Sample	Viscosity/viscosity of water (13°)
Untreated serum	2.52-2.88
Serum with formaldehyde and ammonia	3.21-3.32
Heated at 70° for 10 min.	7.35
Heated at 70° for 20 min.	17.3
Heated at 70° for 30 min.	32.1

DISCUSSION

All these results are consistent with a progressive alteration of the serum proteins by heating with formaldehyde and ammonia. There is an increasing heterogeneity with respect to particle size and to electrophoretic mobility; the heterogeneity accounts for the 'disappearance' of the fractions as seen in the sedimentation diagrams. The fall of osmotic pressure shows that the mean particle size increases. The increase of viscosity is, however, out of proportion to the increase in particle size; the absence of structural viscosity in the heated samples shows

that the abnormal rise in viscosity cannot be due to a greatly increased asymmetry of the particles and it must, therefore, be ascribed to an increased solvation of the altered particles. This applies only to that fraction of the protein which does not appear in the sedimentation diagrams, since the sedimentation constants of the recognizable components are little changed.

SUMMARY

1. Bovine serum heated with formaldehyde and ammonia over a range of conditions shows loss of specific properties and a progressive aggregation of its proteins to form a polydisperse system of larger particles.

2. The osmotic pressure, viscosities and electrophoretic and ultracentrifugal characteristics of a range of samples have been measured.

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The Nucleoprotein Content of Fibroblasts Growing *in vitro*

2. THE EFFECT OF TISSUE EXTRACTS

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We have already described experiments on the nature of some of the factors which influence the growth of fibroblasts *in vitro* (Davidson & Waymouth, 1943). In these experiments fibroblast cultures were grown in roller tubes according to the technique of Willmer (1942), and the amount of growth was assessed by making chemical estimations of the nucleoprotein phosphorus (NPP) content of the cultures. In this paper the original observations have been continued and extended.

The technique of estimating the growth of tissue cultures by measuring the increase in nucleoprotein phosphorus (NPP) in the growing tissue was introduced by Willmer (1942) and was recommended by

Cunningham & Kirk (1942). Willmer presumed that such an estimation might give a measure of the amount of nuclear material present, but he pointed out that the presence of nucleic acid as a cytoplasmic constituent in certain tissues was a possible drawback to the method. We have recently shown (Davidson & Waymouth, 1944*a, b*) that mammalian and avian tissues contain two types of nucleic acid, deoxyribonucleic acid in the nucleus and ribonucleic acid in the cytoplasm and that in many tissues the ribonucleic acid is the greater in amount. In liver, for example, some 60-80% of the total nucleic acid is present as the cytoplasmic ribonucleic acid (Davidson & Waymouth, 1944*c*). In the rapidly

growing tissues of the embryo the total nucleic acid content is greater than in the corresponding adult tissue, but for any given tissue the ratio of ribonucleic acid to deoxyribonucleic acid is of the same order in the embryo as in the adult. This ratio is not known with any degree of certainty for connective tissue or fibroblasts. It can, however, be presumed that NPP which includes both ribonucleic acid and deoxyribonucleic acid will give a reasonably accurate measure, in any one type of tissue, not of nuclear material but of total cellular material (cf. Carruthers & Suntzeff, 1942), and that it can legitimately be used for the assessment of the growth of tissue cultures.

It must be emphasized that any tissue-culture method for the investigation of the tissue growth-promoting activity of a substance is relatively crude in comparison with the recognized methods for examining growth-promoting factors for yeast or many types of bacteria. In the case of many micro-organisms the investigator can start with a basal medium of known composition containing the necessary known growth-promoting factors. The growth response with such a medium under standard conditions is dependent on the concentration of the factor under test. In a tissue-culture test such simple conditions are not possible. In the first place, growth occurs in a two-phase system consisting of plasma clot and fluid medium. The plasma varies in composition from bird to bird and contributes unknown factors to the medium, so that comparison of the growth-promoting activity of two media can be made only when a single batch of plasma is used throughout. Secondly, the growing tissue requires a large number of factors, nutrient and biocatalytic, whose nature is unknown. Thirdly, the tissue itself cannot be implanted in quantitatively accurate amounts as can a cellular suspension (of yeast or bacteria) be used to seed a medium. And unless a pure strain of cells is available (and it was not in the present investigation), the tissue used for the original explants will be variable in composition and in potential growth capacity. For these reasons the present investigation is aimed at detecting only gross differences in the growth-promoting power of the materials tested.

METHODS

Fresh explants of the 9-day chick embryo heart were grown in plasma clot in roller tubes by the method previously described (Davidson & Waymouth, 1943, 1944*d*). For the first 2 days the fluid phase in the roller tubes consisted of Tyrode solution alone. At the end of this period the Tyrode was replaced by the test solution, and the tubes allowed to rotate for a further 2 days. The NPP in the tissue was then determined as previously described (Davidson & Waymouth, 1943). The change in NPP during the 2 days of the test is taken as a measure of the amount of growth which has occurred.

In agreement with results previously recorded, it was found that when the fluid phase during the 2 days of the test proper consisted of Tyrode solution alone, a slight fall in NPP occurred. When the fluid phase contained embryo extract, the NPP rose. These observations have a bearing on the method by which the results are best expressed (*vide infra*).

Preparation of embryo extract. The preparation of sterile chick embryo extract has already been described (Davidson & Waymouth, 1943).

In winter it has been possible to obtain large quantities of sheep embryos 9–10 weeks old. Extract was prepared by mincing the embryos and allowing the pulp to stand overnight in the refrigerator with an equal volume of water, Tyrode solution or 0.9 % NaCl. The extract was then filtered through muslin and centrifuged. It was then sterilized, usually by the use of large Berkefeld candles. The extracts are viscous and tend to clog the candles easily, but with candles of a large surface area, sufficient sterile material could be obtained before the candles became clogged. A loss of about 40 % total N in the extract resulted from filtering thus in some cases. The sterile extract was suitably diluted with sterile Tyrode solution before use.

In a few cases sterilization was avoided by preparing the extract aseptically from very small embryos. The complete uterus was brought from the slaughter-house to the laboratory and opened aseptically. The embryo was transferred to a sterile glass vessel and minced with sterile scissors. An equal volume of sterile Tyrode solution was added and the mixture centrifuged. The sterile '50 % embryo extract' was then pipetted off and stored in sterile tubes.

Method of expressing results. The results of a series of ten individual experiments with chick embryo extract (nitrogen concentration = 28–31 mg./100 ml.) (N=30)* are shown in Table 1, arranged in decreasing order of initial

Table 1. Increase in NPP in tissue grown in embryo extract (N = 30) during 2-day test period

(Plasma blanks subtracted.)

Test no.	NPP content			
	Initial (μg.)	Final (μg.)	Increase (μg.)	Relative increase
25	1.53	1.96	0.43	0.28
45	1.50	1.91	0.41	0.27
44	1.34	1.71	0.37	0.28
49	1.23	1.68	0.45	0.36
56	1.14	1.56	0.42	0.37
82	1.12	1.44	0.32	0.29
61	0.90	1.29	0.39	0.43
69	0.89	1.22	0.33	0.37
75	0.89	1.33	0.44	0.49
67	0.86	1.23	0.37	0.43

Mean and standard error 0.39 ± 0.014 0.36 ± 0.024

NPP values. These initial values cover a wide range (0.86–1.53 μg.). The increase in NPP in the course of 24 hr. is expressed in two ways: (i) as an absolute increase per roller tube, and (ii) as a relative increase $\frac{\text{Final NPP} - \text{Initial NPP}}{\text{Initial NPP}}$.

* This convention will be used to express the concentration of total N in mg./100 ml. in the 0.5 ml. fluid added to the roller tubes at the beginning of the test period.

These ten tests involved the use of 35 roller tubes, i.e. 215 pieces of tissue with the corresponding plasma blanks. It can be seen (Table 1) that there is a smaller range of variation in the increases in NPP expressed as an absolute increase than in those expressed as a relative increase. The effect of embryo extract therefore seems to be to cause, during the 2-day growth period, an increase in NPP which is not proportional to the amount of tissue initially present. The limiting factor for growth lies, under these conditions, in the medium and not in the tissue.

Individual figures for a series of experiments, in which tissue is grown with Tyrode solution alone as fluid phase, are shown in Table 2, again arranged in descending order of

Table 2. *Change in NPP in tissue grown in Tyrode solution alone during 2-day test period*

(Plasma blanks subtracted.)

Test no.	NPP content			
	Initial ($\mu\text{g.}$)	Final ($\mu\text{g.}$)	Change ($\mu\text{g.}$)	Relative change
33	1.75	1.29	-0.46	-0.26
39	1.70	1.19	-0.51	-0.30
45	1.46	1.38	-0.08	-0.05
44	1.42	1.01	-0.41	-0.29
50	1.36	1.25	-0.11	-0.08
47	1.35	1.34	-0.01	-0.01
48	1.25	1.08	-0.17	-0.14
46	1.22	1.22	± 0	± 0
38	1.20	1.25	+0.05	+0.04
55	1.20	0.92	-0.28	-0.23
91	1.19	1.14	-0.05	-0.04
68	1.12	0.99	-0.13	-0.12
42	1.06	1.20	+0.14	+0.13
41	1.04	1.10	+0.06	+0.06
34	0.99	0.99	± 0	± 0
61	0.86	0.79	-0.07	-0.08
67	0.78	0.70	-0.08	-0.10
69	0.56	0.59	+0.03	+0.05
60	0.55	0.44	-0.11	-0.20

Mean and standard error -0.11 ± 0.04 -0.08 ± 0.03

initial NPP values. In this case the change in NPP (usually a fall, Davidson & Waymouth, 1943) is greater for higher initial values of NPP. Where the initial figures are high ($>1.0 \mu\text{g.}$) the absolute differences are greater, and where the initial figures are low ($<1.0 \mu\text{g.}$) the absolute differences are less than the relative differences. Here the tissue itself, in the absence of a growth-promoting medium, controls the magnitude of the change in NPP.

It has therefore been concluded that the absolute increases in NPP per roller tube during the growth period express satisfactorily the effects of growth-promoting media derived from embryonic tissue, and it has been assumed that the method is valid for other types of growth-promoting medium. It should be emphasized that the differences in initial NPP values are, of course, never so gross in a single test as in the range shown in Table 2.

In the experiments recorded here, large differences are sought and a positive change of $>0.2 \mu\text{g./roller tube}$ in the 2-day period is taken as indicating definite growth-promoting properties in the medium under test. A rise of $>0.5 \mu\text{g.}$ indicates very good growth. With each medium tested at least two, and in most cases three or more, roller

tubes are used at each time, i.e. the number of pieces of tissue is at least 12 and in most cases is 18 or more. The number of tubes which can be carried on the drum is limited to 20 for any one test. Plasma blanks were therefore dispensed with in some experiments, after it had been established that the correction was insignificant for the preparations under test.

RESULTS

(1) *Comparison of chick embryo extract with sheep embryo extract* (Table 3, tests 128/1 and 130/1)

A comparison was made of the growth-promoting power of chick and sheep embryo extracts. Both were prepared aseptically from sterile embryos (i.e. sterilization was avoided) by mixing the embryo pulp with an equal volume of Tyrode solution and centrifuging. The '50%' chick embryo extract prepared in this way had a total N content of 60–90 mg./100 ml., while '50%' sheep embryo extract contained 200–300 mg. N/100 ml. They were both diluted to the same nitrogen content and tested at N=20, 30 and 50. Chick embryo extract was more effective under these conditions than sheep embryo extract.

(2) *The effect of drying*

Sheep embryo extract was dried by a number of methods: (i) Portions of 30–50 ml. were dried in the frozen state in an apparatus similar to that described by Christensen (1942) and by Hays & Koch (1942). The dried product dissolved completely in water ('Frozen-dried extract'). (ii) A large batch of about 5 l. was sent in a refrigerated container to the Low Temperature Research Station at Cambridge where it was dried from the frozen state in a large-scale apparatus. It was dissolved from the walls of the apparatus in a small amount of water and the solution was dried in vacuum desiccators. A large part of the product did not dissolve in water ('Cambridge-dried extract'). (iii) A large amount of material was dried at room temperature in thin layers in Petri dishes in vacuum desiccators over concentrated sulphuric acid. The dry product was scraped off the dishes and finely powdered. A large proportion was insoluble in water ('Vacuum-dried extract'). (iv) Thin layers of extract dried at room temperature in flat dishes in the blast of an electric fan according to the method of Fischer (1941b) gave a similar material containing a large amount of insoluble matter.

In most cases the dried material was suspended in water, the insoluble residue centrifuged off, the chloride content determined, and the salt concentration corrected by addition of suitable amounts of double-strength Tyrode solution. The reconstituted extract was diluted to a suitable total N con-

centration with ordinary Tyrode solution and sterilized by passage through a small filter candle. In some cases the dried extract was reduced to a fine powder by grinding and sieving and was extracted with benzene to remove lipid materials before being reconstituted.

Small portions (50–100 mg.) of the dry powder were also sterilized by allowing them to stand for some days under benzene in sterile, plugged 15 ml. conical centrifuge tubes (cf. Fischer, 1941b). The bulk of the benzene was then poured off and the plugged tube placed in a vacuum desiccator to remove the last traces of benzene. The sterile powder was then suspended in sterile distilled water, allowed to stand 1–2 hr. and the insoluble residue centrifuged down. This treatment not only sterilized the powder but also removed lipid material from it.

An attempt was made to dry embryo extract in the spray-drier described by Edwards, Kay & Davie (1940), but even a '50 % extract' contained insufficient solids to yield satisfactory results. Attempts at spray-drying at the Low Temperature Research Station in Cambridge were also unsuccessful.

Examples of some results obtained with dried embryo extracts are shown in Table 3 (tests 148/1, 95/1, 98/1).

Chick embryo extract, when dried in the frozen state, sterilized under benzene, and reconstituted,

gave an extract which was more potent than the original fresh material at the same N concentration (test 148/1). The increase in activity may be due in part to the removal of inhibitory lipid materials by the benzene (*vide infra*).

Sheep embryo extract, 'vacuum-dried' or 'Cambridge-dried', gave preparations which contained a large amount of insoluble material. The activity of the solutions from these powders was low but was enhanced by previous extraction of the dried powder with benzene (*vide infra*) (tests 95/1, 98/1).

(3) The effect of defatting

(i) *By ether.* The removal of lipid material from liquid embryo extract was carried out by freezing with ether according to the method used by McFarlane (1942) for serum. Popják & McCarthy (1943) have shown that this treatment removes from serum all the cholesterol, approximately two-thirds of the phospholipids and two-thirds of the neutral fats.

100 ml. sheep embryo extract were shaken with 30 ml. ether and frozen to a temperature below -25° by immersion of the flask in a solid CO_2 -ethanol mixture. Freezing and thawing were carried out rapidly, and a top layer containing fat and some denatured protein was obtained. The clear lower aqueous layer was sucked off and the extraction with ether repeated. The clear extract was freed

Table 3. Comparison of the growth-promoting effects of fresh, dried, defatted and stored embryo extracts

Test no.	Species	State	Defatted	Sterilized	N concentration (mg./100 ml.)	Change in NPP of cultures ($\mu\text{g.}$)
128/1	Chick	Fresh	—	Prepared aseptically	30	+0.44
	Sheep	"	—	"	30	+0.24
130/1	Chick	"	—	"	50	+1.00
	"	"	—	"	20	+0.37
	Sheep	"	—	"	50	+0.46
	"	"	—	"	20	+0.16
95/1	Sheep	Vacuum-dried	—	Berkefeld candle	—	+0.18
	"	"	—	"	—	-0.05
	"	"	Benzene	Benzene	—	+0.36
98/1	Sheep	Cambridge-dried	—	Berkefeld candle	9	+0.11
	"	"	Benzene	"	9	+0.18
127	Sheep	Fresh	—	"	30	+0.23
	"	Frozen-dried	Ether	L3 candle	30	+0.58
	"	"	"	Berkefeld candle	30	+0.48
129/1	Sheep	Fresh	—	"	40	+0.29
	"	Frozen-dried	Ether	"	40	+0.61
148/1	Chick	Fresh	—	Prepared aseptically	20	+0.31
	"	Frozen-dried	Benzene	Benzene	20	+0.60
151/2	Chick	Fresh	Ether	Prepared aseptically	50	+1.16
125/1	Sheep	Stored sterile for 9 months at -30°	—	Berkefeld candle	30	+0.73
	"	Stored 9 months at -30° and then sterilized	—	"	30	+0.38
	"	Prepared from embryos stored for 9 months at -30°	—	"	30	+0.16

from ether in a vacuum desiccator, refrozen, thawed and filtered through paper. In one instance the total N of sheep embryo extract before defatting was 247.5 mg./100 ml. and phospholipid P was 1.41 mg./100 ml. After defatting, the total N was 210.0 mg./100 ml. and phospholipid P was negligible. Defatted extracts filtered easily through a Berkefeld candle, but it was difficult to remove the last traces of ether from them. Such extracts which had been dried in the frozen state (and therefore completely freed from ether) and reconstituted were more active than fresh extract at the same N concentration (Table 3, test 127).

The results of a test with chick embryo extract defatted by freezing with ether under aseptic conditions (i.e. sterilization of the defatted solution was unnecessary) are shown in Table 3, test 151/2. The preparation had good growth-promoting activity, though a direct comparison with ordinary chick embryo extract at the same N concentration was not made (but cf. test 130/1) (see also § 6, and Table 4, test 90).

(ii) *By benzene.* As we have already shown, extraction of dried embryo extract with benzene before reconstitution enhanced the activity of the preparations (Table 3, tests 95/1, 98/1).

(4) *Extracts dried after defatting*
(Table 3, test 125/1)

Extracts from which lipid material had been removed by freezing with ether as described in § 3 (i) were also dried from the frozen state. When an extract was defatted in this way, frozen-dried and reconstituted, the powder dissolved very readily to give a solution which filtered easily through a candle and had high activity (Table 3, tests 127, 129/1). The most active dry preparations of sheep embryo extract were made in this way.

(5) *Effect of storage on embryo extract*

The effect of storage on the growth-promoting properties of sheep embryo extract was examined. Storage was carried out for 9 months in a cold

chamber at -30° in the Torrey Research Station. Sterile sheep embryo extract, sealed up in ampoules and stored, retained powerful activity after 9 months. When embryo extract was stored in the non-sterile state and Berkefeld-filtered after storage for 9 months, the activity was relatively good. When whole embryos were stored for 9 months and then converted into extract and filtered, the extract was still less active (Table 3, test 125/1). It is preferable, therefore, to store embryo extract in the sterile state in sealed ampoules.

(6) *Comparison of the growth-promoting properties of extracts from various organs and tissues of the embryo*

(i) *Sheep embryos.* Extracts were prepared by mincing the different embryonic organs finely in a large Latapie mincer, adding 1 vol. Tyrode solution, allowing the mince to stand overnight at 0° and centrifuging. The extracts were sterilized by filtration and tested at the same N concentration (Table 4, tests 39, 36, 83, 84/1).

A test was also made with extracts which had been defatted by freezing with ether (Table 4, test 90). None of the extracts of individual tissues gave a better response than whole embryo extract. Extracts of muscle, heart and cartilage were possibly slightly superior to those of viscera, e.g. liver.

(ii) *Chick embryos.* Nine-day chick embryos were too small for the preparation of extracts of individual organs. Aseptic dissection of embryos 12 or 13 days old was possible, and extracts of muscle, cartilage, brain, viscera and of the carcass from which brain and viscera had been removed, were prepared and tested at the same N concentration ($N=20$). The results were not completely conclusive, but they did not indicate that the activity of chick embryo extract as a growth-promoting medium could be predominantly attributed to any particular tissue.

(7) *Ethanol precipitates from tissue extracts*

(i) *From embryo tissue extracts.* Sheep embryo extracts were treated in the cold with 4 vol. of ice-cold ethanol. The precipitate was centrifuged

Table 4. *Comparison of the growth-promoting properties of extracts of various organs and tissues of the sheep embryo*

Test no.	N concentration of extract (mg./100 ml.)	Change in NPP content of cultures ($\mu\text{g.}$) induced by extract of							
		Whole embryo	Viscera	Brain	Cartilage	Heart	Liver	Muscle	Lung
39	42	—	+0.04	—	+0.29	—	—	+0.22	—
36	30	—	—	—	+0.26	—	—	—	—
83	10	—	—	—	—	—	+0.12	+0.31	—
84/1	10	—	—	± 0.0	+0.04	+0.27	—	—	+0.14
90	Varied with the tissue used; '50% extracts' diluted 1 in 4	+0.51*	—	+0.45†	—	+0.40†	+0.12†	+0.44†	—

* Extracts defatted by freezing once with ether.

† Extracts defatted by freezing twice with ether.

down quickly and dried *in vacuo* over CaCl_2 . In some cases the precipitate was washed with cold absolute ethanol and ether before being dried. The dried precipitates were ground finely and extracted once with ether. Powders were made in this way from individual minced sheep and calf embryo organs and also (*vide infra*) from adult sheep heart by extracting with 1 vol. of 0.9% NaCl and precipitating the extracts with ethanol. Such ethanol powders were found to be only slightly soluble in water.

(a) The powders were allowed to stand in 100 mg. portions with 10 ml. Tyrode solution for 4 hr. at 0° . The suspensions were then centrifuged and the supernatant fluids passed through L3 Pasteur-Chamberland candles. Total N was determined in the sterile extracts. Such extracts from embryo brain, heart, liver and muscle powders were found to contain 13, 21, 28 and 39 mg. N/100 ml. respectively.

(b) To obtain a more concentrated solution and to sterilize the solution without the use of filter candles, portions of these ethanol powders were dissolved in water with the aid of heat, and the supernatant fluid obtained on centrifuging was sterilized by heating to 100° for 10 min. After cooling, 1 vol. of double-strength Tyrode solution was added.

(c) To avoid the use of candles or of heat in obtaining a sterile preparation, portions of 50 mg. of the ethanol powders were transferred with the aid of a sterile platinum scoop (Packchianian, 1942) to a sterile 15 ml. pyrex centrifuge tube which was then filled with Analar benzene, stoppered with a sterile plug of cotton-wool and allowed to stand for several days. The benzene was decanted off and the powder dried *in vacuo*. To the dry powder, now sterile, 5 ml. sterile Tyrode solution were added and the tube set aside for 4 hr. at 0° before being centrifuged. The supernatant fluid was analyzed for total N.

The results (Table 5, tests 130/2, 131, 133, 134) show that active preparations can be prepared from these ethanol precipitates both from whole sheep embryo extract and from extracts of individual organs. Extracts of brain and muscle were superior to extracts of liver (test 133). The results obtained with preparations heated or sterilized by benzene were similar to those from Berkefeld-filtered preparations.

Precipitation of sheep embryo extract with acetone in place of ethanol was also tried. Ice-cold sheep embryo extract was treated with 4 vol. ice-cold acetone and the precipitate immediately centrifuged down and dissolved in ice-cold water. Almost the whole precipitate dissolved, yielding a reddish solution with no trace of the brown colour which develops when the operation is carried out at room

temperature. The solution was frozen-dried and a fluffy powder obtained. It was freely soluble in water and was sterilized by a Berkefeld candle. The solution had no activity.

(ii) *From adult and embryo sheep heart extracts.* Extracts of adult heart tissue from the fowl (Doljanski & Hoffman, 1939; Hoffman, Tenenbaum & Doljanski, 1939, 1940) and from the sheep, cow, rabbit and dog (Doljanski, Hoffman & Tenenbaum, 1942) (but not those from the rat (Hoffman, 1940) unless lipid material is removed from the tissue before preparation of the extract (Werner, 1944)), including ethanol precipitates from such extracts (Werner & Doljanski, 1942; Werner, 1944) are believed to exert a growth-promoting action on tissue cultures, and these extracts and precipitates have recently been applied to the healing of wounds (Doljanski & Auerbach, 1944; Auerbach & Doljanski, 1944; Mandl & Maybaum, 1943; Mandl, 1944; Hoffman & Dingwall, 1944; Kerr & Werner, 1944). We have accordingly prepared extracts and precipitates from adult sheep heart by the method described by Werner & Doljanski (1942), and by Kerr & Werner (1944).

Fresh adult sheep heart was finely minced, extracted with 5 vol. of physiological saline solution and the extract precipitated with 4 (in some batches 2) vol. of ethanol. The precipitate was centrifuged down and dried *in vacuo* over CaCl_2 . The dry powder was extracted with ether and dried again. The amount of material which could be brought into solution from the adult heart powders was even less than from embryo tissue powders. Extracts from powders prepared in this way were compared with extracts from powders prepared in the same way from sheep embryo heart and whole sheep embryo extract, using the methods described in (i) above. The results are shown in Table 5, tests 130/2, 131, 132/1, 135, 137, 139/1.

In general, preparations from adult sheep heart were inferior to preparations of the same N concentration from embryo sheep heart and to the corresponding preparations from whole embryo extract. In one instance (test 137), where such preparations were compared at low N concentrations ($N=6$), the adult sheep heart appeared to be more active.

In order to obtain a more soluble preparation from the ethanol precipitate of adult sheep heart extract, we extracted 10 g. of the dry powder with 100 ml. 0.01N-ammonia as recommended by Werner & Doljanski (1942). After standing at room temperature for 15 hr., the insoluble residue was centrifuged down and the extract dried in a vacuum desiccator. The resulting material (270 mg.) was powdered and sterilized under benzene. It dissolved fairly readily in sterile Tyrode solution. After a small amount of insoluble material had been re-

Table 5. *Comparison of the growth-promoting properties of ethanol precipitates from tissue extracts*

Test no.	Tissue of origin	Method of sterilization	N concentration (mg./100 ml.)	Change in NPP of cultures (μg.)
130/2	Adult sheep heart	L3 candle	20	+0.11
	Embryo sheep heart	"	24	+0.37
131	Whole sheep embryo	L3 candle	36	+0.33
	"	"	14	+0.11
	"	Heated	36	+0.33
	Whole chick embryo	L3 candle	1	-0.03
	"	Heated	8	-0.14
	Adult sheep heart	L3 candle	14	+0.07
132/1	"	Heated	7	+0.05
	Whole sheep embryo	L3 candle	25	+0.17
	Adult sheep heart	"	25	+0.20
	"	"	25	+0.09
	"	"	25	+0.01
133	"	"	45	+0.15
	Sheep embryo brain	L3 candle	13	+0.54
	Sheep embryo heart	"	13	+0.25
	Sheep embryo liver	"	13	+0.18
	Sheep embryo muscle	"	13	+0.24
	Sheep embryo heart	"	21	+0.44
	Sheep embryo liver	"	21	+0.22
	Sheep embryo muscle	"	21	+0.55
134	Whole sheep embryo	Benzene	12	+0.57
	"	L3 candle	12	+0.14
	Sheep embryo brain	"	12	+0.17
	Sheep embryo heart	"	12	+0.11
	Sheep embryo muscle	"	12	-0.02
	"	"	35	+0.36
135	Whole sheep embryo	Benzene	25	+0.23
	Adult sheep heart	"	25	+0.06
	"	"	46	-0.02
137	Adult sheep heart	Benzene	6	+0.45
	Whole sheep embryo	"	6	+0.35
	Embryo calf muscle	"	8	+0.41
	Embryo calf heart	"	8	+0.24
	Embryo calf brain	"	8	+0.21
139/1	Adult sheep heart	Benzene	8	+0.04
	Embryo sheep heart	"	44	+0.31
	Whole sheep embryo	"	30	+0.17
	Embryo calf muscle	"	16	+0.25
159/1	Adult sheep heart—fraction soluble in dilute ammonia	Benzene	30	+0.44

moved at the centrifuge, the supernatant fluid was tested at N=30 (Table 5, test 159/1). It had moderately good growth-promoting power.

(8) *The nucleoprotein fraction of embryo extract*

In view of the statements by Fischer (1939, 1940, 1941b) that growth-promoting activity is associated with the nucleoproteins of embryo extract, we have prepared the nucleoprotein fraction by the method of Hammarsten (1920) as employed by Fischer (1941b). This fraction has been termed 'embryonin' by Fischer & Astrup (1943).

'Cambridge-dried' sheep embryo extract (§ 2, ii; 3 g.) was suspended in 50 ml. ice-cold 0.06N-HCl and allowed to stand with occasional stirring in the refrigerator for 2 hr. The suspension was centrifuged

and the supernatant fluid discarded. The residue was washed twice with ice-cold glass-distilled water. The residue was treated with a little ice-cold distilled water and, while cooled in ice, was treated with cold 0.06N-NaOH till the reaction was just alkaline (faint blue to litmus; pH < 9). Almost the whole residue dissolved. The solution while cooled in ice was then adjusted, with cold 0.1N-HCl, to pH 5.6-5.8, and the precipitate of nucleoprotein centrifuged down. Addition of another drop of HCl to the supernatant fluid gave no further precipitate. The nucleoprotein while cooled in ice was redissolved in water by cautious addition of 0.06N-NaOH, centrifuged, and the clear supernatant fluid (4.5 ml.) was dried from the frozen state. The dried material was a fluffy yellowish powder. It contained 10.96% N and 0.52% P.

The powder was treated with Tyrode solution and a small amount of insoluble material centrifuged down and discarded. The clear yellowish supernatant fluid could be sterilized by means of an L3 or small Berkefeld candle although some of the nucleoprotein was adsorbed by the candle. The sterile solution had good growth-promoting power (Table 6, test 125/2).

With most preparations, Berkefeld-filtration was avoided by sterilizing the powder under benzene. The sterile powder was then treated with sterile Tyrode solution, insoluble material was centrifuged down and discarded, and the sterile supernatant fluid was tested at various N concentrations (Table 6,

sterilized by heat and have already been shown to have the power of causing a marked rise in the NPP of tissue cultures (Davidson & Waymouth, 1944d). The mixture of nucleoprotein and boiled kidney extract had very powerful growth-promoting power (Table 6, test 125/2). The addition of peptone was not so effective as the addition of kidney extract.

The nucleoprotein fraction prepared from liquid embryo extract by two methods was also tested. (i) *Greenstein's method*. The nucleoprotein fraction of fresh sheep embryo extract was prepared by the method of Greenstein & Jenrette (1940). This method is designed to avoid, as far as possible, protein denaturation during the isolation of the

Table 6. *The effect on the nucleoprotein phosphorus (NPP) content of tissue cultures of nucleoprotein prepared from dried sheep embryo extract by the method of Hammersten (1920)*

Test no.	Test substances	Method of sterilization of nucleoprotein	N concentration (mg./100 ml.)	Change in NPP of cultures (μ g.)
125/2	Nucleoprotein	L3 candle	10	+0.67
	"	Berkefeld candle	7	+0.41
	Nucleoprotein + boiled kidney extract	"	4 + 25	+1.58
	Boiled kidney extract	"	25	+0.97
143	Nucleoprotein	Benzene	12	+0.17
	"	"	4.5	+0.32
	Nucleoprotein + peptone	"	4.5 + 15	+0.52
	Peptone	"	15	+0.29
148/2	Nucleoprotein	Benzene	5	+0.42
	Nucleoprotein + peptone	"	5 + 7.5	+0.22
	Peptone	"	7.5	+0.24
151/1	Nucleoprotein	Benzene	5	+0.44
	Nucleoprotein + peptone	"	5 + 7.5	+0.57
	Peptone	"	7.5	+0.46

tests 143, 148/2, 151/1). A typical solution contained 9.8 mg. N/100 ml. and 0.70 mg. organic P/100 ml. The proportion of P to N was thus higher in the solution than in the original powder. It had good growth-promoting power at low concentrations (N=5). Higher concentrations (N=12) appeared to be less effective (test 143). Hopkins & Simon-Reuss (1944) found that of two concentrations of hypoxanthine used, the lower was favourable to the growth of tissue cultures.

When estimations of tissue growth by means of NPP are made on cultures to which an amount of nucleoprotein corresponding to several μ g. of NPP has been added, the question naturally arises, whether the increases in NPP observed might be due to adsorption of added nucleoprotein on to the plasma coagulum, and not to actual incorporation of the nucleoprotein in the living tissue. It was found that much higher concentrations of nucleoprotein than were used here were required to cause a significant rise in NPP in plasma blanks.

The nucleoprotein was tested also in conjunction with solutions of boiled kidney extract prepared by the method of Fischer & Astrup (1942), and with solutions of peptone. Both these solutions were

nucleoprotein fraction. The product which was nevertheless poorly soluble was dried from the frozen state, dissolved in Tyrode solution and Berkefeld-filtered. It had no activity. (ii) *Acid precipitation*. The precipitate obtained by acidifying embryo extract at 0° to pH 4.3–4.6 with acetic acid, when dissolved in the minimum amount of cold dilute alkali and sterilized by filtration after addition of an equal volume of double-strength Tyrode, had little or no activity.

A considerable proportion of the total nitrogen of these nucleoprotein preparations was retained by the filter candles. Seitz filtration resulted in almost complete adsorption of the nucleoprotein on the filter pad.

To avoid the use of candles and filters which adsorb the active materials, preparations of nucleoprotein were also made aseptically by acid precipitation. To ice-cold sterile chick embryo extract, ice-cold dilute HCl (0.001–0.01N) or acetic acid was added to give a pH of 4.3 and the precipitate centrifuged down. It was washed with sterile ice-cold water (slightly acidified) and dissolved in the minimum amount of ice-cold sterile 0.01N-NaOH. All operations were, of course, conducted with sterile

glassware. To the neutral solution, 1 vol. of double strength Tyrode solution was added. It had no activity.

(9) Histone

In view of recent suggestions (Stedman & Stedman, 1943 *a, b, c*, 1944 *a, b*; Stedman, 1944) that the histone of the cell nucleus plays a part in controlling cellular proliferation, and particularly that a high content of histone 'produces an inhibition of both the processes which lead to mitosis and those which lead to the synthesis of chromosomin necessary for the duplication of the chromosomes' (Stedman & Stedman, 1943*c*), it was thought desirable to test the effect of histones on the growth of tissue cultures.

Histones from two sources were prepared by two different methods. (a) Thymus histone was prepared from calf thymus by the method of Felix & Harteneck (1926). The histone was free from phosphorus. (b) Nucleohistone was prepared from sheep spleen by the method of Mirsky & Pollister (1942). The histone was separated from the nucleic acid by the chloroform-octanol method of Sevag, Lackman & Smolens (1938).

solution was very small in all cases. In test 146, the N concentration was slightly higher than in other tests, owing to the presence of a small amount of histone in suspension.

In all cases histone alone had no effect on the NPP of the cultures. It was found that the addition of histone to embryo extract regularly produced a greater increase in NPP than did embryo extract alone. This effect was not an additive one, as the increases were generally greater than the sum of the NPP increases caused by embryo extract and by histone separately (Table 7).

DISCUSSION

It has been recognized since the early tissue culture work of Carrel (1913) that embryonic extracts can promote rapid growth of explanted tissue and can provide an adequate medium for prolonged growth *in vitro*. The form of test adopted here is designed to detect large and rapid increases, occurring within 2 days, in the nucleoprotein content of the growing fibroblasts. Fresh explants from the 9-day chick embryo heart have been used, and no attempt has

Table 7. *Effect of histone from sheep spleen and from calf thymus on the nucleoprotein phosphorus (NPP) content of cultures grown with and without chick embryo extract*

Test no.	Test substance	Method of sterilization of histone	N concentration (mg./100 ml.)	Change in NPP of cultures (μg.)
124	Thymus histone + chick E.E.*	L3 candle	6.5 + 30	+0.71
	"	"	1 + 30	+0.92
	Spleen histone + chick E.E.	"	4 + 30	+0.79
	"	"	1 + 30	+0.35
	Thymus histone + chick E.E.	Heat	4 + 30	+0.61
	Spleen histone + chick E.E.	"	5 + 30	+0.77
146	Chick E.E. alone	" —	30	+0.56
	Thymus histone	Benzene	11	-0.07
	Spleen histone	"	1.5	-0.05
	Thymus histone + chick E.E.	"	11 + 28	+0.75
	Spleen histone + chick E.E.	"	1.5 + 28	+0.57
	Chick E.E. alone	" —	28	+0.49
150	Spleen histone	Benzene	8	-0.07
	Spleen histone + chick E.E.	"	8 + 30	+0.83
	Chick E.E. alone	" —	30	+0.61
158	Thymus histone	Benzene	6.5	+0.15
	Thymus histone + chick E.E.	"	6.5 + 30	+0.54
	Chick E.E. alone	" —	30	+0.29

* E.E. = embryo extract.

Several methods were used for obtaining sterile solutions of the histones. In the first experiment, histones were dissolved in water, and the solutions sterilized by heating at 100° for 15 min., cooled and treated with an equal volume of double-strength Tyrode solution. Another portion of each histone was dissolved in Tyrode solution and passed through an L3 Chamberland candle. In subsequent experiments the dried histone preparations were sterilized under benzene and dissolved in Tyrode solution. The amount of histone which could be brought into

been made to maintain pure strains over long periods of time.

Both chick and sheep embryo extracts are active in producing rapid increases in NPP, though a comparison of extracts at the same N concentration showed that extracts of chick tissue were superior. For this comparison the 50% sheep extract had to be considerably diluted, and it is possible that comparable concentrations of the active factors in the two extracts are not associated with equal N concentrations. Previous workers have not com-

pared the potencies of extracts of different species on any quantitative basis, though heterologous extracts have often been successfully used (Fischer, 1924; Mottram, 1927; Fischer, 1941a; Carrel & Ebeling, 1923; Landsteiner & Parker, 1940; Gey & Gey, 1936). Fischer (1941a, 1942a, b) noted that higher degradation products of homologous proteins were more effective than those of heterologous tissues. Removal of fat from liquid extracts by freezing with ether, besides removing lipid materials which may be inhibitory (cf. Mayer, 1936; Baker & Carrel, 1925), reduces the viscosity of the extracts, which can then be much more easily passed through a filter candle.

It is apparent that sterile embryo extract can be stored at -30° for long periods without losing its activity. At this temperature bacterial action (Reay, private communication) and enzyme action are almost negligible.

Of all the dried preparations made, only those dried from the frozen state were easily soluble. Those dried at room temperature contained much insoluble matter, although the soluble part retained some activity. Treatment with benzene improved the activity, again suggesting the removal of inhibitory lipids, and also provided a convenient method of sterilizing small amounts of powder.

Much of the material present in ethanol precipitates of embryo tissue extracts was also insoluble, though the small amount which did dissolve showed a definite growth-promoting activity. Fischer (1941b) found that no fraction of embryo extract was as active as untreated extract, although precipitation with ethanol 'gave a rather good result'. On the other hand, ethanol precipitates prepared in the same way from extracts of adult sheep heart showed very poor activity under the conditions of our experiments. This is contrary to the findings of Doljanski who states that, under his conditions, certain adult organs (including heart) activate the growth of fibroblast colonies *in vitro* to a greater extent than embryonic extract of the same concentration, and that the activity shown by ethanol precipitates is not inferior to that of the native extract (Auerbach & Doljanski, 1944). He does not, however, record any direct comparison between materials prepared from the embryo heart and from the adult heart, although extract of adult fowl heart is stated (Doljanski & Hoffman, 1939) to be more potent than chick embryo extract in increasing the area of tissue cultures. When a comparison is made between ethanol precipitates similarly prepared from adult and embryo sheep hearts, embryo material is found to be much more potent in rapidly increasing the NPP of the cultures.

Comparison of extracts of different embryonic organs confirms the inhibitory effect of liver extracts (cf. Walton, 1914; Heaton, 1926; Brues, Subbarow,

Jackson & Aub, 1940). Of the other tissues, cartilage, skin and muscle were all effective. It may be significant that all these contain large amounts of muco- or sulpho-polysaccharides. The high activity of sheep embryo muscle extracts is in contrast to the almost complete absence of activity found by Trowell & Willmer (1939) in extracts of adult fowl muscle. Of the different parts of the chick embryo tested by Fischer (1941b) extracts of brain were intermediate in activity between those of the body which were most active and those of the internal organs. The nucleic acid contents of sheep embryo tissues have already been recorded (Davidson & Waymouth, 1944a), and there appears to be no relationship between the nucleic acid content (or the nucleotide content) of a tissue and the growth-promoting activity of extracts of that tissue. Although the nucleoprotein fraction prepared from fresh liquid embryo extract is inactive, nucleoproteins prepared by Hammarsten's method (1920) from dried sheep embryo extract have a definite growth-promoting power at low concentrations. These preparations resemble the 'embryonin' prepared from dried beef embryo pulp by Fischer & Astrup (1943), who have stressed the importance of using dried embryonic tissue as the starting material. Drying is reported to render insoluble a large amount of accompanying inactive protein.

According to the hypothesis of Stedman (1944), the amount of basic protein in the nucleus may condition the ability of a cell to divide, so that when sufficient histone is present to combine with all the nuclear nucleic acid, the formation of a nucleic acid-acidic protein (chromosomin) complex with self-reproducing properties is precluded. This hypothesis has been supported (Stedman, Stedman & Pettigrew, 1944) by evidence of the retardation of the growth of tumour material which had been injected as an emulsion with histone into experimental animals. Solutions of histone included in the tissue culture media were found to have no inhibitory effect on nucleoprotein synthesis in the presence of embryo extract, and indeed generally caused a slightly greater rise in NPP than did the extract alone. The maximum concentration of histone which we could obtain in Tyrode solution at pH 7.4 was much less than the 2% present in the emulsion of Stedman *et al.* (1944). It is, moreover, by no means certain that the histone in the form added to the medium was available to the tissue, since the isolated protein differs in physical condition and in chemical association (cf. Macheboeuf, 1944) from the histone *in vivo*. A similar uncertainty must exist about the availability of the isolated nucleoproteins and the fact that the nucleoprotein fraction from dried embryo extract is active again raises the question of the nature of the growth-activating principles of embryo juice.

The recent work of Fischer (1939, 1940, 1941*a, b*) and of Fischer & Astrup (1943) suggests that some factor in 'embryonin' acts as a growth-promoting biocatalyst or growth hormone, but whether such a factor is present in the same form in fresh embryo juice is not yet clear. A distinction between nutrient and purely catalytic functions is difficult to draw. The experiments of Fischer (1941*a*) with dialyzed culture media have shown, for example, that embryo extract is ineffective in the absence of the appropriate amino-acids, which are required to maintain the life of the tissue. It is certain that growing cells have higher energy requirements than resting cells, for 'growth is... a process which involves a high rate of metabolism and consumes much energy, but the actual source of that energy is comparatively unimportant' (Willmer, 1935), and it is conceivable that the provision of unnaturally high concentrations of the normal metabolites might itself constitute a primary stimulus to cell division (Calkins, Bullock & Rohdenburg, 1912). Needham (1942), in this connexion, states that the 'growth-promoting factor is probably no more than a right conjunction of nutrient materials and the appropriate capacities for making use of them'. The 'appropriate capacities' may still include specific substances of growth hormone type, but they will certainly depend for their effectiveness on the nutritive requirements of the cells being first fulfilled. Growth catalysts might, moreover, be of two types; those which increase the rate of growth by decreasing the mitotic and/or intermitotic period and those with the true growth-promoting effect of stimulating an increased number of cells to undergo division.

In the presence of the essential nutrients (and as Berrill (1943) points out, the failure of an adult cell to grow *in vivo* is 'clearly not due to lack of nutritive substances or to any deficiency on the part of the cell') proliferation will occur only if the nutrients are available to the cell. According to Berrill, who lays emphasis on the physical as well as the chemical conditions in the tissue, cell division 'is the climax of a complex process of growth and duplication of a multitude of cell components', which must 'occur when cell size reaches a certain critical value', in order to maintain certain volume-surface ratios. The question must still remain open whether one specific tissue component is the limiting factor determining cell division or whether the provision of an optimum environment is itself sufficient stimulus. Nucleoproteins have been considered from this point of view, and it has been suggested that a high concentration of nucleic acid is required for mitosis to take place (Caspersson, 1936; Darlington & La Cour, 1940; Darlington, 1942). Nucleoprotein synthesis in growing tissue cultures may be very rapid and precede cell division by some hours (Willmer, 1942, 1943; Davidson & Waymouth, 1944*d*), and the

effectiveness of embryo extract may be due to its capacity to provide both the energy requirements of the tissue and the materials for nucleoprotein synthesis in a readily available form.

SUMMARY

1. The effect of various tissue extracts on the nucleoprotein phosphorus (NPP) content of chick heart fibroblasts growing *in vitro* has been examined. It has been found preferable to express the results as absolute increases in NPP rather than as relative increases.
2. Chick embryo extract has a greater growth-promoting power than sheep embryo extract at the same N concentration.
3. The activity of the extracts is easily impaired by procedures which denature the proteins.
4. Increased activity results from defatting the extracts. Defatted extracts are further improved by drying from the frozen state.
5. Of various procedures adopted for drying embryo extracts, only drying from the frozen state gives satisfactory products.
6. Finely divided powders can be sterilized by standing under benzene or ether, and can then be redissolved aseptically. This procedure obviates the use of filter candles for sterilizing extracts.
7. Sheep embryo extract retains good growth-promoting activity when stored at -30° for 9 months in the sterile state.
8. The growth-promoting activity of chick or sheep embryo extract cannot be attributed to any particular organ or tissue.
9. Ethanol precipitates of embryonic tissue extracts are more active than similar preparations from adult sheep heart.
10. A nucleoprotein fraction prepared from dried sheep embryo extract is effective in low concentrations.
11. Solutions of histone in low concentrations do not impair, but appear to increase, the effect of embryo extract on the NPP of fibroblasts.
12. It is stressed that the normal nutritive requirements of a tissue must be satisfied before the effects of growth stimulants can be assessed, and that these effects may depend on physical as well as purely chemical characteristics of the stimulating materials.

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