erant of skin grafts from the Cb strain. Late mortality (21 to 60 days post-irradiation), after good early protection (21 days) against doses of whole body X-irradiation above the LD_{100}, did not occur in mice protected with isologous bone marrow (where neither the host nor the donor can react immunologically against each other). It occurred in mice protected with homologous marrow from a foreign strain (where both the host strain and the donor strain can potentially react immunologically against each other). It did not occur in mice protected with marrow from an F_1 hybrid of the irradiated strain (where the host strain can potentially react against the donor strain but the donor strain has no potential to react against the host strain). It occurred in some but not all combinations of irradiated F_1 hybrids protected with marrow from a parent strain (where the host has no potential to react against the donor strain but the donor strain can potentially react against the recipient strain).

The terms "homologous disease" and "heterologous disease" are proposed to designate the complications specifically attributable to successful transplantation of homologous or heterologous, as opposed to isologous or autologous, bone marrow or lymphoid tissue into a tolerant host.

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Production of Hyaluronic Acid in Tissue Culture by Fibroblasts Growing from Intestinal, Stomach, Liver and Kidney Explants.* (23415)

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The isolation of hyaluronic acid, and the description of its enzymatic hydrolysis by Meyer(1) brought to light the enzyme-substrate nature of the spreading reaction, first found in skin and subcutaneous tissue(2). In many tissues in which the spreading reaction was demonstrated, hyaluronic acid was found to be present and to be hydrolyzed by hyaluronidase. On the other hand, spreading factors were described which were not found to be related to hyaluronic acid and its hydrolysis, so - called hyaluronate - inactive spreading factors.

Favilli(3) and Duran-Reynals(2) demonstrated the spreading reaction in organs other than skin and subcutaneous tissue, such as stomach, intestine, and striated muscle, while no conclusion could be reached concerning spreading reaction in hepatic and renal tissue. It was, however, unknown whether the spreading reaction found in those organs was

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TABLE I. Positive Mucin Clot Test in Supernates of Cultures of Fibroblasts Growing from Intestinal, Stomach, Liver and Kidney Explants.

Organ	Growth	Tubes	Tubes after 10 days		Tubes after 30 days		After 38-96 days	
			Good growth	Mc +	Good growth	Mc +	Good growth	Mc +
Liver	Mostly fibroblasts; some liver epithel.	34	28	14	26	24	16	14
Stomach	Fibroblasts and epithel. membranes	30	22	18	24	18	9	8
Intestine	Epithel. and fibroblasts	12	10	7	11	8	4	4
Kidney	Fibroblasts; tubuli and epithelial sheets	10	10	6	10	8	7	5

hyaluronate-active and, if so, whether or not hyaluronic acid present in those organs was produced locally. These questions could most adequately be studied in tissue culture experiments.

Methods. Tissue cultures were prepared from liver, stomach, intestine, and kidney of 10- to 12-day-old chick embryos on a thin layer of chicken plasma, to which after coagulation, a liquid supernate was added consisting of 20% chick embryo extract, 30% horse serum, 20% chick amniotic fluid, and 30% Earle's balanced salt solution. The medium was renewed every 3 to 4 days in the first 3 weeks of cultivation, later every 6 days. For testing the presence of hyaluronic acid in the culture medium, the method of mucin clot (mc) formation and its prevention by testicular hyaluronidase was used. Formation of a tight mc on addition of 0.2 ml of 1 N acetic acid to 1 cc of the culture medium indicated the presence of hyaluronic acid, if formation of the mc could be prevented by hyaluronidase. Since chondroitin sulfates and partially depolymerized hyaluronic acid do not yield a mc on addition of acetic acid, this test has proven convenient for obtaining prompt information concerning the presence of highly polymerized hyaluronic acid in small samples of tissue culture supernates(4).

The minimal concentration of hyaluronic acid which gives a positive mc test is about 30 μg/ml. Fresh undiluted EE_{50} prepared in this laboratory gave a mc which could be prevented by hyaluronidase. EE_{50} contained about 105-120 μg hyaluronic acid per ml; 20% of it contained about 20 μg/ml, which is below the minimal concentration for the mc test and in fact never gave a mc. On the other hand, it has been found that hyaluronic acid, contained in chick EE in any concentration, is depolymerized at 37° in the first few days of incubation (Table II). Consequently, a positive mc test after that time always indicates the presence of hyaluronic acid newly produced *in vitro* by fibroblasts. Chick EE may thus be used in any concentration in the culture medium in which production of hyaluronic acid *in vitro* will be tested by the mc method.

Results. Growth of cultures from chick embryo stomach and intestine was luxuriant and large epithelial membranes predominated. Each culture, however, showed also more or less growth of fibroblasts. Explants from chick embryo liver gave abundant growth predominantly of fibroblasts, and about 40% of the cultures showed, in addition, growth of typical liver epithelium, which, however, degenerated in 1 to 2 weeks while fibroblasts continued to grow.

The explants from kidney of 10- to 12-day-old chick embryos were either mesonephric or metanephric, or both. Microscopical observation of cultures showed profuse growth of epithelial sheets typical in cultures of embryonic kidney, with elongated cells (kidney epithelium is of mesodermal origin), but rather small nuclei. In most cultures, growth of mostly strait tubuli were observed, and in some cultures of metanephros scattered epithelial islets. In addition, in most cultures scarcely growing typical fibroblasts were observed.

Starting from the 3rd or 4th, sometimes only after the 7th renewal of the medium, the

supernate of those of the cultures which showed good growth, on addition of a few drops of 1 N acetic acid, yielded a tight mc, the formation of which could be prevented by hyaluronidase. The feeding solution gave no mc before incubation (Table I). Estimation of hyaluronic acid produced in cultures of the named tissues, using the turbidimetric method, gave values ranging from about 36 to 210 μg per ml in various cultures, and at different times and intervals between feedings. The production rate was evidently dependent on the state of the cultures. Factors influencing production were not further analyzed in these experiments.

Discussion. In previous reports(4,5), production of hyaluronic acid in tissue culture by fibroblasts growing from explants of human synovia, human embryo skin, bone, tendon, epiphysis, aorta, and pericardium; rat subcutaneum; chick embryo bone and heart, and beef embryo bone was demonstrated. The present results show that hyaluronic acid is produced in tissue culture of liver, stomach, intestine, and kidney of chick embryos by cells of mesenchymal origin, since all cultures, the supernate of which gave an mc, showed either growth of fibroblasts alone, or growth of fibroblasts mixed with epithelial growth, while the supernate from cultures with pure epithelial growth never gave an mc. The present findings also indicate the local production of hyaluronic acid in chick embryo liver, stomach, intestine and kidney.

It should be emphasized that, for studies of hyaluronic acid production *in vitro,* the use of primary cultures is the method of choice. Such cultures have been producing hyaluronic acid for nearly one year and under suitable conditions may perhaps produce indefinitely. If production stops (for unknown reasons), it may soon start again, under more suitable conditions of growth. On the other hand, replica cultures of cell strains of mesenchymal origin sooner or later irreversibly lose their capacity of hyaluronic acid production *in vitro,* as is the case, for instance, with strain L, deriving from a mouse fibroblast, and with similar strains of mesenchymal origin. It appears that such strains (as a result of trypsinization or other procedures leading to a loosening of cell connections) gradually lose their ability to produce mucopolysaccharides as precursors of ground substance, or in other words, gradually lose their capacities to form connective tissue.

It has yet not been possible to identify factors which in tissue culture specifically promote hyaluronic acid production. In general, conditions which are required for obtaining good growth are also necessary for continuous production of hyaluronic acid. Deterioration of cultures due, for instance, to pH changes or to starvation will impair both growth and production of hyaluronic acid. Similarly hydrocortisone in high concentrations which inhibit growth promptly inhibit also production of hyaluronic acid. On the other hand, it appears that there are specific factors which promote hyaluronic acid production only but not growth. In preliminary work cultures of fibroblasts were kept for several weeks at room temperature. In such stationary cultures, despite significant or complete depression of growth, production of hyaluronic acid continued in some cultures. By cultivation at lower temperatures, factors promoting hyaluronic acid production as distinct from growth factors, could thus more conveniently be studied.

Summary. 1. Tissue cultures of chick embryo liver, stomach, intestine and kidney, in which both epithelium and fibroblasts were growing for over 3 months produced hyaluronic acid, as shown by a positive mucin clot test and by the prevention of mucin clot formation by hyaluronidase. These findings are consistent with the demonstration of the spreading reaction in some of these organs and indicate that they belong to the group of hyaluronate-active spreading reactions. 2. In primary cultures production of hyaluronic acid may take place for very long periods of

TABLE II. Incubation of Chick Embryo Extract (EE_{50}) at 37°C.

No. of samples	Before incubation	Days of incubation	No mucin clot
7	Mucin clot +	1	4
14	"	2	12
11	"	3	9
12	"	4	11
25	"	5	22

TABLE IIa. Incubation of Supernates from Tissue Cultures.

Tissue	Medium tested before feeding	After 5 days of growth	Days of incubation of supernates alone at 37°C	# samples	Mc +	No mc
Rat subcut.	No Mc	Mc +	4 - 18	9	9	—
Chick embryo bone	"	"	8 - 33	41	38	3
Rat tail tendon	"	"	4 - 55	7	6	1
Rat subcut.	"	"	30*-195*	3	3	—
Chick embryo bone	"	"	6*- 65*	22	21	1

* Kept in refrigerator at 4°C.

time, usually as long as the cultures do not deteriorate, while replica cultures, for instance strain L, sooner or later lose the ability to produce hyaluronic acid, despite their excellent appearance and good growth. 3. Although it has not yet been possible to separate factors promoting mucopolysaccharide production from factors promoting growth, it has been observed that cultures kept at room temperatures with considerably reduced growth, may sometimes continue to produce hyaluronic acid. 4. Hyaluronic acid of chick embryo extract is depolymerized in the first few days of incubation at 38°C (Table II), while hyaluronic acid produced in tissue culture is not depolymerized for months under the same conditions (Table IIa). A positive mucin clot test after a few days of incubation thus always indicates the presence of hyaluronic acid newly produced *in vitro* by fibroblasts. Chick embryo extract may therefore be used in any concentration in the culture medium in which production of hyaluronic acid *in vitro* will be tested by the mucin clot method.

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Calcification XIX. Calcification of Transplanted Rachitic Bone.*† (23416)

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The purpose of this investigation was to study calcification of rachitic bone slices that were transplanted subcutaneously to rats with the thought that transplantation would be a useful approach to the study of the calcifying mechanism as was the case with *in vitro* calcification(1-10). By standardizing both the rachitic sections and the host animal, a transplantation technic would permit the study of calcification after experimental modification of either the rachitic section or the composition of body fluid in the host animal.

Experimental. Method of preparation of animals for the transplants. Each animal was

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