Pseudoisoenzymes of Rabbit Muscle Phosphoglucose Isomerase*

(Received for publication, October 14, 1971)

MICHAEL N. BLACKBURN; JOHN M. CHIRGWIN, GORDON T. JAMES, THOMAS D. KEMPE, THOMAS F. PARSONS; ADELE M. REGISTER, KLAUS D. SCHNACKERZ, & ERNST A. NOLTSMANN

From the Department of Biochemistry, University of California, Riverside, California 92502

SUMMARY
Rabbit muscle phosphoglucose isomerase, either in crude muscle extract or after isolation and crystallization, has been found to occur as three different species when chromatographed on carboxymethyl Sephadex. The three forms are indistinguishable with respect to over-all amino acid composition except that they differ in accessibility of their sulfhydryl groups to p-mercuribenzoate. They can be interconverted both by treatment with dithiothreitol and by exposure to oxidative conditions, indicating that they represent different states of the same protein rather than genuinely dissimilar protein species as claimed in another report (YOSHIDA, A., AND CARTER, N. D. (1969) Biochim. Biophys. Ada, 194, 151). When subjected to isoelectric focusing, these "pseudoisoenzymes" yield characteristic isoelectric points which correspond to their elution positions on cation exchange columns.

Rabbit skeletal muscle phosphoglucose isomerase (p-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9) has in recent years been the object of detailed studies in this laboratory (1-6). Work on the structure and mechanism of this enzyme has in the past been carried out with five times crystallized preparations (7, 8) which had been shown to be homogeneous by various criteria (6). The extent to which the data obtained represent meaningful analytical information, however, was rendered doubtful when Yoshida and Carter (9) reported that they could resolve three isoenzymes from a commercial preparation of crystalline rabbit muscle phosphoglucose isomerase. On the basis of different amino acid compositions and different peptide maps obtained for two of their chromatographic species, these authors suggested (9) that their isomerase isoenzymes might have different primary structures and thus originate from different genes in the rabbit.

We will show in this paper that phosphoglucose isomerase from skeletal muscle of the rabbit, when chromatographed under the conditions given by Yoshida and Carter (9), does not resolve into the isoenzyme species described by these authors; that under conditions entirely different from those of Yoshida and Carter, partially purified as well as crystalline phosphoglucose isomerase can be separated into a number of chromatographic forms the relative amounts of which depend on the oxidation state of available sulfhydryl groups; that these chromatographic enzyme species, in contrast to the isoenzymes described by Yoshida and Carter (9), do not differ significantly in over-all amino acid composition and that they are, in fact, interconvertible; and that these multiple chromatographic species cannot, therefore, be isoenzymes, but rather represent different states of the same molecular species of rabbit muscle phosphoglucose isomerase.

EXPERIMENTAL PROCEDURE

Assay—Phosphoglucose isomerase activity was measured at 30° by coupled spectrophotometric assay as previously described (7, 8). Enzyme units are defined as micromoles of fructose 6-phosphate converted to glucose 6-phosphate per min at 30°. Protein concentrations were determined by measuring the absorbance at 280 nm in 0.01 M sodium phosphate of pH 7.0. Under these conditions, an absorbance of 1.0 for a 10-mm light path corresponds to 0.76 mg of enzyme per ml (6).

Enzyme Isolation—Detailed descriptions of the isolation and crystallization of phosphoglucose isomerase from rabbit skeletal muscle have been provided earlier (7, 8). In the present work, these procedures were followed as described when preparing the crystalline enzyme; crystallizations were allowed to occur very slowly (6). When only partially purified enzyme was desired as starting material for further chromatographic studies, the original method was followed through completion of Fraction III, i.e. the collection of the precipitate at 0.63 ammonium sulfate saturation, which was then dialyzed against the starting buffer of the subsequent chromatographic column. Other changes of the standard purification procedure, necessitated by the conditions required for a particular experiment, are described in the text or in the legends to the corresponding figures. The rabbits were obtained from several different local rabbit farms without selection or specification as to origin.

Preparation of Muscle Extracts for Chromatography on Carboxymethyl Sephadex—Ground rabbit muscle (usually 100 g) was
Fig. 1. Chromatography of crystallized rabbit muscle phosphoglucone isomerase on carboxymethyl Sephadex according to the method of Yoshida and Carter. Upper portion, data redrawn from their paper; middle portion, twice crystallized enzyme (37 mg) chromatographed under conditions identical with those described by Yoshida and Carter, i.e., CM-Sephadex column (1.5 x 30 cm) eluted with a linear NaCl gradient (0 to 100 mM in 600 ml of 10 mM phosphate buffer, pH 6.8); lower portion, rechromatography of pooled peak fractions (after concentration) from the column depicted in the middle portion.  •, enzyme activity; ○, absorbance at 280 nm.

Fig. 2. Chromatography of twice crystallized enzyme on CM-Sephadex with a sodium phosphate gradient, 9 to 18 mM in Na+, in the absence of DTT. Column dimensions, 2.5 x 15 cm. Gradient: sodium phosphate, pH 6.90, Na+ increasing linearly from 9 to 18 mM in a total volume of 800 ml; flow rate, 24 ml per hour. Upper portion, 63 mg of enzyme (~300 units per mg); lower portion, rechromatography of 7 mg of the pooled fractions from the column depicted in the upper portion. Symbols as for Fig. 1.

days. The gel was then washed extensively with buffer to ensure complete equilibration. After degassing, the gel slurry was poured into a column of 2.5 cm inner diameter, equipped with flow adaptors, to bed heights ranging from 15 to 35 cm. For large preparations, columns of 5.0 cm inner diameter were used which could accommodate bed heights of up to approximately 40 cm. Final equilibration of the column was achieved by pumping a minimum of 1 bed volume of starting buffer through the column.

Elution buffers were prepared by mixing solutions of monobasic and dibasic sodium phosphate to a final pH of 6.90 ± 0.02 at 25°C ± 1°C and to sodium concentrations of 12 and 24 mM for the starting buffer and the final gradient buffer, respectively. Extreme care was exercised in preparing these buffers, since deviation in pH by more than 0.05 pH unit from the required value resulted in failure of the enzyme either to bind to the gel or to elute within the gradient. Measurements of the pH were made with a Beckman model 1019 research pH meter permitting pH readings to 0.001 unit, which was calibrated before and after each determination.

The protein solution was pumped directly onto the column and washed in with starting buffer. Elution with starting buffer was continued until the absorbance at 280 nm had returned to near the baseline value, at which time the column was developed with a linear gradient of sodium phosphate. 1

1 The abbreviations used are: CM, carboxymethyl; DTT, dithiothreitol; PMB, p-mercuribenzoate.
Fig. 3. Chromatography of twice crystallized enzyme in the presence of DTT. All conditions were as described for Fig. 2, except that the enzyme was dialyzed and chromatographed in the presence of 1 mM DTT. Upper portion, 112 mg of twice crystallized enzyme; lower portion, rechromatography of 17 mg of the pooled fractions from the column depicted in the upper portion. Symbols as for Fig. 1.

Fig. 4. Chromatography of crude muscle extract in the presence of DTT. Crude rabbit muscle extract was prepared with 10 mM KCl-5 mM DTT, concentrated to 80 mg per ml, dialyzed against the gradient starting buffer containing 1 mM DTT. The sample (total protein, 2.9 g) was applied to a CM-Sephalac column (2.5 × 15 cm), which was washed with 6 mM sodium phosphate, pH 6.90, until the initial bulk protein peak had passed through and then eluted with 800 ml of a linear sodium phosphate gradient, pH 6.90 (6 to 24 mM in phosphate, 12 to 24 mM in Na+), all solutions containing 1 mM DTT. Upper portion, initial extract; middle portion, rechromatography of the "wash-through" peak (Pool 1); lower portion, rechromatography of the G-I peak (Pool 2). Symbols as for Fig. 1.
to 24 mM in Na+, in a total volume of 1200 ml. At the end of the gradient the column was washed with 1 M NaCl. These standard conditions were used for the column measuring 2.5 X 20 cm. For columns of different size, concentrations and volumes of the gradient buffers were adjusted accordingly to obtain maximum resolution.

Isoelectric Focusing—Isoelectric points of the different enzyme species were measured at 4°C by isoelectric focusing in an LKB model S101 electrosfocusing column (capacity, 110 ml). Sucrose density gradients were prepared with an LKB model 8121 gradient mixer and pH gradients from pH 7 to 9 were established in the column with LKB Ampholine ampholyte solution kept for 96 to 120 hours at a constant voltage of 600 volts. At the end of the focusing period the column contents were removed at a flow rate of 30 ml per hour with the aid of a Harvard model 1201 peristaltic pump. Fractions of approximately 1 ml were collected and their protein content, enzymatic activity, and pH were determined.

Amino Acid Analyses—Aliquots of the various chromatographically separated fractions were hydrolyzed for 20 hours in 6 N HCl and analyzed with the aid of a Beckman-Spinco automatic amino acid analyzer model 120C as previously described in detail (6).

Determination of Sulphydryl Groups—Sulphydryl assays were performed at 30°C in 0.05 M sodium phosphate, pH 7.1, by Boyer's p-mercuribenzoate method (10) with care not to exceed a ratio of 1.5 eq of PMB per titrable —SH group (1). The increase in absorbance due to mercaptide formation was followed spectrophotometrically at 255 nm with use of a molar absorptivity coefficient of 0.62 X 10^4 (1, 10). Matched sets of 2.0-ml cuvettes were used throughout. Initial enzyme concentrations ranged from 1.5 to 4.5 μM, initial PMB concentrations ranged accordingly from 25 to 75 μM, in a total volume of 2.0 ml. Contents of the blank cuvette were identical with those of the sample cuvette except that phosphate buffer was substituted for the enzyme solution. The reaction was allowed to proceed for 40 min; the absorbance difference was extrapolated back to zero time and this value was taken as representative of the immediately available —SH groups (Type I, cf. Reference 1). Then 0.20 ml of 5% sodium dodecyl sulfate (in the same phosphate buffer) was added to both blank and sample cuvette to obtain the absorbance change reflecting the total sulphydryl content (1). To obtain the number of —SH groups per molecule, before and after addition of sodium dodecyl sulfate, the measured absorbance values were corrected for self-absorption at 255 nm by both enzyme and PMB and for volume changes caused by addition of the denaturant.

Determination of Total Half-cystine Content—The total half-cystine content was measured as cysteic acid after performic acid oxidation according to the procedure of Moore (11). The oxidized enzyme samples were hydrolyzed in 6 N HCl under the usual conditions and the cysteic acid was quantitated by chromatography on the automatic amino acid analyzer.

RESULTS

Earlier work in this laboratory on the chromatographic behavior of rabbit muscle phosphoglucone isomerase on both DEAE- and carboxymethyl celluloses had given strong indication that multiple peaks of enzymatic activity obtained by these methods were most likely artifacts and not real isoenzymes. It had neither been possible to reproduce identical elution profiles nor had conditions been found at that time under which a previously single peak would not elute as multiple species on rechromatography. The project was therefore discontinued. However, diverging results have recently been published by Yoshida and Carter (9), who concluded from their data that phosphoglucone isomerase isolated from rabbit skeletal muscle can be resolved into what they feel are genuine isoenzymes with different primary structures.

These authors reported (9) that they could resolve a commercial preparation of crystalline rabbit muscle phosphoglucone isomerase into three active enzyme peaks (and one inactive protein peak) by chromatography on carboxymethyl Sephadex with elution of the isoenzymes by means of a NaCl gradient from 0 to 0.1 M in 0.01 M phosphate buffer of pH 6.8. Their elution profile is redrawn in the top portion of Fig. 1. They also state that both their major components (“Isomerase I” and “Isomerase II”) on rechromatography under the same conditions eluted in the same positions as observed for the first chromatography (9). Numerous attempts in this laboratory to reproduce their separation pattern have failed with either two or five times

3 K. D. Schnackerz, M. N. Blackburn, C. Mahany, and E. A. Noltemann, unpublished experiments, 1968.

4 Attempts made by this laboratory to obtain a sample of the enzyme preparation used by Yoshida and Carter were unsuccessful. According to information from the company quoted by these authors as the source of their preparation, the original supplier made only one delivery after which he discontinued manufacture of the enzyme.
crystallized enzyme or with muscle extract as starting material for the first chromatography. Examples of these attempts are presented in the middle and bottom portions of Fig. 1, showing chromatography of twice crystallized enzyme and rechromatography of the peak with enzymatic activity, respectively, all runs being made exactly under the conditions of column size, buffer, pH, and NaCl gradient as described by Yoshida and Carter (9). It is apparent that chromatography under these particular conditions failed to produce any significant resolution of activity peaks.

Resolution of Multiple Enzyme Species with 6 to 12 mm Sodium Phosphate Gradient—After it was found impossible to reproduce the chromatographic patterns of Yoshida and Carter, a systematic study of the elution behavior of rabbit muscle phosphoglucose isomerase was begun in which very shallow sodium phosphate gradients of different pH were explored. This led to the conditions illustrated in Fig. 2, i.e. equilibration of the column, application of the protein sample, and subsequent washing of the column with 6 mm sodium phosphate (9 mm in Na⁺), pH 6.90, followed by a sodium phosphate gradient from 6 mm (9 mm...
in NaOH to 12 nm (8 5 mm in NaOH) of the same pH. The system was found to be extremely sensitive to pH. Whereas pH values of 7.0 or higher would not allow the enzyme to be initially adsorbed, values of pH 6.8 or lower would yield progressively poorer resolution of the activity peaks. The upper portion of the figure shows the elution profile of twice crystallized enzyme, prepared by the standard isolation procedure (8). Except for small amounts of activity which were eluted whenever larger quantities of inert protein appeared in the effluent (in the initial "wash-through" and in the final NaCl wash to strip the column), phosphoglucoisomerase activity appeared in two peaks about half-way through the gradient, designated as G-I and G-II. 

On rechromatography of the pooled fractions of Peak G-II, enzyme activity was again resolved into the two major peaks, G-I and G-II, with some indication of splitting of G-I into separate components. Thus, enzyme form G-II when pooled, concentrated, and rechromatographed without additional protective measures, will be partially converted to form G-I. (This is in contrast to the findings of Yoshida and Carter (9) who state that on rechromatography their "isoenzymes" remain single peaks.) Elution patterns analogous to those shown in Fig. 2 were also obtained when rabbit muscle extract, prepared according to the standard isolation procedure (8), was chromatographed in the same system. Except for some phosphoglucoisomerase which eluted with a huge amount of inert protein in the initial "wash-through" peak (for explanation, see below), the majority of the activity appeared in the elution profile where the G-I and G-II peaks are observed for the crystalline enzyme.

**Table I**

**Amino acid analysis of various chromatographic forms of skeletal muscle phosphoglucoisomerase**

| Amino acid | Peak G-I A (3 analyses) | Peak G-IB (3 analyses) | Peak G-II (11 analyses) | Five times crystallized enzyme, extrapolated values (from Reference 6) | "Isomerase II" calculated from Yoshida and Carter (9) | "Isomerase I" calculated from Yoshida and Carter (9) | Phosphoglucoisomerase of human muscle calculated from Carter and Yoshida (12) |
|------------|------------------------|-----------------------|------------------------|-------------------------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------------------------|
| Aspartic acid | 121 ± 2.4             | 119 ± 2.4             | 117 ± 2.2             | 116 ± 1.8             | 116 ± 1.8             | 116 ± 1.8             | 118 ± 1.8             |
| Threonine   | 73.0 ± 3.6             | 76.6 ± 1.4            | 74.9 ± 2.0            | 81.8 ± 3.4            | 75.9 ± 2.4            | 69.8 ± 3.4            | 79.9 ± 2.4            |
| Serine      | 73.8 ± 4.4             | 76.4 ± 2.2            | 74.8 ± 3.0            | 76.6 ± 2.2            | 70.8 ± 2.2            | 66.2 ± 2.2            | 65.8 ± 2.2            |
| Glutamic acid | 128 ± 8.8              | 129 ± 3.8             | 128 ± 3.2             | 127 ± 1.4             | 122 ± 1.4             | 116 ± 1.4             | 135 ± 1.4             |
| Proline     | 47.8 ± 2.8             | 50.2 ± 2.4            | 53.2 ± 2.8            | 50.8 ± 2.4            | 53.2 ± 2.4            | 44.0 ± 2.4            | 43.8 ± 2.4            |
| Glycine     | 92.6 ± 3.4             | 91.8 ± 1.6            | 88.4 ± 2.4            | 87.9 ± 1.0            | 86.8 ± 1.0            | 89.6 ± 1.0            | 89.0 ± 1.0            |
| Alanine     | 70.4 ± 5.0             | 70.4 ± 0.4            | 80.0 ± 1.2            | 84.3 ± 1.6            | 74.8 ± 1.6            | 88.2 ± 1.6            | 85.0 ± 1.6            |
| Valine      | 70.2 ± 1.8             | 68.2 ± 3.2            | 68.4 ± 1.4            | 68.0 ± 2.0            | 70.2 ± 1.4            | 80.0 ± 2.0            | 61.6 ± 2.0            |
| Methionine  | 28.2 ± 0.4             | 28.0 ± 0.6            | 29.2 ± 0.8            | 28.5 ± 1.3            | 26.8 ± 1.3            | 27.0 ± 1.3            | 26.2 ± 1.3            |
| Isoleucine  | 62.2 ± 1.4             | 64.6 ± 2.2            | 67.4 ± 1.0            | 68.4 ± 2.8            | 66.6 ± 2.8            | 61.6 ± 2.8            | 59.8 ± 2.8            |
| Leucine     | 113 ± 1.8              | 113 ± 2.0             | 115 ± 1.2             | 115 ± 1.0             | 115 ± 1.0             | 115 ± 1.0             | 112 ± 1.0             |
| Tyrosine    | 24.6 ± 0.6             | 23.6 ± 1.4            | 23.0 ± 0.8            | 24.4 ± 0.6            | 25.0 ± 0.6            | 28.6 ± 0.6            | 26.8 ± 0.6            |
| Phenylalanine | 56.0 ± 1.8             | 59.8 ± 1.6            | 61.2 ± 1.4            | 60.6 ± 1.4            | 58.6 ± 1.4            | 54.6 ± 1.4            | 59.6 ± 1.4            |
| Lysine      | 86.8 ± 2.2             | 86.2 ± 3.2            | 81.0 ± 2.6            | 81.6 ± 2.2            | 82.8 ± 2.2            | 97.4 ± 2.2            | 78.2 ± 2.2            |
| Histidine   | 48.8 ± 0.6             | 46.0 ± 1.0            | 47.8 ± 1.8            | 47.4 ± 0.8            | 45.2 ± 0.8            | 45.8 ± 0.8            | 45.0 ± 0.8            |
| Arginine    | 45.8 ± 0.6             | 43.6 ± 1.6            | 42.4 ± 1.6            | 42.5 ± 0.6            | 45.0 ± 0.6            | 45.8 ± 0.6            | 64.6 ± 0.6            |

a Values represent recoveries after 20 hours of hydrolysis in 6 N HCl. The deviations are the average of the absolute deviations from the mean.

b Because of the difficulty of obtaining samples of Peak G-I A, analyses had to be performed at the limit of sensitivity of the analytical method. The values in this column must therefore be considered to be less reliable than those for Peaks G-IB and G-II.

c Values for the five times crystallized enzyme were calculated for a molecular weight of 132,000 from previously published data (6). They represent the best data from a study of the recovery of the individual amino acids as a function of hydrolysis time. The deviations for this column are the maximum values that have been found for any set of analyses performed on protein hydrolysates for any one single time period.

d Although Yoshida and Carter (9) and Carter and Yoshida (12) have given values for the molecular weight of rabbit muscle and human muscle phosphoglucoisomerase differing slightly from the value of 132,000 obtained in this laboratory (6), the latter is used here for all of the species to allow a direct comparison of the respective amino acid analyses.

e Isoleucine values are not corrected for the small amount (approximately 2%) that is converted to alloisoleucine (6).
of Fig. 4. The uppermost profile represents the chromatogram of the original extract prepared in the presence of 5 mM DTT and dialyzed and chromatographed at a 1 mM concentration of the thiol. This figure also provides an example of the experimental problems encountered in chromatographing enzyme species comprising only a small fraction of the total protein applied to the column (in this case, CM-Sephadex). Part of the enzyme protein, either unspecifically bound to or merely swept along with the mass of protein not adsorbed to the ion exchanger but eluted from the column in the initial “wash-through,” appeared as an artificial activity peak. On rechromatography, however, with substantially less total protein present on the column, the enzyme activity was located in the normal Peak G-II position, preceded by some activity in the G-I region (midportion of Fig. 4). This may be interpreted as indicating that, there was still a small amount of the enzyme present in the original extract, that had not been reduced. The bottom portion of Fig. 4 finally shows that the G-II form of the enzyme remains a single discrete species if it is kept in contact with the thiol reagent.

Chromatography of Five Times Crystallized Rabbit Muscle Phosphoglucose Isomerase—After the studies described in the preceding sections had shown that variable mixtures of chromatographically distinct enzyme species can be obtained from both crude muscle extract and two times crystallized phosphoglucone isomerase, it was of paramount interest to investigate the chromatographic pattern of the five times crystallized rabbit muscle enzyme which had served as the standard source material for all of the chemical and physical studies previously reported from this laboratory. To achieve maximal resolution, buffer volumes and column dimensions were modified (for details, see legends to figures) to further decrease the Na⁺ gradient. As a result, Peak G-I resolved into two subfractions, G-IA and G-IB, well separated from the main peak, G-II (Fig. 5). The chromatogram shown in Fig 5 was obtained without DTT and reflects the distribution of the various species in a routine preparation of fifth crystals of phosphoglucone isomerase, i.e. 17, 21, and 62% for Fractions G-IA, G-IB, and G-II, respectively. Approximate average contributions of the three forms estimated for the various analyzed preparations ranged from 7 to 17% for Fraction G-IA, 15 to 25% for Fraction G-IB, and 60 to 75% for Fraction G II.

Resolution and Interconversion of Fractions G-IA, G-IB, and G-II from Partially Purified Muscle Extract—To provide larger quantities especially of Fractions G-IA and G-IB for more detailed chemical analysis, the standard phosphoglucone isomerase preparation (8) was carried through the first two steps, i.e. to the end of the ammonium sulfate fractionation, and then chromatographed on a column (5 x 41 cm) of CM-Sephadex with a sodium phosphate gradient of pH 6.90, 12 to 36 mM in Na⁺ (Fig. 6A). The effluent fractions were pooled as indicated in the figure and concentrated by membrane ultrafiltration. The combined (G-IA)/(G-IB) pool was divided into two equal portions, one of which was kept in 10 mM DTT for 6 days, the other of which was rechromatographed without further treatment and without DTT present during chromatography. The thiol-treated sample was dialyzed and chromatographed under the same conditions except for the presence of 1 mM DTT. The untreated, combined G-I pool yielded the elution profile shown in Fig. 6B, which completely lacks a G-II peak. In contrast,

**Table II**

| CM-Sephadex column | Without SDS | With SDS | Without SDS | With SDS | Without SDS | With SDS |
|---------------------|------------|----------|-------------|----------|-------------|----------|
| 53                  | 4.9        | 8.1      | 6.2         | 11.5     | 6.3         | 13.3     |
| 54                  | 4.5        | 10.1     | 4.6         | 11.1     | 6.3         | 13.3     |
| 56                  | 2.7        | 7.1      | 4.0         | 10.6     | 5.7         | 13.1     |
| 57                  | 4.9        | 9.5      | 5.5         | 12.5     | 5.5         | 12.3     |
| 58                  | 5.0        | 12.4     | 5.3         | 12.8     | 5.3         | 12.8     |
| 59                  | 6.0        | 11.5     | 4.1         | 12.1     | 4.6         | 12.6     |
| 60                  | 4.0        | 10.8     | 3.8         | 11.5     | 4.8         | 12.4     |
| 61                  | 4.1        | 11.6     | 4.5         | 12.6     | 4.7         | 12.2     |
| 62                  | 3.3        | 9.6      | 3.6         | 11.1     |
| 63                  | 3.1        | 9.2      | 3.4         | 10.6     |
| Average             | 3.8 ± 0.5  | 9.8 ± 1.1| 4.5 ± 0.6   | 11.5 ± 0.6| 5.5 ± 0.4  | 12.9 ± 0.4|

a Sodium dodecyl sulfate.
exposure of the same material to DTT resulted in approximately 30% conversion of G-I species to the reduced G-II form (Fig. 6C). The G-II pool was made 1x with DTT, dialyzed, and kept in that medium for 12 days, then rechromatographed in the presence of the thiol. It yielded a single peak in the G-II position (Fig. 6D), indicating that the reductive conditions had prevented any conversion into faster eluting species.

**Amino Acid Composition of Chromatographically Separated Enzyme Species**—Although the conversion experiments left little doubt that the various chromatographic species could not differ substantially in their primary structure, it was felt necessary to perform amino acid analysis on the individual enzyme species to counteract Yoshida and Carter's claim (9) that the resolved phosphoglucose isomerase "isoenzymes" have different amino acid compositions. Numerous amino acid analyses were carried out under rigorously controlled conditions (6); the results are summarized in Table I. The data show remarkably small deviations both among each other and when compared with the amino acid analysis of the five times crystallized enzyme previously reported (6). Certainly, the differences are not large enough to warrant the postulate that the various chromatographic forms possess different primary structures.

**Sulphydryl and Total Half-Cystine Content**—The pronounced effect of dithiothreitol on the distribution pattern of the chromatographic fractions as well as its ability to convert the G-I chromatographic species to the G-II form suggested that the varying extents of oxidation of sulphydryl groups might be responsible for the different chromatographic behavior. Numerous sulphydryl analyses were therefore performed on the three chromatographic forms isolated from a substantial number of different preparations and the results are shown in Table II. In addition, aliquots of the three forms from several chromatographic experiments were also subjected to total half-cystine analysis to ascertain whether a priori differences exist in the total cysteine-cystine content. These data are summarized in Table III. The results of both types of analysis are presented in their entirety, rather than only as averages with their deviations, to illustrate the difficulty of obtaining representative measurements. Although the final averages demonstrate convincingly a difference in the number of titrable sulphydryl groups, it is also apparent that this conclusion can only be drawn from the sum of the experiments, whereas a particular single analysis might not always conform to the pattern. Even though all possible precautions were taken to perform the sulphydryl analyses under conditions which would minimize oxidation after chromatographic isolation and during the manipulations required for the assay, a disconcerting scatter of the data could not be avoided. Nevertheless, it is clear from Tables II and III that (a) the various chromatographic species do not differ in their total half-cystine content, (b) the number of -SH groups accessible to PMB in the native state increases by about 1 per molecule in the order G-I, G-II, G-II, and (c) the number of -SH groups that can be titrated in the denatured enzyme species increase in the same order by about 2 per molecule. Notably, for the G-II fraction the number of total titrable -SH groups is equal to the total half-cystine content.

From an analytical point of view, it is gratifying to note that this detailed study of the sulphydryl and total half-cystine content confirmed the routine analyses previously made on five times crystallized rabbit muscle phosphoglucose isomerase, which contained variable amounts of the three chromatographic species (depending upon the extent of sulphydryl oxidation inadvertently produced in the course of isolation). A total cysteine content of 12.5 residues per molecule by PMB assay and of 11.5 (12.2 after correction) after performic acid oxidation had previously been reported for the five times crystallized enzyme (6).

**Isoelectric Focusing Experiments**—To characterize the individual enzyme species in terms of their over-all net charge, pooled mixtures as well as individual chromatographic species were subjected to isoelectric focusing. Fig. 7 represents a typical elution profile obtained on focusing a mixture of the three species in an ampholyte pH gradient from pH 7 to 9. The isoelectric points measured by this technique were within the ranges of 8.05 ± 0.10 for Fraction G-I, 8.25 ± 0.15 for Fraction G-II, and 8.50 ± 0.05 for Fraction G-II. For comparison, the isoelectric point of five times crystallized rabbit muscle enzyme had previously been determined to be 8.5 (extrapolated to zero ionic strength) by free boundary electrophoresis in various buffers as a function of ionic strength (4). A notable observation made during isoelectric focusing was that the experimental conditions apparently induced oxidation of the enzyme species. In the absence of added DTT, a larger percentage of the G-I species was always found on elution from the ampholyte column than would be expected from the known composition of the mixture applied to the column. On the other hand, addition of DTT to the am-
mice (21, 22), and fish (23). In these instances, however, heavy species and tissues, e.g. human (17-19) and rabbit blood (20), this paper for the rabbit skeletal muscle enzyme. Claims for the other cases, multiple forms may merely be artifacts, as shown in genuine isoenzymes (e.g. brewers' yeast (16)) but for which, in some organisms, may appear in the form of controlled conditions of extraction and storage.

phoretically obtained multiple activity bands with genetically preceding tissue work-up and storage procedures (14). An multiple electrophoretic bands with little or no consideration isoenzymes is derived solely from activity staining patterns of overlooked when evidence for postulating genetically different logical" conditions. This prerequisite, however, is frequently protein species which are not interconvertible under "physiological" conditions. This exception of arginine, almost within the experimental error to be

isoenzymes appear to be the example of an enzyme deserves to be called isoenzymes as is old as the observation that the same enzymatic activity may be associated with different physically separable protein species. The semantic aspects of the problem have been amply reviewed (for an informative appraisal refer, for example, to Wieme (13)), but the main controversy has remained, i.e. disagreement concerning a satisfactory definition for characterizing an "isoenzyme." Independent of which doigma one wishes to follow, the one single criterion that would appear to be essential is that isoenzymes must be stable, isolatable protein species which are not interconvertible under "physiological" conditions. This prerequisite, however, is frequently overlooked when evidence for postulating genetically different isoenzymes is derived solely from activity staining patterns of multiple electrophoretic bands with little or no consideration for possible artifacts created by the technique itself or by the preceding tissue work-up and storage procedures (14). An admirable exception is the report by Kirkman and Hanna (15) who caution against the practice of uncritically equating electrophoretically obtained multiple activity bands with genetically determined isoenzymes since the former may originate from "uncontrolled conditions of extraction and storage.

Phosphoglucose isomerase appears to be the example of an enzyme which, in some organisms, may appear in the form of genuine isoenzymes (e.g. brewers' yeast (16)) but for which, in other cases, multiple forms may merely be artifacts, as shown in this paper for the rabbit skeletal muscle enzyme. Claims for the existence of isoenzymes have been made for a number of other species and tissues, e.g. human (17-19) and rabbit blood (20), mice (21, 22), and fish (23). In those instances, however, heavy reliance was placed on activity staining without further characterization of the various protein species. To what extent these electrophoretic bands correspond to real isoenzymes, as opposed to artifacts, will have to await further study. It is interesting to note that aging of mammalian phosphoglucone isomerase preparations had previously been observed to generate new activity bands in starch gel electrophoresis, which could be made to disappear by addition of β-mercaptoethanol (9, 20). These bands were considered to be the result of sulphydryl oxidation, and it was suggested that a heat-stable protein factor might be responsible for their production (9).

No explanation is forthcoming for the difference between the data presented here and those of Yoshida and Carter (9). It has not been possible to repeat their chromatographic separation under the conditions described in their paper. From the behavior of rabbit muscle phosphoglucone isomerase in the chromatographic systems developed in this laboratory it may be speculated that their "Isoenzyme I" could have been contaminated by inert protein since their gradient was much steeper and must have had less resolving power than the one described here. This possibility would appear to be supported by two points.

(a) The amino acid composition reported by Yoshida and Carter for their "Isoenzyme II" is somewhat similar to that found for any of the species analyzed in this work, whereas the data for their "Isoenzyme I" are substantially different (cf. Table I).

(b) On starch gel electrophoresis in the presence of 8 M urea they observed two bands for "Isoenzyme I" but only one for "Isoenzyme II" (with use of protein stain!) which they interpreted to indicate that "Isoenzyme II" is composed of two identical subunits, whereas "Isoenzyme I" has dissimilar subunits (9). However, the second band in the case of "Isoenzyme I" could have equally been a protein contamination.

Another quite puzzling feature is that the differences in amino acid compositions reported by Yoshida and Carter for their two "isoenzymes" (9) are larger than those between human muscle phosphoglucone isomerase published by them (12) and the rabbit muscle enzyme found in this laboratory (cf. Table I and Reference 6). In fact, the agreement between man and rabbit muscle enzyme regarding all those amino acids that can be reliably determined by the limited number of analyses performed for the human muscle enzyme by Carter and Yoshida (12) is, with the exception of arginine, almost within the experimental error to be expected between duplicates of the same protein.

All of the evidence found in this laboratory indicates that the three chromatographically separable forms of rabbit muscle phosphoglucone isomerase do not qualify as isoenzymes: (a) they do not remain single species on rechromatography; (b) they are interconvertible on treatment with either dithiothreitol or exposure to oxygen; (c) they have approximately the same specific activity; (d) their over-all amino acid compositions are indistinguishable within the precision of the analyses; and (e) if the entire isolation procedure is performed in the presence of dithiothreitol, only one chromatographic species is obtained. Aside from the differences in net charge which are reflected in small differences in the isoelectric pH and which determine the different elution volumes on ion exchange chromatography as well as the different positions in the pH gradient attained on isoelectric focusing, the only property found by which the individual enzyme species could be distinguished was a different accessibility to PMB and variation in the extent of oxidation of their sulphydryl groups. The total half-cystine content, however, was found to

Fig. 7. Effluent profile of rabbit muscle phosphoglucone isomerase pseudoenzymes after isoelectric focusing in an ampholyte pH gradient from pH 7 to 9. A mixture of enzyme species G-IA, G-IB, and G-II, previously isolated by chromatography on CMSephadex, was dialyzed against water to make it isionic. An aliquot corresponding to a total of 10 mg of protein was then added to the "light solution" used for creating the ampholyte-acrocose gradient. After focusing for 96 hours at 600 volts, the contents of the columns were removed and assayed as described under "Experimental Procedure."
be the same in all three forms and equal to the maximally available sulfhydryl groups of the most extensively reduced species. These findings are analogous to those reported by Morikofe-Zwez et al. (24) for three fractions of human and horse erythrocyte catalase. These authors found similarly characteristic differences in chromatographic behavior and accessibility of sulfhydryl groups to PMB which they attributed to secondary alterations of the enzyme molecule during purification.

It is therefore concluded that the different chromatographic species of rabbit muscle phosphoglucose isomerase represent different states of the same enzyme protein and not, as implied by Yoshida and Carter (9), genuinely different isoenzymes with genetically different primary structures. To that extent, they are artifacts and thus appropriately designated as "pseudoisoenzymes." It should finally be stressed that the renewed evidence presented here for the primary chemical homogeneity of chromatographically prepared rabbit muscle phosphoglucose isomerase supports the validity of previous studies performed with the five times crystallized preparation.

REFERENCES

1. Chatterjee, G. C., and Noltmann, E. A. (1967) J. Biol. Chem., 242, 3440.
2. Chatterjee, G. C., and Noltmann, E. A. (1967) Eur. J. Biochem., 2, 9.
3. Dyson, J. E. D., and Noltmann, E. A. (1968) J. Biol. Chem., 243, 1401.
4. Dyson, J. E. D., and Noltmann, E. A. (1969) Biochemistry, 8, 3333.
5. Dyson, J. E. D., and Noltmann, E. A. (1969) Biochemistry, 8, 3544.
6. Pon, N. G., Schnackerz, K. D., Blackburn, M. N., Chatterjee, G. C., and Noltmann, E. A. (1970) Biochemistry, 9, 1506.
7. Noltmann, E. A. (1964) J. Biol. Chem., 239, 1545.
8. Noltmann, E. A. (1966) Methods Enzymol., 9, 557.
9. Yoshida, A., and Carter, N. D. (1969) Biochim. Biophys. Acta, 194, 151.
10. Boyer, P. D. (1954) J. Amer. Chem. Soc., 76, 4331.
11. Moore, S. (1963) J. Biol. Chem., 238, 235.
12. Carter, N. D., and Yoshida, A. (1969) Biochim. Biophys. Acta, 181, 12.
13. Wieme, R. J. (1968) in N. Van Thoai and J. Roux (Editors), Homologous enzymes and biochemical evolution, p. 19, Gordon and Breach, Publishers, New York.
14. Noltmann, E. A. (1972) in P. D. Boyer (Editor), The Enzymes, Vol. VI, Ed. 3, p. 271, Academic Press, New York.
15. Kirkman, H. N., and Hanna, J. E. (1968) Ann. N. Y. Acad. Sci., 151, 133.
16. Nakagawa, Y., and Noltmann, E. A. (1967) J. Biol. Chem., 242, 4782.
17. Schwartz, M. K., and Bodansky, O. (1966) Amer. J. Med., 40, 231.
18. Detter, J. C., Ways, P. O., Giblett, E. R., Baughan, M. A., Hopkinson, D. A., Povet, S., and Harkie, H. (1968) Ann. Hum. Genet., 31, 329.
19. Fitch, L. I., Parr, C. W., and Welch, S. G. (1968) Biochem. J., 110, 50F.
20. Welch, S. G., Fitch, L. I., and Parr, C. W. (1970) Biochem. J., 117, 525.
21. Carter, N. D., and Parr, C. W. (1967) Nature, 215, 511.
22. Delorenzzi, R. J., and Ruddle, F. H. (1969) Biochem. Genet., 3, 151.
23. Scopes, R. K., and Gosselin-Rey, C. (1968) J. Fish. Res. Bd. Canada, 25, 2715.
24. Morikofe-Zwez, S., Cantz, M., Kaufmann, H., von Waart-Burg, J. P., and Aebi, H. (1969) Eur. J. Biochem., 11, 49.
Pseudoisoenzymes of Rabbit Muscle Phosphoglucone Isomerase
Michael N. Blackburn, John M. Chirgwin, Gordon T. James, Thomas D. Kempe, Thomas F. Parsons, Adele M. Register, Klaus D. Schnackerz and Ernst A. Noltmann

*J. Biol. Chem.* 1972, 247:1170-1179.

Access the most updated version of this article at [http://www.jbc.org/content/247/4/1170](http://www.jbc.org/content/247/4/1170)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/247/4/1170.full.html#ref-list-1](http://www.jbc.org/content/247/4/1170.full.html#ref-list-1)