PURIFICATION AND CHARACTERIZATION OF GLYCOGEN SYNTHASE FROM *ASCARIS SUUM*

THESIS

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Glycogen synthase, the enzyme that catalyzes the rate-limiting reaction of glycogen synthesis, has been purified and characterized from Ascaris suum muscle. Glycogen in the crude extract was digested to release the enzyme, eluted from a DE52 cellulose column and then applied to a Sepharose 4B gel filtration column. Further purification was accomplished by a glucosamine 6-phosphate Sepharose affinity column. The purified Ascaris enzyme was found to be homologous to the mammalian enzyme with regard to subunit and holoenzyme M_r, allosteric activation, substrate affinity and covalent modification. However, the association between Ascaris glycogen synthase and endogenous glycogen differed from that in mammalian systems.

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CHAPTER I

INTRODUCTION

Ascaris lumbricoides is an intestinal parasitic roundworm (Nematoda) which infects 25% of the world's human population, with most of the infection being in underdeveloped areas where sanitary conditions are poor (1). Ascaris lumbricoides var. suum is found in approximately 80% of the pig population of the United States (2).

The adult Ascaris consists of a long unbranched intestinal tract running the length of the organism. The intestine consists of a single layer of tall columnar epithelial cells which secrete and are supported by a relatively permeable basement membrane (basal lamella). The parasites are dieocious and the female (only females are used in these experiments) has its uterus, oviduct and ovaries free floating in its pseudoceolomic cavity. The eggs leave the body through a genital pore located approximately one-third the distance from the anterior region. The muscle in the worm is longitudinal and underlies a protective hypodermis layer. The hypodermis secretes the relatively thick and impermeable cuticle which supports it (3).

About 70% of the wet weight of the worm consists of muscle which contains large quantities of glycogen compared with mammalian skeletal muscle. Up to 15% of the muscle's wet weight consists of glycogen compared to less than 1% in mammalian muscle (3). As a free living anaerobic parasite, Ascaris is dependent on glycolysis to extract energy needed for muscle contraction. When the worm's host is not feeding, the

fuel for glycolysis must come from these large glycogen stores. For this reason the regulation of enzymes controlling glycogen metabolism, including glycogen synthase, is important in maintaining muscle contraction during changes in feeding states of the host.

Glycogen synthase (UDP-glucose: glycogen -4-glucosyltransferase, E.C. 2.4.1.11) is the regulatory enzyme in glycogen synthesis. It catalyzes the transfer of the glucose moiety from UDP-glucose to the nonreducing end of a glycogen molecule forming an $\alpha(1\rightarrow4)$ linkage. This reaction can be written as:

$$\text{UDP-glucose} + (\text{glycogen})_n \longrightarrow \text{UDP} + (\text{glycogen})_{n+1} \quad (4)$$

The enzyme has been studied in a wide variety of organisms including mammals, fish (5), mussels (6), snails (Biomphalaria glabrata) (7), and Ascaris suum (8). Glycogen synthase has been purified from several mammalian sources, among them rabbit skeletal muscle (9,10), human leukocytes (11), rat and rabbit liver (12,13) and pig and rat adipocytes (14,15). The enzymes are substantially similiar with respect to their physical, catalytic and regulatory properties.

All of the animal glycogen synthase enzymes studied to date are regulated by two mechanisms, covalent phosphorylation and allosteric modification. Modification by phosphorylation allows the enzyme to exist in two interconvertable forms, one which has high amounts of covalently bound phosphate and the other which has lesser amounts of phosphate (4). The highly phosphorylated form has very low activity in the absence of its allosteric effector, glucose 6-phosphate (G 6-P), while the lesser phosphorylated form is active in the absence of G 6-P. The dependence on G 6-P as an activator is reflected in the names given to each form,

dependent (D) and independent (I), respectively. Glucose also acts as a positive effector while AMP, cyclic AMP and glycogen act as inhibitors (4).

The regulation of glycogen synthase is reciprocally coordinated with that of the enzyme that degrades glycogen to glucosyl units, glycogen phosphorylase. Phosphorylase is activated by phosphorylation and inactivated by dephosphorylation. The effect is that when phosphorylase is active, glycogen synthase is inactive, and when glycogen synthase is active, phosphorylase is inactive. The opposing effects of phosphorylation on these two enzymes insure that glycogen synthesis and degradation do not occur simultaneously. The phospho- and dephospho- forms of both enzymes are interconvertable depending on the organism's metabolic need for glucose mobilization or glycogen storage (4).

The mechanism by which glycogen synthesis and mobilization is regulated has been established by using purified components of the system. Regulatory events are initiated by the binding of an extracellular messenger molecule (epinephrine or glucagon) to its specific membrane receptor. This binding activates the associated adenylate cyclase to produce cyclic adenosine 3',5'-monophosphate (cyclic AMP). Cyclic AMP is the positive modulator for cyclic AMP-dependent protein kinase, which catalyzes the transfer of phosphate from ATP to phosphorylase kinase. Phosphorylase kinase, in turn, catalyzes phosphorylation of glycogen phosphorylase thereby effecting glycogen degradation. The cyclic AMP-dependent protein kinase also phosphorylates glycogen synthase to inhibit glycogen synthesis (4).

The activity of this cascade mechanism has been demonstrated in intact organisms (16) and tissues (17) as well as perfused tissues (18) and cultured cells (19). In fed and fasted rats Danforth *et al.* have shown a

positive correlation between glycogen synthase activity and the feeding status of the animal (20). It was found that approximately 80% of the total glycogen synthase pool in fed rats was in the active form, while only 20% of the enzyme in fasted rats was in the active form. This suggested that when an abundant energy source was available, glycogen synthase was actively synthesizing glycogen from the excess fuel not needed for immediate consumption. When epinephrine, a hormone that increases heart rate, blood pressure and raises blood glucose levels, was given to fed rats, active glycogen synthase was present in levels less than 20%. These data would suggest that energy sources previously used in glycogen synthesis were being redirected to fuel the increase in metabolic rate stimulated by the epinephrine.

Experiments correlating hormonal exposure with glycogen synthase activity and degree of enzyme phosphorylation were performed on rat hepatocytes by Ciudad et al. (21). Cells incubated with [^{32}P] phosphate were exposed to epinephrine, glucagon or vasopressin, followed by extraction of glycogen synthase by immunoprecipitation. All three hormones effected a decrease in glycogen synthase activity with a concominent increase in phosphate content of the enzyme.

The peptide hormone insulin is known to be an inducer of glycogen synthase activity, but the mechanism of action has not been elucidated (22). Perfusion of rat hearts with [^{32}P] phosphate and insulin and glucagon was done by Ramenchandran et al. (23). These workers found that insulin-induced activation correlated with an increase in glycogen synthase I from 25% to 40% along with a 22% decrease in phosphate content. Conversely, perfusion with glucagon caused a 50% increase in phosphate content as well as

inactivation of the enzyme.

Similar studies correlating hormonal action with specific phosphorylation sites were done by DePauli-Roach et al. (24). Glycogen synthase from insulin- and epinephrine-treated rat hemidiaphragms was cleaved with cyanogen bromide to yield two major polypeptide fragments. It was found that although insulin caused a 40 to 50% reduction of phosphate equally in each fragment, epinephrine incorporation of unequal amounts of phosphate into each fragment.

Elucidation of the molecular mechanism by which glycogen synthase is regulated has utilized glycogen synthase purified from muscle and liver sources. Glycogen synthase from rabbit skeletal muscle was purified to near homogeneity by Soderling et al. in 1970 (9). The procedure these workers developed began with a glycogen precipitation from the soluble muscle fraction in order to separate the glycogen bound proteins, including glycogen synthase, from other proteins. This was accomplished by lowering the pH of the solution to 6.1 with acetic acid. The isolated protein and glycogen complex was then digested with amylase to allow solublization of the enzyme. Subsequent ion exchange and gel filtration chromotography yielded glycogen synthase purified 500-fold with a specific activity of 4.7 units/mg and a recovery of 9% from the muscle extract. Fifty to sixty percent of the enzyme was present in the I form. Subunit M_r was found to be 90,000 to 100,000 while holoenzyme M_r was found to be 400,000, indicating a tetrameric structure.

Glycogen synthase has been purified from another glycogen-rich source, rat liver, by Jett et al. in 1979 (12). This purification procedure differed from the rabbit muscle procedure in the following ways. First, the

acid precipitation step was unnecessary, presumably because the glycogen concentration in liver is much higher than in muscle. Next, an incubation step was added to facilitate conversion of the enzyme totally to the I form. Finally, the gel filtration step was replaced with affinity chromotography. Rat liver glycogen synthase purified by this procedure was recovered 100% in the I form. Purification was 1,300-fold with a specific activity of 50 units/mg and a 20% recovery. Structural analysis indicate a subunit M_r of 85,000 and a holoenzyme M_r of 156,000 to 171,000 which suggests that the liver enzyme, in contrast to the muscle enzyme, may exist as a dimer.

Purified mammalian glycogen synthase has been shown to possess multiple phosphorylation sites, seven of which have been found on each subunit of rabbit skeletal muscle enzyme (25). Several different kinases, collectively known as the glycogen synthase kinases, incorporate different amounts of phosphate into glycogen synthase with varying effects on activity. Synergistic effects and less than additive affects resulting from combinations of kinases have been reported by DePauli-Roach et al. in 1983 (26). The physiological significance of this multisite phosphorylation has not been determined.

Since glycogen has been shown to be the sole source of carbon for maintenance of the cellular energy charge during periods of nonfeeding in Ascaris, the glycogen cascade was extensively characterized in the muscle of this organism. The enzymes that have been demonstrated in Ascaris muscle to date are phosphorylase (28), glycogen synthase (29) and cyclic AMP-dependent protein kinase (30).

Donahue et al. has correlated feeding states of Ascaris with changes in glycogen synthase activity in the muscle. When the worm is starved,

glycogen synthase activity decreases and muscle glycogen is depleted (29,30). When this muscle is perfused with glucose, glycogen synthase activity is increased and incorporation of glucose into glycogen occurs (28). Effects of acetylcholine and 5-hydroxytryptamine (serotonin) were also investigated. Perfusion with acetylcholine caused muscle contraction, an increase in phosphorylase activity and a decrease in glycogen synthase activity. Serotonin also increased phosphorylase activity and decreased glycogen synthase activity but did not elicit muscle contraction. However, perfusion with serotonin did result in increased levels of cyclic AMP three-fold from control values (31). This suggests that two mechanisms exist for the initiation of glycogen mobilization and the inactivation of glycogen synthesis in Ascaris, one of which is cyclic AMP-dependent and one which is cyclic AMP-independent. The cyclic AMP-dependent mechanism appears to be similar to the epinephrine cascade in mammals except that serotonin is the extracellular signal.

Collectively, these data indicate that regulation of glycogen synthesis and metabolism by cyclic AMP occurs through a cascade highly similiar or identical to that reported in mammalian skeletal muscle. However, no evidence has been accumulated which resolves the importance of multisite phosphorylation in the regulation of glycogen synthase activity in vivo. Further, the Ascaris perfusion system offers a mechanism by which the response of glycogen synthase to controlled physiological stimuli may be quantitated. Since the regulation of glycogen mobilization and subsequent glycolytic flux is essential to the survival of the organism, contributions to the current knowledge of Ascaris glycogen metabolism will result in more efficient and economical chemotheraputic control of this parasite. In view of the central role of glycogen synthase in the regulation of Ascaris metabolism,

this study of Ascaris skeletal muscle glycogen synthase was initiated.

The specific aims of this project were to purify glycogen synthase from Ascaris suum and to characterize the enzyme by substrate specificity, allosteric binding effects, hysteretic properties, molecular weight, and pH and temperature optimums.

CHAPTER II

MATERIALS AND METHODS

Tissue source.-- A. suum were collected at a slaughterhouse and transported to the laboratory in a salt solution (8). The muscle was dissected out and flash frozen in liquid nitrogen. The frozen muscle was stored at -80°C until use in enzyme preparations.

Purification of rabbit skeletal muscle glycogen synthase.-- Rabbit glycogen synthase was purified according to the method of Soderling et al. (9). All procedures were done at 4°C unless otherwise indicated. Fresh rabbit skeletal muscle was ground in a meat grinder and then homogenized in buffer A (1:3;w/v). Buffer A is 50 mM tris(hydroxymethyl)aminomethane-HCl (TRIS-HCl), pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM $MgCl_2$, and 15 mM β-mercaptoethanol (BME). The homogenate was centrifuged at 10,000 x g for 10 minutes using a Sorvall RC-5B centrifuge and a GSA rotor at 8,500 rpm. The resulting supernatant was filtered through glass wool and then titrated with 1 N acetic acid to pH 6.1 or until a cloudy precipitate appeared. This was stirred for 10 minutes and centrifuged again at the same force for 30 minutes. The glycogen and protein pellet was resuspended in buffer B: 100 mM β-glycerophosphate, pH 8.0, 30 mM BME and 4 mM EDTA. The sample was diluted with the same volume of buffer B at pH 7.5 and centrifuged at 105,000 x g for 1 hour using a Beckman LC-75 ultracentrifuge and a Ti 45 rotor at 36,000 rpm. The pellet was resuspended in buffer C: 50 mM TRIS-HCl, pH 7.5, 1 mM EDTA, 10 mM $MgCl_2$, 40 mM BME and 5% sucrose. Total protein was determined using the Bradford

method (34). Human salivary amylase was then added to the preparation at a ratio of 7.5×10^{-5} ug per mg protein. The mixture was incubated for 1 hour at $30^{o}C$ and then centrifuged at 105,000 x g for 1 hour. The supernatant was applied to a DE52 column equilibrated with buffer C. The column was washed with two column volumes of buffer C and then eluted with a linear KCl gradient of 50 mM to 400 mM. The fractions were assayed for activity and the peak fractions were concentrated with ammonium sulfate (45% saturation). The resulting protein pellet was solubilized in buffer D: 50 mM β-glycerophosphate, pH 7.0, 2 mM EDTA, 40 mM BME and 10% sucrose. After dialysis in the same buffer, the sample was applied to a Sepharose 4B-200 column equilibrated with buffer D. The active fractions were concentrated on a small DE52 column and the purified enzyme was frozen at $-20^{o}C$.

Glycogen synthase activity assay.-- Glycogen synthase activity was assayed by a modification of the method of Thomas et al. (30) which measures the incorporation of UDP[^{14}C]glucose into glycogen. The sample to be assayed was added to a mixture containing a final concentration of 6.7 mM UDP[^{14}C] glucose, 50 mM TRIS-HCl, pH 7.5, and 1% glycogen (Ascaris unless otherwise noted.) This mix measured I form activity only. To measure I + D or total activity, 10 mM glucose 6-phosphate was added to the mixture. The reaction mixture was allowed to incubate at $30^{o}C$ for 10 minutes. The reaction was stopped by pipetting 50 ul aliquots onto 1 cm^2 Whatman ET 31 papers and washing the papers in 7 to 8 ml per paper of cold 66% ethanol twice for 10 minutes each. The papers were then washed in acetone for 5 minutes, dried and [^{14}C] incorporation into glycogen was measured by liquid scintillation counting. Radioactivity measured as 23,000 cpms corresponded to 0.670 units of enzyme activity. One unit of activity was defined as that

amount of enzyme which will catalyze the incorporation of 1 umol [^{14}C]glucose transferred into glycogen per minute at 30^{o}C. Oyster glycogen purchased from Sigma and Ascaris glycogen extracted by the method of Bueding and Orrell (31) were used in the assays as indicated.

Glycogen extraction.-- Ascaris glycogen was extracted by a modification of the cold water method of Bueding and Orrell (31). Whole worms were homogenized at 4^{o}C in 4 volumes of 0.2 M glycine buffer pH 10.5 plus two volumes of chloroform. This was centrifuged at 180 x g for 5 minutes and the upper aqueous phase was decanted. The lower chloroform phase was extracted twice more with 2 volumes of glycine buffer and the 3 resulting aqueous phases were combined and centrifuged at 180 x g for 5 minutes. The resultant supernatant was centrifuged at 70,000 x g for 16 hours. The gelatinous residue was homogenized with enough water to liquify. The solution was then stirred 5 minutes with one-third of its volume with a mixture of chloroform and octanol in a 3:1 ratio. The upper aqueous phase was aspirated after low speed centrifugation for 5 minutes. This was treated with the chloroform-octanol 3 more times, stirring for 1 hour, 2 hours, and overnight, respectively. The aqueous phases were combined and 0.05 volume of 1 M citrate buffer (pH 6.1) plus 1 mM LiCl were added. Glycogen was precipitated by the addition of 1 volume 100% ethanol and collected by centrifugation at 1,600 x g 15 minutes. The pellet was washed with 100% ethanol, dried on a vacuum flask overnight and ground to a powder with a mortar and pestle. Oyster glycogen was obtained from Sigma Chemical Co. and repurified over an Dowex M31 mixed ion exchange column (32).

Glucose determination.-- Free glucose as a product of glycogen degradation was determined using the glucose oxidase kit from Sigma

Chemical Co. This kit uses a modification of the method of Raabo and Terkildsen (33).

Glucosamine 6-phosphate Sepharose affinity column.--Cyanogen bromide activated-Sepharose 4B purchased from Sigma Chemical Co. was coupled to glucosamine 6-phosphate by the method of Pharmacia Fine Chemicals (44). Application and elution of glycogen synthase was done by a modification of the method of Jett et al. (12).

Other Methods.-- Protein was determined by the dye-binding method of Bradford (34) using bovine serum albumin as a standard. Gel electrophoresis in the presence of SDS was carried out following the method of Laemmli (35).

Other materials.-- DE52 cellulose anion exchange resin and ET 31 paper were purchased from Whatman-Bodman Company. Sepharose 4B 200 gel and Concanavalin-A Sepharose 4B affinity resin were obtained from Pharmacia Fine Chemicals. Glucose 6-phosphate, glucosamine 6-phosphate, uridine diphosphoglucose, Dowex M31 resin, human salivary amylase and amyloglucosidase were all products of Sigma Chemical Co. Uridine diphospho[^{14}C]glucose was purchased from ICN Pharmaceuticals, Inc. All other materials used were of the highest quality available.

CHAPTER III

RESULTS

Purification of rabbit skeletal muscle glycogen synthase.-- Rabbit skeletal muscle glycogen synthase was purified according the method of Soderling et al. (1970) (see Materials and Methods). The rabbit glycogen synthase was used as a control enzyme and was used for comparative purposes only in this study.

Glycogen synthase activity in the Crude Extract from Ascaris.-- In applying the rabbit muscle procedure to Ascaris muscle, differences in appearance, composition and enzyme activities of the 10,000 x g supernatant were observed. Table I compares data from extracts of Ascaris muscle, rabbit skeletal muscle (9) and rat and rabbit liver (12,13). Typically the protein concentration for the Ascaris extract was 63 g per kg of muscle. This is nearly two-fold greater than the 33 g protein per kg muscle from mammalian skeletal muscle. The specific activity for Ascaris at this step was 7840 units/mg protein which is greater than that for rabbit muscle (419 units/mg) or rat liver (1506 units/mg). Ascaris glycogen synthase K_a for glucose 6-phosphate was 0.5 mM which is comparable to that for mammalian muscle enzyme (0.2 to 0.5 mM) but much greater than that for mammalian liver enzyme (0.007 to 0.06 mM). The percent of the total enzyme pool present in the active or I form in the Ascaris extract varied from 19-54%. This variation may reflect the fed state of the worm at time of dissection and freezing. Comparable values for the percent I from mammalian muscle and liver preparations of 31% and 12%, respectively, were observed.

TABLE I

COMPARATIVE PROPERTIES OF GLYCOGEN SYNTHASE ACTIVITY IN CRUDE EXTRACTS

Crude extracts of A. suum muscle were prepared as described in Methods and glycogen synthase activity was determined in the presence of 10 mM glucose 6-phosphate. All results cited are for comparable preparations.

Tissue	Protein	Glycogen	Glycogen Synthase Activity	% GSI	Ka (G 6-P)
	(g/kg)	(g/kg)	(units/kg)		(mM)
A. suum Muscle	63	150	7840	10-54	0.5
Rabbit Skeletal Muscle	33[a]	10	419[a]	24[c]	0.2-0.5[a]
Rat[b]/Rabbit[c,d] Liver	37[b]	46	1506[b]	33[b]	0.007-0.6[d]

[a]Soderling et al. (9)

[b]Jett and Soderling (12)

[c]Sheorain et al. (36)

[d]Camici et al. (13)

Differences in glycogen concentrations were observed in that the Ascaris extract contained 150 g glycogen per kg tissue while mammalian muscle contained 10 g/kg and liver contained 46 g/kg.

Differences in the appearances of Ascaris muscle and mammalian muscle extracts were also observed. When rabbit muscle homogenate was centrifuged at 10,000 x g, the resulting pellet was firm while the supernatant (Crude Extract) was bright pink in color and slightly cloudy. In contrast, the 10,000 x g pellet from Ascaris homogenate was much less firm and the supernatant was very cloudy. This greater turbidity of the Ascaris extract is most likely due to the greater protein and glycogen content of Ascaris muscle. A change in the homogenization buffer concentration (from 50 mM to 20 mM TRIS-HCl) and a decrease in the buffer pH (from 7.5 to 7.0) caused the Ascaris pellet to become firmer. Addition of exogenous glycogen or increases in salt concentration (up to 0.7 M KCl) did not significantly affect glycogen synthase activity in this fraction.

In the purification of both Ascaris and rabbit glycogen synthase this Crude Extract was titrated to pH 6.1 with 1 N acetic acid. The decrease in pH precipitated the glycogen so that soluble enzymes would be removed by centrifugation at 10,000 x g, while the glycogen synthase remained with the pellet. Table II compares several components of this pellet from Ascaris, rabbit skeletal muscle and rat liver. Protein in the Ascaris pellet was 42 g/kg muscle, greater than that for either mammalian tissue. The specific activity of glycogen synthase was 1376 units/kg tissue in Ascaris which is nearly five times greater than that of rabbit muscle but comparable to that of rat liver. The percent of total glycogen synthase in the I form at this step was 85% in Ascaris, comparable to 67% in mammalian muscle and 82% in

TABLE II

COMPARATIVE PROPERTIES OF GLYCOGEN SYNTHASE ACTIVITY FROM THE GLYCOGEN PELLET

Glycogen pellets from A. suum muscle prepared as described in Methods. Glycogen synthase activity was determined in the presence of 10 mM G6-P. All results cited are for comparable preparations.

Tissue	Protein g/Kg	Glycogen Synthase Units/Kg	% GSI
Ascaris	42	1376	85
Rabbit Skeletal Muscle	1.27[a]	290[a]	61[c]
Rat Liver	0.54[b]	1008[b]	32[b]

[a] Soderling et al. (9)

[b] Jett and Soderling (12)

[c] Nimmo et al. (10)

liver.

In rabbit preparations this precipitation was accompanied by an obvious increase in turbidity. In Ascaris preparations, however, the solution was already very cloudy and the pH was lowered to 5.5 to effect any change in turbidity, if at all. The supernatant from the centrifugation of the titrated fraction also differs in rabbit and Ascaris tissue. The rabbit supernatant was relatively clear whereas the Ascaris supernatant remained cloudy. These differences are also attributed to the much higher glycogen and protein content of Ascaris muscle.

Binding of Ascaris Glycogen Synthase to DE52 Cellulose.-- Glycogen synthase, like other enzymes of glycogen metabolism, is closely associated with the glycogen granule (37). The glycogen-enzyme complex will not bind to DE52 presumably because the weight of the particle overcomes the ionic interactions. This inability to bind was demonstrated for Ascaris enzyme by experiments using resuspended pH 6.1 pellet from 105,000 x g centrifugation and DE52 cellulose resin. One milliliter aliquots of the sample were mixed with the same volume of DE52 resin equilibrated in buffer C. The mixture was centrifuged at approximately 12,000 x g for 3 minutes to sediment the resin. The supernatant was removed, the resin was washed with the same buffer plus 0.35 M KCl, and then again with buffer without salt. Each of the washes was assayed for glycogen synthase activity. Binding was expressed as the percentage of original activity recovered in the supernatant. The results showed that 0.04% of the enzyme bound to the DE52 resin, while 100% of the activity remained unbound in the first supernatant. This indicated that most of the glycogen synthase activity did not bind. In order to give the binding reaction more time, equal volumes of sample and resin

were stirred slowly overnight at 5° C. The experiment proceeded as above and the results showed only a 0.07% binding. These experiments indicated that the glycogen synthase was not binding to DE52.

A third experiment was performed in which the resuspended pellet from the 105,000 x g centrifugation was stirred overnight in the cold with DE52 equilibrated in buffer C. Again the resin was centrifuged at low speed and washed with buffer C plus 0.7 M KCl. After assaying the washes for glycogen synthase activity, 16% of the original activity was found in the high salt wash, indicating that only 16% of the glycogen synthase in the pellet had bound to the resin.

Solubilization of Ascaris Glycogen Synthase.--To facilitate enzyme binding to DE52, alternative methods for digestion of endogenous glycogen were investigated. Human salivary amylase was added to aliquots of resuspended glycogen pellet in ratios of 0.19, 0.38, 0.76 and 1.52 mg/kg muscle. Similar concentrations to these were used by Soderling (9) for rabbit skeletal muscle. The aliquots were allowed to incubate for 1 hour at 30°C and then centrifuged at 12,000 x g for 20 minutes. The resulting supernatants were assayed along with the control sample which was incubated without amylase. The results are expressed in terms of the percent activity in the supernatant which was recovered from the control incubate. Percent solubility ranged from 47% to 60%, however, these values did not show a correlation with the increasing amounts of amylase added.

Amylase was next tested over a wider range of concentrations. In order to be sure that all particulate glycogen was being pelleted and that true solubilization was being measured, it was necessary to centrifuge the amylase treated samples at 105,000 x g for 1 hour. Incubation of glycogen

pellet with a wide range of amylase concentrations (1/5000x to 50,000x the ratio of 0.19 mg per kg muscle) was followed by centrifugation. Again, solubility is expressed as the percentage of activity in the control incubate recovered in the 105,000 x g experimental supernatants. Table III shows the results of these experiments which demonstrate that no more than 31% of glycogen synthase activity was being solubilized with the addition of amylase. This value was not significantly greater than the control value of 25%.

Since the glycogen synthase was not apparently solubilized by amylase alone, experiments were designed to solubilize glycogen synthase using both amylase and amyloglucosidase. Amyloglucosidase, which hydrolyzes glycogen at the 1 6 branch points, (4) was added to the glycogen pellet. This enzyme was added with and without amylase, using the same unit equivilants of amyloglucosidase (4.8 mg/kg muscle) as used for amylase. In experiments where the amyloglucosidase concentration was increased, no significant differences were found between experimental and control values. When a similar experiment was performed varying amyloglucosidase concentration with a fixed concentration of amylase (1.9 mg/kg muscle), the solubility of the experimental samples averaged only a moderate increase (34%) over the controls (29%). During the purification procedures the percent solubility of glycogen synthase from the Ascaris glycogen pellets varied from 16-40%. It was found that repeating the incubation and centrifugation of the pellet twice more yielded about 40% of the activity in the supernatant. The digestive enzymes were added to each incubation, although the data did not clearly demonstrate an effect on enzyme solubilization. The three supernatants were pooled and applied to a DE52 column. This procedure improved the overall yield of the preparation but was very time consuming and more efficient alternatives were desired and investigated.

TABLE III

SOLUBILIZATION OF *ASCARIS* MUSCLE GLYCOGEN SYNTHASE BY AMYLASE DIGESTION

Amylase mg/kg Muscle	Percent Solubilized
None	25
3.8×10^{-5}	29
3.8×10^{-4}	24
1.9×10^{-3}	31
1.9×10^{-1}	29
9.2×10^{2}	26
9.2×10^{3}	6.6

In order to determine whether or not the amylase was able to digest native Ascaris glycogen, the glucose oxidase test was employed. This test employs glucose oxidase and peroxidase coupled with a chromogenic oxygen accepter colormetrically determine glucose concentration. The concentration of glucose is proportional to the intensity of the orange color produced from an originally colorless solution. Since glucose is one product of glycogen digestion, an increase in glucose concentrations would indicate that the amylase was effective. The glucose oxidase test was used with a standard glucose solution and color change was found to be linear with regard to glucose concentration and time. If BME were present in the sample to be tested, no color change was apparent and the glucose oxidase reaction was inhibited. When BME was added to a solution with glucose standard, no color change resulted and, therefore, subsequent experiments were performed in the absence of BME. Amylase digests on both pure oyster and pure Ascaris glycogen (see Materials and Methods) were tested for free glucose. Both treated glycogen samples exhibited an intense orange color. When the glucose oxidase test was performed on pure Ascaris glycogen not treated with amylase, the color was much less intense. When absorbance was read at $O.D._{450}$, a value of 0.340 was obtained for the pure Ascaris glycogen treated with amylase and a value of 0.190 was obtained for the untreated Ascaris glycogen (see Table IV). This meant there was greater concentration of glucose in those samples after digestion with amylase than controls, indicating that the amylase itself was effective. Resuspended Ascaris glycogen pellet was then incubated with amylase. When this sample was tested for an increase in free glucose, no significant difference in the absorbance value was found from that of pure untreated Ascaris glycogen (see Table IV). These results indicated that because amylase was able to digest pure Ascaris

TABLE IV

GLUCOSE OXIDASE TEST ON PURE AND NATIVE *ASCARIS* GLYCOGEN AMYLASE DIGESTS

One percent solutions of *Ascaris* glycogen were incubated with and without salivary amylase. Glycogen digestion by the glucose oxidase reaction where an increase in the OD_{450} correlated directly with an increase in free glucose concentration. BME was not present.

Glycogen (1%)	Amylase (mg/ml)	OD_{450}
Pure *Ascaris*	none	0.19
Pure *Ascaris*	0.01	0.34
Ascaris 6.1 Pellet	0.01	0.13

glycogen, but not native Ascaris glycogen in the 5.5 pellet, either a component present in the glycogen pellet was inhibiting amylase activity or the native glycogen differed in some way from the purified Ascaris glycogen.

In order to solubilize Ascaris glycogen synthase by other means, digestion of glycogen using endogenous glycogen phosphorylase was explored. One millimole AMP was added to resuspended glycogen pellet in order to allosterically activate phosphorylase and allowed to dialyze at 25^{o}C and 5^{o}C overnight. The samples were then centrifuged at 105,000 x g and assayed for release of glycogen synthase activity. Glycogen synthase in the sample held at 25^{o} had a solubility of 13.5% and that maintained at 5^{o} had a solubility of 26%. These values were lower than those obtained by using amylase and/or amyloglucosidase and therefore reliance on endogenous phosphorylase was not sufficient to induce complete solubilization of glycogen.

Separation of glycogen synthase from the glycogen pellet was next tested by using a Concanavalin A Sepharose 4B affinity column. Concanvalin A is a plant lectin which binds α-glucans such as glycogens, amylopectins and dextrans (45). The strategy employed was to bind the glycogen and protein complex to the Concanavalin A affinity column, and thereby remove all other soluble proteins. Possibly the factor which was interfering with amylase activity would also be removed and solubilization of glycogen synthase would therefore be greatly facilitated. The glycogen pellet from 15 g of Ascaris muscle was resuspended in 50 mM TRIS-HCl, pH 7.4, 1 mM DTE, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. This suspension was added to 25 ml (settletd volume) of the affinity resin and the mixture was allowed to sit for 1 hour at 5^{o}C. The mixture was then poured into a

column, the resin was washed with two column volumes of the above buffer and the glycogen was eluted with the same buffer plus 0.1 M glucose. Recovery of activity was 13%. When the active fractions were pooled and centrifuged at 105,000 x g, 87% of the activity was in the pelleted glycogen. This pellet was then resuspended, incubated with amylase and again centrifuged at 105,000 x g. This time 70% of the glycogen synthase activity was found in the supernatant. In subsequent preparative experiments using larger volumes of muscle and resin, enzyme activity was lost within several hours of column elution. Also, the amount of resin needed to bind all the available glycogen was economically unfeasible. For these two reasons, the use of the Concanavalin A affinity column was abandoned. An interesting observation made during these experiments was that the enzyme applied to the column was 77% in the I form whereas the enzyme eluted from the column was only 18% in the I form.

Modification of the original procedure to Ascaris muscle.--Modifications to the original rabbit muscle enzyme procedure were made in accordance with the differences found in the Crude Extract. First, buffer A was changed to buffer A': 20 mM TRIS-HCl, pH 7.0, 5 mM EDTA and 15 mM BME. This change precipitated the insoluble fraction of the homogenate into a firmer pellet so as to avoid contamination of the desired soluble fraction with insoluble particles. Next, the pH during the acid titration was lowered to 5.5 rather than 6.1. As discussed previously, a difference in the turbidity of the soluble muscle fraction was not discernable at this step because the initial turbidity was so great. Again, this high turbidity was probably due to the large glycogen and protein content of the Ascaris muscle as compared to rabbit mammalian skeletal muscle. Finally, buffer C rather than buffer B was used to resuspend the glycogen pellet as it simplified the procedure and

activity was not affected.

Figure 1 is a typical DE52 column profile. The column was loaded with 218 units of glycogen synthase activity which was eluted with a linear gradient of 0.04 to 0.4 M KCl. The active fractions were pooled and concentrated with ammonium sulfate at 45% saturation. The concentrated protein was dissolved in buffer D (1/40 the original volume) and applied to and eluted from a Sepharose 4B column equilibrated in buffer D (Figure 2). Twenty-seven units of activity were applied to the column and the active fractions were pooled and concentrated on 2 ml of DE52 equilibrated in buffer D. Table V gives the purification scheme for this procedure. Recovery was 0.15% and purification was 15-fold from the soluble muscle fraction. The specific activity was 1.16 units/mg.

Since a large portion of the activity was lost during this procedure, certain steps were modified to improve the amount of recovered activity. The pellet fraction from the 105,000 x g centrifugation consisted of two distinct parts. The glycogen pellet was clear in color and was packed very tightly against the centrifugation tube. The other pellet was grayish in color and was much softer in consistency. It was that discovered 60% of the activity remained in the 105,000 x g supernatant and 30% remained in the soft pellet when the acid precipitation step was omitted. Hereafter the precipitation step was omitted and the activity was recovered from the supernatant and the soft pellet rather than the glycogen pellet. These two portions were resuspended together and incubated 1 hour at $30^{o}C$ with 1 mM AMP, 5 mM K_2HPO_4, and 10 mM $MgCl_2$ to enhance endogenous phosphorylase activity. The amylase was omitted from the incubation. The incubate was applied directly to a DE52 column of increased size (40 ml to 200 ml),

Figure 1. Anion exchange chromotography of Ascaris muscle glycogen synthase. Ascaris glycogen synthase prepared as for rabbit glycogen synthase (see Methods) from 500 g muscle was applied to a column (40 x 5 cm) of DE52 cellulose equilibrated in buffer C. Glycogen synthase was eluted with a linear KCI gradient of 0.04-0.40 M. Fraction volumes were 3.8 ml. Assays were done in presence of 10 mM G 6-P.

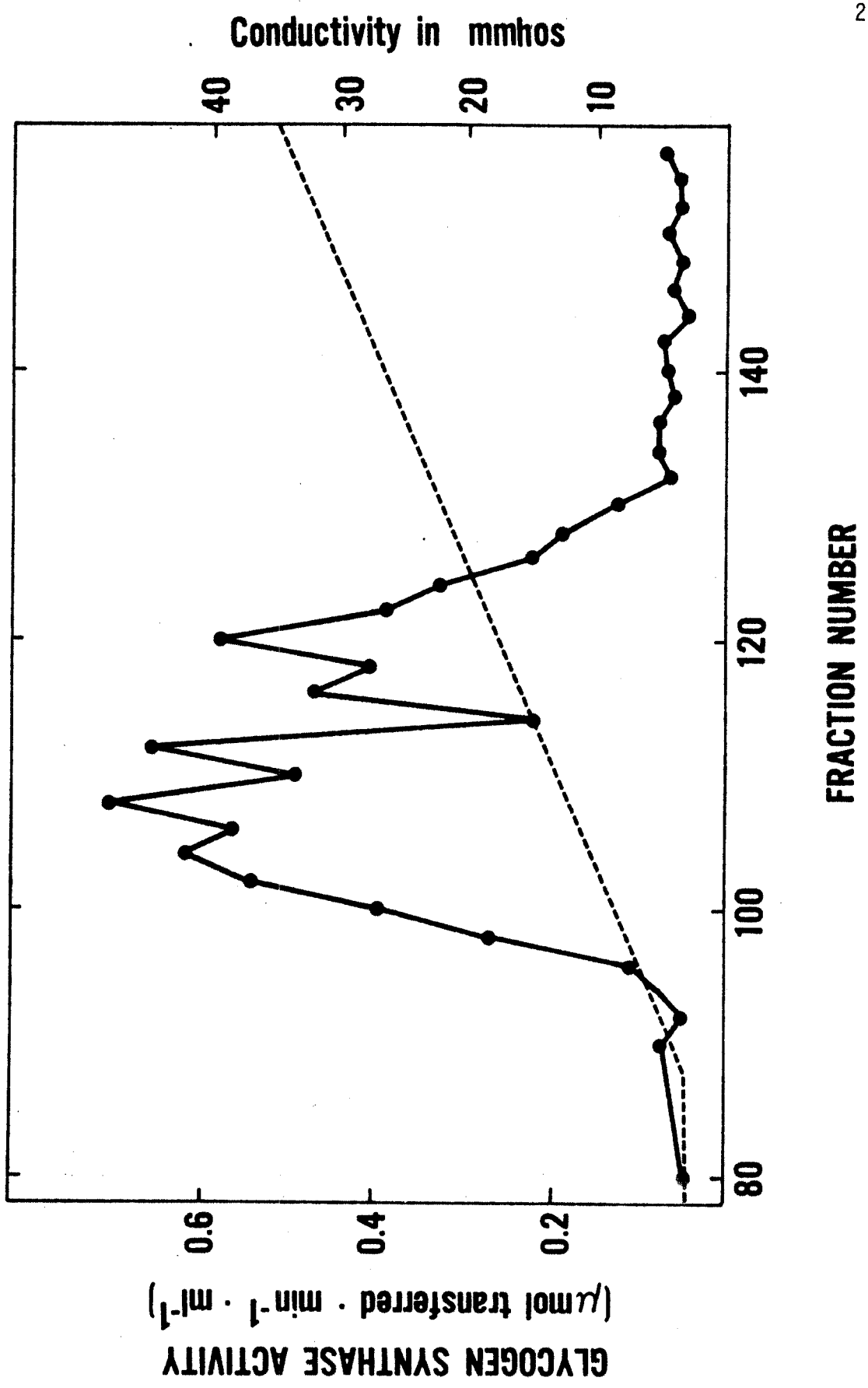

Conductivity in mmhos
40
30
20
10
0.6
0.4
0.2
80
100
120
140
FRACTION NUMBER
GLYCOGEN SYNTHASE ACTIVITY
(μmol transferred · min⁻¹ · ml⁻¹)

Figure 2. Gel filtration chromotography of Ascaris muscle glycogen synthase. Ascaris glycogen synthase concentrated from DE52 (see Methods) was applied to a column (100 x 2.5 cm) of Sepharose 4B equilibrated in buffer D. Fraction volumes were 3.8 ml. Assays were done in the presence of 10 mM G 6-P.

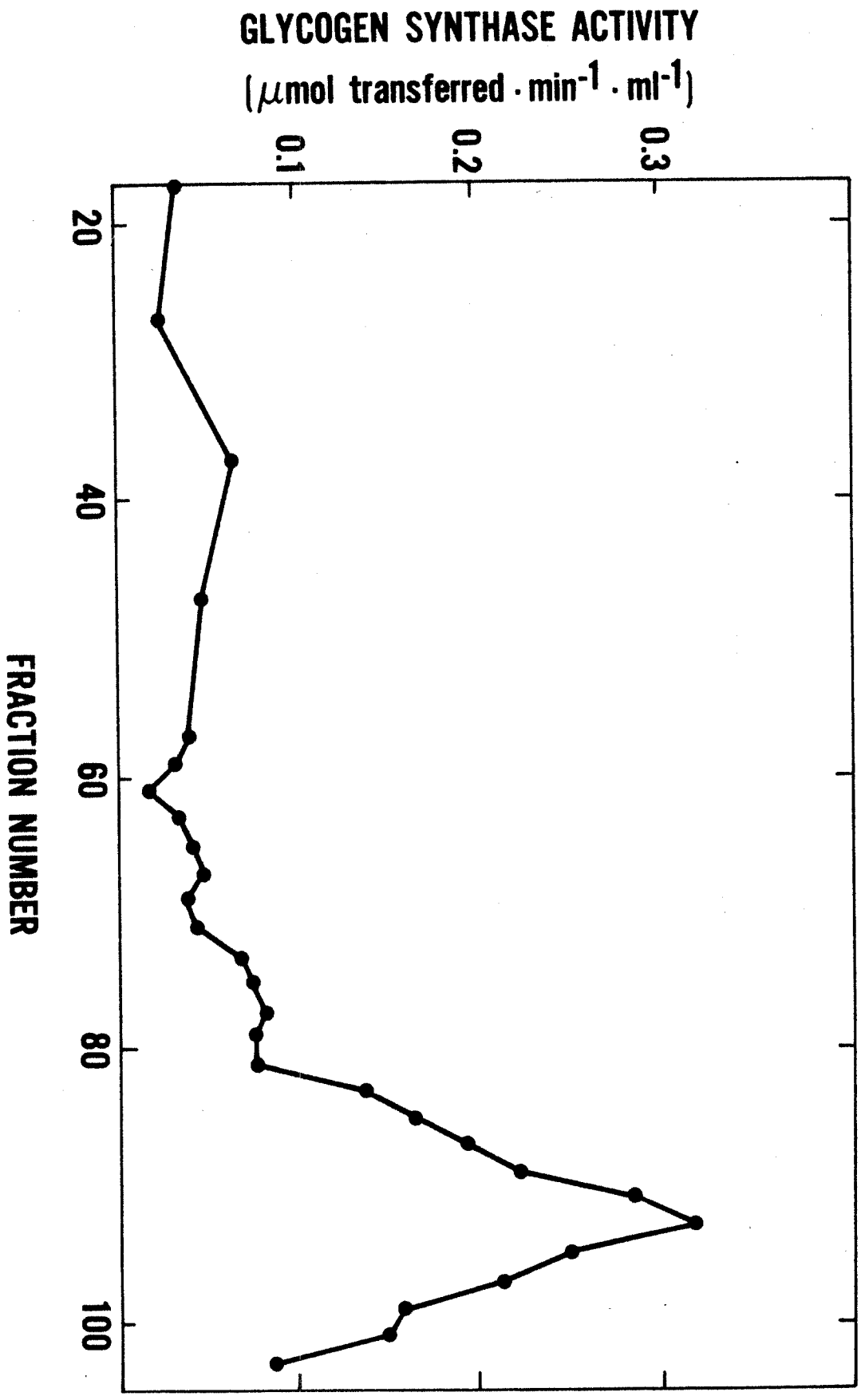

GLYCOGEN SYNTHASE ACTIVITY
(μmol transferred · min-1 · ml-1)
0.1
0.2
0.3
20
40
60
80
100
FRACTION NUMBER

TABLE V

PURIFICATION OF *ASCARIS SUUM* MUSCLE GLYCOGEN SYNTHASE

Fraction	Volume	Protein	Glycogen Synthase Activity		
			Total Units	Units/mg	Recovery
	ml	mg/ml	µmol/min		%
Crude Extract	2000	17.6	2764	0.078	100
Glycogen Pellet	252	22	689	0.125	25
Solubilized from Amylase	200	1.6	218	0.680	8.0
DE52 Eluant (after concentration)	5	8.0	26.7	2.66	0.3
Sephorase 4B Eluant (after concentration)	2.3	1.5	4.0	1.16	0.15

washed with Buffer C and eluted with a 400 ml linear KCl gradient of 50 to 500 mM. Glycogen synthase activity eluted from the column at a conductivity from 13 to 23 mmhos. The active fractions were concentrated with 45% ammonium sulfate and the procedure continued as previously described for the rabbit enzyme.

Figure 3 shows the DE52 column profile for this modified procedure. One hundred-eleven units of activity were applied to the column. Fourteen percent of the activity was recovered in the eluted fractions and 24% was recovered in the wash buffer. Both pools of activity were applied separately to the Sepharose 4B column after concentration with ammonium sulfate. Figure 4 shows the column profiles for the elutions of both pools. Peak activity for the DE52 purified pool eluted in fraction 98, whereas peak activity for the pool that did not bind to DE52 eluted in fraction 74, suggesting that the latter activity was associated with a larger molecular weight particle.

A further modification to the purification procedure was the addition of a glucosamine 6-phosphate Sepharose affinity column as described by Jett et al.(12). After concentration and dialysis of the gel filtration eluant the glycogen synthase was slowly loaded on a 1 ml column of glucosamine 6-phosphate affinity resin equilibrated with 25 mM glycerophosphate, pH 6.8, 2 mM EDTA, 40 mM BME and 10% sucrose. The column temperature was allowed to equilibrate to 20^{o} and the column was eluted with the same buffer plus 10 mM G 6-P also at 20^{o}. The active fractions were dialyzed to remove G 6-P and frozen at -30^{o}.

Table VI gives the purification scheme for this modified procedure. Recovery was 3.1%, specific activity was 4.3 units/mg and the purification

Figure 3. Anion exchange chromotography of *Ascaris* muscle glycogen synthase. *Ascaris* glycogen synthase prepared according to modified procedure (see Results) from 50 g muscle was applied to a column (60 x 2.0 cm) of DE52 cellulose equilibrated in buffer C'. Glycogen synthase was eluted with a linear KCI gradient of 0.05-0.50 M. Fraction volumes were 3.8 ml. Assays were done in the presence of G 6-P.

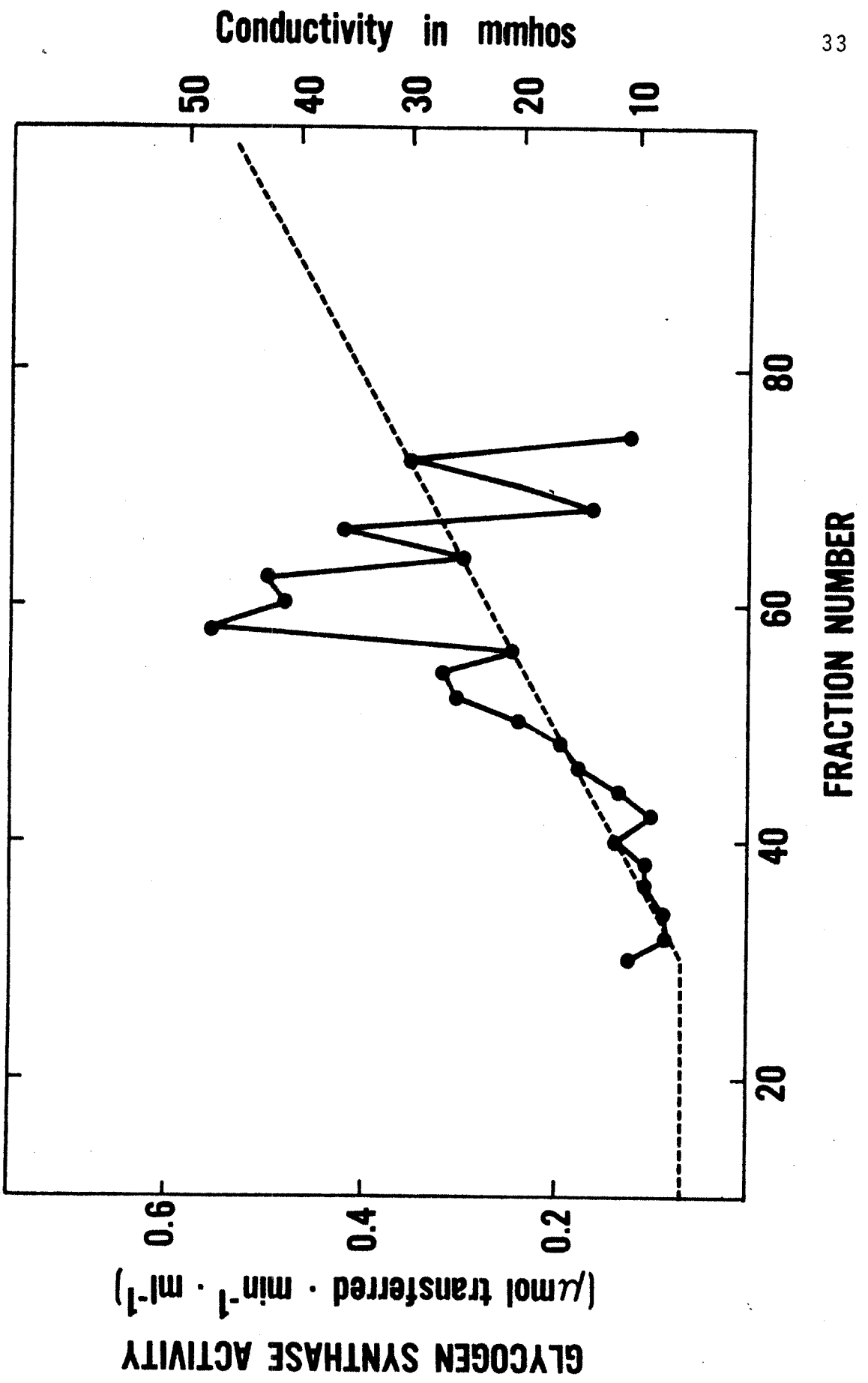

Conductivity in mmhos
50
40
30
20
10
80
60
40
20
FRACTION NUMBER
0.6
0.4
0.2
(μmol transferred · min⁻¹ · ml⁻¹)
GLYCOGEN SYNTHASE ACTIVITY

Figure 4. Gel filtration chromotography of Ascaris muscle glycogen synthase. Ascaris glycogen synthase eluted from (—▲—) or passed through (—●—) a DEAE cellulose column was concentrated and applied to a column (100 x 2.5 cm) of Sepharose 4B. Fraction volumes were 3.8 ml. Assays were done in the presence of 10 mM G 6-P.

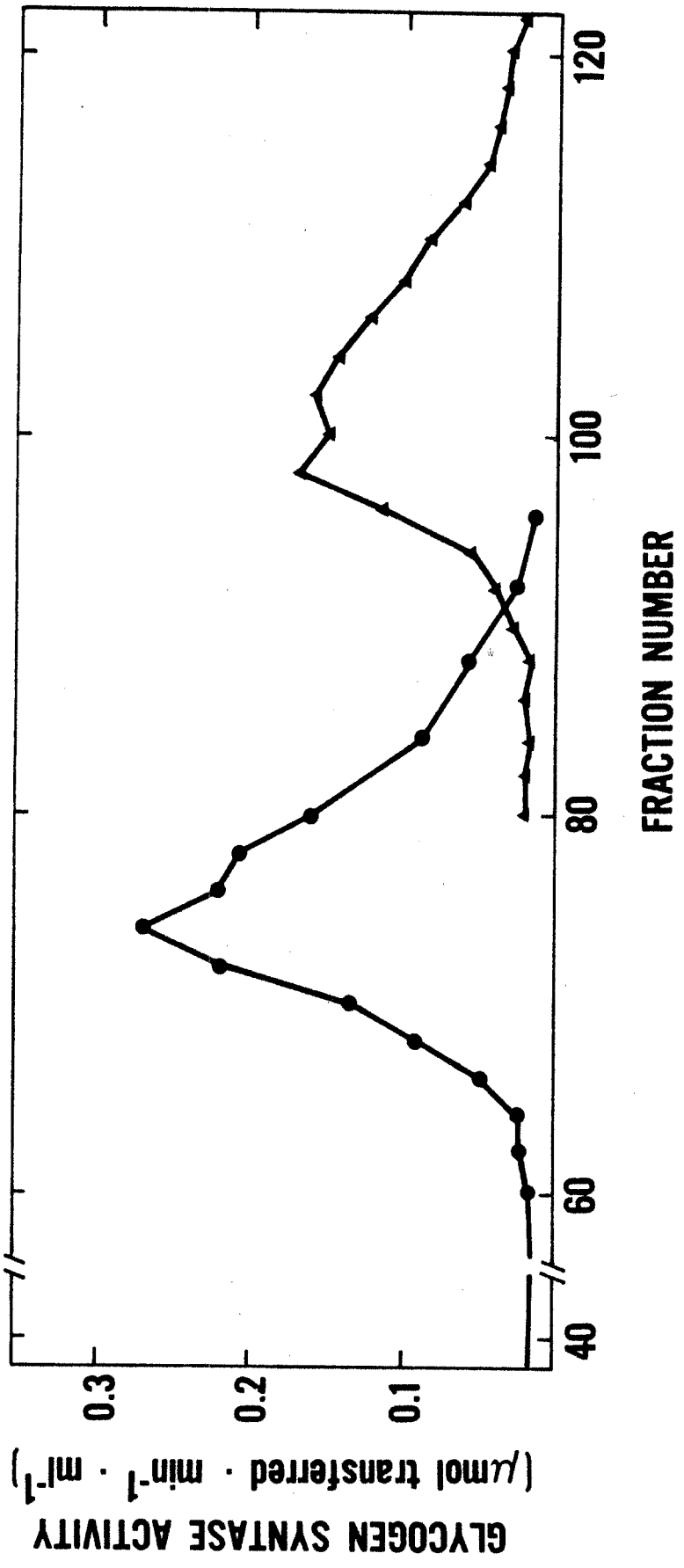

GLYCOGEN SYNTASE ACTIVITY
(μmol transferred · min⁻¹ · ml⁻¹)
0.3
0.2
0.1
40
60
80
100
120
FRACTION NUMBER

TABLE VI

MODIFIED PURIFICATION OF *ASCARIS SUUM* MUSCLE GLYCOGEN SYNTHASE

Fraction	Volume	Protein	Glycogen Synthase Activity		
			Total Units	Units/mg	Recovery
	ml	mg/ml	µmol/min		(%)
Crude Extract[a]	210	14.7	392	.127	100
Supernatant Fraction (105,00 xg)	260	13.3	359	.104	92
Solubilized Fraction	260	13.3	306	.088	78
DE-52 Eluant (after concentration)	7.1	16.9	84.1	0.7	21
Sepharose 4B Eluant (after concentration)	6.2	1.98	27.0	2.2	6.9
Glucosamine 6-phosphate	2.83	1.01	12.3	4.3	3.1

[a]Crude Extract was obtained from 50 g fresh muscle.

was 34-fold from the Crude Extract.

Conversion from D form to I form.--One goal of the project was to purify glycogen synthase 100% in the I form. This reaction occurs through dephosphorylation by endogenous glycogen synthase phosphatase. Jett and Soderling have shown that the presence of $MgCl_2$ increases the rate of this reaction (12). In an experiment to determine whether amylase also had an effect on this conversion reaction, rabbit and Ascaris glycogen pellets were resuspended in Buffer C plus 20 mM $MgCl_2$ and divided into aliquots. The samples were incubated for 1 hour at $30^{o}C$ plus and minus amylase. The results in Table VII show that all samples, regardless of the presence or absence of amylase, demonstrated glycogen synthase in nearly 100% I form after incubation. Glycogen synthase not incubated but containing amylase remained at 50% I form. It was concluded that incubation with $MgCl_2$ was more effective in converting the enyzme to the I form than the presence of amylase. Typically, during Ascaris glycogen synthase preparations the percent of glycogen synthase I in the Crude Extract ranged from 19 to 54%, but by the end of the procedure it had increased to 85- 100%.

Characterization of Ascaris Glycogen Synthase.--Ascaris glycogen synthase I recovered from the Sepharose 4B gel filtration column had a specific activity of 0.92 units/mg and was greater than 95% pure. This enzyme was used to perform a number of characterization experiments. Using oyster glycogen as substrate, a wide range of enzyme concentration was assayed (0.1 to 5.0 ul) as described in Methods in the presence of 10 mM G 6-P. The curve generated from this experiment is shown in Figure 5. Its sigmoidal shape suggest that a cooperative mechanism may be involved in the activation of the glycogen synthase.

TABLE VII

GLYCOGEN SYNTHASE D TO I CONVERSION
IN RABBIT AND *ASCARIS* pH 6.1
GLYCOGEN PELLETS

Glycogen pellets were prepared according to Methods and incubated for 60 min at 30° in the presence of 10 mM $MgCl_2$. Glycogen synthase assays were done ± 10 mM G 6-P. When amylase was present concentration was 0.19 mg/kg muscle.

Conditions	Percent GS I in Pellet	
	Rabbit	*Ascaris*
+Amylase +Incubation	100	82
-Amylase +Incubation	95	81
-Amylase -Incubation	50	53

Figure 5. Effect of *Ascaris* glycogen synthase concentration on activity. Glycogen synthase (0.1 - 3µg) was assayed in a final volume of 0.1 ml as described in Methods. Enzyme was diluted (1:10 v/v) with buffer C containing 0.25 mg/ml serum albumin and 0.5% *Ascaris* glycogen and aliquots (1-30 µl) were added to initiate the reaction. G 6-P was 10 mM.

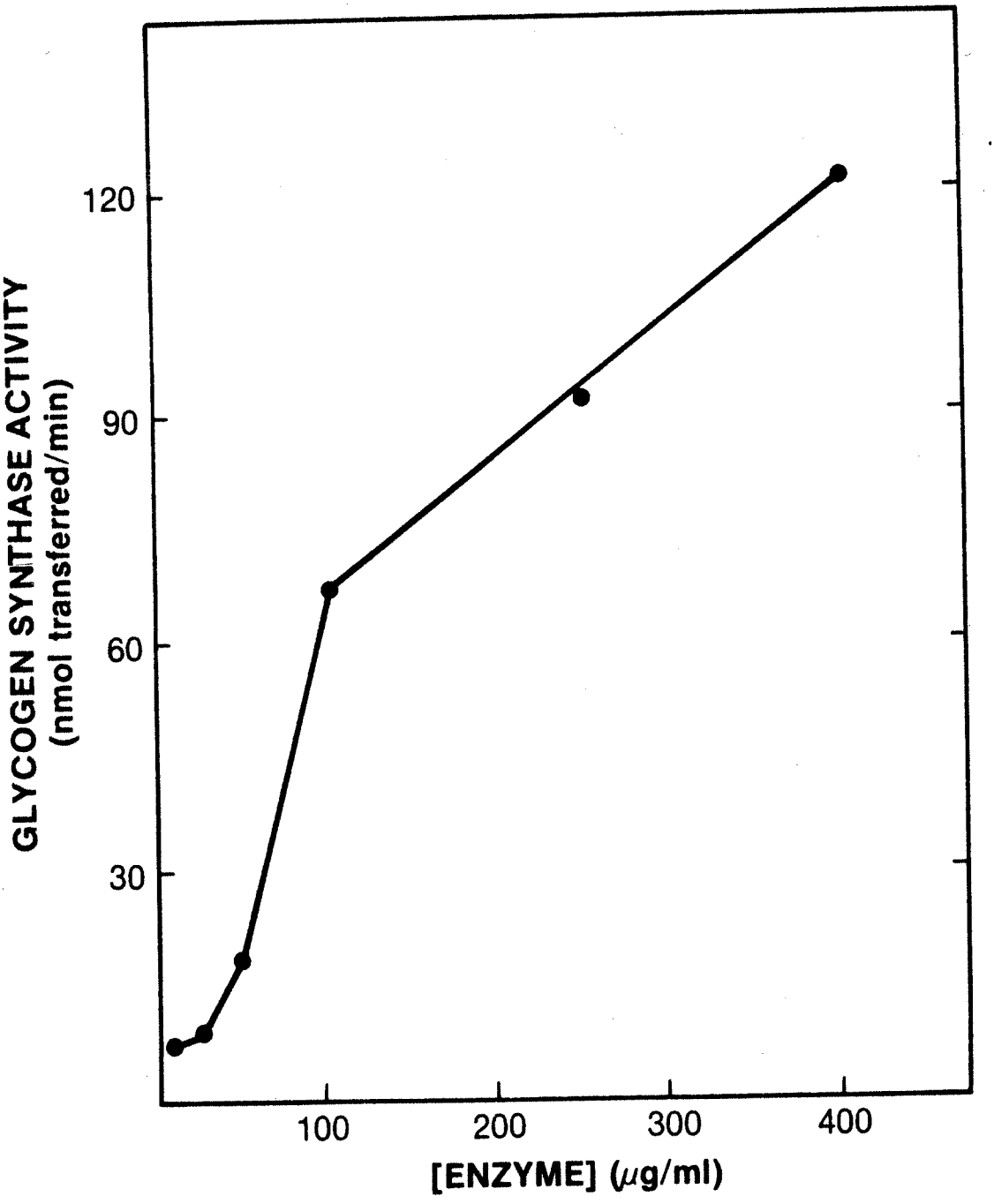

GLYCOGEN SYNTHASE ACTIVITY
(nmol transferred/min)
120
90
60
30
100
200
300
400
[ENZYME] (μg/ml)

Figure 6. Apparent K_a of Ascaris glycogen synthase for G 6-P. Glycogen synthase (3.0 µg) was assayed with G 6-P (0.01-0.1 mM) in a final volume of 0.1 as described in Methods. Correlation coefficient was 0.99.

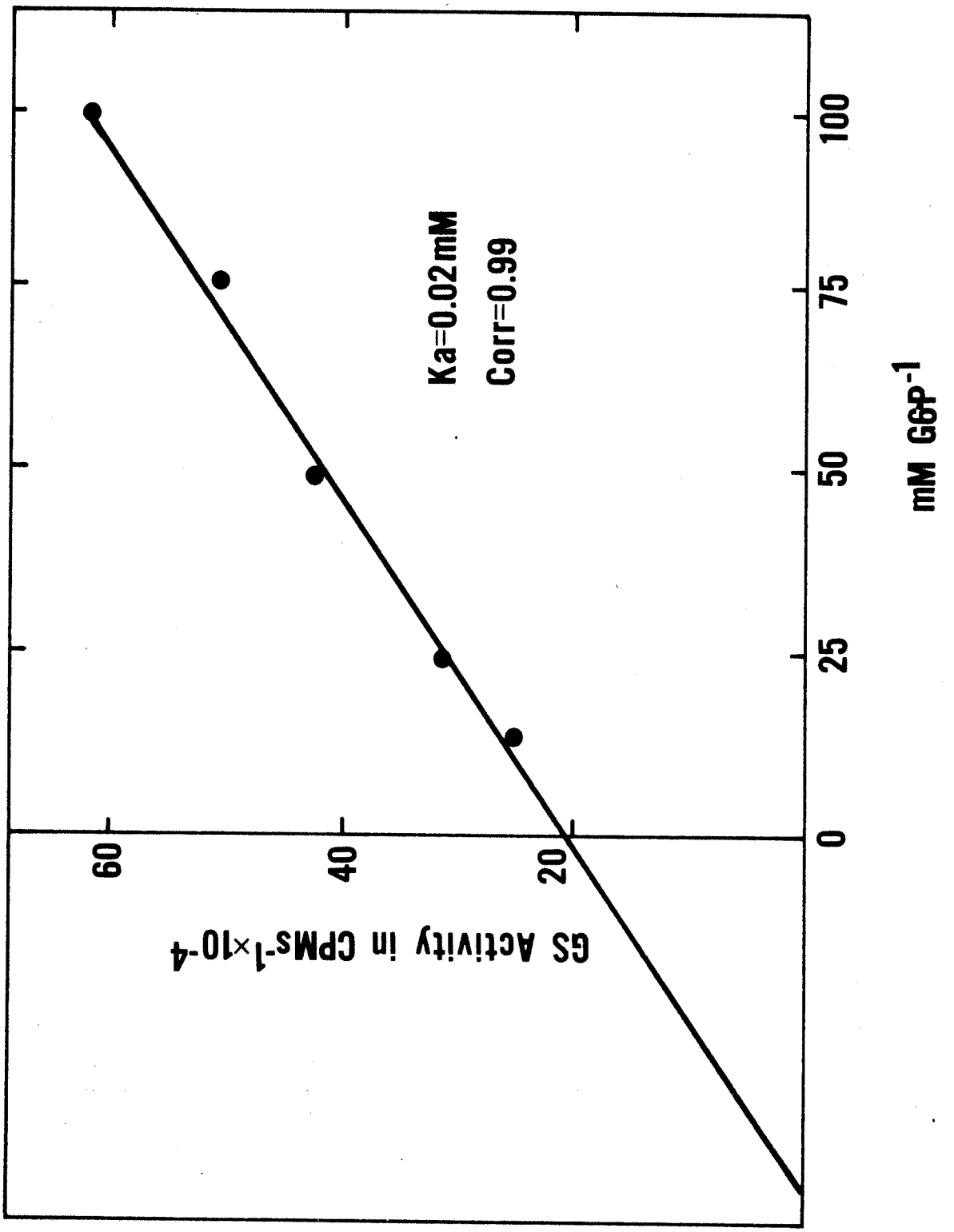

GS Activity in CPMs-1×10-4
60
40
20
0
25
50
75
100
mM G6P-1
Ka=0.02mM
Corr=0.99

Figure 6 shows a double reciprocal plot of Ascaris glycogen synthase activity versus concentration of its allosteric activator glucose 6-phosphate (G 6-P). The levels of G 6-P ranged from 0.01 to 0.1 mM and the apparent K_a was found to be 0.02 mM. This is in contrast to an apparent K_a of 0.5 mM for G 6-P in the Crude Extract at which point the enzyme was 70 to 80% in the D form.

Because glycogen synthase has two substrates, glycogen and UDPG, two sets of experiments were done to measure K_m values. In the first experiment, levels of UDPG ranged from 0.05 to 0.7mM and the apparent K_m was determined to be 0.6 mM, as shown by Figure 7. Next, an experiment was carried out in which Ascaris and rabbit glycogen synthase were assayed with both oyster and Ascaris glycogen. Figures 8-11 show the double reciprocal plots from these experiments. Glycogen concentrations are expressed in terms of percentage (g/100 ml) and ranged from 0.0025 to 0.025%. The apparent K_m for Ascaris enzyme and Ascaris glycogen was 0.0187%; for oyster glycogen it was 0.008%. The apparent K_m for rabbit enzyme and Ascaris glycogen was 0.006%; for oyster glycogen it was 0.004%.

Figure 12 shows the relation between Ascaris glycogen synthase assayed plus and minus 10 mM G 6-P and assay temperature which ranged from $4^{o}C$ to $41^{o}C$. The results show that total glycogen synthase (assayed plus G 6-P) increased with an increase in temperature. Glycogen synthase I activity (assayed minus G 6-P) paralleled that of the total glycogen synthase activity until maximum activity was reached at $30^{o}C$. With further increases in temperature, the I activity decreased and then began a slight rise after reaching $34^{o}C$. These results were comparable to those obtained by Solling and Esmann (11).

Figure 7. Apparent K_m of *Ascaris* glycogen synthase for UDPG. Glycogen synthase (3.0 µg) was assayed with UDPG (0.05-2.0 mM) in a final volume of 0.1 ml as described in Methods. Concentration of G 6-P was 10 mM. Correlation coefficient was 0.99.

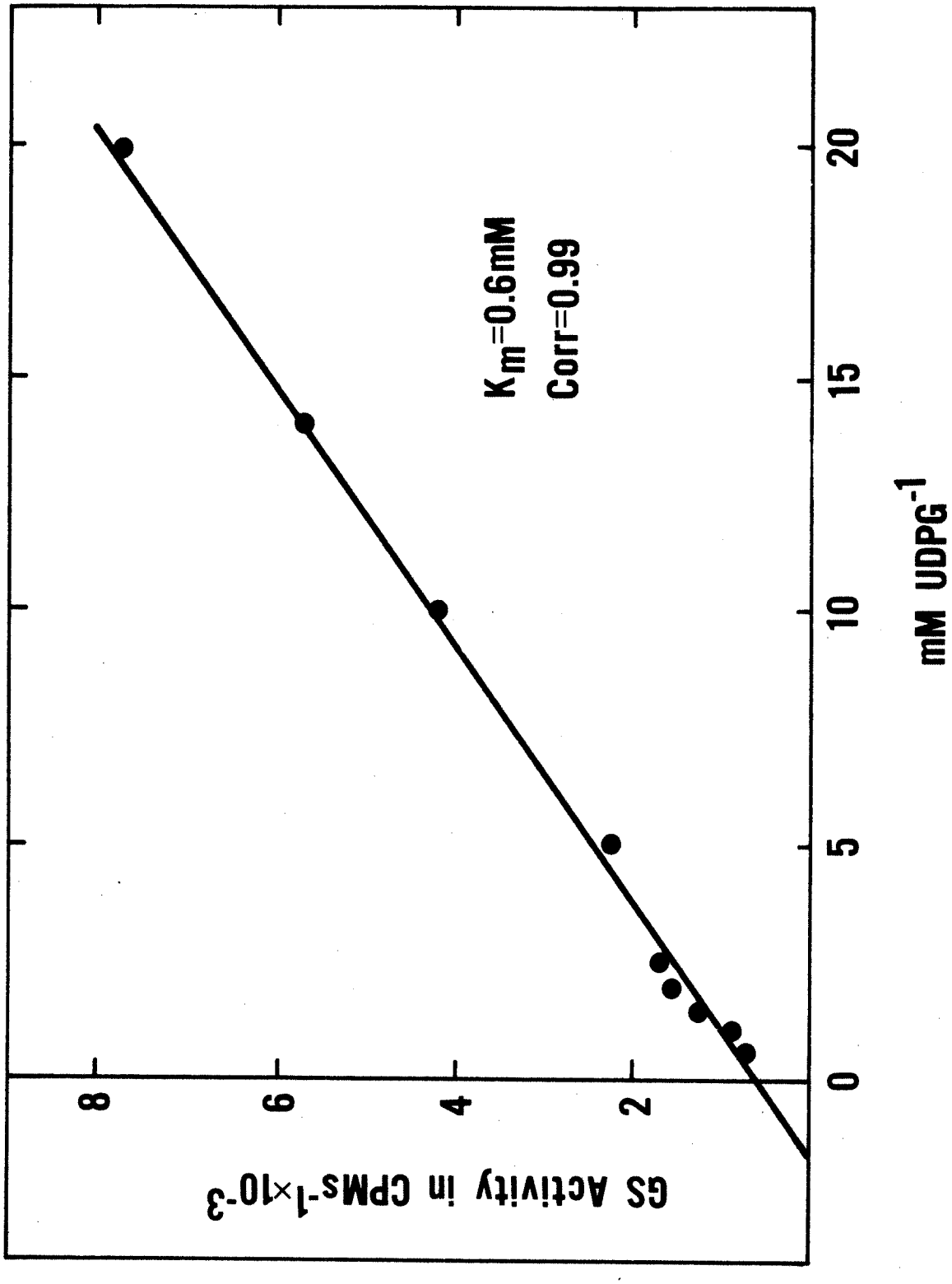

GS Activity in CPMs$^{-1}\times10^{-3}$
8
6
4
2
0
5
10
15
20
mM UDPG^{-1}
K_m=0.6mM
Corr=0.99

Figure 8. Apparent K_m of Ascaris glycogen synthase for Ascaris glycogen. Ascaris glycogen synthase (12.0 µg) was assayed with Ascaris glycogen (0.0025%-0.025%) in a final volume of 0.1 ml as described in Methods. Concentration of G 6-P was 10 mM. Correlation coefficient was 0.99.

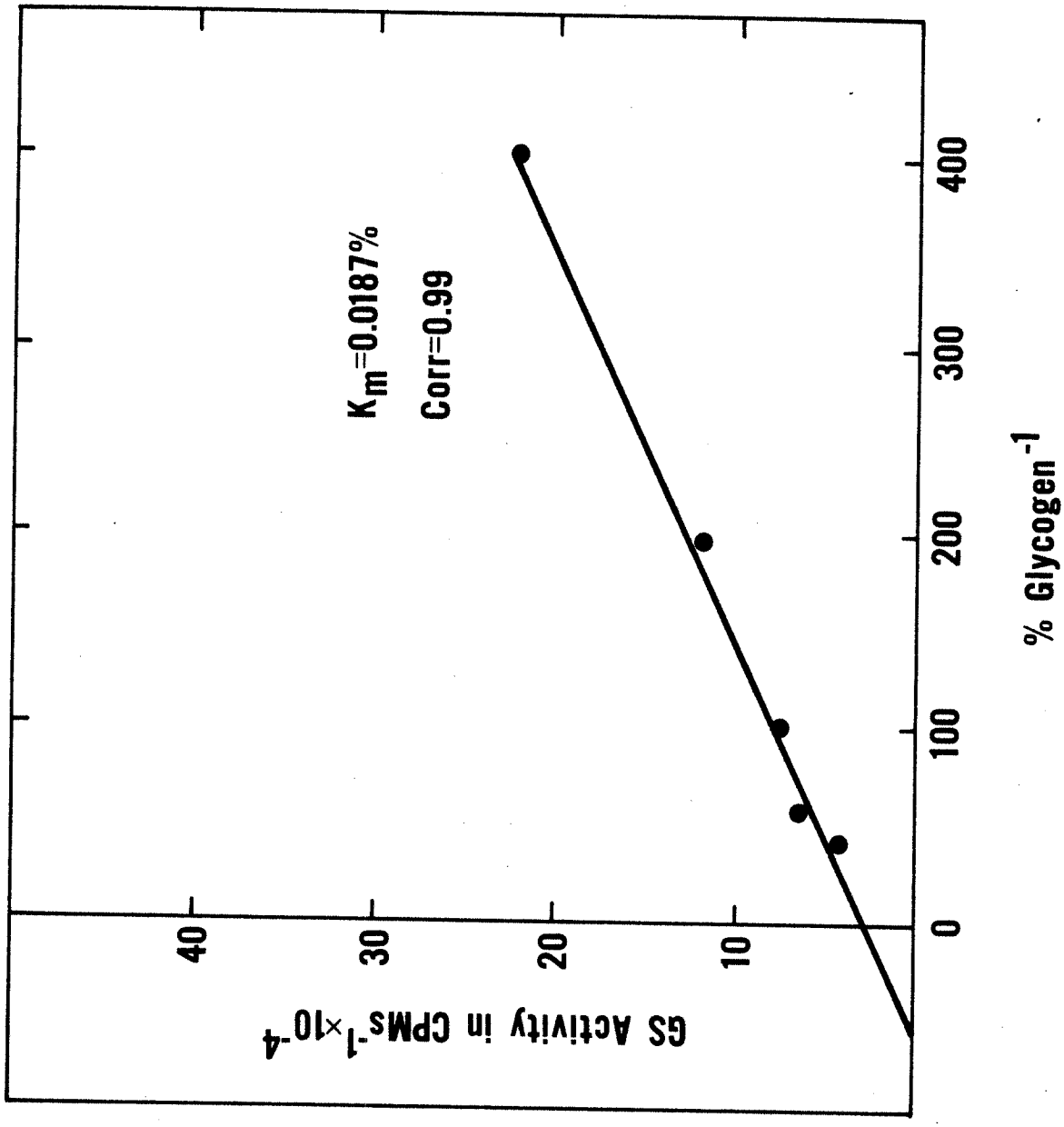

GS Activity in CPMs^{-1}×10^{-4}
40
30
20
10
0
100
200
300
400
% Glycogen^{-1}
K_m=0.0187%
Corr=0.99

Figure 9. Apparent K_m of *Ascaris* glycogen synthase for oyster glycogen. *Ascaris* glycogen synthase (12.0 µg) was assayed with oyster glycogen (0.0025%-0.025%) in a final volume of 0.1 ml as described in Methods. Concentration of G 6-P was 10 mM. Correlation coefficient was 0.99.

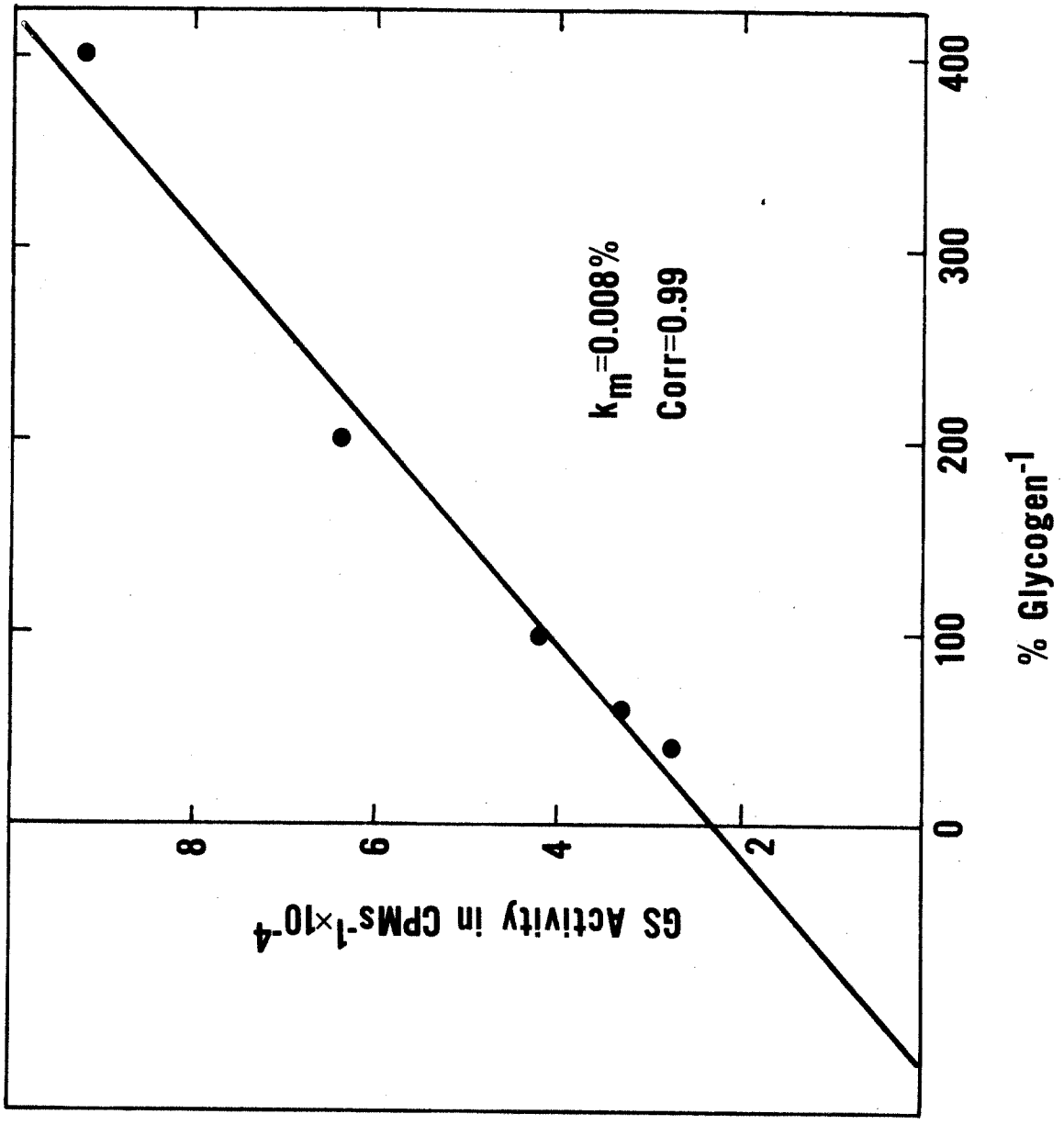

GS Activity in CPMs$^{-1}\times10^{-4}$
8
6
4
2
0
100
200
300
400
% Glycogen^{-1}
k_m=0.008%
Corr=0.99

Figure 10. Apparent K_m of rabbit muscle glycogen synthase for Ascaris glycogen. Rabbit muscle glycogen synthase (5.0 µg) was assayed with Ascaris glycogen (0.0025%-0.025%) in a final volume of 0.1 ml as described in Methods. Concentration of G 6-P was 10 mM. Correlation coefficient was 0.98.

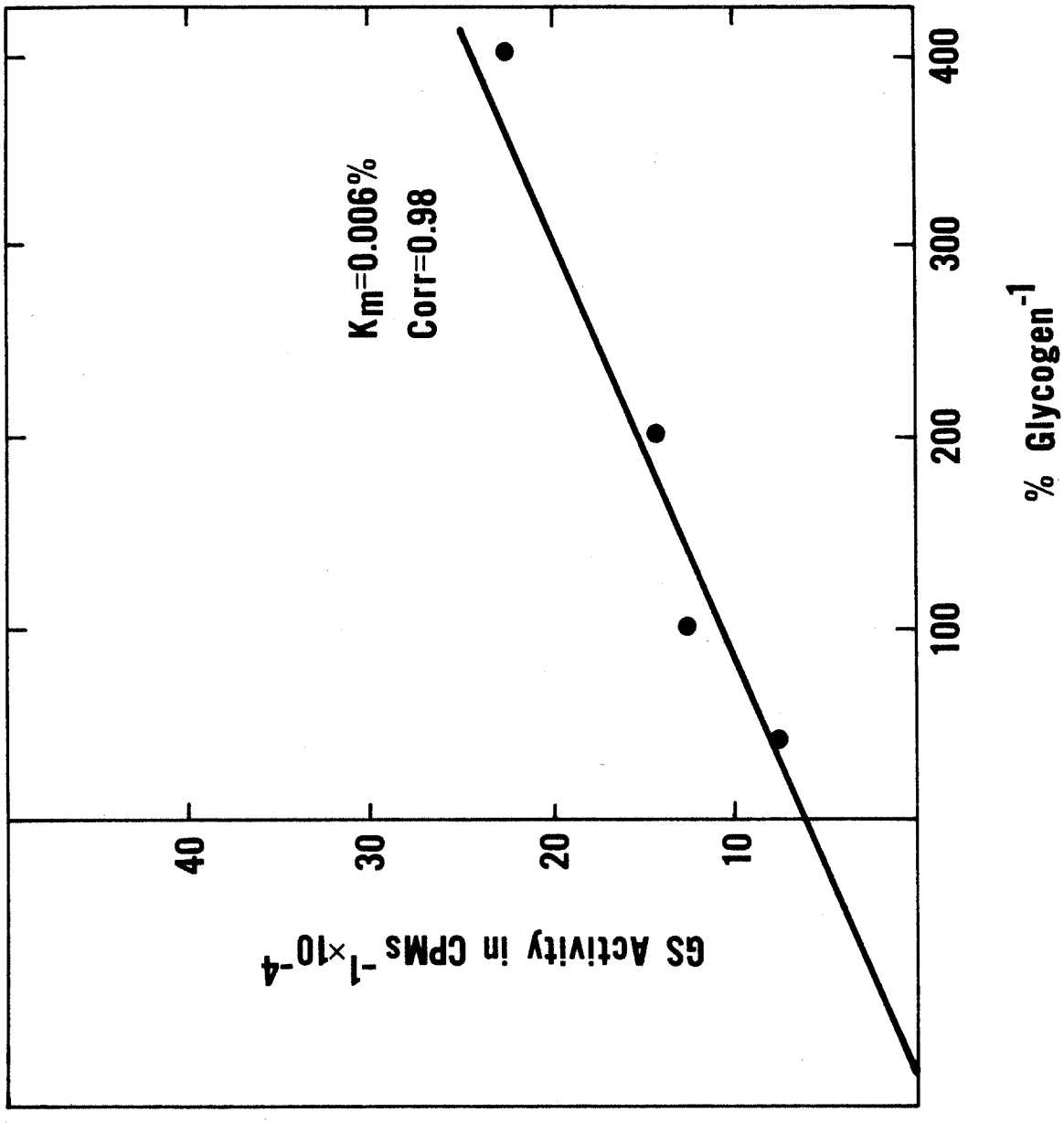

GS Activity in CPMs $^{-1}\times10^{-4}$
40
30
20
10
100
200
300
400
% Glycogen^{-1}
K_m=0.006%
Corr=0.98

Figure 11. Apparent K_m of rabbit muscle glycogen synthase for oyster glycogen. Rabbit muscle glycogen synthase (5.0 µg) was assayed with oyster glycogen (0.0025%-0.025%) in a final volume of 0.1 ml as described in Methods. Concentration of G 6-P was 10 mM. Correlation coefficient was 0.98.

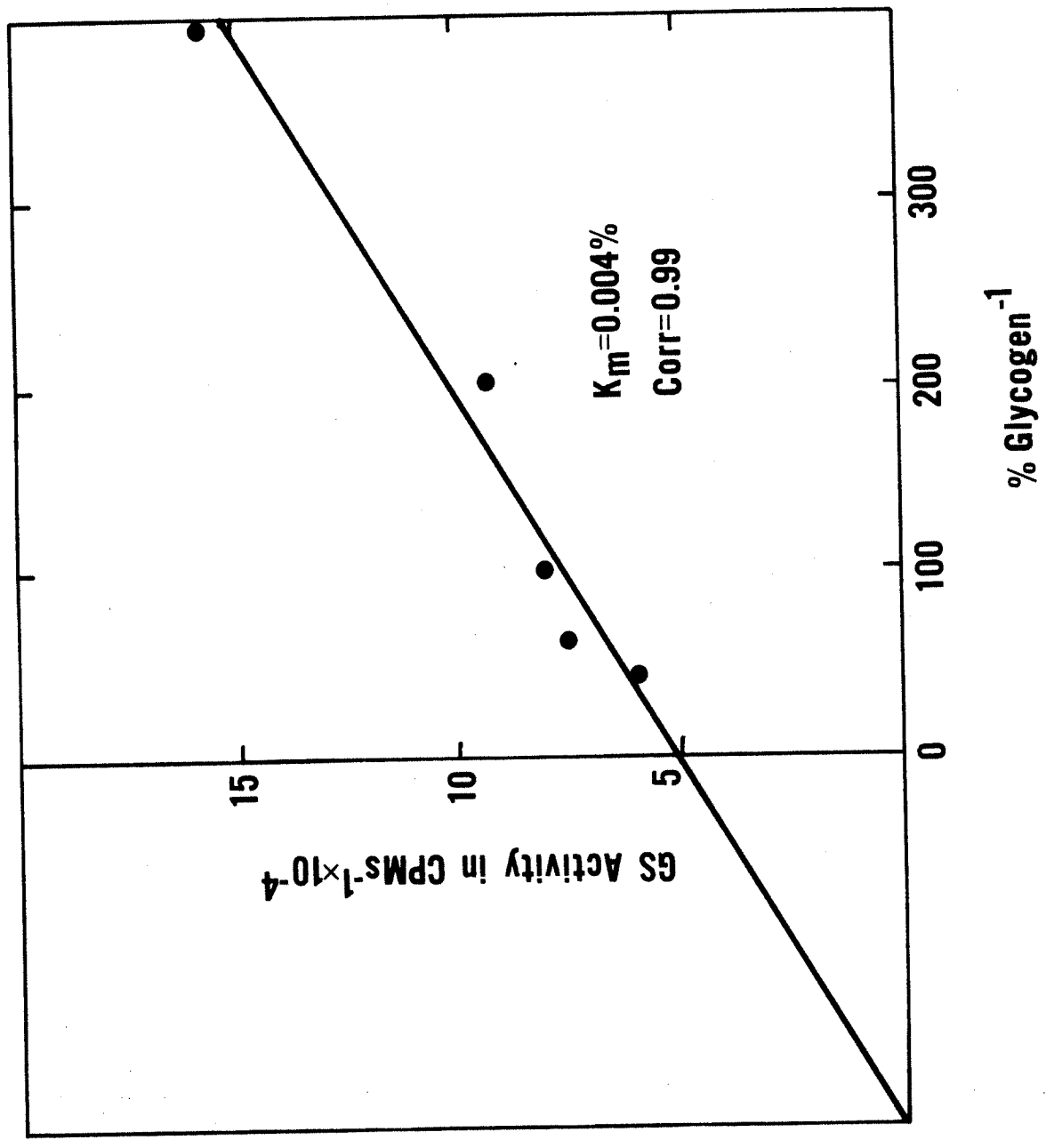

GS Activity in CPMs$^{-1}\times10^{-4}$
15
10
5
0
100
200
300
% Glycogen^{-1}
K_m=0.004%
Corr=0.99

Figure 12. Effect of temperature on Ascaris glycogen synthase activity. Glycogen synthase (4.0 µg) was assayed in the presence (—●—) or absence (—○—) of 10 mM G 6-P at temperature ranging from 5 - 39°. The initial velocity is nmol/min. mg.

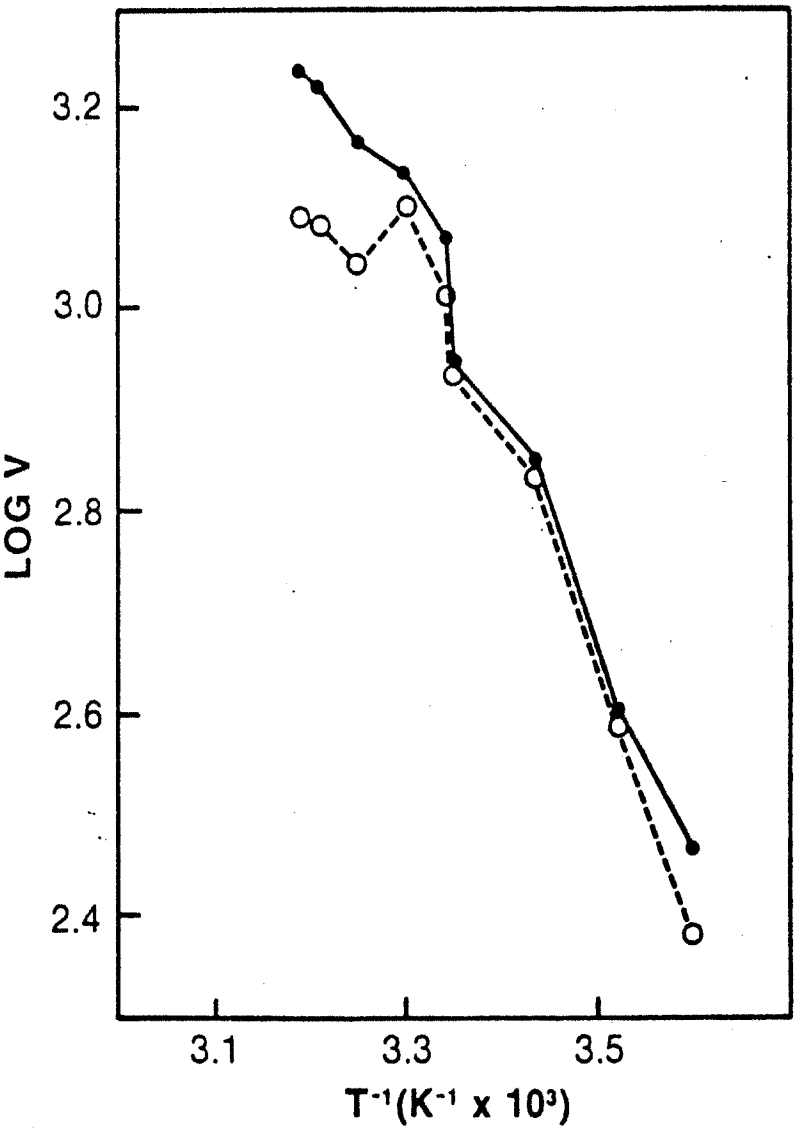
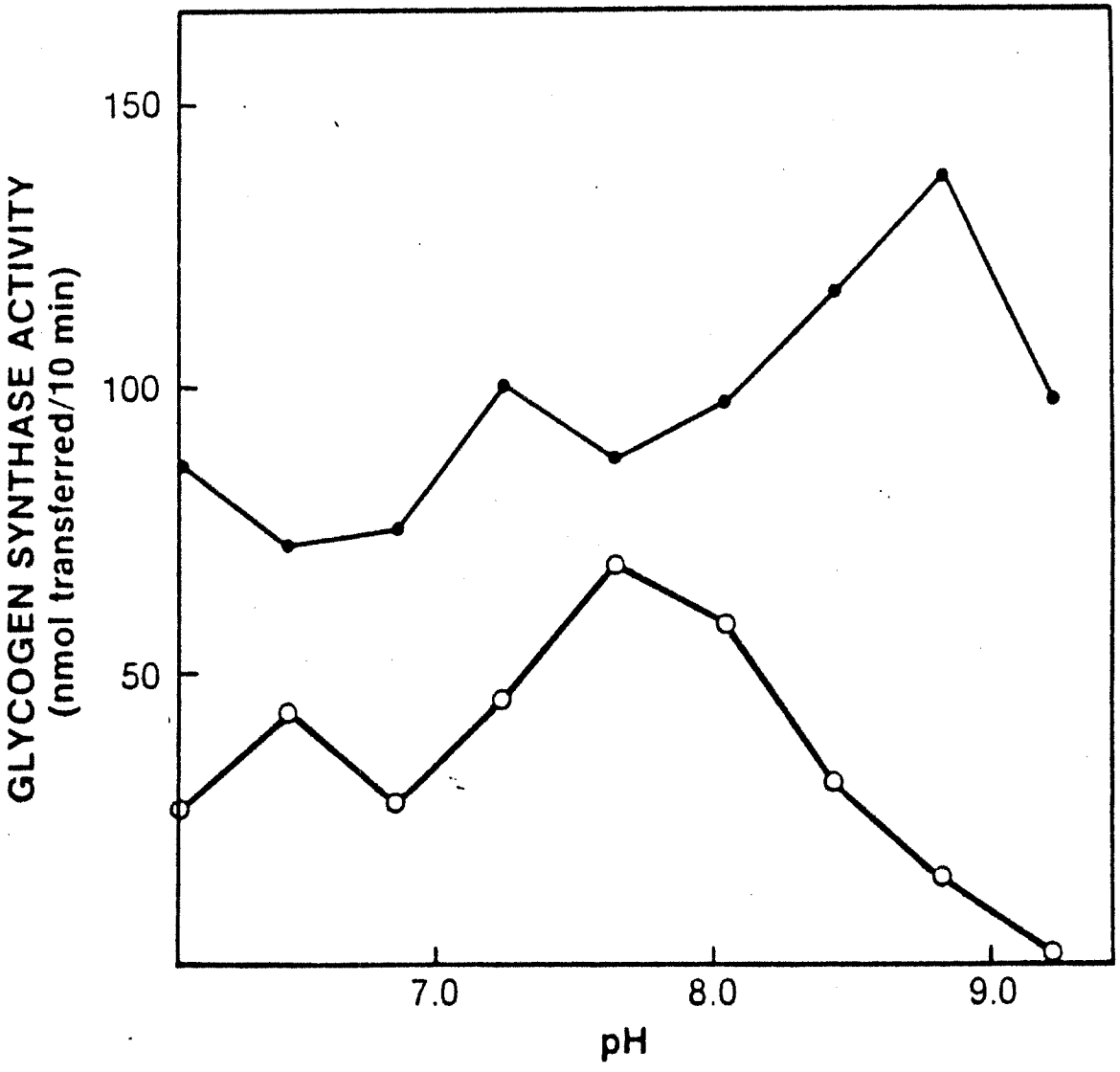

3.2
3.0
2.8
2.6
2.4
LOG V
3.1
3.3
3.5
T⁻¹(K⁻¹ x 10³)

Figure 13. Effect of pH on Ascaris glycogen synthase activity. Glycogen synthase (2 µg) was assayed in the presence (—●—) or absence (—o—) of 10 mM G 6-P. The buffer pH was maintained by 50 mM TRIS-HCl.


GLYCOGEN SYNTHASE ACTIVITY
(nmol transferred/10 min)
150
100
50
7.0
8.0
9.0
pH

In Figure 13 the relation between enzyme activity and assay pH are shown. The optimal pH for glycogen synthase I was 7.4. Total activity was highest at pH 8.8.

Brown and Larner (41) reported that purified rabbit muscle glycogen synthase in the absence of glycogen as a stabilizer was capable of aggregating into a less active form at a temperature of $3^{o}C$ and dissociating into an active form when the temperature was raised. To test Ascaris glycogen synthase for this property, the following experiments were performed. The purified enzyme was first pre-incubated for 30 minutes over a range of temperatures (4^{o} to $41^{o}C$) before assaying for 10 minutes plus and minus G 6-P at $30^{o}C$. The results of this experiment in Figure 14 showed that enzyme activity remained stable until 31^{o} where a drop in glycogen synthase activity occured followed by a slight increase. Reactivation of a cold aggregated complex was not indicated. In the second experiment the enzyme was diluted over a range of up to 15-fold and then assayed so that the enzyme concentration was constant in each assay. The results from this experiment in Figure 15 showed no increase in activity due to dilution of the enzyme, but, in fact, activity was decreased as the dilution increased. This experiment also did not give evidence for cold aggregation of Ascaris glycogen synthase.

Solling and Esmann (39) demonstrated hysteretic activation of glycogen synthase purified from human leukocytes by incubation with glycogen over time. Ascaris glycogen synthase was tested for this property by incubating the enzyme over a time period of 6 hours with two concentrations of Ascaris glycogen, 0.004 % and 2.5%, and including a control with no glycogen. Activity was measured every two hours and 2.5% glycogen was added to the

Figure 14. Effect of incubation temperature on Ascaris glycogen synthase activity. Glycogen synthase (2 µg) was incubated at temperatures ranging from 4-40° in buffer C' containing 0.25 mg/ml serum albumin and 0.5% Ascaris glycogen for 30 minutes. Enzyme was assayed with (—●—) and without (—▲—) 10 mM G 6-P at 30° and was initiated by 6.7 mM UDPG.

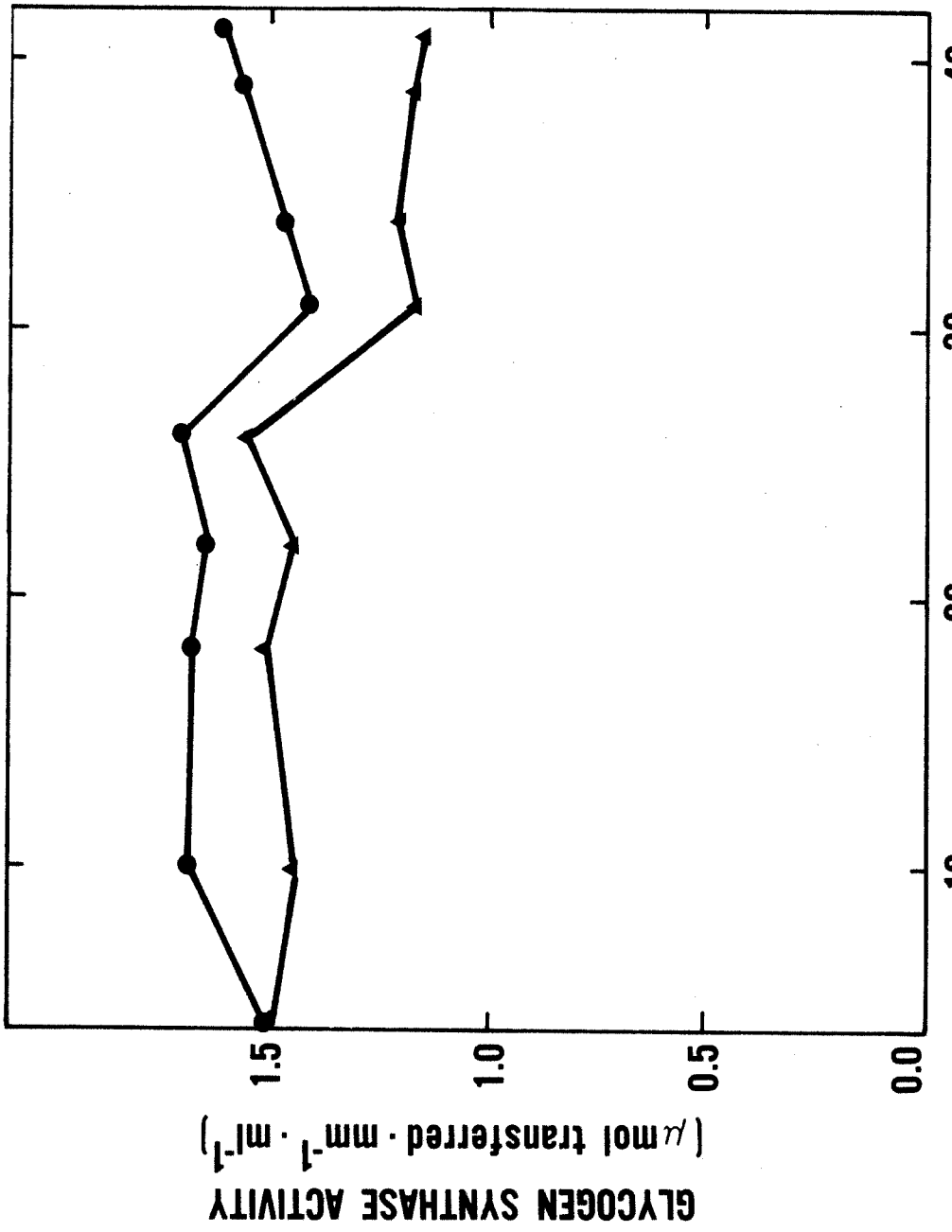

GLYCOGEN SYNTHASE ACTIVITY
(μmol transferred · mm⁻¹ · ml⁻¹)
0.0
0.5
1.0
1.5
10
20
30
40
Preincubation Temperature C°

Figure 15. Effect of dilution of Ascaris glycogen synthase activity. Glycogen synthase was diluted with buffer C' containing 2.5 mg/ml serum albumin and 0.5% glycogen by factors of 1, 2, 5, 10 and 15. Total concentration of enzyme (3 µg) remained constant in each assay. Concentration of G 6-P was 10 mM.

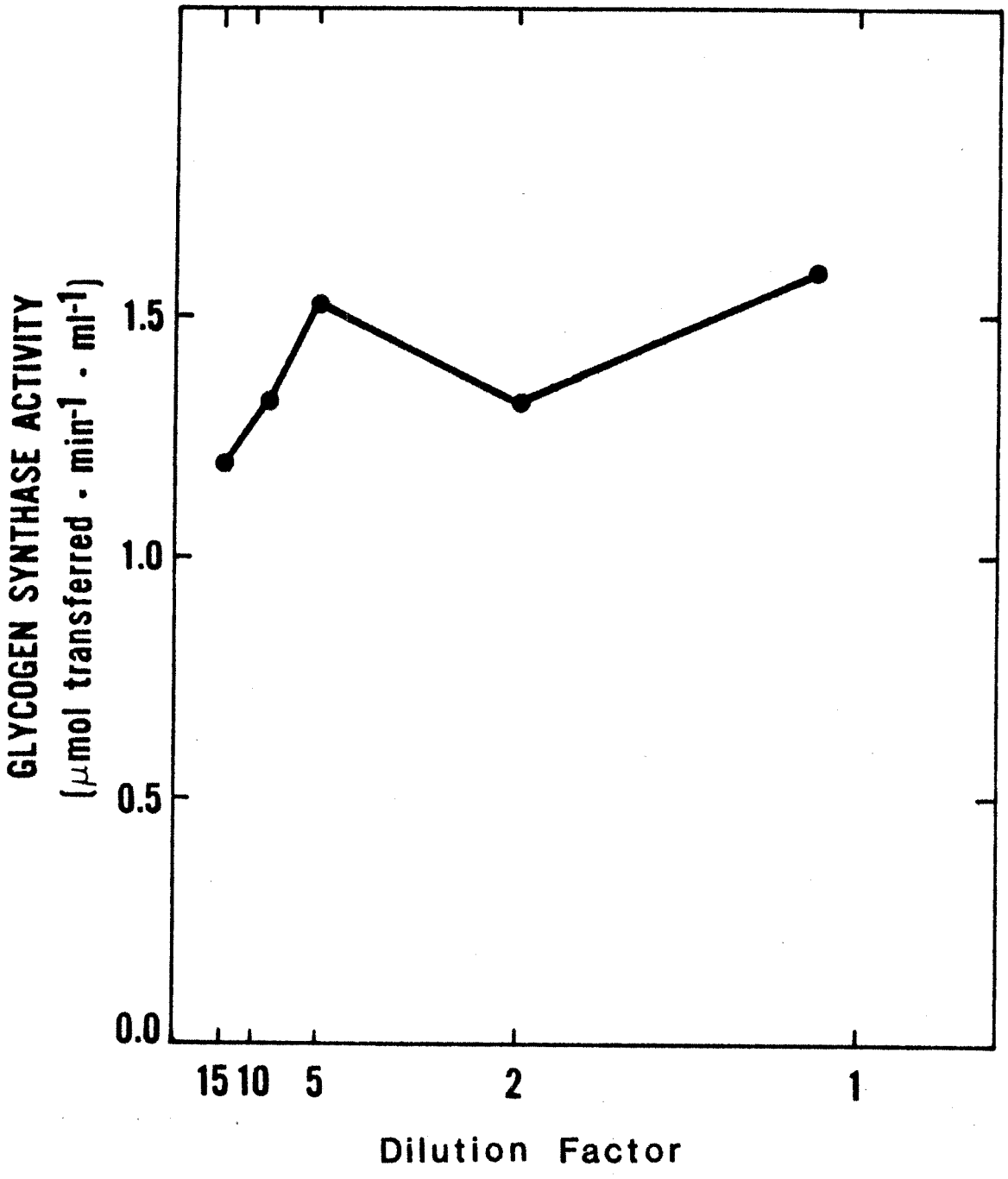
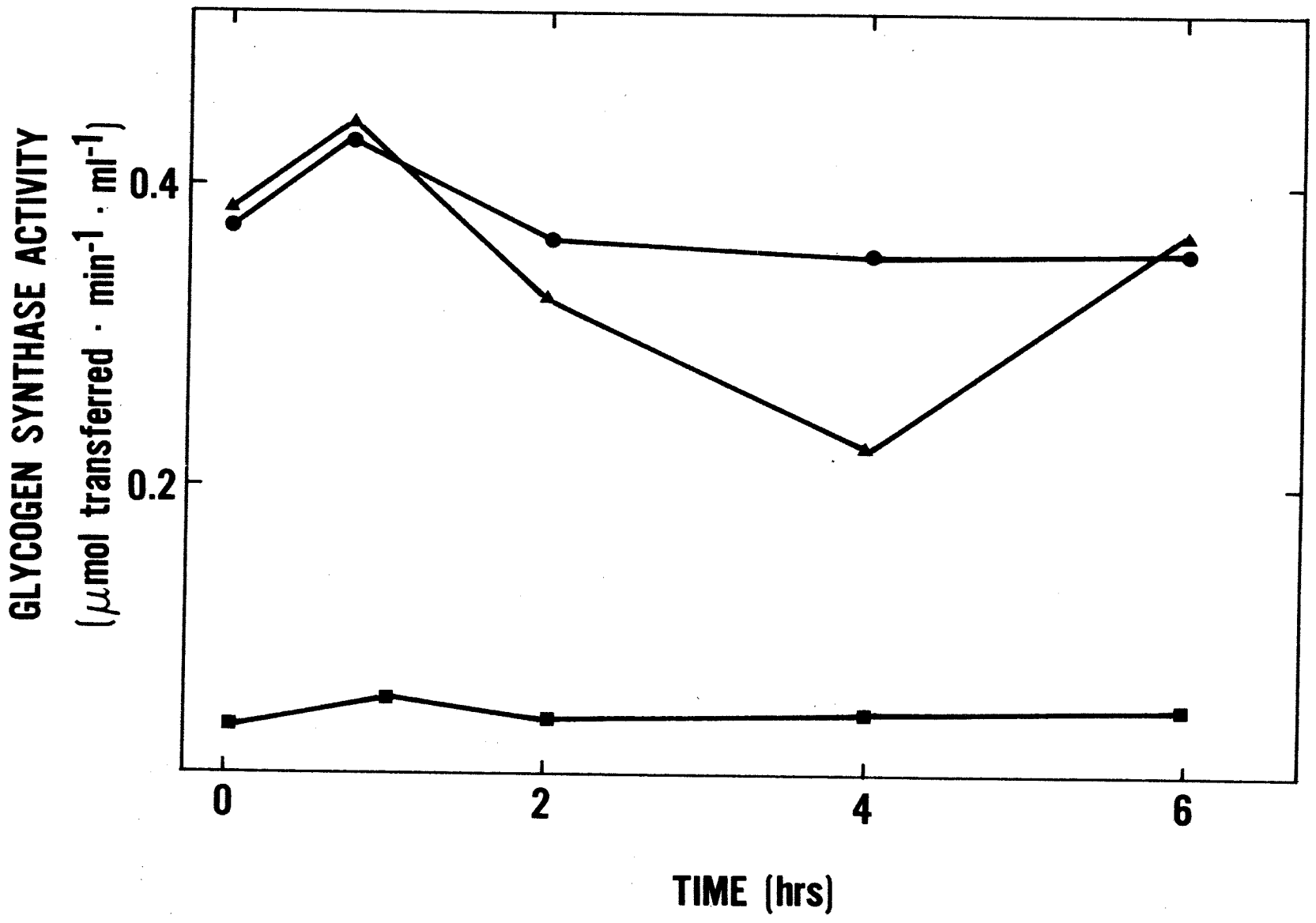

GLYCOGEN SYNTHASE ACTIVITY
(μmol transferred · min⁻¹ · ml⁻¹)
1.5
1.0
0.5
0.0
15 10 5 2 1
Dilution Factor

Figure 16. Effects of glycogen incubation on Ascaris glycogen synthase activity. Glycogen synthase was incubated at 30° for the indicated times with 2.5% (—▲—), 0.004% (—■—) and no glycogen (—●—) and assayed as described in Methods in the presence of 10 mM G 6-P. 2.5% glycogen was added to no glycogen (—●—) at time of assay.


GLYCOGEN SYNTHASE ACTIVITY
(μmol transferred · min-1 · ml-1)
0.4
0.2
0
2
4
6
TIME (hrs)

control immediately prior to the assay. The results in Figure 16 show that both samples containing 2.5% glycogen at the time of assay had similar increases in activity after 1 hour, but this activity then returned to the original level or below. The sample containing 0.004% glycogen showed constant but very low activity, possibly because the substrate was limiting. Evidence for a time dependent activation was not conclusive.

The next experiment involved preincubation of Ascaris glycogen synthase plus and minus G 6-P. Increasing concentrations of enzyme (1.0 - 30 ug/ml) were incubated in assay mixture for 30 minutes at 30°C. The enzyme was first diluted in the following buffer as described by Soderling (9), 50 mM TRIS, pH 7.5, 45 mM BME, 1 mM EDTA, 0.25 mg/ml bovine serum albumin and 0.5% glycogen. The reaction was initiated with the addition of UDPG and 10 mM G 6-P was added to half of the minus G 6-P samples at this time. The results in Figure 17 show that samples preincubated in the presence of G 6-P demonstrated more activity than samples preincubated in its absence, even if G 6-P was present during the assay reaction. These results clearly show evidence of a time dependent reaction.

The holoenzyme M_r was shown to very close to that for rabbit skeletal muscle glycogen synthase by gel filtration. Figure 18 shows coelution of the rabbit and Ascaris enzymes on a Sepharose 4B column. A subunit M_r of 88,200 was determined by gel electrophoresis in the presence of sodium dodecyl sulfate as shown in Figure 19. The gel also indicated that the purified Ascaris glycogen synthase was 95% homogeneous.

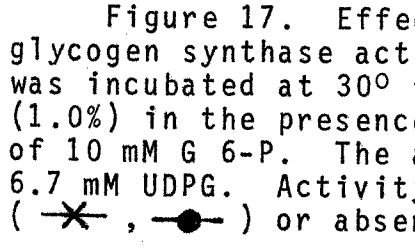


Figure 17. Effect of G 6-P incubation on Ascaris glycogen synthase activity. Glycogen synthase (0.1-3 µg) was incubated at 30° for 30 minutes with Ascaris glycogen (1.0%) in the presence (✕) or absence (●, ○) of 10 mM G 6-P. The assay was initiated by addition of 6.7 mM UDPG. Activity was measured in the presence (✕ , ●) or absence (○) of G 6-P.

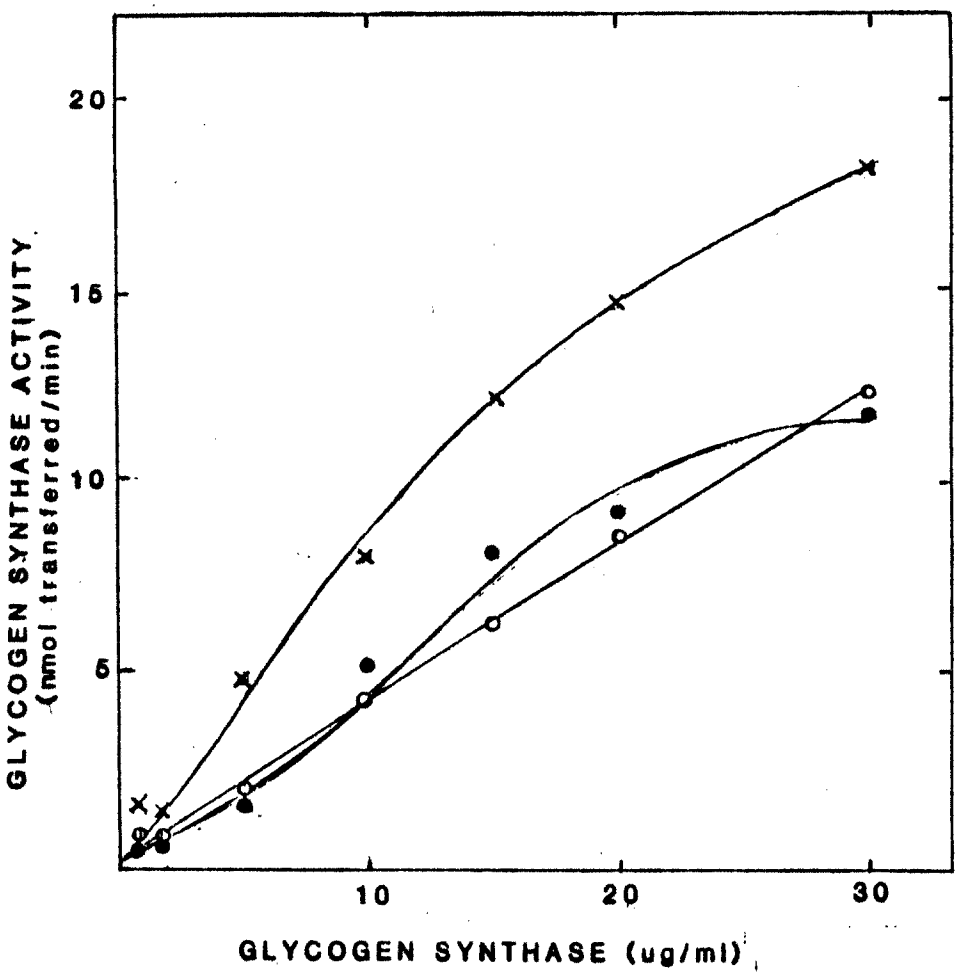

GLYCOGEN SYNTHASE ACTIVITY
(nmol transferred/min)
20
15
10
5
10
20
30
GLYCOGEN SYNTHASE (ug/ml)

Figure 18. Gel filtration chromotography of *Ascaris* muscle and rabbit muscle glycogen synthase. *Ascaris* glycogen synthase (—●—) prepared as described in Results from 50 g muscle or rabbit glycogen synthase (—○—) prepared according to Sodering *et al.* (1970) from 1.2 kg muscle was applied to a column (100 x 2.5 cm) of Sepharose 4B equlibrated in Buffer D. Fraction volumes were 3.8 ml. Assays are in the presence of 10 mM G 6-P.

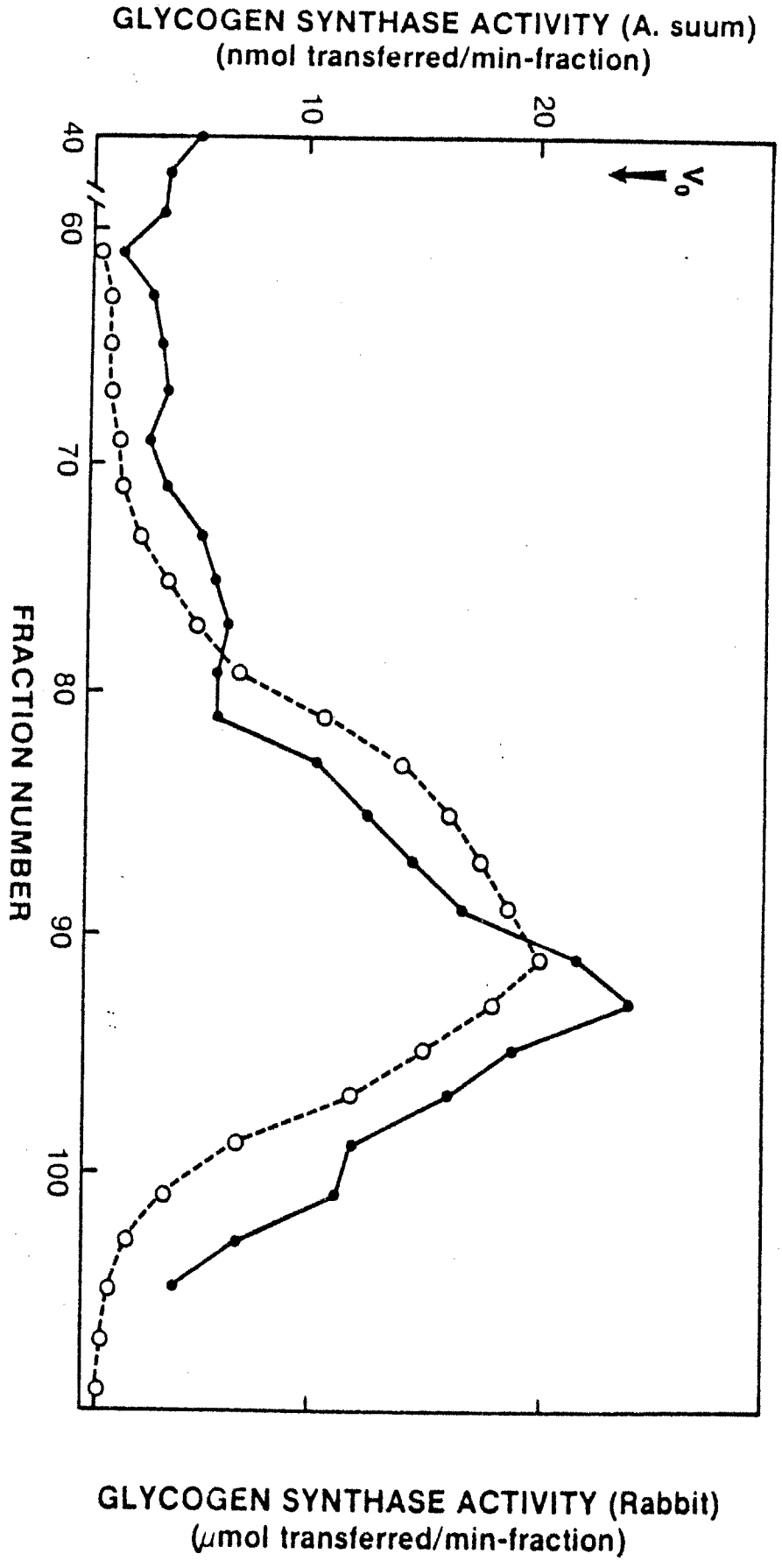
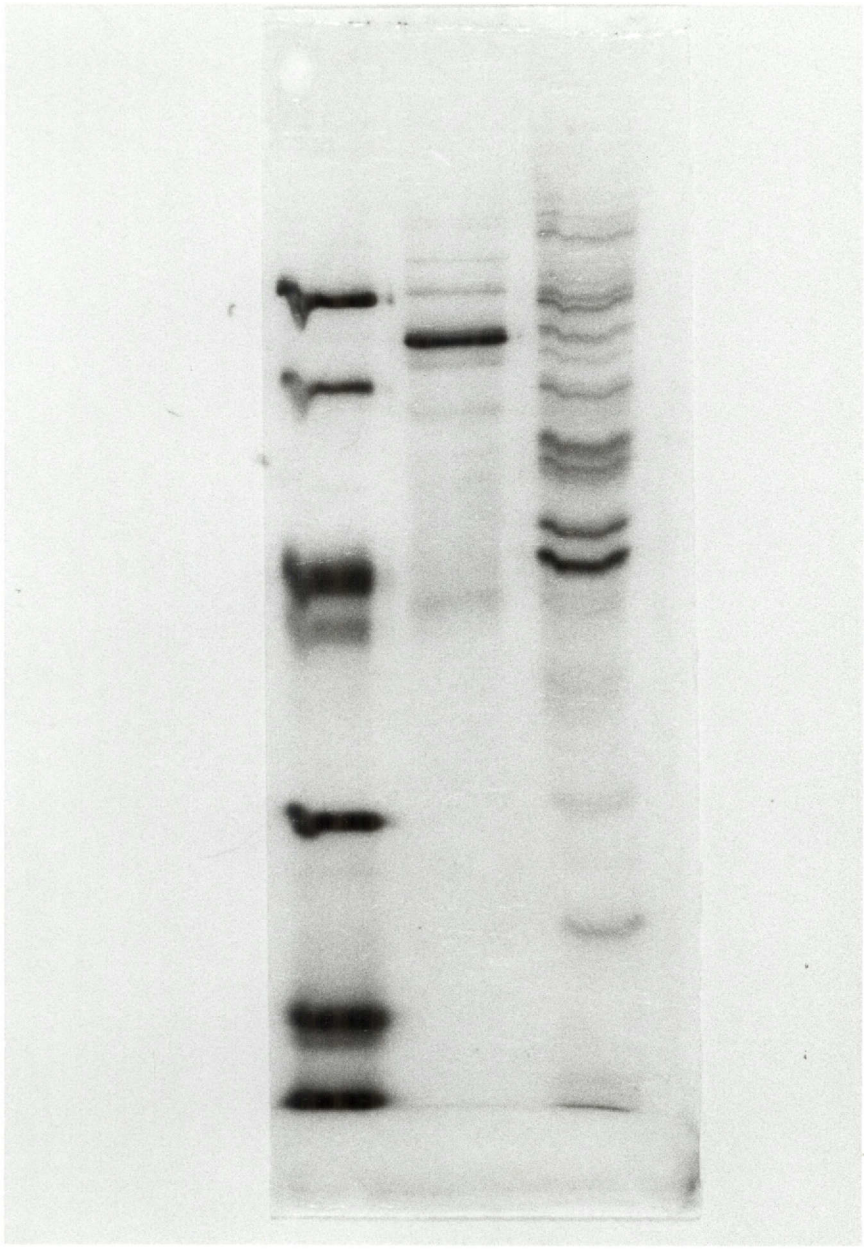

GLYCOGEN SYNTHASE ACTIVITY (A. suum)
(nmol transferred/min-fraction)
10
20
V_o
40
60
70
80
90
100
FRACTION NUMBER
GLYCOGEN SYNTHASE ACTIVITY (Rabbit)
(μmol transferred/min-fraction)

Figure 19. SDS Polyacrylamide Gel Electrophoresis of Purified Ascaris Glycogen Synthase. Glycogen synthase obtained after (B;10 μg) and before (C;150 μg) chromatography on glucosamine 6-phosphate Sepharose as shown in Table VI was analyzed by 12.5% polacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Standards (A;M_r) were phosphorylase (94,000), serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (22,000) and lysozyme (14,400).



A B C

CHAPTER IV

DISCUSSION

These studies describe the development of a purification scheme and the characterization of glycogen synthase from the muscle of Ascaris suum.

A comparison of crude extracts of Ascaris muscle and rabbit skeletal muscle reveals that the Ascaris tissue contains twice as much protein, 15 times more glycogen and 18 times more glycogen synthase activity. Therefore, comparable amounts of activity could be recovered from 50 g Ascaris muscle as could be recovered from 500 g of rabbit muscle. Comparison with mammalian liver extract shows, again, twice as much protein, but three times more glycogen and five times more glycogen synthase in Ascaris muscle. The greater amount of glycogen and glycogen synthase present in Ascaris tissue as compared in the mammalian tissues is indicative of the importance of glycogen as the sole source of stored fuel for Ascaris muscle. In contrast are the mammalian tissues which have the capacity to oxidize fatty acids, amino acids and other metabolites through aerobic respiration in addition to glycolysis.

Over 66 percent of the glycogen synthase activity in the rabbit muscle extract is retained in the 6.1 glycogen pellet, while less than 25 percent of the Ascaris glycogen synthase activity remains with the pellet. The Ascaris enzyme may be present in a more soluble state or much of the enzyme may be associated with smaller sized glucan particles as opposed to regular glycogen granules. These particles would not be large enough to sediment

with ultracentrifugation but could interfere with protein binding in ionic exchange chromotography as will be discussed later. The existence of smaller glucan particles with associated enzyme activity suggests the possibility of a primer glycoprotein molecule necessary for de novo glycogen synthesis hypothesized by Krisman and Barengo (37). Further investigation in this area is currently being done in this laboratory.

One of the most outstanding differences between the behavior of rabbit and Ascaris muscle fractions was the high resistance of the Ascaris glycogen to digestion as compared with rabbit muscle glycogen and glycogen from other animal sources. Digestion of glycogen solubilizes the glycogen synthase and it is only in the soluble state that the enzyme will bind to DE52 cellulose. This was found to be true in our laboratory. Soderling et al. (9) and Nimmo et al. (10) also report that glycogen synthase will not bind to DE52 unless the glycogen is first solubilized. While Soderling used amylase to promote glycogen digestion, Nimmo and workers employed phosphate and AMP to activate endogenous phosphorylase and debranching enzyme. Camici et al. (13) applied resuspended glycogen pellet from rabbit liver directly to DE52 without first removing the glycogen. The protein and glycogen complex were reported to have bound and to have been eluted with a NaCl gradient. This is one of the few papers in the literature which does not emphasize the need to solubilize the glycogen synthase prior to ionic exchange chromotography.

Soderling et al. reports 68 percent solubility from amylase digests of rabbit glycogen pellets (9). Nimmo et al. reports 60 percent solubility of glycogen synthase activity from phosphate digests of rabbit glycogen pellets (10). My results, in working with Ascaris glycogen pellets, showed solubilities

from a wide range of amylase concentrations not significantly higher than controls (25%). Addition of amyloglucosidase, either alone or in conjunction with amylase did not improve results. When it was further observed that amylase was successful in digesting purified Ascaris glycogen, it was concluded that either a factor in the 6.1 pellet caused inhibition of the amylase activity or that the structure of the purified glycogen was more accessible to the amylase than that of the native glycogen. A different approach was needed in order to obtain soluble glycogen synthase.

Several investigators have used Concanavalin-A Sepharose affinity chromotography to isolate the glycogen-protein complex with success (13,38,39). In working with human leukocyte glycogen synthase, Solling and Esmann achieved an 80 percent recovery of enzyme activity applied to a Concanavalin-A column (39). Camici et al. report a 40 percent recovery of glycogen synthase in the D form from rabbit liver (13). In this laboratory only 13 percent activity was recovered from Ascaris preparations. However, of the total activity recovered, only 18 percent was in the I form. The original activity applied to the column had been 77 percent in the I form, and this suggested that a conversion from I to D had taken place while the glycogen synthase was bound to the column or that a preferential elution had occurred. Elution of only the D form would also account for the low recovery of the column and perhaps the phosphorylation state of the enyme influences the binding affinity of glycogen to the Concanavalin A. Other workers using this technique did not report loss of glycogen synthase activity after elution from Concanavalin-A chromotography as did this laboratory.

The most successful approach in obtaining soluble Ascaris glycogen synthase was to recover the soluble enzyme activity present in the Crude Extract without first precipitating the glycogen with acetic acid. The high speed supernatant from this fraction was incubated with the addition of potassium phosphate and AMP for activation of endogenous phosphorylase to encourage further solubilization. This supernatant fraction yielded a greater percentage of glycogen synthase activity from the Crude Extract (61-74%) than had 6.1 glycogen pellets incubated with amylase (25%). Approximately 30% of the soluble activity was recovered from DE52 cellulose chromotography while 60% was recovered in the column flow through. Overall yield was improved from the previous method by 35 fold. The presence of soluble glycogen synthase in the Crude Extract indicates that glycogen-free as well as glycogen-containing enzyme may occur in vivo in Ascaris suum muscle.

The presence of small glucan particles associated with glycogen synthase activity was suggested by column chromotography profiles. As discussed earlier, a large portion of the glycogen synthase activity applied to DE52 cellulose was either recovered as flow through or as a separate pool eluted at a lower salt concentration. Proteins associated with the particles described would bind the resin either not at all or more weakly than the proteins alone. When these pools were applied to Sepharose 4B gel filtration, glycogen synthase activity eluted earlier than authentic enzyme, indicating the presence of a higher molecular weight complex. These pools of activity may represent products of incomplete glycogen digestion or the hypothesized glycogen synthase primer (37).

Once purified, the structural and kinetic characteristics of Ascaris glycogen synthase were comparable to mammalian glycogen synthase. Structural analysis of Ascaris glycogen synthase by gel electrophoresis in the presence of sodium dodecyl sulfate revealed a subunit M_r of 88,200 which is consistent with previous results obtained for mammalian enzymes (9-12). Ascaris glycogen synthase comigrated with authentic rabbit muscle glycogen synthase on Sepharose 4B gel filtration indicating a similar holoenzyme M_r of 360,000. This suggests that Ascaris glycogen synthase, like the mammalian enzyme, consists of a tetramer of four identical subunits.

Conversion of Ascaris glycogen synthase D to the I form was found to occur similarly to that of rabbit glycogen synthase both purified in this laboratory and as reported by others (9,12).

Kinetic constants of Ascaris glycogen synthase for G 6-P and UDPG compared favorably to values found in the literature. The apparent K_a of glycogen synthase I for G 6-P was 0.02 mM, while that for glycogen synthase D (in the Crude Extract) was 0.5 mM. These values are comparable to those obtained from mammalian glycogen synthase, ranging from 0.05 to 0.002 mM G 6-P for glycogen synthase I (21), and 0.2 to 0.5 mM for glycogen synthase D (9). The apparent K_m of Ascaris glycogen synthase for UDPG of 0.6 mM was identical to that obtained by Takeda et al. for rabbit skeletal muscle enzyme (41). This value was well within the range of 2.0 to 0.11 mM reported for mammalian skeletal muscle and liver glycogen synthase (13,21). The decrease in affinity of glycogen synthase D for G 6-P may be explained by conformational change caused by incorporation of phosphates which subsequentially interferes with allosteric binding. The simultaneous occurance of both levels of inhibition assures that glucose will not be shunted into

glycogen synthesis when the cell's immediate priority is the mobilization of fuel substrates.

The temperature profile for Ascaris glycogen synthase activity was nearly identical to that generated by Solling and Essmann (1977). At temperatrues higher than 30^{0} C, glycogen synthase D began to exhibit thermal inactivation while total activity continued to increase. The pH profile was similar, but not identical, to the results of these workers. Glycogen synthase D was more stable at pHs above 7.4 than the I form which suggests that the presence of phosphate acts as a stabilizer at high pH.

A negative cooperative effect on activity was seen by titrating the enzyme at low concentrations. Between concentrations of 10 and 100 ug/ml glycogen synthase activity increased sigmoidally, but once the concentration reached 10 ug/ml, the activity became linear. This behavior can be explained as aggregates of glycogen synthase molecules which form at a particular concentration inhibiting optimal activity. Aggregation of the mammalian enzyme is known to occur as a function of temperature. Solling and Essmann found that aggregation of human leukocyte glycogen synthase occurred at 3^{0} and that incubation at 30^{0} would induced an increase in activity (39). Similar experiments with Ascaris enzyme did not produce an activation effect. However, since a cooperative effect has been seen in the Ascaris enzyme through changes in concentration, the possibility of a cold effected aggregation has not been eliminated.

Dramatic activation by preincubation with glycogen as seen by Brown and Larner (40) was not evident with Ascaris glycogen synthase. However, the presence of glycogen in a preincubation mixture had the effect of straightening the sigmoidal curve generated previously without preincubation.

The loss of the negative cooperativity resulting from the preincubation may be explained by glycogen molecules which segregate the enzyme molecules sterically so as to effect optimal activity. In contrast to this was another type of activation seen after preincubating with the allosteric affector G 6-P. An increase in activity of approximately 50 percent was seen when the enzyme was first preincubated with 10 mM G 6-P.

The nature of the association between Ascaris glycogen synthase and the native glycogen may differ from mammalian systems. Because both enzymes appear highly homologous, a difference in the glycogen may contribute to the tighter binding seen with the Ascaris complex. Even though placement of branch points are reported to be comparable with other glycogens (42), Ascaris glycogen may differ structurally in terms of molecular size or packaging. The apparent K_s of Ascaris glycogen synthase for Ascaris glycogen was higher than that for oyster glycogen. This may reflect the greater physiological concentration of glycogen in Ascaris muscle.

Collectively, the data presented in this thesis demonstrate that glycogen synthase from Ascaris suum muscle is homologous to mammalian skeletal muscle glycogen synthase but that the enzyme-glycogen complex differs from that in mammalian systems. Such properties as allosteric activation, pH and temperature dependence, aggregation and substrate affinity do not differ substantially between the invertebrate and vertebrate enzyme. Other studies in this laboratory have shown that Ascaris glycogen synthase is regulated by multi-site phosphorylation as well (43). The classic tetrameric structure of mammalian skeletal muscle glycogen synthase of four identical subunits of M_r 88,000 each, was also seen in the Ascaris enzyme. This homology is not surprising considering, first, that Ascaris muscle is obliquely

striated and very skeletal-like, and second, that Ascaris is a parasite of mammalian systems. Characteristics which set the Ascaris system apart from mammalian systems include a much greater amount of muscular glycogen, a corresponding increase in the amount of glycogen synthase activity, the resistance of native Ascaris glycogen to digestion, and the presence of smaller glucan particles which remained associated with glycogen synthase activity.

To date, no other workers have purified and characterized glycogen synthase from an invertebrate. This research will provide a foundation for continued work on invertebrate glycogen metabolism and specifically for that of Ascaris suum. New knowledge in these areas is vitally important for the development of more efficient and effective chemotherapies and, therefore, better control of this and other economically important parasites.

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