Iron Replacement of Lactalysate and Embryo Extract in Growth of Cell Cultures.* (26782)

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A serumless medium for the growth of mammalian cell cultures has been described (1). A critical constituent and the only undefined component of this medium was lactalysate, a commercial enzymic digest of lactalbumin. In further studies, requirements of avian cell cultures for serum and embryo extract were partially replaced by components of the serumless medium. A specific function performed by lactalysate and by embryo extract was the counteraction of a toxicity of serum for the avian cells(2).

The present communication describes the effectiveness of iron and iron-bearing materials in replacement of lactalysate and embryo extract requirements for growth of cell cultures and for overcoming a serum toxicity.

Materials and methods. Procedures have been described previously(1,2,3). In the present studies, 5000 Walker carcinosarcoma 256 cells from stock cultures or 60 to 80,000 white Leghorn chick embryo (14 day) lung cells prepared by trypsinization were established (plated) overnight in 1 ml of medium in 15 × 125 mm roller tubes placed in an inclined position in racks. Incubation was at 37°C in an atmosphere of 92% air—8% carbon dioxide. After 16 to 24 hours, the medium was withdrawn from the tubes which were then rinsed with Earle's solution. One ml of test basal medium containing graduated quantities of test substances at 6 to 9 levels was pipetted into duplicate tubes. Repeated experiments confirmed the results in all cases. Incubation was continued for 3 or 4 days. The cultures were examined microscopically and growth was measured by protein determination according to the procedure of Oyama and Eagle(4).

The medium used to establish Walker tumor cells contained 0.2 mM pyruvate, 10 μg adenosine per ml, 5% bovine serum and amino acids, vitamins, glucose, minerals, antibiotics and phenol red in the quantities specified for serumless medium(1). This medium, supplemented with 5% embryo extract, was used to establish chick embryo cultures.

The test basal medium for Walker carcinosarcoma 256 cells was the serumless medium described previously(1) with omission of mucic acid and lactalysate and with replacement of Tween 80 by Tween 20. The latter material has been found in this laboratory to be less toxic than Tween 80 for cell cultures (5). Methyl oleate was decreased to 10 μg/ml. This test basal medium, modified to contain 5 μg methyl oleate and 5 μg insulin per ml, was utilized for studies of avian cultures.

Transferrin† was prepared by modification of the procedure of Surgenor *et al.*(6) from pooled human plasma. Bovine hemoglobin (2 × recrystallized) and horse heart cytochrome C (type III) were obtained from Sigma Chemical Co., St. Louis, Mo. Horse heart myoglobin and horse spleen ferritin were obtained from Pentex Incorporated, Kankakee, Ill.

Lactalysate was Edamin DR, an enzymatic digest of lactalbumin supplied by the Sheffield Chemical Co., Norwich, Conn.

The relatively non-dialyzable portion of lactalysate (lactalysate residue) was prepared by dialysis overnight against running tap water followed by dialysis for 24 hours against several changes of an excess of demineralized water. The lactalysate residue was freeze-dried. Hydrolysates of lactalysate residue were prepared by autoclaving 100 mg in 5 ml 6 N HCl in sealed tubes for 16 hours at 15 lb pressure. The HCl was removed by flash evaporation. The hydroly-

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TABLE I. Growth Response of Walker Carcinosarcoma 256 Cells to Lactalysate, Lactalysate Residue, Hydrolyzed Residue, Ash and Ferric Nitrate.

Lactalysate		Lactalysate residue		Hydrolyzed lactalysate residue		Lactalysate ash		Lactalysate residue ash		Fe^{+++}	
μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml*	μg cell protein/tube	μg/ml†	μg cell protein/tube	μg/ml	μg cell protein/tube
0	4	0	4	0	4	0	4	0	4	0	4
100	6	5	26	5	32	600	6	50	4	.056‡	42
200	44	10	46	10	40	1,200	8	100	5	.28	51
500	73	20	69	20	64	3,000	13	200	6	.56	57
1,000	74	50	52	50	59	6,000	36	500	11	1.12	59
2,000	66	100	59	100	57	15,000	12	1,250	19	2.24	47
										4.48	14

Inoculum was 5000 cells/ml. Growth period was 72 hr.
* Equivalent of lactalysate. † Equivalent of lactalysate residue. ‡ Equivalent to a concentration of 0.001 mM ferric nitrate.
Individual data points represent means of 2 tubes. Avg stand. dev. per single point was estimated to be ± 5.2 μg cell protein/tube. Avg stand. error of individual means was estimated to be ± 3.7 μg cell protein/tube.

sate was taken up in ethanol and again flash evaporated. An aqueous solution of the hydrolysate then was decolorized with Darco charcoal, grade S-51.

Iron determination was by the procedure of Gubler *et al.*(7).

Results. The comparative stimulatory effects on growth of Walker carcinosarcoma 256 cells by lactalysate, lactalysate residue, lactalysate residue hydrolysate, ash preparations and ferric nitrate are shown in Table I. It is apparent that the growth-promoting effect of lactalysate was concentrated in the non-dialyzable residue which comprised about 8% of the original material. The dialysate was inactive. The activity of the lactalysate residue persisted after drastic hydrolysis. The ash of both lactalysate and lactalysate residue contained slight activity, but excessive inactive salts present might have interfered with the bioassays. A number of trace elements were tested for activity, and iron as ferric nitrate was found to stimulate growth markedly. The iron content of lactalysate and lactalysate residue were determined to be 0.011% and 0.091% respectively, which was sufficient to account for activity on the basis of iron content. Salts of manganese, zinc, copper, and cobalt, as well as sodium nitrate, were inactive either alone or in combination over a wide range of concentrations.

The growth response of Walker carcinosarcoma 256 to lactalysate, transferrin, and several iron-containing proteins and compounds is shown in Table II. Minute quantities of transferrin, hemoglobin, myoglobin and hemin stimulated growth. Inhibition was produced at higher levels. Substantially greater concentrations of cytochrome C were required for stimulation. In further investigation, sodium iron versenate stimulated at 1-5 μg/ml. Ferrocyanide, ferricyanide, catalase, and ferritin were inactive.

Lactalysate, lactalysate residue, lactalysate residue hydrolysate and embryo extract earlier were observed to stimulate chick embryo cell cultures as well as to overcome a toxicity of bovine or chicken serum(2). Continuation of the present studies demonstrated that iron and iron-bearing materials substi-

TABLE II. Growth Response of Walker Carcinosarcoma 256 Cells to Various Iron-Bearing Materials.

Lactalysate		Human transferrin		Bovine hemoglobin		Horse myoglobin		Horse cytochrome C		Hemin	
μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube
0	10	0	10	0	10	0	10	0	10	0	10
100	30	.1	18	1	15	1	22	1	11	.1	11
200	45	.5	32	2	22	2	29	2	8	.5	36
500	51	1	43	5	36	5	46	5	13	1	22
1000	50	5	47	10	45	10	52	10	13	2	6
2000	32	10	46	50	31	50	54	50	15	5	4
		50	41	100	32	100	6	100	13	10	6
				500	6	500	6	500	46		
								1000	39		

Inoculum was 5000 cells/ml. Growth period was 72 hr.

Individual data points represent means of 1 to 3 tubes. Avg stand. dev. per single point was estimated to be ± 5.3 μg cell protein/tube. Avg stand. error of individual means estimated to be ± 3.7 μg cell protein/tube.

tuted for lactalysate and derivatives and for embryo extract in promotion of growth and inhibition of the toxic serum effect (Table III). Iron, hemoglobin, and myoglobin supported growth comparable to that with lactalysate residue (superior to lactalysate for avian cultures) or with 5% dialyzed embryo extract. Of particular interest was the ineffectiveness of human transferrin. As with the Walker tumor cells, 0.5 to 1 μg hemin, 1-5 μg sodium iron versenate and 500-1000 μg cytochrome C stimulated, while ferrocyanide, ferricyanide, catalase, and ferritin were inactive. These effects were most notable in media containing bovine serum. Additional toxicity perhaps of a different nature in chick serum was observable even after addition of embryo extract(3) and diminished responses to iron-bearing materials in the presence of this serum.

In Fig. 1 is shown the appearance of the chick embryo lung cultures in different media. Fig. 1a is the plated inoculum. Growth in basal serumless medium (Fig. 1b) is improved by either lactalysate residue (Fig. 1c) or by ferric nitrate (Fig. 1d). Inclusion of 5% dialyzed bovine serum in the basal medium results in destruction of the cells (Fig.

FIG. 1. Chick embryo lung cultures. ×30. Fixed with Bouin's and stained with Giemsa. Inoculum was 80,000 cells/ml. Growth period was 4 days.

(a) Inoculum plated 24 hr.
(b) Basal medium devoid of lactalysate or iron.
(c) Basal medium + 250 μg lactalysate residue/ml.
(d) Basal medium + ferric nitrate at 0.02 mM.
(e) Basal medium + 5% dialyzed bovine serum.
(f) Basal medium + 5% dialyzed bovine serum & 5% dialyzed embryo extract.
(g) Basal medium + 5% dialyzed bovine serum & 250 μg lactalysate residue/ml.
(h) Basal medium + 5% dialyzed bovine serum & ferric nitrate at 0.02 mM concentration.

TABLE III. Growth Response of Chick Embryo Lung Cells to Various Iron-Bearing Materials.

Lactalysate residue		Fe^{+++}		Human transferrin		Bovine hemoglobin		Horse myoglobin	
μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube
Basal medium without added dialyzed bovine serum									
0	19	0	19	0	19	0	19	0	19
10	58	.028*	34	1	20	2	46	2	54
50	72	.056	46	5	17	10	72	10	68
100	78	.28	64	10	12	20	72	20	74
250	78	.56	80	50	14	50	82	50	82
500	86	1.12	74	100	12	100	94	100	86
1000	90	2.8	80	500	16	500	108	500	94
Basal medium with 5% dialyzed bovine serum									
0	30	0	30	0	30	0	30	0	30
10	94	.028*	36	1	38	2	64	2	80
50	158	.056	34	5	32	10	78	10	80
100	166	.28	94	10	30	20	124	20	134
250	184	.56	128	50	36	50	146	50	180
500	190	1.12	158	100	30	100	158	100	172
1000	170	2.8	158	500	28	500	118	500	160

Inoculum was 80,000 cells/ml. Growth period was 4 days. Growth in basal medium plus 5% dialyzed embryo extract — 76 μg cell protein/tube. Growth in basal medium plus 5% dialyzed embryo extract plus 5% dialyzed bovine serum = 183 μg cell protein/tube.

* Equivalent to concentration of 0.0005 mM ferric nitrate.

Individual data points represent means of 2 tubes. Avg stand. dev. per single point for basal media without added dialyzed bovine serum was ± 4.1 μg cell protein/tube. Avg stand. error of individual means was estimated to be ± 2.9 μg cell protein/tube. For basal medium with dialyzed bovine serum, avg deviation was estimated to be ± 12.1 μg cell protein/tube. Avg stand. error of individual means estimated to be ± 3.6 μg cell protein/tube.

1e). This is readily overcome by lactalysate residue (Fig. 1g), dialyzed chick embryo extract (Fig. 1f), or by ferric nitrate (Fig. 1h).

Discussion. The stability of a growth-promoting factor of lactalysate to drastic hydrolytic conditions and detection of activity in ashed samples, despite possible interference of excessive salts, suggested that trace metals might be stimulatory. Iron and iron-bearing compounds, indeed, were found to simulate the effects of lactalysate and derived products in 2 different culture systems. Growth of both Walker carcinosarcoma 256 cells in serumless medium and chick embryo lung in a similar medium with or without added serum was stimulated. Furthermore, like lactalysate and embryo extract, iron and iron-bearing materials specifically overcame a toxic effect of serum through which chick embryo cells failed to multiply and underwent dissolution. The retention of activity in the non-dialyzable portion of lactalysate and embryo extract indicated that these residues bind, stabilize or transport iron; in fact, following dialysis, the iron content of lactalysate residue was increased approximately 8 to 9-fold and corresponded to an increased activity. It is evident that at least one major contribution of lactalysate and dialyzed embryo extract to cell growth was related to iron requirements. Of course, embryo extract and lactalysate may provide amino acids and vitamins to cultures in media in which these nutrients are not otherwise incorporated. The growth of the Walker tumor cells with lactalysate or chick embryo lung cells with lactalysate residue or embryo extract often was slightly superior to that obtained with other supplements as may be seen from the tables. This suggests that the properties of these materials serve particularly well the processes of iron metabolism or contribute additional special nutrients or non-specific factors such as buffering capacity. Other functions that might be served by lactalysate preparations and embryo extract as well as their effects on other cell strains and cells in long term culture are at present under investigation.

In earlier work, Morgan, Morton, and Parker included iron in their Medium 199 de-

signed to prolong survival of chick embryo cultures(8). White observed beneficial effects on strain "L" mouse fibroblasts in semi-synthetic media(9). Waymouth stimulated growth of these cells in synthetic media with iron(10). Ham found iron to be essential for growth of isolated Chinese hamster cells in media containing serum albumin and fetuin(11). The current studies quantitatively confirm these findings on mammalian and avian cell cultures. Hemoglobin also has been observed earlier to stimulate growth of cell cultures(12,13,14).

A marked difference between mammalian and avian cells was the incapacity of the latter to respond to human transferrin. The toxic sera presumably already contained high concentrations of transferrin which was not utilized by the avian cells or actually was involved in the toxicity of the serum through interference with availability of minute quantities of iron.

Summary. Iron salts and iron-bearing materials replaced lactalysate in stimulation of growth of Walker carcinosarcoma 256 cells in serumless medium and replaced lactalysate and embryo extract in stimulation of chick embryo lung cultures in a similar medium with or without further addition of serum. Presumptive evidence is presented that a stimulatory activity of lactalysate is due to iron and iron-bearing materials. A specific function of lactalysate and embryo extract in overcoming toxicity of serum for embryo cell cultures was served equally well by iron or iron-bearing materials. The avian cells revealed in their incapacity to respond to transferrin a characteristic distinguishing them from the Walker tumor cells.

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Mammary Lobulo-Alveolar Growth Induced by Anterior Pituitary Hormones in Adreno-Ovariectomized and Adreno-Ovariectomized-Hypophysectomized Rats.*† (26783)

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Studies in ovariectomized-hypophysectomized or in adreno-ovariectomized-hypophysectomized rats have indicated that hormones from the anterior pituitary and ovaries are both essential for inducing mammary lobulo-alveolar growth(1-3). The adrenals are believed to be of secondary importance, although it is recognized that they secrete several steroids which can influence mammary growth(3). Only a small degree of mammary growth was produced by injecting anterior pituitary extracts into ovariectomized

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