groups were slightly lower than comparable figures for DCI pretreated animals statistical treatment indicated that the apparent differences were not at all significant.

It would appear from this series of experiments that the unique type of adrenergic blockade produced by DCI(5,6) is not effective against either fever or heat loss. It is concluded that some mechanism other than body temperature is concerned in the protective action of DCI which has been observed in mice challenged with *E. coli* endotoxin(7).

These data also suggest that the pyrogenicity of an endotoxin is not directly correlated with its toxicity. This is an important observation since many investigators use as an assay for endotoxin its ability to produce hyperthermia in experimental test animals. If the assay employed fails to measure the originally significant property of an endotoxin, *i.e.*, its toxicity, then the value of the assay needs thoughtful evaluation.

*Summary.* 1) Pretreatment of rabbits with DCI failed to prevent the pyrogenic reaction to injected bacterial endotoxin. 2) The hypopyrexia produced in mice by endotoxin likewise was unaffected. 3) It is concluded that the type of adrenergic inhibition produced by this substance is not germane to the problem of body temperature changes induced by bacterial endotoxins.

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## Partial Replacement of Serum and Embryo Extract Requirements for Growth of Avian Cell Cultures.* (26500)

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Embryo extract has been employed extensively in tissue culture(1) since the discovery of its stimulatory effects by Carrel(2). Modern developments have eliminated this material in a majority of instances from the requirements of mammalian cells(3); nevertheless, avian cultures, upon which the initial observations were made, have continued to require embryo extract or components of embryo extract for satisfactory growth(4,5,6). Serum, traditionally necessary for mammalian cells, also has been essential to avian cultures in addition to the embryo extract(4,5,6).

The present report concerns the growth response of avian cultures to constituents of a serumless medium devised for cultivation of mammalian cells(7).

*Methods and materials* have been described previously(6). In brief, cell suspensions of 14-16 day white Leghorn chick embryo lung or heart were prepared by stirring the minced tissue 2-4 hours with 0.2% Difco 1:250 trypsin in Earle's balanced salt solution at 37°C. The cell suspension filtered through gauze and collected by centrifugation was resuspended in a complete medium containing serum and embryo extract(6). One ml amounts of the suspension containing 50,000 to 100,000 cells per ml were pipetted into 15 × 125 mm roller tubes placed in a slanted position in racks. The cultures were established

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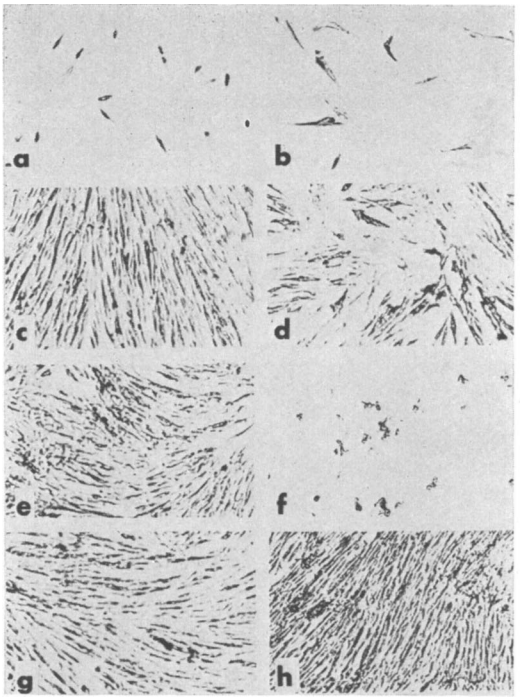


FIG. 1. Response of chick embryo lung cells to different media. Growth period, 4 days. Cells fixed with Bouin's and stained with Giemsa. × 30. a. Plated inoculum. b. Basal medium A. c. Basal medium A with 5% dialyzed bovine serum and 5% dialyzed embryo extract. d. Basal medium A plus special additives. e. Basal medium A plus special additives and 5% dialyzed bovine serum. f. Basal medium A plus special additives plus 5% dialyzed serum, no lactalysate residue. g. Basal medium A plus special additives, 5% dialyzed serum and 5% dialyzed embryo extract, no lactalysate residue. h. Basal medium A plus special additives, 5% dialyzed serum and 5% dialyzed embryo extract, lactalysate residue included.

(plated) overnight by incubation at 37°C in an atmosphere of 8% carbon dioxide—92% air. The medium was removed and the tubes rinsed with Earle's solution. The cells then were overlaid with one ml of basal medium containing graduated quantities of test materials. After further incubation for 3-5 days growth was measured by the protein determination method of Oyama and Eagle(8). The basal test medium (Medium A) for these studies consisted of all the constituents of the serumless medium for mammalian cultures(7) with the exception of the special additives which were incorporated as indicated for individual experiments. The preparation of dialyzed bovine serum and dialyzed embryo extract used has been described(6).

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 non-dialyzable residue then 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. HCl was removed by flash evaporation. The hydrolysate was taken up in ethanol and again flash evaporated. An aqueous solution of the hydrolysate was then decolorized with Darco, grade S-51.‡

*Results.* The appearance of typical chick embryo lung cultures in various media is shown in Fig. 1. The plated cells of the inoculum (Fig. 1a) increased in size but did not multiply in Medium A (Fig. 1b). Supplementation with dialyzed embryo extract and dialyzed serum promoted rapid growth (Fig. 1c). Addition of pyruvate, insulin, salmine, lactalysate (or derived preparations), folinic acid and methyl oleate, which were special constituents of serumless medium, enabled the cells to grow into sheets (Fig. 1d). Addition of dialyzed serum to the medium containing the special additives produced rapid growth (Fig. 1e) comparable with media containing embryo extract and serum. In the presence of serum, omission of lactalysate or derivatives resulted in a marked toxicity (Fig. 1f). Embryo extract prevented this toxicity (Fig. 1g) as did the lactalysate preparations and good growth proceeded in medium containing embryo extract, serum, and special additives together with the non-dialyzable residue of lactalysate (Fig. 1h). Inclusion of whole lactalysate in the most complex medium often resulted in a characteristic clumping of cells and less growth. In all further studies lactalysate residue was used rather than whole lactalysate.

Investigation of the effect of special constituents added singly or in combination to

† A pancreatic digest of lactalbumin, Edamin DR, Sheffield Chemical Co., Norwich, N. Y.

‡ A. H. Thomas Co., Philadelphia, Pa.

TABLE I. Effect of Insulin, Salmine, Pyruvate, and Lactalysate Residue on Growth of Chick Embryo Heart and Lung Cell Cultures.

| Insulin | | | Salmine | | | Pyruvate | | | Lactalysate residue | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | μg cell prot./tube | | | μg cell prot./tube | | | μg cell prot./tube | | | μg cell prot./tube | |
| μg/ml | 0 serum | 5% serum | μg/ml | 0 serum | 5% serum | mM | 0 serum | 5% serum | μg/ml | 0 serum | 5% serum |
| | | | | | Chick embryo heart | | | | | | |
| 0 | 4 | 138 | 0 | 30 | 48 | 0 | 30 | 102 | 0 | 22 | 18 |
| .05 | 10 | 121 | .1 | 30 | 74 | .01 | 36 | 116 | 25 | 54 | 56 |
| .1 | 10 | 121 | .5 | 38 | 46 | .05 | 36 | 138 | 50 | 44 | 98 |
| .5 | 20 | 138 | 1 | 38 | 84 | .1 | 32 | 166 | 100 | 98 | 144 |
| 1 | 36 | 150 | 3 | 44 | 166 | .2 | 38 | 168 | 200 | 52 | 184 |
| 2 | 34 | 178 | 5 | 38 | 172 | .5 | 42 | 182 | 500 | 42 | 190 |
| 5 | 40 | 190 | 8 | 38 | 166 | 1 | 46 | 184 | 1000 | 46 | 186 |
| 10 | 46 | 178 | 10 | 44 | 154 | 2 | 48 | 164 | | | |
| | | | | | Chick embryo lung | | | | | | |
| 0 | 36 | 82 | 0 | 96 | 36 | 0 | 100 | 132 | 0 | 12 | 12 |
| .05 | 38 | 80 | .1 | 96 | 40 | .01 | 112 | 128 | 25 | 28 | 42 |
| .1 | 38 | 80 | .5 | 112 | 40 | .05 | 118 | 126 | 50 | 48 | 72 |
| .5 | 46 | 88 | 1 | 118 | 78 | .1 | 108 | 128 | 100 | 60 | 92 |
| 1 | 52 | 94 | 3 | 126 | 156 | .2 | 118 | 130 | 200 | 60 | 104 |
| 2 | 54 | 102 | 5 | 118 | 170 | .5 | 118 | 152 | 500 | 68 | 128 |
| 5 | 58 | 116 | 8 | 102 | 182 | 1 | 112 | 158 | 1000 | 66 | 118 |
| 10 | 64 | 88 | 10 | 104 | 158 | 2 | 118 | 162 | | | |

Inoculum was 80,000 cells/ml. Growth was 4 days at 37°C in basal medium A together with insulin, salmine, pyruvate, lactalysate residue, folinic acid and methyl oleate with and without 5% dialyzed serum.

medium A with and without serum demonstrated the stimulatory effects of lactalysate and derivatives, pyruvate, insulin, and salmine. Methyl oleate and folinic acid were not found to be consistently stimulatory. Mucic acid and galacturonic acid at no time appeared beneficial and were omitted from the media. In Table I is shown the growth response of chick embryo lung and heart cultures to graduated quantities of insulin, salmine, pyruvate and lactalysate residue. The basal medium was medium A, containing, unless otherwise specified, 5 μg insulin, 5 μg salmine sulfate, 250 μg lactalysate residue, 1 mM pyruvate, 0.1 μg folinic acid, and 5 μg methyl oleate per ml with and without 5% dialyzed bovine serum. It will be noted that omission of any one of the additives evaluated resulted in decreased growth. Optimal levels were approximately the quantities included in the complete medium (as listed above) and which were determined by preliminary experiment. The heart and lung cultures responded in a similar fashion. Growth was greatest in media containing serum. Variation of the serum concentration over a range of 3 to 10% did not affect results appreciably.

The role of lactalysate or derivatives was especially important in that these overcame the toxicity noted previously, which was characteristic of all samples of bovine serum from animals of relative maturity or chicken serum tested whether dialyzed or undialyzed. This toxicity of serum was manifested in the simpler medium A as well as in presence of the special constituents. In either case lactalysate, its derivatives, or embryo extract overcame the toxicity. On occasion, lactalysate was not wholly satisfactory in media containing samples of serum which were exceptionally toxic. Examination of the lactalysate revealed that stimulatory and protective factor(s) reside chiefly in the non-dialyzable portion comprising about 8% of the total. This "lactalysate residue" at 250 μg/ml substituted completely for 1-5 mg of lactalysate to stimulate growth and consistently overcame toxicity of *all* sera tested. Although the non-dialyzable property of the active portion of lactalysate suggested a proteose or peptone, this concept was dispelled by the equivalent activity of lactalysate residue subjected to acid hydrolysis (Table II). Identification of the active factor(s) is being investigated.

Bacto-peptone displayed activity but was

TABLE II. Relative Activity of Lactalysate, Lactalysate Residue and Its Acid Hydrolysate for Growth of Chick Embryo Lung Cultures.

| Lactalysate | | Lactalysate residue | | Lactalysate residue (acid hydrolysate*) | |
|---|---|---|---|---|---|
| mg/ml | μg cell protein/tube | mg/ml | μg cell protein/tube | mg/ml | μg cell protein/tube |
| 0 | 12 | 0 | 12 | 0 | 12 |
| .1 | 12 | .025 | 106 | .025 | 102 |
| .5 | 30 | .05 | 138 | .05 | 128 |
| 1.0 | 60 | .1 | 172 | .1 | 158 |
| 2.0 | 100 | .2 | 186 | .2 | 166 |
| 3.0 | 122 | .5 | 194 | | |
| 5.0 | 142 | 1.0 | 186 | | |

* Autoclaved 16 hr in 6 N HCl. Inoculum was 80,000 cells/ml. Growth was 4 days at 37°C in basal medium A with special additives and 5% dialyzed bovine serum.

much less potent than lactalysate. Bactotryptone and Difco proteose-peptone were not significantly active.

Bovine fetal serum containing essentially a gamma protein which was 39% α-globulin,§ presumably largely fetuin, proved to be an exception among all sera tested in that it was non-toxic. Table III gives a comparison of growth of chick embryo lung cultures in media containing dialyzed fetal calf serum and dialyzed bovine serum. The contrast in toxic properties of the 2 sera may be noted. Addition of lactalysate or embryo extract was unnecessary for maximal growth with fetal serum. However, growth was inferior to that in media containing either of these factors together with the customary bovine serum. Fetuin stimulated growth in absence of serum as did the lactalysate residue or embryo extract, although it did not overcome toxicity of bovine serum. The effectiveness of fetal serum apparently is not attributable to its high content of fetuin.

Relative growth rates of chick embryo lung cultures on different media are shown in Fig. 2. Inclusion of the special additives with medium A accelerated growth. Further addition of 5% dialyzed serum supported growth rates comparable to those in media containing 5% dialyzed serum and 5% dialyzed embryo extract.

Freshly prepared chick embryo lung cells were found to plate out and grow in media containing the special additives and 5% dialyzed serum without previous establishment in media containing embryo extract. These cells were grown through several subcultures. Growth rates declined, however, and it became apparent that surviving cells would represent a selected or adapted population, and the cultures were abandoned.

*Discussion.* Of particular interest in growth of the avian cultures were the beneficial effects of the special additives even in presence of serum. Salmine, which enables cells to adhere to surfaces of culture vessels (9), possibly complemented the action of suboptimal concentrations of naturally occurring "sticking" or "spreading" factors. Insulin may also be required in relatively high

TABLE III. Comparison of Growth of Chick Embryo Lung Cultures in Media Containing Dialyzed Fetal Calf Serum and Dialyzed Bovine Serum.

| Dialyzed bovine serum (%) | Addition to basal medium:* None | 250 μg lactalysate residue/ml | 2 mg fetuin/ml | 5% Embryo extract |
|---|---|---|---|---|
| | (μg cell protein/tube) | | | |
| 0 | 16 | 114 | 74 | 64 |
| 2 | 38 | 158 | 92 | 172 |
| 5 | 36 | 202 | 56 | 178 |
| 10 | 46 | 224 | 44 | 206 |
| Dialyzed bovine fetal serum (%) | | | | |
| 0 | 16 | 108 | 82 | 58 |
| 2 | 130 | 140 | 114 | 136 |
| 5 | 148 | 138 | 132 | 118 |
| 10 | 146 | 166 | 146 | 128 |

Inoculum was 80,000 cells/ml. Growth was for 4 days at 37°C.

* Basal medium A containing insulin, pyruvate, salmine, oleate and folinic acid.

§ Obtained from Colorado Serum Co., Denver, Paper electrophoretic analysis by Dr. A. I. Schepartz, Merck, Sharp and Dohme.

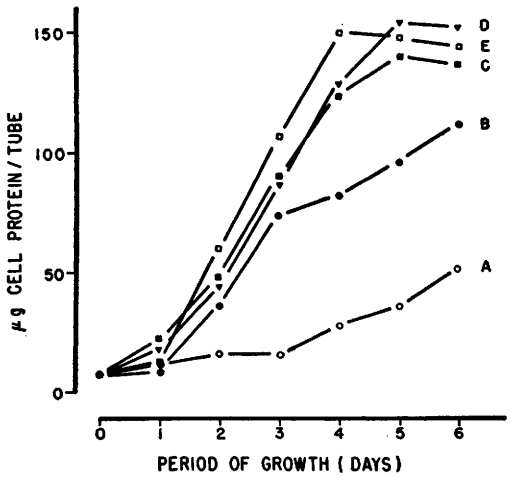


FIG. 2. Growth rates of chick embryo lung culture in different media. A. Basal medium A. B. Basal medium A plus special additives. C. Basal medium A plus 5% dialyzed bovine serum and 5% dialyzed embryo extract. D. Basal medium A plus special additives and 5% dialyzed bovine serum. E. Basal medium A plus special additives, 5% dialyzed bovine serum, 5% dialyzed embryo extract, no lactalysate residue.

concentrations for maximal growth rates under *in vitro* conditions. Pyruvate promotes growth of isolated Walker carcinosarcoma 256 cells(10) and cells in serumless media (7). Oleate and folinic acid did not significantly stimulate growth presumably due to their presence in the serum or to metabolic capacities of the cells. Lactalysate apparently not only provided additional growth factor(s) as indicated by stimulation either in presence or absence of serum, but it also functioned specifically to overcome severe toxic effects of serum. Embryo extract, too, was highly effective in preventing serum toxicity which may account in part for the requirement for this material. Toxic fractions have been prepared from serum by Cohn fractionation procedures(11). Fetal calf serum was observed to be non-toxic although its growth-supporting potential was inferior to other bovine serum.

Conceivably, unusual specimens of serum from more mature animals but containing maximal concentrations of sticking factors and other stimulatory substances and minimal toxic substances would support satisfactory growth of avian cells in absence of embryo extract, lactalysate or derivatives. Harris and Kutsky grew cultures of chick myoblasts which required both serum and nucleoprotein prepared from embryo extract(5). Subsequently, Harris grew myoblasts in a specimen of serum to which the addition of embryo extract or nucleoprotein was unnecessary(12). He further demonstrated a requirement for the dialyzable portion of the serum or for Bacto-peptone. In the present studies the sera tested with the exception of fetal calf serum, whether of chicken or bovine origin, whole or dialyzed, supported satisfactory growth of the chick lung or heart cells only in presence of embryo extract, lactalysate or its derivatives. Serum dialysates were not found to be active. Limited growth could be obtained without embryo extract or lactalysate factor(s) by employment of large inocula (300,000/ml), perhaps due to conditioning of the medium. Proteose-peptones were found to be highly stimulatory as substitutes for embryo extract for growth of explants in plasma clots(13). An anti-toxic action was not ascribed to such materials. Although in the present studies the activity of lactalysate was concentrated in the non-dialyzable portion, the capacity of the lactalysate residue to stimulate growth and to overcome toxicity of serum was not destroyed by acid hydrolysis. Evidently the active factor(s) was not peptone in character.

*Summary.* Salmine, pyruvate, insulin and lactalysate or its derivatives have been shown to replace partially requirements of avian cell cultures for serum and embryo extract. In addition to these factors, however, serum was required for growth comparable to that attained in the basal medium containing both serum and embryo extract. A function of lactalysate and embryo extract was inhibition of a toxic effect of serum. The stimulatory and "anti-toxic" effects of lactalysate were confined largely to the non-dialyzable portion. Nevertheless, the activity of acid hydrolysates of the lactalysate residue demonstrated that these properties were not dependent on a proteose or peptone.

We wish to acknowledge the excellent technical assistance of Miss Joan L. Molowski.

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## Racial Variations in Sweat Gland Distribution. (26501)

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Ogata(1) reported the existence of both active and inactive sweat glands in man. Although no difference between these 2 types of glands is seen histologically, only the active glands appear to be stimulated to secretion by either pharmacological agents or thermal stress. Because the number of sites of secretions was used as an index of the number of sweat glands, the data reported here are assumed to be applicable to only the active sweat glands, by Ogata's distinction.

In Japanese subjects Kawahata(2) showed that the number of sweat glands is smaller at birth than at 2 years of age, and suggested that initially inactive glands may be recruited into use during the first years of life. In support of this suggested environmental influence on eventual number of sweat glands, he subsequently demonstrated that Japanese born and living in tropical areas have a greater number, and consequently a greater density of sweat glands than members of the same racial group inhabiting temperate zones. Seemingly further weight is added to these suggestions by Kawahata's finding that Caucasian (Russian) subjects living in northern Manchuria have fewer active sweat glands, despite larger average body size, than do Japanese from temperate climates. In making the latter comparison, however, Kawahata noted that possible racial differences might have influenced these data. Kawahata and Sakamoto(3) have shown that Ainos in Hokkaido, Japan, have fewer sweat glands than do Japanese inhabiting the same island. Both factors modifying sweat gland distribution, racial and environmental, merit additional study.

*Methods.* The subjects sampled consisted of male Negroes and male and female Caucasians and Eskimos. The Negro subjects were born and lived in the midwestern United States. The origins of the Caucasian group were varied as shown in Table II. The Eskimo group was sampled in their native village (Anaktuvak Pass) in the Brooks mountain range in interior Alaska. The ages of all subjects are reported with the observed sweat gland data in Tables I to III.

Sweat gland measurements were made in heated chambers maintained at 41°-42°C after the subject had been in the room for 30-50 minutes and was sweating maximally. Sweat glands were counted by Jurgensen's method as modified by Kawahata(2). This method consists of counting under magnification (10×) the sites of sweat secretion on the skin surface covered with tinted cedar oil. The secretion sites corresponding to the openings of the underlying glands are counted within a 0.09 $cm^2$ area stamped onto the skin

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