Hammarsten, E. (1920). *Hoppe-Seyl. Z.* **109**, 141.
Hammarsten, O. (1894). *Hoppe-Seyl. Z.* **19**, 19.
Hevesy, G. & Ottesen, J. (1943). *Acta physiol. scand.* **5**, 237.
Javillier, M. & Allaire, H. (1926*a*). *C.R. Acad. Sci., Paris*, **183**, 162.
—— —— (1926*b*). *Bull. Soc. Chim. biol., Paris*, **8**, 924.
—— —— (1931). *Bull. Soc. Chim. biol., Paris*, **13**, 678.
Jones, W. (1920). *Nucleic Acids*, p. 9. London: Longmans Green and Co.
Jones, W. & Perkins, M. E. (1924–5). *J. biol. Chem.* **62**, 290.
Jorpes, E. (1924). *Biochem. Z.* **151**, 227.
—— (1928). *Act. med. scand.* **68**, 253, 503.
—— (1934). *Biochem. J.* **28**, 2102.
Kerr, S. E. (1940). *J. biol. Chem.* **132**, 147.
—— (1942). *J. biol. Chem.* **145**, 647.
—— & Blish, M. E. (1932). *J. biol. Chem.* **98**, 193.
Kiesel, A. & Belozerski, A. N. (1934). *Hoppe-Seyl. Z.* **229**, 160.
Kossel, A. (1882). *Hoppe-Seyl. Z.* **7**, 7.
Kosterlitz, H. W. & Cramb, I. (1943). *J. Physiol.* **102**, 18*P*.
Kunitz, M. (1940). *J. gen. Physiol.* **25**, 15.
Le Breton, E. & Shaeffer, G. (1923). *Variations biochimiques du rapport nucléoplasmique au cours du développement embryonnaire*. Paris: Masson et Cie.
Levene, P. A. & Jorpes, E. (1930). *J. biol. Chem.* **86**, 389.
—— & Mandel, J. A. (1907). *Hoppe-Seyl. Z.* **49**, 262.
Lohmann, K. (1928). *Biochem. Z.* **194**, 306.
Loofbourow, J. R. (1942). *Biochem. J.* **36**, 631, 737.
Loring, H. S. (1939). *J. biol. Chem.* **130**, 251.
Malan, A. I. & Green, H. H. (1928). *J. agric. Sci.* **18**, 391.
Mandel, J. A. & Levene, P. A. (1905). *Hoppe-Seyl. Z.* **46**, 155.
Marshak, A. (1940). *Science*, **92**, 460.
—— (1941). *J. gen. Physiol.* **25**, 275.
Masing, E. (1911). *Hoppe-Seyl. Z.* **75**, 135.
Mathison, G. C. (1909). *Biochem. J.* **4**, 233.
Mendel, L. B. & Leavenworth, C. S. (1908). *Amer. J. Physiol.* **21**, 77.
Menke, W. (1938). *Hoppe-Seyl. Z.* **257**, 43.
Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
Mirsky, A. E. (1943). *Advances in Enzymology*, **3**, 1.
—— & Pollister, A. W. (1942). *Proc. nat. Acad. Sci., Wash.*, **28**, 344.
Mitchell, J. S. (1942). *Brit. J. exp. Path.* **23**, 285, 296, 309.
Needham, J. (1942). *Biochemistry and Morphogenesis*, p. 631. Cambridge: University Press.
—— & Needham, D. M. (1930). *J. exp. Biol.* **7**, 317.
Neuberg, C. (1904). *Ergebn. Physiol.* **3**, 373.
Ostern, P., Terszakowec, J. & Hubl, S. (1938). *Hoppe-Seyl. Z.* **255**, 104.
Painter, T. S. & Taylor, A. N. (1942). *Proc. nat. Acad. Sci., Wash.*, **28**, 311.
Plimmer, R. H. A. & Kaya, R. (1910). *J. Physiol.* **39**, 409.
—— & Scott, F. H. (1908). *J. chem. Soc.* **93**, 1699.
Potter, V. R. & Albaum, H. G. (1943). *J. gen. Physiol.* **26**, 443.
Reeves, R. E. & Munro, J. (1940). *Industr. Engng Chem.* (Anal. ed.), **12**, 551.
Reis, J. (1937). *Enzymologia*, **2**, 110, 183.
Rosenthal, O. & Drabkin, D. L. (1943). *J. biol. Chem.* **150**, 131.
Schultz, J. (1941). *Cold Spr. Harb. Symp. quant. Biol.* **9**, 55.
Sevag, M. G., Smolens, J. & Lackman, D. B. (1940). *J. biol. Chem.* **134**, 523.
Stanley, W. M. (1939). *J. biol. Chem.* **129**, 405.
—— & Knight, C. A. (1941). *Cold Spr. Harb. Symp. quant. Biol.* **9**, 255.
Stedman, E. & Stedman, E. (1943*a*). *Nature, Lond.*, **152**, 267.
—— —— (1943*b*). *Nature, Lond.*, **152**, 556.
Steudel, H. (1936). *Hoppe-Seyl. Z.* **241**, 84.
Taylor, A. R., Sharp, D. G., Beard, D. & Beard, J. W. (1942). *J. infect. Dis.* **71**, 115.
—— —— —— —— (1943). *J. infect. Dis.* **72**, 31.
—— —— & Woodhall, B. (1943). *Science*, **97**, 226.
Thomas, P. & Berariu, C. (1924). *C.R. Soc. Biol., Paris*, **91**, 1470.
Tuttle, L. W., Erf, L. A. & Lawrence, J. H. (1941). *J. clin. Invest.* **20**, 57.
Vowles, R. B. (1940). *Ark. Kemi Min. Geol.* **14** B, no. 10.
Wells, H. G. (1925). *Chemical Pathology*, 5th ed., pp. 20, 31. Philadelphia: W. B. Saunders.
Willmer, E. N. (1942). *19th Ann. Rep. Brit. Emp. Cancer Campaign*, p. 53.
Wohlgemuth, J. (1903). *Biochem. Zbl.* **1**, 533.

# The 'Activation' of Phosphatase

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Following the demonstration that ligature of the common bile-duct led to an enormous increase in the amount of phosphatase in the blood (Armstrong, King & Harris, 1934), we investigated the possibility that the enhanced plasma phosphatase values found in jaundice and certain bone diseases might be due to the presence of a powerful activator rather than to an actual accumulation of phosphatase in the blood stream. Since none of our experiments gave evidence of the first possibility the results were not published, but in view of a renewal of interest in the subject and of several claims to have demonstrated an activator (Thannhauser, Reichel, Gratton & Maddock, 1938; Cantarow, 1940), it seemed advisable to complete our observations and report the conclusions.

Our first experiments, following the observation by Armstrong *et al.* (1934) of large amounts of

phosphatase in the bile and faeces of dogs, sought for an activator in bile and faecal phosphatase preparations. Others were a repetition of part of the work of Thannhauser *et al.* (1938). A final set of tests was devised to exclude as rigidly as possible all variables such as pH, concentration of reactants, etc., which might have contributed to results which appeared to indicate the presence of an activator.

## METHODS

*Preparation of phosphatase solutions.* The kidney and intestinal preparations were made by grinding the tissue with 10 times its weight of 0·9% NaCl solution saturated with chloroform, followed by filtration through glass wool. The bile was diluted with 0·9% NaCl to give an activity of 20–50 units/100 ml. Faecal phosphatase solutions were prepared according to Delory & King (1943).

*Determination of phosphatase activity.* (1) Phenyl phosphate method of King, Haslewood & Delory (1937)—M/100 $Na_2(C_6H_5)PO_4$ substrate and M/10 $Na_2CO_3$-$NaHCO_3$ buffer of pH 9·9. The enzymically liberated phenol is taken as a measure of the phosphatase activity (King & Armstrong (1934) units). Folin & Ciocalteu's phosphotungstic-phosphomolybdic acid reagent (diluted 1 in 3) is used to precipitate the proteins and to estimate the free phenol. 1 ml. of 20% $Na_2CO_3$ is added to 4 ml. of filtrate to develop the phenol blue colour.

(2) Glycerophosphate method of Bodansky (1933)—10 ml. of 5% $Na_2$-$\beta$-glycerophosphate in M/10 veronal buffer are incubated at 37° with a stated amount of serum; trichloroacetic acid is added and free phosphate determined in the filtrate.

## EXPERIMENTS

*Attempted activation of intestinal phosphatase by bile.* If of two enzymes one has a higher activity than the other owing to the presence of an activator, then the activity of a mixture of the two should be greater than the sum of the separate activities. The phosphatase activities of bile, of an intestinal preparation and of a mixture of these were estimated. Some typical results are given in Table 1; these do not suggest the presence of any activating substance. Negative results would also be obtained if each of the preparations contained the optimum amount of activating substance, but this possibility is extremely unlikely. The figures in columns A, B and C show that, if any activator be present, it must be heat-labile.

*Attempt to activate plasma phosphatase by highly active preparation from faeces and kidney.* 1 ml. of a kidney or faecal phosphatase preparation was heated together with 5 ml. of water to destroy the enzyme activity. After cooling, 1 ml. of a normal plasma was added. The solution was diluted to 15 ml. with water (solution A).

1 ml. of plasma + 13 ml. of water were heated for 5 min. in a boiling water-bath, cooled, and after addition of 1 ml. phosphatase the solution was diluted to 15 ml. (solution B).

Solution C was a mixture of equal parts of A and B.

Table 1. *Effect of diluted bile on intestinal phosphatase activity*

| Activity (mg. of phenol liberated/100 ml. of phosphatase preparation) of | | | | | |
|---|---|---|---|---|---|
| A | B | C | D | E | |
| Intestinal phosphatase + dilute bile | Intestinal phosphatase + dilute bile (inactivated by heat) | Intestinal phosphatase | Dilute bile | Calc. C + D | % activation $\frac{A-E}{A} \times 100$ |
| 66 | 45 | 46 | 24 | 70 | −6 |
| 74 | 41 | 42 | 31 | 74 | 0 |
| 110 | 57 | 56 | 52 | 108 | 2 |

Table 2. *Effect of addition of kidney and faecal phosphatase preparations on plasma phosphatase activity*

| | Activity (mg. phenol liberated/100 ml. of enzyme solution) of | | | | |
|---|---|---|---|---|---|
| | A | B | C | D | |
| Phosphatase | Phosphatase (inactivated by heat) + plasma | Phosphatase + plasma (inactivated by heat) | Mixture of equal parts of A and B | Calc. $\frac{1}{2}A + \frac{1}{2}B$ | % activation $\frac{C-D}{C} \times 100$ |
| Kidney | 1·8 | 11·7 | 6·7 | 6·7 | 0 |
| | 1·7 | 11·6 | 6·7 | 6·65 | 0·8 |
| | 1·4 | 3·1 | 2·4 | 2·25 | 6·2 |
| Faecal | 5·2 | 15·2 | 10·0 | 10·2 | −2·0 |
| | 4·8 | 14·6 | 9·5 | 9·7 | −2·1 |
| | 3·7 | 17·1 | 10·4 | 10·4 | 0 |

Table 3. *Effect on phosphatase activity of addition of serum from cases of Paget's disease and obstructive jaundice*

(Figures in brackets indicate pH)

| | | mg. of inorganic P liberated during 1 hr. hydrolysis | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Observed | | | | Observed | | |
| No. | Nature of high phosphatase serum | 0·5 ml. normal serum | 0·1 ml. high serum | 0·5 ml. normal +0·1 ml. high | Calc. as sum of components | 0·2 ml. high serum | 0·5 ml. normal +0·2 ml. high | Calc. as sum of components |
| 1 | Paget's disease | 0·032 (8·5) | 0·054 (9·0) | 0·096 (8·5) | 0·086 | 0·087 (9·0) | 0·148 (8·4) | 0·119 |
| 2 | Paget's disease | 0·048 (8·5) | 0·052 (9·0) | 0·120 (8·5) | 0·100 | 0·120 (9·0) | 0·168 (8·4) | 0·160 |
| 3 | Paget's disease | 0·099 (8·7) | 0·050 (9·1) | 0·160 (8·5) | 0·149 | | | |
| 4 | As 3, pH adjusted | 0·101 (8·5) | 0·062 (8·5) | 0·160 (8·5) | 0·161 | | | |
| 5 | Paget's disease | 0·071 (8·7) | 0·100 (9·1) | 0·172 (8·5) | 0·171 | | | |
| 6 | As 5, pH adjusted | 0·070 (8·5) | 0·118 (8·5) | 0·175 (8·5) | 0·189 | | | |
| 7 | Icteric | 0·025 (8·7) | 0·099 (9·1) | 0·120 (8·5) | 0·124 | | | |
| 8 | As 7, pH adjusted | 0·025 (8·5) | 0·100 (8·5) | 0·125 (8·5) | 0·130 | | | |
| 9 | Icteric | 0·059 (8·7) | 0·410 (9·1) | 0·460 (8·5) | 0·469 | | | |
| 10 | As 9, pH adjusted | 0·059 (8·5) | 0·430 (8·5) | 0·460 (8·5) | 0·489 | | | |
| 11 | Paget's disease | 0·52 (8·7) | 0·86 (9·1) | 1·25 (8·5) | 1·38 | 24 hr. hydrolysis | | |
| 12 | As 11, pH adjusted | 0·48 (8·5) | 0·95 (8·5) | 1·31 (8·5) | 1·43 | | | |

5 ml. portions of the buffer substrate (phenyl phosphate) were placed in a water-bath for 5 min. to allow them to attain the temperature of the bath (37°). 2 ml. of A, B or C, previously warmed, were added and the hydrolysis allowed to proceed for exactly 30 min., when 3 ml. of Folin & Ciocalteu's reagent were added.

The results obtained are given in Table 2. It will be seen that there was no activator in the preparations from either kidney or faeces.

*'Activation' of plasma phosphatase by the procedure of Thannhauser* et al. (1938). High phosphatase sera were mixed with low phosphatase sera, and their activities measured according to the method of Bodansky. The pH of the mixture was noted. In some experiments the pH of the components was adjusted to the same value before mixing. Some of the results (Table 3) indicate an apparent activation, i.e. the activity of the mixed sera is greater than the sum of the activities of its components. But this 'activation' is only occasional, and is probably due to causes considered in the Discussion.

*Attempt to demonstrate activation under closely controlled conditions.* In order to make a properly controlled comparison of the phosphatase activity of several sera and mixtures of sera, several criteria must be satisfied:

(1) The pH must be the same in all instances.

(2) The proportion of serum, substrate and buffer must be the same in all of the incubated mixtures.

(3) Care must be taken to insure against a variable destruction of the enzyme, due to the alkaline reaction of the medium.

All these rules for legitimate comparison are violated in the preceding experiment. In the following tests a more reliable result has been achieved by using constant amounts of buffer, serum, substrate, identical conditions of pH, and by employing

Table 4. *Attempt to demonstrate activation of phosphatase in normal serum by addition of high phosphatase serum*

| | | Phosphatase activity in King-Armstrong units/100 ml.: (*a*) without preliminary incubation, and (*b*) after preliminary incubation | | | |
|---|---|---|---|---|---|
| Nature of high phosphatase serum | | Normal serum | High phosphatase serum | 5 Parts normal +1 part high | Calc. as sum of components |
| Paget's disease | *a* | 4·9 | 78·1 | 17·0 | 17·1 |
| | *b* | 5·1 | 79·2 | 16·4 | 17·5 |
| Icteric | *a* | 5·0 | 72·1 | 14·5 | 16·1 |
| | *b* | 4·6 | 72·8 | 15·5 | 16·0 |
| Paget's disease | *a* | 10·4 | 80·2 | 23·8 | 22·0 |
| | *b* | 9·5 | 79·4 | 23·6 | 21·1 |
| Fortified with faecal phosphatase | *a* | 4·1 | 141·2 | 24·5 | 26·9 |
| | *b* | 5·0 | 137·3 | 26·4 | 27·0 |
| Fortified with faecal phosphatase | *a* | 4·5 | 81·4 | 19·0 | 17·4 |
| | *b* | 5·9 | 84·1 | 19·1 | 18·9 |
| Paget's disease | *a* | 11·6 | 130·0 | 29·9 | 31·1 |
| | *b* | 11·4 | 128·3 | 30·7 | 31·0 |
| Icteric | *a* | 4·5 | 53·4 | 13·0 | 12·6 |
| | *b* | 4·8 | 53·0 | 12·4 | 12·8 |
| Icteric | *a* | 7·2 | 61·2 | 16·2 | 16·2 |
| | *b* | 7·9 | 62·4 | 15·7 | 15·3 |
| | | | | 1 part normal +1 part high | |
| Icteric | *a* | 10·1 | 56·9 | 32·8 | 33·5 |
| Icteric | *a* | 9·5 | 82·3 | 46·0 | 45·6 |
| Icteric | *a* | 11·2 | 61·7 | 36·4 | 35·9 |
| Paget's disease | *a* | 6·2 | 85·3 | 46·3 | 45·7 |

short periods of incubation. The possibility of a slow activation was covered by incubating samples of the normal with the icteric or Paget's serum for 24 hr. before determination of enzyme activity. The estimation of phosphatase was carried out by the phenylphosphate method.

The determinations were performed on high phosphatase sera, normal sera and on mixtures of five parts normal serum with one of high phosphatase serum, this last being claimed by Thannhauser *et al.* (1938) to give the maximum activation. Samples of the three specimens were then kept at 37° for 24 hr., the pH's checked, and the determinations repeated. All estimations were carried out in duplicate and the means of typical results are recorded in Table 4, which also includes the results of experiments made with a mixture of equal parts of the normal and high-phosphatase sera without preliminary incubation.

## DISCUSSION

The experiments recorded are only a few of many we have performed, but they are representative and give a fair picture of the results obtained. We have been unable in any of our work to demonstrate the presence of an activating substance in plasma of high phosphatase content. This is contrary to the findings of Thannhauser *et al.* (1938), who claimed that normal serum was activated by the addition of serum from a patient with Paget's disease. Cantarow (1940) claimed that icteric sera had no effect on the activity of normal sera when mixed in proportions varying from 1/5 to 5/1 with an incubation of 1 hr., but that with an incubation period of 24 hr. there was evidence of activation of the normal by the icteric serum. In one of our experiments we attempted to repeat Thannhauser's tests but this was somewhat difficult, owing, as Allcroft & Folley (1941) have pointed out, to the lack of experimental detail given. Thannhauser measured the amount of phosphate enzymically liberated by 0·5 ml. of normal serum and by 0·1 ml. of high phosphatase serum and compared these figures with the amount of hydrolysis obtained with the two sera together. In this type of experiment the pH is not the same in the three mixtures, nor is there a constant proportion of buffer, substrate and serum, so that it seems possible that the results obtained by Thannhauser *et al.* and by Cantarow were a result of errors inherent in their technique. In our experiments on this model (Table 3), we were not able to demonstrate any consistent activation of the low phosphatase by the high. Tests 3 and 4 (Table 3) seemed to suggest that the apparent activation was due to the fact that the high phosphatase serum was acting at pH 9·1, while the normal serum was acting at pH 8·7 and the mixture at a still lower pH of 8·5, but later experiments did not confirm this. The table does show, however, the variable results obtained by this procedure. Much better agreement is obtained by our final method (Table 4).

Our failure to obtain any evidence of the presence of an activating substance in high phosphatase serum is supported by the somewhat similar experiment of Albers (1940), and by Williams & Watson (1941), who measured the reaction velocities for the activity of purified plasma phosphatase preparations to which had been added optimum amounts of the known activators, Mg salts and glycine.

Allcroft & Folley (1941) found no evidence in favour of the suggestion that the very high blood phosphatase values found in certain breeds of cows might be due to the presence of an activator. It was also noted that one of them (Folley) had considered the possibility in 1935 that the high blood phosphatase of jaundice was due to an activation phenomenon, but had abandoned the idea on the basis of evidence obtained at that time.

Serum phosphatase values of over 100 King-Armstrong units/100 ml. have been encountered in generalized bone disease and values as high as 250 units/100 ml. have been recorded in bone sarcoma and in obstructive jaundice. In these results we are dealing with an increase over the normal which is between tenfold and twenty-fivefold, beside which Thannhauser's 'activation' of 35% appears insignificant.

## SUMMARY

No evidence has been found to support the hypothesis that the high serum phosphatase values found in generalized bone disease and in jaundice are due to the presence of an activator and it is concluded that there is an actual increase in the amount of enzyme present in the serum in these conditions.

## REFERENCES

Albers, D. (1940). *Hoppe-Seyl. Z.* **266**, 1.
Allcroft, W. M. & Folley, S. J. (1941). *Biochem. J.* **35**, 254.
Armstrong, A. R., King, E. J. & Harris, R. I. (1934). *Canad. med. Ass. J.* **31**, 14.
Bodansky, A. (1933). *J. biol. Chem.* **101**, 93.
Cantarow, A. (1940). *Amer. J. clin. Path.* **10**, 858.
Delory, G. E. & King, E. J. (1943). *Biochem. J.* **37**, 547.
King, E. J. & Armstrong, A. R. (1934). *Canad. med. Ass. J.* **31**, 376.
—— Haslewood, G. A. D. & Delory, G. E. (1937). *Lancet*, **1**, 886.
Thannhauser, S. J., Reichel, M., Gratton, J. F. & Maddock, S. J. (1938). *J. biol. Chem.* **124**, 631.
Williams, H. L. & Watson, E. M. (1941). *J. Lab. clin. Med.* **26**, 1333.