Studies on UDPG-α-Glucan Transglucosylase. III. Interconversion of Two Forms of Muscle UDPG-α-Glucan Transglucosylase by a Phosphorylation-Dephosphorylation Reaction Sequence*

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Paper I of this series presented evidence for two forms of UDPG-α-glucan transglucosylase. The first was a glucose-6-P independent (*I*) form, the second was a glucose-6-P dependent (*D*) form. The biphasic curve (Fig. 1) in the present paper suggested the two-way interconversion of these forms. Early experiments supported the idea that these were changes of the enzyme that were molecular in nature. The conversion of the *I* form to the *D* form required ATP and Mg^{++}. This reaction was studied in purified enzyme preparations and an apparent K_m for ATP of 7×10^{-5} M was estimated. The reaction appeared to be enzymic in nature and was shown to involve transfer of the terminal phosphate from P^{32}-labeled ATP to the enzyme. The reverse conversion of the *D* form to the *I* form when performed in the presence of labeled enzyme was accompanied by the loss of P^{32} as P_i from the enzyme.

We have previously reported that after rat diaphragms had been exposed to insulin the activity of UDPG-α-glucan transglucosylase (transglucosylase)[1] was increased in extracts of the tissue when measured without added glucose-6-P (Villar-Palasi and Larner, 1960, 1961; Larner, 1960). When the enzyme was fully stimulated by adding this cofactor in excess, no difference was detected between extracts prepared from control and insulin-treated diaphragms. It was shown that the activation by insulin was not explainable by the increased content of glucose-6-P, which had been observed previously (Larner *et al.*, 1960), or by any other soluble cofactor (Villar-Palasi and Larner, 1961). To explain the activation it was postulated that the enzyme might exist in two forms and that insulin might act to regulate their interconversion. The existence of two such forms was established in paper I of this series (Rosell-Perez *et al.*, 1962) by their preparation from rat muscle in a partially purified state and their differentiation by several criteria. One form (*D* or dependent form) was dependent upon glucose-6-P, and the other (*I* or independent form) acted independently of glucose-6-P.[2] We have now found that these two forms are interconvertible; the *D* form is converted to the *I* by a dephosphorylation reaction, and the *I* to the *D* by an ATP and Mg^{++}-dependent phosphorylation of the enzyme. A preliminary note reporting these findings has appeared (Friedman and Larner, 1962).

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[1] Abbreviations used are UDPG (uridine diphosphate glucose), glucose-6-P (glucose-6-phosphate), ATP (adenosine triphosphate), ADP (adenosine diphosphate), AMP (adenylic acid), DEAE (diethylaminoethyl), Tris-HCl (tris-(hydroxylmethyl)aminomethane, neutralized with HCl), EDTA (ethylene diaminetetraacetic acid), TCA (trichloroacetic acid), P_i (inorganic phosphate), transglucosylase (UDPG-α-glucan transglucosylase), *I* activity, *D* activity, total activity (see footnote 2).

[2] *I* activity is measured in the absence of glucose-6-P. Total activity is determined in the presence of excess added glucose-6-P. *D* activity is estimated by subtracting *I* activity from total.

METHODS AND MATERIALS

Enzyme Purification.—Extracts containing transglucosylase were prepared as follows: Muscle was dissected from the hind legs and backs of fed Wistar strain rats and placed into a tray chilled in ice. The muscle was trimmed of fat, divided with scissors, and homogenized in the Waring Blendor in 6 volumes (v/w) of 0.25 M sucrose, 0.05 M Tris-HCl, *p*H 7.5. The homogenate was then centrifuged for 20 minutes at 2600 × *g*, the precipitate was discarded, and the supernatant fluid was passed through two layers of cheesecloth. This extract was used in the experiment described in Figure 1. For the further purification of the enzyme this low-speed centrifugation could be omitted.

The crude extract was purified by a series of centrifugations, all of which were done at 0°. It was first centrifuged at 17,000 × *g*, and the *p*H of the supernatant fluid was adjusted to 7.7 with 1.0 M Tris-HCl, *p*H 8. It was then centrifuged for 3 hours at 100,000 × *g* in 12-ml tubes, and the supernatant fluid was discarded. The small pellet, which was glassy in appearance and pale yellow in color, contained the enzyme, which could be stored in the deep freeze at −20° for a month or more with little loss in activity. On the day of the experiment, pellets were suspended in 2–5 ml of 0.05 M Tris-HCl, *p*H 7.5. containing 0.05 M mercaptoethanol, incubated for 30 minutes at 30° and centrifuged at 34,000 × *g* for 30 minutes. This time the pellet was discarded. The supernatant solution contained the transglucosylase purified about 25-fold.

The enzyme was purified further by DEAE-cellulose column chromatography.[3] Twenty ml of extract containing 18 mg protein was applied to the column (diameter 1 cm, height 9 cm) and the effluent liquid was collected in 7-ml fractions. The column was then eluted with 20 ml of 0.05 M Tris-HCl, *p*H 7.8, 0.05 M mercaptoethanol containing added 0.05 M NaCl. This was followed by 65 ml of the same Tris-mercaptoethanol buffer containing 0.1 M NaCl, and finally by 30 ml of the same buffer containing 0.25 M NaCl. The transglucosylase, purified 150-fold, was eluted with the last concentration.

[3] The general procedure for the DEAE-cellulose column was worked out for purification of the glucose-6-P-dependent activity by M. Rosell-Perez with the technical assistance of Mr. Norman Brown in our laboratory.

The two most active fractions were pooled, and at this point were treated with P^{32}-labeled ATP and Mg^{++} in order to prepare labeled enzyme. EDTA was added to a concentration of 0.005 M and the enzyme was precipitated by adding solid $(NH_4)_2SO_4$ to bring the concentration to 75% saturation. The solution was allowed to stand at 2° for 30 minutes and then was centrifuged for 20 minutes at 12,000 × *g*. The precipitate was purified further over either a second DEAE-cellulose column with gradient elution or a phosphocellulose column.

When the enzyme was purified over a second DEAE-cellulose column the $(NH_4)_2SO_4$ precipitate was dissolved in 5 ml 0.05 M Tris-HCl, *p*H 7.8, containing 0.05 M EDTA and 0.05 M mercaptoethanol. It was then passed over a DEAE-cellulose column (diameter 0.7 cm, height 2.5 cm). The column was eluted with 10 ml of the same buffer containing 0.1 M NaCl followed by a NaCl gradient between 0.1 M and 0.33 M. Sixty ml of buffer containing 0.1 M NaCl in the lower chamber was mixed continuously with buffer containing 0.9 M NaCl in the upper reservoir. The mixture was passed onto the column, which had 3 ml of buffer above the resin. Fractions of 1.2 ml were collected in calibrated tubes. The peak transglucosylase fractions had a total purification of 200- to 400-fold by this method.

In early attempts at purification by means of a phosphocellulose column it was found that changes in the salt concentration of the eluting buffers led to large *p*H changes in the effluent. When the salt concentration was increased the *p*H dropped, and vice versa. These could be explained by the ion exchange properties of the resin. It was also observed that the buffering capacity of the column was an important determinant of the effluent *p*H. Applying these properties of the resin it was found that a *p*H gradient in the effluent was established when only one buffer at a constant *p*H was passed over the column. The rate of change of the gradient could be delicately regulated by small changes in the salt concentration or *p*H of this eluting buffer. The *p*H of all buffers used was measured at 2° with a Radiometer *p*H meter, Model 22.

The $(NH_4)_2SO_4$ precipitate was first dissolved in 3 ml of a solution of 0.001 M Tris-HCl, 0.05 M mercaptoethanol, and 0.005 M EDTA at *p*H 7.8. It was then diluted with 3 ml of a solution of 0.004 M maleate buffer, 0.05 M mercaptoethanol, and 0.005 M EDTA at *p*H 5.5. The final *p*H was 6.1. The extract was passed over the phosphocellulose column (diameter 1.2 cm, height 3.5 cm), which was then eluted with 0.01 M Tris-HCl containing 0.1 M NaCl, 0.005 M EDTA, and 0.05 M mercaptoethanol at *p*H 7.6. Air pressure was used to maintain a flow rate of 2–3 ml/minute. Under these conditions, the *p*H of the effluent rose slowly to 5.4–5.6, and then more rapidly to 7.6. The enzyme was eluted during the phase of rapid *p*H rise. This procedure gave a further purification of 4- to 6-fold, yielding an over-all purification of 600- to 800-fold.

Preparation of P^{32} Labeled ATP.—Radioactive ATP labeled in both terminal phosphates was prepared with rat liver mitochondria by the method described by Colowick and Kaplan (1957). This was modified by adding bovine serum albumin (0.066% and a catalytic amount of ATP (1×10^{-4} M) to the incubation mixture. The labeled ATP was isolated from a Dowex-1-Cl^- column (Cohn, 1957) by elution with 0.1 M HCl–0.2 M KCl. Labeled ADP was eluted prior to this with 0.05 M and 0.1 M HCl. The ATP was then adsorbed onto charcoal (8 mg/ml), eluted with 50% ethanol containing 0.05 M Tris-HCl, *p*H 8.0, and lyophilized. To detect possible cross contamination the preparations of labeled ADP and ATP were separately examined by paper chromatography. Carrier ATP and ADP were added to an aliquot of the labeled ATP. By use of the solvent system of Paladini and Leloir (1952), it was found by scanning the paper chromatograms that all the radioactivity was associated with the ATP, with none detected in the ADP. Conversely, when the labeled ADP was examined in this manner, no radioactivity was detected in the ATP. β-labeled ATP was prepared enzymically by incubating the labeled ADP with unlabeled creatine phosphate and ATP creatine transphosphorylase (Pressman, 1960). The enzyme prepared by the procedure of Noda *et al.* (1955) was carried through to the lyophilization step. The absence of myokinase was confirmed by incubating the labeled ADP with the enzyme in the absence of creatine P. Under these conditions no labeled ATP was demonstrated by paper chromatography. The specific activity of the β,γ-labeled ATP was 183×10^6 cpm/μmole adenine, while the specific activity of the β-labeled was 92×10^6 cpm/μmole adenine.

Analytical.—Transglucosylase activity was determined by measuring the amount of radioactivity incorporated into glycogen from C^{14}-glucose-labeled UDPG. The method described by Villar-Palasi and Larner (1961) was used. The reaction mixture used was modified slightly and was as follows. UDPG, 0.0044 M (5000–7000 cpm/μmole); glycogen, 0.36%; glucose-6-P (when present), 0.013 M; Tris-HCl, 0.04 M–EDTA, 0.0066 M (*p*H 8.2); enzyme preparation, 0.05 ml. The total volume was 0.15 ml and incubations were done at 30° for 5–10 minutes. Phosphorylase was assayed spectrophotometrically in the manner of Larner and Villar-Palasi (1959). The reaction mixture for this assay which was modified slightly was as follows: glycylglycine buffer, *p*H 7.4, 0.04 M; EDTA, 0.0016 M; $MgSO_4$, 0.0056 M; TPN, 0.0016 M; phosphoglucomutase crystalline, specific activity 2.7 μmoles/min/ml, 0.066 ml; glucose-6-P dehydrogenase, 0.05 mg; cysteine, 0.015 M; glycogen, 0.4%; AMP (when present), 0.005 M; inorganic phosphate, 0.005 M. Glucose-1,6-diphosphate was found not be to required.

Protein determinations were carried out by the method of Lowry *et al.* (1951). P_i was estimated by the method of Lowry and Lopez (1946). P^{32} was identified as P_i by precipitation with magnesia mixture (Sacks, 1949) or by extraction of the phosphomolybdic acid with isobutanol (Weil-Malherbe and Green, 1951). The P^{32} incorporated into protein was determined with aliquots of the column fractions in the following manner: 1.0 mg bovine serum albumin was added first as carrier, followed by 10 ml cold 6% TCA. The protein was allowed to precipitate for 10 minutes in an ice bath and then was centrifuged for 15 minutes at 3000 rpm in the International refrigerated centrifuge. After draining for 10 minutes, the precipitates were dissolved in 1.0 ml of 0.1 M NaOH. The protein was precipitated and redissolved as above two more times. The dissolved protein was then plated and radioactivity counted. The efficacy of this washing procedure in removing nonprotein-bound radioactivity was demonstrated by the fact that only traces of protein-bound radioactivity were found in the column fractions which were known to contain excess labeled ATP, prior to precipitation of the protein.

Chemicals.—DEAE-cellulose (type 20, capacity 0.86 meq/g) was obtained from Brown Company. It was washed with distilled water, removing the fines by decantation. A slurry was applied to the columns,

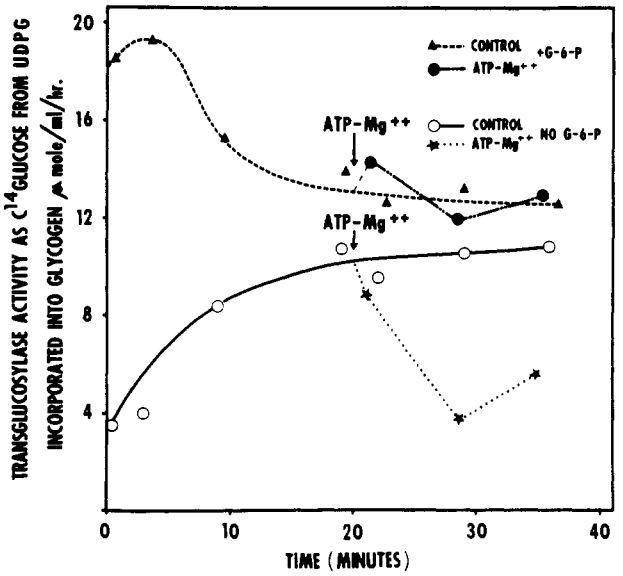

FIG. 1.—Time course of enzyme activation and inactivation. The preparation of the extract, a 2600 × *g* supernatant of the homogenate, was described in the text. Two ml of the extract was incubated at 30° with no additions. At 20 min., 0.5 ml was removed and added to a tube containing 0.10 ml of a mixture of ATP and $MgCl_2$ so that the final concentrations were 5 × 10^{-3} M and 1 × 10^{-2} M, respectively. Distilled water was added to the control extract. At the times indicated 0.05-ml aliquots were removed and added to 0.10 ml of a test mixture for the assay of transglucosylase with and without glucose-6-P. The results are recorded as transglucosylase activity in μmoles/ml extract/hour.

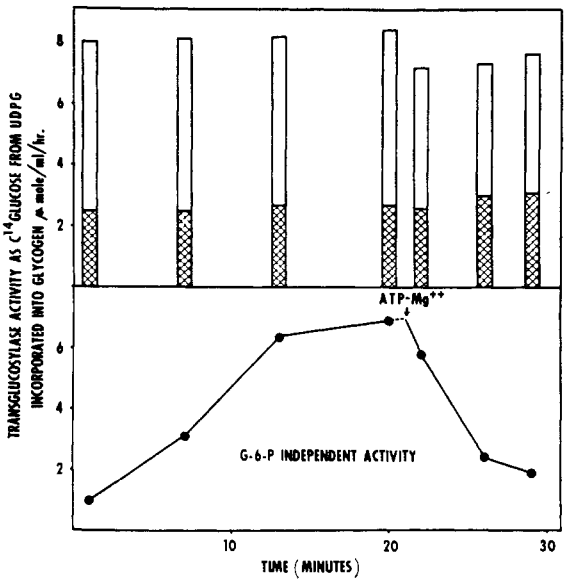

FIG. 2.—Addition of boiled extracts prepared at different points in the time course of enzyme activation and inactivation. A crude extract prepared as in Fig. 1 was incubated at 30° in seven one-ml portions. At 21 minutes a mixture of ATP and $MgCl_2$ was added to three of the tubes so that the final concentrations were 5 × 10^{-3} M and 1 × 10^{-2} M, respectively. At the indicated times 0.05-ml aliquots were removed and added to test mixture for the assay of *I* activity (no glucose-6-P). These results are shown in the lower curve. The remaining 0.95 ml was immediately placed on a boiling water bath and was heated for 10 minutes. The protein was removed by centrifugation, and 0.20 ml of the supernatant was mixed with 0.20 ml of a purified particulate preparation of transglycosylase. The mixture was assayed for transglucosylase activity. These results are depicted by the bar graphs. The entire bar represents total activity; the crosshatched area is *I* activity. Results are recorded as transglucosylase activity in μmoles/ml extract/hour.

which were allowed to pack by gravity. Prior to use the columns were washed with 15 bed volumes 0.05 M Tris-HCl *p*H 7.8, followed by 3 bed volumes of the same buffer containing 0.05 M mercaptoethanol. Phosphocellulose (Cellex P, 0.79 meq/g) was obtained from the Bio Rad Company. It was prepared by cycling with 0.5 N NaOH–0.5 M NaCl, 1 N HCl, 0.1 N NaOH, and 0.1 N HCl, and was finally washed with large volumes of 0.002 M maleate buffer *p*H 6.1 until the *p*H reached 6.1. A slurry was applied to a column which was packed by gravity. Before use, the column was washed with 25 ml of 0.002 M maleate buffer containing 0.005 M EDTA and 0.005 M mercaptoethanol at *p*H 6.1. The presence of EDTA caused the *p*H of the effluent to drop from 6.1 to about 5.0. ATP, ADP, and AMP were obtained from Sigma Chemical Company. P^{32} inorganic phosphate was acquired from Oak Ridge National Laboratory. The source of other chemicals is cited in paper I of this series (Rosell-Perez *et al.*, 1962).

RESULTS

Initial Evidence for Interconversion.—It was found that when crude extracts (Methods and Materials) were incubated at 30°, transglucosylase I activity increased 3-fold in 20 minutes while the total activity[2] decreased (Fig. 1). The ratio of activities (total/*I*) decreased from 5.8 to 1.3. If ATP and Mg^{++} were then added, *I* activity decreased rapidly while total activity did not change as compared with the control (second half of Fig. 1). The ratio (total/*I*) changed from 1.3 to 3.2 and then back to 2.3. Similar results were observed with homogenates which had not been centrifuged and with extracts after centrifugation at 17,000 × *g*. However, when transglucosylase was purified further by centrifugation at 100,000 × *g*, only the ATP-Mg^{++} effect was present in the pellet with transglucosylase. This indicated that a system responsible for the increase in *I* activity was removed or rendered inactive by the latter centrifugation.

In order to determine whether the above changes in transglucosylase were due to changes in glucose-6-P (or any other heat stable cofactor), boiled extracts were prepared at different points of the time course of Figure 1. These boiled extracts were then tested for their ability to activate (or inhibit) transglucosylase by addition to a purified preparation of the enzyme which had been obtained by centrifugation at 100,000 × *g* (Fig. 2). No differences in *I* activity were observed when boiled extracts were added.[4] This indicated that the changes noted in Figure 1 were not due to changes in glucose-6-P content or in any other heat stable factor directly affecting enzyme activity.

The Nature of the ATP-Mg^{++}-Dependent Transformation.—Two additional early experiments suggested that the ATP and Mg^{++} transformation might be a direct molecular change of the enzyme. First, if crude extracts were treated with ATP and Mg^{++}, and then centrifuged at 100,000 × *g* for 3 hours, the isolated pellets contained transglucosylase with higher ratios of activities (total/*I*) than untreated controls. In other words, the effect of ATP-Mg^{++} was retained when the enzyme was separated from the supernatant

[4] There was some inhibition of total activity by the boiled extracts which contained ATP and Mg^{++}. This was probably due to the inhibition of *D* activity by Mg^{++} as reported previously (Rosell-Perez *et al.*, 1962).

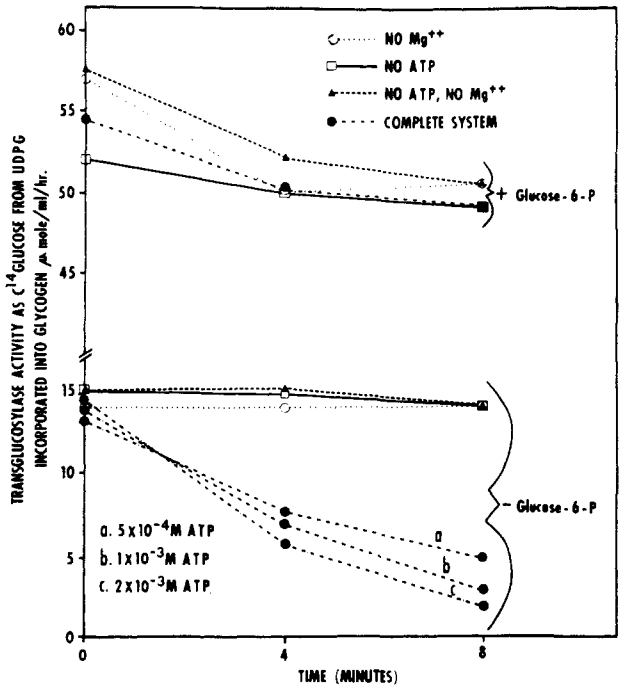

FIG. 3.—Time course of ATP-Mg^{++} transformation in a 25-fold purified enzyme preparation. The complete incubation mixture contained 0.44 ml of extract prepared from pellets suspended in 2 ml of buffer as described in the text, 2×10^{-3} M $MgCl_2$, and varying concentrations of ATP. The final volume was 0.50 ml. Incubation was at 30°. At the times indicated 0.05-ml aliquots were removed and added to a test mixture with and without glucose-6-P for the assay of transglucosylase. Results are recorded as transglucosylase activity in μmoles/ml incubation mixture/hour.

fluid. Second, if in the 100,000 × *g* pellet the ATP-Mg^{++} effect were allowed to proceed and then EDTA added in excess to complex the Mg^{++}, the transformation reaction had already stopped when assayed 1 minute after adding EDTA. This indicated that there was a Mg^{++}-dependent reaction that was directly upon transglucosylase rather than on some other enzyme which acted on transglucosylase. In the latter case, the reaction would have been expected to continue after the addition of EDTA.

Specificity and Kinetic Properties of the ATP-Mg^{++}-Dependent Transformation.—It was observed in the 25-fold purified enzyme preparations (Fig. 3) that either Mg^{++} or ATP alone was completely without effect.[5] However, when the two were added together, the usual transformation was noted, that is, a decrease in the *I* activity with no change in the total activity. The effect of varying the concentration of ATP is also shown in Figure 3. It is seen that the amount of transformation is a function of the amount of ATP added. From similar experiments, with measurements at 2 minutes after ATP addition to approach initial velocity and to minimize effects of adenosine triphosphatase, an apparent K_m for ATP of 7×10^{-5} M was estimated. When the ATP concentration was 5×10^{-4} or 1×10^{-3} M, the amount of Mg^{++} required for the maximum effect was found to be 2–4 times the concentration of ATP. ADP was almost as active as ATP in the crude extracts possibly due to the action of myokinase. It was found to be only one-tenth as effective as ATP in the 25-fold purified particulate preparations described in paper I. AMP, which was also tested, was ineffective at 5×10^{-4} M (see next section).

Phosphorylase Controls.—The possibility arose that ATP and Mg^{++} could have been acting to convert phosphorylase *b* to phosphorylase *a* through phosphorylase *b* kinase (Fischer and Krebs, 1955). The transformation which we noted could therefore have been a reflection of this increased phosphorylase activity. Such a proposal did not explain the fact that the total transglucosylase activity was not decreased by ATP-Mg^{++}.

A number of additional studies disproved this possibility. First, glycogen was dissolved from planchets used in the transglucosylase assays. No measurable decrease of glycogen had occurred after ATP treatment indicating no gross breakdown by phosphorylase. This result, however, did not rule out changes in phosphorylase leading to a specific breakdown of newly added glucose units too small to measure chemically. Second, in an experiment in which added ATP-Mg^{++} decreased transglucosylase *I* activity in terms of glycogen synthesis by 0.32 μmole/ml of reaction mixture, P_i was measured by the method of Lowry and Lopez (1946). In order for phosphorylase to be responsible for this difference, assuming complete utilization of P_i for hexose monophosphate formation, at least 0.32 μmole/ml P_i would have been required. Only 60% of this value was found. Furthermore, addition of P_i (0.7 μmole/ml) to the reaction mixture stimulated the transglucosylase as measured in the absence of glucose-6-P rather than inhibiting it.[6] Third, 5×10^{-4} M AMP, a quantity that is 10-fold greater than the K_m for activation of phosphorylase *b* (Madsen and Cori, 1957), was without detectable effect on the transglucosylase I activity when added with or without P_i. Finally, it was possible to separate transglucosylase from phosphorylase on DEAE-cellulose columns (Fig. 4). Both phosphorylase *a* and *b* were eluted in the 0.05 M NaCl and 0.1 M NaCl fractions while transglucosylase *I* and *D* were eluted in the 0.25 M NaCl fraction. The ATP-Mg^{++}-dependent transformation system was present in this 150- to 200-fold purified transglucosylase fraction although the reaction was slower than in cruder preparations.

Incorporation of P^{32} from ATP into Transglucosylase during Conversion of I to D.—Evidence already cited for a direct molecular transformation of the two activities and for the greater effectiveness of ATP compared to ADP suggested that the observed transformation was produced by a phosphorylation of the enzyme. This hypothesis was examined by using ATP labeled with P^{32}. When incubated with the 150-fold purified DEAE fraction, radioactivity was incorporated into protein coincident with a conversion of the *I* activity to the *D*. The enzyme was then precipitated with $(NH_4)_2SO_4$ and passed over either a second DEAE-cellulose column or over a phosphocellulose column. In the first case a salt gradient was used and in the second case a *p*H gradient was used. It was seen that the radioactivity was associated with transglucosylase protein in both cases (Figs. 5 & 6). The amount of incorporated phosphate calculated as P_i on both DEAE- and phosphocellulose columns ranged in the peak tubes from 1.0 to 2.4 × 10^{-3} μmole/unit of transglucosylase converted from *I* to *D* (Table I). One unit is defined as that amount of enzyme catalyzing the addition of one μmole of glucose from UDPG to

[5] A time dependent decrease in the *D* transglucosylase activity has been observed in the presence of Mg^{++} in higher concentrations than used in Figure 2. It is not known whether this is related to the inhibition observed without incubation (see footnote 4).

[6] This stimulation by anionic compounds has been previously noted in highly purified preparations by M. Rosell-Perez (1962).

TABLE I

QUANTITATIVE RELATIONSHIP OF P^{32} INCORPORATION TO CONVERSION OF I TO D

The percentage of *D* that was derived from *I* was obtained from a parallel unlabeled ATP experiment which was performed simultaneously with the labeled experiment. It is assumed that the two incubations were comparable. This percentage is multiplied by the number of units of *D* activity in the peak column fraction to give the number of units of *D* activity in the column fraction that were derived from *I* activity. This latter value is divided into the amount of P^{32} incorporated into protein in the column fraction to give the P^{32} incorporated/unit enzyme converted from *I* to *D*.

Experiment	Column	Labeled ATP	P^{32} Incorporated into Protein of Column Fraction[a] (mμmole/ml)	*D* Activity in Column Fraction[a] (units/ml)	*D* Activity Derived from *I* (%)	*D* Activity in Column Fraction Derived from *I*[a] (units/ml)	P^{32} Incorporated/ Unit Enzyme Converted (mμmoles P^{32}/ unit enzyme, converted)
1[b]	DEAE	AMP $P^{32}P^{32}$	0.036	0.104	15.1	0.0157	2.3
2[b]	DEAE	AMP $P^{32}P^{32}$	0.199	0.471	22.0	0.104	1.9
3	DEAE	AMP $P^{32}P^{32}$	0.082	0.183	18.6	0.0340	2.4
4	Phospho-cellu-lose	AMP $P^{32}P^{32}$	0.022	0.101	17.5	0.0177	1.2
			0.019	0.106		0.0186	1.0
5[b]	DEAE	AMP $P^{32}P$	0.004	0.727	21.8	0.158	0.03

[a] Results were calculated from the column fraction (second column) containing the highest level of enzyme activity. [b] In these experiments the P^{32}-labeled enzyme was not precipitated with $(NH_4)_2SO_4$ as described in Methods and Materials. Instead it was diluted 1:5 with 0.05 M Tris-HCl, 0.05 M mercaptoethanol before passing over the second column.

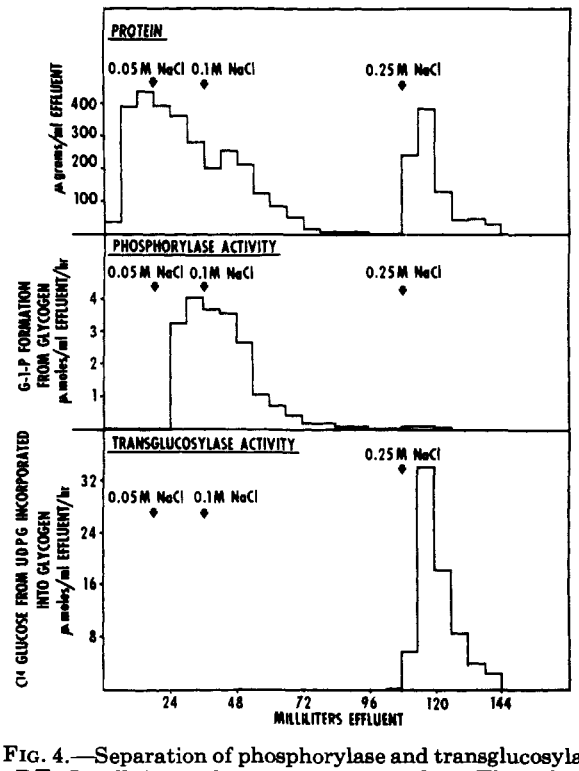

FIG. 4.—Separation of phosphorylase and transglucosylase by DEAE-cellulose column chromatography. The column procedure and assay methods are described in Methods and Materials. Only the total phosphorylase (sum of *a* and *b*) and total transglucosylase activities are shown. In both cases the elution pattern for the inactive (dependent) activity coincided with that of the total activity.

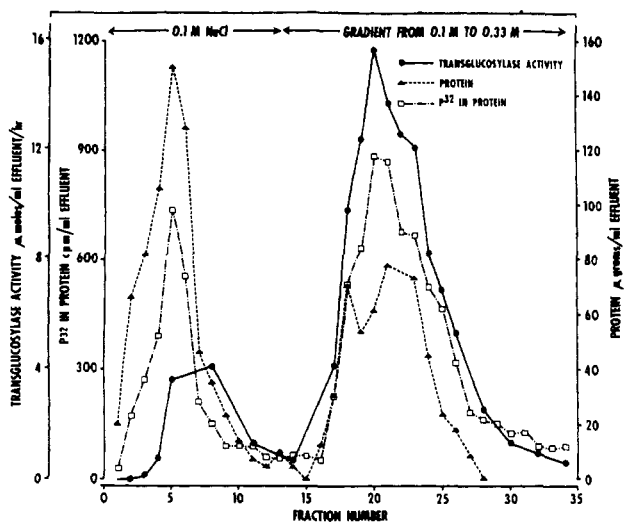

FIG. 5.—DEAE-cellulose column chromatography of P^{32}-labeled transglucosylase, second column. The 150-fold purified transglucosylase from the first column was incubated at 30° for 20 minutes in the presence of 2.2×10^{-4} M ATP, 21,800,000 counts/min/μmole adenine and 1×10^{-3} M $MgCl_2$. The reaction was followed by an incubation with unlabeled ATP. The labeled mixture was then treated with EDTA, precipitated with $(NH_4)_2SO_4$, and passed over the second DEAE-cellulose column, the results of which are shown above. The details of these procedures are described in Methods and Materials. Only total transglucosylase activity is shown. The elution pattern of the *I* activity coincided with that of the total activity.

glycogen in one minute under the specified assay conditions. Because of the greater purification of transglucosylase achieved on the phosphocellulose columns it is felt that the lower values are the more accurate. When ATP labeled only in the β position was used instead of doubly β,γ-labeled ATP, only about 1–3% of this incorporation was observed.

Release of P^{32} from Transglucosylase during Conversion of D to I.—The results of the P^{32} incorporation experiments indicated that the enzyme was phosphorylated during the conversion of the *I* form to the *D*. The question of whether the reverse conversion of the *D* form to the *I* was a dephosphorylation was investigated using P^{32}-labeled enzyme isolated by either of the two column procedures. The peak column fractions were added to a crude homogenate as prepared in Methods in order to study the conversion of the *D* form to the *I* in the presence of P^{32}-labeled enzyme. There was a complete correspondence between conversion of *D* to *I* and release of P^{32} from protein (Fig. 7).

The P^{32} which was released was identified as P_i by (1) precipitation with magnesia mixture and (2) isobutanol extraction of phosphomolybdic acid. In

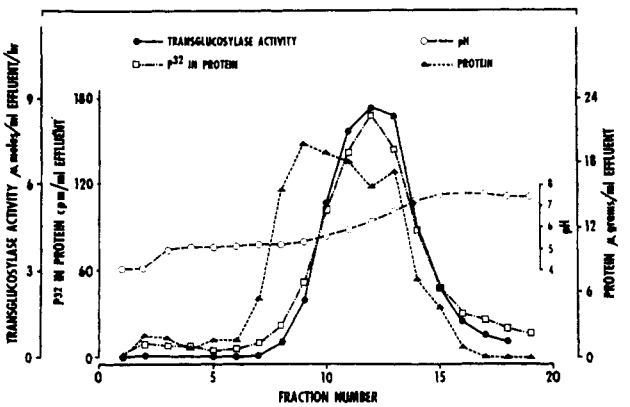

FIG. 6.—Phosphocellulose column chromatography of P^{32}-labeled transglucosylase. The 150-fold purified enzyme was incubated with 5×10^{-4} M ATP, 15,100,000 counts/min/μmole adenine and 1×10^{-3} M $MgCl_2$ as in Figure 5. The isolation procedure culminating in the above column is described in Methods and Materials. Only total transglucosylase activity is shown. The elution pattern of the *I* activity was similar to that of the total activity.

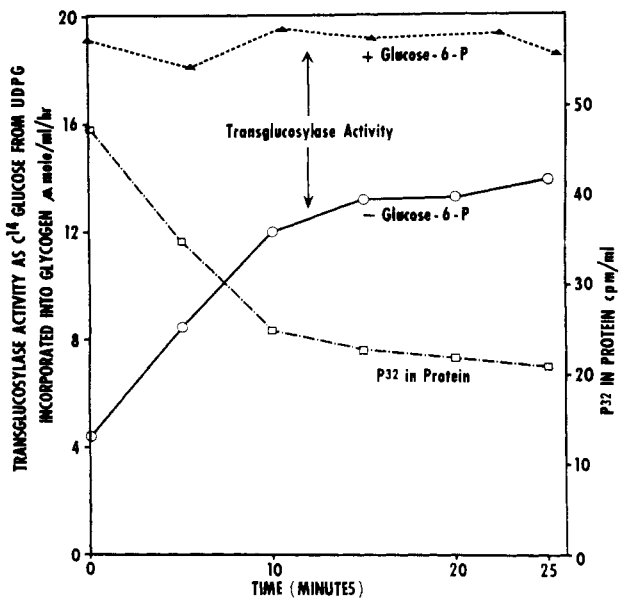

FIG. 7.—Release of P^{32} from transglucosylase enzyme during conversion of *D* to *I*. The P^{32}-labeled transglucosylase was prepared as in Figure 6. The most active fractions from the phosphocellulose column were pooled and contained 150 counts/min/ml P^{32} in protein. Three ml was mixed with 6 ml of crude extract. The extract, a 2600 $\times$ *g* supernatant of the homogenate, was prepared as described in Methods and Materials except that only 5 volumes of Tris-sucrose were used in the homogenization. The mixture of crude extract and labeled enzyme were incubated at 30°. At the times indicated by points in the graph 0.05-ml aliquots were removed and assayed for transglucosylase activity, by addition to test mixture with or without added glucose-6-P. At the same time, 1-ml aliquots were removed and added to 0.03 ml 70% perchloric acid. The protein was isolated by centrifugation, dissolved in 1 ml of 0.2 M NaOH and plated, and radioactivity was counted.

these experiments (Table II) unlabeled P_i (0.01 M) was added in order to minimize loss of radioactive P_i into organic compounds. By both methods it is clear that the released P^{32} was P_i. The reservation must be held that this might still be a phosphate ester which is rapidly cleaved enzymically or by cold perchloric acid to P_i.

TABLE II

IDENTIFICATION OF P^{32} RELEASED FROM TRANSGLUCOSYLASE DURING CONVERSION OF *D* TO *I*

The conditions were similar to those in Figure 7. The enzyme labeled with P^{32} was isolated from a phosphocellulose column in experiment 1, and from a DEAE-cellulose column in experiment 2. P^{32} in protein was determined as in Figure 7. The perchloric acid supernatants were treated with either magnesia mixture or ammonium molybdate and isobutanol. In the former case, the precipitate was isolated by centrifugation, dissolved in 0.2 M HCl, and plated. In the latter case, the isobutanol extract was plated directly. Corrections were made for self-absorption and other losses by use of internal standards.

Experiment	Time (min)	P^{32} Released from Protein (cpm/ml)	Released P^{32} Precipitated by Magnesia Mixture (cpm/ml)	Released P^{32} Extracted into Isobutanol (cpm/ml)
1	0	0	0	0
	10	15	12	19
	20	19	23	15
	30	20	18	23
2	0	0	0	0
	20	16	26	16

Enzymic Nature of the Phosphorylation Reaction.—When the 600- to 800-fold purified transglucosylase from the phosphocellulose column was incubated with 5×10^{-3} M ATP and 1×10^{-2} M Mg^{++} no interconversion reaction was detected. This suggests that the phosphorylation is enzymically catalyzed, and that the kinase responsible has been separated or rendered inactive during the phosphocellulose column purification.

DISCUSSION

The results presented in this paper support the following two reactions:

(1) Transglucosylase *I* + *n*ATP $\xrightarrow[Mg^{++}]{\text{kinase}}$ transglucosylase *D* + *n*ADP

(2) Transglucosylase *D* $\xrightarrow{\text{phosphatase}}$ transglucosylase *I* + *n* P_i

Reaction (1) was supported by the demonstration of the incorporation of P^{32} from ATP into transglucosylase during the conversion of *I* to *D*. The coincidence of the radioactivity with transglucosylase was established on two different types of columns, DEAE-cellulose and phosphocellulose. The extent of reaction was shown to be a function of the amount of ATP added. An apparent K_m for ATP of 7×10^{-5} M was estimated. The formation of ADP as the other reaction product was suggested by the finding that the γ- but not the β-labeled P of ATP was incorporated into the enzyme. The observation that the enzyme purified 600- to 800-fold over the phosphocellulose column did not undergo conversion with ATP and Mg^{++} supports the idea that an enzyme is required for this reaction, presumably a phosphoprotein kinase. Reaction (2) was studied by the release of P^{32} during the conversion of *D* to *I*. By two methods the radioactivity released from the enzyme was shown to be P_i.

As a result of these studies an analogy between transglucosylase and glycogen phosphorylase is apparent. Both enzymes have been shown to exist in two forms, one of which depends on a cofactor for activity (Cori and Green, 1943). From the present work it appears that the two forms of transglucosylase,

like those of phosphorylase (Sutherland and Rall, 1960), are interconvertible. Furthermore, the present evidence indicates that, like the interconversions of the phosphorylase system, the interconversions of the transglucosylase system are via phosphorylation and dephosphorylation reactions. A difference between the two systems lies in the fact that the interconversion reactions occur in an opposite sense. Thus, for example, the ATP-Mg^{++}-dependent phosphorylation reaction leads to the formation of the glucose-6-P-dependent or less active form of transglucosylase, but to the "active" or AMP-independent form of phosphorylase.

From the above considerations we are led to consider two possibilities with regard to control mechanisms. Both phosphorylase and transglucosylase could be subject to simultaneous regulation through control of a common kinase (or phosphatase). A second possibility would be control through a mechanism which could act in an identical manner on two separate but similar kinases (or phosphatases). Either mechanism of control would then lead to an activation of one of the glycogen metabolizing enzymes and a coincident inhibition of the other. Since phosphorylase serves principally a glycogenolytic function *in vivo*, whereas tranglucosylase acts in glycogen synthesis, this would mean that the synthesis of glycogen would be inactivated when its breakdown was activated, and vice versa. Belocopitow (1961) already has presented evidence in preliminary form in general agreement with these speculations. His results indicated that adenosine 3′,5′-cyclophosphate inhibited transglucosylase activity but activated phosphorylase.

The activating action of insulin on transglucoyslase can now be delineated in terms of either an activation of the dephosphorylation reaction or an inhibition of the phosphorylation reaction. Either of these or a combination of the two would lead to the observed result, namely, an increased activity of *I* with no significant difference in total activity. It is of interest to note that Randle (1960) already postulated an action of insulin on sugar transport that is mechanistically of the same reaction type and in the same direction as the action on transglucosylase. These workers suggested that insulin's action on transport could be through the stimulation of a dephosphorylation or by the inhibition of a phosphorylation of a carrier. Although the similarity in these two proposals may be coincidental, it is of interest to point them out at present in the context that they may represent a common denominator in these two apparently separate actions of insulin.

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