bolic rate.

The results reported here may be related, however, to observations of Saegesser,[6] who reported a depression by cholesterol of thyroxine effects on the rate of metamorphosis of tadpoles, and on the oxygen requirement of rats in the oxygen deficiency test ("Sauerstoffmangelversuch") of Asher and Streuli.[7]

[6] Saegesser, M., *Klin. Wochenschr.*, 1933, **12**, 672.

Summary. Dietary cholesterol significantly decreased the oxygen consumption of thyroid-fed rats, but the reduction was not sufficient for the metabolic rate to approach normal levels. This effect of cholesterol cannot be attributed to a depression of the food intake.

[7] Asher, L., and Streuli, H., *Biochem. Z.*, 1918, **87**, 359.

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17181. Calcification of Cell Cultures in the Presence of Embryo Juice and Mammalian Sera.

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Upon undertaking to learn if leprosy bacilli would multiply in cells cultured from leprous lesions, the mistake was made of trying to use the serum and chick embryo juice in proportions suitable for the cultivation of chick tissues. Following the first or second renewal of a liquid phase composed of human serum 25% and chick embryo juice 15%, the central explants or transplants became opaque to transmitted light and a brownish precipitate accumulated in the surrounding plasma. Once this happened a further renewal of the same type of medium caused a dense precipitate to appear among the growing cells, inhibiting growth and usually resulting in loss of the cultures. In the meantime the central fragments have acquired, by reflected light, a whitish color due to the accumulation of bone salts, and could be heard to crunch when cut with a knife. Though the phenomenon described may have been an extreme example of rapid calcification in cell cultures, similar difficulties have plagued other investigators. Earle[1] reported trouble with opaque plasma (in this case in areas removed from the colony sites) during the cultivation of Walker's rat carcinoma in horse serum and chick embryo juice. Gey and Gey[2] reported unfavorably on the use of chick embryo juice with human cord serum and later stated[3] that the difficulty was associated with turbidity in the plasma. Parker[4] reports that "these precipitates have been encountered in many laboratories and in every sort of culture. In Fischer's laboratory, in Berlin-Dahlem, where there was a lot of it at one time, the general feeling developed that it was a calcium compound and related in some way to the Tyrode's solution that was being used."

Since chick embryos were the only source of tissue extract available at Culion, the factors causing calcification were studied and means developed for avoiding or minimizing these difficulties in the presence of mammalian sera.

Methods. Cultures were prepared as previously described,[5] except that 2% embryo juice was used to transfer the 1.5 mm explants of human skin, leproma, or divided tissue culture to the 13 mm tubes containing one drop of chicken plasma 50%. After adding 0.5 ml of liquid phase and equilibration under 30 mm (4%) CO_2, the tubes were incubated in a

* The greater part of the work was done in the Leonard Wood Memorial Laboratory, Culion, Philippines, 1941-43.

[1] Earle, W. R., *Am. J. Cancer*, 1931, **24**, 566.

[2] Gey, G. O., and Gey, M. K., *Am. J. Cancer*, 1936, **27**, 45.

[3] Gey, G. O., personal communication.

[4] Parker, R. C., personal communication.

[5] Hanks, J. H., *J. Cell. and Comp. Physiol.*, 1948, **31**, 235.

slanting position at 34°C. Supernatant fluids were replaced each 7 to 14 days, *i.e.*, as the pH fell from 7.5 to 7.0. The plasma was patched at these intervals as required. All cultures were examined each week and, until their shape became irregular as they almost filled the plasma, measured along two diameters by means of an ocular micrometer. For the present purpose additional notes were made on the pH and the turbidity of the media, the occurrence of precipitate in the liquid phase, and on the opacity of the explants and of the plasma.

The chicken plasma *(CH)* contained 0.2% sodium citrate. Chick embryos were pressed through a Latapie grinder and extracted with 2 ml balanced salt solution (lacking $NaHCO_3$) per gram of embryo. These suspensions were spun immediately in the SP angle centrifuge for 30 minutes at 3000 RPM. The supernatants, after having been respun in the same way, were remarkably clear except for an upper stratum of lipoid. When adjusted to neutrality and buffered with 0.125% $NaHCO_3$, the concentration of these extracts became 30% (EM_{30}). The salt solution, Sol. A, (otherwise as before)[5] contained 0.2 g per liter of $CaCl_2$ and 0.06 g each of $Na_2HPO_4 \cdot 2H_2O$ and of KH_2PO_4. After adding to the autoclaved salt solution 10% by volume of sterile 1.4% $NaHCO_3$, the concentration of bicarbonate became 0.127%, of Ca 6.5 mg%, and of inorganic P 2.2 mg%. A similar *Sol. N* (no Ca or P) and other modifications will be mentioned in the text.

In the early work placental serum (PLS) was collected as available and preserved by Chryochem dehydration. When reconstituted, 0.1% glucose and 0.002% phenol red were included and the serum further diluted to 50% in the balanced salt solution. It was sterilized by pressure filtration (5% CO_2) through a Seitz filter. The Seitz pads were found to contribute Mg ions and alkaline radicals, and this method was replaced by aseptic collection of serum. The composition of fluid media will be indicated in code, *e.g.*, $PLS_{25}EM_{15}$ referring to placental serum 25%, embryo juice 15%.

Chemical determination. Both calcium and inorganic phosphate were determined in trichloracetic acid filtrates. Calcium was determined by Tisdall's modification[6] of Kramer and Tisdall's method and phosphorus by Brigg's[7] modification of the Bell and Doisy method. Values for Ca and inorganic P in mg% will be written as exponents. Ca x P will designate the product of these values. For example, Ca x P of the salt solution: $Ca^{6.5} \times P^{2.2} = 14.3$.

Experimental results. After many early attempts to employ 15% chick embryo juice in the presence of 25% human serum, it became evident that several conditions influenced the calcification. These factors included: the age of the embryos employed, the proportion of embryo juice mixed with the serum, and different methods of preparing, storing, or treating the embryo juice.

Age of Embryos. Various batches of extract from pools of 10-day-incubated embryos differed remarkably in their ability to cause calcification. Since Philippine chickens hide their nests, overage eggs were unavoidable among the freshest obtainable. On one occasion marked calcification resulted from a single application of medium containing embryo juice from a pool of 5 embryos, one of which was perhaps 12 or 13 days actual age. From such observations it became apparent that calcification *in vitro* was related to the development of the calcifying mechanisms of the chick embryo. Data eventually collected on Ca and P values in relation to the age and weight of embryos (see Table I) revealed that the Ca x P of the embryo juice averaged 68.5 by the 11th day of incubation and reached approximately 100 by the 12th day. It will be noted that the typical Ca : P ratios at 11 days average 1:2.4 and *are inverted by comparison with mammalian ratios.*

Comparisons between extracts from 9- and 11-day embryos from the same lots of eggs revealed that the juice from the 11-day embryos (in $PLS_{25}EM_{15}$) usually induced calcification after second renewal of the medium while juice from the 9-day embryos permitted 3 or 4 renewals. 9-day extracts, however, were inferior to 11-day juices for growth stimulation and for continuous main-

[6] Tisdall, F. F., *J. Biol. Chem.*, 1923, **56**, 439.

[7] Briggs, A. P., *J. Biol. Chem.*, 1922, **53**, 13.

TABLE I.
Effect of Age of Pooled Embryos on the Ca and P Values of Fresh EM_{30}.

Pool No.	Incubation of eggs (days)	Avg wt* of embryos (g)	Ca (mg %)	P (mg %)	Ca x P	Ratio Ca : P
1	8	1.0	4.0	7.4	29.6	1: 1.9
2	10	1.5	4.1	10.2	41.8	1: 2.5
3†	11	1.9†	6.2†	8.7†	53.9†	1: 1.4†
4	11	2.3	5.4	12.3	66.4	1: 2.3
5	11	2.4	5.2	13.1	68.1	1: 2.5
6	11	2.6	5.5	12.9	71.0	1: 2.3
7	12	3.0	7.0	14.9	104.2	1: 2.1
Avg values for No. 4-6 (11-day Em)		2.4	5.4	12.8	68.5	1: 2.4

* These eggs were much smaller than American eggs.
† Unusual Ca and P values and not averaged with No. 4-6; the only 11-day EM studied which failed to cause calcification when used at 15% levels.

tenance of human fibroblasts. It was decided to continue the work with extracts prepared from 11-day embryos.

Varying proportions of serum and embryo juice. As may be seen in Table II, the use of varying proportions of 50% serum and 30% embryo juice in a series of experiments revealed that $PLS_{17}EM_{20}$ usually produced calcification after a second application (following one renewal): $PLS_{25}EM_{15}$ ordinarily permitted 3 applications; $PLS_{33}EM_{10}$ was tolerated for 4 or sometimes 5 applications, while $PLS_{42}EM_{5}$ could be employed during intervals up to 8 weeks. The latter medium was the only one which assured that long-term cultures would not be lost due to calcification during repeated transplantation. In it the explants or transplants sometimes turned opaque but the precipitate did not spread among the growing cells.

From the Ca and P data in the table it is apparent that the media with higher proportions of embryo juice contained P in excess of Ca. Furthermore, the higher Ca x P values occur where there is the least serum protein to stabilize these compounds in solution.[8] It was only when the embryo juice was reduced to 5% that Ca was appreciably in excess of P and the "potential" Ca x P could be expected to remain below 60.

Since P is liberated during incubation of the cultures, and both Ca and P are absorbed in the plasma and the explants, these potential values could not be determined without destruction and analysis of cultures being maintained for another purpose. Evidence that fresh embryo juice is saturated with respect to Ca and P and that active phosphatases liberate still more P during incubation will be presented in justification of the calculations employed.

Embryo juice as a solution saturated with Ca and P. Though the actual levels of Ca and P tended to vary in different lots of extract from 11-day embryos, these values were usually related inversely to each other and showed fairly good correlation between the Ca x P products and the average weights of the embryos employed. The relatively clear extracts were observed to become more opalescent or actually turbid during refrigeration. When incubated at 37° for a few days, they quickly passed through a cycle of opalescence, turbidity and sedimentation. In 9 extracts which were allowed to pass through this cycle for 3 to 5 days at 37° the average initial values of $Ca^{5.3}$ x $P^{12.6}$ = 66.8 had changed to $Ca^{4.2}$ x $P^{16.1}$ = 67.6 after incubation. These products indicate that the fresh embryo juice was saturated. The increase in P had been more than the indicated 28%, since some had been involved with the Ca precipitated.

Other evidence of saturation appeared upon trying to suspend fresh embryo pulp in salt solutions designed to lower or modify the levels of Ca and P. 4 batches of 11-day embryos were pulped without diluent. One

[8] Rosenheim, A. H., *Biochem. J.*, 1934, **28**, 699.

TABLE II.
Ca and P Values Upon Mixing Various Proportions of Placental Serum 50% with 11-day Chick Embryo Juice 30%, and Their Relation to Calcification of Tissue Cultures.

Formula of medium %	Proportion of each component	Ca and P contributed by each		C : P ratio	Initial Ca x P product of medium	Applications required to produce calcification	Potential Ca x P product of medium
		Ca mg %	P mg %				
PLS 50*	6/6	9.0	5.0	1: 0.6	(45.0)		
EM 30*	6/6	6.0	13.0	1: 2.2	(78.0)		
PLS 17	2/6	3.0	1.7				
EM 20	4/6	4.0	8.7	1: 1.5	72.8	2	79.3
		T 7.0	10.4				
PLS 25	3/6	4.5	2.5				
EM 15	3/6	3.0	6.5	1: 1.2	67.5	3	74.8
		T 7.5	9.0				
PLS 33	4/6	6.0	3.3				
EM 10	2/6	2.0	4.3	1: 1.0	60.8	4 or 5	67.5
		T 8.0	7.6				
PLS 42	5/6	7.5	4.2				
EM 5	1/6	1.0	2.2	1: 0.8	54.4	6 to 8	58.1
		T 8.5	6.4				

* Composition of ingredients used to prepare the medium.
Method of calculating initial Ca : P and Ca x P shown in detail.
T = Total Ca and P upon making mixtures. Potential Ca x P calculated in similar manner, using the Ca and P values of EM_{30} which has been incubated for one week, *i.e.*, $Ca^{4.6}$, $P^{16.9}$.

half of the pulp was suspended in the usual *Sol A* and the other half in *Sol N* (no Ca or P). The extracts were centrifuged immediately. The extracts in *Sol A* averaged $Ca^{5.3} \times P^{13} = 68.9$, while those in *Sol N* averaged $Ca^{4.1} \times P^{16.1} = 66$. In this brief interval the pulp had practically saturated *Sol N* with these elements, P exceeding Ca by approximately 4 times. When employed in serum media of the type illustrated in Table II, the extracts in *Sol N* produced more rapid calcification than those in *Sol A*. Calculation shows that this was to have been expected from the high Ca x P values produced upon mixing the high Ca of the serum and salt solutions with tissue extracts having P values around 16.

Correct Ca and P ratios (but high products) could be produced in fresh extracts by diluting the pulp in balanced salt solution containing elevated Ca and no P. Upon incubation, however, these extracts precipitated excessively. Calculation indicated that this modification was impractical, since each mg% of additional P liberated by phosphatases must now be multiplied by a higher initial level of Ca.

Since P is dialyzable, correct values and ratios for Ca and P should be obtained by dialysis of embryo juice. A fresh embryo extract containing $P^{10.7}$ was diluted with 2 volumes of balanced salt solution containing $Ca^{7}P^{0}$, to produce an anticipated value of $Ca^{6.5}$ and $P^{3.6}$. Upon being rapidly concentrated to the original volume in a pressure dialyzer modified after Simms,[9] the first half of the filtrate contained 3.5 mg% of P, the second half 3.6, and the residue in the bag 3.7 mg% of P. Though this procedure can produce embryo juice of any desired P value, it is not practical as a routine and also washes out small molecules which are important to nutrition.

Pasteurization to inactivate phosphatases. Inactivation of phosphatases by pasteurizing the embryo juice at 60° for 30 minutes, which causes some coagulation of protein, reduced the initial P values by 1-2 mg% and the Ca x P products by approximately 6 to 12. The P values did not rise on incubation of this juice alone and rose only slowly during the incubation with serum, presumably due

[9] Simms, H. S., and Stillman, N. P., *J. Gen. Physiol.*, 1937, III, **20**, 649.

to serum phosphatase. These extracts, when used in $PLS_{25}EMP_{15}$ induced good growth and permitted continuous maintenance of several series of cultures in which all of the control cultures in unheated embryo juice were lost by calcification. The pasteurization of embryo juice has previously been shown to inhibit the liberation of free lipoid and to promote greater longevity of chick embryo cultures than is possible with fresh embryo juice.[5]

Calcification studies in plasma blanks with 4 types of serum. Though growing cell colonies obviously play a role in phosphate liberation and CaP deposition near the central explants, blank tests in the plasma base with serum-embryo juice mixtures renewed at weekly intervals provided a convenient method for comparing different degrees of supersaturation, for example in systems containing serum from different animal species. These experiments were undertaken because Carrel had warned against the opacity which develops promptly if rabbit serum is employed in tissue cultures, because our attempts to substitute bovine for human serum had always increased calcification, and because the embryo juices referred to in this study had not been causing calcification in chick embryo tissue cultures.

The data in Table III summarize the observations on 3 batches of embryo juice in combination with 4 animal sera. Though the human and bovine sera gave comparable results in 2 of the trials, it is evident from the summarized scores, together with the experience in tissue cultures, that the 4 sera may be arranged as follows with respect to calcification: rabbit> bovine> human> chicken.

During these tests in the absence of cell colonies it became evident that a slowly increasing opalescence of the supernatant media containing mammalian sera provided the most favorable circumstance for calcification of the plasma layer, but that chicken serum media could become highly opalescent without inducing calcification. When incubated in plain glass tubes for several weeks, both rabbit and chicken serum media liberated more free lipoid (which appeared as a scum on the surface) than the bovine or human sera. The relation of free lipoid to lipoid accumulation in cells has been emphasized elsewhere.[5]

TABLE III.
Calcifying Tendency of Serum from 3 Mammalian Species and from Chickens, as Revealed in Plasma Blanks.

Embryo juice No.	Opacity in plasma following weekly renewal with serum 25% and embryo juice 15% (sera)			
	Rabbit	Bovine	Human	Chicken
63	034	012	012	000
67	044	023	023	000
70	144	023	002	001
Total scores*	222	58	37	1

* The 3 values for each serum represent the opacity rating on a plus scale at the end of the first, second and third weeks of the experiment. For example, 034 indicates no opacity after 1 week, 3+ opacity after 2 weeks and 4+ after 3 weeks. To illustrate the difference between the sera, these values are simply added as a total "score."

Preparation of media with acceptable Ca and P content. The data in Table IV provide a basis for estimating the total Ca and P content of media composed of human placental serum and chick or beef embryo juice. The ingredients differ from those used at Culion. The salt solution (prior to the addition of buffer) contains only 5 mg% of Ca, the approximate concentration of diffusible Ca in human serum. When mixed with any proportion of serum it provides ionized Ca levels similar to those in body fluids of corresponding protein content. Both chick and beef embryo juice should be prepared at 50% concentrations. Dilute extracts will tend to be saturated with Ca and P. If such extracts comprise a large portion of the final medium, it is impossible to dilute or modify their inorganic constituents to appropriate levels. When embryo juice is employed in concentrations greater than 5%, it is not necessary or desirable to incorporate P in the salt solution used for diluting the medium.

Since 10:1 ratios of serum and embryo juice seem to be optimal for good growth and quality of mammalian cells, the examples in the table are based on these ratios. With formulae such as $PLS_{60}EM_6$ the initial Ca x P may be nearly 55, while the "po-

TABLE IV.
Data for Estimation of Initial Ca and P Products of Media Which Combine Human Serum with Chick or Beef Embryo Juice.

	Placental serum			EM_{50}, chick (11-day embryos)			EM_{50}, beef* (4-inch embryos)			Balanced salt solution		
	%	Ca	P	%	Ca	P	%	Ca	P	%	Ca	P
1.	100	12.0	5.5	50	4.3	13.9	50	10.5	12.0	100	5.0	2.4
2.				15†	1.3	4.2	15†	3.2	3.6			
				10	0.9	2.8	10	2.1	2.4			
	60	7.2	3.3	6†	0.5	1.7	6†	1.3	1.4	28	1.4	0.7
	50	6.0	2.8	5	0.4	1.4	5	1.1	1.2	40	2.0	1.0
	40	4.8	2.2	4	0.3	1.1	4	0.8	1.0	52	2.6	1.3
	30	3.6	1.7	3	0.3	0.8	3	0.6	0.7	64	3.2	1.5
	20	2.4	1.1	2	0.2	0.6	2	0.4	0.5	76	3.8	1.8
3.	10	1.2	0.6	1	0.1	0.3	1	0.2	0.2	88	4.4	2.1

1. Ca and P of constituents employed.
2. These concentrations of embryo juice, though satisfactory with chicken serum, are not recommended in the presence of mammalian sera.
3. If using 10:1 ratios of serum and embryo juice simply add the values on appropriate horizontal line, using data for the embryo juice employed. Otherwise add the values for the actual concentrations being used.

Example

	%	Ca	P	Ca x P
PLS_{40}	40	4.8	2.2	
EM_4 (beef)	8	0.8	1.0	
Salt sol	52	2.6	1.3	
		8.2	x 4.5 =	36.9

* Data from Dr. Geo. O. Gey, extract prepared in salt solution with $Ca^{9.9}$ and $P^{3.6}$.

† Values take into account the fact that 50% extracts occupy twice the volume indicated by the percentage concentrations.

tential" Ca x P will exceed 60. The suitability of such formulae presumably depends on the fact that the increased concentration of serum protein raises the levels of Ca x P which can be kept in solution.[8]

Discussion. The calcification of tissue cultures in mammalian sera as here described should not be confused with the Liesegang rings of calcium salts which were observed in "aerobic" chick embryo cultures by Hueper and Russell[10] and others to whom they refer. Calcification does not occur in chick cell cultures at appropriate pH, even in high concentrations of embryo juice and in the presence of 40% oxygen. The phenomenon of Hueper and Russell[10] must be attributed to excessive alkalinization in cotton stoppered vessels.

Since mixtures of embryo juice and chicken serum may become highly opalescent without inducing calcification, the difference between chicken serum and the mammalian sera may be related in part to the unusual capacity of chicken serum to carry high Ca and P values.[11] Further indication of differences between chick and mammalian systems is afforded by the observations of Fell and Robinson[12] on the resistance of the chick femur to calcification.

The calcification of mammalian tissue cultures in the presence of mammalian sera under similar circumstances is explained primarily by the high P values contributed by embryo juice and by the lower levels of bone salt required for saturation, while the differences between the 3 mammalian sera are doubtless related to differences in the natural levels of total and diffusible calcium.[13]

It would appear that cultivated mammalian tissues may differ in their phosphatase activity *in vitro*. With rat tissues in the media here described the plasma tended to remain clear

[10] Hueper, W. C., and Russell, M. A., *Am. J. Med. Sciences*, 1933, **186**, 383.

[11] Greenberg, D. M., Lawson, C. E., Pearson, P. B., and Burmester, B. R., *Poultry Science*, 1936, **15**, 483.

[12] Fell, H. B., and Robinson, R., *Biochem. J.*, 1934, **28**, 2243.

[13] Schmidt, C. A., and Greenberg, D. M., *Physiol. Rev.*, 1935, **15**, 297.

in the region of the cell colonies and to become opaque elsewhere. Similar observations have been recorded by Earle.[1]

In the presence of human tissues the freshness and potential phosphatase activity of the embryo juice influenced the type of calcification. If embryo juice was mixed with undiluted serum during refrigeration, the precipitation cycle and liberation of P was inhibited. The media subsequently prepared possessed excellent growth stimulating properties and the cell colonies appeared to play a role in phosphatase activation, since calcification centered around the transplants and among the growing cells. If, on the other hand, media were prepared from incubated juices which had passed through the precipitation cycle, the growth promoting properties were greatly diminished and calcification occurred diffusely throughout the plasma as though resulting from ionic exchange and simple supersaturation.

The present suggestions concerning the desirability of keeping the Ca x P products below 60 and the serum and embryo juice ratios at 10:1 serve only as an approximation of permissible thresholds under average circumstances. The work of Robinson and coworkers[8] reveals the manner in which protein, glucose, magnesium and the serum from different animal species influences the *in vitro* calcification of cartilage. From these and other data on the solubility of Ca and P in body fluids,[14,15] it is apparent that the permissible Ca x P will be related to the concentration of serum protein in the medium.

Several precautions should be borne in mind during the preparation of embryo juices and of serum. These include: (1) the use of 10- or 11-day chick embryos and exclusion of any which appear over-age; (2) the selection of beef embryos which do not exceed 4 inches in length; (3) the preparation and storage of extracts at 50% concentrations so that the final dilution will permit modification of the Ca and P to desired levels; (4) the maintenance of the lowest practical temperatures during extraction and centrifugation; (5) storage of the extracts in a frozen state. The loss of protein during pasteurization of 50% juices is excessive. If this procedure is employed to inactivate phosphatases, the juice should be diluted with an equal volume of salt solution just prior to heating. Hemolysis of red cells during serum production must be expected to elevate the P values[7] while filter pads which liberate magnesium, alkali, or other inorganic radicals should be avoided.

When growth inhibition or plasma opacity are shown to be due to calcification (as evidenced by darkening of the central transplants, by data on the Ca and P values of the medium, or by solubility of the opacity in N/10 HCl) the interim steps which should be taken are: (a) rapid transplantation with exclusion of the central portion of the colony or (b) tryptic digestion and removal of the opaque plasma if the colonies are to be retained in the original vessels. Re-evaluation and correction of the inorganic composition of the medium is necessary to prevent recurrence of the difficulty.

Summary and conclusions. 1. Chick embryo juice, in concentrations suitable for chick cell cultures, induces calcification of the cell colonies and plasma in mammalian cell cultures.

2. Fresh embryo pulp and extract are saturated with Ca and P. Ca and particularly P increase with age of the embryos, their ratios being 1:2.4 at 11 days. Upon incubation the P increases an additional 30%.

3. Pasteurization of embryo extracts inactivates the phosphatases and prevents calcification in cell cultures receiving high concentrations of embryo juice.

4. Calcification of cultures in the presence of mammalian sera is due to combining the Ca of serum and salt solution with the high levels of P in embryo juice, to the further liberation of P during incubation, and to the limited Ca x P products held in solution by mammalian sera.

5. The calcifying propensity of chicken serum is much less than that of 3 mammalian sera, which may be arranged as follows: human<bovine<rabbit.

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[14] Peters, J. P., and Eiserson, L., *J. Biol. Chem.*, 1929, **84**, 155.

[15] McLean, F. C., and Hastings, A. B., *J. Biol. Chem.*, 1935, **108**, 285.