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Enzyme Patterns in Single Human Muscle Fibers*

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Individual muscle fibers from the biceps of five individuals were analyzed for 10 different enzymes. For each person, lactic dehydrogenase and adenylokinase were first measured in 50 to 100 fibers. From each large group, 20 or more fibers, representing the full range of enzyme activity, were analyzed in duplicate for the other eight enzymes (glycogen phosphorylase, P-fructokinase, pyruvate kinase, glycero-P dehydrogenase, citrate synthase, fumarase, β-hydroxyacyl-CoA dehydrogenase, and creatine kinase). On the basis of the enzyme patterns, two clearly defined fiber types and several subtypes could be distinguished. The high lactic dehydrogenase group (type II) had high levels of other glycogenolytic enzymes and low levels of mitochondrial enzymes; the low lactic dehydrogenase group (type I) had the reverse enzyme complement.

The high lactic dehydrogenase fibers consisted of two partly overlapping subgroups, containing high or medium adenylokinase and low or medium levels of mitochondrial enzymes. The low lactic dehydrogenase fibers, although not so clearly separable into distinct subgroups, were heterogeneous in respect to enzymes of glycogenolysis, which varied in a coordinate manner over a wide range of values. In contrast, they were comparatively homogeneous with respect to the mitochondrial enzymes. No consistent correlation was seen between creatine kinase activities and fiber type. The results demonstrate the presence among neighboring muscle fibers of a broad spectrum of highly coordinated enzyme activities and illustrate the advantages of measuring many enzymes on the same individual fibers.

During development skeletal muscle fibers differentiate into at least three distinct types which vary in speed of contraction and enzyme composition. These may be designated slow twitch red (type I), fast twitch red (type IIA), and fast twitch white (IIB). Various other names have also been applied (1-12). (Naming the fiber types presents difficulties (8, 9).) Although most muscles contain an intimate mixture of all three fiber types, in certain muscles one or another type predominates. Enzyme analyses of these specialized muscles have shown that type I fibers are adapted to aerobic metabolism of fats and carbohydrates, type IIB fibers are suited to anaerobic glycolysis, and type IIA fibers display both capacities.

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Such analyses of whole muscles indicate the average composition of the major fiber types, but mask individual differences in enzyme composition and possible subgroupings within the types. It, therefore, seemed important to analyze individual fibers for representative enzymes of glycolytic and oxidative pathways. This would also permit correlation of enzyme levels on a fiber to fiber basis and provide an unusual opportunity to observe the range and pattern of phenotypic expression within a single differentiated cell class.

For this pupose a methodology was developed for measuring the enzyme composition of individual human muscle fibers. It combines microchemical techniques from this laboratory with a convenient method of fiber dissection developed by Essén *et al.* (13). With these procedures it is possible to perform assays for as many enzymes as desired on replicate samples from a single fiber. The results indicate a remarkable range in composition among normal fibers, with fairly stringent restrictions concerning co-variation among different enzymes.

EXPERIMENTAL PROCEDURES

The muscle was obtained by open biopsy from the biceps and frozen immediately in liquid N_2 brought to its freezing point by partial evaporation under vacuum. (This increases speed of freezing by minimizing bubble formation on contact.) Portions of the biopsy, roughly 1 mm in diameter and 5 to 10 mm long, were dried under vacuum at −40°C. These were brought to room temperature under vacuum, and single fibers were teased apart at room temperature with the aid of stiff hair loops and razor blade knives (Ref. 14, p. 231). Each fiber (usually 2 to 5 mm long) was then stored separately in a 16-hole aluminum holder (an aluminum plate with holes drilled through and sandwiched between two microscope slides (Ref. 14, p. 225)). The surfaces of the aluminum plate were ground very flat with fine carborundum to prevent loss of fibers when vacuum is applied for storage. Before freeze-drying, the biopsy samples were stored at −70°C. After drying, samples and single fibers (in the above holders) were stored under vacuum in glass vacuum tubes at −70°C. (Storage at −25°C would probably be adequate.)

At the time of analysis, the single fibers were warmed to room temperature under vacuum. Samples were cut off one end with a razor blade knife, weighed on a quartz fiber balance (Ref. 14, p. 236), and analyzed. The average sample weighed 15 ng; this is the equivalent of about a 20-μm slice of the fiber (usually a 30- to 50-μm slice cut in half). Thus, a fiber 2 mm long could provide up to 100 samples for as many different purposes. The remainder of each fiber was returned to low temperature vacuum storage. Exposure to room air was kept to a minimum by keeping the racks under vacuum as much as possible when out of the freezer, although tests showed that none of the enzymes studied thus far is very sensitive to room air (see below).

All but one of the enzymes used in the analyses were obtained from Sigma Chemical Co. or Boehringer Corp. The AMP aminohydrolase (EC 3.5.4.6) was kindly supplied by Dr. Barry Ashby (Department of Biochemistry, Washington University, St. Louis, Mo.) who prepared it according to the procedure of Smiley *et al.* (15).

Analytical Methods

General—The initial analytical step in all cases was conducted in a small droplet under oil in an "oil well rack." (For specific details of

the "oil well technique," including loading of samples, see Ref. 14, p. 253.) The sample was loaded through the oil, either into the complete reagent or in most cases into a preincubation reagent with the complete reagent added 30 to 60 min later. Preincubation presumably increases accessibility of the reagent to the enzyme (see below). The reaction was stopped by adding a measured volume of acid or alkali 60 min after sample and complete reagent had been brought together. Incubations were conducted at dissecting room temperature (about 20°C). Activities were corrected to exactly 20°C when necessary by temperature factors determined for each enzyme. All of the methods are based directly or indirectly on pyridine nucleotide enzyme reactions. In some cases the enzyme to be measured had sufficient activity so that the reduction or oxidation of the nucleotide could be measured directly. In the other cases, the reduced or oxidized nucleotide had to be amplified by enzymatic cycling.

The need for preincubation and the best composition of the preincubation reagent was determined by preliminary experiments with samples from single fibers treated in alternative ways. With some enzymes the results were quite low and erratic if the preincubation was omitted. This was especially true for glycogen phosphorylase and citrate synthase. In general the problem seemed to be that muscle samples do not disintegrate in aqueous droplets the way most tissues do. Therefore, the preincubation reagent contained 0.6 M KCl to dissolve the myosin and sometimes was made more alkaline than the specific reagent to favor solution of other proteins. Triton X-100 was not helpful with any of the enzymes studied here.

Table I is a flow sheet for the methods. The composition of complex reagents and notes on individual methods are as follows.

Pyruvate Kinase (EC 2.7.1.40)—The preincubation reagent contained 50 mM imidazole-HCl buffer, pH 7.0, 0.6 M KCl, and 0.05% bovine serum albumin. This can be stored at −70°C. The specific reagent contained the same buffer with 0.05% bovine serum albumin, 0.1 M KCl, 3 mM $MgCl_2$, 1 mM ADP, 10 mM 5′-AMP, 1 mM P-pyruvate, 500 μM NADH, and 2 μg/ml of beef heart lactic dehydrogenase. The reagent minus the last three ingredients can be stored at −70°C. The 5′-AMP was added to the reagent to block adenylokinase activity.

P-fructokinase (EC 2.7.1.11)—The specific reagent contained 50 mM Tris-HCl buffer, pH 8.1, 1 mM fructose-6-P, 1 mM ATP, 2 mM $MgCl_2$, 10 mM K_2HPO_4, 1 mM 5′-AMP, 150 μM NADH, 1 mM β-mercaptoethanol, and 10 μg/ml each of muscle aldolase (EC 4.1.2.7), triose-P isomerase (EC 5.3.1.1), and glycero-P dehydrogenase (EC 1.1.1.8). The reagent minus NADH, mercaptoethanol, and auxiliary enzymes can be stored at −70°C.

Preincubation in reagent with 0.6 M KCl added, but fructose-6-P and enzymes omitted, gave 20% lower results than if samples were added directly to the specific reagent without preincubation.

Lactic Dehydrogenase (EC 1.1.1.27) and Adenylokinase (EC 2.7.4.3)—These enzymes were measured on the same sample with a combined reagent. The preincubation reagent was the same as for pyruvate kinase. The specific reagent contained 100 mM imidazole-HCl buffer, pH 7.0, 0.05% bovine serum albumin, 2 mM sodium pyruvate, 300 μM NADH, 3 mM ADP, 2 mM $MgCl_2$, 2 mM glucose, 10 μg/ml of yeast hexokinase (EC 2.7.1.1), and 20 μg/ml of AMP aminohydrolase (EC 3.5.4.6). The indicator reagent ("G6P rgt," Table I) for adenylokinase contained 50 mM Tris-HCl buffer, pH 8.1, 50 μM $NADP^+$, 1 mM EDTA, 0.2 μg/ml of baker's yeast glucose-6-P dehydrogenase (EC 1.1.1.41), and 1 μg/ml of muscle P-glucoisomerase (EC 5.3.1.9). The isomerase was added so that any glucose-6-P converted to fructose-6-P during the specific reaction step would be converted back to glucose-6-P and hence to 6-P-gluconate.

The AMP aminohydrolase was added to the specific reagent to prevent product inhibition. The use of a combined specific reagent did not affect adenylokinase activity, but caused a 6% decrease in lactic dehydrogenase activity. As others have stressed (17), during storage NADH solutions may accumulate an inhibitor of lactic dehydrogenase. To avoid this we used a stock 25 mM NADH solution in 100 mM carbonate buffer, pH 10.6, stored in small quantities at −70°C. Each portion was heated 5 min at 95°C before use (to eliminate any NAD^+) and afterwards discarded.

Creatine Kinase (EC 2.7.3.2)—The preincubation reagent contained 100 mM imidazole acetate buffer, pH 7.0, 0.6 M KCl, 0.05% bovine serum albumin, and 0.5 mM dithiothreitol. More concentrated dithiothreitol made loading difficult due to a reaction with the albumin which caused the surface of the 1-μl droplet to become very "sticky." The specific reagent contained the same buffer with 25 mM P-creatine, 1 mM ADP, 10 mM $MgCl_2$, 3 mM glucose, 0.02% bovine serum albumin, 5 mM dithiothreitol, 20 mM 5′-AMP, and 20 μg/ml of yeast hexokinase. The 5′-AMP was added to block adenylokinase as proposed by Oliver (18). The indicator reagent ("G6P rgt," Table I) contained 50 mM Tris-HCl buffer, pH 8.1, 100 μM $NADP^+$, 1 mM EDTA, and 0.5 μg/ml of glucose-6-P dehydrogenase. It would have been preferable to add P-glucoisomerase, as was done for adenylokinase (see above). However, tests showed that no detectable fructose-6-P was formed during the reaction. Apparently, tissue P-glucoisomerase is sufficiently inhibited by the reagent components.

Glycogen Phosphorylase (EC 2.4.1.1)—The preincubation reagent contained 50 mM Tris-HCl buffer, pH 8.1, 0.6 M KCl, 0.2% bovine serum albumin, 1 mM 5′-AMP, 10 mM K_2HPO_4, and 0.5 mM dithiothreitol. This can be stored at −70°C. Low results were obtained without preincubation in KCl or with lower albumin concentration or if preincubation was done at pH 7 (the pH of the specific reagent) instead of at pH 8. The other ingredients were not tested exhaustively but seemed to have favorable effects.

The specific reagent contained 50 mM imidazole-HCl, pH 7.0, 0.2%

TABLE I

Protocols for the enzyme analyses

Other details, including the composition of the specific reagents, are given under "Analytical Methods." Abbreviations are β-OH-Acyl-CoA DH, β-hydroxyacyl-CoA dehydrogenase; G6P, glucose-6-P; FBP, fructose-1,6-bisphosphate; RT, room temperature; rgt, reagent; cycl, cycling; preinc, preincubation.

	Preincubation	Specific reagent	Stop reaction	Step 4 (3)	Step 5 (4)	Standards[a]
Pyruvate kinase	1 μl	10 μl	2 μl of 0.5 N HCl, 10 min RT	10 μl into 1 ml of 6 N NaOH[b]		0.5 and 1.5 mM pyruvate
P-fructokinase	No preinc	10 μl	As above	As above		5 and 10 μM FBP
Lactic dehydrogenase	1 μl	10 μl	3 μl of 0.3 N HCl, 10 min RT	6 μl into 1 ml of 6 N NaOH[b]		0.5 and 1 mM NAD^+
Adenylokinase	Same sample	Same sample	Same sample	6 μl into 1 ml of G6P rgt		2.5 and 5 mM ATP
Creatine kinase	1 μl	10 μl	2 μl of 0.5 N NaOH, 15 min RT	10 μl into 1 ml of G6P rgt		4 and 8 mM ATP
Phosphorylase	1 μl	4 μl	1 μl of 0.5 N NaOH, 20 min 80°C	1 μl into 50 μl of 1000× cycl rgt	1 ml 6PG rgt	25 and 50 μM glucose-1-P
Glycero-P-dehydrogenase	1 μl	4 μl	5 μl of 0.1 N HCl, 10 min RT	1 μl into 100 μl of 2000× cycl rgt	1 ml malate rgt	25 and 50 μM NAD^+
Citrate synthase	1 μl	3 μl	See text	1 μl into 50 μl of 1000× cycl rgt	As above	25 and 50 μM citrate
Fumarase	No preinc	1 μl	See text	2 μl into 100 μl of 500× cycl rgt	As above	50 and 150 μM malate
β-OH-Acyl-CoA DH	1 μl	10 μl	2 μl of 0.3 N HCl, 10 min RT	2 μl into 100 μl of 1000× cycl rgt	As above	30 and 60 μM NAD^+

[a] These were provided in the first reagent at the concentrations shown, in the same volume used for the samples.

[b] This contained 10 mM imidazole to prevent light sensitivity (16). The fluorescence was read after heating 20 min at 60°C.

bovine serum albumin, 50 mM (0.8%) rabbit liver glycogen, 20 mM K_2HPO_4, 0.5 mM $MgCl_2$, 1 mM 5'-AMP, 50 μM $NADP^+$, 2 μM glucose-1,6-P_2, 0.5 mM dithiothreitol, 3 μg/ml of muscle P-glucomutase (EC 2.7.5.1), and 2 μg/ml of glucose-6-P dehydrogenase. The glycogen and P_i levels are rather high compared to the Michaelis constants for rabbit muscle phosphorylase; however, reduction of glycogen to 20 mM (as glycosyl units) or reducing P_i to 5 mM each gave 25% lower results with assays on samples from the same individual frozen dried human muscle fibers. This may be the result of hindered access of the substrates to the enzyme.

Glycero-P Dehydrogenase (EC 1.1.1.8)—The preincubation reagent is the same as for pyruvate kinase. Preincubation may not be essential. However, addition of 120 mM KCl, either from the preincubation reagent or by inclusion in the specific reagent, gave about 60% higher activity. The specific reagent contained 50 mM imidazole buffer, pH 7.0, 0.05% bovine serum albumin, 200 μM dihydroxyacetone-P, and 100 μM NADH. The NADH stock solution was stored and treated as described for lactic dehydrogenase.

Citrate Synthase (EC 4.1.3.7)—The activity was determined by a procedure in which acetyl-CoA is allowed to react with oxalacetate; the oxalacetate excess is then destroyed with hot alkali and the citrate measured with citrate lyase (EC 4.1.3.6). This indirect procedure has distinct advantages. It allows the use of optimal amounts of the substrates and ends with a pyridine nucleotide step which permits amplification.

The preincubation reagent contained 50 mM 2-amino-2-methyl-1,3-propanediol, pH 8.8 (acid:base ratio 1:1), 0.25% citrate-free (19) bovine serum albumin, and 0.6 M KCl. The specific reagent contained 50 mM Tris-HCl buffer, pH 7.8 (acid:base ratio 2:1), 0.4 mM acetyl-CoA, 0.5 mM oxalacetate, and 0.25% citrate-free bovine serum albumin. (The buffer ratios and strengths of the two reagents are such as to bring the final pH to 8.1.) The stock acetyl-CoA was stored at −70°C at pH 4.7. The oxalacetate was stored at −70°C as a 1 M solution in 0.5 M HCl.

After the specific step (Table I), the reaction was stopped with 1 μl of 0.17 N NaOH and the oil rack heated at 95°C for 30 min. After cooling, 5 μl of citrate reagent were added which contained 200 mM Tris-HCl, pH 7.0 (acid:base ratio 10:1), 50 μM NADH (see "Lactic Dehydrogenase" method), 80 μM $ZnCl_2$, 0.02% bovine serum albumin, 4 μg/ml of *Aerobacter aerogenes* citrate lyase, and 1 μg/ml of beef heart malic dehydrogenase (EC 1.1.1.37). This was incubated 20 min at room temperature and then the excess NADH destroyed with 2 μl of 0.5 N HCl. After at least 10 min at room temperature, the procedure was continued according to Table I.

Preincubation conditions proved to be particularly important for reproducibility as well as maximal activity. Preincubation at pH 8 gave 40% higher results if KCl (0.6 M) was present and still higher results by 25% at pH 8.8 than at pH 8.1. The high albumin concentration (0.25% as compared to 0.05%) also increased activity and reproducibility.

Fumarase (EC 4.2.1.2)—Preincubation was omitted because it decreased results by 20 to 40% with or without KCl present. The specific reagent contained 50 mM phosphate buffer (K^+ salts), pH 7.0, 50 mM sodium fumarate, and 0.05% bovine plasma albumin. (Reducing fumarate to 25 mM gave 25% lower results with frozen dried samples although this would not be expected from the K_m, which is about 2 mM.)

The malate formed was oxidized by NAD^+ in a separate step to avoid the danger of enzymatic reoxidation of NADH. After incubation in specific reagent, 1 μl of 0.05 N NaOH was added and the oil well rack heated 20 min at 80°C. After cooling, 5 μl of malate reagent were added which contained 50 mM 2-amino-2-methyl-1-propanol buffer, pH 9.6 (acid:base ratio 2:1), 200 μM NAD^+, 10 mM glutamate, 5 μg/ml of malic dehydrogenase, and 2 μg/ml of glutamic oxalacetate transaminase (EC 2.6.1.1). This was allowed to react 20 min at room temperature, then 5 μl of 0.2 N NaOH were added followed by heating 20 min at 80°C to destroy excess NAD^+. The assay was completed according to Table I.

β-Hydroxyacyl-CoA Dehydrogenase (EC 1.1.1.35)—The preincubation reagent was the same as for pyruvate kinase. Preincubation increased measured activity about 25%. The specific reagent contained 150 mM imidazole-HCl, pH 6.0 (acid:base ratio 9:1), 50 μM NADH, 50 μM acetoacetyl-CoA, 1 mM EDTA, and 0.05% bovine serum albumin.

Effect of Freeze-drying on Enzyme Activity—We have not compared the activity of these enzymes in fresh and freeze dried muscle. However, experience over many years with brain, liver, and kidney has shown that most enzymes *in situ* are unaffected by freeze-drying. Spamer and Pette (20) tested this for lactic dehydrogenase and P-fructokinase in rabbit muscle and found no loss.

Stability of the Enzymes in Frozen Dried Muscle Fibers at 20°C—Samples from the same individual fibers were stored in the dissecting room (20°C) in air or vacuum and then assayed (Table II). Of the seven enzymes tested, only one, pyruvate kinase, showed a significant decrease (20%) during 7 days under vacuum, and only one, fumarase, showed a significant decrease (20%) after 24 h in room air. Two enzymes, lactic dehydrogenase and phosphorylase, actually gave somewhat higher values after storage for a day at 20°C under vacuum. Altogether, the results indicate that the exposure to room temperature required for preparing samples for analysis does not introduce serious errors.

Reproducibility—Fig. 1 illustrates the reproducibility for duplicate assays of 47 fibers from one individual. Each sample was analyzed for both lactic dehydrogenase and adenylokinase (see "Analytical Methods"). The standard deviations were 4% for both enzymes (expressed as percentage of the average activity). There is some sign that differences between duplicates are in the same direction for both enzymes. This may be due to small systematic errors due to weighing, or slight evaporation before aliquoting, or true differences between the samples. The methods for some of the enzymes have not quite this same degree of reproducibility, but when duplicates differed by more than 10 or 15%, the analyses were repeated.

RESULTS

Muscle Fiber Types—A large number of fibers from five

TABLE II

Storage test of muscle fiber enzymes

All of the analyses for a given enzyme were made on samples from a single fiber. Results are recorded as mol kg^{-1} (dry) h^{-1} at 20°C (± S.E.) for four samples at each storage time. Control samples were kept under vacuum at −70°C. Deviations from controls with $p \leq 0.05$ are italic. The abbreviation is β-OH-acyl-CoA, β-hydroxyacyl-CoA dehydrogenase.

	Control	1 day 20°C air	1 day 20°C vacuum	7 days 20°C vacuum
Lactic dehydrogenase	38 ± 1	41 ± 1	44 ± 2	37 ± 2
Adenylokinase	136 ± 2	136 ± 2	144 ± 5	132 ± 9
Pyruvate kinase	21.9 ± 0.6	20.9 ± 0.7	*20.0* ± 0.7	*17.7* ± 0.6
P-fructokinase	1.88 ± 0.13	1.75 ± 0.13	2.09 ± 0.25	2.28 ± 0.26
Glycogen phosphorylase	0.63 ± 0.03	0.64 ± 0.04	*0.77* ± 0.04	0.64 ± 0.02
Fumarase	2.17 ± 0.09	*2.56* ± 0.19	2.58 ± 0.35	2.93 ± 0.20
β-OH-acyl-CoA	3.10 ± 0.37	2.96 ± 0.19[a]	3.89 ± 0.27[a]	3.16 ± 0.12[a]

[a] These samples were stored at 20°C 1 day longer than the rest.

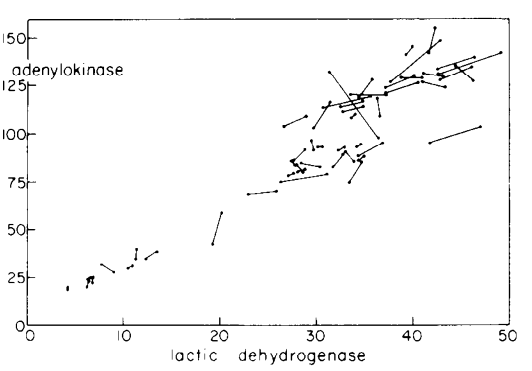

FIG. 1. Adenylokinase and lactic dehydrogenase measured on the same duplicate samples from 47 different fibers from C (see Fig. 2 legend). The lines connect the duplicates. Activities are recorded as mol kg^{-1} (dry weight) h^{-1} at 20°C.

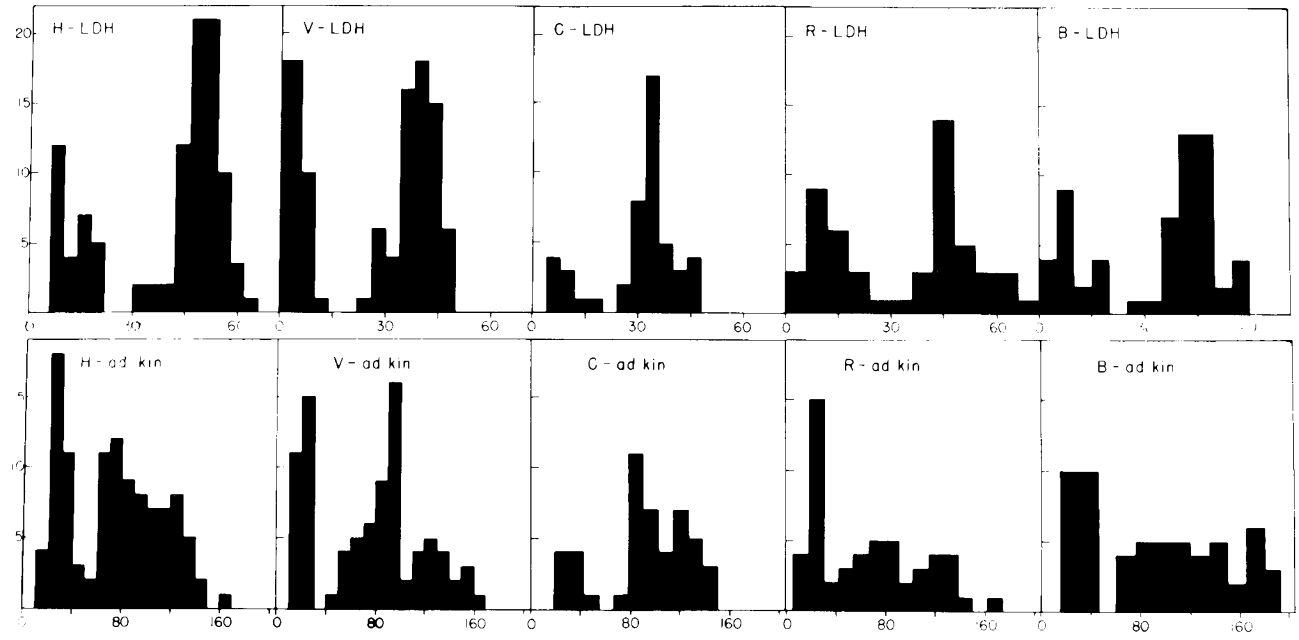

FIG. 2. Histograms of lactic dehydrogenase (*LDH*) and adenylokinase (*ad kin*) activity in single fibers from the biceps of five persons. There were about 100 fibers from the first two people and about 50 from the other three. Each fiber was analyzed in duplicate for each enzyme. Activities are recorded as mol kg^{-1} (dry weight) h^{-1}. Note the intervals chosen are not the same in every case. All individuals were males with muscle diagnosed as normal. *H* and *V* were volunteers, aged 66 and 34, respectively. The other three were patients biopsied to rule out muscle disease; their ages were 15 (*C*), 50 (*B*), and 79 (*R*).

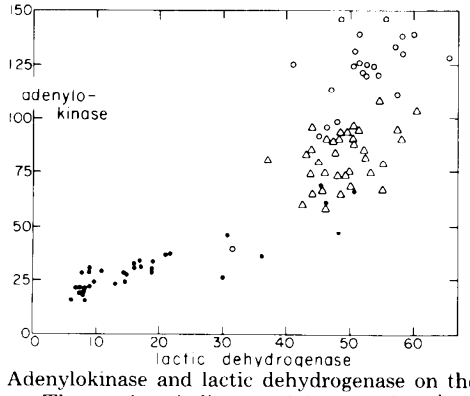

FIG. 3. Adenylokinase and lactic dehydrogenase on the same 89 muscle fibers. The numbers indicate activity as mol kg^{-1} (dry weight) h^{-1}. The symbols indicate the range of β-hydroxyacyl-CoA dehydrogenase activity for the same fibers in the same units: ○, <1.60; △, 1.61 to 2.75; ●, >2.75.

persons were analyzed for both lactic dehydrogenase and adenylokinase to see if the values fall into separate classes corresponding to known fiber types. Histograms of lactic dehydrogenase activity show two distinct groups in each case (Fig. 2), but only two. However, when the lactic dehydrogenase values are plotted against adenylokinase (Fig. 3), the fibers with high lactic dehydrogenase appear to fall into two subgroups, one with high the other with medium adenylokinase levels. The identification of two separate groups among fibers with high lactic dehydrogenase is confirmed by measurements of a third enzyme, β-hydroxyacyl-CoA dehydrogenase (Fig. 3). The high lactic dehydrogenase and high adenylokinase fibers had consistently lower values for this third enzyme than the medium adenylokinase fibers. Furthermore, a general trimodal adenylokinase distribution is shown by histograms for this enzyme in three of the five cases (Fig. 2). The pattern is not so clear for the other two, but in neither case do the upper values fit a pattern of normal (Gaussian) distribution around a single mean. Thus, there appear to be at least two classes of high lactic dehydrogenase fibers, although these may overlap to a considerable degree. The possible existence of two classes among the fibers in the low lactic dehydrogenase-adenylokinase range will be discussed below.

Correlation among Five Enzymes of Glycogenolysis Individual Fibers—On the basis of the lactic dehydrogenase and adenylokinase values, four to nine fibers were selected from each of five regions of the enzyme spectrum (designated A to E). Each fiber was analyzed for four other enzymes of glycogenolysis (we include glycero-P dehydrogenase in this category), for three enzymes involved in oxidative processes, and for creatine kinase. The level for each enzyme in each fiber was plotted against lactic dehydrogenase. Fig. 4 contains these graphs for one of the five individuals; Fig. 5 summarizes the results for all five persons. It seems probable from the above, and from data to follow, that groups A to C are "fast twitch" and groups D and E slow twitch fibers. In *panel A* of Fig. 4 are shown the initial adenylokinase-lactic dehydrogenase selections. Pyruvate kinase (*panel B*) does not follow the adenylokinase pattern, instead it rises steeply at the low lactic dehydrogenase end, then levels off at the high end. This pattern is similar in the other persons (Fig. 5, *panel B*) although in one case (*R*) there was a nearly linear pyruvate kinase-lactic dehydrogenase relationship.

The differences in adenylokinase:pyruvate kinase ratios among the low lactic dehydrogenase fibers (groups D and E of Fig. 4) indicate that this group is inhomogeneous. This ratio groups averages 1.93 ± 0.13 for all D groups and 3.18 ± 0.49 for all E groups. The difference is statistically significant ($p \leq 0.02$). However, at this time it is not possible to divide the low lactic dehydrogenase fibers into two distinctly separate classes.

P-fructokinase and glycogen phosphorylase patterns (*panels C* and *D*) are very similar to those for pyruvate kinase. The curves for glycero-P dehydrogenase are intermediate between those for pyruvate kinase and adenylokinase (Figs. 4*E* and 5*E*).

All six enzymes so far discussed vary together in the same general direction. However, the range of values differs sub-

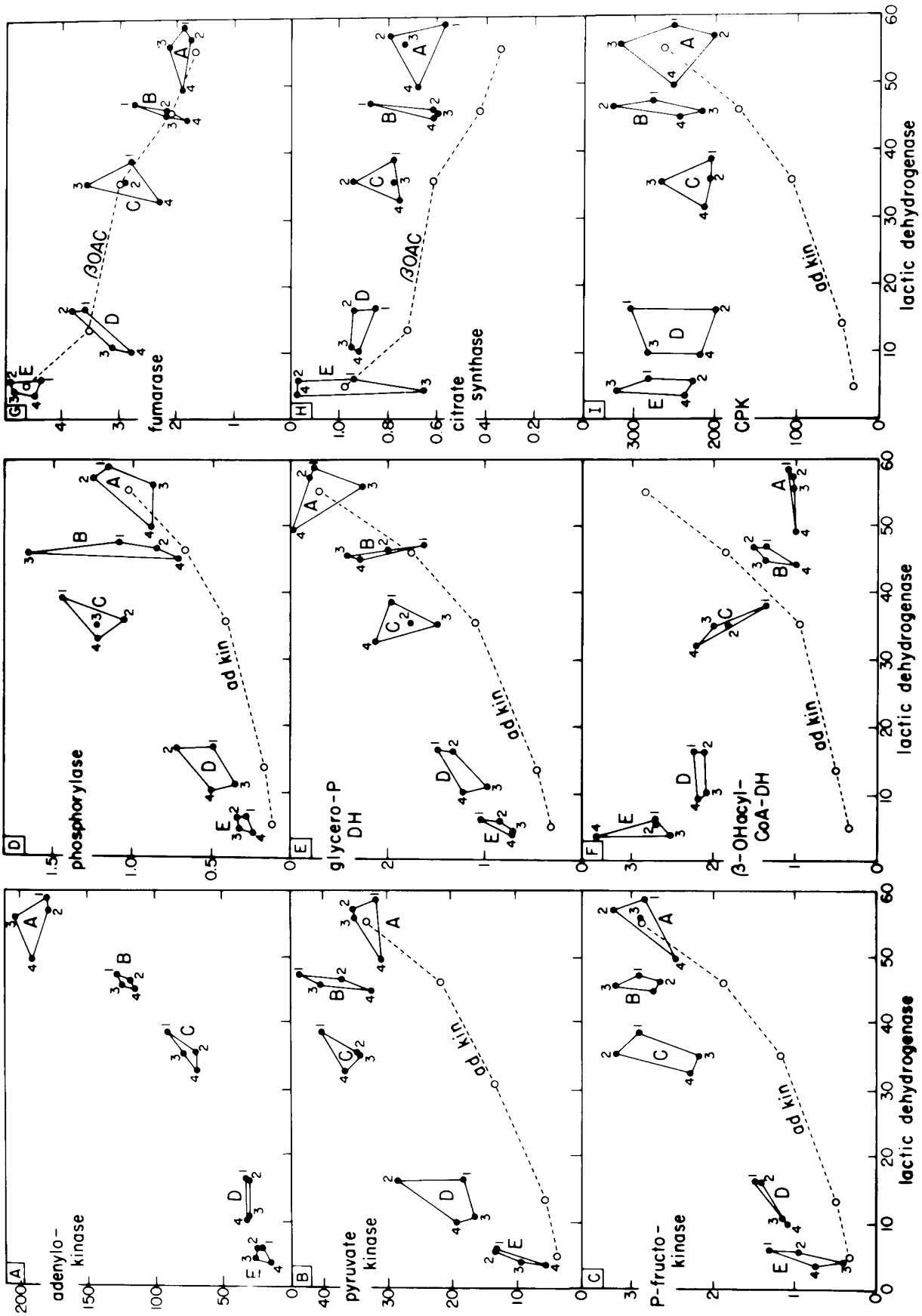

FIG. 4. Correlation of nine enzymes with lactic dehydrogenase among individual fibers. Twenty muscle fibers from one person were analyzed in duplicate for the 10 enzymes, and the activities of each of the other nine were plotted in turn against lactic dehydrogenase activity. Each fiber is identified by the group letter and the number (*1* to *4*) within that group. All activities are recorded as mol kg^{-1} (dry weight) h^{-1}. In six of the panels, the average adenylokinase values from *panel A* have been repeated for comparison (*ad kin*). The scale for adenylokinase has been adjusted in each case so that the mean value for group A coincides with that of the enzyme in question. Similarly, in two panels β-hydroxyacyl-CoA dehydrogenase values from *panel F* have been repeated (*βOAC*). Creatine kinase is abbreviated *CPK*.

stantially among the six. This may be important as a sign that different constitutive and adaptive factors are involved. Table III compares the average ratios between the highest and lowest values among individual fibers (for each person) and among the mean values for the five fiber groups. The range among groups is greatest for lactic dehydrogenase (9-fold) and the least for P-fructokinase, phosphorylase, and glycero-P dehydrogenase (about 3-fold). Individual values showed sub-

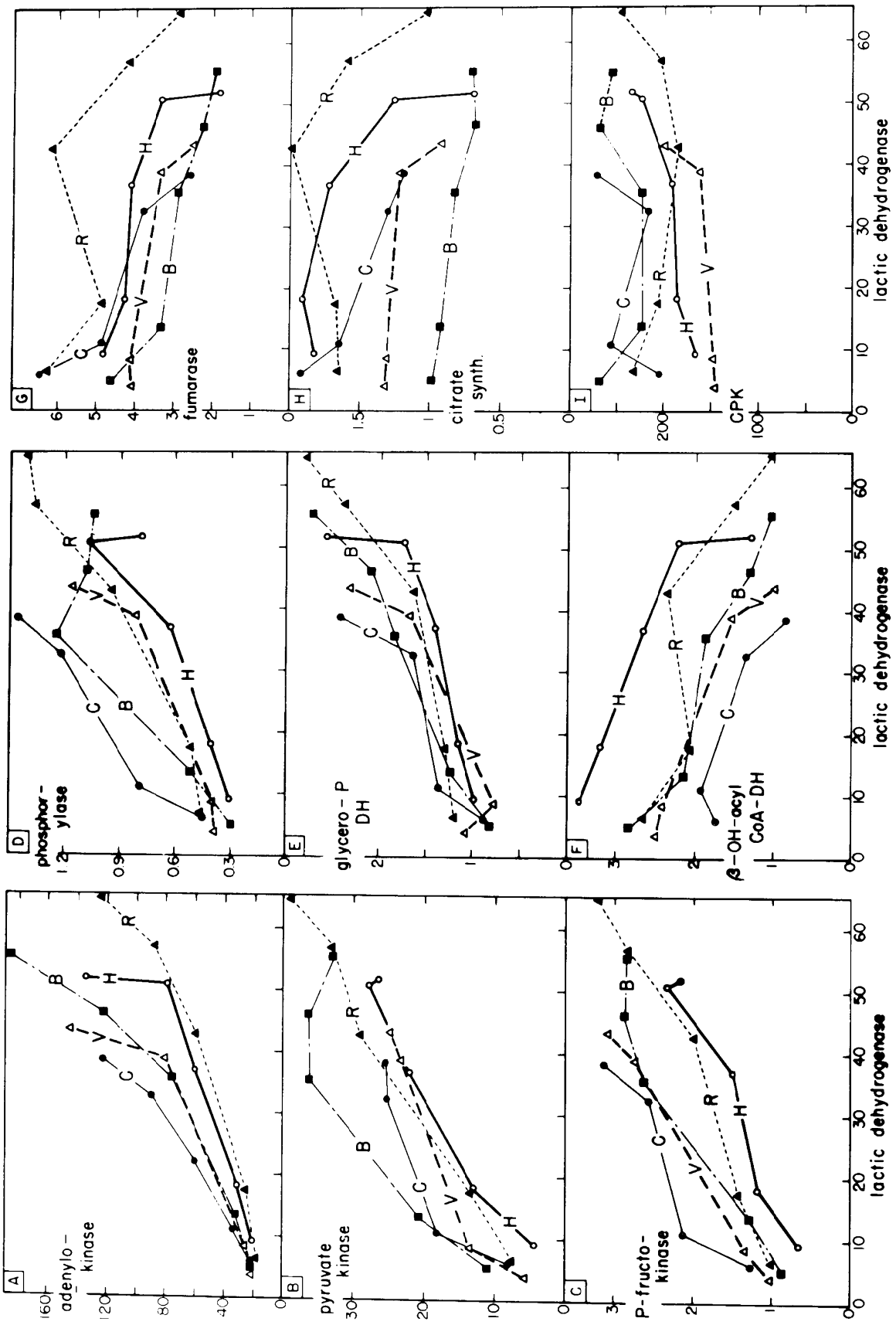

FIG. 5. Correlation of nine enzymes with lactic dehydrogenase among fiber groups from five people (*B,C,H,R,V*). The figure is a summary of data obtained and treated in an identical manner with that shown for one person (*B*) in Fig. 4. Each point represents the mean for a group of four to nine individual fibers, each of which was analyzed for all 10 enzymes. *CPK*, creatine kinase.

stantially wider ranges, but the differences among enzymes are in the same rank order. There was some arbitrariness in the selection of the five fiber groups from the random sample. This could have introduced a bias, *i.e.* the groups were selected for the greatest spread for lactic dehydrogenase and adenylokinase. Conceivably if they had been selected on the basis of glycero-P dehydrogenase, for example, a wider range of values for this enzyme might have been seen. However, since 25 to

50% of the fibers in each random sample were analyzed for every enzyme, the bias can not be great.

Related to this phenomenon of differences in range is the fact that in the case of at least three of the enzymes (adenylokinase, phosphorylase, and glycero-P dehydrogenase), plots against lactic dehydrogenase do not extrapolate through the origin (Fig. 5 *A*, *D*, and *E*).

Three Mitochondrial Enzymes—β-Hydroxyacyl-CoA dehydrogenase and the two members of the citric acid cycle were all negatively correlated with lactic dehydrogenase, as expected (Figs. 4 and 5). However, the range of values was less than for the enzymes of glycogenolysis (Table III). When the *A* to *E* groups were compared, there was an average range of only about 2-fold for citrate and fumarase. Even among individual fibers the average range was only about 3-fold. The difference between the group ratios for β-hydroxyacyl-CoA dehydrogenase and citrate synthase is statistically significant, which suggests a difference in mitochondrial composition. In the case of β-hydroxyacyl-CoA dehydrogenase for one person (*H*) the possibility of the low ratio being due to a biased selection was ruled out by analyzing all of the fibers of the random sample. The high:low ratio was only 5.2, compared to 10.6 for lactic dehydrogenase and 9.4 for adenylokinase.

Creatine Kinase—This enzyme, one of the most active muscle enzymes, varied little among the different fibers in any of the five persons and was not correlated with lactic dehydrogenase or with any of the other eight enzymes (Figs. 4*I* and 5*I*). This is in striking contrast to adenylokinase which varied 15-fold and was highly correlated with fiber type. These two enzymes share a common function: they both can make available additional high energy phosphate when ATP falls. Perhaps, adenylokinase is important not so much for salvaging high energy phosphate from ADP as for ensuring that 5′-AMP rises when ATP falls. AMP has important positive regulatory functions, including activation of phosphorylase and P-fructokinase, both of which must be turned on more rapidly in fast twitch than in slow twitch fibers.

Summary of Fiber Differences—Fig. 6 summarizes the differences for all 10 enzymes among four of the five selected fiber groups. It shows that group B differs statistically from group A in having lower glycero-P dehydrogenase and creatine kinase and higher fumarase and β-hydroxyacyl-CoA dehydrogenase. (Since the groups were selected on the basis of the lactic dehydrogenase and adenylokinase levels, the differences for these two enzymes have no statistical meaning.) All of the differences between B and D fibers are significant, except those for fumarase and creatine kinase. Likewise all of the differences between D and E fibers are significant except for

TABLE III

Ratio between highest and lowest muscle fiber enzyme levels for five persons

For the "Individual basis" column, the ratio between highest and lowest enzyme level was calculated for each person and these ratios were averaged. The standard errors of these five ratios are shown. The fibers were those which provided the data for Fig. 5 (20 to 30 for each person). For the "Group basis" the ratios between the highest and lowest of the four or five groups of Fig. 5 were calculated for each person and then averaged the same way.

	Individual basis	Group basis
Lactic dehydrogenase	15.8 ± 3.1[a]	9.0 ± 1.2
Adenylokinase	9.9 ± 1.1[a]	6.8 ± 0.5
Pyruvate kinase	7.9 ± 0.9	5.2 ± 0.5
P-fructokinase	6.8 ± 0.6	3.32 ± 0.20
Glycogen phosphorylase	6.8 ± 0.3	3.14 ± 0.20
Glycero-P dehydrogenase	4.2 ± 0.2	2.80 ± 0.17
β-OH-acyl-CoA dehydrogenase	4.4 ± 0.6	2.57 ± 0.08
Fumarase	3.4 ± 0.4	2.27 ± 0.17
Citrate synthase	2.9 ± 0.4	1.82 ± 0.25
Creatine kinase	1.86 ± 0.14	1.31 ± 0.04

[a] Even larger high to low ratios were obtained for the original random samples (50 to 100 fibers each, Fig. 2). These averaged 22:1 for lactic dehydrogenase and 12:1 for adenylokinase.

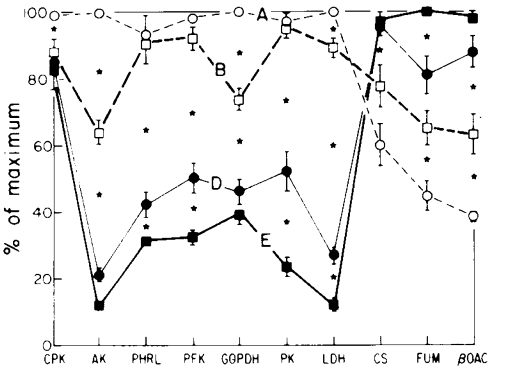

FIG. 6. Activities of 10 enzymes in four groups of fibers from five persons. The figure is based on the data of Fig. 5. The group designations refer to the highest lactic dehydrogenase groups (*A* and *B*) and the two lowest lactic dehydrogenase groups (*D* and *E*) of Fig. 5 (see also Fig. 4.). Each group value for a particular person was calculated as the percentage of the highest group value for that person. The average of these percentages for all five persons are shown, together with *error bars* (±S.E.). Statistically significant differences ($p \leq 0.05$) from one group to the next are indicated by stars. New abbreviations are: *CPK*, creatine kinase; *AK*, adenylokinase; *PHRL*, phosphorylase; *GOPDH*, glycero-P dehydrogenase; *PK*, pyruvate kinase; *CS*, citrate synthase; and *FUM*, fumarase.

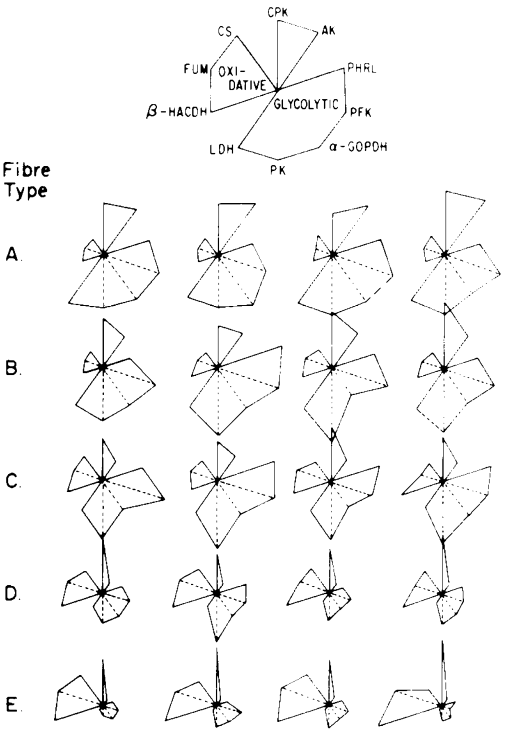

FIG. 7. Relative activities of 10 enzymes in 20 fibers from one individual (*B*). The activity of each enzyme is represented by the distance from the center. However, the scale for each enzyme is chosen so that the radius of the circle at the top is equal to the average of the highest group value (for that enzyme) for each of the five persons (Fig. 5). Abbreviations are the same as for Fig. 6 except for β-hydroxyacyl-CoA dehydrogenase (*β-HACDH*).

β-hydroxyacyl-CoA dehydrogenase, glycerol-P dehydrogenase, and creatine kinase.

Interrelationships among 10 Enzymes at the Single Fiber Level—The way the data for individual fibers were presented in Fig. 4 shows the relationships of each enzyme to one other enzyme (lactic dehydrogenase) but fails to show the extent to which all of the enzymes may be interrelated in individual fibers. Fig. 7 is an attempt to do this for one of the individuals. The profiles shown are remarkably similar for most of the fibers in a given group, but are quite dissimilar between different groups.

DISCUSSION

In all species, fast white fibers are high in enzymes of glycogenolysis and low in oxidative enzymes. Slow red fibers contain low levels of the enzymes of glycogenolysis. In rodents and rabbits these are intermediate in regard to oxidative enzymes, but in man, as shown by histological stains (8) and by succinic dehydrogenase activity (13), they are highest in oxidative capacity. Fast red fibers contain intermediate to high levels of glycogenolytic enzymes and in rodents are highest in oxidative enzymes. However, in man, they appear to contain intermediate levels of oxidative enzymes (8, 13).

Based on these criteria, we would identify our group A fibers as fast white (IIB), whereas groups B and C fit the criteria for fast red fibers (IIA). It was seen, however, that the borderline between A and B fibers is somewhat blurred. Preliminary attempts to distinguish groups A and B on the basis of ATPase staining (8) were only partially successful, although all group A to C fibers were clearly type II. Groups D and E are sharply separated from the other groups and presumably slow twitch red, and both groups were type I according to ATPase staining. Although there is no sign of distinct separation of the D and E groups, taken together the fibers are very heterogeneous in regard to enzyme levels. For example, within the combined D and E groups there was a range of lactic dehydrogenase of at least 3-fold and for one person the range was 9-fold.

Fiber Heterogeneity—The most striking feature of the data presented is the extreme range of individual values, particularly for the enzymes of glycogenolysis and for adenylokinase. The high degree of correlation, direct or inverse, among the different enzymes in each fiber, clearly rules out any possibility of this being due to random variation and suggests that differences are physiologically meaningful. A fiber especially low in lactic dehydrogenase is also especially low in pyruvate kinase, P-fructokinase, phosphorylase, and glycero-P dehydrogenase and, conversely, especially high in the mitochondrial enzymes (Figs. 4 and 7). Pette (11, 12) has stressed the coordination which is found between groups of enzymes on the same pathway (glycogenolysis, oxidation, *etc.*) when whole muscles from different parts of the body are compared. The current findings show that this enzyme-group coordination extends down to the individual fiber.

However, although overall correlation of enzyme levels is evident, constant ratios between related enzymes are seen in only a few cases and in only part of the enzyme spectrum. Of the five enzymes which correlate with lactic dehydrogenase, it was seen that plots against lactic dehydrogenase for three of them do not extrapolate through the origin. At imaginary zero lactic dehydrogenase, there would remain (compared to highest levels) roughly 15% of the adenylokinase, 20% of the phosphorylase, and 30% of the glycero-P dehydrogenase. This suggests the possibility that these percentages are constitutive and that higher levels are adaptive.

A skeletal muscle is made up of many motor units, each of which is composed of as many as 100 or more fibers innervated by a single motor neuron. Edström and Kugelberg (21) have shown in the anterior tibial muscle of the rat that the members of a motor unit are rather widely dispersed, so that the domain of a single motor unit would include fibers from at least 10 to 12 other units. They also showed that (*a*) there were many shades of difference apparent in the combined array of fibers stained for three enzymes, but that (*b*) all of the fibers of the same motor unit gave indistinguishable enzymatic stains.

Extrapolating from rat muscle data to the present situation, it is possible that the broad spectrum of enzyme patterns observed could result from the mixing of fibers from many motor units, with each contributing fibers of nearly identical composition. To account on this basis for dispersions of values, such as those in Figs. 1 and 3, would require representation from 10 to 15 varieties of motor unit. The results of Edström and Kugelberg (21) make this a reasonable possibility.

Our data would fit with the concept that in a muscle such as the biceps there are three and possibly four distinct fiber types, but that within each type there are substantial differences which tend to cause some overlap in composition.

Regardless of the number of discrete fiber types, the extreme heterogeneity found among different fibers, located next to each other in the same muscle, must have functional significance. This heterogeneity may contribute to the ability to perform movements which are finely controlled as to speed and position, whether under light or heavy loads. To produce a vibrato on a violin string may require contraction of fibers with different metabolic machinery from that needed for a series of fast punches by a prize fighter.

Comparison with Other Single Fiber Data—Essén *et al.* (13) measured P-fructokinase and succinic dehydrogenase on individual human muscle fibers. The fibers were classified as type I and type II by ATPase stains. There is quite close agreement between their average P-fructokinase values for types I and II with our groups D plus E and A plus B, respectively (Fig. 6). They also observed a very wide range of activity within each group. The average succinic dehydrogenase activities in group II were 60% of those for group I, a difference comparable to what we found on the average for three other mitochondrial enzymes for our groups E plus D *versus* groups A plus B.

Thorstensson *et al.* (22) made a similar study of three of the enzymes reported here. However, instead of analyzing the fibers separately, they were pooled for analysis according to type, as shown by ATPase staining. The ratios between activities of type II and type I were 2.3:1 for lactic dehydrogenase, 1.8:1 for adenylokinase, and 1.27:1 for creatine kinase. The first two ratios are much smaller than expected from our data.

Spamer and Pette (20) measured P-fructokinase and lactic, malic, and glyceraldehyde-P dehydrogenases in single muscle fibers from two different muscles in the rabbit. The most striking finding was that there are marked differences between the psoas and soleus muscles in regard to average enzyme levels in fast fibers (classified by ATPase staining). In the soleus, fast and slow fibers were rather similar in enzyme composition, whereas in the psoas most of the fast fibers had higher lactic dehydrogenase and P-fructokinase and lower malic dehydrogenase than the slow fibers. These authors were also impressed by the many different levels of enzyme activity found among different fibers.

Single Muscle Fiber Analysis—As pointed out under "Experimental Procedures," a 2-mm length of an average fiber can provide as many as 100 samples of the size used here. Since the dry fibers can be stored indefinitely without deterioration, there is almost no limit to the number of different

analyses that can be performed on each fiber. It should be useful to extend the present approach not only to other enzymes, but to metabolites and cofactors as well. Methods are available to do this without increasing the size of the tissue sample (14). The present results demonstrate the advantage of comparing the levels of different enzymes on a fiber to fiber basis rather than between averages for different fibers or fiber pools. Useful information about control of muscle fiber metabolism might be anticipated from comparisons between metabolite levels among fibers with different enzyme compositions.

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