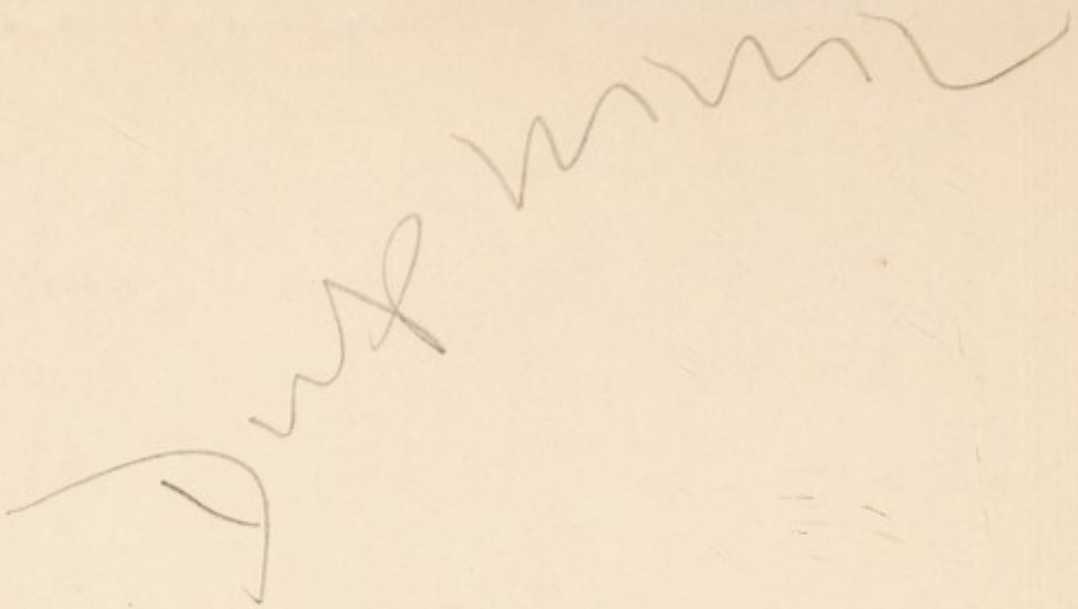
The significance of phosphoric esters in metabolism / by Robert Robinson.



Christian A. Herter Lectureship on
Pathological Chemistry
New York University

THE SIGNIFICANCE OF PHOSPHORIC ESTERS IN METABOLISM

THE HERTER LECTURESHIP

The late Dr. Christian A. Herter, former professor of pathological chemistry in the University and Bellevue Hospital Medical College, through the Council of New York University, provided a fund for an annual course of lectures upon some subject related to pathological chemistry.

The SIGNIFICANCE *of* PHOSPHORIC ESTERS *in* METABOLISM

By

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THE NEW YORK UNIVERSITY PRESS
WASHINGTON SQUARE EAST · NEW YORK CITY
LONDON · HUMPHREY MILFORD · OXFORD UNIVERSITY PRESS
1932

Printed in the United States of America
Waverly Press Inc.,
Baltimore, Maryland

FOREWORD

IT is a very pleasant duty to acknowledge how greatly I appreciated the invitation of the Herter Lecture Foundation to deliver these lectures in 1931, in the New York University and Bellevue Hospital Medical College.

To the Dean and Faculty of the College I owe cordial thanks for their hospitality and kindness which made my visit a very happy one. I am much indebted to them also, and particularly to Professor R. K. Cannan, for their encouragement to publish the lectures and for taking upon themselves much of the responsibility and labour involved.

My thanks are due to Professor A. Harden, F.R.S., Professor V. Korenchevsky, the Medical Research Council of England, and Professor O. Meyerhof, for kind permission to reproduce figures and illustrations from their publications, and to the Biochemical Society of England for the loan of blocks of figures and plates that have appeared in their *Journal*.

Finally, I would thank those whose collaboration and help I have enjoyed in my work. Especially, I would acknowledge my debt for the friendly interest shown through twenty years by Professor Harden, to whom I owe my first curiosity as to the significance of phosphoric esters in metabolism.

R. R.

London, July 8, 1932.

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THE SIGNIFICANCE OF PHOSPHORIC ESTERS IN METABOLISM

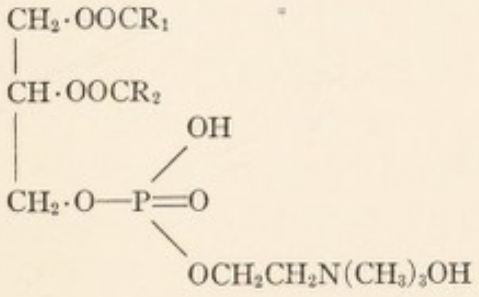
I

THE OCCURRENCE OF PHOSPHORIC ESTERS IN NATURE

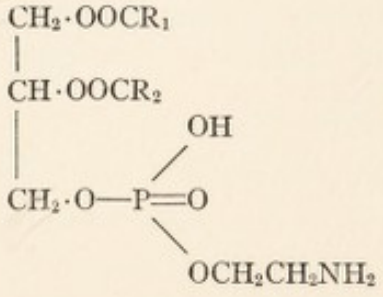
THE occurrence of phosphoric esters among the products of metabolism was recognised as early as 1850, when Gobley demonstrated the presence of glycerophosphoric acid in a substance which he had isolated from egg yolk (1846, 1847) and had named lecithin. He showed that this substance also contained fatty acids and a nitrogenous constituent which was later identified by Strecker (1868) as the base choline. Strecker held that the fatty acids are joined to the glycerol, and choline to the phosphoric acid, by ester linkages; and this view has become generally accepted.

Compounds of this type were discovered to be most widely distributed in animal tissues; but for many years the chemistry of this group was in great confusion, from which it emerged largely through the investigations of H. MacLean and of Levene and his colleagues. Besides lecithin itself a second compound, kephalin, occurs with it in all tissues. It contains the base aminoethyl alcohol in place of choline, but in other respects its structure is probably similar to that of lecithin although this is not yet beyond dispute.

Lecithin



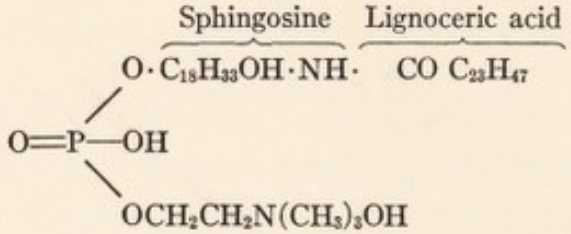
Kephalin



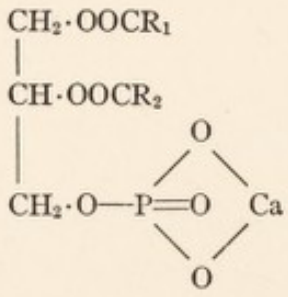
It is probable that various lecithins and kephalins exist, each containing one radicle of a saturated and one of an unsaturated

fatty acid and differing from one another only in the nature of these fatty acids. There is some evidence also for the existence of lecithins derived from β-glycerophosphoric acid in which the phosphoric radicle is attached to the central C atom of glycerol (Bailly, 1915).

Sphingomyelin, which occurs in brain, in red blood corpuscles, and many other tissues, also belongs to this class of ether-soluble compounds, the phospholipins or phosphatides. It contains no glycerol; but, according to Levene (1916), the phosphoric acid is joined by ester linkages with choline and with another hydroxy-base, sphingosine, while lignoceric or another fatty acid is attached to the sphingosine through the amino group. Levene's formula for sphingomyelin is here shown with the composition of sphingosine altered from C_{17} to C_{18} in accordance with the recent work of Klenk (1929).



The phospholipins of plant tissues have been less thoroughly investigated than those derived from animal sources, but there is evidence that both lecithins and kephalins are synthesised by plants (Levene and Rolf, 1925, 1926; Daubney and Smedley-MacLean, 1927). Chibnall and Channon (1927) have isolated from cabbage a phospholipin, which resembles lecithin in containing fatty acids joined to α-glycerophosphoric acid but contains no choline or other base. This ester, named phosphatidic acid, occurs in the form of its calcium salt which is soluble in ether.



A complex phospholipin obtained from tubercle bacilli has been investigated by Anderson (1927, 1929), with the collaboration, on the pathological side, of Sabin, Doan, and Forkner (1927–1930). It has been shown that the pathogenic activity of the tubercle bacillus is associated with this phospholipin and more particularly with one of the fatty acids, phthioic acid, obtained from it by hydrolysis.

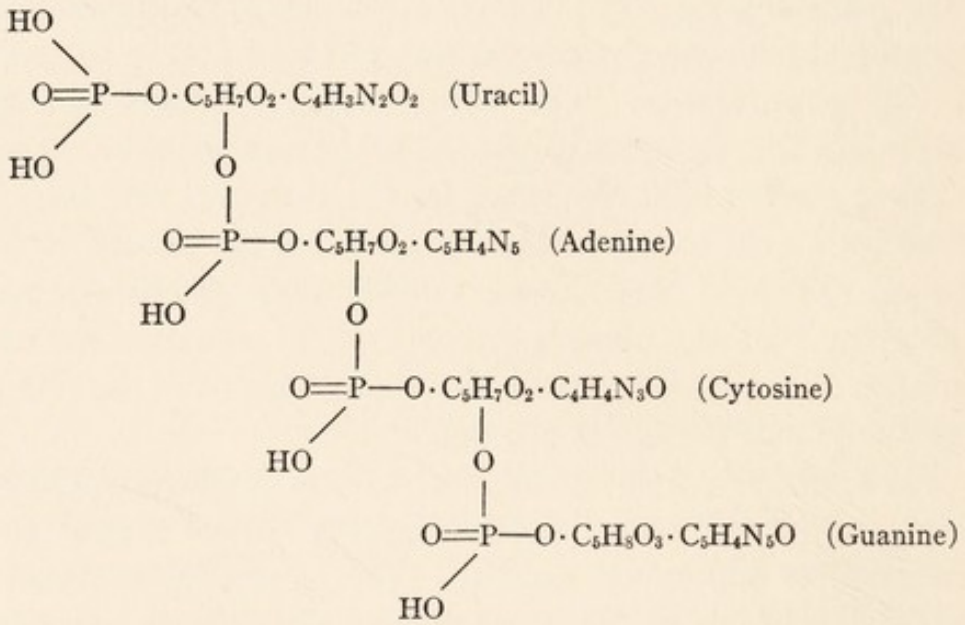
It is not surprising that the universal presence of phospholipins in living tissues, the ease with which they undergo oxidation, their curious behaviour in contact with water and other interesting properties should have given rise to much speculation as to their function in the organism. It is held that they play important rôles in the transport of the fatty acids and in their oxidation in the animal body, in the coagulation of blood and in the control of cell permeability. The experimental evidence for these beliefs is in the aggregate considerable but is in no case conclusive.

These esters might also serve for the transport and storage of phosphoric acid in a non-ionised form readily accessible to all tissues. Plimmer and Scott (1909) have published results indicating that the phosphate required for calcification of the skeleton in the fowl embryo is derived largely from the phospholipins originally present in the egg.

The hydrolysis of phospholipins by tissue enzymes would probably repay further investigation having special regard to the liberation of individual radicles. We have evidence that the partial hydrolysis of these esters may give rise to compounds which are active in the body. Thus, Delezenne and Fourneau (1914) and Levene and Rolf (1923) have shown that cobra venom removes the unsaturated fatty acids from lecithin and kephalin forming the strongly haemolytic lysolipins.

The discovery of another type of phosphoric esters synthesised by the living organism originated with the work of Miescher, who, in 1868, obtained nucleoprotein from pus cells and, a few years later, nucleic acid from the heads of salmon spermatozoa. Kossel's (1881) investigations of the nucleic acids prepared from

the thymus gland and from yeast gave the first insight into their chemical constitution; and our knowledge has been extended by later workers among whom Levene and Walter Jones must be specially mentioned. It is now generally accepted that nucleic acids from other sources are identical with one or other of the two compounds derived from thymus and from yeast. Each nucleic acid is built up of four different nucleotides, but the manner in which these are linked together has not yet been definitely settled. Levene and Bass (1931) give the following formula as representing the structure of yeast nucleic acid, the four nucleotides being each composed of a pentose, d-ribose, joined to a purine or pyrimidine base and to phosphoric acid:



In animal nucleic acid, thymine takes the place of uracil while the sugar is a reduced pentose, d-2-desoxyribose. Until recently this sugar was thought to be a hexose, and its identification was accomplished only within the last year by Levene and his colleagues (Levene, Mikeska, and Mori, 1930).

Certain individual mononucleotides also occur in animal tissues; for example, adenylic acid has been found in the red corpuscles, in muscle (in combination with pyrophosphate), and in the kidney. These animal nucleotides contain, however, d-ribose, the sugar of yeast nucleic acid.

The hydrolysis of nucleic acids by various tissue juices and extracts has been studied in considerable detail; and a number of different enzymes have been named as responsible for the scission of the various linkages. Much of the work that has been done on the rôle of these compounds has been concerned with the metabolism of the purines, and lies outside the scope of these lectures.

What part the phosphoric acid group may play in the synthesis or the degradation of the purine or of the pentose fraction of the molecule is as yet unknown; but, in the light of other knowledge that we possess, the synthesis of these pentose-phosphoric esters by the cell remains of great interest.

The phosphoproteins, whose constitution has still to be unravelled, form another group of phosphoric esters occurring in the milk of mammals, the eggs of birds, and the roe of fishes. They are thus synthesised specifically for the nutrition of the young animal (Rimington and Kay, 1926).

Phytin, which is present in the seeds of many plants, is probably a hexaphosphoric ester of the cyclic alcohol, inositol, which occurs also in animal tissues. It provides a rich reserve of phosphate for the needs of the young developing plant.

Although the physiological significance of the different esters so far enumerated has been the subject of much experimental study, we have little information regarding the part played by the phosphoric acid radicle in the phenomena with which they are concerned. Does this esterified phosphoric acid serve merely as a phosphate reserve, or does it fill some more specific rôle? The first definite evidence of such a specific rôle of phosphate was supplied by Harden and Young's investigations of the alcoholic fermentation of sugar by yeast juice.

In the closing years of the last century, Eduard and Hans Buchner, with their assistant Martin Hahn, succeeded, where many previous investigators had failed, in preparing from yeast a cell-free juice which possessed the power to ferment sugar. The method they employed was to grind the yeast with sand and

kieselguhr, and, after the addition of more kieselguhr, to press out the mass in a hydraulic press. It was shown that this juice was capable of fermenting various sugars with production of carbon dioxide and alcohol as in fermentation by living yeast. This power was not destroyed by filtering the juice through a Berkefeld filter or by addition of chloroform, benzene, or sodium arsenite. From these facts Eduard Buchner concluded "that the production of alcoholic fermentation does not require so complicated an apparatus as the yeast cell and that the fermentative power of yeast juice is due to the presence of a dissolved substance" to which he gave the name zymase.

It is recorded by Harden (1931) that his own investigations of alcoholic fermentation began in 1903 as a result of attempts by Macfadyen to prepare an antizymase by injecting Buchner's yeast juice, into animals. As a preliminary to these experiments, Harden examined the effect of normal serum on the fermentation by yeast juice, and found that both the rate of fermentation and the total amount of sugar fermented were greatly increased. In further experiments it was found that boiled autolysed yeast juice exerted a similar favourable influence, and this effect was ultimately traced to two independent factors: (a) the inorganic phosphates in the juice; (b) the presence in boiled juice of a thermostable and dialysable co-enzyme, indispensable for fermentation. For this co-enzyme the name co-zymase was later suggested by von Euler.

The favourable effect of sodium phosphate on fermentation by yeast juice had been observed by Wroblewski (1901) and by Buchner (1903), but had been ascribed to the alkalinity of the solution. Harden and his colleague Young proved that it was in fact a specific effect of the phosphate. They showed that the addition of sodium or potassium phosphate to a fermenting mixture of glucose, fructose, or mannose with yeast juice causes the rate of fermentation to rise rapidly to a value which may be as high as twenty times the original rate (Fig. 1, curve 1). After a time the rate falls again to a value approximately the same as,

but usually slightly higher than, the original rate (Fig. 1, curve 2). If, at this point, a further quantity of phosphate is added, the rate again rises and falls; and this cycle can be repeated a number of times until the fermentative activity of the juice becomes diminished through dilution and other causes. For each preparation of juice there is an optimum concentration of inorganic phosphate giving the maximum rate of fermentation. If phosphate in excess of this is added at one time, the rate will

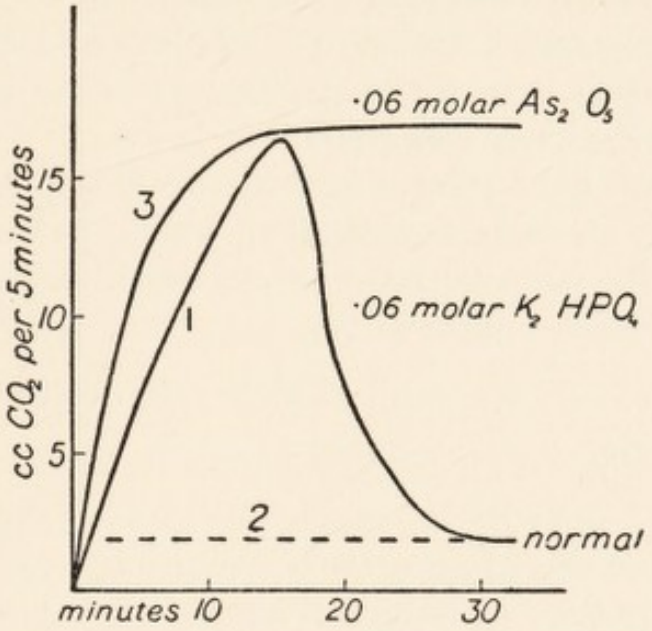


Fig. 1. Fermentation of yeast-juice. Reproduced from Harden's Nobel Lecture, Stockholm, 1929.

rise more slowly to a lower maximum or, if enough phosphate is added, fermentation may be entirely stopped.

Harden and Young measured the evolution of carbon dioxide throughout the whole course of the fermentation, and discovered that, during the period of increased rate after each addition of phosphate, the amount of carbon dioxide in excess of that which would have been evolved during the same period at the normal rate is equivalent, molecule for molecule, to the phosphate added (Harden and Young, 1905, 1906). They also showed that the production of alcohol is increased in similar degree, the

excess of alcohol over that produced at the normal rate being also equivalent to the amount of added phosphate.

On examining the fermentation liquid when the rate had again fallen to the normal level, they found that almost the whole of the inorganic phosphate had disappeared; and from this solution Young (1907, 1909) isolated a compound which he showed to be a hexosediphosphoric ester, the hexose being probably fructose. He showed, moreover, that the same compound was obtained whether the sugar fermented was glucose, fructose, or mannose.

When fermentation slackens through lack of sugar, inorganic phosphate again increases at the expense of the hexosediphosphate which gradually disappears.

From these facts Harden and Young (1908) deduced their equations of alcoholic fermentation, which, however, take no account of the intermediate reactions which undoubtedly form part of this complex phenomenon:

1. $2C_6H_{12}O_6 + 2R_2HPO_4 = 2CO_2 + 2C_2H_5OH + 2H_2O + C_6H_{10}O_4(PO_4R_2)_2$

2. $C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4$

Equation (1) shows the changes taking place during the stage of rapid fermentation in presence of inorganic phosphate. Equation (2) shows the hydrolysis of the hexosediphosphate which takes place simultaneously and is due to a phosphatase present in the juice.

As soon as all the phosphate has been esterified, it is this hydrolysis which, in their view, controls the rate at which inorganic phosphate is again liberated to take part with a further quantity of sugar in a fresh cycle of changes, and it is this hydrolysis, therefore, which controls the normal steady rate of fermentation. Both yeast and yeast juice contain phosphate; and the further experiments of Harden and Young (1910) supplied strong evidence that this phosphate is essential for fermentation.

The normal rate of fermentation by yeast juice is very much lower than that given by the equivalent quantity of fresh yeast;

but the rate of fermentation with fresh yeast is scarcely affected by the addition of phosphate, and with the optimum amount of phosphate the maximum rates are of the same order of magnitude in both cases. It was concluded, therefore, that living yeast possesses high phosphatase activity so that in the cell the reaction represented by equation (2) proceeds as rapidly as the series of reactions included in equation (1). A great part of this phosphatase activity must disappear during the grinding and pressing of the yeast, either through loss of phosphatase itself or through the destruction of some activator.

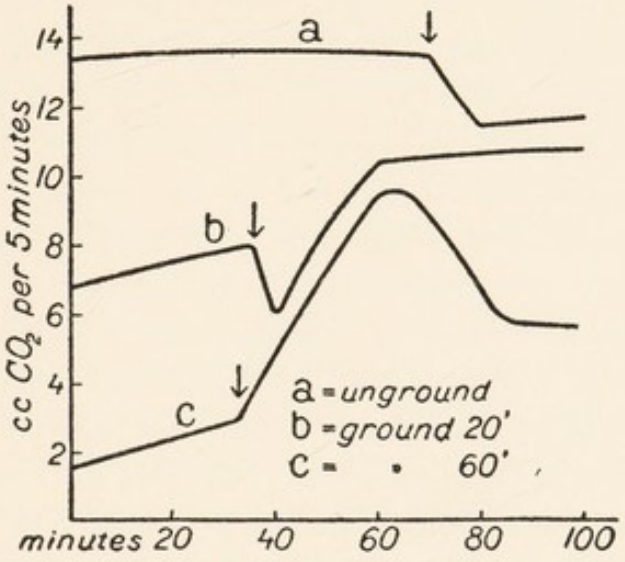


Fig. 2. Fermentation of yeast-sand mixture. At ↓ an addition of phosphate was made. (Harden and Macfarlane, 1930).

Harden and Macfarlane (1930) have recently obtained confirmation of this by grinding yeast with sand for different periods of time and examining its fermentative power and response to phosphate. The phosphatase activity was reduced by 60 minutes grinding to 15% of its original value while the zymase activity was not greatly affected (see Fig. 2).

Harden and Young (1911) found that the addition of arsenate brought about a similar increase in the rate of fermentation by yeast juice (Fig. 1, Curve 3); but in this case there was no subsequent fall from the high rate, nor did synthesis of an arsenic

ester occur. They concluded that the effect of arsenate was to stimulate the phosphatase and thus increase the rate of hydrolysis of the hexosediphosphate, so that the whole cycle of changes shown in equations (1) and (2) is passed through more rapidly. According to Meyerhof (1927), however, the hexosediphosphate undergoes two types of change, one a simple hydrolysis and the other a fermentation, and only the latter is stimulated by arsenate. This view is supported also by recent work of Macfarlane (1930, 1931).

The formation of hexosediphosphoric ester was independently observed by Ivanov (1907, 1909), who, however, considered it to be a triosemonophosphate. The ester was subsequently obtained by Lebedev (1909, 1910) and by Euler and Fodor (1911). The experiments of these and other workers have confirmed and extended the conclusions arrived at by Harden and Young. Many different types of yeast have been used in these investigations and various preparations of these, in addition to Buchner's juice, e.g., acetone yeast or zymin, yeast dried in air at 37°, maceration juice prepared by digesting dried yeast with warm water, and, finally, fresh yeast in presence of toluene.

Some differences are observed in the behaviour of these various preparations and may be attributed to the different proportions in which zymase, co-zymase, phosphatase, and possibly other enzymes are present. Their behaviour is also affected to some extent by the varying amounts of inorganic phosphate and glycogen which they contain.

In 1914 Professor Harden and I found that hexosediphosphate was not the only ester formed during fermentation of sugars by yeast juice. We obtained a second compound, which, though somewhat impure, appeared to be a hexosemonophosphate; similar products were obtained from the fermentation of both glucose and fructose. Work on this substance was interrupted by the war; but in 1922 I was able to describe the isolation of the ester in analytically pure condition and to give an account of its properties. It differed from hexosediphosphoric acid in the

much greater solubility of its barium, calcium, and lead salts, in its greater reducing power to Bertrand's solution, and in its higher dextro-rotatory power. On acid hydrolysis it gave a dextro-rotatory syrup which appeared to contain both glucose and fructose. I formed the opinion that this compound was a mixture of isomeric hexosemonophosphoric esters, probably those of glucose and of fructose; and this conclusion was supported by later experiments of Meyerhof and Lohmann (1927) who employed the iodimetric reduction method of Willstätter and Schudel (1918) to estimate the percentage of the aldose component.

Another hexosemonophosphate was prepared by Neuberg in 1918 by partial acid hydrolysis of hexosediphosphate, one of whose phosphoric acid groups is much more readily split off than is the other. The properties of this ester differentiated it sharply from the hexosemonophosphate isolated from fermentation products.

Meyerhof and Lohman (1927) concluded that both these esters and also hexosediphosphate are mixtures of aldose and ketose derivatives; but whereas the ketose component largely preponderates in hexosediphosphate and in the Neuberg monophosphate, the aldose component preponderates in the Robison ester.

From the results obtained with highly purified specimens of hexosediphosphate and Neuberg hexosemonophosphate by a somewhat different iodimetric method (Macleod and Robison, 1929), I am inclined to doubt whether these compounds contain any aldose derivatives. The reduction values are, it is true, slightly higher than those for the equivalent amount of pure fructose; but it is quite possible that the esters are more easily oxidised than the free sugar. The iodimetric values obtained for the Robison ester indicated the presence of aldosemonophosphate to the extent of about 60%. Lohmann (1928) was able to effect a partial separation of this aldosemonophosphate by a method of fractional hydrolysis with boiling hydrochloric acid, the ketose

ester being more rapidly hydrolysed than the other. Dr. King and I also made use of this method, but again with only partial success. We have been more successful in our attempts to separate the components by recrystallisation of the brucine salts from ethyl and methyl alcohols. Two years ago we obtained in this way an ester containing over 90% of an aldosemonophosphate estimated iodimetrically (Robison and King, 1929).

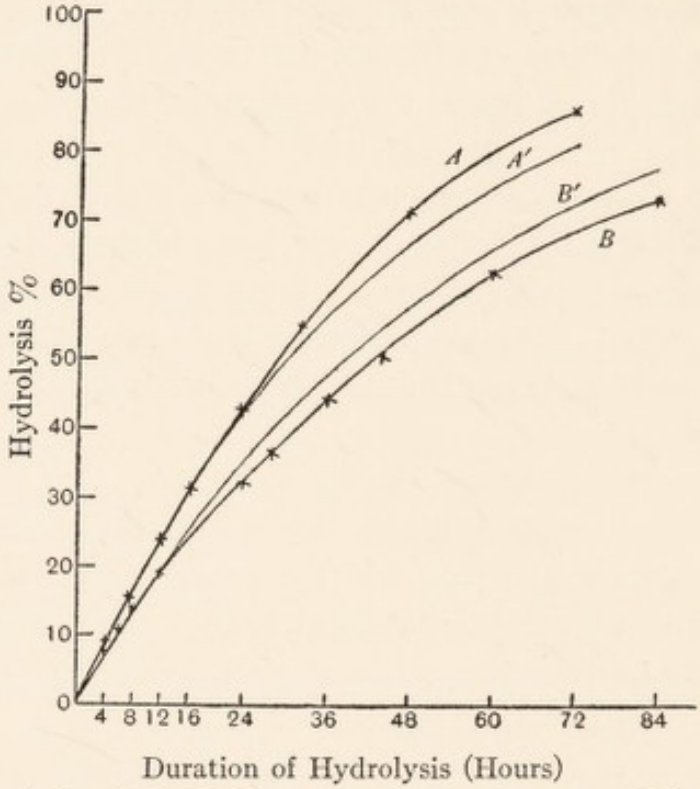


Fig. 3. *A*. Autolysis of 0·03 *M* aldosemonophosphoric ester at 100°. *A'*. $k = 0.167 \times 10^{-3}$. *B*. Hydrolysis of 0.03 *M* aldosemonophosphoric ester in *N* H_2SO_4 at 100°. *B'*. $k = 0.13 \times 10^{-3}$.

The fractionation of the brucine salt was continued; and during the last year we have isolated the aldosemonophosphoric ester in pure condition (Robison and King, 1931).

From the mother liquors we obtained a ketose ester very similar in its properties to the Neuberg hexosemonophosphate, which is, indeed, from its relationship to hexosediphosphate, most likely to be present in the fermentation products. We also obtained strong evidence of the presence of one or more other

esters not yet identified; but we cannot as yet say whether they form any considerable proportion of the original hexosemonophosphate.*

It seems probable, however, that the principal component of this ester is the aldosemonophosphate which has now been isolated. This ester is very resistant to acid hydrolysis; but it is remarkable that hydrolysis proceeds more rapidly when the free aldosemonophosphoric acid is heated in aqueous solution alone than in the presence of *N* sulphuric acid (Fig. 3). The only explanation I can suggest for this is that the rate of hydrolysis may be affected by the position of the oxygen bridge in the hexose molecule and this in turn may be influenced by the acidity of the solution.

The sugar produced by acid hydrolysis was identified as d-glucose. Further evidence that the ester is a glucosemonophosphate was obtained by converting it by oxidation with bromine into a phosphohexonic acid, which, by treatment with bone phosphatase, gave d-gluconic acid.

	$(H_2PO_4)C_5H_{10}O_4CHO$	phosphoglucose.
bromine oxidation	↓	
	$(H_2PO_4)C_5H_{10}O_4COOH$	phosphogluconic acid.
bone phosphatase	↓	
	$C_5H_{11}O_5COOH$	d-gluconic acid.

The osazone prepared from this pure ester was identical with that obtained by Young from hexosediphosphoric acid.

A more precise knowledge of the constitution of hexosediphosphoric and hexosemonophosphoric esters is of importance if we are to understand what part these esters may play in the degradation of the sugar molecule. It seemed probable from the work of Young that hexosediphosphate is an ester of fructose, although Young himself drew no definite conclusion on this point, since the sugar he obtained by acid hydrolysis had a lower specific rotation than that of fructose. Pure fructose was later obtained by

* One of these esters has since been isolated and shown to be a derivative of mannose, probably mannose-6-phosphate: it yields a crystalline, sparingly soluble phenylhydrazone (Robison, 1932).

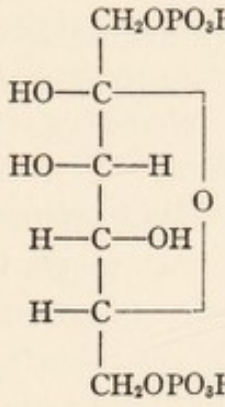
Neuberg and his co-workers (1917), who considered it the only sugar present in the ester. From the fact that this ester yielded a phenylhydrazone in which both phosphoric acid groups were still present while in the formation of the osazone one of these groups was split off, Young (1911) concluded that this group was attached to the C atom adjacent to the carbonyl group. The position of the second phosphoric acid group was still undecided when, in 1927, Morgan prepared the methylhexosidediphosphoric acids and separated the α and β isomers by fractional crystallisation of the brucine salts. He further prepared a fully methylated derivative hoping to oxidise this and identify the product; but this step proved unexpectedly difficult of accomplishment. By the use of the bone phosphatase, however, it was found possible to remove both phosphoric groups from the isomeric methylhexosidediphosphates without disturbing the methoxy group (Morgan and Robison, 1927, 1928). The properties of the methylhexosides obtained in this way and their behaviour on acid hydrolysis proved them to be derivatives of the reactive butylene oxide or γ form of fructose, in Haworth's nomenclature, fructofuranose. This was confirmed by further methylation of the methylhexosides, tetramethylfructofuranose being finally obtained.*

Subsequently Morgan (1929) showed that the same methylhexosidediphosphoric acids are obtained whether the methylation is carried out at room temperature or at 60°, the latter condition being favourable to the formation of methylfructopyranose, containing the stable 1:5 or amylene oxide ring. Dr. Morgan and I concluded from these facts that the second phosphoric acid group is probably attached to the terminal C atom, thereby preventing the formation of the stable 1:5 oxide ring. A similar conclusion was arrived at by Levene and Raymond (1928) also from the results of methylation experiments. If this is correct,

* The Neuberg ester, obtained by acid hydrolysis of hexosediphosphate, is probably 6-phosphofructofuranose. Macleod and Robison (1932, in the press) have obtained, though not yet in pure condition, another hexosemonophosphate by partial hydrolysis of the diphosphate with bone phosphatase. The properties of this ester, which is strongly laevo-rotatory and very rapidly hydrolysed by acids, suggest that it may be 1-phosphofructopyranose.

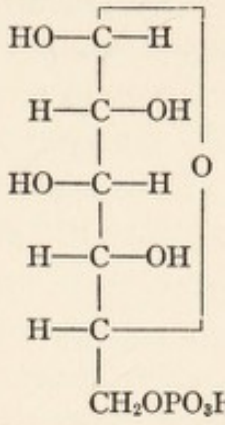
hexosediphosphoric ester is a derivative of γ-fructose, namely, 1:6-diphosphofructofuranose.

Hexosediphosphoric ester.



γ-fructose-1:6-diphosphate
1:6-diphosphofructofuranose

Aldose component of
Hexosemonophosphoric ester.



glucose-6-phosphate
6-phosphoglucopyranose

The methylhexosides of the glucosemonophosphate isolated from the Robison ester have been investigated by King, Morgan, and McLaughlin (1929, 1931), who in this case obtained two different products according to the temperature at which the methylation was carried out. The properties of these methylated products and of the methylhexosides obtained from them by removal of the phosphoric group with bone phosphatase showed them to be derivatives of the reactive and stable forms of glucose respectively. It was, therefore, judged that the phosphoric acid group in glucosemonophosphoric ester cannot be in either position 4 or 5. Position 2 is excluded by the formation of a phospho-osazone; while the identity of this osazone with that obtained from fructosediphosphate indicated that the phosphoric acid group is attached to the same C atom, No. 6, in both esters. This conclusion is supported by the fact that Dr. King and I (1931) were unable to obtain a phosphosaccharic acid by vigorous oxidation of the ester.

A synthetic glucose-3-phosphate has recently been prepared by Josephson and Proffe (1930) and by Levene and Raymond (1930), and proved to be quite different in properties from the ester of fermentation. All the evidence so far considered, therefore, goes to show that the aldose component of hexosemono-

phosphate of fermentation is glucose-6-phosphate, or, in Haworth's terminology, 6-phosphoglucopyranose.

Levene and Raymond (1930) have, however, synthesised another ester which they consider to be, very probably, glucose-6-phosphate. This resembles the Robison ester in many respects but differs from the pure aldose component in its specific rotation. Further information as to this synthetic ester must, therefore, be awaited before the constitution of fermentation glucosemonophosphate can be considered as finally settled.*

In the course of these investigations large amounts of the phosphoric esters were prepared; and it was found that while yeast juice as a rule gave the best yield of the hexosemonophosphate, which might form up to 80 or 90% of the total esters, dried yeast on the other hand gave a much larger proportion of the diphosphate. In working up the residual fractions of soluble barium salts from the dried yeast preparations, Dr. Morgan and I discovered the presence of a new ester with a very high specific rotation. After laborious fractionations the compound was finally isolated in pure condition through its crystalline brucine salt, and proved to be the monophosphoric ester of a non-reducing disaccharide (Robison and Morgan, 1928). From its very high dextro-rotatory power, +185°, it was suspected that the ester might be derived from trehalose, a non-reducing glucose glucoside found in trehala manna which is secreted by the insect *Larinus maculatus*. Trehalose has been found also in certain fungi and in dried yeast from which it was isolated by Koch and Koch (1925). We submitted a small portion of the new ester to the action of bone phosphatase by which the phosphoric acid group was rapidly removed. The sugar product was non-reducing, showing that the glucoside linkage had not been affected; and from the solution of this syrupy product in warm alcohol, pure trehalose was obtained in lustrous rhombic prisms.

Trehalosemonophosphoric acid is only very slowly hydrolysed

* In a subsequent communication Levene and Raymond (1931) state that by further purification of their synthetic ester its specific rotation has been raised to a value very close to that of the fermentation glucosemonophosphate.

by normal sulphuric acid at 100°. The rupture of the phosphoric ester linkage (b) takes place much more slowly than that of the glucoside linkage (a), so that glucosemonophosphoric acid accumulates during the first twenty hours of hydrolysis. This ester has been isolated and appears to be the same as the aldose component of hexosemonophosphate; but the identity is not yet definitely established.

Hydrolysis of trehalosemonophosphoric ester.

$C_6H_{11}O_5$——O——$C_6H_{10}O_4$——O——PO_3H_2

a b

(acid)	Glucose	:	Glucosemonophosphoric ester.		
	Glucose	:	Glucose	:	Phosphoric acid.
(bone phosphatase)	Trehalose			:	Phosphoric acid.

Trehalosemonophosphoric ester has not so far been found in the products of yeast juice. It is found in the products of fermentation of fructose and of glucose by dried yeast and also by zymin, and has in some cases amounted to over 50% of the monophosphate fraction and 12% of the total esterified phosphorus. It appears to be formed chiefly towards the end of the fermentation (Robison and Morgan, 1930; Boyland, 1929).

It was shown that the amount of trehalosemonophosphoric ester in the fermentation products could not have been present in the dried yeast itself, nor could it have been derived to any great extent from preformed trehalose. It must, therefore, have been synthesised during the course of the fermentation. Whether its formation plays any part in the fermentation process is not known. It may be that esterification with phosphoric acid facilitates the linking up of two hexose molecules and thus plays a part in the building up of the complex reserve polysaccharides from the simple sugars. It would be premature to do more than indicate this as an interesting possibility; but it may be noted that Neuberg and Leibowitz (1928) have reported the formation of another disaccharidemonophosphate by the action of *B. delbrücki* on hexosediphosphate.

Some properties of the phosphoric esters of fermentation are shown in the following table.

	$[\alpha]^{20°}_{5461}$		Reducing Power Calculated as Percentage of Hexose in Molecule.	
	Free Acid.	Ba. Salt.	Hagedorn and Jensen.	Iodimetric.
Fructosediphosphate.....	+3.4°	+3.5°	40	7
Hexosemonophosphate.....	+29.5°	+14.4°	66	55
Glucosemonophosphate.....	+41.4°	+21.2°	78	100
Fructosemonophosphate.....	+1.5°	+0.7°	78	4
Trehalosemonophosphate....	+185°	+132°	0	0

Hexosemonophosphates differing in properties from the above have been described by Euler, Myrbäck, and Runehjelm (1928), by Nilsson (1930), and by I. Neuberg and Ostendorf (1930). These esters have not, however, been obtained in pure condition, and for the present no opinion as to their nature can be formed.

As I have already indicated, these esters are found in the fermentation products in extremely varying proportions which depend chiefly on the character of the yeast preparation, to some extent on the conditions of fermentation, and possibly on the nature of the sugar fermented. While these proportions seem to be not entirely independent of the rate at which fermentation, i.e. production of carbon dioxide and alcohol, takes place, the connection is not easy to trace. What then is the significance of these esters in the fermentation process? It is clear that the production of a large proportion of monophosphoric esters renders necessary some modification in Harden and Young's first equation of alcoholic fermentation.

Equivalence between the carbon dioxide formed and the phosphorus esterified, originally demonstrated by Harden and Young, was confirmed at a later date by Euler and Johanssen (1913) who also showed that, when equivalent quantities of sugar and phosphate were taken, all the sugar had disappeared when all the phosphate had become esterified. The question has recently been submitted to renewed and critical investigation by Harden and Henley (1927, 1929), who found that, while the ratios of carbon dioxide to hexosediphosphate and to hexosemonophos-

phate taken separately may vary within wide limits, the ratio of carbon dioxide to total phosphorus esterified is always very close to unity. The extreme values found by them were 0.85 and 1.09; but somewhat lower ratios (0.6–0.7) have been found by Kluyver and Struyk (1928).

Any scheme that is formulated to express the reactions occurring in alcoholic fermentation must, therefore, take account of this approximate, or possibly complete, chemical equivalence between the molecules of phosphoric acid esterified and those of carbon dioxide and alcohol formed.

All the phosphoric esters that have been isolated from the fermentation products are themselves fermented by yeast juice, dried yeast, and zymin; but whereas hexosediphosphate is fermented at the relatively slow basal rate, the monophosphate is fermented very rapidly, the initial rate being equal to that given by fructose in presence of phosphate. The rate, however, falls off when only a small fraction of the ester has been converted into carbon dioxide and alcohol. Unpublished results of further experiments by Dr. Harden and myself show that the presence of inorganic phosphate is an essential condition for this rapid fermentation of the monophosphate and that the Neuberg and Robison esters behave similarly in this respect. I have suggested that hexosemonophosphoric ester may be an intermediate stage in the formation of hexosediphosphate (Robison, 1922); and a similar notion has entered into several schemes of fermentation put forward within the last few years. The central idea of all these schemes is that the entrance of the phosphoric acid group renders the hexose molecule labile. Kluyver and Struyk (1926, 1927, 1928) suggest that glucose and fructose form different monophosphoric esters, which are derivatives of the reactive forms of the respective sugars. These hexosemonophosphates then break up into triosemonophosphate and glyceraldehyde, or dihydroxyacetone respectively. Two molecules of the triosemonophosphate join together to form hexosediphosphate, while the glyceraldehyde or dihydroxyacetone is fermented to carbon

dioxide and alcohol. These authors have adopted Meyerhof's view, that the Robison ester is a stabilised form of the primary, reaction product, the "active" hexosemonophosphate.

In Euler and Myrbäck's (1928) scheme, which is here shown, less specific assumptions are made as to the position of the phosphate group or the nature of the 3-carbon atom residue:

$$C_6H_{12}O_6 + PO_4HR_2 = C_3H_6O_3 + C_3H_5O_2\ PO_4R_2 + H_2O$$

$$2C_3H_5O_2PO_4R_2 = C_6H_{10}O_4(PO_4R_2)_2$$

Two molecules of hexosemonophosphate undergo mutation with formation of one molecule of hexosediphosphate and compounds containing three carbon atoms. It is suggested that a triosemonophosphate is formed as an intermediate product, and that pyruvic acid may be one of the 3-carbon compounds, being then fermented in accordance with the scheme of Neuberg and Kerb (1913).

Meyerhof's (1926, 1930) theory supposes that an active form of hexosemonophosphoric ester not identical with either the Robison or Neuberg esters is the first product (equation A) and this undergoes further change in three different ways. One part passes directly into the Robison ester without fermentation, another portion may be converted into diphosphate by simple esterification, while the remainder is fermented at high rate in a coupled reaction (equation B), one molecule being broken down to carbon dioxide and alcohol while its phosphoric acid group converts a second molecule into hexosediphosphate. In the second phase of slow fermentation the latter also undergoes two different types of change, one a direct fermentation requiring very little coenzyme, and the second a simple hydrolysis to hexose and phosphoric acid. Only the first reaction (α) is stimulated by arsenate.

Phase 1.

Phosphate period. Rapid Fermentation. High concentration of coenzyme necessary.

A. $2C_6H_{12}O_6 + 2R_2HPO_4 = 2C_6H_{11}O_5PO_4R_2(\text{active}) + 2H_2O$

B. $2C_6H_{11}O_5PO_4R_2(\text{active}) + 2H_2O = 2CO_2 + 2C_2H_5OH + C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O$

Phase 2.

Ester period. Slow fermentation. Low concentration of coenzyme sufficient.

α. $C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = 2CO_2 + 2C_2H_5OH + 2R_2HPO_4$

β. $C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4$

The schemes of Kluyver and Struyk, of Euler and Myrbäck, and of Meyerhof all fail to account for the fact that the carbon dioxide produced is proportional, not to the hexosediphosphate or to any other single ester but to the total number of molecules of phosphoric acid esterified. According to these schemes the production of the stable monophosphate (Robison ester) is not accompanied by any corresponding evolution of carbon dioxide, whereas in fact, this monophosphate may amount to 80% or 90% of the total phosphoric esters while the ratio of carbon dioxide produced to phosphate esterified remains close to unity.

Harden, in his Nobel lecture (1929), has emphasised this radical objection to all these theories in which some one hexosephosphate is assumed to possess an intermediate character. He considers rather that "a coupled reaction of some kind occurs, as the result of which the introduction of two phosphate groups into certain sugar molecules—either into the same molecule or one each into two different ones—induces the decomposition of another molecule." He suggests further that the small heat production which Meyerhof and Suranyi (1927) have shown to accompany esterification may have some significance for the occurrence of the coupled reaction.

Harden represents the formation of hexosemonophosphate and diphosphate in accordance with this conception by modifying his original equations as here shown. Equations I(a) and I(b) denote independent reactions, each of which is coupled with

the reaction shown in equation II. Equations III(a) and III(b) represent the second stage of the fermentation in which the phosphoric esters are hydrolysed to hexose and inorganic phosphate which then take part in a fresh cycle of changes.

MODIFIED EQUATIONS OF ALCOHOLIC FERMENTATION

Harden, 1931.

Equation (1) becomes:

I(a) $2C_6H_{12}O_6 + 2PO_4HR_2 = 2C_6H_{11}O_5PO_4R_2 + 2H_2O$

I(b) $C_6H_{12}O_6 + 2PO_4HR_2 = C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O$

II $C_6H_{12}O_6 = 2CO_2 + 2C_2H_5OH$

Reaction I(a) or I(b) is coupled with Reaction II.

Equation (2) becomes:

III(a) $C_6H_{11}O_5PO_4R_2 + H_2O = C_6H_{12}O_6 + PO_4HR_2$

III(b) $C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2PO_4HR_2$

I(a) and I(b) represent the formation of hexosemonophosphate and hexosediphosphate respectively.

II represents the reaction coupled with I(a) and also with I(b) in which one molecule of sugar is simultaneously converted into carbon dioxide and alcohol.

III(a) and III(b) represent the hydrolysis of the monophosphoric and diphosphoric esters by the yeast phosphatase, with liberation of hexose and phosphoric acid.

These equations are empirical statements of the process and give no information as to which route the reaction will take—via the monophosphate or the diphosphate—or as to the intermediate reactions involved in equation II.

Of the four schemes which have been described, that of Harden alone is not contradicted by any of the facts at present known to us, but until more evidence has been accumulated and the full tale of the esters formed in fermentation is known, it would be premature to reject the possibility that the entrance of the phosphoric acid group increases the reactivity of the hexose molecule and is the direct cause of its breakdown to smaller molecules.

In this connection it is important to learn what we can of the mode of formation of the different esters, and I will therefore reconsider for a moment certain of the experimental facts which are summarised below:

1. glucose / or / fructose / or / mannose → + phosphate → { glucosemonophosphate + fructosemonophosphate + fructosediphosphate } + alcohol + carbon dioxide (high rate)

2. glucosemonophosphate / or / fructosemonophosphate → + phosphate → fructosediphosphate + alcohol + carbon dioxide (high rate)

bone phosphatase

3. glucosemonophosphate / or / fructosediphosphate → glucose + fructose + phosphate

1. In the fermentation of glucose, fructose, or mannose by non-living yeast preparations, coincident with the rapid production of carbon dioxide and alcohol, three phosphoric esters are synthesised, glucosemonophosphate, fructosemonophosphate, and fructosediphosphate.

The ratio of monophosphoric to diphosphoric esters may vary widely; but the ratio of the two monophosphoric esters to each other appears to be more constant, which suggests that both are formed in the same stage of the process.

2. Both the monophosphoric esters react with inorganic phosphate, giving rise to a rapid fermentation and, probably, fructosediphosphate.

Fructosediphosphate does not react with inorganic phosphate, and is fermented at a much slower rate than the monophosphoric esters. This rate, however, is catalytically accelerated by arsenate and possibly by a substance or favourable conditions present in living yeast.

3. On hydrolysis with bone phosphatase both glucosemono-

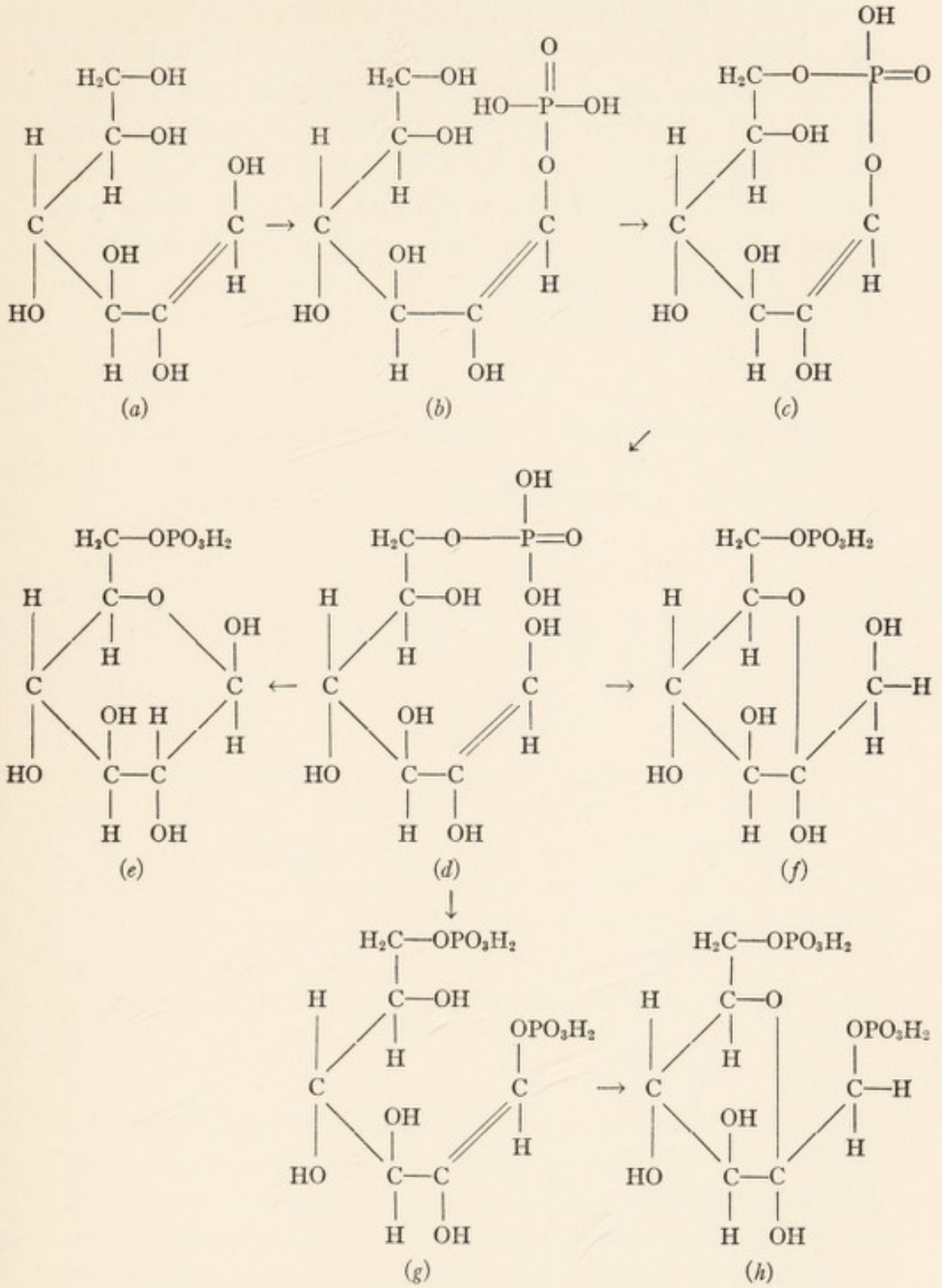
phosphate and fructosediphosphate give mixtures of sugars containing glucose and fructose.

These facts seem to show that the reducing group and its neighbouring carbon atom (atoms 1 and 2) are involved in both stages of the esterification with phosphoric acid and also in the enzymic hydrolysis of the esters, although, as we have seen, the phosphoric group is most probably attached to carbon atom 6 in both the monophosphoric esters finally isolated from the fermentation products.

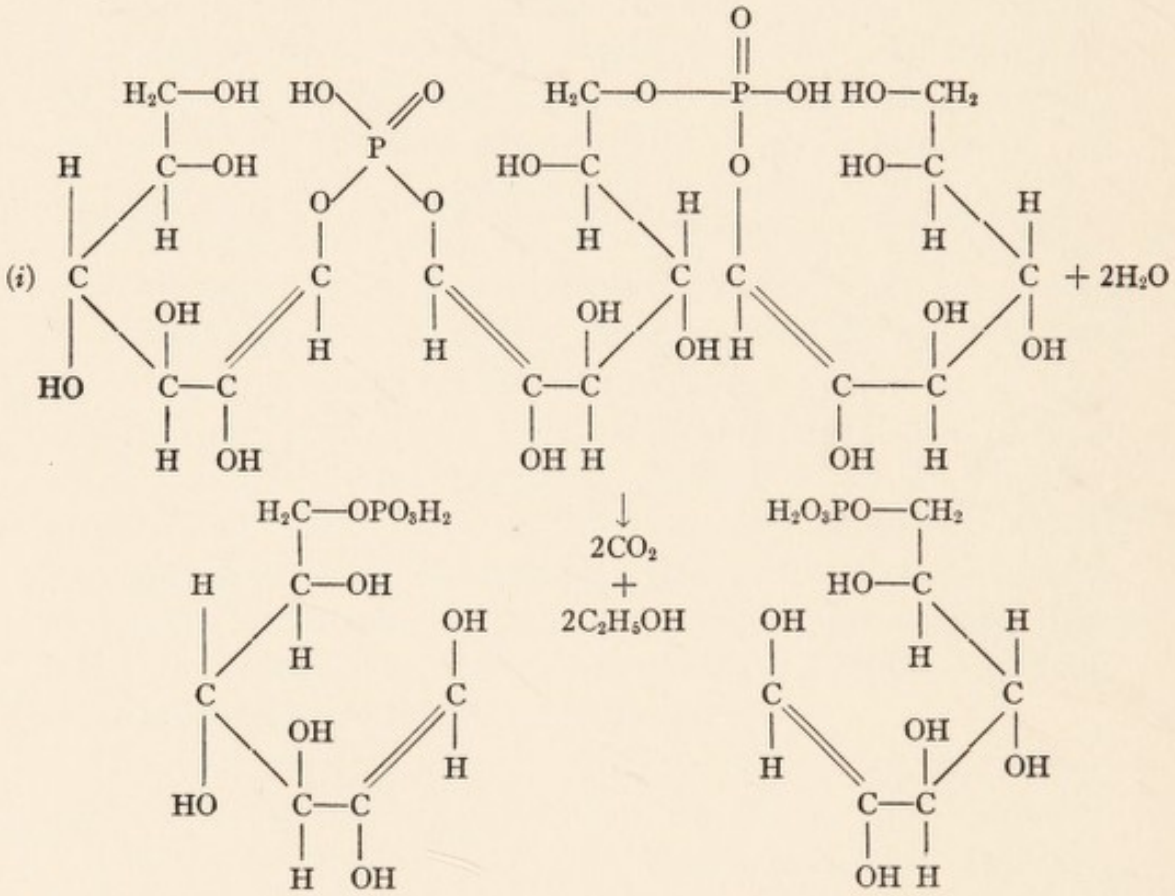
This conclusion lends support to the suggestion put forward by E. F. Armstrong in 1904, that the substance actually fermented by yeast is the enolic compound common to glucose, fructose, and mannose. We may suppose that this enolic compound (*a*) reacts rapidly with phosphoric acid, one molecule of which becomes attached to carbon atom 1 (*b*). This phosphoric group then wanders to carbon atom 6 via the intermediate compound (*c*). Such a change would be comparable with the wandering of the acetyl group in 1:2:3:4 tetraacetyl-β-glucose under the influence of alkali (Fischer 1920; Haworth, Hirst, and Teece, 1930). The resulting ester (*d*) by addition of water to carbon atoms 1 and 2, would yield hydrated forms of both glucose- and fructose-phosphates. Subsequent removal of water from the hydrated glucose ester would give 6-phosphoglucopyranose (*e*), while the hydrated fructose ester could yield only the γ-derivative, 6-phosphofructofuranose (*f*).

The enolic group of ester (*d*) could, however, react with a further molecule of phosphoric acid, giving the diphosphoric ester (*g*) which would yield 1:6 diphosphofructofuranose (*h*), the hexosediphosphate of Harden and Young.

During the hydrolysis of these esters by phosphatases (of yeast or of bone) this series of changes may be supposed to take place in the reverse direction.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

Although this scheme accounts for the formation of the three chief phosphoric esters of fermentation, it does not explain why the introduction of each phosphoric group should be accompanied by the decomposition of *half* a molecule of hexose into alcohol and carbon dioxide. It is conceivable that three molecules of hexose become linked together through two molecules of phosphoric acid and that the complex ester (i) so formed then breaks down, yielding two molecules each of hexosemonophosphate, carbon dioxide, and alcohol.



Two molecules of monophosphate could similarly become linked with another molecule of hexose through two additional molecules of phosphoric acid, and this new complex ester, on disruption, would yield two molecules each of hexosediphosphate, carbon dioxide, and alcohol. The ratio CO_2 produced/PO_4 esterified would then be equal to unity for both stages of esterification.

Such assumptions, however, extend beyond our present experimental knowledge, and it must be left for the future to provide the complete answer to this problem.

One of the most interesting analogies in Biochemistry is that which has been demonstrated between the reactions undergone by carbohydrates in animal muscle and those occurring in yeast. After the discovery of hexosediphosphate, Embden and his associates (1914, 1917, 1921) showed that the addition of this ester to muscle press juice gives rise to increased formation of lactic and phosphoric acids. They concluded that the precursor of lactic acid in muscle, to which Embden had given the name "lactacidogen," is hexosediphosphate or some closely allied compound. In 1924 Embden and Zimmermann isolated a hexosediphosphoric ester from muscle juice after this had been allowed to act on glycogen in presence of sodium fluoride; but later (1927), on repeating this experiment without the addition of fluoride, they obtained no hexosediphosphate but instead a hexosemonophosphate having a somewhat higher dextro-rotatory power than the Robison ester of alcoholic fermentation. These facts were confirmed by Pryde and Waters (1927, 1929), who isolated hexosemonophosphoric acid from normal muscle juice from rabbit, goat, and donkey. Hexosediphosphate was obtained only after fermentative re-synthesis had been allowed to take place in the presence of sodium fluoride. Pryde and Waters showed that the muscle hexosemonophosphate consisted, to the extent of 90%, of an aldose ester which proved to be a derivative of d-glucose. It is probable that this ester is identical with the glucosemonophosphate obtained from the products of fermentation by yeast; but this is not yet quite certain. Meanwhile it had been shown by Meyerhof that the ultimate source of the lactic acid produced during muscular contraction is the muscle glycogen which is broken down by the muscle enzymes with simultaneous esterification of phosphoric acid. It was further shown that during the recovery stage about four-fifths of the lactic acid becomes reconverted into glycogen, phosphates again playing a part in the proc-

ess. The energy required for this resynthesis is derived from the simultaneous oxidation of the remaining fifth of lactic acid or the equivalent amount of carbohydrate, to carbon dioxide and water (*v.* Hill, 1923; Meyerhof, 1923).

Our knowledge of lactic acid fermentation in muscle has been very greatly advanced in the last few years through the further researches of Meyerhof (1926–1930), who has obtained aqueous extracts of the lactic enzyme complex in high concentration and entirely free from all the muscle carbohydrates.

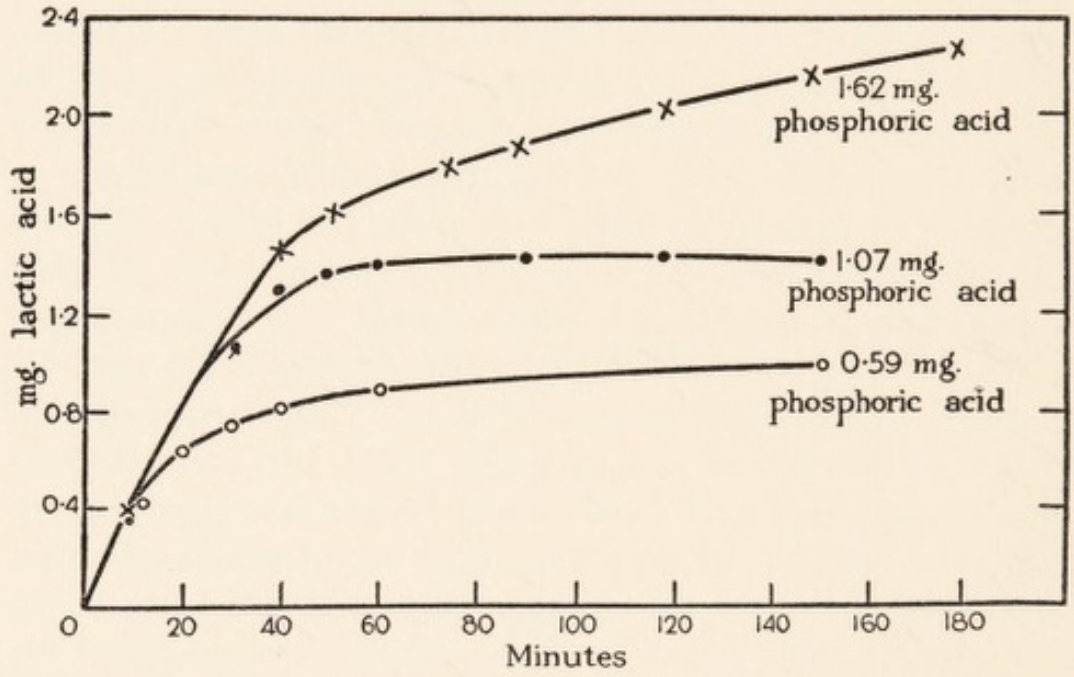


Fig. 4. The course of lactic acid formation in muscle extract. 0.5 cc. extract corresponding to 0.2g. frog muscle at 20° and pH 7.2. Glucose + hexokinase + different amounts of phosphate. (After Meyerhof. 1926).

This enzyme complex contains a thermostable and dialysable coenzyme which is mutually exchangeable and is probably identical with the co-zymase of yeast. In a series of delightful experiments Meyerhof has studied the formation of lactic acid from different carbohydrates by these extracts and the part played by phosphates in this fermentation. Certain carbohydrates such as cane sugar and galactose are not fermented; glucose, fructose, mannose, are normally fermented only by

certain extracts, especially those from rabbit muscle. These hexoses are, however, rapidly fermented in presence of a very small amount of an activator, to which Meyerhof has given the name "hexokinase" and which he obtained from autolysed yeast

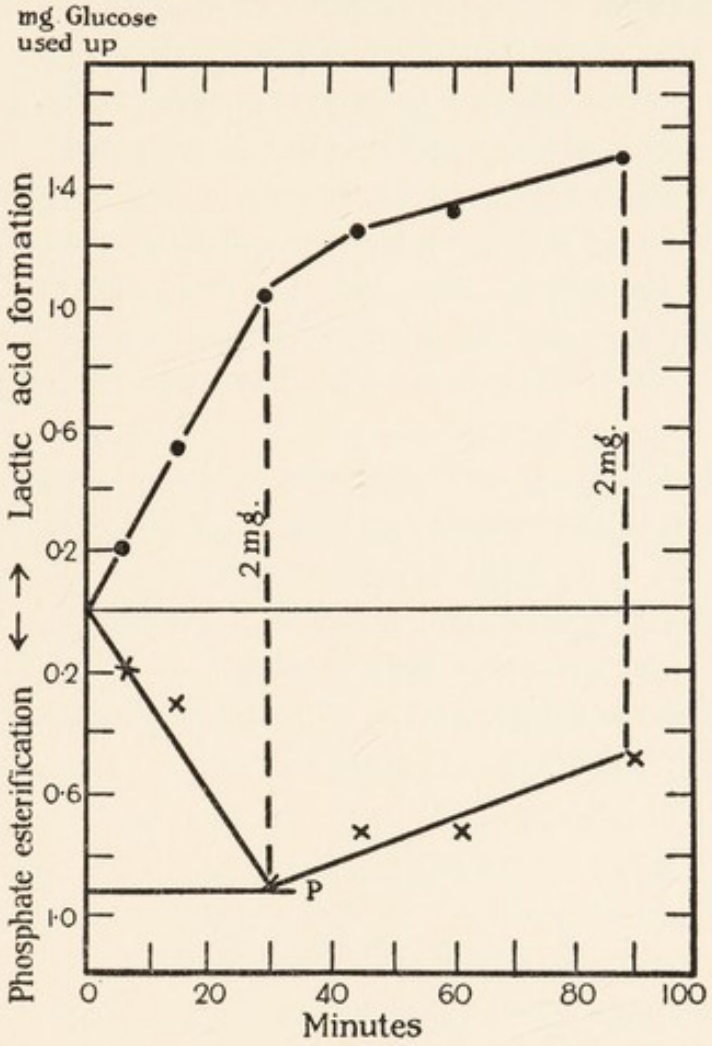


Fig. 5. The course of lactic acid production and phosphate esterification in muscle extract to which 2 mg. glucose (+ hexokinase) has been added. Both changes are calculated in the same terms, namely of sugar used. The horizontal line P shows the equivalent of inorganic phosphate originally present. (After Meyerhof, 1926).

by precipitation with alcohol. This hexokinase is thermolabile and is not identical with either insulin or glucokinin.

The carbohydrates of a third group consisting of starch, glycogen, and other polysaccharides, are fermented without addition of hexokinase, while hexosediphosphate is fermented by

extracts which, through partial loss of coenzyme, are unable to ferment hexoses or polysaccharides. The influence of phosphates in the course of the fermentation is shown in figures 4 and 5 which are reproduced from Meyerhof's original paper (1926).

Figure 4 shows the effect of different amounts of phosphate on the lactic acid production from glucose activated by hexokinase. The rate of fermentation falls very considerably at the point where the whole of the phosphate has become esterified.

This is very clearly seen in figure 5 which shows the production of lactic acid and the equivalent esterification of phosphate, followed by the second phase, when all the sugar has been esterified or fermented, and the slow hydrolysis or fermentation of the phosphoric ester takes place with liberation of inorganic phosphate.

Both the Neuberg and Robison hexosemonophosphates are fermented by these muscle extracts at an initial high rate, phosphate at the same time becoming esterified. From these and other results Meyerhof concludes that the course of lactic acid fermentation of glucose in muscle extracts is strictly analogous to that of alcoholic fermentation by yeast preparations, as shown by the following scheme (Meyerhof, 1930):

A.

$$2C_6H_{12}O_6 + 2H_3PO_4$$

$$\downarrow$$

$$2C_6H_{11}O_5(H_2PO_4) + 2H_2O$$

$$\swarrow \qquad \searrow$$

$$2CO_2 + 2H_2O + 2C_2H_5OH + C_6H_{10}O_4(H_2PO_4)_2 \qquad 2C_3H_6O_3 + 2H_2O + C_6H_{10}O_4(H_2PO_4)_2$$

B.

$$2C_6H_{10}O_4(H_2PO_4)_2 + 2H_2O$$

$$\swarrow \qquad \searrow$$

$$2CO_2 + 2C_2H_5OH + 2H_3PO_4 \qquad 2C_3H_6O_3 + 2H_3PO_4$$

yeast muscle

An active monophosphate is first synthesised and part of this ester may pass directly into the stable monophosphate found in muscle juice while another portion is converted into hexosedi-

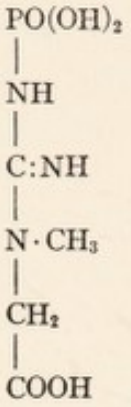
phosphate and lactic acid. This hexosediphosphate is fermented to lactic acid and does not normally accumulate in muscle.

Although, as I have already pointed out, the scheme suggested by Meyerhof is, in the case of alcoholic fermentation, open to certain serious objections, his investigations and those of Harden and Young prove beyond doubt the complete parallelism between these two fermentative processes by which carbohydrate is utilised for the requirements of a unicellular plant and of a highly organised animal tissue. Meyerhof considers it probable from the work of Dakin and Dudley (1913) and Neuberg (1913) that methylglyoxal is formed directly from the active monophosphate and that this yields lactic acid in muscle and pyruvic acid in alcoholic fermentation by yeast.

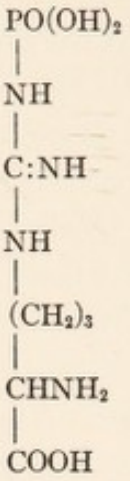
It has recently been shown by Lohmann (1930) that under the influence of sodium fluoride, oxalate, or citrate, Harden and Young's hexosediphosphate is converted by muscle extracts into another diphosphoric ester much more resistant to acid hydrolysis. The same ester is formed from hexosemonophosphate under similar conditions, a second molecule of phosphoric acid being esterified. It is formed when minced muscle or muscle extract acts on glycogen in presence of fluoride—the condition employed by Embden and Zimmerman. Yeast maceration juice in presence of fluoride converts hexosediphosphate and also starch into the same ester, but much more slowly than muscle extract. Lipmann and Lohmann (1930) have also obtained a similar ester by the action of diluted frog's muscle juice on hexosediphosphate. These new esters do not seem to be formed in the normal conversion of glycogen into lactic acid by muscle extracts.

Quite recently the central position in the chemistry of muscular contraction, so long occupied by the reversible system glycogen-hexosephosphate-lactic acid, has been partly usurped by a new phosphoric ester, phosphagen, discovered in the muscle of vertebrates by Eggleton and Eggleton (1927, 1928) and shown by Fiske and Subbarrow (1927, 1929) to be a derivative of creatine. A second compound of similar type, arginine-phosphoric acid,

was subsequently isolated by Meyerhof and Lohmann (1928) from the muscles of invertebrates.



Phosphagen.
Creatine-phosphoric acid.



Arginine-phosphoric acid.

The Eggletons showed that a large part of the phosphate previously estimated as inorganic, represents, in the resting muscle, this ester phosphagen, which is very readily hydrolysed by acids and is hydrolysed also in the muscle during contraction. Meyerhof and his colleagues (*v.* Meyerhof 1930) have studied the ratio of phosphagen breakdown to lactic acid production in muscle under different conditions, and the relative effect of certain chemical substances on these reactions. These researches have shown that the liberation of inorganic phosphates from phosphagen is proportional to the speed of the excitation processes and that resynthesis of phosphagen also goes on during and after the contraction. It has been shown by Lundsgaard (1930) that a muscle poisoned by iodoacetic acid can continue to contract without any lactic acid being formed; but that, during contraction, the phosphagen is hydrolysed and when this is used up the muscle goes into rigor. The phosphate liberated from this hydrolysis of phosphagen reacts with glycogen or with hexoses derived from it to form hexosephosphoric esters. This work has led Meyerhof to conclude that the formation of lactic acid during activity is a process only indirectly connected with the mecha-

nism of contraction and that the hydrolysis of creatine-phosphoric acid has an importance as an energy-producing reaction equal to that of the formation of lactic acid. He considers that lactic acid formation may possibly supply the energy for a continuous resynthesis of phosphagen at the moment of contraction.

In discussing the fermentation of carbohydrate in yeast and muscle, I have confined myself to those early stages in which esterification with phosphoric acid is involved. So far as we know, phosphoric esters play no part in the later reactions by which carbon dioxide and alcohol or lactic acid are formed from the intermediate products.

A few words more must be said in conclusion about the co-enzyme. Euler and Myrbäck, who have carried out extended investigations into the nature of co-zymase of yeast (*v.* Euler, 1930), found that their most active preparations consisted largely of a phosphoric ester, adenine nucleotide, and that when these were treated with dried liver or pancreas their activity diminished in proportion to the amount of phosphate set free. (Euler and Myrbäck, 1929.) Meyerhof and Lohmann have recently brought forward evidence that adenylpyrophosphate, while it will not alone discharge the functions of the co-enzyme, yet forms an essential part of the system both in muscle and in yeast. According to the latest report from Lohmann (1931), the second constituent of the co-enzyme of muscle is no more complex a substance than the element magnesium.

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II

CALCIFICATION OF CARTILAGE AND BONE

IT may seem a far cry from alcoholic fermentation to the formation of bone in the animal body; but, in my own case, it was the investigation of the phosphoric esters of fermentation that led directly to a study of calcification in the skeleton. While working on the hexosemonophosphate whose isolation was described in the previous lecture, I studied the hydrolysis of this ester by various enzymes. For these experiments the readily soluble calcium and barium salts were used, and, when hydrolysis occurred, the liberated inorganic phosphate was precipitated as the insoluble calcium or barium phosphate on the sides of the vessel. The sight of the precipitated phosphate in these enzyme tests suggested to me the possibility that such a mechanism might be concerned with the deposition of calcium salts in bone. To test this idea, I placed portions of the bones of young rats in solutions of barium hexosemonophosphate and in a few hours found precipitates of barium phosphate which showed that considerable hydrolysis had occurred. Further experiments carried out with the proper controls soon confirmed the fact that bone does indeed contain a very active phosphatase, capable of effecting the hydrolysis not only of hexosephosphates but also of other phosphoric esters. The tests showed, moreover, that this phosphatase is absent from cartilage of the small-celled type in which ossification is not taking place (Robison, 1923). Before describing the investigations which followed these preliminary tests, I must give some account of the histological and chemical changes which together produce the fully calcified bone.

In the formation of bone from cartilage in the embryo, the cartilage cells around some centre first become enlarged and arranged in columns. At the same time a change takes place in

the character of the matrix which becomes more basophilic in its staining properties. In this matrix the granules of the bone salt are deposited until the intercellular spaces have been densely calcified.

Simultaneously, changes take place in the periosteum involving the development of osteoblasts and, arising from these, the deposition of a layer of osteoid tissue beneath the periosteum and surrounding the cartilage. In the ground substance of this osteoid layer, calcium salts are deposited. Later, this sub-periosteal tissue invades the calcified cartilage which becomes absorbed, the marrow cavities are formed, and fresh deposits of osteoid tissue are laid down and calcified. This process of erosion has been ascribed to the activities of giant cells, the osteoclasts; but some histologists hold that the osteoblasts themselves are concerned in the mechanism of bone-absorption as well as in that of bone-formation. The processes of erosion and redeposition do not cease when the bone is fully formed but continue in the adult animal.

In the case of membrane bones such as the parietals, ossification does not take place in cartilage at all but in fibrous connective tissue, in which osteoblasts develop and give rise to the formation of osteoid ground substance which then becomes calcified.

For the study of the chemistry of calcification, certain facts derived from the histological study of bone would appear to be of outstanding importance.

1. In the normal animal the power to effect the deposition of inorganic salts is not a property of cartilage as such but only of cartilage at a certain stage in its development, this stage being characterised by enlargement of the cells and the formation of a strongly basophilic ground substance.

2. Similar power is exhibited by certain cells in the periosteal and endosteal tissues, which also bring about the formation of strongly basophilic ground substance. It is by the agency of these cells, the osteoblasts, that true bone is finally formed, with or without the previous co-operation of cartilage cells.

3. In the formation of adult bone, processes of absorption, including the redissolving of the deposited calcium salts, are of equal importance with the production of new osteoid tissue and its calcification. The osteoblasts are probably concerned in both processes. The processes of absorption and redeposition of calcium salts continue after the bone has been fully formed.

The adult bone contains on the average somewhat less than half its weight of water and anything up to 25% fat; but the amounts of both water and fat vary within wide limits in different bones, and depend also on the age and state of nutrition of the animal. The composition of the dry fat-free matter is much more constant, being approximately one third organic and two-thirds inorganic material.

The composition of the inorganic portion is also remarkably constant, both in bones and teeth, of the same and of different animals. This can be seen from Gabriel's (1894) analyses, which are shown in Table 1.

TABLE 1.

	Human Bone. %	Ox Bone. %	Ox Teeth. %	Goose Bone. %
CaO	51.31	51.28	50.76	51.01
MgO	0.77	1.05	1.52	1.27
K_2O	0.32	0.18	0.20	0.19
Na_2O	1.04	1.09	1.16	1.11
Water of crystallisation	2.46	2.33	2.21	3.05
P_2O_5	36.65	37.46	38.88	38.19
CO_2	5.86	5.06	4.09	4.11
Cl	0.01	0.04	0.05	0.06
Water of constitution	1.32	1.37	1.27	1.07
Total	99.74	99.86	100.14	100.06

In addition to the above constituents small amounts of fluoride are commonly found both in bones and teeth.

In pathological conditions such as rickets or osteomalacia, very considerable variations in the relative proportions of the organic and inorganic constituents may occur; but even in such cases the composition of the inorganic portion is not greatly altered.

The elementary composition of the ash as shown in these figures

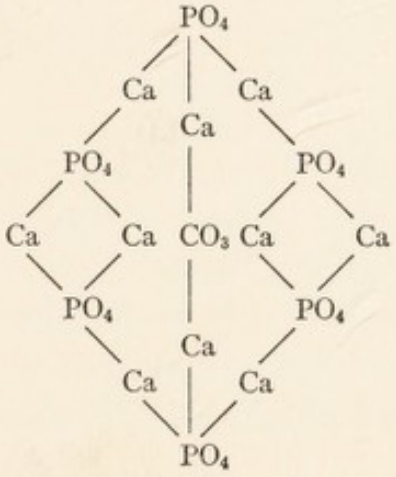
unfortunately does not tell us all that we want to know about the chemical constitution of the inorganic compound or compounds present in bone.

We can, of course, calculate how much calcium phosphate, calcium carbonate, magnesium phosphate, and so on, would correspond with these analytical data. But we do not know that any one of these individual compounds exists separately in bone. In place of such a mixture of half a dozen salts, the inorganic bone substance more probably consists of some complex compound in whose molecule most, or all, of these elements find a place.

Many years ago Hoppe-Seyler (1862) noticed that the relative amounts of calcium and phosphorus in bones and teeth corresponded very nearly with ten atoms of calcium to six atoms of phosphorus. This ratio is also found in the most important mineral phosphate, apatite, which exists in a variety of forms, occurring together in isomorphous mixtures, namely:

Fluoro-apatite	$3Ca_3(PO_4)_2CaF_2$
Chloro-apatite	$3Ca_3(PO_4)_2CaCl_2$
Hydroxy-apatite	$3Ca_3(PO_4)_2Ca(OH)_2$
Oxy-apatite	$3Ca_3(PO_4)_2CaO$
Carbonato-apatite	$3Ca_3(PO_4)_2CaCO_3$

Hoppe-Seyler was so struck by this resemblance of bone to the mineral phosphate that he suggestedthe following as the molecular constitution of the bone compound:



This elegant geometrical design must, however, be considered merely as an interesting guess at the possible structure of the compound.

Gabriel (1894), from his own analyses, also concluded that the bone substance consisted of a complex salt with this empirical formula:

$$Ca_3(PO_4)_2 + Ca_5HP_3O_{13} + aq.$$

with 2–3% of Ca replaced by Mg, K, Na,
and 4–6% of PO_4 " " CO_3, Cl, F.

Gassmann (1910) considered the bone salt to be a carbonato-apatite and has prepared artificially a compound of the same composition (1913, 1928), while Bassett (1917), using physico-chemical methods of investigation, came to the conclusion that the essential constituent of the bone deposit was hydroxy-apatite and that this is mixed with calcium carbonate which is independently precipitated, while the small amounts of other salts are carried down by adsorption.

Quite recently the method of X-ray analysis has been applied to the study of the bone salts. Such analyses have been carried out by de Jong (1926), Taylor and Sheard (1929), and by Roseberry, Hastings, and Morse (1931), all of whom conclude that bone contains a complex salt belonging to the apatite series, a conclusion extended also to the inorganic constituents of teeth. No evidence was obtained of the presence of calcium carbonate or of the acid phosphate $CaHPO_4$, which has been supposed by some authors to be the salt first deposited during calcification. My own experiments on calcification *in vitro* have also supplied evidence that carbonate is not separately precipitated but as part of a complex carbonato-phosphate. Experience in the precipitation of the salts of phosphoric esters leads me to believe that the small amounts of other inorganic basic and acidic radicles present in bone, namely, magnesium, sodium, potassium, chloride, fluoride, and lastly hydroxyl, are not carried down merely by adsorption but more probably as part of the carbonato-

phosphate replacing equivalent amounts of calcium, phosphate, or carbonate, in this complex molecule.*

It cannot, however, be said that the constitution of the bone salt is yet definitely settled; and, on the chemical side, improved methods for the complete analysis of bone, including estimations of sodium, potassium, and magnesium, are badly needed. The available data, which are chiefly confined to estimations of calcium, phosphate, and carbonate, reveal rather wide variations in the ratio of carbonate to phosphate. In some, though not all, types of rickets, this ratio appears to be increased. This was observed by Howland, Marriott, and Kramer (1926) and by Kramer and Shear (1928). High values of the ratio Ca/P had been previously noted by Robison and Soames (1925) and by Chick, Korenchevsky, and Roscoe (1926) in bones of rachitic rats fed on the McCollum diet 3143, which is very low in inorganic phosphate and gives rise to a low phosphate level in the blood. When cod-liver oil was added to the diet, the level of phosphate in the blood was raised, and the ratio of Ca to P in the bones fell to a value approximately the same as that required by carbonato-apatite.

Kramer and Shear also found that in the bones of rats the ratio of calcium carbonate to phosphate increased with the age of the rat. In young animals this ratio was close to the value for apatite, but in adult animals the proportion of carbonate was decidedly higher. It is not easy to draw conclusions from these data in absence of information respecting the other basic and acidic radicles present; but, if it is accepted that the main constituent is apatite, it would seem probable either that calcium carbonate may in certain conditions be separately precipitated or that carbonate can replace one of the phosphate groups in the complex carbonato-apatite molecule. The different basicity of the two acids is, however, a difficulty in the latter supposition.

It is a serious handicap in our attempt to understand the

* See also recent papers by Morgulis (1931), who considers that the principal component of bone ash is a complex salt $Ca[\{Ca_3(PO_4)\}_6]OH_2$, and by Bogert and Hastings (1931), who conclude that the chief inorganic constituent is a crystalline salt, $CaCO_3$, $nCa_3(PO_4)_2$, where n approximates the value 2.

calcification process that we do not certainly know whether we have to account for the deposition of one salt or of a mixture of salts involving a correspondingly greater number of factors.

When we consider the physico-chemical relationships of the tissue fluids to the bone deposit, we are met by an even more serious uncertainty. Michaelis (1922), in some pungent sentences of his book on hydrogen ion concentration, has pointed out the absurdity of propounding theories to account for calcification before it has been discovered whether the blood is indeed supersaturated with calcium salts or not. And yet, in spite of much valuable work that has been carried out since that date, this fundamental question is still in some doubt.

Let us assume for the moment that the deposit consists solely of tricalcium phosphate; then the equilibrium between this salt and its saturated solution in water may be thus expressed:

	Saturated Solution			
	Dissolved ionised		Dissolved unionised	Solid
(1)	$Ca^{++} + Ca^{++} + Ca^{++} + PO_4^{\equiv} + PO_4^{\equiv}$	$\rightleftarrows$	$Ca_3(PO_4)_2$	$\rightleftarrows$ $Ca_3(PO_4)_2$
(2)	$[Ca^{++}]^3 \times [PO_4^{\equiv}]^2 = [Ca_3(PO_4)_2] = s$, solubility product.			

Equilibrium of the reversible reactions shown in line 1 is expressed by the equation in line 2 in which the product of the ion concentrations is equal to "s," the solubility product of the salt in the medium. We owe the conception of this solubility product to Nernst, and its usefulness is still acknowledged although the modern theories of the complete dissociation of strong electrolytes call for some modification in the definitions of the symbols here employed. The important point is that saturation is determined by the concentrations of the ions, or by their activity, and not by the concentration of total calcium and phosphate.

In the above system, the addition of calcium or phosphate ions, from whatever source, would cause the reactions to proceed towards the right, and fresh calcium phosphate would be deposited. Removal of calcium or phosphate ions, by whatever means, would result in more of the solid passing into solution

until equilibrium is restored. Addition of any compounds of calcium or phosphate which do not yield Ca^{++} or $PO_4^{=}$ ions would not effect the equilibrium or cause precipitation to occur.

Phosphoric acid is dissociated in three stages, thus:

$$H_3PO_4 \rightleftarrows H^+ + H_2PO_4^- \quad \left| \quad \frac{[H^+] \times [H_2PO_4^-]}{[H_3PO_4]} = K_1 = 1.2 \times 10^{-2} \text{ (a strong acid)} \right.$$

$$H_2PO_4^- \rightleftarrows H^+ + HPO_4^= \quad \left| \quad \frac{[H^+] \times [HPO_4^=]}{[H_2PO_4^-]} = K_2 = 2.2 \times 10^{-7} \text{ (a weak acid)} \right.$$

$$HPO_4^= \rightleftarrows H^+ + PO_4^{\equiv} \quad \left| \quad \frac{[H^+] \times [PO_4^{\equiv}]}{[HPO_4^=]} = K_3 = 1.7 \times 10^{-12} \text{ (an extremely weak acid)} \right.$$

$$[H^+] = \frac{K_1[H_3PO_4]}{[H_2PO_4^-]} = \frac{K_2[H_2PO_4^-]}{[HPO_4^=]} = \frac{K_3[HPO_4^=]}{[PO_4^{\equiv}]}$$

The relative proportions of the three phosphate ions are, therefore, dependent on the values of the dissociation constants K_1,K_2, and K_3, and on the hydrogen ion concentration of the medium. In plasma at pH 7.4, about 20% of the total phosphate is present as $H_2PO_4^-$ and 80% as $HPO_4^=$ ions. The concentration of $PO_4^{\equiv}$ ions is very small but it is not on this account negligible. It is given by the following equations: the second form (b) being stated in terms of the Debye-Hückel theory and quoted from Sendroy and Hastings (1927).

$$[\text{Total } PO_4] = [H_3PO_4] + [H_2PO_4^-] + [HPO_4^=] + [PO_4^{\equiv}]$$

$$\text{(a)} \qquad [PO_4^{\equiv}] = \frac{[\text{Total } PO_4]\, K_1K_2K_3}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}$$

or

$$\text{(b)} \qquad [PO_4^{\equiv}] = \frac{[\text{Total } PO_4]K_1'K_2'K_3'}{(\alpha_{H^+})^3 + (\alpha_{H^+})^2K_1' + \alpha_{H^+}K_1'K_2' + K_1'K_2'K_3'}$$

The equilibrium between these ions and those of calcium in plasma is given by the equations:

Ions.		Undissociated salt.	Solubility product.
$[Ca^{++}] \times [H_2PO_4^-]^2$	=	$K_1[Ca(H_2PO_4)_2]$	s_1 (very soluble)
$[Ca^{++}] \times [HPO_4^{=}]$	=	$K_2[CaHPO_4]$	s_2 (sparingly soluble)
$[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$	=	$K_3[Ca_3(PO_4)_2]$	s_3 (very slightly soluble)

If the concentrations of calcium and phosphate are sufficiently increased, one of these salts will ultimately be deposited, depending on which of the three values s_1, s_2, s_3 is first reached. From the experiments of Bassett (1917) and others, we know that solid $CaHPO_4$ is in equilibrium with such solutions only at a pH below 7; and it is therefore very unlikely that this salt could be deposited from the tissue fluids. The solid in equilibrium with neutral and alkaline solutions was found by Bassett to be more basic than tricalcium phosphate and had the composition of hydroxy-apatite.

In plasma the equilibria are complicated by the presence of carbonate and bicarbonate ions; and it seems probable that the solubility product first reached is not that of any of the simple calcium phosphates but of a complex salt of the apatite type formed from the interaction of calcium, phosphate, and carbonate ions.

Human plasma normally contains about 9–11 mg. of calcium per 100 c.c. and from 2.5 to 5 or 6 mg. P as inorganic phosphate, the concentration of phosphate being highest in infancy. Similar values for the calcium content of plasma or serum have been recorded for other mammals; but in rabbit serum the calcium concentration is higher, 13–17 mg. per 100 c.c. The phosphate content of the serum also varies somewhat in different animals. In young rats it may exceed 10 mg. P per 100 c.c.

If these amounts of calcium and inorganic phosphate were introduced into water at the pH of the plasma, the solution would be highly supersaturated and would at once deposit a calcium phosphate. The presence of the other inorganic constituents of plasma increases the solubility to some extent; and it is possible with care to prepare aqueous solutions containing such salts and calcium and inorganic phosphate in concentrations similar to

those in plasma. The solutions are, however, definitely supersaturated and deposit a calcium salt if left for some days, or weeks, at 37° and pH 7.3–7.4. Some experiments carried out by Miss Maclean and myself may be quoted to illustrate this. Solutions were prepared containing sodium chloride and bicarbonate, potassium, and magnesium, in concentrations similar to those in plasma, but with varying concentrations of calcium and phosphate. After adjusting the pH to 7.4, the solutions were left at 37° for two months. At the end of this time solutions and precipitates were analysed, some of the results being shown in Table 2.

TABLE 2.

Composition of Solution. mg. per 100 c.c.					
Initial.		Final.		Precipitate after 2 months	Ratio $\frac{Ca}{P}$ in precipitate
Ca	P	Ca	P		
5	4	5	4	−	−
10	2	9.2	1.8	trace.	−
10	3	4.5	0.5	+	2.19
10	4	4.2	1.5	+	2.10
10	5	2.8	1.2	+	2.13
				Carbonato-apatite requires	2.15

In solutions containing 10 mg. Ca and 3 mg. P per 100 c.c., precipitation usually commenced in a few days, and ultimately the concentration of calcium fell to 4.5 and that of phosphorus to 0.5 mg. per 100 c.c. But solutions containing 10 mg. Ca and 2 mg. P did not give any appreciable precipitate during 2 months. The addition to such solutions of small quantities of precipitate obtained from other solutions did, however, cause further precipitation to take place very slowly. It is clear that tendency to deposit the solid salt in such aqueous salt solution is slight until a considerable degree of supersaturation is reached.

The composition of the precipitates was in general similar to that of the bone salt, the ratio of calcium and magnesium to phosphate and carbonate lying within the limiting values which have been found for bone. The analyses indicated the presence

of a certain proportion of closely bound water not driven off by gentle ignition, as was also noted by Gabriel in bones and by Bassett in his precipitates.

The concentrations or activities of the ions responsible for the formation of the bone salt are affected by the other ions present in the tissue fluids, but certain of these appear to have a much more powerful effect than others. The amount of magnesium in plasma is only one quarter of that of calcium; but, even in such low concentrations, magnesium markedly reduces the tendency of the calcium salt to precipitate. This may perhaps be due to the formation of a soluble but sparingly ionised magnesium phosphate.

The conditions in plasma are further complicated by the presence of proteins and other organic substances some of which have been shown to influence the calcium ionisation, while the effects of others are still undetermined.

It was shown by Rona and Takahashi (1913) that the inorganic phosphate is present in plasma in completely diffusible form. This does not, of course, imply that the phosphate is completely ionised, for these authors themselves demonstrated the diffusibility of salts such as $CaHPO_4$ whose ionisation even in such low concentrations is far from complete.

Grollman (1927) has recently obtained the same result for mammalian blood, but records that the phosphate is not completely diffusible in the blood of lower animals. He ascribes this difference to the higher calcium content of the latter since artificial increase of the calcium in mammalian blood causes part of the inorganic phosphate to become non-diffusible.

Eichholtz and Starling (1925) found that the isolated kidney, when perfused by means of a heart-lung preparation, did not excrete inorganic phosphate, and concluded from this that a great part of the inorganic phosphate of the serum is present in the form of unionised colloidal calcium phosphate to which the glomerular membrane is impermeable. Brull (1928) also, from his own experimental results, considered the occurrence of this colloidal phosphate in plasma to be very probable.

In addition to the inorganic phosphate, blood contains a much larger store of phosphorus in the form of phosphoric esters which do not yield $PO_4^{\equiv}$ ions. These are present for the most part in the corpuscles.

The degree of ionisation of calcium in blood is of importance not only for the process of calcification but for other physiological phenomena—the irritability of muscle and nerve, the coagulation of blood. Rona and Takahashi (1913), Cushny (1920), and others have shown that only a fraction, between 50% and 70%, of the total calcium in serum will pass through collodion membranes by diffusion or by ultrafiltration under slight pressure. Rona and his co-workers further proved that the non-diffusible calcium was bound to the serum proteins and that the proportion of such bound calcium depended on the pH of the fluid in relation to the isoelectric point of these proteins. Thus at pH 5, more calcium was found in the external liquid than in the residual serum within the collodion membrane.

Cameron and Moorhouse (1925) estimated the concentration of calcium in the cerebrospinal fluid of dogs, and found it to be only 53% of that in plasma. They considered that this fluid represents a natural diffusate or ultrafiltrate of blood, a conclusion which has received a good deal of experimental support, although it appears to be contradicted by some recent results of Morgulis and Perley (1930), who find that changes in the calcium concentration in blood due to injection of calcium salts and parathyroid-hormone are not accompanied by corresponding changes in the cerebrospinal fluid.

The diffusible calcium is not, however, wholly ionised. Brinkman and van Dam (1920) attempted to estimate the fraction by finding the minimum amount of oxalate that sufficed to give a faint turbidity in a measured volume of serum. They obtained the value 2.2 mg. ionised calcium per 100 c.c. of serum and a similar figure was subsequently obtained by Freudenberg and Budde (1924), using the same method. This concentration is the same as that calculated by Rona and Takahashi (1913) for a

saturated solution of $CaCO_3$ under similar conditions of pH and CO_3 concentrations.

The ionised calcium has been determined electrometrically by Neuhausen and Marshall (1922) using a calcium amalgam electrode and by Corten and Estermann (1928) using a different electrode system. In both cases the values found were about 2 mg. per 100 c.c. Only one third of the diffusible calcium is, therefore, ionised; and part of the total calcium must be combined with some constituent of the blood forming a sparingly ionised but diffusible compound. Bernard and Beaver (1926) have shown that in electrodialysis of serum some of this calcium moves to the anode and is, therefore, bound up in a negatively charged complex.

Table 3 is quoted from Klinke's (1928) review of recent work in this field and gives the average values obtained for the different fractions of total calcium obtained from data of many investigators.

TABLE 3.

mg. Calcium in 100 c.c.	Serum	Tissue Fluids.	Cerebrospina Fluid.
Total	10–12	10	5–6
Ionised	2	2	2
Dialysable	6–7	Unknown	All
Unionised-dialysable	4–5	"	3–4
Combined with protein	4–5	"	0

Detailed and valuable studies of the physico-chemical relationships of the serum and bone salts have been carried out by two groups of American workers, Holt, La Mer, and Chown (1925), and Hastings, Murray, and Sendroy (1927). Holt, La Mer, and Chown concluded from their data that blood serum is normally supersaturated with tertiary calcium phosphate to the extent of 200% and in experimental confirmation of this conclusion showed that on shaking serum with calcium phosphate most of the serum calcium was precipitated. Both Klinke and Sendroy and Hastings have pointed out that in these experiments

the precipitate was not calcium phosphate but, for the most part, calcium carbonate.

TABLE 4.

	Millimols per litre.			
	Ca	P	CO_3	pH
Klinke.				
Human Serum	2.9	1.0	30.2	7.36
" " after shaking with $Ca_3(PO_4)_2$	1.3	1.0	28.0	7.31
Precipitated	1.6	0	2.2	
Human serum	2.6	0.9	34.8	7.39
" " after shaking with $Ca_3(PO_4)_2$	0.9	0.8	32.8	7.32
Precipitated	1.7	0.1	2.0	
Sendroy and Hastings.				
Horse serum	3.2	0.79	32.2	7.47
" " after shaking with $Ca_3(PO_4)_2$	0.6	0.86	27.4	7.37
Precipitated	2.6	−0.07	4.9	

Table 4 shows some of the results obtained by Klinke (1928, 1929) and by Sendroy and Hastings (1927) proving this fact, namely, that when serum is shaken with tertiary calcium phosphate there is hardly any further precipitation of this salt, as would be the case if the solutions were supersaturated. Instead, calcium carbonate is precipitated, although if serum is shaken with solid calcium carbonate no precipitation of calcium takes place. I think that these facts are most simply explained by supposing that the added calcium phosphate had combined with the carbonate and calcium of the serum to form the still less soluble carbonato-phosphate. This would be somewhat analogous to the formation of hydroxy-apatite by the action of water on tricalcium phosphate as shown by the early experiments of Warrington (1873) and those of Bassett (1917).

An apparent objection to this view is that the amounts of calcium and carbonate brought down are not strictly equivalent. The CO_3 is greater; but this result may perhaps be linked up with the increased proportion of carbonate in adult bones, and explained by the formation of a complex molecule having a

higher proportion of carbonate than is present in carbonato-apatite. Klinke also reports that some precipitation of calcium carbonate occurred when serum was shaken with freshly precipitated barium sulphate but not with dried barium sulphate; and these facts are not easy to explain. The conclusion arrived at by Sendroy and Hastings is that the theory of supersaturation is inadequate and that calcium exists in serum in abnormal amounts, bound to some substance or substances which hold it in solution in unionised form. A very similar opinion has been expressed by Klinke. Experiments which I shall describe in the next lecture point to the possibility of glucose combining with calcium in the serum to form an un-ionised compound. The evidence is at present only indirect, and I do not wish to be more definite until further work has been completed.

It would seem from the balance of evidence now available that we are not justified in postulating that the tissue fluids are supersaturated with calcium phosphate in order to account for the calcification of the skeleton.

At the time when my own experiments were begun, much less information was available and the theory of supersaturation was, perhaps, more commonly though not universally accepted. I did not find this theory altogether reasonable on the following grounds.

Calcification does not normally take place in any tissue other than cartilage and bone, and only in cartilage at a certain stage in its development. A supersaturated solution of calcium phosphate might, it is true, be sufficiently stable to deposit its excess of calcium salt only in presence of the solid phase, that is, where calcification had already begun. We should still have to explain the beginning of calcification, the formation of the first crystal in the matrix of the hypertrophic cartilage and osteoid tissue, and the further fact that precipitation, once begun, does not extend to the adjacent non-osteoid tissue. The continuous reabsorption and redeposition of the calcium salt are both essential processes in the shaping and growth of bones, and continue

throughout life. Moreover bone forms a calcium and phosphate reserve which is rapidly drawn upon for any special need of the organism, e.g., in pregnancy or during lactation. Aub and his colleagues (Bauer, Aub, and Albright 1929) have shown how readily the bony trabeculae give up their calcium salt in hyperparathyroidism. All these facts speak for a mobile and rapidly adjustable equilibrium between the blood and tissue fluids and the calcified bone.

Many theories have been put forward to account for calcification of the skeleton. In 1900 Grandis and Mainini suggested that by the breaking down of nucleic acids in the cartilage cells, phosphate may be liberated and combine with the calcium of the blood. This hypothesis was rejected for the sufficient reason that the total phosphorus in the cells is quite inadequate to account for the bone phosphate. Pauli and Samec's (1909) explanation depended on the increased solubility of calcium phosphate and carbonate in the tissue fluids owing to the high concentration of proteins and the precipitation of these calcium salts in consequence of a hypothetical breakdown of the proteins by hydrolysis.

Pfaundler (1904) suggested that a constituent of cartilage, formed at a certain stage in its development, has a special affinity for calcium salts which are adsorbed and are then precipitated on the further breakdown of this organic ground substance. This idea was developed at a later date by Freudenberg and György (1920–1924), who showed that, if cartilage is soaked in a solution of calcium chloride, thoroughly washed, and thereafter placed in a solution of sodium phosphate, it becomes calcified, while if the cartilage is placed in these solutions in the reverse order no deposit is formed. The scheme put forward by Freudenberg and György (1923) is here shown:

Cartilage-protein + Ca = Ca—cartilage-protein
Ca—cartilage-protein + phosphate = Ca—cartilage-protein—phosphate
Ca—cartilage-protein—phosphate = calcium phosphate + cartilage-protein

In this attempt to explain the calcification process by a

property which is shared by non-ossifying cartilage a most essential point has, I think, been overlooked.

Hofmeister (1910) suggested that variations in the amount of carbon dioxide in the lymph might account for the precipitation of the calcium salts. A higher concentration of carbon dioxide enables the lymph to take up more calcium salts with which the tissues become impregnated. When the carbon dioxide content decreases, the excess of calcium salts is deposited. This deposit is not redissolved when the carbon dioxide again increases since re-solution is, he considered, a much slower process than deposition. With fluctuating levels of carbon dioxide in the tissue fluid the deposit of calcium salt thus gradually increases.

Wells (1911), though accepting this as a possible cause of certain abnormal deposits, did not consider it a likely explanation of calcification either in ossifying cartilage or in necrotic tissues. He believed that calcium is carried in the blood in amounts not far from the saturation point and that calcification is initiated by specific colloidal adsorption of the calcium salts by the cartilage matrix.

Watt (1925), after comparing the shape of particles of calcium phosphate and carbonate precipitated in colloids with the appearance of the calcium salts in bone, concluded that the bone salts are not deposited by simple precipitation but are taken up by the osteoblasts and deposited in the matrix by a secretory process.

None of these hypotheses gives, in my opinion, a satisfactory explanation of the mechanism of calcification, though some of them may well contain a portion of the truth. The scheme which I have suggested may be stated as follows:

Blood is to be considered normally as a saturated solution of the bone salt, which for simplicity in discussion we may think of as tricalcium phosphate. In addition to the inorganic phosphate, blood contains a phosphoric ester whose calcium salt is soluble. As this ester cannot yield $PO_4^{\equiv}$ ions on dissociation, it does not affect the saturation of the blood with respect to calcium phosphate. The tissues which become calcified, namely the hyper-

trophic cartilage and osteoid, contain an enzyme, phosphatase which hydrolyses this ester, setting free inorganic phosphate whereby the concentration of $PO_4^{=}$ ions is increased and the product of the concentrations of $PO_4^{=}$ and Ca^{++} ions then exceeds the solubility product of calcium phosphate which is deposited in the organic ground substance of the tissue.

SCHEME OF CALCIFICATION

Blood plasma = saturated solution of bone salt.

$[Ca^{++}]^3 \times [PO_4^{=}]^2 = s$, solubility product.

$C_6H_{11}O_5PO_4R_2 \rightleftarrows C_6H_{11}O_5PO_4^{=} + 2R^+$

Tissue Fluid (hypertrophic cartilage and osteoid tissue)

by the action of bone phosphatase on the phosphoric ester, becomes supersaturated with bone salt.

$C_6H_{11}O_5PO_4R_2 + H_2O \xrightarrow[\text{phosphatase}]{} C_6H_{12}O_6 + R_2HPO_4$

$\downarrow$

$2R^+ + H^+ + PO_4^{\equiv}$

$[Ca^{++}]^3 \times [PO_4^{=}]^2 > s$

$\downarrow$

Calcified tissue. $Ca_3(PO_4)_2$ solid phase deposited

To simplify this statement the bone salt is here assumed to be tricalcium phosphate and the ester, hexosemonophosphate. The scheme applies equally well if the bone salt is a more complex phosphate such as carbonato-apatite, while any other phosphoric ester available to these tissues and hydrolysable by the phosphatase would serve in place of the hexosephosphate.

I have already told how this scheme was suggested by experiments on the enzymic hydrolysis of calcium hexosemonophosphate and how the first tests carried out with the bones of young rats revealed the presence of an active phosphatase such as I have imagined (Robison, 1923). I have now to describe the subsequent investigations and the results so far obtained. If the problem no longer appears quite so simple as at first conceived, this only adds to the interest of the search.

The distribution of the phosphatase and its more important properties have been studied with the collaboration of Miss Soames and Miss Martland. The bone phosphatase was found to effect the hydrolysis of hexosemonophosphates and diphos-

phates, glycerophosphates, nucleotides, and many other esters, but not those such as diethylphosphate, in which two alcoholic groups are attached to the phosphoric acid. It was found to be present in greatest activity in the ossifying cartilage, bones, and teeth, of very young animals, the activity per unit weight of tissue decreasing with age. Cartilage of the small-celled, non-ossifying type, such as costal and tracheal cartilage, was almost or entirely inactive. The ability of certain other animal tissues, especially kidney and intestine, to hydrolyse phosphoric esters had previously been demonstrated by other workers (Grosser and Husler, 1912; Plimmer, 1913; Tomita, 1922). Our own experiments (Robison, 1923; Robison and Soames, 1924) showed that in very young animals the phosphatase activity of the developing bone is even higher than that of the kidney, while the liver, spleen, pancreas, and muscle are very much less active.

Table 5 shows the relative amounts of sodium hexosemonophosphate hydrolysed at 37° and pH 9.0, by the same weight of various tissues of a rat 27 days old. The superiority of the epiphysial cartilage in phosphatase activity was even more pronounced than appears from these figures, since a very large proportion (78%) of the total substrate was hydrolysed by this tissue.

TABLE 5.

Tissues of rat 27 days old.	Relative phosphatase activity at 37°, pH 9.0., 18 hours. Substrate: hexose-monophosphate
Epiphyses of long bone (ossified)	100
Rib cartilage (unossified)	1
Kidney	47
Spleen	11
Liver	5
Muscle	5
Pancreas	1

In Table 6 are shown the relative phosphatase activities of the cartilage and bones of a $5\frac{1}{2}$-months human foetus (Martland and Robison, 1924). The femora and tibiae were dissected out

and divided into three portions, the ossified shaft, the epiphysial cartilage in which no calcification was apparent, and the junction between these two, including the line of calcification. The results show that this region of advancing calcification and ossification is even more active, weight for weight, than the fully calcified bone, while the resting epiphysial cartilage is quite inactive.

TABLE 6.

Phosphatase activity of human cartilages and bones.
Substrate: glycerophosphate (3.1 mg. P.)
1 c.c. 5% tissue extract: 18 hours. 38°. pH 8.4.

				Ester hydrolyzed %
Skeleton of 5½ months human foetus:				
	Femur	Shaft	Ossified	60
	"	Junction	Ossified	71
	"	Epiphysis	Unossified	0.3
	Tibia	Shaft	Ossified	47
	"	Junction	Ossified	56
	"	Epiphysis	Unossified	0.4
	Parietal		Ossified	42
Infant, full term	Costal cartilage		Unossified	0
	Costochondral junction		Ossified	59
	Patella		Unossified	0
Infant, full term	Patella		Unossified	0
Infant, 7 months old	Patella		Unossified	0
Child, 6 years old	Patella		Partly ossified	
		Half containing ossification centre		70
		Half containing no ossification centre		0

The parietal was also examined as a type of membrane bone and was found to contain phosphatase equally with the bones formed in cartilage. In the human skeleton the ossification centres appear for the most part during the second and third months of foetal life. In the patella, however, ossification does not begin until 3 years or more after birth, when a single centre of ossification appears, from which bone formation spreads slowly, becoming completed about puberty. We, therefore, examined patellae obtained *post mortem* from children of different

ages. The results, which are given at the foot of Table 6 show that before the appearance of the ossification centre the cartilage is devoid of phosphatase, but that this enzyme is present whenever active ossification is taking place.

From these and other experiments it was considered legitimate to conclude that the production of the enzyme is a part of those cellular activities which result in the formation of bone. The presence of the phosphatase in teeth is very significant in view of the close similarity in the chemical composition of the inorganic portions of teeth and bones, which renders it probable that the same mechanism of calcification functions in both. The decrease which occurs in the phosphatase activity of bones with increasing age may be explained partly by the larger proportion of inorganic matter in the older bones; but there may also be a reduction in the number and activity of the enzyme-secreting cells.

The enzyme was found to be present also in the bones of rachitic rats; and indeed the activity of such bones is usually greater than that of normal bones, which may probably be explained by their much lower content of inorganic matter.

These bones of rachitic animals served for our experiments on calcification *in vitro*, which will be described in the next lecture. In solutions of calcium salts of phosphoric esters, the zone of hypertrophic cartilage became fully calcified; and, from the location of the new deposits as well as for other reasons, I concluded that the enzyme is secreted both by osteoblasts and also by the hypertrophic cartilage cells characteristic of the early stages of ossification in cartilage.

The fact that the kidney tubules and the intestinal mucosa also secrete phosphatase in relatively high degree may seem to provide an argument against the hypothesis that this enzyme plays a part in the calcification of bone. The presence of a phosphatase among the numerous intestinal enzymes is scarcely surprising since it is probably required for the hydrolysis of phosphoric esters occurring in much food material. Its presence in the kidney is more interesting since this tissue does not normally

become calcified, although it is a frequent site of pathological ossification. It must be remembered, however, that the hydrolysis of phosphoric esters in any tissue will not necessarily be followed by the calcification of that tissue unless other factors such as the concentration of calcium ions and the pH of the tissue fluid are also favourable. We showed that in very young animals the phosphatase activity of the bones was usually much higher than that of the kidney, while the reverse might be true in adult animals. The same facts were subsequently demonstrated very clearly by Kay (1926), who found that in the animal embryo the phosphatase activity of the kidney was almost nil, while that of the developing bone was extremely high. During growth these values approached one another and, in the case of the rabbit, became approximately the same ten or twelve weeks after birth.

The significance of the phosphatase in kidney was investigated by Eichholtz, Brull, and myself (1925), using Starling's heart-lung-kidney technique. I have already referred to the fact that the excised kidney when perfused with defibrinated blood secretes no inorganic phosphate. The addition of a phosphoric ester to the blood was followed by the secretion of inorganic phosphate in the urine in high concentration, which led us to suggest that the kidney phosphatase may be concerned in the normal secretion of phosphates *in vivo*. The subsequent work of Brull (1928) and of Brain, Kay, and Marshall (1928) indicates, however, that the bulk of the urinary phosphates most probably do not arise in this manner but are derived from the inorganic phosphates in the blood.

For the preparation and investigation of bone phosphatase, the long bones of young rabbits are split longitudinally, and, after removal of the marrow, are placed in 5 times their weight of chloroform water, where they are left for 10 days or more at room temperature. Dry preparations can readily be obtained by precipitation from these aqueous extracts with alcohol-ether, and these retain their activity unimpaired for many months.

Such preparations have proved of great service as a biochemical reagent in the investigation of phosphoric esters (Martland and Robison, 1929).

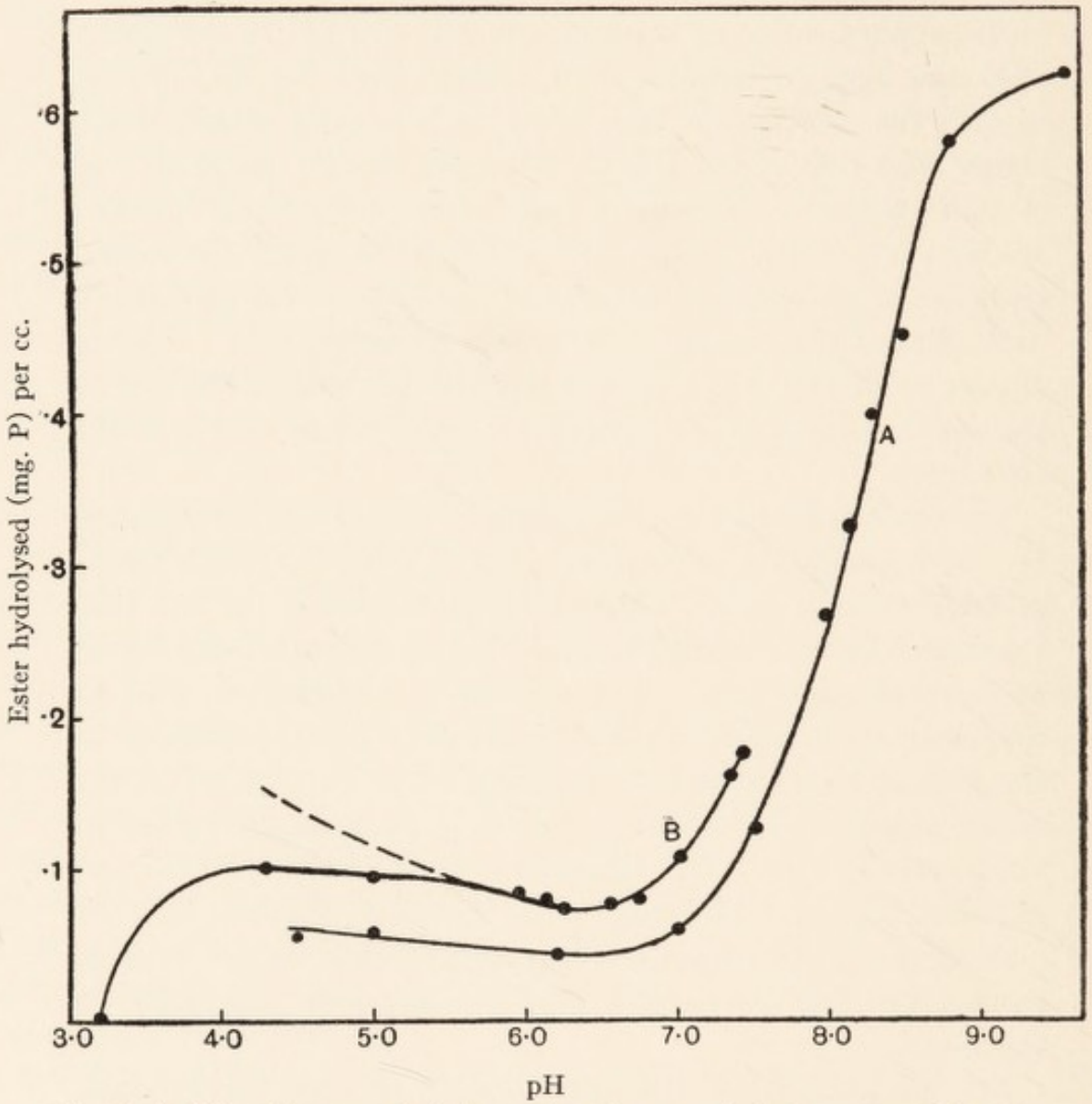


Fig. 1. Activity-pH curve of the bone phosphatase. Activity measured by the hydrolysis of 0.1 *M* sodium glycerophosphate and given as the amount of ester hydrolysed (mg. P) per cc. in 1 hour. Temp. 39.5°.

The first determinations of the optimum pH of the enzyme showed a very rapid increase in activity between pH 7 and pH 8.4, but no further change up to pH 9.4, above which the activity fell rapidly (Robison and Soames, 1924). This wide optimum

range was afterwards discovered to be due to the partial inactivation of the enzyme in the more alkaline solutions during the 18-hour tests. When the activity was measured over shorter periods of time it was found to increase with the pH up to 9.4 (Fig. 1). The pH activity curve was of the S type like those found by Michaelis and Davidsohn (1911) for invertase. The resemblance to the usual titration curve of a weak base or acid suggests that the hydrolysis is governed by the dissociation either of the enzyme itself or of its compound with the phosphoric ester. (Martland and Robison, 1927).

As with other enzymes the activity of the phosphatase is affected by the presence of the products of hydrolysis, but in this case the effect of phosphate is far greater than that of the second product, hexose, glycerol, or other hydroxy compound.

Even in very low concentrations inorganic phosphate markedly reduces the rate of hydrolysis of the ester, which indicates that the enzyme possesses a strong affinity for the phosphate radicle (Figure 2). In the older theories of calcification there is much talk of a calcium fixation by some constituent of cartilage, as in Freudenberg and György's scheme. We have here an example of phosphate fixation and I have speculated whether this property of the enzyme, quite apart from its hydrolytic activity, might not be utilised in the calcification process. Perhaps both properties should be considered together. A molecule of phosphoric ester is seized upon by the enzyme molecule and combined with it; the alcohol group is split off leaving the phosphate group attached. This then combines with wandering calcium and carbonate ions to form the bone salt.

The phosphatase has a much lower affinity for hexose, glycerol, or other hydroxy-compounds; and, in low concentration, these do not appreciably affect its activity in aqueous solutions. In higher concentrations, however, the reverse reaction, that of ester synthesis, becomes apparent. Miss Martland and I (1927) were able to demonstrate the synthesis of phosphoric esters by bone phosphatase from glycerol, glycol, mannitol, glucose, and

fructose in presence of inorganic phosphate. The maximum synthesis amounted to 25% of the total phosphate.

Although this synthetic action of the enzyme becomes manifest *in vitro* only when the concentration of hydroxy-compound is large and that of water is greatly reduced, it may, none the less be of great significance in the living tissues. We know that

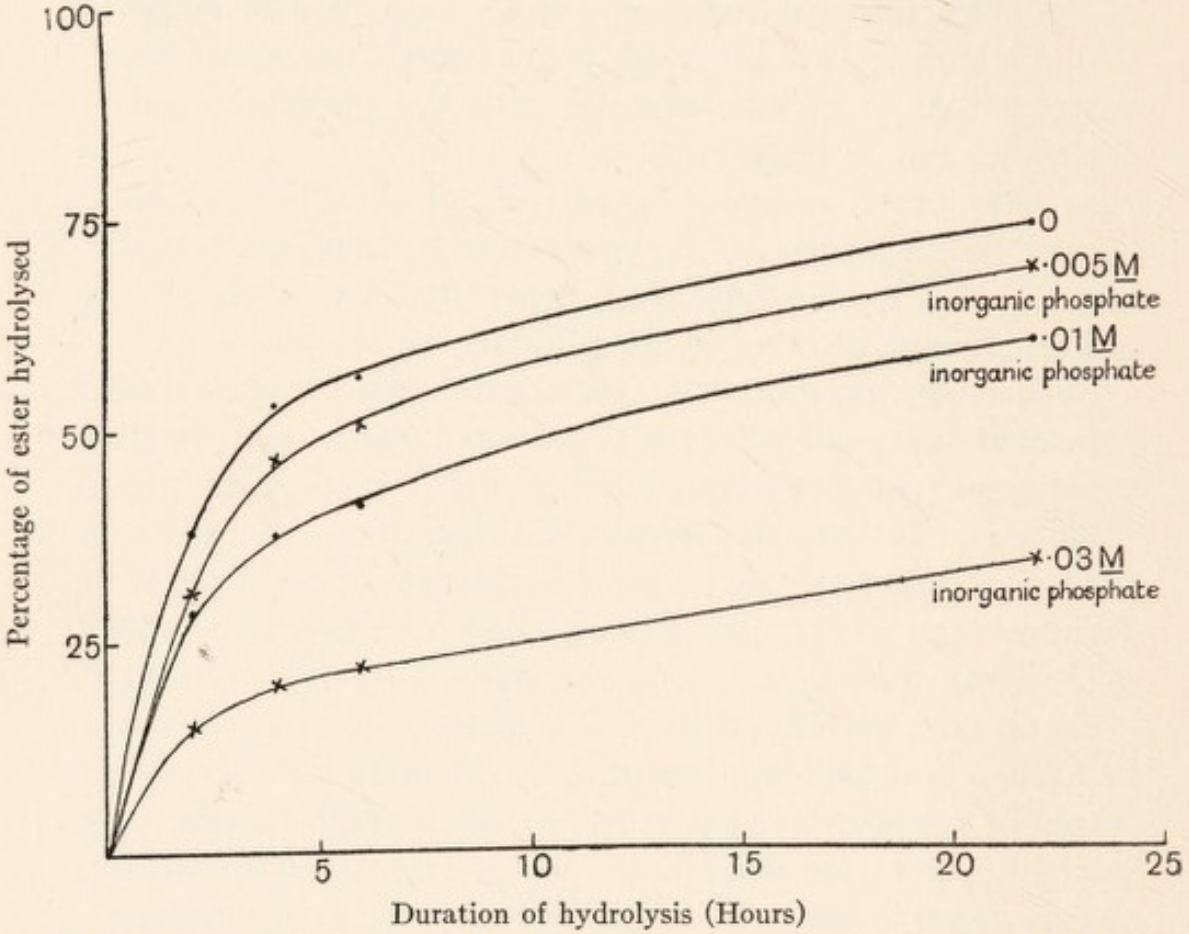


Fig. 2. Effect of inorganic phosphate on the rate of hydrolysis of glyceroposphhoric ester by the bone phosphatase. Concentration of ester 0.03 *M*. Concentration of added inorganic phosphate as shown on each curve. Temp. 38.0°.

glycogen is synthesised or hydrolysed by the liver and muscle enzymes, according to the immediate requirements of the body; and it seems to be equally possible that phosphoric esters should be synthesised or hydrolysed by the phosphatase of the osteoblasts according to the immediate requirements of the bone, and that this might be in part the explanation of the process of resorption and redeposition of the bone salt.

The high optimum pH of the bone phosphatase suggested that a second mechanism might conceivably play a part in the calcification process. If the alkalinity of the fluid in the hypertrophic cartilage and osteoid tissue is raised above that of blood, not only would the activity of the phosphatase be increased but the concentrations of $PO_4^{=}$ and $CO_3^{=}$ ions would also be increased at the expense of the more acid ions $HPO_4^{=}$ and HCO_3^{-}. All these changes would lead towards the same result, the supersaturation of the fluid with the bone salt, while the separate precipitation of a portion of the calcium carbonate might also be accounted for by such a mechanism. It is conceivable that such a difference in the pH might arise from the activities of the cells or from some Donnan equilibrium between the dense colloidal matrix and the surrounding fluids; but we have too little information as to the permeability of this ground substance to justify more detailed argument. It is not at all necessary that the pH of the matrix should be as high as 8.4. A very small upward shift would be effective.

In spite of many attempts, I have not succeeded in obtaining any satisfactory evidence either for or against this notion. The introduction of indicators into calcified cartilage or bone is likely to lead to erroneous conclusions since the re-solution of the bone salt itself could give rise to an alkaline solution. Dr. Fell and I are hoping to gain information through the cultivation of ossifying cartilages in media containing suitable indicators, some of which seem to be tolerated fairly well by the growing tissues. It may perhaps be as well to emphasise that the precipitation of the basic bone phosphate necessarily involves a fall in the pH of the residual fluid and this must be made good by the cells or by the buffers of the blood, whether before the precipitation or after.

Even should the pH of the cartilage and osteoid matrix be no higher than 7.4 there is no lack of phosphatase for the task required of it. It is unlikely that we have ever succeeded in extracting the full amount of enzyme present in a growing bone; but from our results it may be calculated that the phosphatase in

the bone of a young rabbit could in one hour at the temperature and pH of the blood hydrolyse enough phosphoric ester to precipitate an amount of bone salt equal to half the total weight of the bone.

Having described the bone enzyme we must now consider its substrate, the phosphoric ester, of which, unfortunately, we have

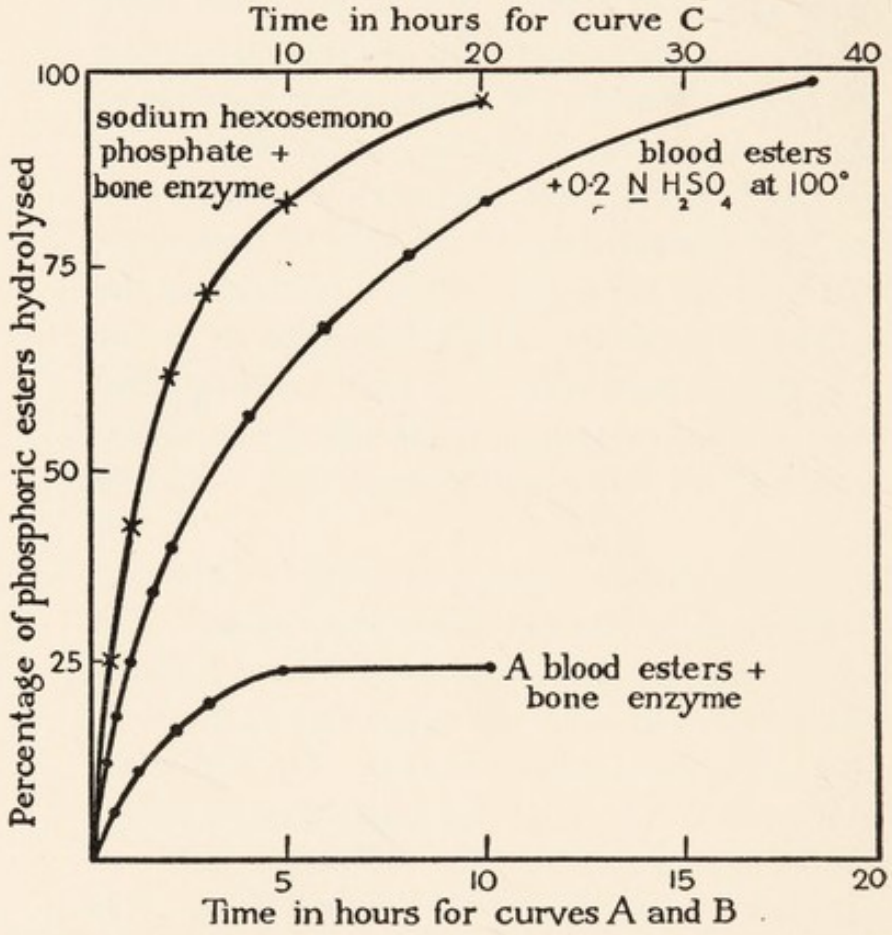


Fig. 3. Hydrolysis of the phosphoric esters of blood by bone phosphatase

less definite knowledge. It was known from the work of Greenwald (1916), Bloor (1918), Iversen (1920), and others, that the blood contains phosphoric esters of more than one type, ether-soluble phospholipins, present in both corpuscles and plasma, and certain acid-soluble phosphoric esters occurring chiefly in the corpuscles. Very little was known as to the nature of these acid-soluble esters when this investigation was begun.

In collaboration with Dr. Kay (1924), I investigated the effect of the bone phosphatase on these acid-soluble esters, and found that a fraction varying from 14% to 36% was readily hydrolysed while the residual fraction was scarcely affected by the enzyme but could be hydrolysed by boiling with acids. In Figure 3, Curve A represents the action of the bone phosphatase on the acid-soluble phosphoric esters from the blood of an adult rabbit. In this case about 25% of the total esters, reckoned as P, was hydrolysed in 5 hours after which there was no further liberation of phosphate. Curve B shows the almost complete hydrolysis of hexosemonophosphate by the same bone extract, while Curve C represents the hydrolysis of the blood ester by sulphuric acid.

TABLE 7.

Distribution of phosphorus in the acid-soluble compounds of blood.

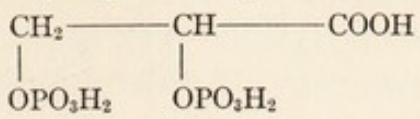
		mg. P per 100 g. blood.				
Animal.		Total acid soluble.	Inorganic.	Organic.	Hydrolyzed by bone phosphatase.	% of organic P hydrolyzed by bone phosphatase.
Cockerel	6 mths.	30.1	7.1	23.0	3.2	14
Rats	24 days.	38.5	10.9	27.6	9.5	34
"	3 mths.	27.8	5.0	22.8	7.4	32
"	9 mths.	29.1	5.4	23.7	5.4	23
Rabbit	3½ mths.	44.1	8.5	35.6	9.9	28
"	12 mths.	38.4	6.2	32.2	10.5	32
"	Adult venous	32.0	5.1	26.9	8.5	32
	arterial.	32.3	7.8	24.5	8.7	35
Child.	6 yrs.	21.9	4.3	17.6	6.4	36
"	11 yrs.	22.8	4.1	18.7	5.7	30
Man		25.2	2.6	22.6	6.0	27
"		26.6	3.3	23.3	7.0	30
"		21.9	3.2	18.7	6.1	33

Table 7 shows some of the results obtained by the application of this method to the blood of different animals. It was proved in this way that at least two different acid-soluble esters were present in all these samples of whole blood, and that one of these esters, if it is diffusible into the cartilage and osteoid ground substance, could serve as a substrate for the bone phosphatase in the calcification process. The bulk of these phosphoric esters are,

however, present in the corpuscles, mostly in the red corpuscles, though Kay (1926) has shown that leucocytes and blood platelets are relatively richer in these esters. The presence in the plasma of even the small amount (0.5 mg.-1 mg. P per 100 c.c.) reported by Greenwald and by Bloor has been disputed, but Miss Martland and I (1926), after a thorough examination of the sources of error in our estimations of total phosphorus, satisfied ourselves that such amounts are actually present. We examined the plasma from human blood and from that of rabbits, drawn both from veins and arteries at various intervals after food or after large quantities of sodium phosphate had been taken, but could find no regularity in the amounts of ester present. The highest value we found was 1.27 mg. organic P per 100 c.c., while the more usual value was from 0.4 to 0.5 mg. McKellips, De Young and Bloor (1921), who had previously obtained similar values for the plasma of adults, found much larger amounts in that of young infants up to two weeks old, the highest figure being 3.(mg. with an average of 2.2. We showed, however, that this small amount of acid-soluble ester in plasma is completely hydrolysable by the bone phosphatase. The phospholipins, on the other hand, though present both in plasma and corpuscles in large amounts, are not attacked to any extent by aqueous extracts of bones, but if the choline group were removed by any other enzyme in the blood or tissues it is possible that the phosphate group could then be split off by the bone phosphatase.

It was suggested by Bass in 1914 that adenine existed in blood in the form of the nucleotide, adenylic acid. In 1924 this compound was isolated in somewhat impure form by Jackson from human blood and in the following year from pig's blood by Hoffman (1925), who obtained the pure crystalline compound. About the same time Greenwald (1925) isolated from pig's blood and also from dog's and human blood a new compound, the diphosphoric ester of laevo-glyceric acid, in amounts representing from 36% to 55% of the organic acid-soluble P.

Diphospho-laevo-glyceric acid.



It may be noted that adenine nucleotide is hydrolysed by the bone phosphatase but diphosphoglycerate is less readily attacked.

Some progress had been made in the separation of the blood ester by Mr. Goodwin and myself (1924) when these discoveries were published. A large non-reducing fraction had been obtained, which, though not then identified, was found to consist chiefly of Greenwald's diphosphoglycerate. A much smaller fraction, possessing marked reducing properties, was thought to contain a hexosephosphate, though obviously in very impure condition or else combined with some large molecule; but the work had to be put on one side for a time and I am still somewhat uncertain whether a free hexosephosphate is present or not. Lawaczeck (1924) has also described the presence of a hexosephosphate in blood and has carried out certain investigations (1925, 1928) on the variations in the amount of this ester; but his results are not more conclusive as to its identity than are our own. Further evidence of the occurrence of this ester has, however, recently been obtained by Roche (1930).

Posternak (1928) has claimed that horse blood contains another ester, which reduces Fehling's solution, namely, diphospho-keto-trihydroxyadipic acid, but his published evidence for this is very slight.

It is probable that Greenwald's diphosphoglycerate constitutes a large proportion if not the whole of the fraction of acid-soluble ester not readily hydrolysed by the bone phosphatase, while the hydrolysable fraction contains adenine-ribose-phosphate and possibly a hexosephosphoric ester. Whether the esters present in plasma are identical with the hydrolysable esters of the corpuscles and in equilibrium with them is not known.

It was noted by Bloor and other workers that if blood is allowed

to stand for some hours after withdrawal from the body the inorganic phosphate increases. The cause of this increase was investigated by Lawaczeck (1924) and by Martland, Hansman, and Robison (1924). It was shown to be due to the activity of a phosphatase present in the corpuscles. This enzyme is most active at a pH between 6 and 7 and is, therefore, quite distinct from the bone phosphatase. Furthermore, it hydrolyses those esters in the blood on which the bone enzyme has no action.

The hydrolysis takes place rapidly when blood is laked and also in unlaked blood or corpuscles if the pH is lowered by absorption of carbon dioxide. We confirmed Lawaczeck's finding that in unlaked blood which has lost CO_2 by contact with air a decrease in the inorganic phosphate, indicating synthesis of a phosphoric ester, occurs during the first two or three hours. The relationship of these changes to the pH was studied by Martland (1925) who showed that a delicate equilibrium exists in the intact corpuscles, synthesis of phosphoric esters occurring if the pH of the plasma rises above 7.35 and hydrolysis if the pH falls below 7.3. There is no reason to doubt that this equilibrium exists also in the circulating blood and may account for some of the changes in inorganic phosphate content in conditions of acidosis and alkalosis. It should be remembered, however, that in the living body in such conditions the loss or gain of phosphate from other sources may mask the changes due to the corpuscular phosphatase.

Some evidence was obtained that this synthesis of phosphoric ester may be connected with the process of glycolysis, a question which also has been the subject of numerous investigations by other workers (*v.* Roche, 1930). For the problem of calcification there is one possibility which, although at present no more than a speculation, I would like to mention, namely, that the diphosphoglycerate may, by the action of the corpuscular phosphatase yield a monophosphoric ester which then diffuses into the plasma and tissue fluids and serves as substrate for the bone phosphatase.

While studying this enzyme of the corpuscles we found that the plasma also contains a phosphatase, though in very small amount, and that its optimum pH and other properties are similar to those of the bone phosphatase (Martland and Robison, 1926). It may possibly be derived from bone by slow diffusion, or it may arise from the breakdown of the white cells which have been shown to contain a phosphatase of the same type (Roche, 1931).

The relative phosphatase activity of the plasma in normal and pathological conditions has been investigated by Kay (1929; 1930), who observed a marked increase in certain cases confined almost exclusively to bone diseases, osteitis deformans, generalised osteitis fibrosa, osteomalacia, and rickets of different types. Kay considers, I think rightly, that the abnormally high activity is more probably to be looked upon as the result of the diseased condition than as its cause.

I have already indicated that there is no lack of phosphatase activity in the bones of animals suffering from experimental rickets. The activity per unit weight of such bones is even higher than that of normal bones. Miss Soames and I (1925) investigated the possibility that there might in such cases be a deficiency in the amount of hydrolysable phosphoric esters in the blood. We examined the blood of rats fed on various rickets-producing diets, but could not detect any significant difference in the percentage of hydrolysable esters in the whole blood from the average values found for normal animals. It is possible that differences may exist in the amount of hydrolysable esters in plasma, but this has not been established and would be difficult to prove without further improvements in our technique.

It would seem, therefore, that the cause of deficient calcification in rickets must be looked for in one or more of the other factors involved, for example, the concentration of ionised calcium or phosphate, the pH of the tissue fluid, or the permeability or other physical properties of the cartilage and osteoid tissues.

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III

CALCIFICATION *IN VITRO*

THE experimental results which were described in the previous lecture appeared to lend considerable support to my conception of the rôle of phosphatase in normal calcification. The fact that bones of rachitic animals were not lacking in this phosphatase led me to try whether the deficient calcification of such bones might not be made good by immersing them *in vitro* in solutions of calcium hexosemonophosphate or other hydrolysable phosphoric esters. In the first experiments, Dr. H. Goldblatt gave generous help on the histological side; and later, Miss K. M. Soames collaborated with me in this part of the work.

Rib junctions or entire heads of the bones of rachitic rats were soaked for periods of 8 to 24 hours in a tenth molar solution of calcium hexosemonophosphate or calcium glycerophosphate at 37°, corresponding rib junctions or bones being placed in solutions of sodium phosphate or calcium chloride as controls. In some cases the bones were cut longitudinally, one half then serving as a control for the other. In these early experiments chloroform was used as an antiseptic. After the immersion the bones were washed, fixed, and stained with silver nitrate (Robison, 1923; Robison and Soames, 1924).

Figure 1 shows a section of the tibia of a normal rat age 42 days. Notice the zone of small-celled epiphysial cartilage, which contains no phosphatase, and below this the zones of proliferating and hypertrophic cartilage, the latter in process of calcification.

Figure 2 is a section of the radius of a rat which had been fed for some weeks on a rickets-producing diet (McCollum, 3143). Note the broad metaphysis of uncalcified hypertrophic cartilage

PLATE I

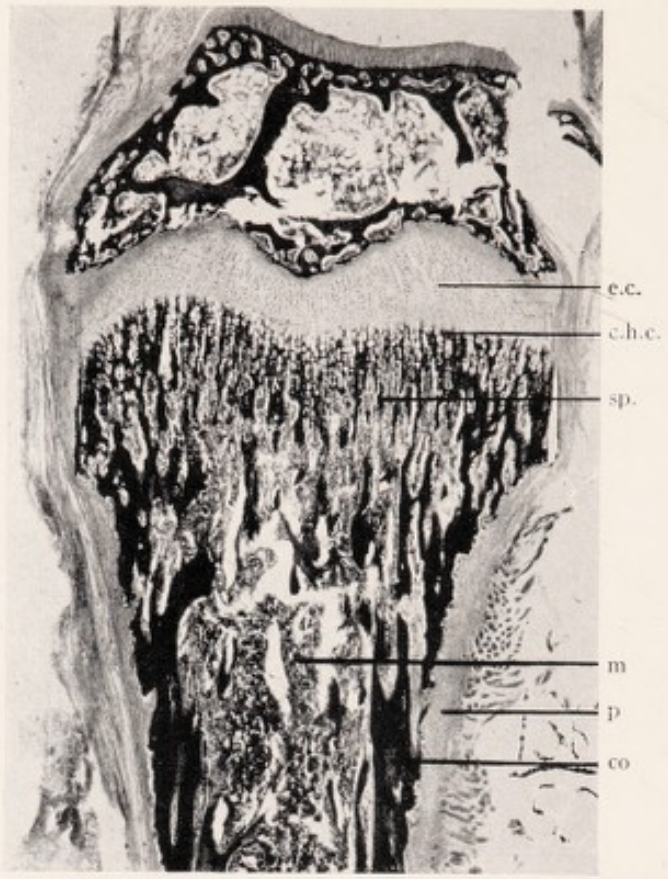


FIG. 1

FIG. 2

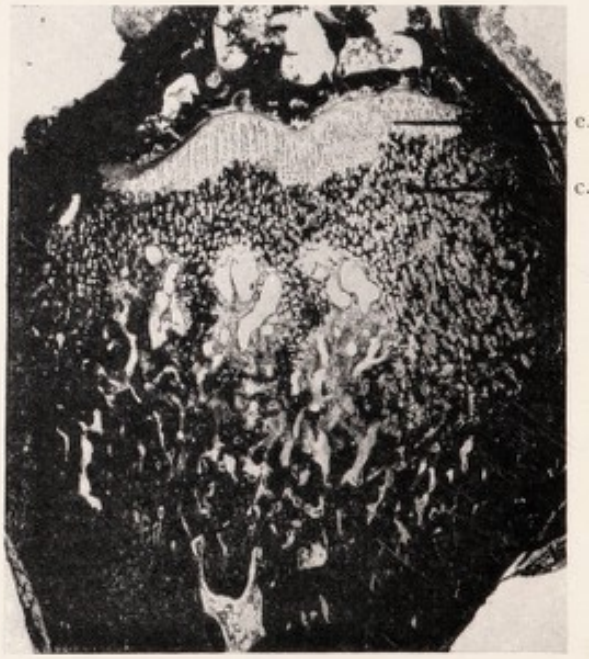


FIG. 3

which is characteristic of rickets. This bone had been immersed for 16 hours in a solution of sodium phosphate and served as a control for the bone shown in the next figure.

Figure 3 shows the other radius of the same rachitic rat. This bone was immersed for 16 hours in a solution of calcium hexosemonophosphate; and the entire zone of hypertrophic cartilage has become densely calcified *in vitro*, while the epiphysial plate of small-celled cartilage has remained quite free from deposit.

In these experiments the conditions were far from physiological; and yet calcification occurred *in vitro*, in the matrix of the hypertrophic cartilage where calcification would have taken place *in vivo* if the animal had been fed on a normal diet. The concentration of calcium in the solutions was much higher than that occurring in plasma, but no inorganic phosphate was present at all until liberated from the phosphoric ester by the action of the phosphatase in the bone itself. It was, therefore, concluded from these results that the enzyme must be located in the hypertrophic cartilage where the deposition occurred and in the osteoid tissue where increased calcification *in vitro* was also observed. From the whole of the experimental evidence I formed the opinion that phosphatase was secreted by osteoblasts and also by cartilage cells during hypertrophy.

Shortly afterwards it was observed by Shipley (1924) that, when slices of the cartilage and bone of a rachitic rat are placed in the serum of normal rats, calcification occurs in a similar manner to that taking place in a living rat fed on a curative diet.

Later, Shipley, Kramer, and Howland (1926) showed that slices of the cartilage and bone of rachitic rats became calcified by immersion in sterile solutions of inorganic salts—sodium chloride, magnesium sulphate, and sodium bicarbonate,—containing calcium and inorganic phosphate in concentrations similar to those in plasma. Thus, with 10 mg. calcium and 4 mg. phosphorus per 100 c.c., calcification occurred regularly in 9 hours, and with 3.5 mg. phosphorus per 100 c.c., in 16 to 24 hours. They found that the calcification was normal in charac-

ter, the deposit being formed in the matrix of the hypertrophic cartilage on the epiphysial side of the metaphysis—the phenomenon observed by Schmorl (1909) in the healing of rickets in children and utilised as the "line test" of McCollum, Shipley, Simmonds, and Park (1922). They noted, however, that the presence of protein to the extent of even 1% or 2% inhibited calcification at these levels of calcium and inorganic phosphate. Calcification was also inhibited by heat, cold, and protoplasmic poisons including chloroform and potassium cyanide. They could give no explanation of the selective deposition of calcium salts, but they considered that the process was not one of simple precipitation but depended on the activity of the living tissue. They rejected my suggestion that phosphatase played any part in the process. These most interesting results undoubtedly proved that the hypertrophic cartilage possesses some property favourable to the deposition of the calcium salt quite apart from its enzymic activity; but, at the same time, I was not able to accept the view that the concentrations of the bone-forming constituents in their experimental solutions resembled those of normal plasma (Robison, 1926). In my opinion, the absence from these solutions of proteins and other organic constituents which greatly depress the ionisation of calcium in plasma largely destroyed the significance of the apparent similarity in the calcium concentrations. The inorganic solutions used in the experiments of Shipley, Kramer, and Howland were undoubtedly highly supersaturated with respect to the bone salt. As Miss Maclean and I have shown, such solutions deposit precipitates similar in composition to the bone salt if left at 37° and pH 7.4 for some days or weeks. Now we have seen that plasma is probably not supersaturated with regard to this bone salt; it cannot in any case be supersaturated to the same degree as the inorganic solutions which bring about the calcification of rachitic bones *in vitro*. It appeared to me more reasonable to retain my first opinion that, by the activity of the bone phosphatase, a local supersaturation is produced in the ground substance of hyper-

PLATE II

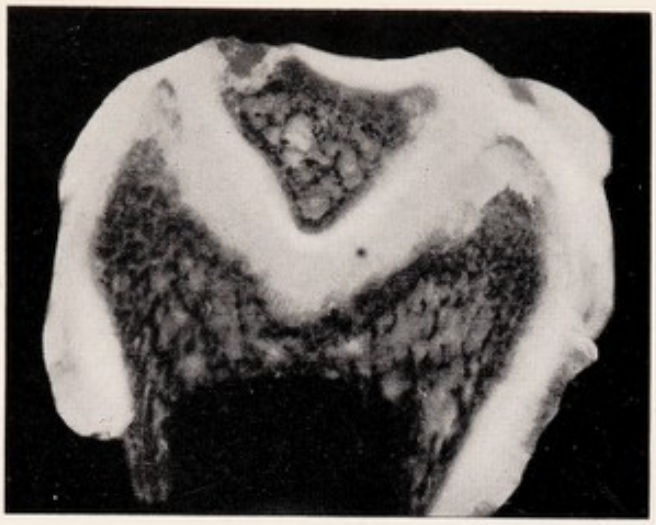


FIG. 4

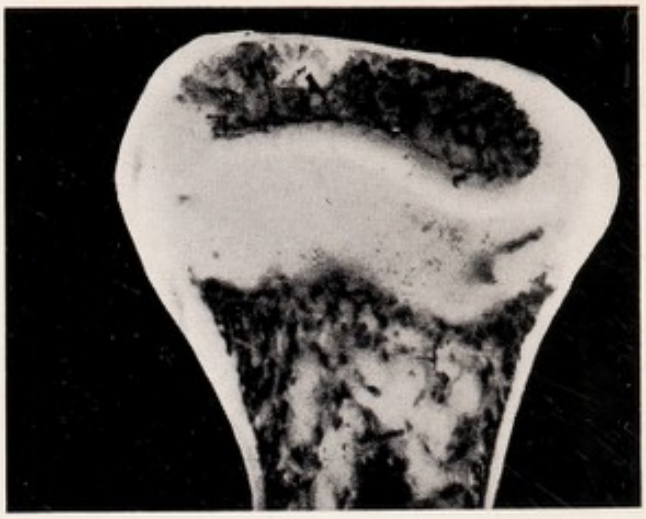


FIG. 7

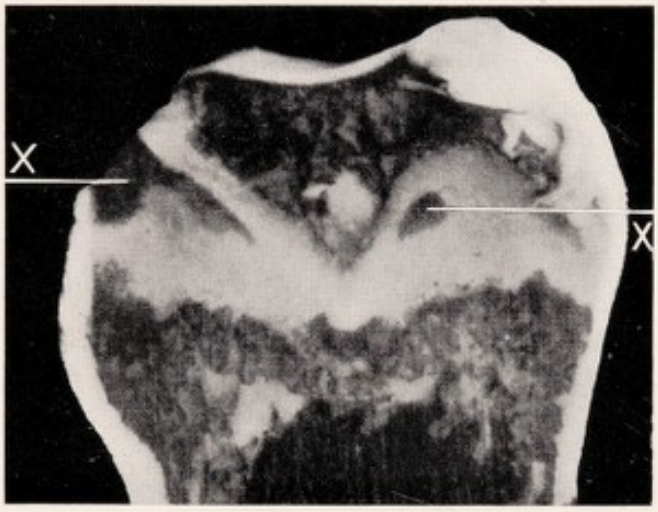


FIG. 5

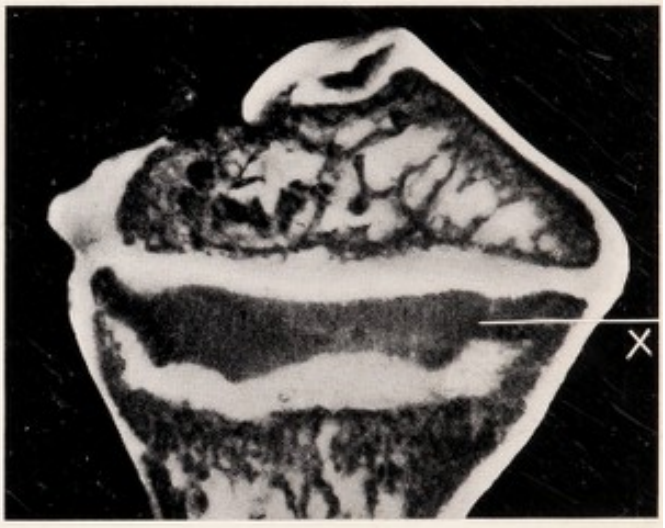


FIG. 8

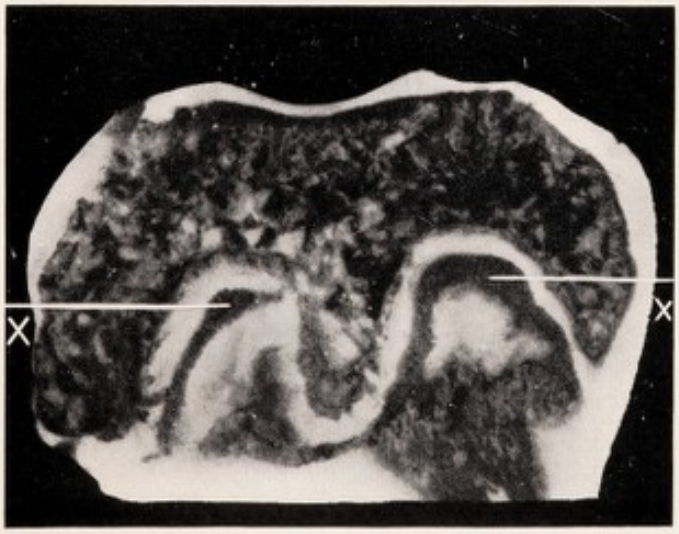


FIG. 6

FIG. 9

trophic cartilage and osteoid tissue, and that a second mechanism possessed also by these tissues assists in the deposition of the salt from this supersaturated solution. In the experiments of Shipley, Kramer, and Howland, the solutions allowed to diffuse into the cartilage matrix were already highly supersaturated, so that the action of the phosphatase was not required.

Meanwhile Miss Soames and I had continued our experiments, reducing the concentration of ester, adding other salts to the solutions, and in general approximating somewhat more closely to physiological conditions. We confirmed the main results of Shipley, Kramer, and Howland with inorganic solutions; but we also showed that, while calcification can undoubtedly be effected in such inorganic solutions provided that the calcium and phosphate levels are sufficiently high, it will take place at lower levels of calcium and inorganic phosphate if a phosphoric ester is also present even in very small amounts (Robison and Soames, 1928, 1930).

Figures 4 to 9 show the results of one experiment in which slices cut under sterile conditions from the distal heads of the femora and the proximal heads of the tibiae of a rachitic rat were immersed in solutions containing the same concentrations of calcium and inorganic phosphate but different concentrations of phosphoric ester. Each slice was placed in a small stoppered flask containing 40 c.c. sterile solution at pH 7.35 and kept at 38° during 16 hours, after which it was washed, stained with silver nitrate, and photographed, without clearing, by reflected light.

The basal solution used in this experiment had the following composition:

	g. per 100 c.c.	m. Mol.
NaCl	0.90	103.0
$NaHCO_3$	0.03	3.6
KCl	0.04	5.4
$MgSO_4$, $7H_2O$	0.025	1.0
Phenol red	0.0003	—

The concentration of calcium in all solutions was 10 mg. per 100 c.c. and of inorganic phosphate 3.5 mg. P per 100 c.c.

The solutions were sterilised and all operations were carried out with precautions to prevent bacterial contamination. Chloroform was not added. No calcification occurred in any of the slices immersed in the solutions in which no phosphoric ester was present; but the addition of ester—in this case glycerophosphate—equivalent to only 0.5 mg. P per 100 c.c. caused a definite though small amount of calcification to occur. The extent of this deposit increased with each further increase in the concentration of ester.

Figure 4 shows one of the femur slices which had been immersed in the solution containing no phosphoric ester. Note the wide zone of uncalcified hypertrophic cartilage in the metaphysis.

Figure 7 is a slice of the tibia immersed in the same solution. In both these slices the picture is the same as that shown by the controls. No new calcification has occurred *in vitro*.

Figure 5 is a femur slice immersed in the solution containing 0.5 mg. organic P per 100 c.c. Notice the definite new calcification, beginning at the epiphysial side of the hypertrophic cartilage.

Figure 6 is a femur slice immersed in the solution containing 1 mg. organic P per 100 c.c. The amount of new deposit is much greater than in the previous picture.

Figure 8 is a tibia slice immersed in the solution containing 2 mg. organic P per 100 c.c. There is a further marked increase in the amount of new calcification.

Figure 9 is a tibia slice immersed in the solution containing 4 mg. organic P per 100 c.c. Almost the entire zone of previously uncalcified hypertrophic cartilage has here become calcified. The narrow zone of small-celled epiphysial cartilage is, however, free from calcium salts as it would be in the tibia of a normal rat at this age.

It is, therefore, clear that, at critical levels of calcium and inorganic phosphate, as little as 0.5 mg. P per 100 c.c. in the form of phosphoric ester, that is, amounts of the same order as have been shown to occur in plasma, can determine the deposition of cal-

cium salts in the matrix of the hypertrophic cartilage, and that with higher concentrations of ester the amount of deposit is correspondingly increased. The "critical levels" of calcium and inorganic phosphate were, in these experiments, still considerably above the concentrations required for a saturated solution of the bone salt (*v.* Lecture II, Table 2); but calcification occurred also at much lower levels of these inorganic constituents if the amount of phosphoric ester was increased.

In comparing the new calcification in these bone slices with that found in healing rickets, two facts are to be borne in mind: first, that *in vitro* the dissolved salts must diffuse into the cartilage through the cut surfaces instead of being distributed throughout the matrix by the blood vessels; and second, that the deposits are formed in a few hours, whereas *in vivo* several days would be required. Owing to tissue degeneration and loss of enzyme, it was rarely found advantageous to increase the period of immersion beyond 24 hrs. It is therefore not to be assumed without question that calcification *in vitro* will occur under such conditions with solutions of the same ionic concentrations as those in tissue fluids.

In drawing conclusions from these experiments as to the influence of the phosphoric ester on calcification, there was one point to which criticism might be directed. During the immersion of the bone slice, a slight increase occurred in the concentration of inorganic phosphate in the surrounding fluid owing to diffusion of phosphatase from the bone. This increase rarely exceeded 0.5 mg. P per 100 c.c., and occurred chiefly toward the end of the period of immersion; but, in order to avoid this complication of the argument, we carried out a large number of experiments in which the sterile calcifying solutions were made to flow continuously over the bone slices or half bones of rachitic rats and rabbits for periods of from 18 to 48 hours. The results entirely confirmed those already shown. In every case calcification was obtained at lower levels of calcium and inorganic phosphate if phosphoric ester was present than in its absence. A further fact which

emerged from these experiments was that bones of different rats varied in their response to these inorganic calcifying solutions, that is, a solution of a certain concentration might effect calcification in bones of one rat but not in those of another rat even from the same litter. These variations might amount to as much as 20% of the minimum level of the product [Ca] × [P] adequate for calcification.

The next step in our investigations was to discover the influence of the various ions and organic constituents of plasma on calcification *in vitro*. It has been shown by Shipley and Holt (1927) and by Kramer, Shelling, and Orent (1926, 1927), that sodium chloride and to a less extent potassium chloride have an inhibitory effect on calcification, i.e., that higher [Ca] × [P] levels are necessary when the concentrations of these salts are increased. They showed also that magnesium has a much more pronounced inhibitory effect even in such relatively low concentrations as are normally found in plasma. These studies were, however, confined to calcification in inorganic solutions, while I was further interested in the effect of these and other radicles on calcification in presence of phosphoric ester.

The first group of experiments I shall describe was carried out in collaboration with Miss M. Macleod and Miss A. H. Rosenheim (1930). The solutions used in the previous experiments had contained only 0.03% sodium bicarbonate. When the bicarbonate concentration was raised to 0.2%, a level approximating more closely to that in the blood, we observed that calcification was regularly taking place at lower levels of the [Ca] × [P] product than before, and traced this favourable influence to the increased amount of bicarbonate.

Table 1 shows the calcification obtained at different [Ca] × [P] levels in the presence of different amounts of bicarbonate.

Solution A contained 0.2% sodium bicarbonate while B and C contained none. In solution C the concentration of sodium chloride was increased from 0.5% to 0.65%, so that the total sodium content was the same as in solution A. In all these

experiments the slices were cleared before examination, and the density as well as the extent of the new calcification was adjudged. In these tables the extent is denoted by a number (maximum 10) and the density by bars (maximum ≣).

TABLE 1.

Effect of bicarbonate on calcification *in vitro*. 16 hrs. 37°C. pH 7.4.

	mg. per 100 c.c.					
Ca	10	10	10	10	8	8
Inorganic P	3	4	2.5	2.5	5	4.5
Organic P	0	0	3	10	0	3
Solution A. 0.5% NaCl, 0.2% $NaHCO_3$		3=	3≣	8≣	3=	7≣
Solution B. 0.5% NaCl, 0 $NaHCO_3$					0	4≡
Solution C. 0.65% NaCl, 0 $NaHCO_3$	0	0	0	6=	0	4=

The fact that calcification regularly occurs at lower levels of calcium and inorganic phosphate when the concentration of bicarbonate is high must be taken, I think, as evidence that a complex carbonato-phosphate, of lower solubility than tricalcium phosphate, is precipitated. Separate precipitation of calcium carbonate would seem to be excluded, since in the absence of phosphate no deposition occurred even at much higher calcium levels.

It will be seen that when much phosphoric ester was present the favourable effect of bicarbonate was somewhat masked owing to the calcification being nearly maximal in all slices. A concentration of 0.2% sodium bicarbonate was used in all the following experiments.

Table 2 shows the effect of sodium chloride on calcification *in vitro*. In inorganic solutions inhibition by 0.2% and 0.5% NaCl was quite marked, as observed by Shipley and Holt (1927).

The presence of ester tended to mask this effect owing to greatly increased calcification in all slices.

TABLE 2.

Effect of sodium chloride on calcification *in vitro*. 16 hrs. 37°C. pH 7.4.

mg. per 100 c.c.	Ca.	8	8	8	8
	Inorg. P.	2.5	3	2.5	2.5
	Org. P.	0	0	3	10
NaCl g. per 100 c.c.:					
0		5=	2=	7≡	9≡
0.2		0	2=	7≡	9≡
0.5		0	0	6=	8≡

Table 3 shows the effect of potassium and magnesium salts. In concentrations up to twice that normally found in plasma, potassium has little or no inhibitory effect; but magnesium in concentrations as low as those which occur in plasma very definitely reduces the tendency of the calcium salt to precipitate.

TABLE 3.

Effect of potassium and magnesium salts on calcification *in vitro*. 19 hrs. 37°C. pH 7.4.

mg. per 100 c.c.	Ca.	8	8	8	8
	Inorg. P.	4	5	3.5	3.5
	Org. P.	0	0	3	30
K.	Mg.				
0	0	5≡	6≡	3≡	9≡
0	2.4	2≡	6≡	1=	7≡
0	4.8	0	2=	1=	6=
19.5	0	6≡	6≡	8≡	6≡
19.5	2.4	4≡	4≡	3=	9≡
19.5	4.8	0	0	1=	7≡

This may perhaps be due to the formation of a sparingly ionised magnesium phosphate. Here again the presence of phosphoric ester gave greatly increased deposits and to some extent masked the inhibitory effect of magnesium.

Table 4. Calcification was obtained in inorganic solutions over a wide range of calcium and phosphate concentrations from

2.5 mg. to 50 mg. of calcium and from 1 mg. to 14 mg. P as inorganic phosphate per 100 c.c. The minimum level of the product of these concentrations required for calcification was, however, always decidedly lower if phosphoric ester was present in the solution, even in such small amounts as normally occur in plasma.

TABLE 4.

Calcification *in vitro* at different levels of calcium and inorganic phosphate.

Inorg. P. mg. per 100 c.c.	Ca. mg. per 100 cc.						
	2.5	5	10	20	30	40	50
1				1−	2=	3=	2=
1.5				3≡	3≡		
2				5=			
2.5			0	8≡			
3			2−				
3.5			4=				
4		0	4=				
5		0	4≡				
6		0					
7		4−					
8	0	4=					
10	0	7≡					
12	0						
14	1−						
16	3−						

Calcification was observed in solutions of pH as low as 6.5 and as high as 8.0, higher concentrations of calcium and phosphate being naturally required in the more acid solutions. The best results, however, were obtained when the pH of the solution approximated to that of blood.

Although the slices were usually immersed in the solutions for periods of about 16 hours, deposits can be obtained in presence of phosphoric ester in as short a time as 2 hours.

This can be seen from the results shown in Table 5, which also furnish an example of the variation in the response of bones from different rats even from those of the same litter fed on identical diets and showing no significant difference in rate of growth. If this variation is due to some factor which is active also in the

living animal it must obviously be of significance for calcification; and we are trying to discover its cause. The difference was much less marked when phosphoric ester was present, indicating that it is not to the phosphatase content of the bone but to the second favouring factor that we must look for an explanation.

TABLE 5.

Time required for calcification of slices of rachitic bones with and without a phosphoric ester.

mg. per 100 c.c.					
Ca.		8		8	
Inorg. P.		5		4	
Org. P.		0		30	
Time (hrs.)	Rat a.	Rat b.		Rat a.	Rat b.
2	0			3–	
3	0			4=	4=
4	0	0		6=	
5		0			5–
6	0			6≡	
7		0			6≡
8		0			8=
10	4=	0		8≡	8≡
23	3≡	0		9≡	7≡

Shipley, Kramer, and Howland (1925) showed that calcification in inorganic solutions did not take place if the bone slices were previously soaked in chloroform, 95% alcohol, ether, or toluene, or if toluene was added to the solution. Now my own first experiments were carried out in presence of chloroform and yet good calcification was obtained. We have, therefore, studied the effect of these organic solvents in considerable detail, and have found that, while the capacity of the cartilage to become calcified in supersaturated inorganic solutions is very much weakened by such treatment, calcification will still occur in the matrix in the normal manner if the solutions contain phosphoric ester.

Table 6 shows some results of these experiments. The bone slices were soaked for 20 hours in the solvents before immersion for 16 hours in the calcifying solutions, other slices of the same

PLATE III

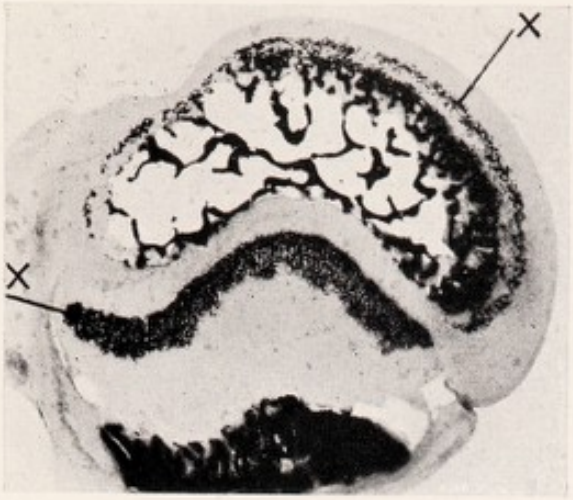


FIG. 10

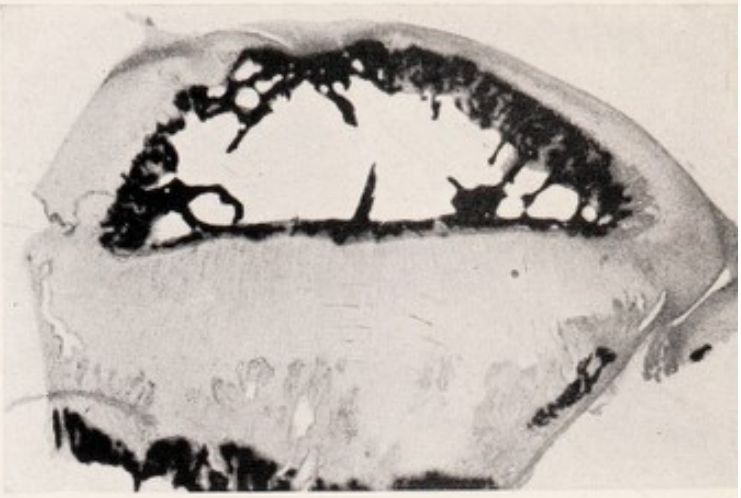


FIG. 11

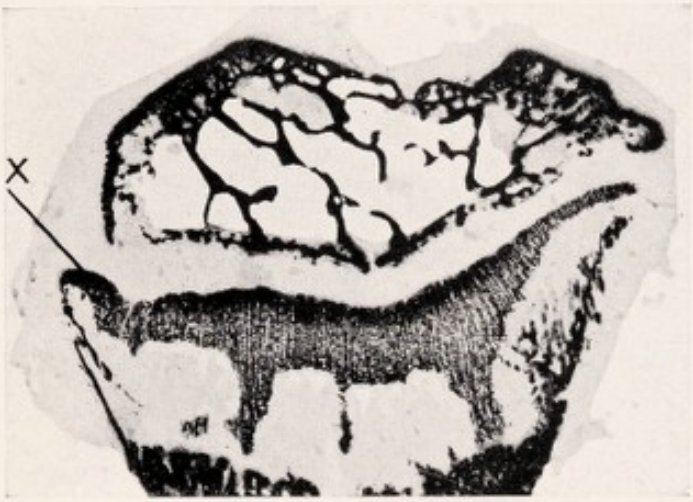


FIG. 12

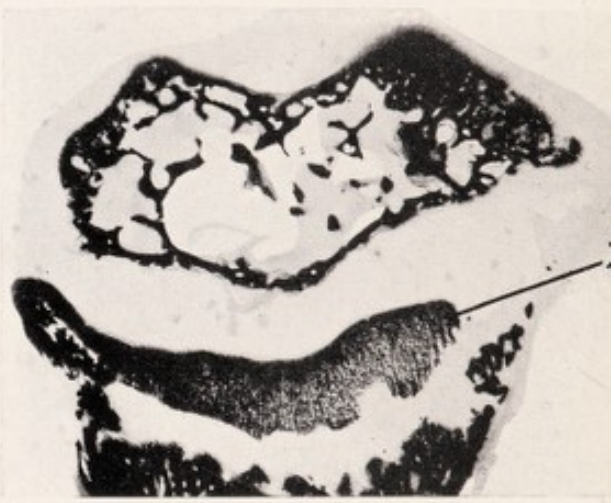


FIG. 13

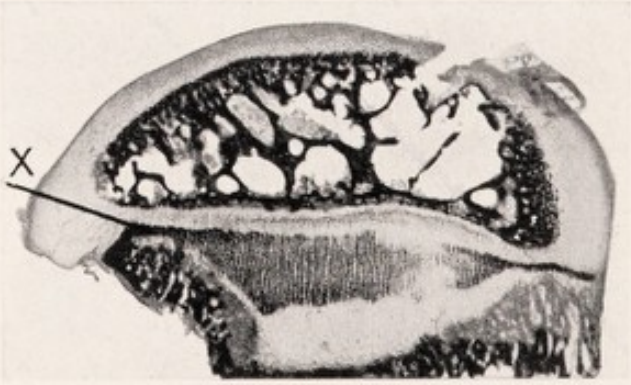


FIG. 14

bones being placed directly in these solutions without previous treatment. You will note that whereas good calcification occurs in the untreated bones in both the inorganic solutions 8:5:0* and 8:6:0, there was little or no deposit in the bone slices which had been treated with these solvents. When phosphoric ester was present, however, calcification occurred in all slices, treated or untreated. Similar results were obtained by dehydrating the bones in an evacuated desiccator overnight before placing them in the solutions. It was surprising to see how rapidly these shrivelled pieces of bone cartilage resumed their normal appearance and how well they calcified in the organic solutions.

TABLE 6.

Effect of 20 hrs. immersion of bone slices in organic solvents on subsequent calcification *in vitro*. 16 hrs. 37°C. pH 7.35.

mg. per 100 c.c.	Ca.	8	8	8
	Inorg. P.	5	6	4
	Org. P.	0	0	30
Untreated		5≡	6≡	8≡
Alcohol		0	0	9≡
Acetone		0	1−	9≡
Chloroform		0	1−	9≡
Dehydrated 24 hrs. *in vacuo*		0	1−	7≡

Figures 10 to 14 show photomicrographs of sections cut from some of these slices without decalcification. Figure 10 is a section of the humerus of a rachitic rat. This slice was not treated with organic solvents but was immersed for 18 hours in the inorganic solution, 8:5:0. The section shows a broad band of newly calcified hypertrophic cartilage adjacent to the small-celled epiphysial cartilage. Note also the corresponding band formed on the articular surface of the epiphysis. This was often noticed in humeri and femora and illustrates the same phenommena of growth and calcification occurring in the epiphysis as in

* The concentrations of calcium, inorganic P, and organic P respectively in mg. per 100 cc. are thus indicated throughout the text.

the shaft, only more slowly so that the breadth of the zone is much less.

Figure 11 is a section of the tibia of the same rat. This slice was immersed for 22 hours in acetone and then for 18 hours in the same inorganic solution 8:5:0. There is here no new deposit at all in the hypertrophic cartilage.

Figure 12 is a section of the tibia of the same rat, not treated with acetone but immersed for 18 hours in the organic solution 8:4:30 (containing glycerophosphoric ester). There is extensive new calcification in the hypertrophic cartilage.

Figure 13 is a section of the tibia of the same rat. This slice was immersed for 22 hours in acetone and then in the organic solution 8:4:30. The new calcification is here almost as extensive as in the untreated slice (Figure 12), showing that the treatment with acetone has little effect on subsequent calcification in solutions containing phosphoric ester.

Figure 14 is a section of another tibia which was immersed for 22 hours in 90% alcohol and thereafter in the organic solution 8:4:30. Note the normal appearance of the new deposit in the matrix of the hypertrophic cartilage.

We found further that the addition of potassium cyanide to the calcifying solution, in concentration as low as M/1000, inhibited calcification in inorganic solutions but scarcely affected the calcification in solutions containing ester. On the other hand, addition of 10% formalin entirely prevented calcification in presence or absence of ester. These results may be correlated with the effect of the different reagents on the bone phosphatase. Acetone, alcohol, and chloroform have little or no effect on its activity. Potassium cyanide has a slight inhibitory effect, but the enzyme is completely inactivated by formalin.

The results of these experiments set in sharp distinction two mechanisms in calcifying hypertrophic cartilage: the phosphatase mechanism which produces in the matrix fluid by hydrolysis of phosphoric ester a condition of supersaturation with respect to the bone salt; and a second mechanism which favours

the deposition of the bone salt from supersaturated inorganic solutions, whether the condition of supersaturation in the cartilage matrix is produced by enzymic hydrolysis of phosphoric ester or by allowing an external supersaturated solution to diffuse into it.

The nature of this second factor is unknown. It may be a slight increase in the pH of the matrix fluid, brought about by some membrane equilibrium—the specific affinity of certain groups in the matrix colloids for calcium or other constituent of the bone salt—some condition of strain causing crystallisation to begin. The fact that it is injured by potassium cyanide in very low concentrations suggests that the activity of the living cell may be necessary for its action. Desiccation of cartilage or treatment with fat solvents greatly reduces its effectiveness while leaving the phosphatase mechanism unimpaired.

I believe that both mechanisms play a part in normal calcification of the skeleton—the phosphatase producing in the matrix of hypertrophic cartilage and osteoid tissue the necessary degree of supersaturation, and the second mechanism assisting in the deposition of the bone salt from this supersaturated solution in some way not yet understood.

It would seem possible that some variation in the power or activity of this favouring mechanism in bones of different animals may account for the different levels of calcium and phosphate necessary for their calcification in inorganic solutions.

The gradual development of the two mechanisms in cartilage which is undergoing hypertrophy was clearly shown by some experiments carried out in collaboration with Dr. Janet Niven, on the calcification *in vitro* of rudimentary cartilage bones of normal rabbit embryos (Niven and Robison, 1931). The rabbits were killed 18 to 19 days after mating, and one tibia and one femur from each embryo were fixed directly as controls, the other tibia and femur being immersed for 16 hours in different calcifying solutions at 37° and pH 7.4.

Figure 15 is a section of a control tibia. In the centre, hyper-

trophic cartilage has developed but has not yet become calcified. Around this zone periosteal bone has begun to form.

Figure 16 is a section of the second tibia from the same embryo. This tibia was immersed in the inorganic solution 8:4:0 (containing no phosphoric ester). Some calcification has taken place *in vitro* in the zone of hypertrophic cartilage, but not to the extreme limit of this zone or of the surrounding periosteal bone.

Figure 17 is a section of a control femur from another embryo. Here again there is a central zone of uncalcified hypertrophic cartilage surrounded by a thin sheath of periosteal bone.

Figure 18 is a section of the second femur from this embryo. This femur was immersed for 16 hours in the organic solution 8:4:10 (containing glycerophosphoric ester). The new calcification is here much denser and involves almost the whole of the zone of hypertrophic cartilage extending to the extreme limit of the periosteal bone.

It is clear that in the cartilage which has most recently emerged from the small-celled stage the activity of the "inorganic" favouring mechanism is not so great as in the later stages of hypertrophy.

From these results and from the location of the new deposit in the experiments with rachitic bones, we may conclude that the calcifying mechanisms are developed in cartilage in parallel with the enlargement of the cells and reach a maximum at a certain stage in this process of hypertrophy, thereafter declining as this stage of maturity is passed.

Miss Rosenheim and I are at present studying the effect of serum proteins, glucose, and the other organic constituents of blood on calcification *in vitro*.

As we expected, the addition of 7% serum protein to the salt solutions made it necessary to raise the concentration of calcium or inorganic phosphate considerably in order that calcification of the bone slices should take place. This can be explained by the combination of the protein with part of the calcium whereby the concentration of calcium ions is diminished. A further result was

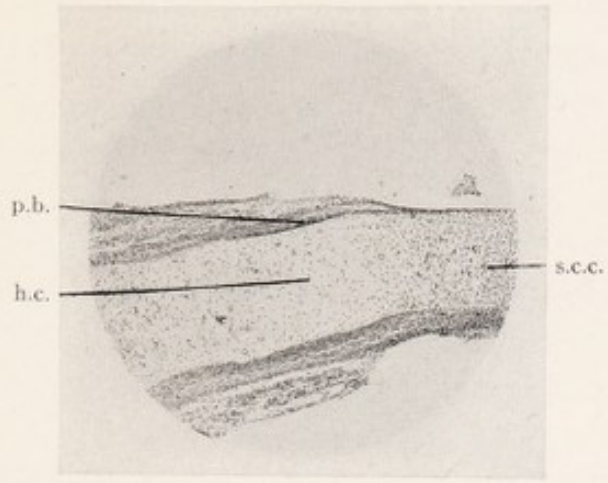


FIG. 15

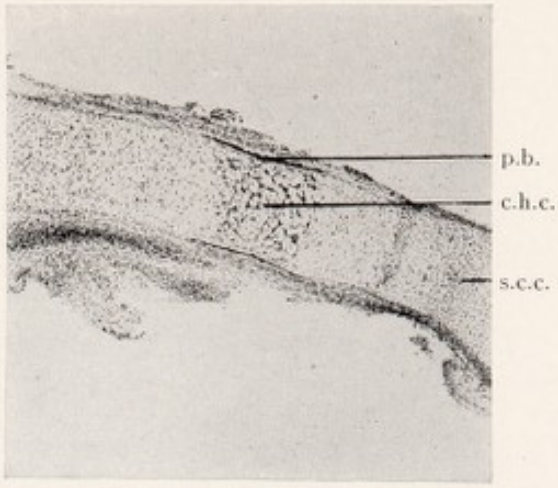


FIG. 16

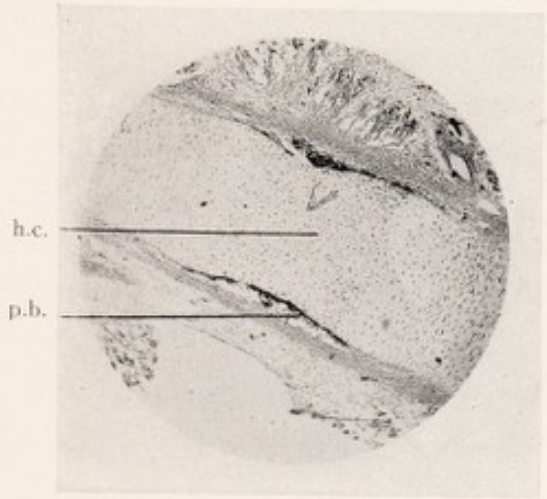


FIG. 17

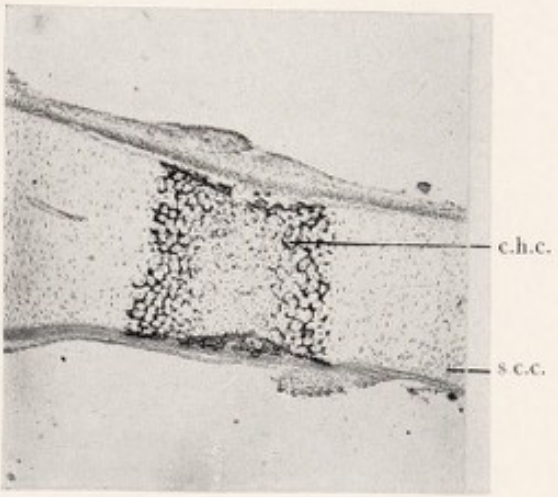


FIG. 18

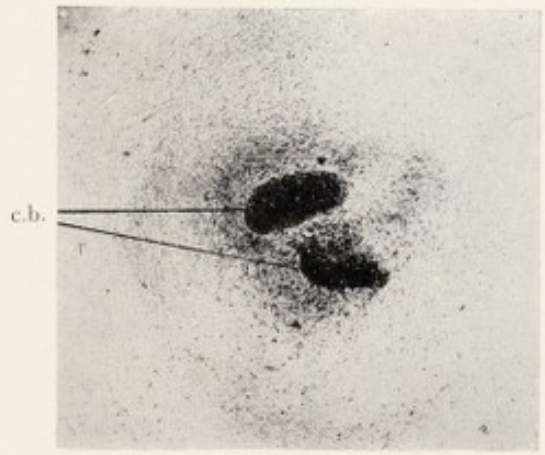


FIG. 38

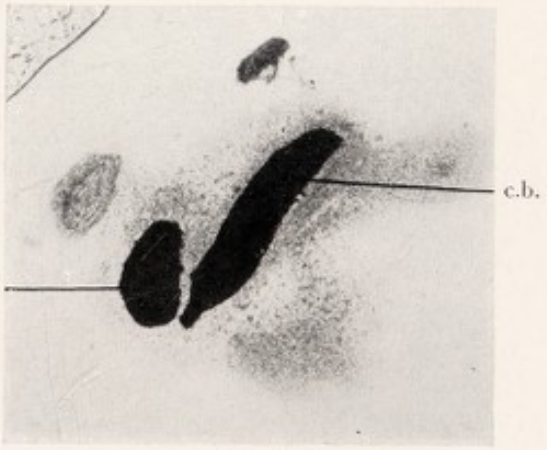


FIG. 39

less clearly foreseen, namely, that glucose in low concentrations exerts a definite inhibitory effect on calcification. This may also be due to a decrease in ionised calcium owing to the formation of a calcium-glucose complex.

In all such solutions, however, the favourable effect of phosphoric ester on calcification has been demonstrated.

Another chapter in this story has been made possible by the collaboration of Dr. Honor Fell of the Strangeways Research Laboratory, Cambridge. Dr. Fell (1925, 1928; Strangeways and Fell, 1926) had previously shown that if terminal fragments of 8-day embryonic limb cartilage of the fowl were explanted *in vitro* they differentiated during subsequent cultivation into a diaphysial portion containing hypertrophied cells and an epiphysis containing smaller cells, bone being in some cases deposited around the diaphysial portion. If, however, undifferentiated limb bud mesenchyme was explanted, cartilage differentiated during cultivation, but with a few exceptions remained of the small-celled type and failed to ossify.

We studied the correlation between the histological differentiation and phosphatase synthesis in such embryonic limb cartilage during cultivation *in vitro* (Fell and Robison, 1929). Femora taken from fowl embryos of $5\frac{1}{2}$–6 days' incubation were cultivated in plasma and embryo extract. After different periods of cultivation some explants were examined histologically while others from the same cultures were used for the determination of phosphatase activity and dry weight by a micro-technique suitable for such minute objects. (In one experiment, during 27 days cultivation, the average dry weight of the femur increased from 0.05 mg. to 0.9 mg.) At the time of explantation the femur consisted of a very early type of small-celled cartilage with no sign of demarcation into epiphysis and diaphysis, no bone and no definite perichondrium. After 21 days' cultivation the cartilage was still remarkably healthy and, as in normal development, had differentiated into well-defined epiphyses of small-celled cartilage, zone of flattened cells, and zone of hypertrophic cells, whilst

a periosteum and compact layer of periosteal bone had also formed *in vitro*. Figures 19 to 24 are reproduced from Dr. Fell's drawings of whole mounts of these explanted femora at different stages of cultivation *in vitro*.

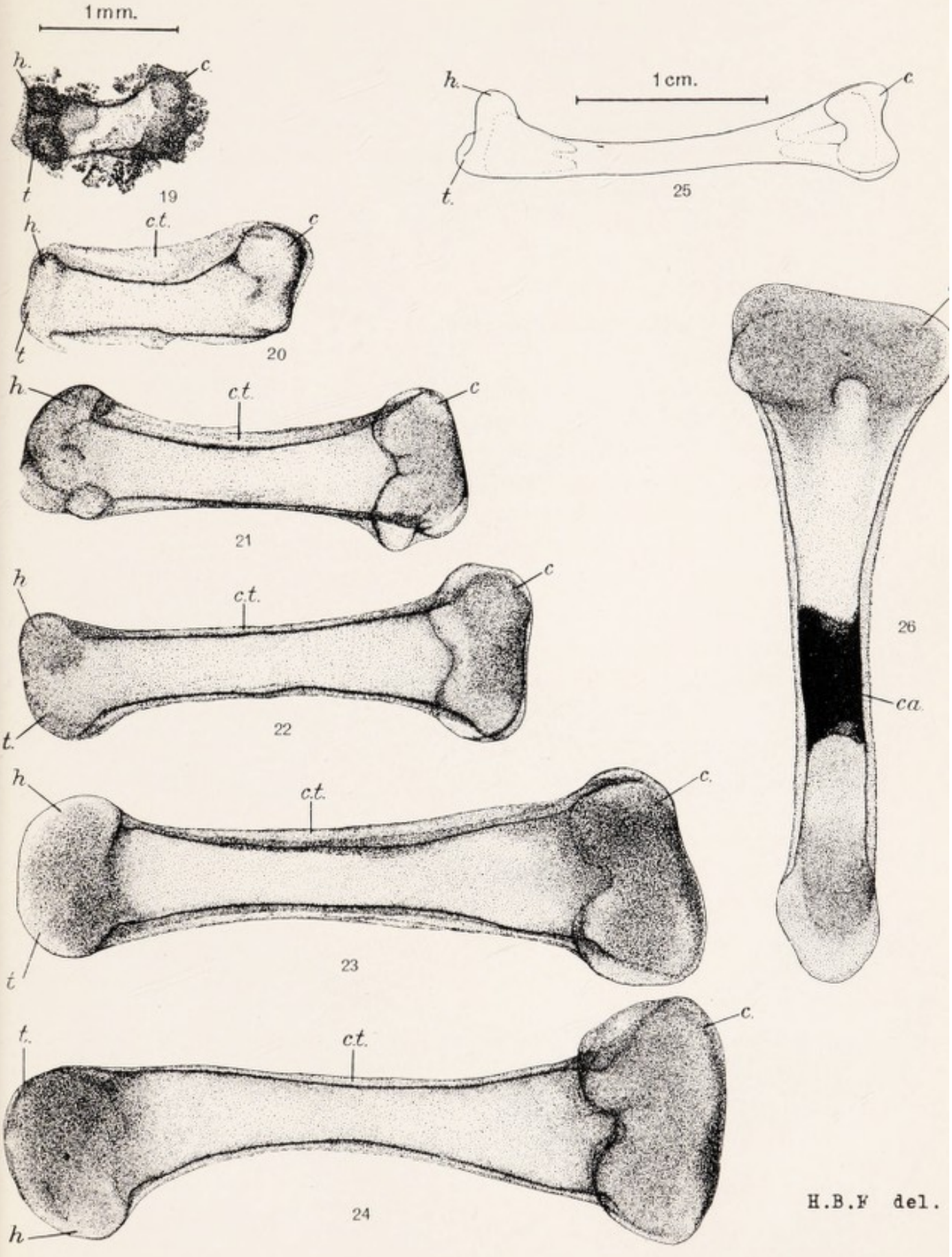
Figure 25 shows a normal embryonic femur from a 21-day fowl embryo, but drawn to a different scale.

Figure 26 shows an explanted femur after 27 days' cultivation *in vitro*, stained with silver nitrate to demonstrate the sheath of calcified periosteal bone enclosing the middle portion of the cartilaginous shaft.

The histological development of these femora is shown by the sections illustrated in Figures 27 to 32. The section of the 6-day embryonic femur (Figure 27) showed a very early type of cartilage with no sign of demarcation into epiphysis and diaphysis, and no trace of bone. After 3 days' cultivation *in vitro* (Figure 28), the beginnings of differentiation into regions of rounded, flattened, and hypertrophied cells was apparent. After 9 days' cultivation (Figure 29), the zone of hypertrophic cells was more distinct and was overlaid by a delicate layer of very early periosteal bone. After 21 days' cultivation (Figure 31), the epiphysis of small-celled cartilage was sharply marked off from the diaphysis by a zone of flattened cells, while the extensive zone of hypertrophic cartilage was covered with a sheath of periosteal bone. A similar picture was shown by the 27-day femur (Figure 32), in which, however, some degenerative changes were apparent.

It can be seen from these drawings that the rudimentary femur when isolated from the embryo and cultivated *in vitro* undergoes a remarkably normal anatomical and histological development. We showed further that it synthesises phosphatase (Figure 33, p. 91). At the time of explantation these cartilages possessed no measurable phosphatase activity; but, after 3 days' cultivation *in vitro*, such activity could be detected, and increased throughout the whole period (Curve A).

The ratio of phosphatase to the dry weight of the femur


1 mm.
h.
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h.
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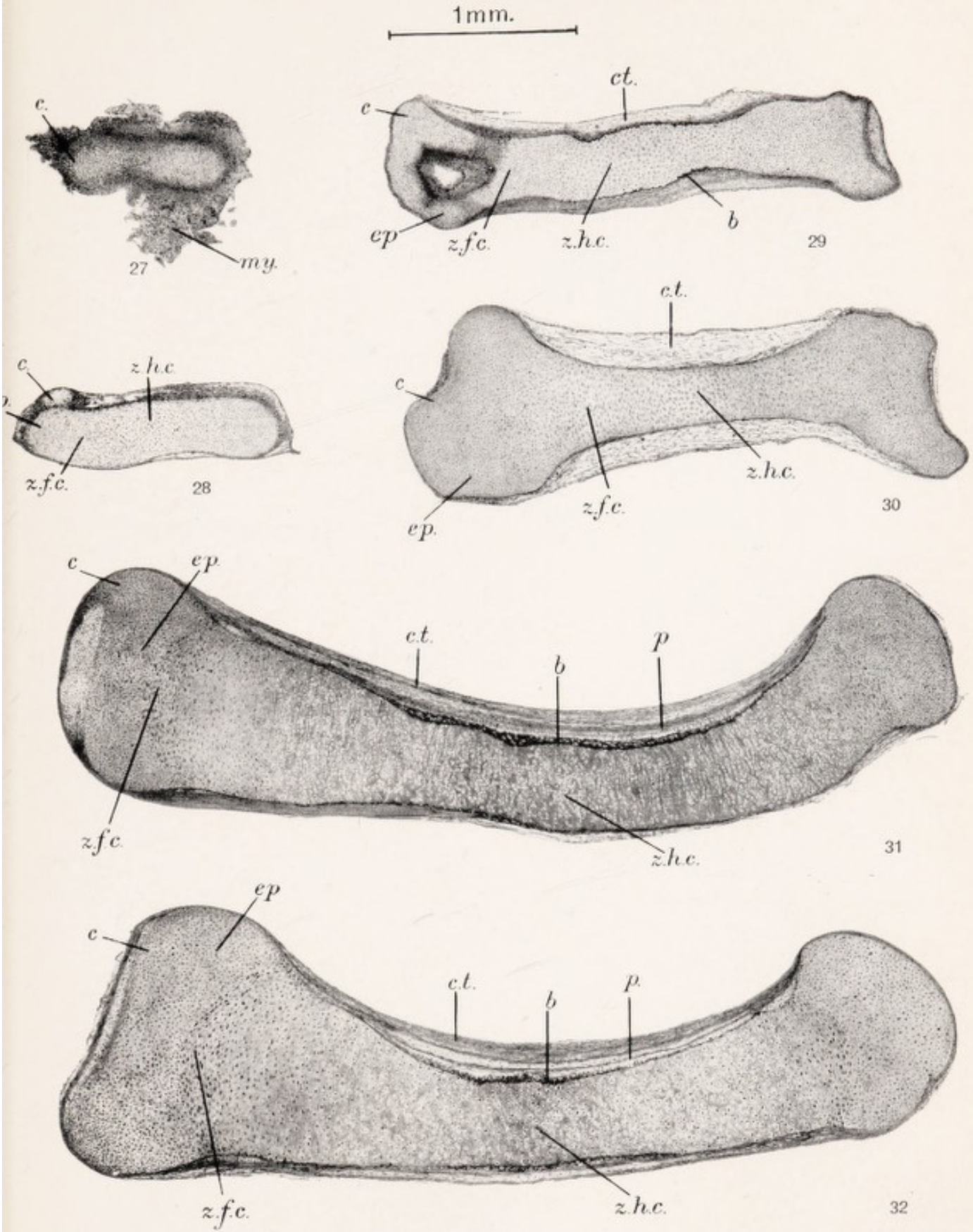
1mm.
c.
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29
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28
c.t.
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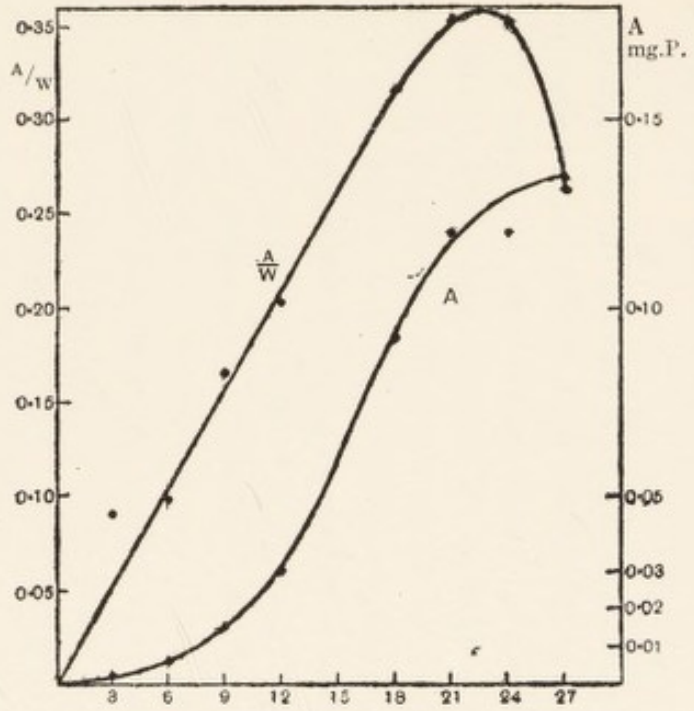

FIG. 33

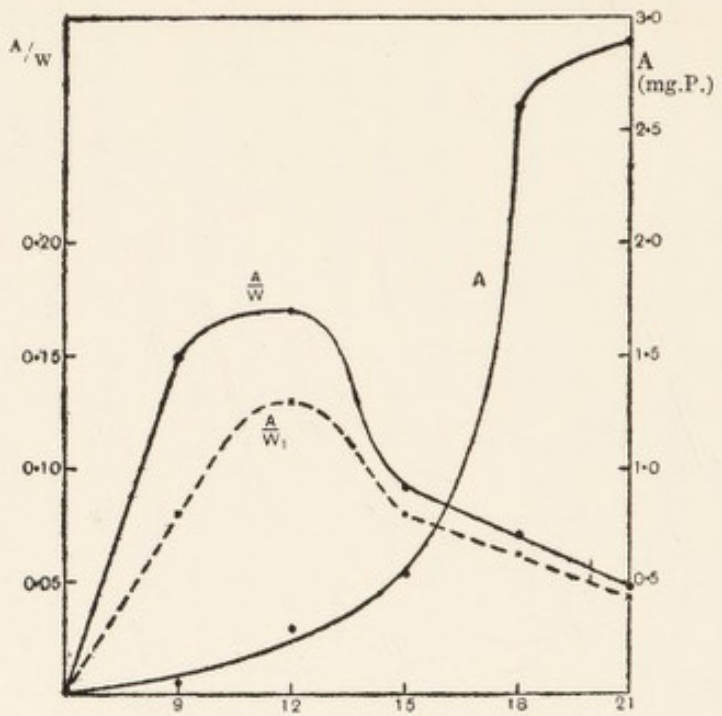

FIG. 34

Fig. 33. Production of phosphatase during the development *in vitro* of femora from 6-day fowl embryos. A. = Phosphatase per femur; given as the amount of hydrolysis (mg. P) of sodium glycerophosphate in 24 hours at 37° and pH 8.5. A/W. = Phosphatase per mg. dry weight of femur (weighed after extraction).

Fig. 34. Production of phosphatase in the femur of the embryonic fowl during normal development *in ovo*. A. = Phosphatase per femur. A/W. = Phosphatase per mg. dry weight of femur (weighed after extraction). A/W_1 = Phosphatase per mg. dry weight of femur (calculated on dry weight of corresponding unextracted femur).

(Figure 33, Curve A/W), increased rapidly from zero to a maximum value of 0.35 at 21 days, falling to 0.26 after 27 days' cultivation. The increase corresponded closely with the progress of the histological differentiation, i.e., with the formation of hypertrophic cartilage and periosteal bone. The fall in activity corresponded with the degeneration observed in the explants after 27 days.

The development of phosphatase activity during normal growth *in vivo* is shown in Figure 34, p. 91. The total activity A reaches a much higher value than *in vitro* corresponding with the more rapid growth of the bone; but the ratio of phosphatase to dry weight (A/W) rises to a maximum which is actually lower than that attained by the explanted femur, and then falls gradually as the percentage of inorganic matter increases.

Explants cultivated from the undifferentiated 3-day embryonic limb-buds gave rise to small-celled cartilage only; and these explants developed no phosphatase activity whatever, thus confirming the view that phosphatase is formed by cartilage, only if hypertrophic cells are present.

We next carried out a similar investigation of the development of two other types of skeletal tissue: first, cartilage that does not normally ossify; and secondly, membrane bone, i.e., bone which develops in a membrane of connective tissue and is not deposited on the surface of cartilage, as in the femur (Fell and Robison, 1930). For these experiments the lower jaw of the embryonic fowl was used. The mandible consists of a rod of cartilage known as Meckel's cartilage surrounded by a sheath of four membrane bones. The greater part of Meckel's cartilage persists throughout embryonic development as an unossified cartilaginous rod, differing from the cartilage of the adjacent palato-quadrate, or of the femur, in the absence of the region of hypertrophic cells characteristic of ossifying cartilage. It was shown that this non-ossifying part of Meckel's rod remains devoid of phosphatase up to an early stage of post-embryonic life while the palato-quadrate like the femur develops a high phosphatase activity. It was

PLATE VIII

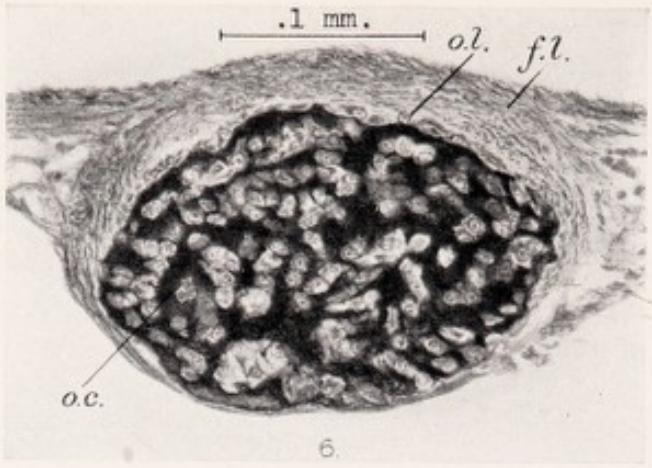

FIG. 36

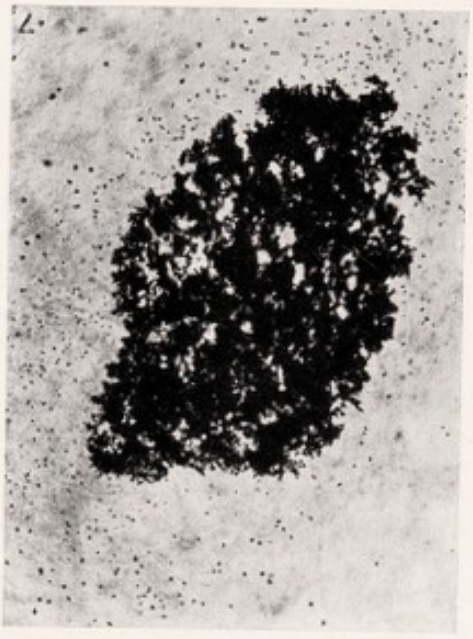

FIG. 37

H. B. F. del.

PLATE VII

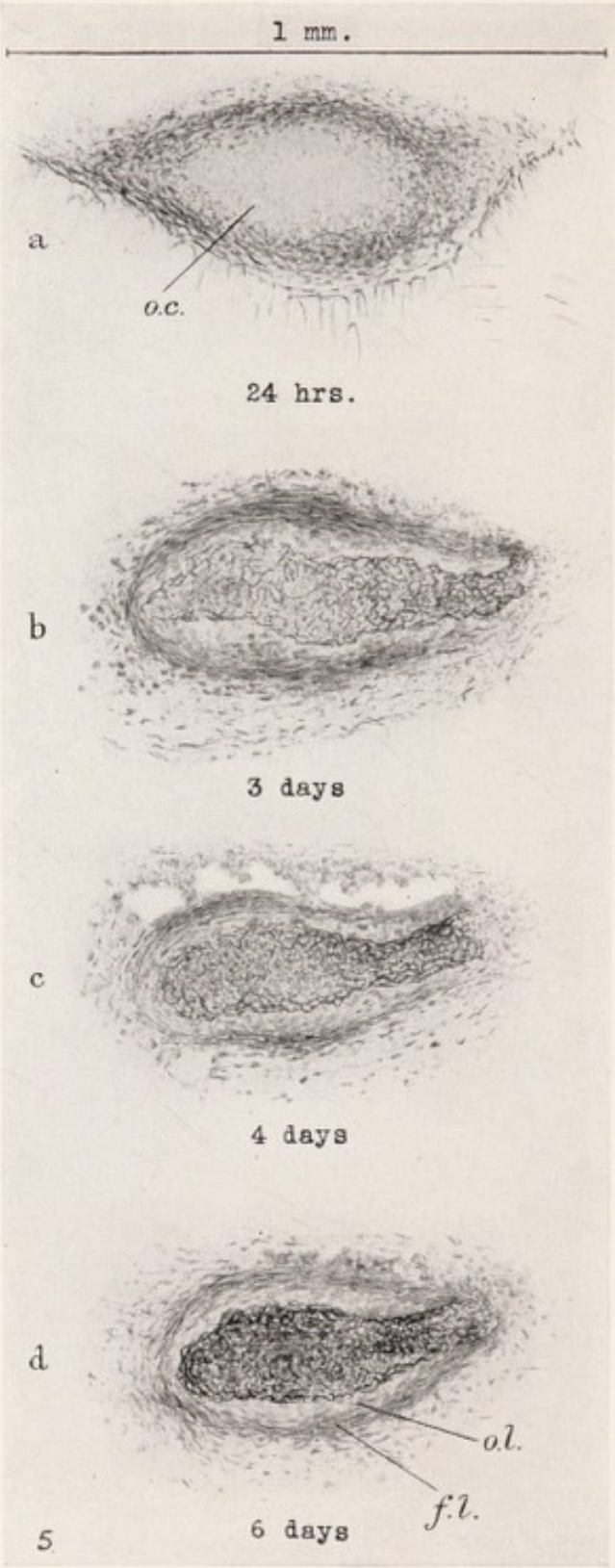

FIG. 35 H. B. F. del.

also shown that the membrane bone surrounding Meckel's cartilage possesses a high phosphatase activity.

Explants of the distal part of Meckel's cartilage from 6-day fowl embryos were cultivated *in vitro* by Dr. Fell's watch glass method, the femora and palato-quadrates from the same embryo being cultivated simultaneously as controls. During 19 days' growth *in vitro* the Meckel's cartilage neither ossified nor formed any region of hypertrophic cells, neither did it synthesise any phosphatase whatever. In the palato-quadrates and femora, differentiation into regions of hypertrophic cells with formation of osteoid tissue occurred, and phosphatase was synthesised in high degree. It was thus shown that during cultivation *in vitro* each of these three cartilage rudiments undergoes the characteristic development associated with its normal growth *in vivo*, while the correlation between histological structure and phosphatase synthesis was once again made evident.

For the cultivation of membrane bone, Dr. Fell removed the mesoderm surrounding the Meckel's cartilage from a large number of 6-day embryos and explanted these *in vitro* by the hanging-drop method. In about 65% of these explants, ossification centres developed during cultivation, and simultaneously phosphatase was synthesised.

Figures 35, a-d, and 36 illustrate the development of an ossification centre in such a culture, while Figure 37 shows that the osteoid tissue formed during 8-days' cultivation *in vitro* had also become partly calcified. We tried the effect of immersing a number of 6-day cultures for a further 16 hours in calcifying solutions such as I had used for the experiments with bones of rachitic rats. After such treatment it was found that the calcification had greatly increased in density and now extended throughout the whole of the osteoid tissue. This result is illustrated in Figures 38 and 39 (Plate IV) which are photographs of two 6-day cultures of mandibular mesoderm in each of which two ossification centres had developed. One of these cultures (Figure 39) had received a subsequent immersion in the solu-

tion 8:3:10, containing phosphoric ester. The cultures were treated with silver nitrate to demonstrate the extent of the calcification.

This greatly increased deposition of calcium salts and the sharp delimitation of the calcified area in the explants after treatment with calcifying solutions appear to indicate that the capacity for undergoing calcification is a special property associated with a definite stage in the development of the intracellular substance.

We are hoping that, by the use of such material as this membrane bone developed during cultivation *in vitro*, we may be able to obtain more light on the nature of the dual mechanism by which calcification is brought about.

It is reasonable to expect that further light on the progress of normal calcification may be gained by a study of different types of pathological calcification and ossification, two distinct phenomena which are yet closely related; for it would seem that the mere presence of solid calcium salts may stimulate osteogenesis in tissues which do not normally form bone. The composition of the inorganic deposit in such abnormally calcified tissues is usually very similar to that in bone itself, but we have as yet little knowledge of their phosphatase activity.

TABLE 7.

Phosphatase activity of transplant of bladder epithelium of dog to the abdominal wall, resulting in heterotopic ossification.

$$\frac{A}{W} = \frac{\text{Phosphatase activity}}{\text{Dry weight of tissue.}}$$

	Controls.		Transplantation experiment.		
	Bladder mucosa.	Rectus sheath.	Rectus sheath adjacent to transplant.	Rectus sheath-bladder epithelium. No bone.	Rectus sheath-bladder epithelium. Bone.
Maximum	0.03	0	0.05	0.05	0.41
Minimum	0.01	0	0	0	0.12
Mean	0.02	0	0.01	0.02	0.20

One interesting result I would like to mention. Dr. C. B. Huggins (1930, 1931) has shown at Chicago University that when bladder epithelium of the dog is transplanted to the rectus

sheath, the connective tissue adjacent to the transplants ossifies, forming calcified bone. Dr. Huggins has recently studied in my laboratory the phosphatase activity of these tissues at different periods after the transplantation, and has demonstrated (Table 7) that the development of this heterotopic bone coincides with the production of phosphatase in very high degree—in fact the ratios of phosphatase activity to dry weight of some of his ossifying tissue fragments were among the highest yet recorded (Huggins, 1931).

In the first of these lectures, I indicated the wide distribution of phosphoric esters in living organisms, and the many fields of research that might justly be included under my title. I have not attempted to give a detailed survey of all these researches, but have told my story from a more personal standpoint, dwelling chiefly on the problems and work in which I am myself most interested and which seem to offer some possibility of discovering how the esterification of phosphoric acid in plant and animal assists in the various processes of their metabolism.

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DESCRIPTION OF PLATES

PLATE I. FIGURES 1–3 (*facing p. 75*)

[e.c., epiphysial (non-hypertrophic) cartilage; h.c., hypertrophic cartilage; c.h.c., calcified hypertrophic cartilage; p., periosteum; co., cortical bone; sp., spongiosa; m., marrow.]

Fig. 1. Section of tibia of a normal quickly growing rat, age 42 days (haematoxylin-eosin). (For this photomicrograph I am indebted to Dr. V. Korenchevsky, from whose monograph, *The Aetiology and Pathology of Rickets*, 1922, it is reproduced.)

Fig. 2. Section of radius of rachitic rat. This bone was immersed for 16 hours in a 0.1 *M* solution of sodium phosphate at 37°. Stained with silver nitrate. Note the broad band of uncalcified hypertrophic cartilage in the metaphysis.

Fig. 3. Section of the other radius of the same rat. This bone was immersed for 16 hours in a 0.1 *M* solution of calcium hexosemonophosphate. The entire zone of hypertrophic cartilage has been densely calcified *in vitro*, while the zone of small-celled, epiphysial cartilage has remained uncalcified.

PLATE II. FIGURES 4–9 (*facing p. 77*)

Figs. 4–9. Slices cut from the distal heads of the femora and proximal heads of the tibiae of a rachitic rat. The slices were immersed 16 hours at 37° in solutions all containing 10 mg. Ca and 3.5 mg. P as inorganic phosphate per 100 c.c., but containing different concentrations of glycerophosphoric ester. Slices stained by von Kossa's silver nitrate method and photographed, without clearing, by reflected light. Mag. × 11.

× = new deposit of calcium salts in the zone of hypertrophic cartilage.

Fig. 4.	Femur slice	in solution	*A*,	Phosphoric ester	= 0	
Fig. 5.	"	"	*B*,	"	= 0.5 mg.	P per 100 c.c.
Fig. 6.	"	"	*C*,	"	= 1 "	
Fig. 7.	Tibia	"	*A*,	"	= 0 "	
Fig. 8.	"	"	*D*,	"	= 2 "	
Fig. 9.	"	"	*E*,	"	= 4 "	

PLATE III. FIGURES 10–14 (*facing p. 85*)

Figs. 10–14. Sections of bone slices after immersion in experimental solutions at 37° and pH 7.35. The sections were cut without previous decalcification. Thickness—sections 1, 2, 3, 5—30μ; section 4—50μ. Sections 1, 2, 3 and 4 were stained with silver nitrate, section 5 was stained with purpurin. Magnification × 11. × denotes hypertrophic cartilage calcified *in vitro*.

Fig. 10. Humerus of rachitic rat. 18 hrs. in inorganic solution 8:5:0. Note the calcification, obtained *in vitro*, in the metaphysis and in the cartilage forming the articular surface of the epiphysis.

Fig. 11. Tibia of the same rat. 22 hrs. in acetone before immersion for 18 hrs. in inorganic solution 8:5:0. Note the complete absence of new calcification in the broad zone of uncalcified hypertrophic cartilage characteristic of rickets.

Fig. 12. Tibia of the same rat. 18 hrs. in solution containing phosphoric ester 8:4:30. Note the extensive calcification, obtained *in vitro*, in the matrix of the hypertrophic cartilage.

Fig. 13. Tibia of the same rat. 22 hrs. in acetone before immersion for 18 hrs. in solution containing phosphoric ester 8:4:30. Note the extensive calcification, obtained *in vitro*, in the matrix of the hypertrophic cartilage, in spite of the drastic treatment of the bone slices. The greater density of the deposit, as compared with that in Fig. 3, is accounted for by the greater thickness of the section.

Fig. 14. Tibia of a rachitic rat. 22 hrs. in 90% alcohol before immersion for 18 hrs. in solution containing phosphoric ester 8:4:30. Note the normal appearance of the new deposit in the cartilage matrix.

PLATE IV. FIGURES 15–18 AND 38 & 39 (*facing p. 88*)

Sections of tibiae and femora of embryos of rabbits, killed 18 to 19 days after mating. One tibia and one femur were fixed directly as controls, the other tibia and femur being immersed for 16 hours in the calcifying solution at 37° and pH 7.4.

Fig. 15. Section of control tibia. A central region of uncalcified hypertrophic cartilage is present, and around this cartilage per-

iosteal ossification has just commenced. Treated with silver nitrate.

Fig. 16. Section of second tibia from the same embryo after immersion in the inorganic solution 8:4:0. The hypertrophic cartilage has been partly calcified *in vitro*. Treated with silver nitrate.

Fig. 17. Section of control femur from another embryo. The appearance is similar to that of Fig. 15 but the layer of periosteal bone is more definite. Stained with purpurin.

Fig. 18. Section of second femur from the same embryo after immersion in the organic solution 8:4:10, containing glycerophosphoric ester. The new calcification is denser than in Fig. 16 and extends almost to the limit of the hypertrophic cartilage and periosteal bone. Stained with purpurin.

Fig. 38. 6-day culture of the soft tissue from a 6-day embryonic jaw. Two ossification centres have developed during the cultivation and have become partly calcified. Treated with silver nitrate.

Fig. 39. A similar culture to the above after immersion for 16 hours in the organic solution 8:3:10. The two ossification centres which had developed during cultivation *in vitro* have become more densely calcified during the subsequent immersion in the solution containing phosphoric ester. The deposit of calcium salts extends throughout the whole of the osteoid tissue. Treated with silver nitrate.

Plates V–VII. Figures 19–37

The outline of all the figures, except Fig. 37, was drawn with the aid of either a camera lucida or a Leitz projection prism. All the figures on Plate V except Fig. 25 are drawn to the same scale and all the figures on Plate VI are to the same scale.

b., bone; *c.*, condyle; *ca.*, calcification; *c.t.*, connective tissue; *ep.*, epiphysis; *f.l.*, fibrous layer; *h.*, head; *h.c.*, hypertrophied chondroblast; *k*, keratin; *my.*, myoblasts; *o.l.*, osteoblastic layer; *p.*, periosteum; *s.c.c.*, small-celled cartilage; *t.*, trochanter; *z.f.c.*, zone of flattened cells; *z.h.c.*, zone of hypertrophied cells; *o.c.*, ossification centre.

Plate V. Figures 19–26 (*facing p. 90*)

Fig. 19. Normal $5\frac{1}{2}$-day embryonic femur as dissected for explantation (whole mount). Small processes representing the condyles, head and trochanter are present.

Fig. 20. Femur from 5½-day embryo after 3 days' cultivation *in vitro* (whole mount). The condyles, head and trochanter are more distinct.

Fig. 21. Femur from 5½-day embryo after 9 days' cultivation *in vitro* (whole mount).

Fig. 22. Femur from 5½-day embryo after 15 days' cultivation *in vitro* (whole mount).

Fig. 23. Femur from 5½-day embryo after 21 days' cultivation *in vitro* (whole mount).

Fig. 24. Femur from 5½-day embryo after 27 days' cultivation *in vitro* (whole mount). Note the relatively normal appearance of this femur as compared with the normal specimen (Fig. 25). It will be seen that the increase in width is much greater in the region of the epiphyses than in the middle of the shaft; this is more marked than in the normal femur.

Fig. 25. Normal femur from 21-day embryo (whole mount).

Fig. 26. Femur from 5½-day embryo after 27 days' cultivation *in vitro* (whole mount). This specimen has been stained by von Kossa's silver nitrate method. Note the sheath of calcified bone enclosing the middle region of the shaft.

PLATE VI. FIGURES 27–32 (*facing p. 90*)

Fig. 27. Section of normal femur from 6-day embryo. The femur is composed of a very simple type of cartilage which shows no hypertrophied cells and no differentiation into epiphysis and diaphysis. (Safranine and picro-indigo-carmine.)

Fig. 28. Section of a 6-day embryonic femur after 3 days' cultivation *in vitro*. More matrix is present than in the previous specimen and indications of a differentiation into epiphysis, zone of flattened cells and zone of hypertrophied cells are seen. (Safranine and picro-indigo-carmine.)

Fig. 29. Section of a 6-day embryonic femur after 9 days' cultivation *in vitro*. The region of hypertrophied cells is now fairly distinct and is overlaid by a delicate layer of very early bony material. (Haematoxylin and van Gieson's stain.)

Fig. 30. Section of a 6-day embryonic femur after 15 days' cultivation *in vitro*. This explant is subnormal in development as compared with other explants; the zone of hypertrophied cells is not quite so

extensive as in the 9-day culture and there is no definite boundary between epiphysis and diaphysis. Note the large size of the epiphysial regions relative to the shaft. (Mallory's triple stain.)

Fig. 31. Section of a 6-day embryonic femur after 21 days' cultivation *in vitro*. The epiphysis is sharply marked off from the diaphysis by the zone of flattened cells; a sheath of bone invests the extensive zone of hypertrophied chondroblasts. (Safranine and picro-indigo-carmine.)

Fig. 32. Section of a 6-day embryonic femur after 27 days' cultivation *in vitro*. (Safranine and picro-indigo-carmine.)

PLATE VII. FIGURE 35 (*facing p. 93*)

Fig. 35. Osteogenesis in a hanging-drop culture of mesoderm from a 6-day embryonic jaw. The figures are drawn from camera lucida sketches of a living culture and were made at different stages of growth to show the development of an ossification centre. After 6 days' cultivation this specimen was fixed and sectioned. A section is shown in Fig. 36.

PLATE VIII. FIGURES 36–37 (*facing p. 93*)

Fig. 36. Histological section of culture shown in Fig. 35 after 6 days' growth *in vitro*.

Fig. 37. Photograph of an 8-day coverslip culture of the soft tissue from a 6-day embryonic jaw. This specimen was stained with silver nitrate and mounted whole. An ossification centre which developed during cultivation, stained black with the silver nitrate indicating that true calcified bone had been formed.

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