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The Nucleoprotein Content of Fibroblasts Growing *in vitro*

3. THE USE OF DEFATTED MEDIA

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It is generally accepted that lipid materials are inhibitory to the growth of tissues *in vitro* (cf. Baker & Carrel, 1925*a*, *b*; Mayer, 1936). We have already shown that the removal of fat from sheep embryo extracts increases the growth-promoting power of such extracts for tissue cultures of chick heart fibroblasts (Davidson & Waymouth, 1945). It has therefore seemed to us desirable that the effect on the growth of cultures of removal of the lipids from the embryo extract of the plasma coagulum or both, should be further investigated. In the course of this work we have prepared fat-free fowl plasma in the form of a sterile powder which can be stored indefinitely (Waymouth & Davidson, 1945). The use of a large batch of such plasma powder enables a long series of cultures to be set up in the same medium and avoids the necessity for the maintenance of a stock of liquid plasma which must be renewed frequently and which may vary in composition from batch to batch.

The use of defatted plasma has also enabled further investigations to be made on a factor in pancreatin which profoundly influences the morphology of fibroblast cultures *in vitro* (Davidson & Waymouth, 1944*a*). This factor appears to be identical with, or related to, the enzyme lecithinase A which hydrolyzes lecithin to lysolecithin. To examine the effect of this enzyme on growing tissue defatted media are obviously desirable.

This paper deals with the phospholipin content of embryonic tissues and of embryo tissue extracts before and after defatting, with the preparation of defatted fowl plasma and its chemical composition, and with the use of these defatted media in tissue culture technique.

METHODS AND MATERIALS

Tissue culture technique

(*a*) *General.* Cultures were grown by the roller tube technique of Willmer (1942) which we have previously employed (Davidson & Waymouth, 1943, 1945). Each roller tube contained 12 pieces of tissue in two rows of 6. As in earlier work all the cultures were made from fresh explants from the 10-day chick embryo heart and consisted therefore to a large extent of the cells which are usually termed 'fibroblasts' in tissue culture work (Mayer, 1939) but which have been more precisely defined as 'mechanocytes' by Willmer (1945). The amount of tissue present was determined by making estimations of nucleoprotein phosphorus (NPP) (Davidson & Waymouth, 1943).

(*b*) *Materials.* Lecithin was prepared from egg yolk by the method of Macfarlane & Knight (1941), and dissolved in ethanol. Insoluble material was centrifuged off and an emulsion prepared by adding 2 ml. of the ethanolic solution to 25 ml. of boiling 0·9% NaCl (Ponder, 1942). The solution was heated for 10 min. in a boiling water-bath to sterilize it and to drive off the ethanol. The concentration of this emulsion was estimated from the P content and suitable dilutions made with Tyrode solution.

Two samples of lysolecithin, of fractions 2 and 7 of King & Dolan (1933), were kindly presented by Prof. E. J. King. 5 mg. portions were dissolved in chloroform, reprecipitated with acetone and dissolved in 1·5 ml. of 0·9% NaCl. The solutions were sterilized by heating in a boiling water-bath at 100° for 10 min., cooled and diluted with Tyrode solution.

The pancreatin preparations were prepared according to the method previously described (Davidson & Waymouth, 1944*a*). They were dissolved in water, sterilized by heating to 100° for 10 min., cooled, and treated with an equal volume of double strength Tyrode solution. Similar preparations were also heated in alkaline solution (pH 8–10) in order to destroy such resistant enzymes as lecithinase A and

ribonuclease. The solutions were then neutralized and treated as above.

Preparations of the heat stable enzyme lecithinase A were obtained from two sources. (i) A sample of Russell's viper venom provided by Boots Pure Drug Co. Ltd. was dissolved in water and sterilized by heating to 100° for 10 min. The denatured protein which appeared during the heating was centrifuged down and discarded. The supernatant fluid was treated with an equal volume of double strength Tyrode solution before use. (ii) From the dry venom of the rattlesnake, *Crotalus t. terrificus*, Slotta & Fraenkel-Conrat (1938*a*, *b*) have isolated the neurotoxic principle as a crystalline protein which they have named 'crotoxin'. This material has powerful lecithinase A activity. A sample of pure 'crotoxin' was kindly put at our disposal by Dr Fraenkel-Conrat who stated that it had been proved to be homogeneous when examined in the ultracentrifuge and in the diffusion apparatus (Gralen & Svedberg, 1938) and when subjected to electrophoresis experiments (Li & Fraenkel-Conrat, 1942). It was dissolved in water and treated in the same way as the Russell's viper venom.

Embryonic tissues and extracts

(*a*) In the preparation of '50%' embryo extract the embryo pulp was mixed with an equal volume of Tyrode solution and was either centrifuged after standing for a short time or was frozen and thawed before centrifuging. Extracts prepared by the latter method contained haemoglobin from the ruptured red blood cells.

(*b*) The embryo extract was defatted as previously described (Davidson & Waymouth, 1945) by freezing with ether according to the method used by McFarlane (1942) for plasma. In the case of chick embryo extract the whole process was carried out aseptically using sterile glassware.

(*c*) The phospholipin-P contents of embryo extract were determined by one of two methods. (i) A 10 ml. sample of embryo extract was added to about 50 ml. ethanol: chloroform (3 : 1), the mixture warmed on a hotplate and filtered. The residue was re-extracted and the combined filtrates made up to a total volume of 100 ml. 10 ml. of the filtrate were taken to dryness in an open dish on a water-bath, the residue extracted with pure chloroform, the extract filtered, and total phosphorus estimated in the chloroform filtrate by the method of Allen (1940). (ii) Embryo extract (2 ml.) was treated with 2 ml. of 20% (w/v) trichloroacetic acid, the residue washed twice with 10% (w/v) trichloroacetic acid, and extracted once with 5 ml. 80% (v/v) ethanol, once with 5 ml. absolute ethanol, three times with ethanol-ether (3 : 1) and once with ether. The ethanol and ether extracts were combined and taken to dryness. Phosphorus was estimated in the extract by the method of Allen (1940).

(*d*) The phospholipin-P content of individual organs and tissues of the 9–10-week sheep embryo and of the adult sheep was also determined. Weighed portions (about 1 g.) of fresh tissue were finely minced and extracted with warm ethanol: chloroform (3 : 1) to a total volume of 100 ml. filtered extract. 10 or 20 ml. of the filtrate according to the amount of phospholipin in the tissue were evaporated to dryness and the residue extracted with chloroform. The chloroform extract was filtered and its phosphorus content determined by the method of Allen (1940).

Fowl plasma

(*a*) *Preparation.* For the preparation of defatted plasma advantage was taken of the observation of Hardy & Gardiner (1910) and of Hewitt (1927) that lipins can be removed from protein-containing solutions without protein denaturation by treatment with ethanol-ether mixture at temperatures below −10°, followed by extraction with ether.

For the present purpose, all manipulations were carried out aseptically and sterile Pyrex glassware was used throughout.

The plasma was collected from the carotid arteries of brown Leghorn cockerels and was stored in sterile waxed test-tubes on ice.

A 500 ml. sterile Pyrex conical flask containing ethanol-ether (7 : 3) mixture (6 vol. of mixture to 1 vol. of plasma) was cooled in a freezing mixture until the contents were below −15°. The plasma was then added slowly from a sterile Pyrex graduated pipette while the flask was gently agitated to ensure thorough mixing. The temperature was not allowed to rise above −5°. The flask surrounded by the freezing mixture was set aside in the cold room for 2 hr. The protein precipitate was then centrifuged down in sterile 50 ml. centrifuge tubes covered with sterile caps, and washed on the centrifuge twice with cold ethanol-ether mixture and three times with cold ether. The ethanol-ether mixture used for washing was kept in a vessel immersed in a freezing mixture and the centrifuge tubes and buckets were chilled in a freezing mixture between runs. The precipitate was then transferred with the aid of a sterile spatula and a little ether to a sterile Soxhlet thimble. Extraction with ether in a sterile Soxhlet apparatus was carried out for two periods of 2 hr., a small amount of sodium being placed in the flasks. The thimble was then drained as nearly free from ether as possible and was transferred aseptically to a sterile glass vessel. The last traces of ether were removed in a vacuum desiccator and the dried plasma proteins were then powdered with a sterile spatula and transferred to sterile tared test-tubes (120–160 mg. per tube) for storage. The yield was about 30 mg. powder per ml. of plasma.

For reconstitution, sterile Tyrode solution was added in the proportion of 1 ml. to 30 mg. powder and the mixture was stirred with a sterile glass rod. It dissolved easily and completely yielding a clear colourless solution which was alkaline (about pH 8) and required the addition of a few drops of sterile 0·02 N-HCl to bring the pH to 7·4 (phenol red).

The reconstituted plasma contained sufficient prothrombin for clotting to occur readily when tissue fragments were added to it.

(*b*) *Analysis of defatted plasma.* Samples of defatted plasma reconstituted in water were compared with corresponding samples of the whole plasma. Total N was determined by the micro-Kjeldahl technique. Chloride was determined by the usual Volhard procedure and calcium by the method of Kramer & Tisdall (1921).

The phosphorus fractions were determined in 4 ml. of plasma which were treated with 4 ml. 10% (w/v) trichloroacetic acid. The precipitated proteins were centrifuged down and washed twice with 5% trichloroacetic acid. In the combined supernatant and washings inorganic-P and total acid-soluble-P were determined by the method of

Allen (1940). The protein precipitate was extracted with ethanol-ether or ethanol-chloroform and the extract used for the determination of lipid-P. The protein residue was ashed for the determination of residual P.

RESULTS

The phospholipin content of embryo tissues and extracts

The results of the estimations of phospholipin-P in the tissues of the embryo and adult sheep are shown in Table 1. On a fresh tissue basis the embryonic tissues have a much lower phospholipin-P concentration than the corresponding adult tissues, the difference being particularly marked in the case of liver and brain. The water content of 9–10-week sheep embryo tissues has already been recorded, and has been shown to be much higher for embryonic than for adult tissues (Davidson & Waymouth, 1944*b*). If the phospholipin-P content of the sheep tissues is calculated on a dry weight basis, it is again found that, except in the case of muscle and heart, the adult tissues are richer in phospholipin than those of the embryo, the difference still being most marked for liver and brain.

Since the phospholipin content of embryonic tissues is low, it is not surprising to find that the phospholipin-P concentration in embryo extracts is not high (Table 2). In the preparation of samples 1, 2 and 3 (Table 2) extracts were made both by simple extraction of the embryo pulp with Tyrode solution and by extraction after freezing and thawing. In all cases the extracts prepared by direct extraction had a slightly higher phospholipin content than those made after freezing.

Samples 4–8 (Table 2) were defatted by freezing with ether. This process removes a considerable amount, but not all, of the phospholipin-P and significantly reduces the nitrogen content. The proteins of chick embryonic tissues appear to be easily denatured and during the defatting process a certain amount of protein is removed on centrifuging. No similar protein loss occurs when plasma or serum is defatted by the same procedure, and the loss is much smaller during the defatting of sheep embryo extract.

Embryo extract contains a small amount of residual P probably derived from ribonucleic acid which is known to be present in embryonic extracts (Davidson & Waymouth, 1944*b*).

Table 1. *Phospholipin-P in sheep tissues*

	mg./100 g. fresh tissue								Mean values for	
									Fresh tissue	Dry tissue
	Embryo									
Liver	56·3	53·0	64·4	46·4	64·9	—	—	—	57·0	334
Heart	42·8	45·6	44·2	48·1	48·8	—	—	—	45·9	353
Muscle	29·9	27·1	29·2	30·6	28·0	—	—	—	29·0	230
Brain	62·2	59·8	53·8	58·6	60·7	—	—	—	59·0	311
Kidney	44·6	48·8	42·9	43·6	43·5	—	—	—	44·7	409
Lung	40·8	31·3	27·9	22·8	28·1	25·5	—	—	29·4	267
	Adult									
Liver	156·5	156·5	168·4	178·0	159·7	157·0	163·6	164·3	163·0	538
Heart	71·6	71·5	68·9	65·2	67·6	69·2	—	—	69·0	309
Muscle	46·7	39·5	44·25	45·9	30·1	27·8	—	—	39·1	123
Brain	166·6	192·9	161·0	147·8	164·2	157·4	—	—	165·0	793
Kidney	68·1	68·0	104·1	105·7	100·2	91·6	90·0	95·0	90·3	539
Lung	84·8	91·0	77·0	87·8	93·2	73·5	—	—	84·6	403

Table 2. *Composition of chick embryo extract*

		mg./100 ml.			
Sample		Total N	Total P	Phospholipin-P	Residual-P
1 *a*		49·0	18·25	2·42	—
b	Frozen-thawed	64·4	16·75	1·38	—
2 *a*		65·3	41·0	1·95	—
b	Frozen-thawed	76·7	42·5	1·90	—
3 *a*		53·5	12·4	1·31	—
b	Frozen-thawed	50·4	12·1	0·90	—
4 *a*	Whole extract	154·0	—	2·49	—
b	Defatted extract	88·5	—	0·44	—
5 *a*	Whole extract	109·0	—	1·68	—
b	Defatted extract	65·0	—	0·08	—
6 *a*	Whole extract	96·0	—	2·28	—
b	Defatted extract	47·0	—	0·51	—
7 *a*	Whole extract	73·8	—	1·89	0·84
b	Defatted extract	56·0	—	0·85	0·01
8 *a*	Whole extract	76·0	17·2	2·24	1·14
b	Defatted extract	48·0	10·3	0·61	0·37

The composition of fowl plasma

This is shown in Table 3. The analytical figures for five samples are recorded, each reconstituted defatted sample being compared with the corresponding whole plasma from which it was made. In

Table 3. *Composition of blood plasma of brown Leghorn cockerels*

	mg./100 ml.							
Sample	Total N	Non-protein-N	Total acid-soluble-P	Inorganic-P	Lipid-P	Residual-P	Chloride (as NaCl)	Calcium
1 A	491	—	2·70	2·45	3·54	0·26	590	—
B	352	—	1·98	1·82	0·34	0·07	37·5	—
2 A	581	—	3·28	3·13	3·63	0·20	683·5	—
B	424	—	2·20	1·99	—	—	14	—
3 A	357	—	3·90	3·30	3·06	0·24	660	8·7
B	265	—	1·90	1·75	0·12	0·08	20	—
4 A	449	27·4	4·52	4·25	2·21	0·10	700	10·7
B	317	12·6	2·40	1·95	0·17	0·04	10	9·1
5 A	599	37·8	3·80	3·60	4·00	0·44	755	10·4
B	352	11·7	3·50	3·30	0·10	0·02	15	8·6

A = whole plasma. B = defatted plasma powder reconstituted with water.

each case the defatted plasma has a lower total nitrogen concentration than the whole plasma. Although a small part of this fall can be accounted for by manipulative losses, the major part appears to be due to a loss of albumin, since the albumin-globulin ratio is much lower in the defatted than in the whole plasma. The defatted plasma still contains an appreciable amount of non-protein nitrogen.

The lipid-P content of whole cockerel plasma is not high and, as might be expected, the amount is greatly reduced in the defatted material. After extraction of acid-soluble-P and lipid-P from whole plasma, a small but fairly constant amount of P still remains. In the defatted plasma the amount of residual-P is negligible.

Of the inorganic constituents of plasma, chloride is present in only small amounts in the defatted material and in no case is more than 7 % of the chloride of the plasma found in the extracted material. On the other hand up to 92 % of the acid-soluble-P of the untreated plasma and of the inorganic-P which forms the greater part of this fraction in the plasma of cockerels, is retained in the reconstituted solution. A large proportion of the calcium is also retained in the defatted material.

The growth of cultures in defatted plasma

The nucleoprotein phosphorus (NPP) content of cultures grown in whole plasma and in defatted plasma is shown in Fig. 1. In confirmation of our previous observations (Davidson & Waymouth, 1943) it is found that cultures maintained in Tyrode solution alone show a marked fall in NPP (test 190, curve *D*). In its initial stages this fall is not prevented by the presence of embryo extract (curve *B*) but can be diminished to some extent in the presence of embryo extract of high concentration ($N = 90$)* (curve *A*). With moderate concentrations of embryo extract ($N = 30$) the NPP at the end of 4 days is much higher than in cultures grown in Tyrode solution alone but is still slightly lower than

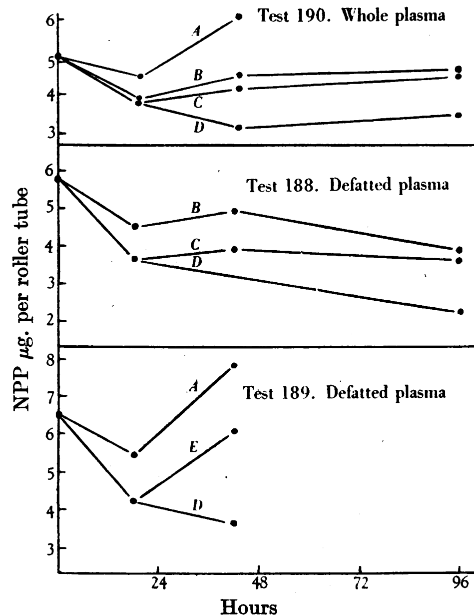

Fig. 1. Effect of chick embryo extract on the nucleoprotein phosphorus (NPP) content of cultures grown in whole plasma and in defatted plasma with and without embryo extract. Each roller tube contained 12 pieces of tissue and the cultures were set up as fresh explants at 0 hr. *A*, embryo extract ($N = 90$) present from time of setting up. *B*, embryo extract ($N = 30$) present from time of setting up. *C*, embryo extract ($N = 30$) added after 16 hr. in Tyrode solution. *D*, Tyrode solution alone as fluid phase. *E*, embryo extract ($N = 90$) added after 16 hr. in Tyrode solution.

when the cultures were first set up and the final figure is the same when the embryo extract is added after 18 hr. as when it is present from the

* This convention is employed as before (Davidson & Waymouth, 1945) to indicate the concentration of total N (as mg./100 ml.) in the fluid added to the roller tubes.

time of setting up (test 190, curves B and C). With high concentrations of embryo extract ($N=90$) a very sharp rise in NPP takes place during the second day and the figure after 2 days is much higher than at the time of setting up (test 190, curve A).

When cultures are grown in defatted plasma (Fig. 1, tests 188 and 189) with Tyrode solution alone as fluid phase (curve D) the fall in NPP in the course of 4 days is much greater than is the case with similar cultures grown in whole plasma, and the fall is much sharper in its initial stages. This sudden decrease in NPP in the course of the first 18 hr. can be abolished by the addition of peptone ($N=25$) and can be reduced by the addition of boiled kidney extract (Fischer & Astrup, 1942) ($N=25$), of malt extract prepared by the method of Astrup & Fischer (1945) ($N=50$), and of pancreatin preparations ($N=25$), but is not affected by lecithin ($N=5$) or choline ($N=4$).

In the presence of embryo extract the NPP of the cultures still shows a fall in the course of the first 18 hr. (test 188, curve B; test 189, curve A) although the fall is not so great as with Tyrode solution alone. With high concentrations of embryo extract ($N=90$) (test 189) a sharp rise in NPP follows the initial fall, and if the embryo extract has been present from the beginning (curve A), the figure for the NPP at the end of 2 days is much higher than at the time of setting up. The use of defatted plasma is therefore compatible with good growth of the explants as measured by increase in NPP. Comparison of curves A and E (Fig. 1, test 189) shows that the rise in NPP during the course of the second day is almost the same when the embryo extract ($N=90$) is added after 18 hr. as when it has been present from the start, although the final figure is of course much lower.

With low concentrations of embryo extract ($N=30$) (test 188, curves B and C) a slight rise in NPP occurs during the second day and this rise is almost of the same magnitude when the embryo extract is added after 18 hr. as when it has been present from the time of setting up, although again the absolute figure is higher in the latter case. During the third and fourth days the NPP falls again and curves B and C tend to converge.

Comparison of curves B and C in tests 188 and 190 (Fig. 1) shows that for $N=30$ whole plasma is more efficient than defatted plasma in maintaining the NPP levels of the cultures and the final values reached after 4 days are similar when the embryo juice is added after 18 hr. and when it is present from the start.

Fig. 2 shows a comparison of whole plasma with defatted plasma in maintaining the growth of cultures in whole and defatted extract. In both cases the test substances were added after the cultures had been maintained for 18 hr. with Tyrode solution alone as fluid phase. In both cases the NPP falls continuously when Tyrode solution alone is the test material (tests 192 and 193, curve D) and the fall is more pronounced with defatted plasma than with whole plasma.

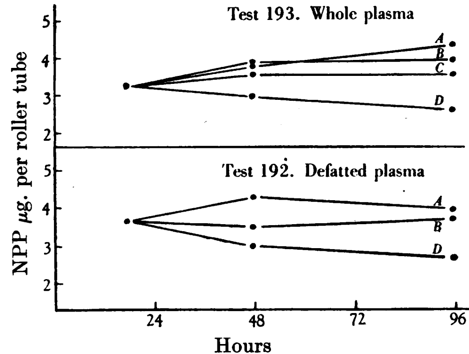

Fig. 2. Effect of whole and defatted chick embryo extract on the nucleoprotein phosphorus (NPP) content of cultures grown in whole plasma and defatted plasma. The cultures were maintained in Tyrode solution alone for 18 hr. before the test substances were added. A, test substance, whole embryo extract ($N=46$). B, test substance, embryo extract defatted by freezing with ether ($N=46$). C, test substance, defatted protein fraction of embryo extract ($N=46$). D, Tyrode solution alone.

Curves A and B in Fig. 2 show a comparison of whole extract with defatted extract. When whole plasma is employed (test 193), in contrast to the results obtained with sheep embryo extract (Davidson & Waymouth, 1945), whole chick extract is slightly more effective than defatted extract in causing a rise in NPP and the figures steadily increase during the 3 days of the test period.

With defatted plasma (test 192) the use of whole extract (curve A) is followed by a rise and then a fall in NPP. This is in accordance with the results obtained in Fig. 1, test 188, curve C. When defatted extract is employed (test 192, curve B) the NPP remains almost constant over the test period. The fall in NPP which occurs with Tyrode solution alone is prevented but no rise takes place. The difference between whole extract and defatted extract is thus more marked with defatted plasma than with whole plasma and defatted chick embryo extract does not appear to be able to cause an increase in NPP in cultures in defatted plasma.

In Fig. 2, test 193, curve C, the test medium was a solution of chick embryo proteins defatted by the method employed in preparing the defatted plasma powder. This defatting procedure caused a certain amount of protein denaturation and the soluble proteins in the final product did not maintain the cultures so well as did embryo extract defatted by freezing with ether.

The effects of lecithinase A and lysolecithin

Previous work has shown (Davidson & Waymouth, 1944*a*) that pancreatin extracts are able to increase the NPP content of tissue cultures and also to cause morphological changes resulting in the production of a dense culture with a sharply demarcated edge. The latter changes are thought to be due to the presence of the enzyme lecithinase A. We have therefore tested the effects on cultures growing in whole and defatted plasma of pancreatin extracts before and after heating in alkaline solution to inactivate the enzyme. Attempts were also made to restore the activity which is abolished by heating in alkaline solution, by the addition of Russell's viper venom and of pure crotoxin. Since it appears probable that lecithinase A exercises its effect by acting on the phospholipin either of the medium or of the cells (e.g. by producing lysolecithin from lecithin) it was thought desirable to test the action of the enzyme on cultures growing in a wholly defatted medium with and without the addition of lecithin. The effect of lysolecithin was also examined. Some of the results are shown in Table 4.

When whole plasma is employed (Table 4, tests 198 and 201) the addition of pancreatin extract to a medium containing embryo extract causes a higher rise in NPP than is produced by a moderate or low concentration of embryo extract alone. A pancreatin preparation which has been heated in alkaline solution is rather less effective in this respect. The addition of lecithinase A or lysolecithin to such a heated preparation results in the appearance of the characteristic morphological changes without however having any marked effect on the NPP. Higher concentrations of lecithinase or lysolecithin than those shown in Table 1 were toxic to the cells and resulted in a fall in NPP in the cultures.

In a medium containing defatted plasma and defatted embryo extract, the addition of pancreatin

Table 4. *The effect on the nucleoprotein phosphorus (NPP) content of tissue cultures of combinations of whole or defatted chick embryo extract (EE) with lecithinase A or associated substances*

(Each roller tube contained 12 pieces of tissue. Cultures were grown for 48 hr. in Tyrode solution alone and for a further 48 hr. in the test media.)

Test no.	Plasma	Test substance	Nitrogen concentration (mg./100 ml.)	Change in NPP of cultures (μg./roller tube)	Morphological change
198	Whole	Whole EE	10	+0·88	
		,, ,, + pancreatin extract	10 + 50	+2·56	
		,, ,, + pancreatin extract (heated)	10 + 50	+1·91	
		,, ,, + pancreatin extract (heated) + crotoxin	10 + 50 + 0·07	+2·04	+ + +
		,, ,, + pancreatin extract (heated) + lysolecithin (50 mg. %)	10 + 50	+1·61	+ +
		,, ,, + pancreatin extract (heated) + Russell's viper venom	10 + 50 + 0·07	+1·36	+ +
201	Whole	Whole EE	38	+2·01	
		,, ,, + pancreatin extract	38 + 50	+2·52	+
		,, ,, + pancreatin extract (heated)	38 + 50	+1·94	
		,, ,, + pancreatin extract (heated) + crotoxin	38 + 50 + 0·07	+2·16	+ +
201	Whole	Whole EE + pancreatin extract (heated) + lysolecithin (50 mg. %)	38 + 50	+2·13	(+)
		,, ,, + pancreatin extract (heated) + Russell's viper venom	38 + 50 + 0·07	+2·61	+ +
200	Defatted	Defatted EE	40	+1·53	
		,, ,, + pancreatin extract	40 + 50	+0·66	
		,, ,, + pancreatin extract (heated)	40 + 50	+1·71	
		,, ,, + pancreatin extract (heated) + crotoxin	40 + 50 + 0·07	+0·61	
		,, ,, + pancreatin extract (heated) + lysolecithin (50 mg. %)	40 + 50	+0·69	
		,, ,, + pancreatin extract (heated) + Russell's viper venom	40 + 50 + 0·07	+1·39	
207	Defatted	Whole EE	20	+0·52	
		,, ,, + lecithin	20 + 1	+0·53	
		,, ,, + lecithin	20 + 5	+1·05	
		,, ,, + crotoxin	20 + 0·07	+0·47	+
		Defatted EE	20	+0·72	
		,, ,, + lecithin	20 + 1	+0·60	
		,, ,, + crotoxin	20 + 0·07	+0·64	+
		,, ,, + crotoxin + lecithin	20 + 0·07 + 1	+0·40	+

extract does not further increase the NPP of the cultures, and morphological changes visible to the naked eye are not seen after the addition of lecithinase A or lysolecithin (Table 4, test 200).

The effect of defatted embryo extract on the NPP of cultures in defatted plasma as measured by the difference between the NPP in the test medium and the NPP in Tyrode solution alone at the end of the growth period, was of the same order as that produced by whole extract (Table 4, test 207). A low concentration of lecithin had no effect in either case and a higher concentration of lecithin had none of the inhibitory effects usually attributed to phospholipins. Crotoxin produced morphological changes when added to these media even in the case of the wholly defatted media though in defatted plasma the effect was seen only on microscopical examination.

DISCUSSION

The phospholipin contents of the tissues in the embryo sheep are in general lower than in the adult. In this respect the sheep embryo resembles the rabbit embryo in which Boyd (1935) found a rise in phospholipin-P during intra-uterine growth. In the pig embryo on the other hand, though the lipin-protein ratio remains constant for a large part of the gestation period, the phospholipin content shows no increase (Gortner, 1945).

We have already observed (Davidson & Waymouth, 1945) that '50%' chick embryo extract has a much lower nitrogen concentration than '50%' sheep embryo extract. The phospholipin-P content of sheep extract is usually 1–2 mg. per 100 ml. and therefore does not differ greatly from that of chick extract.

Although numerous analyses of the blood of laying and non-laying hens have been published, there are few figures on record for the blood of the cockerel. Our figures for the phospholipin-P content of the plasma of the brown Leghorn cockerel are of the same order as those of Boyd & Clarke (1940) and are lower than those of Roepke & Hughes (1935). In the plasma of the laying hen the phospholipin-P content is very much higher. Our figures for inorganic phosphate, which forms a very large proportion of the total acid-soluble-P, are slightly lower than those of Benjamin & Hess (1933) (6·1) and of Roepke & Hughes (1935) (5·4) but are well within the range (1·2–5·6, mean 3·8) quoted by Mayrs (1923). Our figures for calcium agree well with those of Benjamin & Hess (1933) (10·7) and are slightly lower than those of Correll & Hughes (1933) (11·7), of Roepke & Hughes (1935) (11·2), and of Zondek & Marx (1939) (11·2).

The procedure of treating plasma with ethanol and ether at a low temperature removes nearly all the lipid-P. The residual-P is also greatly reduced. Of the inorganic constituents of the defatted plasma reconstituted in water, the chloride content is very low but calcium and inorganic phosphate are not much lower than in whole plasma. This may be due to the relative insolubility of these substances in the plasma-solvent mixture at the low temperature employed or it may be that at least part of the calcium and phosphorus is bound to protein in a loose type of combination which is disrupted by deproteinizing procedures (cf. Grollman, 1927). In this connexion it should be noted that Mayrs (1923) observed that the phosphates of cockerel serum were only partly ultrafilterable, while Greenberg, Larson, Pearson & Burmester (1936) found that the fowl differed from higher vertebrates in having part of its inorganic-P in non-diffusible form.

It is clear from Fig. 1 (test 189, curve *A*) that with a sufficiently high concentration of embryo extract good growth of tissue cultures, as measured by increase in NPP, is possible in defatted plasma. Comparison of tests 188 and 190, however, shows that, in general, cultures deteriorate more quickly in defatted than in whole plasma when the concentration of embryo extract is the same. It appears probable therefore that the plasma clot acts not merely as an inert mechanical support for the growing cells but also contributes factors to their nutritive requirements. Whether these factors are to be found in the lipid fraction or among the non-lipid materials removed in the defatting process is not certain.

Previous work on the effect of lipins on tissue cultures, e.g. by Mayer (1936), who used a mixture of brain lipins, has mostly been carried out with very complex mixtures and the inhibitory effects found have not been attributed to any particular fraction. Carminati (1932) found that a synthetic (distearyl) lecithin had a distinct growth-promoting effect on chick heart cultures. There is thus no evidence that the lipins removed from plasma are necessarily inhibitory.

We have previously found that tissue cultures in whole plasma grow better in defatted than in whole sheep embryo extract (Davidson & Waymouth, 1945). With chick embryo extract, the defatting process slightly reduces the growth-promoting power of the extract and this reduction is more marked when defatted plasma is employed. Cultures do not grow well in a medium consisting of defatted plasma and defatted embryo extract.

The proportion of protein lost in the defatting of chick extract (where the total protein content is relatively low) is much greater than with sheep extract. The difference between the effects of whole and defatted extract may therefore be due not only to the loss of lipin but to alteration in the relative proportions of some of the other constituents.

If the morphological changes produced by extracts of pancreatin are in fact due to the presence

of lecithinase A, it is probable that the enzyme exerts its effect by producing lysolipins from the phospholipins of the plasma, the embryo extract or of the cells themselves. The lysolipins, which produce their effects on erythrocytes and other cells by lowering the surface tension of the medium and of the cells (Bleyl, 1945) may act upon the cells of the culture to produce a change in shape similar to the prelytic swelling and loss of discoidal form of red cells observed by Ponder (1943). Preparations from Russell's viper venom, and also pure crotoxin, in suitable concentrations can act on cultures grown in whole plasma and embryo extract to produce morphological changes similar to those observed with pancreatin extracts. Lysolecithin has a similar effect.

The same concentration of lecithinase A produces very little change in morphology in cells in a defatted medium, and such change as occurs may be due to a direct effect of the enzyme on the phospholipins of the cells, or on the traces of phospholipins remaining in the plasma and embryo extract after treatment with ether.

SUMMARY

1. Estimations have been made of the phospholipin-P concentration in the tissues of the embryo and adult sheep. The phospholipin concentration is in general lower in the embryonic than in the corresponding adult tissue.

2. Cockerel plasma has been prepared in the form of a sterile defatted powder which dissolves freely in water and in Tyrode solution and retains its clotting power. Such defatted material is suitable for tissue culture work.

3. Tissue cultures consisting of fresh explants of the 10-day chick embryo heart can be maintained in a coagulum prepared from defatted plasma, and in the presence of a suitable concentration of embryo extract will show growth as measured by increase in NPP. Cultures deteriorate more quickly in defatted plasma than in whole plasma.

4. Defatted chick embryo extract is less effective than whole chick embryo extract in promoting the growth of cultures. The NPP of cultures grown in defatted plasma and defatted embryo extract is maintained, while in defatted plasma and Tyrode solution there is a marked drop.

5. Changes in the morphology are produced in the cells of cultures in media containing lecithinase A and these changes are probably due to the effect of this enzyme on the phospholipins of the medium and the cells. Lysolecithin produces a similar effect.

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Note added 29 *June* 1946. Since this paper was written we have been able to consult a paper by J. L. Delsal (*Bull. Soc. Chim. Biol.* 1944, **26**, 282) who defatted human and horse serum by Hewitt's method and dissolved the protein powder in water. The reconstituted serum contained nearly all the inorganic-P present in the original material but only 10–20 % of the NaCl.

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