observations. In order to do so it is necessary to postulate a very large number of helices with the helical angle increasing continuously in successive helices. There is, however, no further evidence for such a structure.

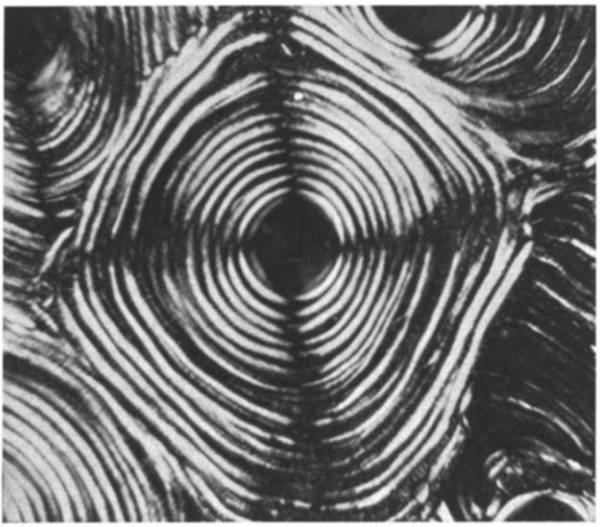

Fig. 3. Single Haversian System in unstained section with Polars crossed showing birefringent and isotropic rings and the dark extinction cross

In Haversian Systems the variation in direction of the fibrils suggested by the structure of pherulites is supported by the similarity in chemical structure of collagen and the artificial polymers. They are all long chain molecles, while nylon, one of the polymers studied by KELLER, and collagen are polypeptides. Furthermore, the formation of Haversian Systems takes place slowly in the primary bone which has been previously laid down and in this respect resembles[1] the formation of spherulites which are obtained when the polymer is kept at a temperature a few degrees below its softening point. Moreover, spherulites of inorganic salts such as barium carbonate are only obtained if the rate of crystallisation is slow, as for instance by allowing a solution of barium chloride to diffuse into a gel containing dissolved sodium carbonate.

This structure offers a straightforward explanation for the existence of Haversian Systems in bone as being a form of crystallisation of collagen analogous to the formation of spherulites from artificial polymers.

*Pathology Research Laboratory,*
*University of Bristol,*
*Bristol (England)*

N. M. BLACKETT

[1] A. KELLER, *Nature*, 169 (1952) 913.
[2] J. B. FINEAN AND A. ENGSTROM, *Biochim. Biophys. Acta*, 11 (1953) 178.
[3] A. A. MAXIMOW AND W. BLOOM, *A Textbook of Histology*, Philadelphia 1952.

Received November 26th, 1954

## On the phosphamidase activity of human seminal phosphate

The idea that the phosphamidases form a group of enzymes distinct from the phosphatases[1,2] seems no longer valid. There do exist perhaps specific phosphamidases that do not attack the P–N compounds and specific phosphamidases that do not attack the P–O compounds analogous to their own substrates, but so far in all cases where the so-called unspecific phospho-mono-esterases have been investigated carefully, they have also displayed phosphamidase activity.

Preparations of alkaline phosphatases, more or less purified, have been shown to catalyze the hydrolysis of P–N compounds[3,4,5,6,7] and also to catalyze the transfer of phosphate from a P–N donor to certain acceptors[4,5,7].

As for the acid phosphatases PERLMANN has demonstrated the P–N splitting abilities of potato and intestinal phosphatase. Transferase and hydrolytic activity of crude seminal plasma using creatinephosphate as substrate has been demonstrated by GREEN AND MEYERHOF[5] and confirmed by MORTON[7] with partially purified human prostatic phosphatase. In contradistinction to these results on acid phosphatases stand the findings of PERLMANN[6]. Using a purified preparation of prostate phosphatase she found no hydrolysis of N- (*p*-chlorophenyl) amido phosphate and only a slight splitting of N-phosphoryl-DL-phenylalanine methyl ester. Likewise, ANAGNOSTOPOULOS *et al.*[8] found practically no splitting of anilidophosphate.

Since prostate phosphatase has been used to characterize the nature of the phosphate linkage in proteins[8,9,10,11,12] it seems desirable to investigate the ambiguity in the demonstration of its phosphamidase activity. Therefore, the following observations on purified human seminal phosphatase with amido-phosphate* ($NaHPO_3NH_2$) as substrate are presented.

The enzyme was prepared and kindly given by Dr. FRANK LUNDQUIST**. It consists of the dialyzed precipitate formed between 40 and 70% saturation with ammonium sulfate, at 0° C in human seminal plasma acetate buffered at pH 5. The phosphatase activity of such preparations is about 5400 Gutman units per ml; in spite of the purification they still display slight proteolytic activity[13].

The cleavage of amidophosphate which is acid consuming was followed by means of a pH-stat[14], in a medium containing 0.5 *M* KCl, 25° C. The substrate ($pK_3 = 7.95$; 25° C, 0.5 *M* KCl (15)) shows a rather high *autohydrolysis*, the first order kinetic constant being 0.0236 $h^{-1}$. ($log_{10}$), 0.5 KCl, 25° C (ROSENBERG's[15] more accurate determination gives 0.0234 $h^{-1}$), in the pH interval 4 to 6.5. Below pH 4 acid catalysis sets in[15], while from pH 6.5 the rate falls off along an S-shaped curve reaching zero at pH 9.5.

The enzyme-catalyzed reaction, 0.5 *M* KCl, 25° C, $1.6 \cdot 10^{-2}$ *M* amidophosphate, followed almost the first order kinetic scheme with a pH-optimum at 6.7.

In order to prevent difficulties with $CO_2$ and enzyme inactivation[16], most of the experiments have been carried out at pH 4.6. Under these conditions an almost linear relationship between enzyme concentration and activity was found.

The Michaelis-Menten constant was found to be $1 \cdot 10^{-3}$ *M* amidophosphate (initial rate, Lineweaver-Burk plot). This is notable since the corresponding values for monophenyl phosphate have been found by LUNDQUIST[17] to be $0.892 \cdot 10^{-3}$ *M*, 25° C, pH 6.0, $\mu = 0.15$ and by SCHØNHEYDER[18] to be $0.906 \cdot 10^{-3}$ *M*, 22° C, pH 5.6, $\mu = 0.3$ (2% NaCl).

The activity at pH 4.6 is not significantly inhibited by one of the reaction products, $NH_4^+$ (0.1 *M*), but measurably so by the other product, phosphate.

On the (not proven) assumption that this inhibition is competitive, the dissociation constant for the enzyme-phosphate complex can be calculated to be $2 \cdot 10^{-3}$ *M*. This is somewhat higher than found by SCHØNHEYDER[18], $1.4 \cdot 10^{-3}$ *M*, 22° C, pH 5.6, $\mu = 0.3$ (2% NaCl). Strong inhibition of the reaction with KF, $10^{-3}$ *M*, pH 4.6 was observed but found not to be instantaneous. At pH 8.6 the inhibition seemed to be much smaller. The following substances had no significant action on the phosphamidase activity at pH 4.6: $BeSO_4$ ($5 \cdot 10^{-4}$ *M*), $CoCl_2$ ($5 \cdot 10^{-4}$ *M*), Versene ($1.4 \cdot 10^{-2}$ *M*), ascorbate ($2.8 \cdot 10^{-3}$ *M*).

A comparison of the activity (expressed in millimols substrate split per minute) of this enzyme measured as a phosphatase *ad modum* LUNDQUIST[19], ($4.55 \cdot 10^{-3}$ *M* monophenylphosphate, but at pH 5.2 and corrected to pH-optimum and 25° C), with the activity measured as a phosphamidase (corrected to pH-optimum and $4.55 \cdot 10^{-3}$ *M* amidophosphate) gives P–N-ase/P–O-nase = 1.3. This result is only to be taken as an order of magnitude, because a comparison is not strictly permissible due to *e.g.* differences in enzyme dilution procedure, presence of citrate in one case only, and so on.

Free and paper electrophoresis of two different enzyme preparations gave three main peaks, the greater part of the activity being accumulated in the middle peak, named B. At 0.5° C, phosphate buffer $\mu = 0.1$, pH 6.5, the mobility of peak B in free electrophoresis was about $-3 \cdot 10^{-5}$ $cm^2$, $volt^{-1}$, $sec^{-1}$ (ascending limb). This is somewhat different from the value $-1 \cdot 10^{-5}$ $cm^2$, $volt^{-1}$, $sec^{-1}$ (descending limb), pH 6.5, $\mu = 0.05$ found by DEROW AND DAVISON[20] and from KUTSCHER AND PANY's[21] value $-10 \cdot 10^{-5}$ $cm^2$, $volt^{-1}$, $sec^{-1}$ in $10^{-3}$ *M* citrate buffer. As seen in Tables I and II the ratio P–N-ase/P–O-ase is practically constant in the electrophoretic fractions and the untreated enzymes.

The enzyme was found to act as a transferase with amidophosphate as donor and glycerol as an acceptor, pH 4.6, 25° C.

As already mentioned the enzyme preparation shows proteolytic activity. HOLTER AND LI[22] have found phosphamidase activity in pepsin, trypsin and rennin, pH-optimum 4.6, 37° C, with *p*-chloro-anilidophosphate as substrate; therefore rennin (BERRIDGE[23] cryst.), pH 4.6, pepsin (porcine, cryst. Armour), pH 4.6 and subtilisin (cryst. GÜNTELBERG AND OTTESEN[24]) pH 4.6 and 8.6, were tested for phosphamidase activity with amidophosphate as substrate, but with negative result.

---

* A gift from Dr. THOMAS ROSENBERG, Nordisk Insulin Laboratorium, Gentofte, Denmark.

** Institute of Forensic Medicine, University of Copenhagen.

The main results of the present study make it highly probable that seminal phosphomonoesterase and phosphamidase are identical, a finding well in accordance with KOSHLAND's[25] theoretical considerations.

TABLE I

FREE ELECTROPHORESIS OF 9.588 ml DIALYZED ENZYME PREP. 3a

| *Fraction* | *Phosphamidase act. per ml arbitrary units* | *Phosphatase act. per ml arbitrary units* | *Ratio* $\frac{\text{Phosphamidase}}{\text{Phosphatase}}$ | *Total Phosphatase act.* |
|---|---|---|---|---|
| (1) ascending limb | 0.42 | 0.055 | 7.6 | 0.20 |
| (2) ascending limb | 61.1 | 9.0 | 6.8 | 32.0 |
| (3) bottom | 82.5 | 12.3 | 6.7 | 30.5 |
| (4) descending limb | 84.0 | 12.8 | 6.6 | 45.6 |
| (5) descending limb | 25.9 | 3.8 | 6.8 | 13.2 |
| | | | | sum: 121.5 |
| Preparation (3a) dialyzed 9.588 ml | 86.3 | 13.2 | 6.5 | 126.6 |

TABLE II

| *Exp. No.* | *Buffer* | *pH* | *Volt per cm* | *Time min* | *B-peak* | | | | *Phosphatase activity recovered* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Total phosphamidase activity* | *Total phosphatase activity* | *Ratio phosphamidase/phosphatase* | *Ratio for untreated (2a) prep.* | *Distribution per cent* | | | *Per cent of initial act.* |
| | | | | | | | | | *peak A* | *peak B* | *peak C* | *peak A + B + C* |
| IV | phosphate | 7.08 | 9.16 | 248 | 1.26 | 0.1943 | 6.5 | 6.5 | 1 | 97 | 2.8 | 71% |
| VIII | veronal acetate sulfate | 7.06 | 3.97 | 740 | 0.400 | 0.0665 | 6.0 | | 1 | 98 | 1.7 | 24%* |

* Bad recovery in this case probably due partly to nature of buffer, partly to exclusion of the paper areas corresponding to the zones of overlapping fractions. In spite of this enzyme loss the activity distribution is the same as in the other experiment.

Further investigations are in progress. A more detailed publication will appear in *Compt. rend. trav. lab. Carlsberg*.

To Dr. FRANK LUNDQUIST and Dr. THOMAS ROSENBERG I am indebted for many helpful suggestions.

I also want to thank Dr. H. HOLTER and Professor K. U. LINDERSTRØM-LANG for suggesting the problem and especially for very valuable advice and discussions.

K. MAX MØLLER

*Carlsberg Laboratory, Copenhagen (Denmark)*

[1] A. SCHÄFFNER, *Die Methoden der Fermentforschung*, II, (E. BAMANN UND K. MYRBÄCK, editors), George Thieme Verlag, Leipzig (1941) p. 1976.
[2] H. HOLTER AND SI-OH LI, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, 27 (1951) 393.
[3] T. WINNICK AND E. M. SCOTT, *Arch. Biochem.*, 12 (1947) 201 and 209.
[4] O. MEYERHOF AND H. GREEN, *J. Biol. Chem.*, 178 (1949) 655, and 183 (1950) 377.
[5] H. GREEN AND O. MEYERHOF, *J. Biol. Chem.*, 197 (1952) 347.
[6] G. E. PERLMANN, *Phosphorus Metabolism*, II, (W. D. MCELROY AND B. GLASS, editors), The Johns Hopkins Press, Baltimore (1952) p. 167.
[7] R. K. MORTON, *Nature*, 172 (1953) 65.
[8] C. ANAGNOSTOPOULOS, M. PACHT, E. BOURLAND AND P. GRABAR, *Bull. soc. chim. biol.*, 33 (1951), 699–704.

[9] G. E. PERLMANN, *J. Gen. Physiol.*, 35 (1952) 711.
[10] G. E. PERLMANN, *J. Amer. Chem. Soc.*, 74 (1952) 6308.
[11] G. E. PERLMANN, *Biochim. Biophys. Acta*, 13 (1954) 452.
[12] G. E. PERLMANN, *Nature*, 174 (1954) 273.
[13] F. LUNDQUIST, unpublished results.
[14] C. F. JACOBSEN AND J. LÉONIS, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, 27 (1951), 333–39.
[15] T. ROSENBERG, unpublished results.
[16] W. KUTSCHER AND A. WÖRNER, *Z. physiol. Chemie*, 239 (1936) 109.
[17] F. LUNDQUIST, *Acta Physiol. Scand.*, 14 (1947) 263.
[18] F. SCHØNHEYDER, *Biochem. J.*, 50 (1951) 378. The results have been recalculated by the author of the present paper.
[19] F. LUNDQUIST, *Acta Physiol. Scand.*, 19, Suppl. 66 (1949) 39.
[20] M. A. DEROW AND M. M. DAVISON, *Science*, 118 (1953) 247.
[21] W. KUTSCHER AND J. PANY, *Z. physiol. Chemie*, 255 (1938) 169.
[22] H. HOLTER AND SI-OH LI, *Acta Chem. Scand.*, 4 (1950) 1321.
[23] N. Y. BERRIDGE, *Biochem. J.*, 39 (1945) 179.
[24] A. V. GÜNTELBERG AND M. OTTESEN, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, 29 (1954) 36.
[25] D. E. KOSHLAND, *The Mechanism of Enzyme Action* (W. D. MCELROY AND BENTLEY GLASS, editors) The Johns Hopkins Press, Baltimore (1954), p. 608.

Received November 10th, 1954

## A new cofactor in the conversion of serine to glycine

Cell-free extracts of an unidentified *Clostridium*, strain HF[1], catalyze the net production of glycine from serine. The fate of the $\beta$-carbon of serine has not yet been determined. Upon extensive dialysis of the extracts, glycine production shows a clear dependence on DPN, $Mn^{++}$ and pyridoxal phosphate (see Experiment I). Following treatment with Dowex 1 HCl and subsequent overnight dialysis, the system can no longer be reactivated by these three cofactors. A fourth factor obtained from boiled extracts of *Clostridium cylindrosporum* is required in addition; it shall be referred to as Co C. Experiment II demonstrates that Co C is essential, and that its presence does not abolish the requirements for DPN, $Mn^{++}$ and pyridoxal phosphate.

Co C is not identical with any of the folic acid derivatives found active in the systems of SAKAMI[2] and BLAKLEY[3]. Experiment II demonstrates that in this system folic acid and anhydroleucoverin are inert, but that citrovorum factor (C F) and tetrahydrofolic acid (*thf*) have an effect. In contrast to Co C, which is active at catalytic levels*, good activity is obtained with C F and *thf* only at substrate concentrations. Furthermore, DPN is not required when these compounds are used, (inhibition by DPN is usually observed) whereas DPN is required for activity with Co C. Another basis for distinguishing Co C from C F and *thf* is that saturation curves reveal that C F can never give as good activity as Co C, and that *thf* can at substrate concentrations give better activity (perhaps as a stoichiometric acceptor for the one carbon piece). Co C is not attacked by potato nucleotide pyrophosphatase. It is absorbed at low pH on Norite, and can be removed by ammoniacal ETOH; it is stable to boiling in dilute alkali (0 05 *N*) and is destroyed under similar conditions in dilute acid. Co C can be distinguished chromatographically from folic acid, C F, anhydroleucoverin, and *thf*; it has repeatedly been recovered from a fluorescent spot on paper chromatograms in various solvent systems.

*Experiment I*

The assay mixture contains the following substances in a total volume of 0.6 ml: 20.0 $\mu M$ DL-serine**, 0.02 $\mu M$ DPN, 2.0 $\mu M$ $MnSO_4$, 0.02 $\mu M$ pyridoxal phosphate, 0.04 ml *M* K-phosphate buffer pH 6.5, and 0.2 ml of an enzyme preparation (20 mg protein/ml) which was dialyzed 43 hours against 0.01 *M* phosphate buffer pH 7.2. The samples were incubated 2 hours at 28° C. The control value in the absence of serine (= 0.046 $\mu M$) is subtracted from each experimental value given.

| *Sample* | *Omitted from assay mix* | *$\mu M$ glycine formed*† |
|---|---|---|
| 1 | — | 0.314 |
| 2 | DPN | 0.078 |
| 3 | $Mn^{++}$ | 0.108 |
| 4 | pyridoxal phosphate | 0.134 |

* The most highly purified preparation of Co C obtained thus far by charcoal and paper chromatography has an absorption peak at 275–280 m$\mu$. Assuming that the molecular extinction coefficient is the same as that for the folic acid derivatives ($\varepsilon_{280(max)}$ = 18,000–26,000) UV absorption at 280 m$\mu$ indicates that Co C is active at less than 0.01 the concentration of the glycine formed.