STUDIES ON PHOSPHOMUTASES

III. THE PHOSPHORIBOMUTASE OF BOVINE UTERUS*

by

ARMAND J. GUARINO** AND HENRY Z. SABLE***

Department of Biochemistry and Nutrition, Tufts College Medical School, Boston, Mass., and Department of Biochemistry, Western Reserve University School of Medicine, Cleveland, Ohio (U.S.A.)

In a preceding paper[2] we reported existence of phosphoribomutases distinct from phosphoglucomutase in extracts of bakers' and brewers' yeast, of uterine muscle (rabbit, bovine, human), and in human blood. In all cases, the phosphoribomutases appeared to have no requirement for a divalent cation, but stimulation by glucose-1, 6-diphosphate could be demonstrated under some conditions.

Further studies on the phosphoribomutase obtained from uterine smooth muscle of cows are to be reported here.

EXPERIMENTAL

Materials and methods

The assay procedures used, the purification of the bovine uterine enzyme, and some of the materials were described in our earlier paper[2]. In most of the studies reported here the "Supt I fraction" which is enriched in phosphoribomutase, but also contains phosphoglucomutase, was used unless otherwise stated. α-GDP§ and β-GDP[3] were gifts of Dr. THEODORE POSTERNAK. α-G-I-P was prepared from potato starch by incubation with inorganic phosphate and potato phosphorylase[2]. β-G-1-P[4] was a gift of Dr. FRANK J. REITHEL. DR-1-P prepared by the action of nucleoside phosphorylase on hypoxanthine deoxyriboside was a gift of Dr. J. O. LAMPEN. R-1-P was prepared by the action of nucleoside phosphorylase on guanosine[2]. DPGA was a gift of Dr. E. W. SUTHERLAND, Jr. PCMB was purchased from the Sigma Chemical Company. IAA was purchased from the Eastman Kodak Co.

RESULTS

Substrate and coenzyme specificity

The phosphoribomutase activity of dialyzed water extracts of uterine muscle was not greatly enhanced by the addition of $10^{-5}M$ α-GDP to the incubation mixture,

* A preliminary report of this work was presented before the American Society of Biological Chemists, April 1954[1]. Some of the data are taken from a dissertation presented by A. J. GUARINO to the Graduate School of Arts and Sciences of Tufts College in August 1953, in partial fulfilment of the requirements for the degree, Doctor of Philosophy.

** Public Health Service Predoctoral Research Fellow of the National Cancer Institute, 1952–1953. Postdoctoral Fellow, 1953–1955. Present address. Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan.

*** Present adress: Department of Biochemistry, Western Reserve University, Cleveland, Ohio.

This author's work was supported in part by a research grant from the National Science Foundation and by the Elisabeth Severance Prentiss Fund.

§ The following abbreviations are used: α-(or β)-GDP, α-(or β)-glucose-1,6-diphosphate; α-(or β)-G-1-P, α-(or β)-glucose-1-phosphate; DR-1-P, deoxyribose-1-P; R-1-P, ribose-1-phosphate; DPGA, 2,3-diphosphoglyceric acid; Versene, ethylenediaminetetraacetic acid; PCMB, *p*-chloromercuribenzoate; IAA, iodoacetic acid; TRIS, tris-(hydroxymethyl)-aminomethane.

References p. 207/208.

while the phosphoglucomutase in these extracts was stimulated several-fold by GDP. Only after several steps in the fractionation which resulted in about 30-fold purification of the phosphoribomutase did a marked stimulation by GDP become apparent (Fig. 1). The enzyme at the third stage of purification[2] (ammonium sulfate precipitate) was stimulated less than 40%. The enzyme obtained after treatment of the ammonium sulfate fraction with calcium phosphate gel (supernatant I) was stimulated four-fold by the α-GDP. One must suppose that the gel, which removed about two thirds of the protein present in the solution, also removed most of the natural coenzyme, presumably ribose-1,5-diphosphate.

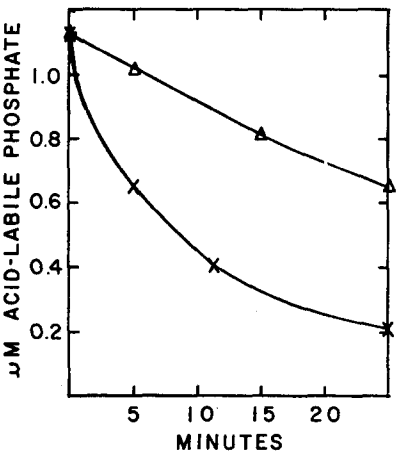

Fig. 1. Effect of α-GDP on phosphoribomutase. Reaction vessels contained 1.1 μM of R-1-P, 0.02 M succinate-TRIS buffer, pH 7.3; and 0.1 ml enzyme (Supt I, 0.11 mg protein). Reactions were stopped by the addition of 1 ml of 10% perchloric acid. Aliquots of protein-free filtrates were analyzed for acid-labile phosphate. ×——× $10^{-5} M$ α-GDP present. △——△ α-GDP omitted.

Several experiments were carried out to determine the specificity of substrate requirement of phosphoribomutase. The usual type of assay[2] was used. When DR-1-P was substituted for R-1-P there was a slow conversion of acid labile phosphate to acid-stable phosphate (the rate was about one-twentieth of that observed for R-1-P). β-G-1-P was not converted to an acid-stable form in experiments in which either α-GDP or β-GDP were added as coenzymes. Likewise β-GDP could not replace α-GDP in stimulating the rate of conversion of α-G-1-P or R-1-P to acid-stable esters.

The participation of α-GDP as a coenzyme for phosphoribomutase suggested that the coenzyme specificity might not be very great. Another similar compound, DPGA, has been described[5] as a coenzyme for phosphoglyceric mutase, and it seemed of interest to test this compound for any effect it might have on phosphoribomutase. The dibrucine salt of DPGA was converted to the potassium salt by addition of KOH followed by repeated extraction of the solution with chloroform, and finally prolonged aeration to remove the chloroform. Preliminary experiments showed that DPGA does not stimulate phosphoribomutase, but is actually inhibitory both in the presence and absence of added α-GDP. Control experiments showed that the inhibition was not due to any substance extracted into the aqueous phase from the chloroform, nor to any residual brucine, since brucine sulfate and water equilibrated repeatedly with chloroform were not inhibitory. A quantitative determination of the effect of DPGA was obtained in an experiment summarized in Table I. All incubations and analyses reported in the table were carried out simultaneously. The inhibition appears to be competitive, although a larger number of determinations would be required for the application of the usual criteria.

Identification of the product of the reaction

The product of the action of phosphoribomutase on R-1-P is an acid-stable phosphate ester. By analogy with phosphoglucomutase the product would be expected to be ribose-5-phosphate. Chromatography has been employed as a means of identifying

References p. 207/208.

TABLE I

THE EFFECT OF DPGA ON PHOSPHORIBOMUTASE

Replicate incubation mixtures contained, in a final volume of 1.0 ml: 0.77 μM R-1-P; 0.022 M TRIS buffer, pH 7.5; 0.0045 M Versene; 0.1 ml enzyme (Supt I, 0.16 mg protein) and in addition the amounts of DPGA and α-GDP indicated below. Reactions were stopped with perchloric acid and the protein-free filtrates analyzed for acid-labile phosphate. The rate is expressed as μM of R-1-P converted in 10 minutes.

DPGA	α-GDP			
	$2.4 \cdot 10^{-6} M$		$2.4 \cdot 10^{-6} M$	
	Rate	Percent inhibition	Rate	Percent inhibition
0	0.27		0.35	
$8.3 \cdot 10^{-6} M$	0.25	7	0.35	0
$2.2 \cdot 10^{-5} M$	0.19	30	0.28	20
$9.1 \cdot 10^{-5} M$	0.15	45	0.24	31

this product. In a large scale experiment, 100 μM R-1-P, 0.009 M TRIS buffer pH 7.61 μM α-GDP, and 10 ml enzyme (Supt 1, 5.9 mg protein) in a final volume of 90 ml were incubated at 37° for 25 minutes. The reaction was stopped by heating in a boiling water bath for 5 minutes, and precipitated protein removed by centrifugation. The supernatant solution was absorbed on a Dowex-1-formate column, and the monophosphate eluted with 0.03 M formate, pH 3.1. Fractions containing pentose were pooled and neutralized with KOH, excess $Ba(OH)_2$ was added, followed by 4 volumes of absolute ethanol. The precipitated barium salts were collected by centrifugation and dried *in vacuo* over $CaCl_2$. The barium salts were dissolved in dilute HCl, barium was removed by precipitation with H_2SO_4, and the precipitated barium sulfate washed twice with water, the washings being combined with the original supernatant. Any residual R-1-P would be hydrolyzed by the acid treatment. After neutralization with KOH, the volume was adjusted to 5 ml and the solution was analyzed for pentose by the method of MEJBAUM[6] with adenosine-5-phosphate as a standard. A total of 50 μM was recovered. The ratio of pentose to acid-stable phosphate in this preparation was 0.91 (Theory 1.00). Paper chromatography of 0.1 ml of this preparation was carried out along with authentic ribose-5-phosphate in the alkaline solvent of BANDURSKI AND AXELROD[7]. The sugars were visualized by a copper reduction method[8]. Authentic ribose-5-phosphate migrated with an R_F of 0.43, while the reaction product gave two spots; one faint spot had an R_F 0.29 and a darker spot had an R_F of 0.43. The nature of the compound of R_F 0.29 was not investigated. The carbazole reaction of DISCHE AND BORENFREUND[9] for ketoses was negative on this preparation of ribose-5-phosphate.

Ion effects

The phosphoribomutases from all sources that were examined[2] appeared to be active in the absence of any added divalent cation, and in the presence of 0.01 M versene. Since all other phosphate-transferring enzymes require the presence of a divalent cation, it seemed important to reinvestigate this question using the partially purified uterine enzyme. Various divalent cations (always as chlorides) were tested at different concentrations. The results of these experiments are summarized in Table II. In no

References p. 207/208.

case is there any real enhancement of activity, and generally inhibition is observed. We do not attach any significance to an apparent stimulation of 13%, nor to apparent inhibitions of 7%. Three trivalent cations were tested at a concentration of $10^{-3}M$. Fe^{+++} and Al^{+++} produced 100% inhibition while Cr^{+++} produced 36% inhibition.

TABLE II

THE EFFECT OF DIVALENT CATIONS ON PHOSPHORIBOMUTASE

The usual assay system was used. The data are assembled from different experiments, but in each case all the assays for a given concentration of divalent ions were carried out simultaneously. The results are expressed as per cent inhibition in the presence of the ions relative to the rate in the absence of ions.

Ion	*Concentration of divalent ion*			
	$8.3 \cdot 10^{-5} M$	$1.7 \cdot 10^{-4} M$	$5.5 \cdot 10^{-4} M$	$4.0 \cdot 10^{-3} M$
Mg^{++}	0	—	0	15
Mn^{++}	0	—	(13*)	7
Ca^{++}	0	—	7	—
Ba^{++}	0	—	7	65
Co^{++}	0	—	0	18
Ni^{++}	—	—	47	65
Cu^{++}	69	—	100	100
Zn^{++}	—	68	93	100

* Mn^{++} at this concentration produced an apparent stimulation of 13%.

Because of the activity of the enzyme in the presence of versene, the mechanism of inhibition by ions could be investigated. Assays were carried out using: (i) enzyme diluted with versene solution; (ii) enzyme preincubated with a solution of Zn^{++} or Cu^{++}; (iii) enzyme preincubated with Zn^{++} or Cu^{++} and then diluted with versene. In these experiments the inhibition produced by the divalent ions was of the same order as that reported in Table II. After preincubation with the cations the enzyme was found to be still fully active when versene was added to the test system.

The relationship between protein-SH group and cation inhibition was investigated. Since the enzyme is not greatly inhibited by thiol-blocking agents (see below), it was possible to study the effect of blocking the protein-SH groups on inhibition by Cu^{++}. Such an experiment is shown in Fig. 2. The ratio of PCMB to Cu^{++} was 3 to 1.

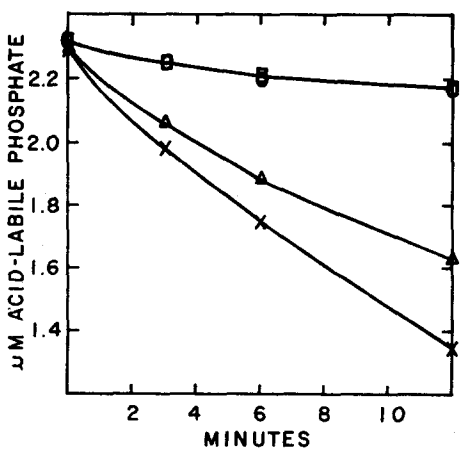

Fig. 2. Relation between free –SH groups of phosphoribomutase and Cu^{++} inhibition. The reaction vessels contained in a final volume of 1.2 ml, 0.02M Tris pH 7.6; 2.3 μM R-1-P; α-GDP $3.6 \cdot 10^{-6}M$; enzyme (Supernatant I, 0.53 mg). ×——× untreated enzyme (preincubated at 0° in water). △——△ Enzyme preincubated with $3 \cdot 10^{-4}M$ PCMB, 10 minutes at 0°. □——□ Enzyme preincubated with $1 \cdot 10^{-4}M$ $CuCl_2$. ○——○ Enzyme preincubated with PCMB for 10 minutes before addition of $CuCl_2$. After the preincubation period the enzyme was added to the other components of the assay mixture described above, and the assay carried out at 30° as usual. The final concentration of Cu^{++} in the assay mixtures was $1.7 \cdot 10^{-5}M$.

References p. 207/208.

Preincubation of the enzyme with PCMB for 10 minutes at 0° produced 25% inhibition, while preincubation with Cu^{++} produced 75% inhibition. When the enzyme was preincubated with PCMB, and Cu^{++} added just before starting the assay, the inhibition was exactly the same as it was in the absence of PCMB. This indicates that protein-SH groups are not the principal sites of cation inhibition.

The effect of the presence of pyrophosphate was tested. In the presence of 0.01 *M* $Na_4P_2O_7$ no phosphoribomutase activity was detectable. In another experiment the enzyme was preincubated with pyrophosphate (final concentration 0.009 *M*), the preparation was then dialyzed against distilled water to remove pyrophosphate, and then assayed in the presence and absence of $3 \cdot 10^{-3} M$ $MgCl_2$. The dialyzed preparation was fully active and no enhancement of activity was produced by the Mg^{++}.

Effect of cysteine and thiol blocking agents

The effect of sulfhydryl reagents was tested with the partially purified enzyme. This has been mentioned in the section on the effects of ions. In various experiments the presence of IAA at concentrations as high as $6 \cdot 10^{-4} M$ or PCMB at $1 \cdot 10^{-4} M$ produced relatively little inhibition. Such an experiment is shown in Fig. 3.

Since the presence of cysteine is known to augment the activity of phosphoglucomutase, a duplicate assay was carried out with partially purified phosphoribomutase with and without 0.004 *M* cysteine to see if any further enhancement of activity could be obtained. The presence of cysteine did not enhance the activity of phosphoribomutase.

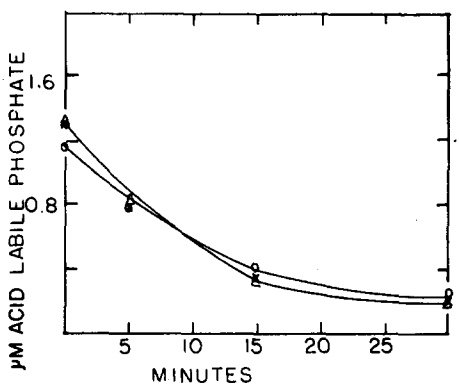

Fig. 3. Effect of IAA and PCMB on phosphoribomutase. Reaction vessels contained 1.2 μM of R-1-P; 0.04 *M* Succinate-TRIS buffer, pH 7.6; 0.2 ml enzyme (55–75 amm. sulf. 0.45 mg protein). Reactions were stopped by the addition of 1 ml of 10% perchloric acid. Aliquots of the protein-free filtrates were analyzed for acid-labile phosphate. ×——× $5.5 \cdot 10^{-4} M$ IAA present. △——△ No added inhibitors. O——O $1.0 \cdot 10^{-4} M$ PCMB.

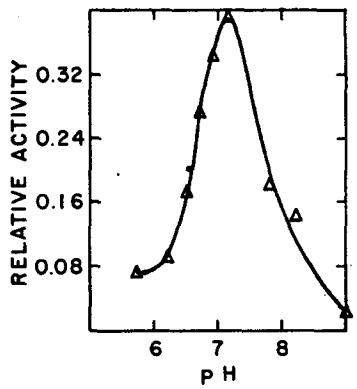

Fig. 4. pH optimum of phosphoribomutase. Reaction vessels contained 1.0 μM R-1-P, 0.1 ml of enzyme (60–80 amm. sulf. 0.4 mg protein) and 0.015 *M* succinate-TRIS buffer of the desired pH. Reactions were stopped by the addition of 1 ml of 10% perchloric acid, and aliquots of the protein-free filtrates were taken for acid-labile phosphate determination. Velocities are expressed as units of enzyme activity per milligram of protein. The pH of incubation mixtures was measured with a Beckman pH meter.

The *pH optimum* of bovine uterine enzyme was found to lie in the vicinity of pH 7 (Fig. 4).

References p. 207/208.

The Michaelis constant

The effect of different substrate concentrations is shown in Fig. 5, in which the data are represented in a form concordant with LINEWEAVER AND BURK's equation four[10]. The velocity of the reaction is expressed as units of enzyme activity per milligram of protein. From these data K_m for R-1-P has been determined, and found to be $1.14 \cdot 10^{-3} M$.

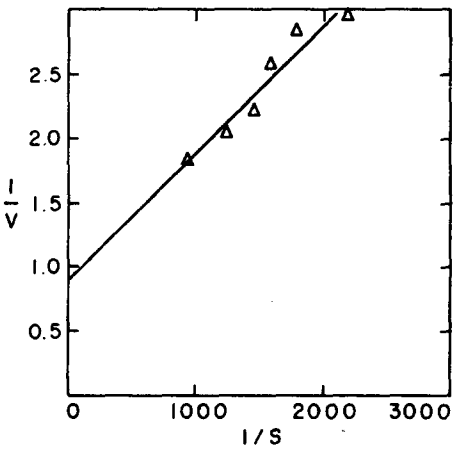

Fig. 5. Effect of varying R-1-P concentration on phosphoribomutase activity. Reaction vessels contained $0.02 M$ succinate-TRIS buffer, pH 7.6, 0.2 ml enzyme (55–75 amm. sulf., 0.45 mg protein), and varying concentrations of R-1-P from $4.0 \cdot 10^{-4} M$ to $6.0 \cdot 10^{-3} M$. Reactions were stopped by the addition of 1 ml of 10% perchloric acid. Aliquots of the protein-free filtrates were analyzed for acid-labile phosphate. Velocities are expressed as units of enzyme activity per milligram of protein.

Equilibrium

Reversibility of the phosphoribomutase reaction was demonstrated in early experiments[11]. The apparent equilibrium point of the reaction has been determined by incubation of a large excess of enzyme with R-1-P for a prolonged period. The amount of enzyme was ten times as great as would be required to convert 90% of the R-1-P in 15 minutes, and the longest incubation period was 40 minutes. The following conditions were employed: TRIS buffer pH 7.4, $0.018 M$; versene $7 \cdot 10^{-3} M$; α-GDP, $4.2 \cdot 10^{-6} M$; and protein, 1.23 mg of Supt I; final volume 3.0 ml. Points on the curve were determined in triplicate and averaged. The amount of R-1-P added was 3.45 μM, and at the end of the reaction 0.23 μM remained. This represents the conversion of 93.3% of the initial amount of R-1-P to an acid-stable form.

DISCUSSION

The configuration of the anomeric carbon atom of R-1-P is unknown. We had assumed at first that in the enzymic synthesis of R-1-P the β-glycosidic configuration of the ribonucleosides[12] would be retained, by analogy with the polysaccharide phosphorylase reaction. One would also have expected that the coenzyme of phosphoribomutase should have the same configuration as the substrate, R-1-P, analogous to the phosphoglucomutase system[14]. Since phosphoglucomutase acts upon R-1-P as well as G-1-P[13] and since esters of known β-configuration were inactive, it became more reasonable to conclude that the R-1-P must have the α-configuration. The same arguments apply to DR-1-P, and LAMPEN's evidence[15] which suggests that DR-1-P has the α-configuration supports our hypothesis*. β-R-1-P[16] has been synthesized and found not to be identical with the natural substance.

The reversal by versene of the inhibition produced by Cu^{++} and Zn^{++} proves that these ions cause no irreversible change in the enzyme protein, such as conversion of –SH to –S–S– linkages. The reactivation by versene also makes it seem unlikely that

* Experiments on the structure of natural R-1-P are in progress and will be reported in the near future.

References p. 207/208.

the inhibitory ions displace a bound cation which normally participates in the reaction. The experiments with pyrophosphate, which is known to form tight complexes with divalent cations, show that its effect is not due to complexing an activating ion, but suggest rather that it may be a competive inhibitor, either for the substrate or the coenzyme. An experiment not detailed here, in which the presence of 0.016 *M* NaF did not affect the rate of the enzymic reaction, is additional support for the earlier findings that divalent cations do not activate phosphoribomutase. While all other phosphate-transferring enzymes require divalent cations, such a discrepancy in metal requirements is not unique, being found also in other classes of enzymes, *e.g.* the aldolases[17,18,19] and the peptidases[20].

Three phosphomutases have been shown not to be inhibited by IAA[5,21,22]. The experiments with the anti-thiol agents show that protein –SH groups are probably not involved in the active center of phosphoribomutase, nor are they the primary sites involved in cation inhibiton. The small inhibition produced by anti-thiol reagents may be due to slight changes in the configuration of the whole protein, due to attachment of new side chains. The inhibition by cations is more difficult to rationalize: it may be due to changes in the charge distribution on the protein, or to formation of an inactive complex between the ion and the coenzyme, for example, but no evidence is available on this point at present.

The equilibrium point cannot be considered to be established until the same equilibrium has been approached starting with ribose-5-phosphate. We have never had a sample of ribose-5-phosphate of whose purity we were sufficiently certain to permit its use in a critical experiment of this nature. If one assumes that the equilibrium determined in the present work is approximately correct, it may be compared with the equilibrium of the phosphoglucomutase reaction[23]. Apparently the free energy change in the reaction ribose-1-phosphate $\rightleftharpoons$ ribose-5-phosphate is very close to that for the reaction glucose-1-phosphate $\rightleftharpoons$ glucose-6-phosphate.

The technical assistance of WILLIAM MORRIS Jr., is some of these experiments is acknowledged.

SUMMARY

A study of the substrate and coenzyme specificity of phosphoribomutase of bovine uterus has been made, and the affinity of this enzyme for ribose-1-phosphate determined. The probable α-configuration for R-1-P is proposed.

The product of the reaction has been identified as ribose-5-phosphate. At equilibrium the ratio of ribose-5-phosphate to ribose-1-phosphate is 93:7.

The optimum pH for the enzyme is near 7.

The effects of various cations and of thiol blocking agents have been investigated. The significance of these findings has been discussed.

REFERENCES

1 A. J. GUARINO AND H. Z. SABLE, *Federation Proc.*, 13 (1954) 222.
2 A. J. GUARINO AND H. Z. SABLE, *J. Biol. Chem.*, 215 (1955) 515.
3 T. POSTERNAK, *J. Biol. Chem.*, 180 (1949) 1269.
4 F. J. REITHEL, *J. Am. Chem. Soc.*, 67 (1945) 1056.
5 E. W. SUTHERLAND, T. POSTERNAK AND C. F. CORI, *J. Biol. Chem.*, 181 (1949) 153.
6 W. MEJBAUM, *Z. physiol. Chem.*, 258 (1939) 117.
7 R. S. BANDURSKI AND B. AXELROD, *J. Biol. Chem.*, 193 (1951) 405.
8 D. FRENCH, D. W. KNAPP AND J. H. PAZUR, *J. Am. Chem. Soc.*, 72 (1950) 5150.

[9] Z. DISCHE AND E. BORENFREUND, *J. Biol. Chem.*, 192 (1951) 583.
[10] H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
[11] Unpublished experiments by H. M. KALCKAR AND H. Z. SABLE.
[12] S. FURBERG, *Acta Chem. Scand.*, 4 (1950) 751.
[13] H. KLENOW, *Arch. Biochem. Biophys.*, 46 (1953) 186.
[14] E. W. SUTHERLAND, M. COHN, T. POSTERNAK AND C. F. CORI, *J. Biol. Chem.*, 180 (1949) 1285.
[15] J. O. LAMPEN, in *Phosphorus Metabolism*, Vol. II, edited by W. D. MCELROY AND B. GLASS, Johns Hopkins Press, Baltimore 1952, p. 363
[16] R. S. WRIGHT AND H. G. KHORANA, *J. Am. Chem. Soc.*, 77 (1955) 3423.
[17] V. JAGANNATHAN AND K. SINGH, *Biochim. Biophys. Acta*, 15 (1954) 138.
[18] R. C. BARD AND I. C. GUNSALUS, *J. Bacteriol.*, 59 (1950) 387.
[19] O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 314 (1943) 149.
[20] E. L. SMITH, N. C. DAVIS, E. ADAMS AND D. H. SPACKMAN, *Mechanism of Enzyme Action*, edited by W. D. MCELROY AND B. GLASS, Johns Hopkins Press, Baltimore, 1954, p. 291.
[21] H. Z. SABLE AND C. W. CALKINS, *J. Biol. Chem.*, 204 (1953) 695.
[22] T. RAMASARMA, J. SRI RAM AND K. V. GIRI, *Arch. Biochem.*, 53 (1954) 167.
[23] S. P. COLOWICK AND E. W. SUTHERLAND, *J. Biol. Chem.*, 144 (1942) 423.

Received November 23rd, 1955