Biochimica et Biophysica Acta, 315 (1973) 317–332

BBA 66958

PROOF OF PHOSPHORYLATION AND EVIDENCE FOR DIFFERENT PRIMARY STRUCTURE OF PHOSPHORYLATED SITES OF GLYCOGEN SYNTHASES FROM RABBIT AND FROG TISSUES*

ADDISON M. ROSENKRANS AND JOSEPH LARNER

Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Va. 22903 (U.S.A.)

(Received January 15th, 1973)
(Revised manuscript received April 18th, 1973)

SUMMARY

Chemical evidence is provided that glycogen synthase extracted from rabbit liver, heart, kidney and brain and from frog liver and muscle all are phosphorylated by [^{32}P]ATP in the presence of protein kinase. After partial acid hydrolysis, serine phosphate together with a number of labeled peptides were observed. The peptide maps were similar and allowed a provisional differentiation of the phosphorylated sites into four separate sites including (a) frog liver, (b) frog muscle, (c) rabbit skeletal muscle, and (d) rabbit heart, brain, kidney and liver.

The major labelled chymotryptic peptides of glycogen synthase and phosphorylase were compared and found to differ markedly in their electrophoretic behavior. On partial rehydrolysis of these chymotryptic peptides with acid, identical phosphopeptide maps were obtained. This proves that within the larger non-identical chymotryptic peptides there is present the identical hexapeptide sequences previously established.

INTRODUCTION

Glycogen synthase has been proven to exist in phosphorylated D and dephosphorylated I forms in skeletal muscle. Recent work has established that the stoichiometry of the I to D interconversion reaction is 6 phosphates incorporated per 90 000 Dalton subunit fully converted[1]. The only other glycogen synthase enzyme that has been demonstrated chemically to have a phosphorylated site is that from heart[2] which has been shown to differ from the muscle enzyme by comparing ^{32}P-labeled peptide maps produced by partial acid hydrolysis.

Although the two forms have been detected kinetically in a wide variety of tissues by assay with and without glucose 6-phosphate[3–9] there has been some

* Some of the se results have been previously presented in meetings[36] and symposia[37,38].

considerable discussion of the actual molecular existance of phosphorylated and dephosphorylated forms in liver and other organs[6,9–14]. Also, of special interest is the case of the enzyme of amphibian organs where only a D form was originally found[15] which on conversion *in vitro*[15] or after insulin administration *in vivo*[12] still retains the characteristics of a D form but now assays with greater total activity, and with a decreased K_m for UDPG [12].

Because of the question of the chemical nature of the two forms of the enzyme in various organs, including amphibian, the present studies were undertaken to establish whether (a) chemically the enzymes of various rabbit and frog organs have phosphorylated sites, and (b) whether these sites are the same, or different. Additional studies were performed in which the chymotryptic peptides of muscle phosphorylase and glycogen synthase containing the phosphorylated sites were compared.

From these studies, it has been concluded that the enzymes from five rabbit organs and two frog organs all purified to virtual homogeneity (with regard to phosphate incorporation) possess phosphorylated sites. Comparison of labeled peptide maps produced by partial acid hydrolysis indicates an overall similarity, but distinguishes at least four separate sites including (a) frog liver, (b) frog muscle, (c) rabbit skeletal muscle, (d) rabbit heart, brain, kidney, and liver. When the major chymotryptic peptides of rabbit muscle phosphorylase and glycogen synthase were compared, they were found to differ markedly in terms of electrophoretic migration, indicating a marked difference in structure. When these were submitted to partial acid hydrolysis and recompared, identical peptide maps were obtained. This proves that the large chymotryptic peptides of phosphorylase and glycogen synthase are non-identical, but contain identical small sequences(s) within the larger non-identical structures.

MATERIALS

Crystalline cyclic AMP, Tris and EDTA were purchased from Sigma Chemical Co. Whatman 3MM paper was used for electrophoresis. DEAE-cellulose, Type 20, was purchased from Brown Co., Research and Development, Berlin, N.H., Kodak RPX-Omat Rapid Processing Medical X-ray film was used for autoradiography. ^{32}P was purchased from New England Nuclear, as $H_3{}^{32}PO_4$ in 0.02 M HCl. Sepharose 4B was purchased from Pharmacia Fine Chemicals. α-Chymotrypsin (45 units/mg) and soybean trypsin inhibitor were purchased from Worthington Biochemicals. For use, 10 mg of chymotrypsin and 1 mg soybean trypsin inhibitor were dissolved in 1 ml distilled water, and the pH adjusted to 8.7 with 0.1 M NH_4OH. The mixture was preincubated for 1 h at 37 °C before use, and stored frozen. Salivary amylase was prepared by the method of Bernfeld[16]. Rabbit liver glycogen was deionized by passage through Amberlite MB-3 mixed bed ion-exchange resin columns before use. Pyridine acetate buffers for electrophoresis were prepared as previously described[17]. DEAE-cellulose was washed with 0.3 M NaOH, 0.1 M HCl, 0.1 M NaOH and water until the pH of the effluent was neutral. [^{32}P]ATP was made by a modification of the method of Post and Sen[18] usually from 20 mCi $H_3{}^{32}PO_4$.

METHODS

Purification of phosphorylase and glycogen synthase from rabbit and frog organs

Glycogen synthase from skeletal muscle, liver, heart, kidney, and brain of rabbits and from skeletal muscle and liver of frogs *(Rana pipiens)* was extracted by homogenizing organs (2.5 v/w) in 50 mM Tris, 5 mM EDTA, 100 mM KF buffer (Tris–EDTA–KF buffer) (pH 8.2) in a Waring blendor and the enzymes precipitated with 95% ethanol (precooled to −60 °C) added to a final concentration of 30% (at −10 °C) according to the procedure of Brown and Larner[19]. After centrifugation at 75 000 × *g* for 2.5 h (Beckman Model L3-40, 30 rotor), the glycogen pellets containing the enzyme were resuspended in 50 mM Tris, 5 mM EDTA, 50 mM mercaptoethanol buffer (Tris–EDTA–mercaptoethanol buffer) (pH 7.8) and were chromatographed on DEAE-cellulose, (0.3 ml of DEAE-cellulose per g of tissue). Phosphorylase was eluted with 20 bed volumes of Tris–EDTA–mercaptoethanol buffer, containing 0.1 M NaCl. The protein peaks were assayed for phosphorylase by the methods of Villar-Palasi and Gazquez-Martinez[20]. Glycogen synthase was next eluted with 5 bed volumes of Tris–EDTA–mercaptoethanol buffer containing 0.3 M NaCl. The protein peaks were assayed for glycogen synthase according to the procedure of Thomas *et al.*[21].

Preparation of ^{32}P-labeled synthases

For conversion to the phosphorylated or D form, the glycogen synthases from various organs, were first precipitated with 95% ethanol (precooled to −60 °C) which was added to a final concentration of 30% (at −10 °C). After centrifugation at 10 000 × *g* for 20 min at −10 °C (Sorval Model RC2-b, GSA rotor), pellets were resuspended in Tris–EDTA–mercaptoethanol buffer (pH 7.8), containing 1.2 ml of 1.1 mM [^{32}P]ATP ($5.4 \cdot 10^9$ cpm/ml), 1 mM cyclic AMP, and 10 mM $MgCl_2$ in a total volume of 2.2 ml. In order to insure the phosphorylation of the synthases, synthase I kinase (protein kinase)* prepared from skeletal muscle was also added. Accordingly, this purified preparation of synthase I kinase was tested first for its capacity to incorporate ^{32}P from [^{32}P]ATP in the absence of added protein substrate. The 50 μg of protein kinase that were used to convert the synthases exhibited less than 3% self-incorporation of the total ^{32}P incorporated in the presence of synthase. The seven synthase enzymes were then incubated for 4 h at 10 °C with added protein kinase. ^{32}P ncorporation was measured by pipetting aliquots of reaction mixtures onto filter papers which were then washed exhaustively in 5 and 10% trichloroacetic acid, 95% ethanol and finally ether according to the procedure of Friedman and Larner[22]. The dried papers were counted in a Packard Tri-Carb liquid scintillation spectrometer, Model 3375.

Preparation of phosphopeptide maps

To denature the ^{32}P-labeled synthases, trichloroacetic acid was added to a final concentration of 10%. The protein was collected by centrifugation and washed thoroughly with 5% trichloroacetic acid, dried with ethanol (66%, 95% and absolute) and finally ether. Partial acid hydrolysis was carried out in 6 M HCl

* Kindly donated by Drs C. Villar-Palasi and L. C. Shen.

(0.1 ml/mg protein) for 20 min at 100 °C. Hydrolyzed peptides were dried in a vacuum dessicator over KOH pellets, redissolved and reevacuated to dryness several times to remove HCl. Peptides were then dissolved in the appropriate buffer (pH 6.5 or 3.5), applied to Whatman 3MM paper, and high-voltage electrophoresis was carried out at either pH 6.5, (50 min at 50 V/cm) or pH 3.5, (40 min at 50 V/cm) in pyridine acetate buffers. Enough material was applied to 3 cm strips so that at least 10^5 cpm of ^{32}P were distributed per cm. After electrophoresis, autoradiographs were prepared by exposing papers to X-ray film for approx. 24 h.

Amylase digestion and Sepharose 4B chromatography

To insure that the phosphopeptide maps produced by the above procedure contain only phosphopeptides derived from the glycogen synthase, each synthase was further chromatographed over Sepharose 4B at room temperature after digestion of glycogen with amylase. With the enzyme from skeletal muscle, this procedure has already been shown to yield essentially homogeneous enzyme[1,19]. Synthases after conversion from I to D forms in the presence of purified protein kinase as described above, were precipated twice with 95% ethanol (precooled −60 °C) to a final concentration of 15% to remove protein kinase[2] and resuspended in approx. 5 ml of Tris–EDTA–mercaptoethanol buffer (pH 6.8).

To each 10 mg of synthase (Folin–Lowry) was added 125 μg of salivary amylase. The mixtures were placed in bags and dialyzed at room temperature for 5 h against several changes of Tris–EDTA–mercaptoethanol buffer (pH 6.8). The enzymes were then transferred to test tubes and the pH adjusted to 7.8 using 2 M Tris base. After incubation for 30 min at 30 °C, the synthases were applied to Sepharose 4B columns (2.5 cm × 85 cm) previously equilibrated with Tris–EDTA–mercaptoethanol buffer (pH 7.8). 5-ml fractions were collected and aliquots of all fractions were assayed for glycogen synthase and protein bound ^{32}P. Aggregated enzyme was excluded from the gel and eluted at the void volume (0.25 bed volume). Active enzyme followed at 0.5–0.7 bed volume. Nucleotide which was still present in association with the enzyme was eluted after the active enzyme.

Large scale synthase I to D and phosphorylase b to a conversions

25 mg of glycogen synthase (30% ethanol fraction following DEAE-cellulose chromatography) were suspended in Tris–EDTA–mercaptoethanol buffer (pH 7.8), containing 10 mm $MgCl_2$, 1 mM cyclic AMP, and 0.25 mg/ml of rabbit liver glycogen. The reaction was begun by the addition of 1 ml of [^{32}P]ATP (1.7 mM, $5.75 \cdot 10^9$ cpm/ml) (total volume of reaction mixture 30 ml) and allowed to proceed for 4 h at 10 °C after which the protein kinase was removed by two consecutive ethanol precipitations (final concn 15%). After resuspending the precipitate in Tris–EDTA–mercaptoethanol buffer (pH 7.8) the synthase was denatured with trichloroacetic acid and dried as described previously.

50 mg of crystalline phosphorylase b was centrifuged at 5000 × *g* for 5 min, and the packed crystal mass resuspended in 5 ml of Tris–EDTA–mercaptoethanol buffer (pH 8.6). 1 mg of phosphorylase b kinase*, magnesium acetate, cyclic AMP, and $CaCl_2$ were added to final concentrations of 10 mM, 1 mM and 1 μM, respectively.

* Kindly supplied by Drs Shen and Villar-Palasi.

Rabbit liver glycogen, 8%, was added to a final concentration of 0.25 mg/ml of reaction mixture. The reaction was begun by the addition of one ml [^{32}P]ATP (1.7 mM, $5.75 \cdot 10^9$ cpm/ml) (total volume of reaction mixture 30 ml) and the reaction allowed to proceed for 4 h at 10 °C. Phosphorylase was then precipitated by adding an equal volume of saturated $(NH_4)_2SO_4$ (pH 6.8), and the pH lowered to 6.8 with 1 M acetic acid. After centrifugation for 15 min, at 10 000 × *g*, the precipitate was dissolved in Tris–EDTA–mercaptoethanol buffer (pH 7.8) and centrifuged at 100 000 × *g* for 1 h (Spinco, Model L 3-40, 30 rotor) to precipitate the phosphorylase b kinase[23]. The supernatant containing the ^{32}P-labeled phosphorylase was denatured with trichloroacetic acid and then dried as previously described.

Chymotryptic digestions

Phosphorylase and glycogen synthase were suspended in 0.1 M NH_4OH and 0.1 M ammonium acetate (1:1, v/v) (pH 8.7), to final concentrations of 10 mg/ml. Chymotrypsin and soybean trypsin inhibitor were added (weight ratio of denatured enzyme to chymotrypsin of 75:1) and the reaction mixtures incubated with continuous shaking for 12 h at 37 °C. The pH was monitored and maintained at 8.7 by the addition of 0.1 M NH_4OH. Reaction mixtures were then directly applied on to Whatman 3MM papers (1 mg protein/cm), air dried, and electrophoresis of peptides and autoradiography were performed as previously described.

RESULTS

Phosphorylation of seven enzymes from rabbit and frog organs

In order to determine whether phosphorylase and glycogen synthase from other organs chromatographed on DEAE-cellulose in a similar manner to the enzymes from skeletal muscle and heart, the respective enzymes were assayed in the 0.1 M NaCl eluates. Phosphorylase was readily identified in the 0.1 M NaCl fraction in all cases. After exhaustive washing (20 bed volumes of 0.1 M NaCl) no remaining phos-

TABLE I

CHROMATOGRAPHY OF GLYCOGEN PHOSPHORYLASE FROM VARIOUS ORGANS ON DEAE-cellulose

Organ	*0.1 M NaCl elution peak*					*After 20 bed volumes of 0.1 M NaCl wash: Total enzyme activity (μmole/min per ml)*
	Weight (g)	*Enzyme activity (μmoles/min per ml)*	*Volume (ml)*	*Total extracted enzyme activity (μmoles/min)*	*Enzyme activity per g organ (μmoles/min per g)*	
Rabbit						
Liver	128	5.5	75	414.0	3.23	Not detected
Heart	41	4.0	20	81.0	1.98	Not detected
Kidney	81	2.4	70	67.2	0.83	Not detected
Brain	75	0.8	35	31.1	0.42	Not detected
Muscle	102	21.0	100	2102.0	20.61	Not detected
Frog						
Liver	13	5.7	10	57.2	4.40	Not detected
Muscle	49	13.1	15	197.4	4.03	Not detected

phorylase was detected (Table I). Phosphorylase contents of the various tissues determined under these conditions varied widely (50-fold) with lowest activity present in the brain (0.4 μmole/min per g) and highest in muscle (20.6 μmoles/min per g). Synthase assays analogously demonstrated that the enzyme from all tissues was eluted in the 0.3 M NaCl fraction (Table II). In all cases yields after chromatography were greater than 50%. Synthase activity was considerably lower than phosphorylase and did not vary as widely (3-fold) with lowest activity again in brain (0.08 μmole/min per g) and highest again in muscle (0.31 μmole/min per g).

TABLE II

CHROMATOGRAPHY OF GLYCOGEN SYNTHASE FROM VARIOUS ORGANS ON DEAE-CELLULOSE

Organ	*Weight (g)*	*Before DEAE-cellulose*		*After DEAE-cellulose*	
		Total extracted enzyme activity (μmoles/min)	*Enzyme activity per g organ (μmole/min per g)*	*Total synthase in 0.3 M NaCl fraction (μmoles/min)*	*Recovery after DEAE-cellulose (%)*
Rabbit					
Liver	128	28.21	0.22	17.73	63
Heart	41	8.10	0.20	7.29	90
Kidney	81	14.34	0.18	7.50	52
Brain	75	6.24	0.08	3.24	52
Muscle	102	31.20	0.31	18.51	59
Frog					
Liver	13	2.79	0.21	1.48	54
Muscle	49	9.73	0.20	5.45	56

When the incorporation of ^{32}P into the various synthase enzymes was examined in the absence of added protein kinase (results not reported), incorporation was low and variable and in some cases, there was no incorporation. This variation was most likely due to the variable activity of protein kinase present in the synthases. Accordingly, additional purified protein kinase was added to increase the incorporation. Therefore, the purified protein kinase was studied without protein substrate in order to determine whether it was capable of "self" incorporating ^{32}P. It was shown that the "self" incorporation of ^{32}P into purified protein kinase in the absence of protein substrate was linear with the amount of enzyme used (Fig. 1). 50 μg of protein kinase, for example, the amount subsequently added to the synthase reaction mixtures, catalyzed the "self" incorporation of 13 000 cpm of ^{32}P from [^{32}P]ATP into itself. In the presence of 50 μg of protein kinase, ^{32}P from [^{32}P]ATP was incorporated into all of the synthase enzymes. ^{32}P incorporation (Table III) into the seven enzymes varied between $4.7 \cdot 10^5$ cpm (0.24%) for frog liver to $3.8 \cdot 10^6$ cpm (1.92%) for kidney. Thus, the $1.3 \cdot 10^4$ cpm of "self" incorporation is at most only 3% of the total and can therefore be neglected.

Phosphorylated sites

Labeled phosphopeptides were produced from the labeled synthases by partial acid hydrolysis. To compare the labeled phosphopeptides with regard to charge and size, they were separated by electrophoresis at pH 3.5 and 6.5. At pH 3.5 (Fig. 2A),

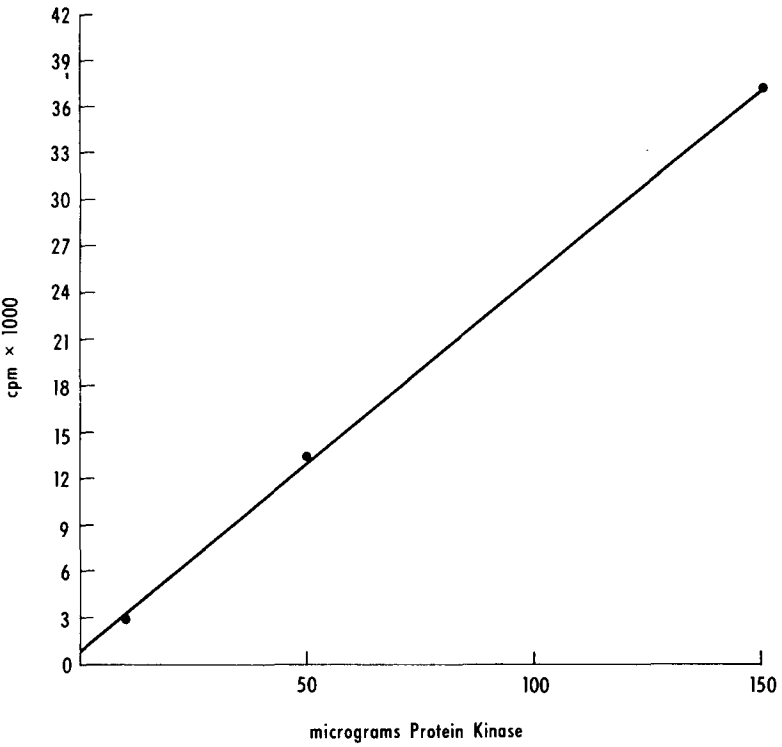

Fig. 1. The incorporation of ^{32}P from [γ-^{32}P]ATP into purified protein kinase. Note the linearity. The reaction was conducted for 4 h at 10 °C.

TABLE III

INCORPORATION OF ^{32}P INTO ORGAN SYNTHASES FROM [^{32}P]ATP

Organ	*Total incorporated (cpm)*	*Total ATP added (cpm × 10^{-8})*	*Incorporation (%)*
Rabbit			
Liver	2 090 700	1.97	1.06
Heart	996 000	1.97	0.51
Kidney	3 793 620	1.97	1.92
Brain	901 140	1.97	0.46
Muscle	1 526 740	1.97	0.77
Frog			
Liver	466 210	1.97	0.24
Muscle	1 690 500	1.97	0.86

the phosphopeptides from rabbit liver, heart, kidney, and brain synthases all contain serine phosphate and appear to have quite similar but distinguishably different electrophoretic patterns from rabbit muscle synthase. For example, rabbit muscle synthase has no phosphopeptides corresponding to A, B, and G of the other rabbit enzymes.

Phosphopeptide maps obtained from frog muscle and frog liver differ in turn slightly from each other and from maps of the rabbit enzymes. Thus frog muscle synthase has phosphopeptide 1′ corresponding to 1′ in frog liver while it has no bands corresponding to 1″ of frog liver. Frog muscle has no bands corresponding to Bands

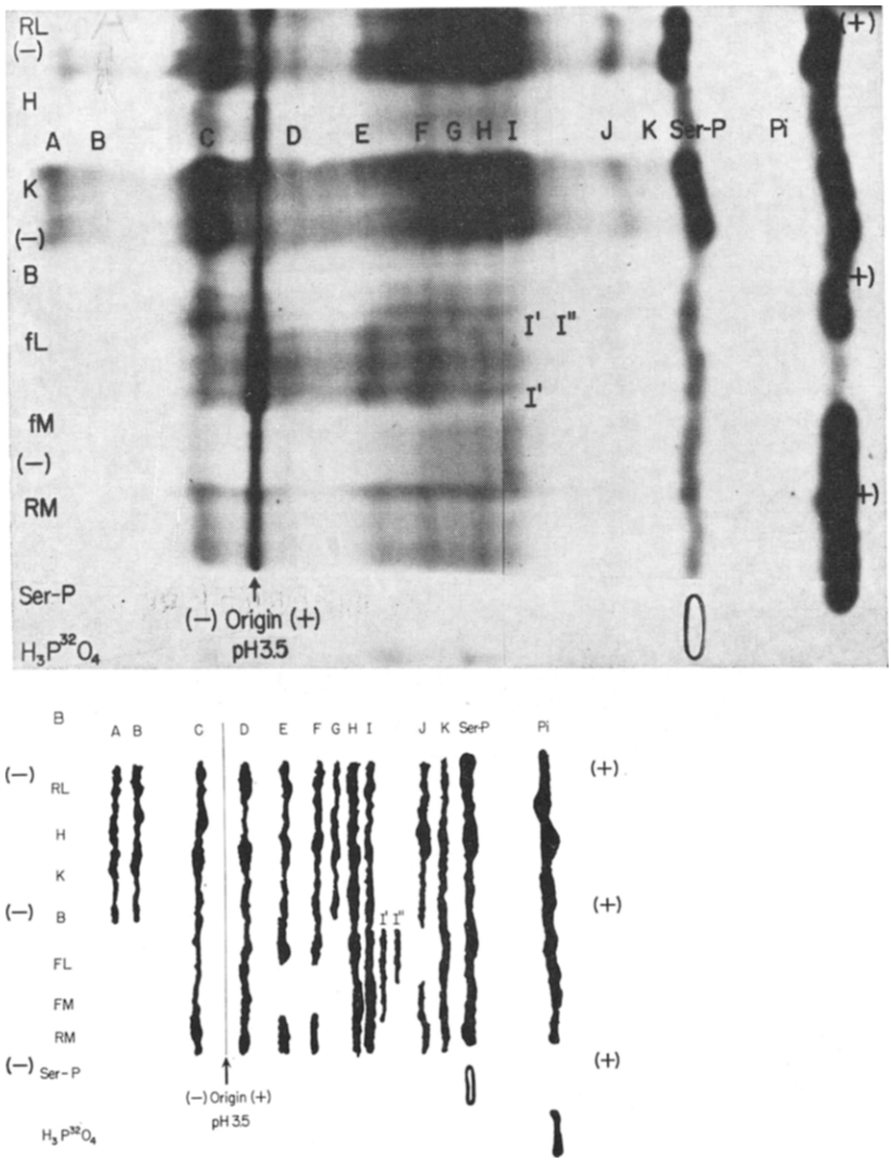

Fig. 2. A. High-voltage electrophoresis of the partial acid hydrolysates of the organ synthases was run for 40 min at 50 V/cm in pyridine acetate buffer (pH 3.5). The line of application is indicated by the arrow. The cathode is on the left and the anode on the right. Phosphoserine and $^{32}P_i$ were used as markers and are labelled phosphoserine and P_i respectively. Other labelled peptides are lettered A–K. RL, rabbit liver; H, rabbit heart; K, rabbit kidney; B, rabbit brain; FL, frog liver; FM, frog muscle; RM, rabbit muscle. B. A drawing made from (A) to clarify the peptide maps.

A, B, E, F, or G of the rabbit enzymes. Frog liver has no bands corresponding to Bands A, B, G and J of the rabbit enzymes. Bands 1′ and 1″ of frog liver do not correspond with bands in the rabbit enzymes. These relationships are seen more clearly in Fig. 2B which is a drawing made from Fig. 2A.

At pH 6.5 (Fig. 3A), maps from rabbit synthases other than skeletal muscle synthase are again quite similar. Bands D′, H′ and M″ in muscle do not correspond with bands in other enzymes. Also, rabbit muscle synthase has no bands corresponding to Bands A, G, H and 1 found in other rabbit enzymes. Maps of frog liver and frog muscle differ slightly from each other and from the maps produced from the rabbit enzymes. In frog muscle, there are no bands corresponding to A, B, G, H, and 1 of the rabbit enzymes. However, frog muscle does contain Bands G′ and J′

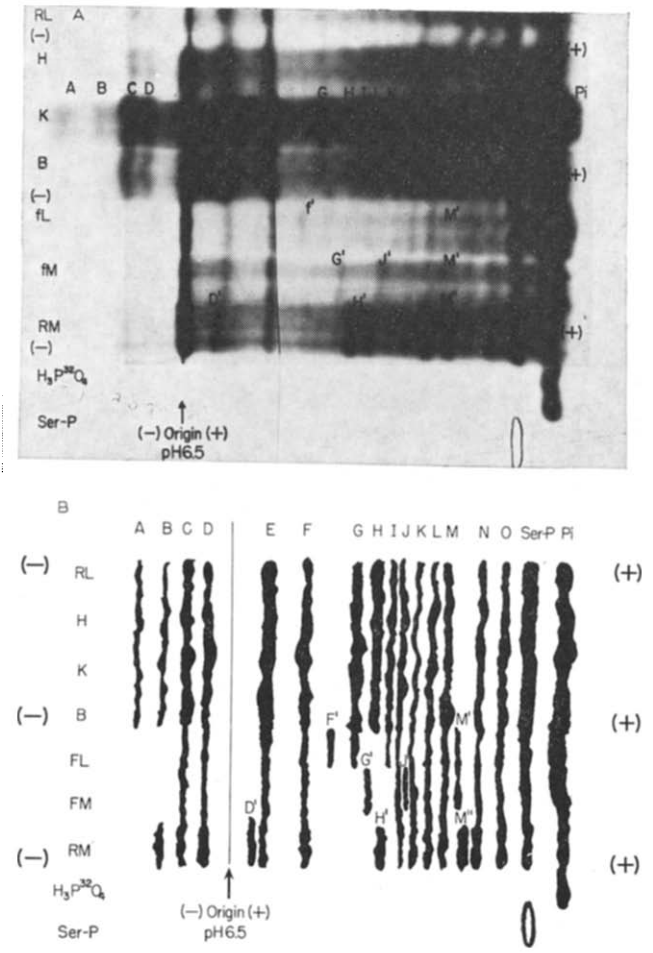

Fig. 3. A. High voltage electrophoresis of the partial acid hydrolysates of the organ synthases was conducted for 50 min at 50 V/cm in pyridine acetate buffer, pH 6.5. All other conditions are identical to those stated for Fig. 2. Phosphoserine and ^{32}P were used as markers and are labelled Ser-P and P_i, respectively. Other labelled peptides are lettered A through O. Other symbols as in Fig. 2(A). B. A drawing made from (A) to clarify the peptide maps.

which do not appear in maps derived from any other enzyme and Band M′ which does not correspond to Band M″ from rabbit muscle. Frog liver has no bands corresponding to A, B and H of the rabbit enzymes. It does contain Bands F′ and M′ not found in any rabbit enzyme.

Rabbit liver, kidney, heart and brain appear to match both at pH 6.5 and 3.5 and would appear therefore to be identical. Again these relationships are even better seen in Fig. 3B which is a drawing made from Fig. 3A.

Sepharose 4B chromatography of synthases

In order to prove that synthase was the only phosphorylated enzyme present, the enzymes from all seven organs were further purified by a procedure which has been shown in the case of skeletal muscle to yield essentially homogeneous enzyme[1,19].

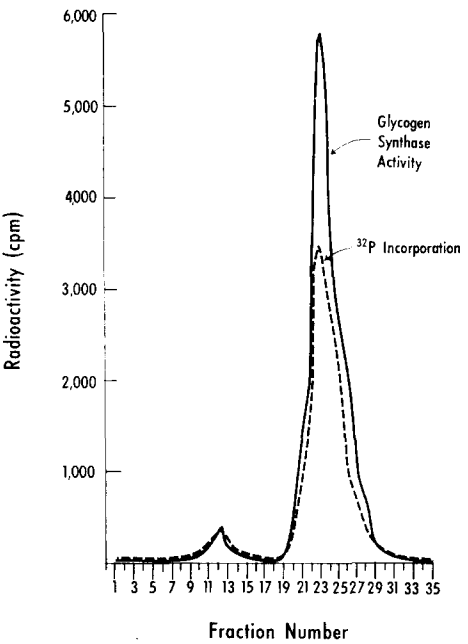

Fig. 4. Sepharose 4B chromatogram of rabbit liver glycogen synthase. Column fractions (5 ml each) were monitored for enzyme activity (– – –) and ^{32}P incorporated into protein (○—○). The peak of the small excluded fraction which contains aggregated synthase occurs at fraction 12, while the peak of the included fraction which contains active enzyme occurs at fraction 23.

The enzyme was first digested with salivary amylase to remove glycogen and then chromatographed over Sepharose 4B at room temperature[19]. Column fractions were monitored both for ^{32}P incorporated and for enzyme activity. A typical chromatogram for the rabbit liver enzyme is shown in Fig. 4. As can be seen for both the small excluded fraction which contains the enzyme in aggregated form, as well as for the larger included active enzyme fraction, there is a complete correspondence between ^{32}P incorporated into protein and enzyme activity. On the other hand, there was no

correlation with ultraviolet absorption, which was most likely due to nucleotides. Although not shown chromatograms from the other organs were similar*.

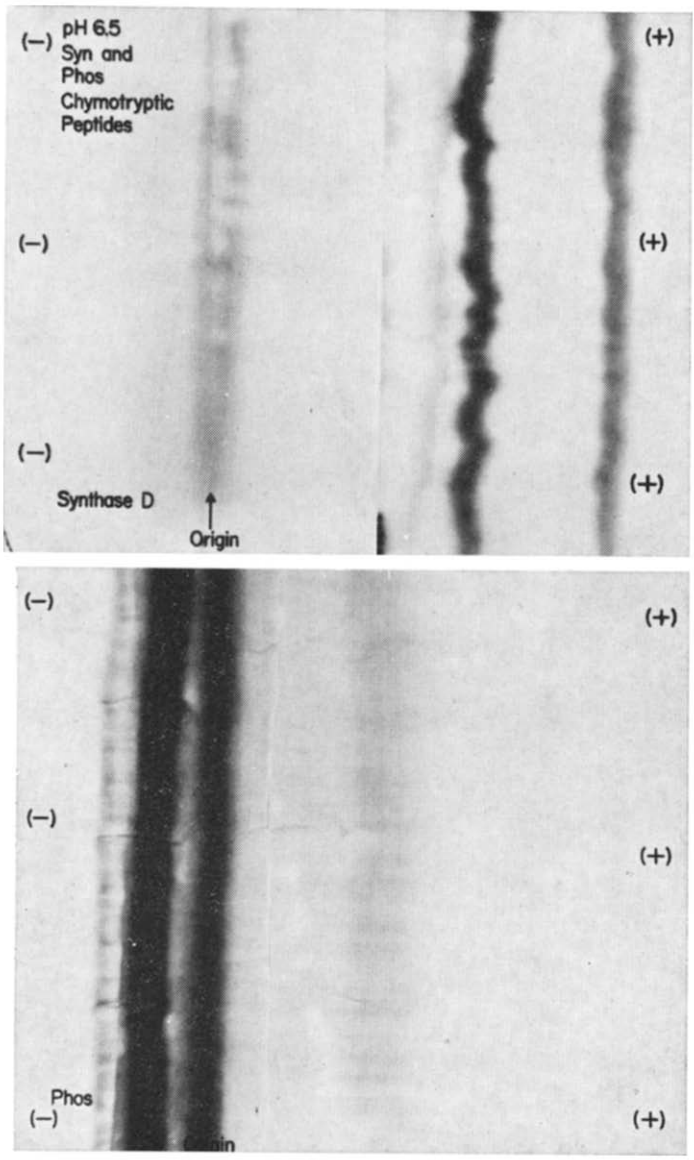

Fig. 5. High-voltage electrophoresis of the chymotryptic phosphopeptides of synthase (D) and phosphorylase a was conducted for 50 min at 50 V/cm in pyridine acetate buffer (pH 6.5). The origin is at the arrow in each case. The synthase chymotryptic phosphopeptide is acidic, migrating to the anode, whereas the phosphorylase chymotryptic phosphopeptide is basic, migrating to the cathode.

* After partial acid hydrolysis, maps of the labeled peptides separated by high-voltage electrophoresis matched those obtained from the labeled enzyme hydrolyzed prior to glycogen removal and chromatography over Sepharose 4B.

Chymotryptic phosphopeptides of synthase and phosphorylase

After chymotryptic digestion of ^{32}P-labeled rabbit muscle glycogen synthase and phosphorylase, electrophoresis at pH 6.5 was performed under the conditions previously described (Fig. 5). It is clear that with both enzymes there was one major radioactive band. The major labeled synthase chymotryptic band was acidic, migrating toward the anode, in contrast to the major labeled phosphorylase band, which was basic, and migrated toward the cathode at this pH. When electrophoresis was carried out at pH 3.5, the synthase chymotryptic band did not migrate and was therefore apparently isoelectric. This was in contrast to the phosphorylase chymotryptic band which was found to have an apparent isoelectric point at pH 8.9. Fig. 6 plots the migration rates at three pH values with $^{32}P_i$ used as a control. The markedly

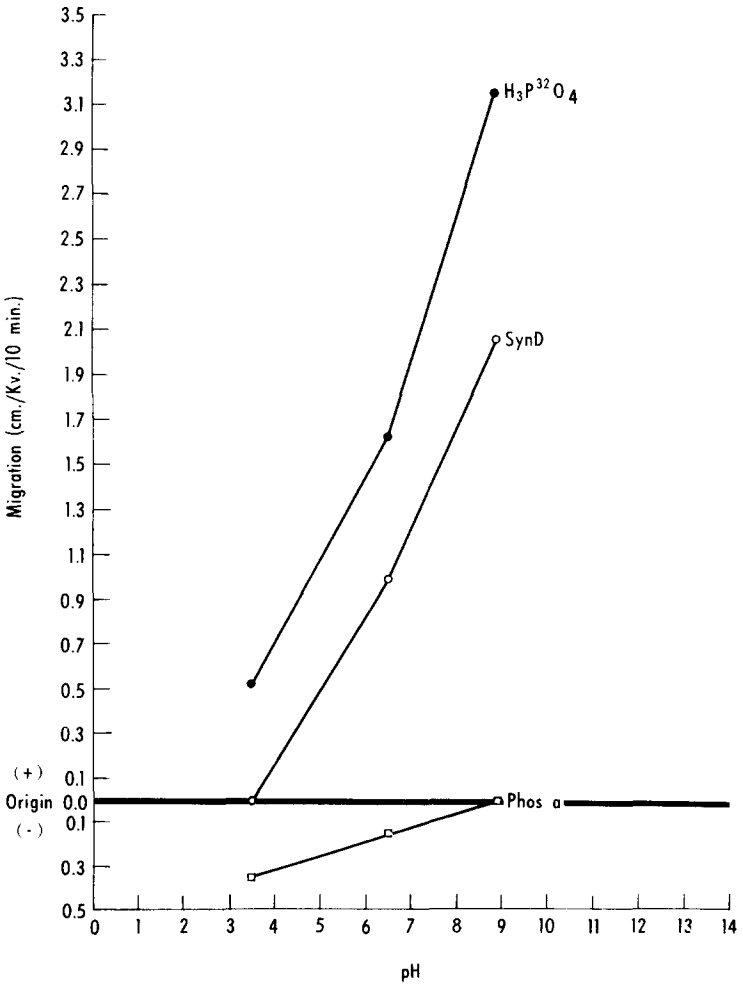

Fig. 6. Plot of migration (cm/kV per 10 min) *vs* pH during electrophoresis of the chymotryptic phosphopeptides of synthase D and phosphorylase a. The anode is towards the top of the origin and the cathode is towards the bottom.

different electrophoretic migrations probably indicate that the two major chymotryptic peptides bands differ in primary structure.

Since it has been previously shown that the hexapeptide sequences containing the phosphoserine residue in phosphorylase and glycogen synthase from rabbit muscle are identical or nearly so[24], the chymotryptic peptides of synthase and phosphorylase were eluted from the papers (10% acetic acid) lyophilized and resubmitted to partial acid hydrolysis in 6 M HCl for 15 and 30 min at 100 °C. Hydrolysates were applied to Whatman 3MM paper and phosphopeptides separated by electrophoresis at pH 6.5, papers dried and autoradiographs prepared. Identical phosphopeptide maps were obtained from the hydrolyzed synthase and phosphorylase chymotryptic peptides at 30 min (Fig. 7) and almost identical maps at 15 min. Note that at 15 min there are still several basic synthase peptides close to the orgin and one basic phosphorylase peptide further from the origin which do not overlap. This proves the large chymotryptic peptides of phosphorylase and glycogen synthase are different but contain identical small sequence(s) within the larger different structures.

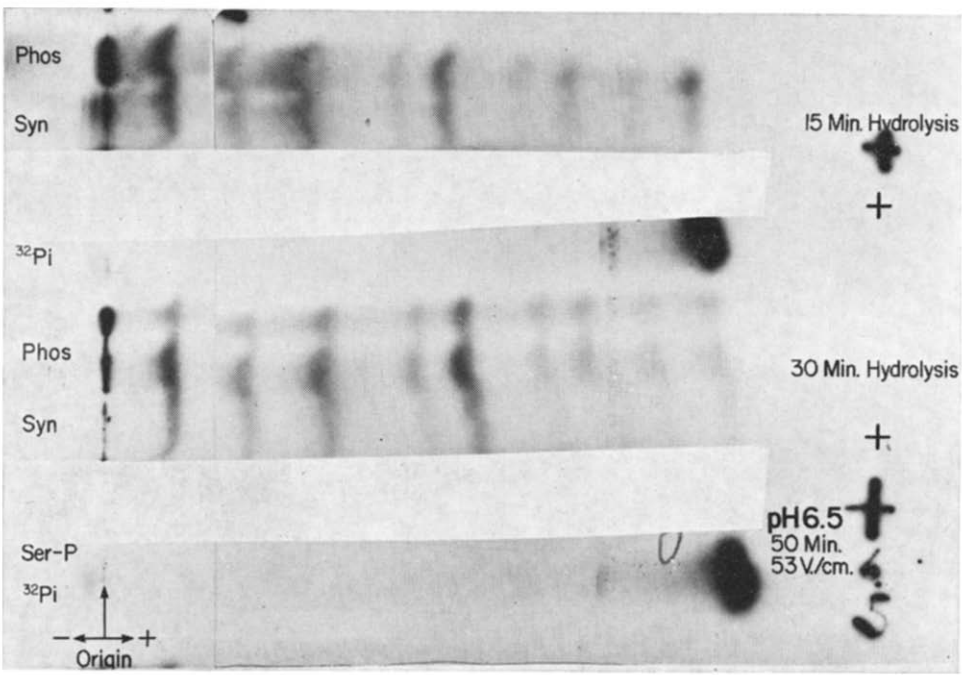

Fig. 7. High-voltage electrophoresis of the partially acid hydrolyzed chymotryptic phosphopeptides of synthase D and phosphorylase *a* was run for 50 min at 53 V/cm in pyridine acetate buffer (pH 6.5). The origin is indicated by the vertical arrow. The anode is to the right of the origin. $^{32}P_i$ and phosphoserine were used as markers.

DISCUSSION

The existence of interconvertable forms of glycogen synthase in muscle was first postulated on the basis of the unusual assay results (± glucose 6-phosphate)

observed after pretreatment of diaphragms with insulin[25]. The presence of the dephospho I and phospho D forms of rabbit skeletal muscle glycogen synthese was demonstrated by the experiments of Friedman and Larner[22] and then confirmed by Rosell-Perez and Larner[26]. The two forms in muscle were originally differentiated by kinetic experiments[27]. These experiments demonstrated that glucose 6-phosphate increased the V of the D or phosphorylated form in the presence of the substrate, UDPG, while glucose 6-phosphate decreased K_m of the I or dephosphorylated form for UDPG.

Both the dephospho and phospho forms of the enzyme are strongly inhibited by the nucleotides ATP, ADP, GTP, and UDP[28]. The phospho form is much more sensitive however to inhibition than is the dephospho form[5,29]. The inhibition by nucleotides is reversed by glucose 6-phosphate, but not as fully in the case of the dephospho form as in the case of the phospho form. In rat liver, Hizukuri and Larner[3] first identified the dephospho I and phospho D forms kinetically. The D form was converted to the I form by a catalyst later shown to be a phosphatase[30]. The reverse reaction was shown to be catalyzed by a kinase[31]. Between the two forms there was a small difference in the K_m for UDPG. Mersmann and Segal[4], Gold[5], and DeWulf *et al.*[6] studied the liver enzymes as concentrated homogenates with concentrations of substrate closer to physiological concentrations. Larger differences in K_m value for UDPG for the dephospho and phospho forms of synthase were observed.

Rabbit heart glycogen synthase has been shown to exist in phospho and dephospho forms. ^{32}P incorporation studies showed that heart synthase incorporates ^{32}P from [^{32}P]ATP in an amount comparable to that observed in skeletal muscle and that the map of the phosphopeptides produced after partial acid hydrolysis is a varient of the corresponding peptide map of skeletal muscle[2].

Goldberg and O'Toole[7] purified synthase from rat brain and have shown that the two interconversion reactions were present. The enzymology of the kidney synthase has been reported[32] and is under current study[33]. Rosell-Perez and Larner[15] demonstrated originally that the amphibian enzyme existed in a dependent form and that the interconversion with mercaptoethanol involved an increase in total activity with little or no generation of independent activity. Sevall and Kim[34,35] recently have purified tadpole liver synthase and found the enzyme totally dependent on glucose 6-phosphate.

In the present study, the ^{32}P incorporation studies and the electrophoretic peptide maps provide chemical evidence that glycogen synthase extracted from every organ studied is capable of being phosphorylated. Thus, in all likelihood, the dephosphorylated and phosphorylated forms of glycogen synthase exist in all of these tissues. The labeled peptide maps indicate that the enzyme is phosphorylated by [^{32}P]ATP on a serine hydroxyl. The larger peptides show little variation when the enzymes from various organs are compared because the size of the peptides possibly masks small sequence differences. The smaller acidic and basic peptides do show differences. Since the small acidic peptides from rabbit muscle and the other rabbit and frog tissues are very similar, it appears that differences in sequence may exist some distance away from the phosphoserine.

The hexapeptide sequence around the serine phosphate in rabbit skeletal muscle was shown by Larner and Sanger[24] to be:

Arg
–Ile–Gln–Ser-*P*–Val–Arg
Lys

Larner *et al.*[2] have shown that the electrophoretic pattern of peptides from rabbit heart synthase differ slightly from the pattern from rabbit skeletal muscle enzyme. This difference was again seen in the present experiments in the acidic peptides which migrated midway to the anode, thus confirming the previous work.

Since the possibility had been raised that the previous identical peptide maps produced by partial acid hydrolysis of phosphorylase and glycogen synthase[24] were due to contamination of the synthase enzyme with inactive phosphorylase, the experiments with chymotrypsin digestion were performed. The present studies in which the peptides produced by chymotrypsin were rehydrolyzed by mild acid rule out the possibility that the previous results could be explained by contamination of synthase by enzymatically inactive but phosphorylatable phosphorylase. The present studies demonstrate that the larger chymotryptic peptides of phosphorylase and synthase are markedly different and therefore in all likelihood differ in sequence beyond the hexapeptide site. After partial acid rehydrolysis of these different chymotryptic peptides identical maps are obtained thus proving that within the larger non-identical peptides there is present the identical hexapeptide sequences previously established.

As for the frog and rabbit synthase, the phosphorylation of the rabbit enzymes results in the conversion of a glucose 6-phosphate independent to a dependent enzyme; yet phosphorylation of the frog enzymes gives rise to a less active glucose 6-phosphate dependent enzyme. This study shows that serine is the phosphorylated site in all enzymes. Therefore, this implies that the same chemical modification on the same amino acid residue of proteins catalyzing identical catalytic functions can lead to different modulation of enzymatic behavior; that is to say a conversion of an independent to a dependent form or a conversion of a more active to a less active dependent form. It is proposed that the primary structure outside of the serine residue governs the properties of the phosphorylated protein, *i.e.* dependency or activation. A detailed comparative study of these enzymes will be of interest in order to elucidate the mechanism of these conversion reactions.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Drs Carl H. Smith, Carlos Villar-Palasi and Laura C. Shen for their advice and assistance throughout this study. This study was supported by the U.S. Public Health Service, National Institutes of Health, Grant AM-14334.

REFERENCES

1 Smith, C. H., Brown, N. E. and Larner, J. (1971) *Biochim. Biophys. Acta* 242, 81–88
2 Larner, J., Villar-Palasi, C. and Brown, N. E. (1969) *Biochim. Biophys. Acta* 178, 470–479
3 Hizukuri, S. and Larner, J. (1964) *Biochemistry* 3, 1783–1788
4 Mersmann, H. J. and Segal, H. L. (1967) *Proc. Natl. Acad. Sci. U.S.* 58, 1688–1695
5 Gold, A. H. (1970) *Biochemistry* 9, 946–952
6 DeWulf, H., Stalmans, W. and Hers, H. G. (1970) *Eur. J. Biochem.* 15, 1–8
7 Goldberg, N. E. and O'Toole, A. G. (1969) *J. Biol. Chem.* 244, 3053–3061

8 Sovik, O., Øye, I. and Rosell-Perez, M. (1967) *Biochim. Biophys. Acta* 124, 26–33
9 Bishop, J. S. and Larner, J. (1967) *J. Biol. Chem.* 242, 1355–1356
10 Kim, K. H. and Blatt, L. M. (1969) *Biochemistry* 8, 3997–4004
11 Blatt, L. M., Sevall, J. S. and Kim, K. H. (1971) *J. Biol. Chem.* 246, 873–880
12 Blatt, L. M. and Kim, K. H. (1971) *J. Biol. Chem.* 246, 2959–2964
13 Sevall, J. S. and Kim, K. H. (1971) *J. Biol. Chem.* 246, 4895–4898
14 Sevall, J. S. and Kim, K. H. (1971) *J. Biol. Chem.* 246, 7250–7255
15 Rosell-Perez, M. and Larner, J. (1962) *Biochemistry* 1, 769–772
16 Bernfeld, P. (1955) *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. I pp. 149–158, Academic Press, New York
17 Naughton, M. A., Sanger, F., Hartley, B. S. and Shaw, D. C. (1960) *Biochem. J.* 77, 149–163
18 Post, R. L. and Sen, A. K. (1967) in *Methods in Enzymology* (Estabrook, R. W. and Pullman, M. E., eds), Vol. X, pp. 762–768, Academic Press, New York
19 Brown, N. E. and Larner, J. (1971) *Biochim. Biophys. Acta* 242, 69–80
20 Villar-Palasi, C. and Gazquez-Martinez, I. (1968) *Biochim. Biophys. Acta* 159, 479–489
21 Thomas, J. A., Schlender, K. K. and Larner, J. (1968) *Anal. Biochem.* 25, 486–499
22 Friedman, D. L. and Larner, J. (1963) *Biochemistry* 2, 669–675
23 Krebs, E. G. (1966) in *Methods in Enzymology* (Neufeld, E. F. and Ginsburg, V., eds), Vol. XIII, pp. 543–546, Academic Press, New York
24 Larner, J. and Sanger, F. (1965) *J. Mol. Biol.* 11, 491–500
25 Villar-Palasi, C. and Larner, J. (1960) *Biochim. Biophys. Acta* 39, 171–173
26 Rosell-Perez, M. and Larner, J. (1964) *Biochemistry* 3, 773–778
27 Villar-Palasi, C. and Larner, J. (1962) *Biochemistry* 1, 763–768
28 Larner, J. and Villar-Palasi, C. (1971) *Curr. Topics Cell. Regul.* 3, 195–236
29 Piras, R., Rothman, L. B. and Cabib, E. (1968) *Biochemistry* 7, 56–66
30 Kato, K. and Bishop, J. (1972) *J. Biol. Chem.* 247, 7420–7429
31 Bishop, J. S. and Larner, J. (1969) *Biochim. Biophys. Acta* 171, 374–377
32 Hidalgo, J. L. and Rosell-Perez, M. (1971) *Rev. Esp. Fisiol.* 27, 343–354
33 Schlender, K. K. (1972) *Biochim. Biophys. Acta*, in the press
34 Sevall, J. S. and Kim, K. H. (1970) *Biochim. Biophys. Acta* 206, 359–368
35 Sevall, J. S. and Kim, K. H. (1972) *Physiol. Chem. Phys.* 4, 370–376
36 Rosenkrans, A. M. and Larner, J. (1971) *Pharmacologist* 13, 271
37 Larner, J. (1972) *Biochemistry of the Glycosidic Linkage*, PAABS Symposium, Vol. 2, pp. 597–619, Academic Press, New York
38 Larner, J. (1972) *Diabetes* 21, 428–438