

# THE CATALYTIC EFFECT OF MOLYBDATE ON THE BREAKDOWN OF PHOSPHOCREATINE\*

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## Summary

The hydrolysis of phosphocreatine in 0.1N HCl at 37 and 65°C. yields, as the principal products, creatine and orthophosphoric acid. Under the influence of molybdate, phosphocreatine in 0.1N HCl at 37 and 65°C. breaks down into creatinine, creatine, and orthophosphoric acid. The relative proportions of the two former compounds are a function of the molybdate concentration. Increase of pH leads to a progressive reduction in this effect of molybdate, which apparently depends upon the degree of ionization.

Of other compounds investigated, only sodium vanadate had a similar effect and then only in a concentration ten times greater than that of ammonium molybdate.

## I. INTRODUCTION

The catalytic effect of molybdate on the acid hydrolysis of phosphocreatine was first described by Fiske and Subbarow (1929) but the products of this hydrolysis were not identified. It is generally supposed that these would be creatine and orthophosphoric acid although Meyerhof and Lohmann (1928) were unable to detect the former by the diacetyl method of Walpole (1911). It was concluded that the creatine liberated was so changed in the presence of molybdate that it no longer gave the diacetyl reaction. The most obvious change which creatine could undergo under such conditions is conversion to creatinine, but in view of the tendency of phosphocreatine to ring closure the formation of creatinine without the intermediate formation of creatine must be borne in mind. The possible reactions involving the hydrolysis of phosphocreatine in acid solution and in the presence of molybdate may be represented as in Figure 1.

The possibility of reaction (I) occurring in the presence of molybdate is not ruled out by the results of Meyerhof and Lohmann (1928). The formation of creatinine from creatine—reaction (II)—occurs in acid solution. Reaction (III), involving the loss of phosphoric acid across the carboxyl group with simultaneous ring closure, apparently occurs in the solid state. Thus Zeile and Meyer (1938) have shown that the calcium salt of phosphocreatine when heated in vacuo and in the presence of dry HCl is transformed into creatinine.

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Such a reaction also occurs in aqueous solution and has formed the basis of the hypothesis developed by Borsook and Dubnoff (1947) to account for the formation *in vivo* of creatinine from phosphocreatine by a non-enzymic reaction. The amounts of creatinine which were formed under the conditions used by these workers were small and would not account for the negative

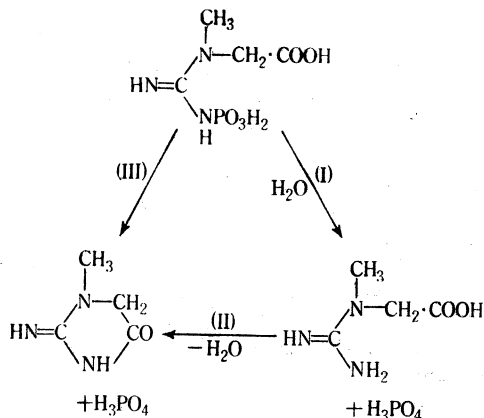


Fig. 1.—Reactions involving hydrolysis of phosphocreatine.

creatine tests reported by Meyerhof and Lohmann (1928). Lundquist (1947) has reported that under more severe conditions (in the presence of HCl or "8/9 saturated picric acid") a small proportion of phosphocreatine is transformed directly into creatinine. No reference has been found to the effect of molybdate on this reaction. It is the purpose of this communication to report the results of investigations designed to show that, under the influence of molybdate, phosphocreatine is transformed into creatinine without the intermediate formation of creatine.

## II. MATERIALS AND METHODS

*Phosphocreatine.*—A preparation of the crystalline sodium salt synthesized as described by Ennor and Stocken (1948). All solutions were prepared immediately before use and the pH adjusted to approx. 7.3 by the addition of 0.1N NaOH.

*Ammonium molybdate.*—Reagent grade.

*Inorganic phosphate.*—A modification by Ennor and Stocken (1950) of the Berenblum and Chain (1938) method was used. This modification makes possible the accurate and direct determination of inorganic phosphate in the presence of phosphocreatine.

*Creatine.*—The method described by Eggleton, Elsdon, and Gough (1943) was used with minor modifications.

*Creatinine.*—Determined by the alkaline picrate method.

## III. RESULTS

As it has been pointed out by Lundquist (1947) that, during the acid hydrolysis of phosphocreatine, there is some formation of creatinine, the initial experiments were designed to determine the mildest conditions which would effect complete hydrolysis and produce minimal amounts of creatinine. Experimentally it was found that these conditions were exposure to 65°C. for 9 minutes in the presence of 0.1N HCl (Table 1).

It was not possible to reduce the amount of creatinine formed and yet attain complete hydrolysis by variation of the time of hydrolysis, pH, and temperature. The effect of molybdate on such a hydrolysis was then determined in a parallel experiment. The technique adopted was to add, at zero time, 2.0 ml. of an approximately  $1.5 \times 10^{-3}M$  solution of sodium phosphocreatine to each of two tubes (one containing 2.0 ml. of 0.4N HCl and 4.0 ml. of water, and the other 2.0 ml. of 0.4N HCl, 1.0 ml. of  $1 \times 10^{-3}M$  ammonium molybdate, and 3.0 ml. water) both of which had been equilibrated at 65°C. At zero + 9 min., 2.0 ml. of 0.4N NaOH was added to each tube, the contents mixed, and cooled in an ice bath. Suitable aliquots were then withdrawn for the determination of P, creatine, and creatinine. As a check on the possible effect of molybdate on creatine in acid solution, 2.0 ml. of a solution containing 35  $\mu g.$  of creatine and 30  $\mu g.$  of P/ml. were added to a third tube containing 2.0 ml. 0.4N HCl, 1.0 ml. of  $1 \times 10^{-3}M$  ammonium molybdate, and 3.0 ml. of water. This was treated in a similar manner to the other tubes. The results (Table 1) show that, in the absence of molybdate, 10.8 per cent. of the phosphocreatine (expressed as creatine) is converted to creatinine, whereas in the

TABLE 1  
PRODUCTS OF HYDROLYSIS OF PHOSPHOCREATINE IN 0.1N HCl AT 65°C. FOR 9 MINUTES  
IN PRESENCE AND ABSENCE OF AMMONIUM MOLYBDATE (FINAL CONC. =  $1.25 \times 10^{-4}M$ )  
All results are the means of duplicate determinations

	Tube 1 Phosphocreatine No Molybdate	Tube 2 Phosphocreatine Plus Molybdate
$\mu g.$ Creatine	129	60.5
$\mu g.$ P	35.4	35.0
$\mu g.$ Creatinine	13.5	71
$\mu g.$ Creatine $\equiv$ creatinine	15.7	82.2
$\therefore \mu g.$ Total creatine	144.7	142.7
Molar ratio P/creatine	1.03	1.03

In tube 3, to which 70  $\mu g.$  of creatine and 60  $\mu g.$  of P had been added, 70  $\mu g.$  were recovered on analysis and no creatinine detected.

presence of a final concentration of  $1.25 \times 10^{-4}M$  molybdate a 57.6 per cent. conversion has occurred. As creatine is unchanged under these conditions, it is evident that, under the influence of molybdate, phosphocreatine is converted directly into creatinine.

The influence of varying concentrations of molybdate on the hydrolysis of phosphocreatine in 0.1N HCl at 65°C. for 9 min. has been determined over the range of 0.025 to 0.5  $\mu$ -moles of ammonium molybdate/ml. These results (Fig. 2) indicate the marked influence of molybdate on the amount of creatine

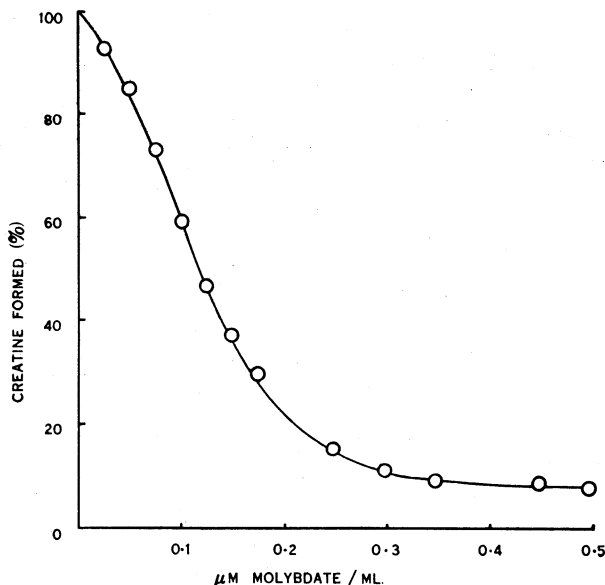


Fig. 2.—Influence of increasing concentration of molybdate ion on the amount of creatine released from phosphocreatine in 0.1N HCl at 65°C. in 9 min. These conditions produce complete hydrolysis. All points on the curve represent the means of at least two determinations. Phosphocreatine conc. 0.13  $\mu$ -moles/ml.

released from phosphocreatine under conditions which, even in the absence of this ion, effect complete hydrolysis. There is, however, a pronounced flattening of the curve at the higher concentrations. Thus with 0.4  $\mu$ -moles of molybdate/ml., 11.4 per cent. of the possible amount of creatine is formed and this is reduced to only 8 per cent. when the amount of molybdate is increased to 0.5  $\mu$ -moles/ml.

It was considered of interest to determine the effect of the molybdate ion upon the hydrolysis of phosphocreatine in 0.1N HCl and under conditions of temperature and time which, in the absence of molybdate did not effect complete hydrolysis. A temperature of 37°C. was chosen for these experiments, and the degree of hydrolysis at the end of each time interval was measured by determining the inorganic P released. The results (Fig. 3) indicate that under these conditions the compound is 50 per cent. hydrolysed in 15 minutes and complete hydrolysis is reached in 90 minutes.

In the experiments employing molybdate a constant hydrolysis time of 30 minutes was chosen as this, in the absence of molybdate, produces incomplete hydrolysis (Fig. 3). The catalytic effect of molybdate upon this hydrolysis is

apparent from Figure 4. In these experiments an experimental technique similar to that employed in the experiments at 65°C. was followed, and the degree of hydrolysis was determined as before.

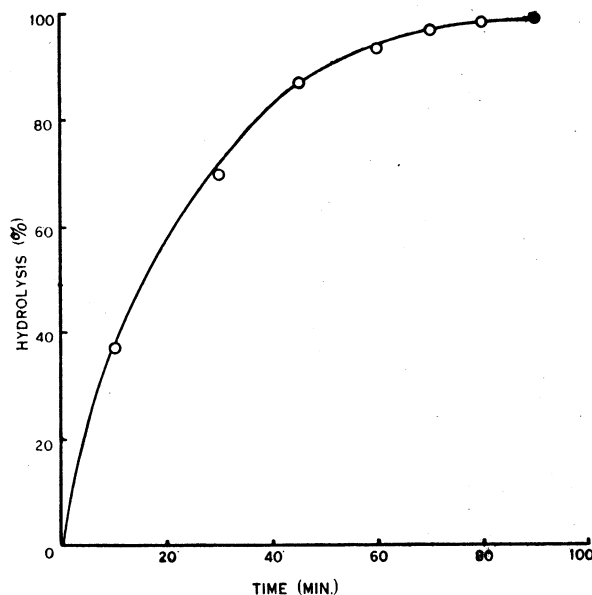


Fig. 3.—Hydrolysis curve of phosphocreatine in 0.1N HCl at 37°C. All points on the curve represent the means of two determinations.

Under these conditions the curve (Fig. 5) illustrating the amounts of creatine produced with varying amounts of molybdate is very similar to that obtained at 65°C. (Fig. 2). It is also of interest to note that, at 37°C. and in the presence of 0.5  $\mu$ -moles of molybdate/ml., virtually 100 per cent. hydrolysis occurs in 30 minutes (Fig. 4), and that again conversion to creatinine is not quite quantitative—about 8 per cent. of the possible amount of creatine still appears (Fig. 5). It has been found impossible to obtain quantitative conversion even by increasing the molybdate concentration to 50  $\mu$ -moles/ml.

The effect of molybdate upon the course of the hydrolysis of phosphocreatine at varying pH has also been determined. In these experiments a potassium hydrogen phthalate buffer was used at a final concentration of 0.1M. pH values were checked with the glass electrode. The experiments were carried out at 37°C., and the time of hydrolysis was in all cases 30 minutes. As before, the degree of hydrolysis was determined by measurement of the inorganic phosphate released, and, in addition, simultaneous determinations were made of the creatine and creatinine formed. As a check on the analytical methods, the molar ratio—P/total creatine, i.e. creatine + creatine equivalent of creatinine—has been calculated and is included in Table 2. It is clear that, irrespective of the degree of hydrolysis and whether or not molybdate is present, the analytical methods used are capable of illustrating the course of the

hydrolysis with reasonable accuracy. With decreasing pH the percentage of phosphocreatine hydrolysed decreases both in the presence and absence of molybdate. At any one pH the catalytic effect of molybdate is apparent although this effect, like that of increased creatinine formation, disappears above pH 4.0; thus at pH 4.6 and 5.4 there is no greater formation of creatinine when molybdate is present, and the degree to which the phosphocreatine is hydrolysed remains affected only by the pH.

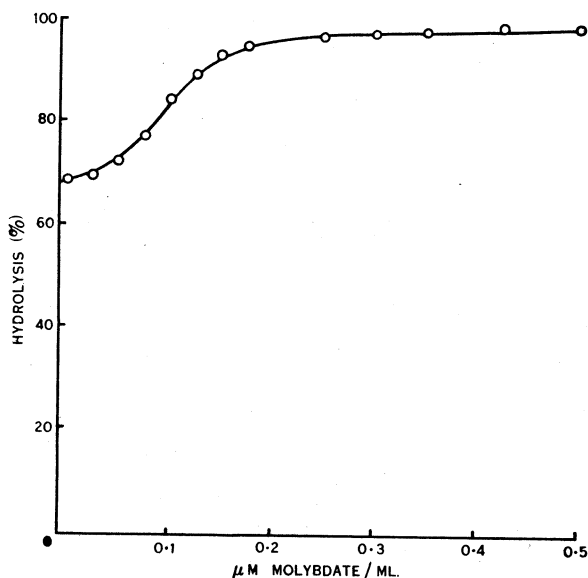


Fig. 4.—Hydrolysis curve of phosphocreatine in 0.1N HCl at 37°C. for 30 min. in the presence of varying amounts of ammonium molybdate. Phosphocreatine conc. 0.13  $\mu$ -moles/ml. All points on the curve represent the means of duplicate determinations.

#### IV. DISCUSSION

The experimental results do not permit of an explanation either of the effect of molybdate as a catalyst in the breakdown of phosphocreatine or of its effect in altering the normal direction of hydrolysis, with the production of larger amounts of creatinine. It seems possible, however, that the reaction depends upon the formation of a molybdate-phosphocreatine complex, as indeed is suggested by the results in Table 2. In this connection it is of interest to note that the hydrolysis of phosphoarginine is actually retarded in the presence of molybdate (Lohmann 1928).

Irrespective of the mechanism involved it is now clear that the failure of Meyerhof and Lohmann (1928) to detect creatine after an acid-molybdate hydrolysis of phosphocreatine was due not to any change in creatine, as was suggested, but to the fact that creatinine, and not creatine, was the principal product of the hydrolysis.

Some experiments have been carried out to determine the effect of other inorganic compounds on the hydrolysis of phosphocreatine. An effect similar to that of ammonium molybdate was found only with sodium vanadate. On an equivalent concentration basis this compound was only about one-tenth as effective as ammonium molybdate.

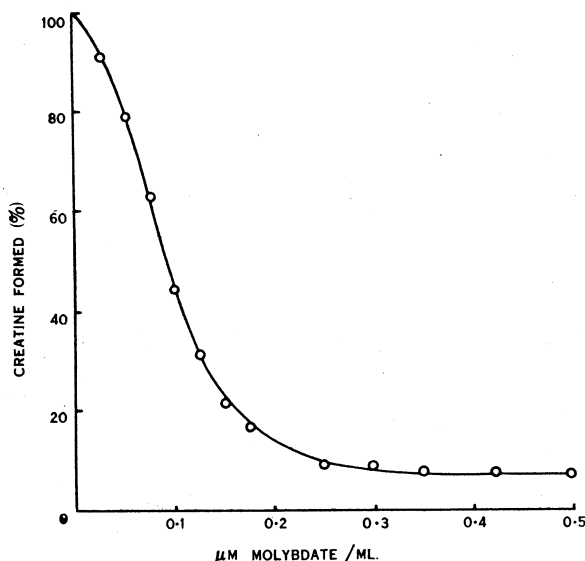


Fig. 5.—Influence of increasing concentration of molybdate ion on the amount of creatine released from phosphocreatine in 0.1N HCl at 37°C. in 30 min. The ordinate gives the amount of creatine released expressed as the percentage of the maximum possible amount under similar conditions of time, temperature, and acid concentration but in the absence of molybdate. All points on the curve represent the means of duplicate determinations.

It seemed possible that the failure of many workers in the past to prepare an enzyme system capable of transforming phosphocreatine to creatinine may have been due to the absence of a coenzyme. Since Mo is a trace metal to which no function in the body has, as yet, been ascribed and because of its effect in a model system *in vitro*, experiments were designed to test the hypothesis that Mo may have such a coenzyme action. These experiments were carried out with dialysed homogenates of skeletal muscle, liver, and kidney, and yielded negative results.

It is possible, since molybdate induces ring closure very readily under extremely mild conditions with phosphocreatine, that it may also be useful in effecting ring closure in other N-phosphorylated compounds. A further application of the effect of molybdate on phosphocreatine is its use as a means of identi-

ying this compound in a mixture of such acid-molybdate-labile organo-phosphate compounds as are found in certain tissue extracts (Barker and Ennor, unpublished data).

TABLE 2

EFFECT OF AMMONIUM MOLYBDATE (FINAL CONC. =  $1.25 \times 10^{-4}M$ ) AND VARYING pH UPON THE HYDROLYSIS OF PHOSPHOCREATINE

Conditions of hydrolysis: 30 min. at 37°C. Buffer, potassium hydrogen phthalate in a final conc. of 0.1M. The following abbreviations are used: CRINE—creatinine; CR—creatine; P—inorganic phosphate

Molybdate	pH	CRINE ( $\mu g.$ )	CR Equiv- alent ( $\mu g.$ )	CR ( $\mu g.$ )	Total CR ( $\mu g.$ )	P ( $\mu g.$ )	Molar Ratio P/CR	Hydro- lysis %
Present	0.1N HCl	31.0	36.0	17.5	53.5	12.9	1.02	89
Absent		4.1	4.8	35.7	40.5	10.2	1.06	70
Present	2.8	22	25.5	25.0	50.5	12.5	1.05	86
Absent		3.3	3.8	37.8	41.6	10.4	1.06	71
Present	3.5	12.4	14.4	30.9	45.3	11.0	1.02	76
Absent		3.6	4.2	37.8	42.0	10.3	1.04	71
Present	4.0	7.9	9.2	33.5	42.7	10.4	1.03	71
Absent		2.9	3.4	34.6	38.0	9.0	1.00	62
Present	4.6	2.9	3.4	26.1	29.5	7.0	1.00	48
Absent		2.9	3.4	26.1	29.5	7.0	1.00	48
Present	5.4	2.3	2.7	10.7	13.4	3.2	1.01	22
Absent		2.3	2.7	10.7	13.4	3.2	1.01	22

#### V. ACKNOWLEDGMENTS

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