14 lots of calf serum

Growth promoting efficiency *	Serum pool number	Total protein (g/100 ml)	Differential proteins (g/100 ml)			
			Albumin	Globulins		
				Alpha	Beta	Gamma
Good	64	7.3	2.55	1.68	0.88	2.19
	67	7.3	2.99	1.17	0.95	2.19
	75	8.0	2.56	1.20	1.20	3.04
	87	5.3	1.48	0.42	1.06	2.33
	90	7.1	2.34	0.50	1.14	3.12
Fair	60	8.1	1.94	3.16	1.05	1.94
	71	6.4	2.11	1.54	0.89	1.85
	72	6.6	2.38	0.99	0.92	2.31
	86	6.3	2.02	0.88	1.07	2.33
	89	7.1	2.91	0.85	1.28	2.06
Poor	68	7.6	1.67	2.73	0.84	2.36
	69	7.0	2.59	1.26	0.91	2.24
	76	8.2	2.87	1.31	1.07	2.95
	77	6.9	1.86	1.93	0.83	2.28

* **Growth Promoting Efficiency** (GPE):
Good: Cells presenting normal morphology and forming monolayers in 3-4 days
Fair : Cells presenting normal morphology but slower multiplication
Poor: Cells presenting altered morphology or granulation and inability to form confluent monolayers

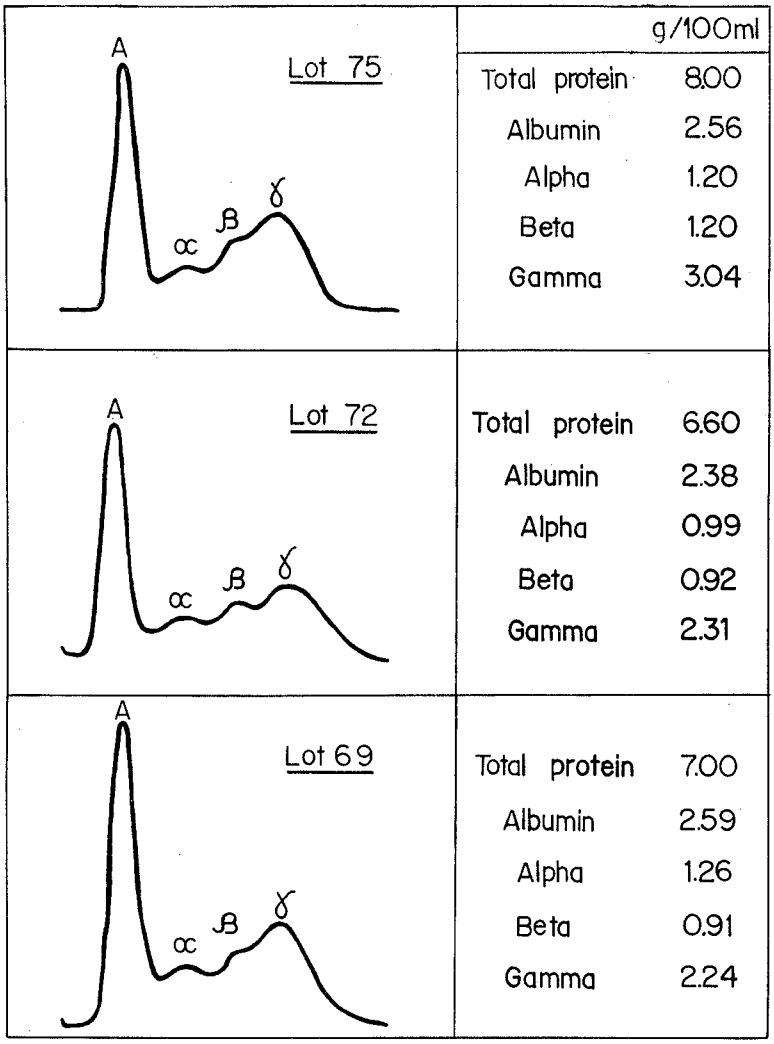
DISCUSSION

The variable quality of serum employed in cell cultivation was reported by CHANG [3] when using individual human sera to cultivate cells of human origin. Such variability, which is related to the efficiency in stimulating cell multiplication, exists even when a serum lot consists of a pool of sera [22]. In our country, the conditions that prevail when young calves are bled to prepare sera for cell culture are usually precarious and should be improved. Blood is collected in slaughter-houses while animals are being killed; often, the agonizing animals vomit, and extraneous materials get mixed with blood. Some laboratories have improved animal bleeding by puncturing blood vessels of large diameter, but even then hygiene is not ideal, since this phase of serum's processing is carried out outdoors, or in stables. Presently, some commercial houses are selling bovine serum prepared under more strict standards but the product, being as expensive as the imported one, makes most cell culture laboratories persist in preparing their own sera. Thus, the necessity of testing such sera in advance to prevent the introduction of lots of questionable quality in the cultivation of cell lines or primary cells, since this may lead to serious consequences and delays.

The best supplement available for cell culture is fetal bovine serum (FBS). Blood harvesting is made aseptically and only for special purposes serum can be: a) heat inactivated (to destroy complement, inactivate putative mucosal disease virus, etc); b) dialysed (to remove low molecular weight nutrients, salts, waste products, etc); c) irradiated by gamma or ultraviolet light (to destroy viruses, mycoplasma, etc). When added to good chemically defined basal medium the low molecular weight fraction present in FBS merely acts as an additional source of nutrients. The high molecular weight fractions, however, play a role in cellular attachment, mitogenic activities which promote optimal growth, and longevity of cells in culture.

There are other unidentified factors present in FBS which also contribute to optimal attachment, growth and survival of cells in culture. Moreover, FBS is devoid of gamma globulin, a point of interest when cell cultures are to be used in Virology.

The choice of FBS for cell cultivation has several advantages, since it does not show as much variability as calf serum. Each lot is capable of promoting good multiplication for a larger number of cell lines in comparison to calf sera. However, it should not be purchased in the presumption that a lot will work for



	g/100ml
Total protein	8.00
Albumin	2.56
Alpha	1.20
Beta	1.20
Gamma	3.04

Total protein	6.60
Albumin	2.38
Alpha	0.99
Beta	0.92
Gamma	2.31

Total protein	7.00
Albumin	2.59
Alpha	1.26
Beta	0.91
Gamma	2.24

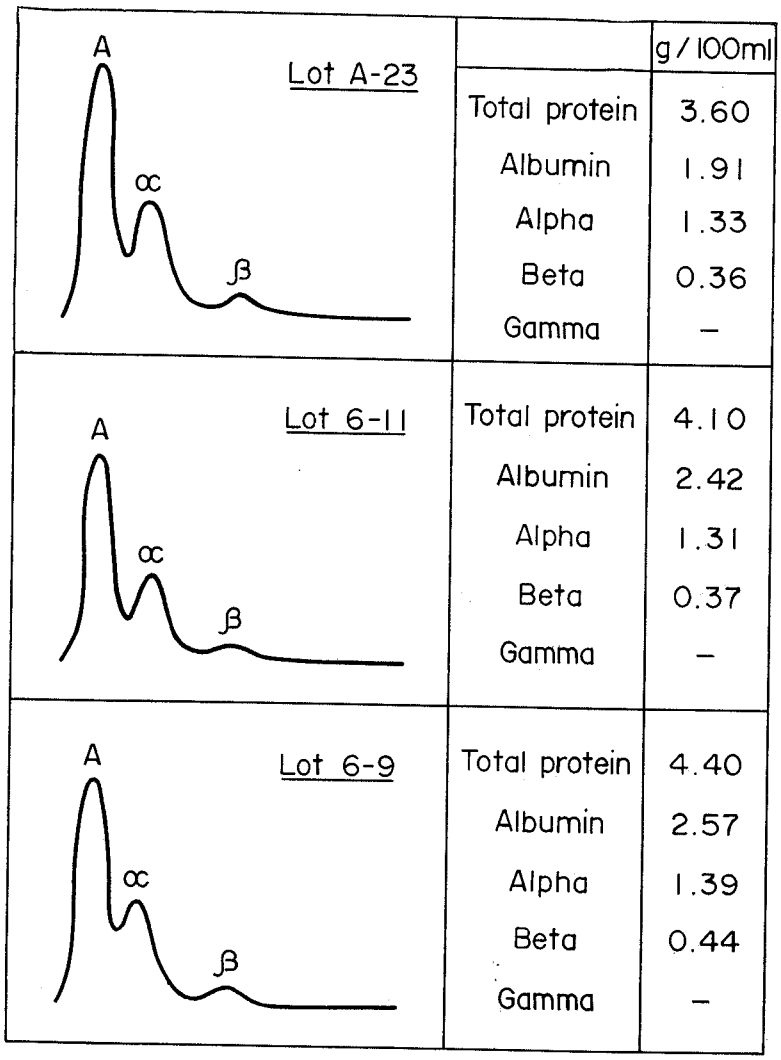
Growth promoting efficiency (GPE)

Serum lot 75 : good
lot 72 : fair
lot 69 : poor

Fig. 1 — Electrophoretic patterns of lots of calf serum with different growth promotion efficiency

all cells and the need for checking the GPE of each lot in advance remains. Being expensive, not all laboratories can afford it and keep on supplementing media with calf serum. Conse quently, for safety reasons, this calf serum should have some parameters established and evaluated such as, GPE, APT, absence of extraneous agents, etc.

Sera listed in Table I and Figure 1 constituted pools obtained from calves of different races, ages and sexes. Although such factors, as well as the season of the year, are likely to influence the protein contents of calf serum, data presented in Table I, referring to total protein values found for the 14 calf serum pools studied, showed no significant differences, as follows: 5.3 — 8.0 g/100 ml for good sera, 6.3



	g/100ml
Total protein	3.60
Albumin	1.91
Alpha	1.33
Beta	0.36
Gamma	–
Total protein	4.10
Albumin	2.42
Alpha	1.31
Beta	0.37
Gamma	–
Total protein	4.40
Albumin	2.57
Alpha	1.39
Beta	0.44
Gamma	–

Fig. 2 — Electrophoretic patterns of lots of fetal bovine serum

— 8.1 g/100 ml for fair, and 6.9 — 8.2 g/100 ml for poor sera. The concentration of differential proteins did not differ greatly either, despite the different capability in stimulating cell multiplication presented by the sera under study, confirming that GPE is not related to the presence of one or more serum fractions in higher or lower concentration[24]. These data agree with early reports by VILLELA et al.[23] on total protein averages of 5.59 g/100 ml and 7.00 g/100 ml, in calves. In an experiment with adult bovines averages similar to those listed in Table I for albumin and globulins were also found by BOTELHO[2]. Exception were the gamma, that occurred in higher concentration in his experiments, which could be accounted for by the fact that being adults, the animals could have received several courses of immunization.

WALLIS et al.[24] found out that for primary monkey kidney cell (MKC) cultures, good sera are those possessing high APT, which expresses the ability of inhibiting proteolytic enzymes synthesized by such cells, rather than the presence of a specific serum fraction in higher or lower concentration. Thus, the serum toxicity so often found could reflect the lack of antiprotease factors in sera, and not the presence of deletereous products. When properly carried out, the inactivation of calf sera by heating does not significantly influence the concentration of serum fractions. However, due to the heat-lability of some antiprotease factors, inactivation

T A B L E II

Antiproteolytic titer of different types of bovine serum

Type of serum	Origin	Lot number	Antiproteolytic titer
Fetal bovine	Unicamp	A	1:1.280
		B	1: 320
		C	1: 320
		D	1: 320
		E	1: 320
		F	1: 320
		G	1: 320
		H	1: 320
		I	1: 320
		J	1: 320
		K	1: 320
		L	1: 320
Young calf	Instituto Adolfo Lutz	1	1: 40
		2	1: 80
		3	1: 80
		4	1: 80
		5	1: 160
		6	1: 80
	Instituto Butantan	79	1: 80
		81	1: 160
		86	1: 160
		89	1: 160
		91	1: 80
		93	1: 80
		95	1: 160
		96	1: 80
		98	1: 80
		100	1: 80
		101	1: 80
		104	1: 80
Calf	Instituto Butantan	23	1: 40
		43	1: 40
		60	1: 40
		63	1: 40
		67	1: 40
		68	1: 40
		69	1: 40
		71	1: 80
		72	1: 80
		73	1: 80
		74	1: 80
		75	1: 40
		76	1: 80
		77	1: 40
		87	1: 40
		90	1: 80
		92	1: 40
		94	1: 40
		97	1: 40

prior to use or prior to sterilization can be responsible for a quality loss since the decrease of the enzymatic concentration may impair the sera capability in promoting the growth of certain cells, such as primary monkey kidney cells [24]. Data from Table III confirmed the negative effect of heating upon the original APT when 75% of the sera tested had their titers lowered from 1:80 to 1:40. This reduction in titer might also render them inadequate for the "in vitro" multiplication of other cell lines. The deletereous effect of inactivation might explain the low APT found for the 19 pools of calf sera (Instituto Butantan) listed in Table II, where 68.4% exhibited titers of only 1:40.

T A B L E III

Antiproteolytic titer of calf serum prior and after inactivation by heat (56°C for 30 min)

Lot number	Calf serum	
	"AS IS"	Heat inactivated
1	1:80	1:80
2	1:80	1:40
3	1:80	1:40
4	1:80	1:40
5	1:80	1:40
6	1:80	1:80
7	1:80	1:40
8	1:80	1:40

When the APT of serum pools obtained from fetuses, young and older calves was compared, it was observed that the former had the highest APT, usually 1:320, with only one exceptional titer of 1:1,280 occurring (Table II). Sera from younger calves had higher APT than sera from older calves, indicating that younger animals present more antiprotease factors in their serum.

Although fetal bovine serum is the very best for growth and maintenance of cells "in vitro", calf serum may replace it for general cell cultivation if its characteristics are known in advance, because a great variability of nutritional needs prevails among the cell lines extensively used in Virology.

RESUMO

Estudo sobre a capacidade promotora de crescimento de soros de vitelas e de soros fetais bovinos para células de origem animal cultivadas "in vitro"

II — Estudo eletroforético e pesquisa da atividade antiproteolítica de soros de vitelas e de soros fetais bovinos

O soro de vitelas e o soro fetal bovino apresentam grande variabilidade no que se re-

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fere à sua capacidade promotora de crescimento (CPC) e são empregados no cultivo de células de origem animal, suplementando os meios de cultura, com a finalidade de estimular a multiplicação das células "in vitro", assim como manter a viabilidade das mesmas. Quando 14 lotes de soro de vitelas apresentando diferentes CPC tiveram seu teor total de proteínas, assim como as percentagens séricas presentes determinados, não foram observadas diferenças significativas que justificassem a variação daquela capacidade.

Na avaliação da atividade antiproteolítica (AA) de 19 lotes de soros de vitelas e de 18 de soros de bezerros de tenra idade, foi constatado que os primeiros apresentavam títulos antiproteolíticos mais baixos (1:40 a 1:80) do que os registrados para os segundos (1:80 a 1:160). Doze lotes de soros fetais bovinos, avaliados em paralelo, exibiram concentração elevada de fatores antiproteolíticos, evidenciada por títulos de 1:320. A inativação a 56ºC durante 30 min. a que são geralmente submetidos os soros de origem bovina (com exceção dos fetais) demonstrou ser responsável pela baixa da AA dos mesmos, o que talvez justifique a incapacidade que certos soros inativados têm, de promoverem a multiplicação de algumas células "in vitro", como é o caso das células primárias de rim de macaco.

Os resultados obtidos salientam a conveniência de se determinar previamente, para cada lote de soro de origem bovina a ser introduzido em cultura celular, certas características, tais como a capacidade promotora de crescimento, a atividade antiproteolítica e também a ausência de agentes adventícios, toxicidade, etc., de modo a minimizar a possibilidade de, ao utilizar lotes de soro de qualidade questionável, incorrer em perda de linhagens celulares, assim como em indesejáveis atrasos.

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REFERENCES

1. BALK, S. D. — Calcium as a regulator of the proliferation of normal, but not transformed chicken fibroblasts in a plasma-containing medium. **Proc. Nat. Acad. Sci. U.S.A. 68:** 271-275, 1971.

2. BOTELHO, G. G. — Proteína total e frações do soro sanguíneo de bovinos submetidos ao cloridrato de xilazina. **Arq. Esc. Vet. U.F.M.G. 29:** 43-48, 1977.

3. CHANG, R. S. — Continuous subcultivation of epithelial-like cells from normal human tissues. **Proc. Soc. Exp. Biol. Med. 87:** 440-443, 1954.

4. CUNNINGHAM, D. D. & PARDEE, A. B. — Transport changes rapidly initiated by serum addition to "contact inhibited" 3T3 cells. **Proc. Nat. Acad. Sci. U.S.A. 64:** 1049-1056, 1969.

5. DAVIES, P. F. & ROSS, R. — Mediation of pinocytosis in cultured arterial smooth muscle and endothelial cells by platelet-derived growth factor. **J. Cell Biol. 79:** 663-671, 1978.

6. DE LUCA, C.; HABEEB, A. F. S. A. & TRISTSCH, G. L. — The stimulation of growth of mammalian cells "in vitro" by a peptide fraction from enzymatic digests of serum. **Exp. Cell Res. 43:** 98-106, 1966.

7. DULAK, N. C. & TEMIN, H. M. — A partially purified polypeptide fraction from rat liver cell conditioned medium with multiplication stimulating activity for embryo fibroblasts. **J. Cell Physiol. 81:** 153-160, 1973.

8. DULBECCO, R. — Topoinhibition and serum requirement of transformed and untransformed cells. **Nature 227:** 802-806, 1970.

9. HADDEN, J. W.; HADDEN, E. M.; HADDOX, M. K. & GOLDBERG, N. D. — Guanosine 3':5'-cyclic monophosphate: a possible intracelular mediator of mitogenic influences in lymphocytes. **Proc. Nat. Acad. Sci. U.S.A. 69:** 3024-3027, 1972.

10. HAUDENSCHILD, C. C.; ZAHNISER, D.; FOLKMAN, J. & KLAGSBURN, M. — Human vascular endothelial cells in culture: lack of response to serum growth factors. **Exp. Cell Res. 98:** 175-183, 1976.

11. HEALY, G. M. & PARKER, R. C. — Cultivation of mammalian cells in defined media with protein and nonprotein supplements. **J. Cell Biol. 30:** 539-553, 1966.

12. HERSHKO, A.; MAMONT, P.; SHIELDS, R. & TOMKINS, G. M. — "Pleiotypic response". **Nature New Bull. 232:** 206-211, 1971.

13. HOFFMANN, R.; RISTOW, H. J.; VESER, J. & FRANK, W. — Properties of two growth-stimulating proteins isolated from fetal calf serum. **Exp. Cell Res. 80:** 275-280, 1973.

14. HOLLEY, R. W.; BALDWIN, J. H. & KIERNAN, J. A. — Control of growth of a tumor cell by linoleic acid. **Proc. Nat. Acad. Sci. U.S.A. 71:** 3976-3978, 1974.

15. HOLLEY, R. W. & KIERNAN, J. A. — "Contact inhibition" of cell division in 3T3 cells. **Proc. Nat. Acad. Sci. U.S.A. 60:** 300-304, 1968.

16. HOLLEY, R. W. & KIERNAN, J. A. — Growth control in cell cultures. CIBA Foundation Symposium (ed. J. Knight & G.E.W. Wolstenhome). London, Churchill, 1971, p. 3.

17. SEFTON, B. & RUBIN, H. — Stimulation of glucose transport in cultures of density-inhibited chick embryo cells. **Proc. Acad. Sci. U.S.A. 68:** 3154-3157, 1971.

18. SEIFEST, W. E. & RUDLAND, P. S. — Possible involvement of ciclyc GMP in growth control of cultured mouse cells. **Nature 248:** 138-140, 1974.

19. TEMIN, H. M.; PIERSON, R. W. & DULAK, N. C. — Growth, nutrition and metabolism of cells in culture. In: G. H. Rothblat, V. J. Christofalo **1:** 49-81. New York, Academic Press, 1972.

20. THORGEIRSSON, G. & ROBERTSON JR., A. L. — Platelet factors and the human vascular wall. Part. 2. Such factors are not required for endothelial cell proliferation and migration. **Atherosclerosis 31:** 231-238, 1978.

21. TODARO, G. J.; LAZAR, G. K. & GREEN, H. — The inhibition of cell division in a contact-inhibited mammalian cell line. **J. Cell Comp. Physiol. 66:** 325-333, 1965.

22. TUCHIYA, H. N.; MENDES, I. F.; MIYAKI, C. & RIZZO, E. de — Study on the growth promotion capacity of calf serum for animal cells "in vitro". I-Test of 56 lots of calf serum against several cell lines and primary cell cultures. **Rev. Inst. Med. trop. São Paulo 22:** 281-285, 1980.

23. VILLELA, L. G.; SILVIA, E. & COSTA VAL, V. P. — Effect of diet on the total protein content in female Gir blood serum. **Arq. Esc. Vet. XXII:** 113-117, 1970.

24. WALLIS, C.; VER, B. & MELNICK, J. L. — The role of serum and fetuin in the growth of monkey cells in culture. **Exp. Cell Res. 58:** 271-282, 1969.

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