

ROLE OF THE SARCOPLASMIC RETICULUM IN GLYCOGEN METABOLISM

Binding of Phosphorylase, Phosphorylase Kinase, and Primer Complexes to the Sarcovesicles of Rabbit Skeletal Muscle

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ABSTRACT

Sarcoplasmic vesicles and β -glycogen particles 30–40 $m\mu$ in diameter were isolated from perfused rabbit skeletal muscle by the differential precipitation-centrifugation method. This microsomal fraction was subjected to zonal centrifugation on buffered sucrose gradients, in a B XIV Anderson type rotor, for 15 hr at 45,000 rpm in order to separate the two cytoplasmic organelles. Zonal profiles of absorbance at 280 $m\mu$, proteins, glycogen, and enzymatic activities (phosphorylase b kinase, phosphorylase b, and glycogen synthetase) were performed. Whereas the entire synthetase activity was found combined with the glycogen particles, 39% of phosphorylase and 53% of phosphorylase b kinase activities, present in the microsomal fraction, were recovered in the purified vesicular fraction ($d = 1.175$). This latter fraction consists of vesicles, derived from the sarcoplasmic reticulum, and of small particles 10–20 $m\mu$ in diameter attached to the outer surface of the membranes. These particles disappear after α -amylase treatment. Incubation of the sarcovesicular fraction with ^{14}C -labeled glucose-1-phosphate confirms the localization of a polysaccharide synthesis at the level of the membranes. "Flash activation" of phosphorylase b, i.e. Ca "activation" of phosphorylase kinase followed by a conversion of phosphorylase b into a, was demonstrated in the purified sarcovesicular fraction. Moreover, the active enzymatic sites were detected on the membranes by electron microscopy. The presence of binding sites between the membranes of the sarcoplasmic vesicles and a glycogen-enzyme complex suggests that this association plays a role in the glycogenolysis during muscle contraction.

INTRODUCTION

Many efforts were devoted to extracting, identifying, and purifying the different cell constituents, and now complementary works strive to reconstitute integrated functional systems similar to those existing in living cells by recombining isolated

elements. Glycogen is an example among many others: it has been extracted from different tissues and then washed or treated to eliminate the contaminating substances. This last step in the purification is significant because the 3–5% proteins

which "contaminate" glycogen prepared with mild methods (Wanson and Drochmans, 1968 *a*) represent mainly enzymes involved in the synthesis and breakdown of the polysaccharide.

The binding of phosphorylase to glycogen has been established very early and its physicochemical properties have been studied by Madsen and Cori (1958). In a quantitative approach to the distribution of phosphorylase in fractions isolated from liver homogenates, Tata (1964) showed that 70–80% of the activity was present in the microsomal fraction and that the majority of the recoverable enzyme was associated with the glycogen particles. More recently, Meyer et al (1970) and, in the same laboratory, Heilmeyer et al (1970) extended the concept of an enzyme-glycogen complex by proposing an integrated functional system which includes particulate glycogen, phosphorylase, and phosphorylase kinase.

A further relationship between particles of glycogen and membrane-bound structures in liver cells has been investigated with very critical assays by Luck (1961). Impressed by the morphological observations of Porter and Bruni (1959), he studied the distribution of the glycogen synthetase in the microsomal components extracted from a liver homogenate. He confirmed the observations of Leloir and Goldemberg (1960), according to which the enzyme involved in the synthesis of glycogen was firmly bound to the polysaccharide, and concluded decisively that the membranes of the smooth endoplasmic reticulum were not directly engaged in the process.

In our laboratory, we met similar problems of distribution and functional association of enzymes with either glycogen or membranous structures (Drochmans, 1964, Wanson and Drochmans, 1968 *a, b*), and we chose the glycogen-sarcovesicular fraction isolated from skeletal muscle to investigate the glycogen-enzyme-membrane interplay. The present paper deals with the enzyme-glycogen complexes and their possible association with membrane-bound structures observed in skeletal muscle.

It will be confirmed that the synthetase presents a strong and specific affinity exclusively for the glycogen particles as already clearly demonstrated for liver glycogen and that significant amounts of phosphorylase and phosphorylase kinase are bound together with small particles of glycogen and sarcoplasmic vesicles. In some experimental

conditions, these "ensembles" are located as active sites on the surface of the membranes.

MATERIALS AND METHODS

Reagents

Adenosine monophosphate (AMP), glucose-1-phosphate, uridine diphosphate glucose (UDPG), and α -amylase, twice crystallized, were purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio); adenosine triphosphate (ATP), glucose-6-phosphate, and commercial shellfish glycogen were purchased from Sigma Chemical Co. (St. Louis, Mo.). Activators and inhibitors of enzymatic reactions, such as sodium fluoride (NaF), magnesium acetate ($Mg^{2+}Ac \cdot 4H_2O$), calcium chloride ($CaCl_2 \cdot 2H_2O$), magnesium chloride ($MgCl_2 \cdot 6H_2O$), and ethylenediaminetetraacetate ($EDTA \cdot 2H_2O$), as well as buffers (tris[hydroxymethyl]aminomethane [Tris], sodium glycerophosphate, cysteine, glycine, maleate), were products of E. Merck AG (Darmstadt, Germany).

Uridine diphosphate glucose- ^{14}C was obtained from NEN Chemicals (Frankfurt, Germany) and α -D-glucose- ^{14}C -1-phosphate from The Radiochemical Centre (Amersham, England). The specific activity of these radioactive compounds was respectively, 230 and 150 mCi/mmmole. Purified crystalline phosphorylase b was a gift from Dr. De Wulf (Laboratoire de Chimie Physiologique, Université de Louvain).

Isolation and Purification of the Sarcovesicular Fraction SV

Normally fed domestic rabbits, weighing 3–4 kg each, were sacrificed by sectioning the blood vessels of the neck, after anesthesia by intravenous injection of Nembutal (1 mg/kg body weight; Abbott Laboratories, North Chicago, Ill.). Bleeding was improved when 300 units/kg body weight of heparine (Liquémine, S. A. Roche, Belgium) were added to the anesthetic. In some experiments, when specified in the text, an additional precaution was taken to prevent amylase activity due to blood which might contaminate the preparations. Therefore, both hind legs were perfused by intraaortic injection of 250 ml of chilled saline. This is enough to wash out most of the blood accumulated in the muscles. The adductor magnus and quadriceps, which consist predominantly of white muscle fibers, were excised, cut into small pieces, and immediately immersed in chilled 0.033 M phthalate buffer, pH 4.8. A microsomal fraction, composed of glycogen particles and vesicles derived from the sarcoplasmic reticulum, was isolated from the skeletal muscles according to the precipitation-centrifugation method previously described (Wanson and Droch-

mans, 1968 *a*) and summarized as follows. (*a*) Muscle pieces were homogenized by treatment for 30 sec with a blender in phthalate buffer, at the final pH of 6.0; (*b*) preliminary centrifugations were performed at 2500 rpm for 15 min (International centrifuge, model PR 1, head 284, International Equipment Co., Needham Heights, Mass.) to discard intact cells, nuclei, and myofibrils and were completed by repeated up-and-down runs at 15,000 rpm (Spinco, model L2 65B centrifuge, No. 30 rotor, Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) to eliminate agglutinated material remaining in suspension; (*c*) centrifugation at 25,000 rpm for 125 min (Spinco, model L2 65B centrifuge, No. 30 rotor) of the postmitochondrial supernatant allowed the recovery of a microsomal fraction, the so-called glycogen-sarcovesicular fraction (G.Sv.F.).

To obtain a purified fraction of sarcovesicles, devoid of glycogen particles, the choice was given between two additional manipulations: either a zonal centrifugation which clearly separates the two components of the G.Sv.F. or an incubation which activates the digestion of the polysaccharide. In method one the zonal centrifugation of the G.Sv.F. was carried out in the Spinco, model L2 65B centrifuge, equipped with a B XIV rotor of the Anderson type (600 ml capacity). In a first step, the running rotor (3000 rpm) was loaded at a flow rate of 15 ml/min by means of a Beckman pump, type 411 (Beckman Instruments, Inc.), with a sucrose density gradient which occupies a volume of 350 ml. The linear density gradient with extreme densities of 1.095 (0.7 M, 22% sucrose) and 1.265 (2.0 M, 55% sucrose) was buffered with 0.010 M imidazole-0.005 M EDTA, pH 7.0. This gradient was layered between a heavy cushion composed of 200-225 ml of 2.0 M sucrose and the sample (25-50 ml) covered by an overlay of 50 ml of the solvent used to suspend the G.Sv.F. Temperature was kept between 3° and 7°C during loading and at 3°C during centrifugation. Once loaded, the speed of the rotor was increased to 45,000 rpm and maintained for 15 hr. The unloading was accomplished by pumping 2.0 M sucrose at a flow rate of 7.5 ml/min into the rotor 60 fractions of 7.5 ml each were collected in a sample collector (Technicon, Dublin, Ireland). The refractive index was measured by means of the Abbe refractometer (Carl Zeiss, Ober Kochen, Germany) and density at 20°C was deduced from these data. In the series of experiments which took advantage of these techniques of centrifugation, the perfusion of the muscle legs mentioned above was a prerequisite manipulation to avoid breakdown of glycogen during the long runs. In method two the incubation of the G.Sv.F. in the presence of amylase, which normally contaminates the fraction obtained from nonperfused muscles, led to vesicles which are easy to separate from the breakdown products. The

degree of degradation of the glycogen was followed, during incubation, by iodine reactions and by glucose determinations according to Nelson (1944). The separation of the sarcovesicles from the oligosaccharides at the end of the incubation, generally after 120 min, was performed by layering the incubated fraction on top of a sucrose density gradient (0.7-2.0 M) and by centrifuging the tubes in swinging buckets (Spinco, rotor SW 27) at 24,000 rpm for 5-15 hr. The gradients were then fractionated in 1 ml fractions from top to bottom.

Analytical Methods

Glycogen was determined by the method of Krisman (1962) in the different fractions isolated by differential and density gradient centrifugation. In some cases, proteins, which interfere with the glycogen-iodine reaction, had to be removed by adding trichloroacetic acid at a final concentration of 5%.

Total protein content was estimated by the micro-method of Lowry et al. (1951), using crystalline bovine serum albumin (Pentex Biochemical, Kankakee, Ill.) as standard. The zonal profile of absorbance at 280 m μ was determined by means of a Uvicord (LKB Produkter, Stockholm, Sweden) and compared with the profile obtained by protein determinations.

Enzymatic Assays

PHOSPHORYLASE b ACTIVITY: The phosphorylase b activity (E.C. 2.4.1.1) was assayed by measuring the release of inorganic phosphate from twice-crystallized 0.1 M glucose-1-phosphate when the subcellular fractions were incubated for 5 and 10 min at 37°C in the presence of 0.02 M sodium fluoride (NaF), 0.003 M adenosine monophosphate (AMP), and 2% commercial shellfish glycogen. The primer was added, although glycogen was present in the fractions under study, except in the experimental conditions stated in the next paragraph. Inorganic phosphate was estimated by the colorimetric method of Fiske and Subbarow (1925), with a Gilford spectrophotometer (model 300 N, Gilford Instrument Labs, Inc., Oberlin, Ohio). The enzymatic activity was expressed in micromoles of phosphorus liberated per minute per milligram of glycogen or per gram of muscle.

The phosphorylase b activity was also determined without adding a primer to the sarcovesicular fractions in order to measure the reaction which takes place only at sites where a phosphorylase-primer complex is present, possibly related to the membranes. This was accomplished by incubating the sarcoplasmic membranes purified by zonal centrifugation or by digestion of the glycogen particles. The test was carried out as follows: 1.1 ml of a suspension of sarcovesicles, containing 0.5 mg protein/ml,

were added to 1.1 ml of the reaction medium composed of 0.1 M labeled glucose-1-phosphate, 0.02 M NaF, and 0.003 M AMP. The ^{14}C -labeled glucose-1-phosphate presented a radioactivity of 2–4 μCi , 100 μM . At various times, samples were taken. Part (0.1 ml) was used to determine the phosphate release and to establish the polysaccharide-iodine spectrum and part (2 ml) was layered on top of a linear imidazole-buffered sucrose gradient and centrifuged at 24,000 rpm for 5–15 hr. The gradient was then fractionated in 1 ml samples and each sample was treated with 1 ml of hyamine hydroxide (Packard Instrument Co., Downers Grove, Illinois) at 50°C for 1 hr and finally added to 13 ml of Bray solution (Bray, 1960) to be counted in a Nuclear liquid scintillation counter, Mark A (Nuclear-Chicago, Des Plaines, Ill.)¹ The counting efficiency, determined against an external standard, reached 40%. For each experiment the quenching curve was established to convert the counts per minute into disintegrations per minute.

Similar incubations of sarcovesicular fractions were carried out with larger quantities of material but with unlabeled substrates to obtain preparations for electron microscopic examinations.

PHOSPHORYLASE KINASE ACTIVITY The phosphorylase kinase activity (E.C. 2.7.1.38) was determined according to the technique proposed by Krebs et al. (1964) and Krebs (1966) and adapted to our experimental conditions. It consists of two steps in a preliminary partial reaction: 0.2 ml of pellet suspension was added to 0.1 ml of 0.125 M Tris-glycerophosphate buffer, pH 8.6, and 0.2 ml of AMP-free phosphorylase b solution containing 130 Hers units of enzyme/ml (Hers, 1964). The reaction started by adding 0.1 ml of 0.06 M Mg^{2+} acetate–0.018 M adenosine triphosphate (ATP), pH 7.0. After 5 min of incubation at 30°C, a 0.1 ml sample was transferred to 0.9 ml of chilled 0.04 M glycerophosphate–0.03 M cysteine buffer, pH 6.8. The second step reveals the phosphorylase activity according to Hers (1964). 50 μl of the last reaction mixture was added to 50 μl of the incubation medium containing 0.1 M glucose-1-phosphate, 2% shellfish glycogen, 0.2 M NaF, and 0.001 M caffeine dissolved in 0.2 M Tris buffer, pH 6.1. Incubation occurred at 37°C for 5 and 10 min. Inorganic phosphate released by the reaction was determined by the method of Fiske and Subbarow (1925). Caffeine was added, as mentioned, to inhibit the phosphorylase b activity (De Wulf, personal communication).

The Ca^{2+} activation of kinase, also called “flash activation” by Heilmeyer et al. (1970), was tested in the G-Sv F and in the zonal purified sarcovesicular fraction. The following system, proposed by these

¹ Measurements were carried out in the Laboratory of Nuclear Medicine, University of Brussels.

authors, was used: 0.9 ml of biological material, 0.05 ml of 0.1 M MgCl_2 , 0.05 ml of 0.02 M CaCl_2 , and 0.02 ml of 0.05 M ATP in order to start the reaction. At various times, samples were removed, diluted 10-fold in 0.1 M glycerophosphate buffer, pH 6.8, and assayed for phosphorylase a activity.

The polysaccharide synthesis produced by phosphorylase a, obtained after flash activation of the phosphorylase kinase, was followed by electron microscopy, and its precise localization on the membrane structures under study was determined. Purified sarcovesicles prepared by zonal centrifugation and reconcentrated to a final concentration of 1.5–4.0 mg protein/ml were incubated in the presence of 5 mM MgCl_2 , 1 mM CaCl_2 , 100 mM glucose-1-phosphate, 20 mM NaF, 1 mM caffeine, 1 mM ATP, and 0.2 M Tris, pH 6.8.

UDPG-GLYCOGEN TRANSFERASE UDPG-glycogen transferase (E.C. 2.4.1.11) was assayed by measuring the incorporation of radioactive glucose from ^{14}C -labeled UDPG into glycogen. Incubation was carried out at 30°C in the presence of the following medium, described by Villar-Palasi and Lerner (1961): 0.025 M UDPG, 0.05 M Tris maleate buffer–0.0025 M EDTA, pH 8.0, 0.02 M glucose-6-phosphate (added when the total transferase activity was measured), and 0.5% commercial shellfish glycogen. ^{14}C -labeled UDPG was added to this medium in such an amount that 15,000 cpm were present in each measured sample. The different tested subfractions adequately diluted were incubated for 5 and 10 min. The enzymatic reaction was stopped by the addition of cold trichloroacetic acid to a final concentration of 5%, and the newly synthesized polysaccharide was precipitated with alcohol after the addition of 10% commercial shellfish glycogen, used as a carrier. The isolated glycogen was once more precipitated, resolubilized in distilled water, and treated with hyamine hydroxide before counting in a Nuclear-Chicago counter, model Mark A (counting efficiency of 45%). Counts were expressed in micromoles of glucose incorporated into glycogen per hour, per milligram of glycogen or per gram of muscle.

Electron Microscopy

The different fractions obtained by differential and density gradient centrifugations were fixed during 2 hr, at 4°C, with 0.1 M phosphate-buffered, distilled glutaraldehyde, 2.5%, pH 7.2, or with 0.1 M phosphate-buffered osmium tetroxide, 1%, pH 7.2. The glutaraldehyde fixation was followed by several short washings in 0.15 M phosphate buffer, pH 7.2, and by a postfixation with 0.1 M phosphate-buffered osmium tetroxide, 1%, pH 7.2.

These fixations were also performed on the pellets obtained from the macroincubation tests: the blocks dehydrated in graded ethanol-water series up to

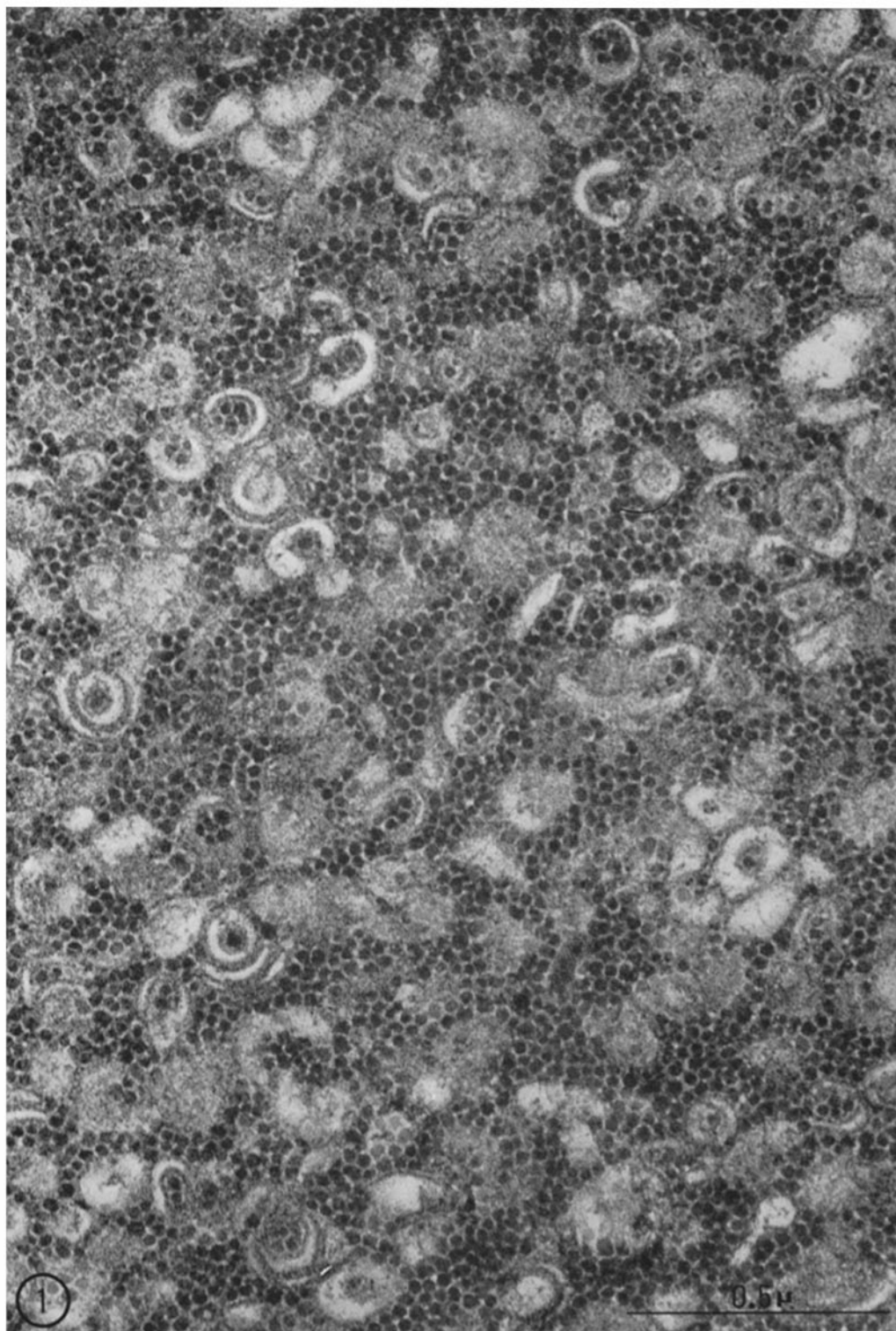


FIGURE 1 Glycogen sarcovesicular fraction. Densely lead-stained β -glycogen particles, 30-40 $m\mu$ in diameter, are packed between the sarcovesicles. This disposition of the sarcoplasmic elements reconstitutes a picture similar to that found in muscle tissue. $\times 80,000$.

100% ethanol were embedded in Epon 812 (Shell Chemical Co, New York), cut on an LKB Ultratome (LKB Produkter), and double stained with uranyl acetate and lead citrate.

Two specific histochemical methods were used to demonstrate the polysaccharide nature of small glycogen particles and of the newly synthesized material obtained after incubation in the presence of glucose-1-phosphate: (a) α -amylase digestions on sections were performed according to the technique proposed by Monneron and Bernhard (1966), using purified, twice-crystallized α -amylase diluted in phosphate buffer, 0.2 M, pH 6.9 at the final concentration of 0.5%. After 3 hr of incubation at 37°C, the treated sections were double stained with uranyl acetate and lead citrate. Control sections were incubated in distilled water at 37°C during the same time. (b) The specific glycogen staining following the method of Perry (1967) was applied on sections they were oxidized for 30 min at room temperature with periodic acid, 1%, rinsed with distilled water, dried, and stained with lead citrate. The sections were examined in a Siemens Elmiskop I electron microscope at 80 kv.

RESULTS

The G.Sv.F. which was used in the present work for further fractionation and enzymatic studies was isolated by differential precipitation-centrifugation of a muscle homogenate. It is composed of two components: glycogen β -particles which constitute most of the compact bottom part of the pellet, and sarcoplasmic vesicles which form

a superficial fluffy layer. Fig. 1 illustrates a section of a pellet in which both components may be distinguished. It reconstitutes a situation which is similar to that found in the muscle sarcoplasm, where vesicles and glycogen particles present close topographical relationships (Wanson and Drochmans, 1968 a).

Distribution of Glycogen, Proteins, and Enzymatic Activities after Differential Centrifugation of a Muscle Homogenate

Chemical data concerning the postmitochondrial, G.Sv.F. (microsomal), and postmicrosomal fractions are listed in Table I. The postmitochondrial supernatant was found to be the most appropriate fraction to refer to, when recovery of enzymatic activities was evaluated.

Half (55%) of the total glycogen content present in the postmitochondrial supernatant was recovered in the G.Sv.F., after discarding of tissue debris, nuclei, mitochondria, and most of the myofilaments by repeated low speed centrifugations. Less was found in the microsomal supernatant. The total glycogen recovery in microsomal and microsomal supernatant amounts to 70%. If we strike the balance of proteins, most of them were recovered in the microsomal supernatant, only 4.5% in the G.Sv.F., the total in the two latter fractions amounting to about 85%. The

TABLE I
Glycogen, Protein Content, and Enzymatic Activities of Different Fractions Obtained by Precipitation-Centrifugation of the Rabbit Muscle Homogenate

Fractions	Glycogen		Protein		Phosphorylase b			Phosphorylase b kinase			UDPG-glycogen transferase		
	mg/g muscle	%	mg/g muscle	%	units enzyme* /mg glycogen	units enzyme/g muscle	%	μ M P/min per mg glycogen	μ M P/min per g muscle	%	μ M glucose/hr per mg glycogen	μ M glucose/hr per g muscle	%
Postmitochondrial supernatant	3.31	100	41.08	100	40.65	143.77	100	70.33	286.2	100	15.40	34.35	100
	± 0.62		± 3.56		± 5.96	± 11.57		± 20.50	± 127.9		± 7.12	± 7.37	
Microsomal fraction G.Sv.F.	1.82	55.0	1.86	4.5	57.61	87.14	60.6	30.60	61.9	21.6	19.00	28.67	83.5
	± 0.27	± 8.1	± 0.15	± 0.4	± 10.14	± 7.96	± 5.6	± 17.72	± 33.1	± 11.6	± 4.23	± 2.86	± 8.3
Microsomal supernatant	0.45	13.6	33.35	81.2	57.80	23.33	16.2	100.0	226.2	79.06	13.50	1.15	3.4
	± 0.30	± 9.0	± 1.27	± 3.1	± 11.43	± 3.26	± 2.3	± 25.1	± 18.1	± 6.5	± 9.03	± 0.22	± 0.6

All the mean values and their standard deviations recorded in this table were calculated from data obtained from 12-20 experiments.

* Units enzyme = micromoles of inorganic phosphorus liberated per minute of incubation at 37°C.

high proportion which remained unsedimented corresponds mainly to soluble contractile proteins.

Most of the phosphorylase b activity (60%) was present in the G.Sv.F. This enzyme was essentially in its inactive b form, displaying less than 5% activity when measured in the absence of AMP. This proportion of 95:5% of the b:a form of the enzyme was systematically found in all the fractions and subfractions isolated by differential and zonal centrifugation. On the other hand, phosphorylase kinase sedimented only at the rate of 21% in the G.Sv.F., most of this latter enzyme activity remaining in the postmicrosomal supernatant (79%). These data expressed in Table I were obtained by measuring the kinase activity at pH 8.6, in the presence of ATP-Mg²⁺. Notice, however, that phosphorylase kinase was not completely in an inactive form in the different fractions isolated by centrifugation: it displayed, when measured at pH 6.8 in the absence of Ca²⁺, 15% of the activity measured at pH 8.6.

UDPG-glycogen transferase activity, assayed in presence of glucose-6-phosphate, was practically entirely detected in the microsomal fraction (83%). The relative proportion of glucose-6-phosphate-dependent and independent forms of enzyme activity corresponded to a constant ratio of 60:40.

No enzyme inactivity or inhibition was detected at this level of the isolation process: a recovery of 90–95% of the original activities present in the first supernatant was obtained for the three above-

mentioned enzymes. It appeared, thus, that the G.Sv.F. is representative of the glycogen content and of the enzymatic activities involved in polysaccharide degradation and synthesis found *in situ*. It constitutes a material of choice for the analysis of enzyme distribution at the level of cell organelles.

Distribution of Enzyme Activities in Relation to the Glycogen Particles and the Sarcoplasmic Vesicles Isolated from the G.Sv.F.

The two main components of the G.Sv.F. were identified after their subfractionation by combined biochemical and morphological examinations. Biochemical data from one experiment, corresponding to the profiles of Fig. 3, are listed in Table II. Per cent values of the total amount present in the G.Sv.F., calculated from data obtained in 12 experiments, are expressed in Table III.

GLYCOGEN: The glycogen particles in all experiments sedimented in the fractionation tubes 35–55 (Fig. 2) at a level where the mean density reaches 1.25, a value which remains far below the density proper to glycogen (1.6). The complete glycogen subfraction represents 46% of the total amount layered on top of the gradient. Although no glycogen was traced in the rest of the gradient, it was possible to recover a signifi-

TABLE II
Glycogen, Protein, and Enzymatic Content of the Different Subfractions Obtained after Zonal Centrifugation of the G.Sv.F. Fraction

Fractions	Glycogen	Protein	Phosphorylase b	Phosphorylase kinase	UDPG-glycogen transferase
	<i>mg</i>	<i>mg</i>	<i>units enzyme</i>	<i>μM P/min</i>	<i>μM glucose/hr</i>
Glycogen sarcovesicular fraction	180.0	150.0	7040	3629	4200
Nonsedimented material	—	41.6	805	520	—
Purified sarcovesicles	5.0	63.0	1421	1850	≤40
Purified glycogen	112.5	25.0	205	153	1680
Recovery	117.5	130.0	2431	2523	1680

Values presented in this table correspond to the total content of different subfractions expressed in milligrams, units enzyme, and micromoles of molecules released by the enzyme activity. They are calculated from the data of one experiment, which enter into the graphic representation of Fig. 3.

cant amount of it in the subfraction of sarco-vesicles (tubes 10-25) by concentrating the material and extracting the polysaccharide from the resulting pellet with potassium hydroxide. This small quantity of glycogen, amounting to 2.5% of that submitted to zonal centrifugation,

responds to an iodine test by giving a usual brown color and a normal spectrum. Similar manipulations carried out on the upper portion of the gradient, including the sample zone fraction, reveal no glycogen.

Parallel examinations under the electron micro-

TABLE III
Distribution of Glycogen, Proteins, and Enzymes in the Different Subfractions Obtained after Zonal Centrifugation of a Microsomal Fraction on a Sucrose Density Gradient

Fractions	Glycogen	Protein	Phosphorylase b		Phosphorylase kinase		UDPG-glycogen transferase	
			Uncorrected	Cor- rected*	Uncorrected	Cor- rected*	Uncorrected	Cor- rected*
			%	%	%	%	%	%
Nonsedimented material	—	29.9 ± 4.5	16.9 ± 3.5	25.5	10.0 ± 3.9	10.0	—	—
Purified sarco-vesicles	2.5 ± 0.3	33.8 ± 4.7	19.2 ± 2.7	39.2	53.0 ± 11.1	53.0	≤1.0	≤1.5
Purified glycogen	46.3 ± 5.4	17.2 ± 0.5	4.1 ± 2.2	11.7	2.3 ± 0.7	4.6	40.0 ± 10.5	80
Recovery	48.8	80.9	40.2	76.4	65.3	67.6	40.0	80

All values are expressed in per cent of the amount of compound present in the microsomal fraction disposed on top of the gradient and are calculated from data obtained in 12 experiments

* Values corrected for inhibition due to sucrose.

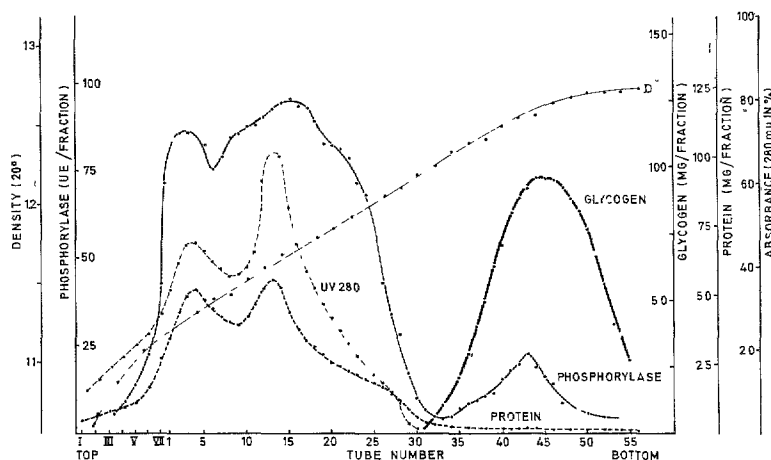


FIGURE 2 Zonal profile of protein, absorbance at 280 m μ , glycogen, phosphorylase, and density at 20°C along the 0.010 M imidazole-0.005 M EDTA buffered sucrose gradient (0.7-2.0 M) after centrifugation of the G Sv F. in the B XIV rotor of the Anderson type, at 45,000 rpm for 12 hr. 60 fractions of 7.5 ml each were collected: β -glycogen particles sedimented in fractionation tubes 35-55, completely separated from sarcoplasmic vesicles, the sarcoplasmic vesicles are isopycnicly banded in fractions 10-25 as demonstrated by the protein profile. The phosphorylase b activity forms three peaks: a first one at the upper region of the gradient corresponds to free proteins; a second and a third one are respectively superposed on the banded sarco-vesicles and on the glycogen particles. Biochemical data corresponding to these profiles are listed in Table II.

scope of the concentrated glycogen subfraction revealed the presence of purified β -glycogen particles, which confirmed previous observations (Wanson and Drochmans, 1968 *a, b*; Wanson and Tielemans, 1971).

SARCOPLASMIC VESICLES: The protein profile and the UV absorption curve of Figs. 2 and 3 show a similar pattern and form three peaks: the first, at the upper low-density region of the gradient, corresponds to free proteins; the second, which extends from fractionation tubes 10–25 with a mean density of 1.17, was identified with the sarcovesicles; and the third, lower in concentration and optical density, was superposed onto the glycogen. By grouping the tubes of each subfraction, it was found (Table III) that about equivalent amounts of proteins were found in the two first subfractions, respectively 30 and 34%, and less (17%) in the glycogen subfraction.

ENZYMES: The distribution of the phosphorylase b activity is superposable on the protein profile. Most of the activity, 39% of that placed on top of the gradient, was detected where the sarcoplasmic vesicles banded, leaving the non-sedimentable material with 25% of the activity; only 11% followed the sedimentation of the glycogen particles.

The phosphorylase-kinase activity was apparently more firmly bound to the sarcoplasmic vesicles since 53% was found in this fraction, only 10% did not sediment, and 46% remained with the glycogen particles.

The UDPG-glycogen transferase (glycogen syn-

thetase) behaved differently, being essentially localized at the level where glycogen sedimented (Fig. 3); this represents 80% of the activity originally placed on the gradient.

Per cent values of enzymatic activities mentioned in the text are corrected for inhibition produced by sucrose. Preliminary assays testing our three enzymes were performed in sucrose solutions of increasing concentration (from 0.5 to 2.0 M). Enzyme inhibition was linearly proportional to sucrose concentration, but a little more intense for phosphorylase b than for the two other enzymes (see Table III).

DEGREE OF BINDING BETWEEN PHOSPHORYLASE AND SARCOPLASMIC VESICLES: It must be noticed that the peaks of the phosphorylase activity were always precisely superposed on the banding of the proteins and the glycogen. Apparently, no enzyme was "floating" throughout the gradient. To test to what extent the enzyme activity remains associated with the vesicular structures isolated by zonal centrifugation, the sarcoplasmic vesicles were centrifuged and the resulting pellet contained still 30–40% of the enzymatic activity. In order to perform this centrifugation, a slight dilution of the suspension of vesicles, which were centrifuged practically at isopycnic equilibrium in the zonal rotor, was necessary. When important and brutal dilutions were imposed, still less, 10–20% enzyme activity, sedimented with the vesicles.

Another test consisted of first treating the G.Sv.F. with α -amylase and then submitting the

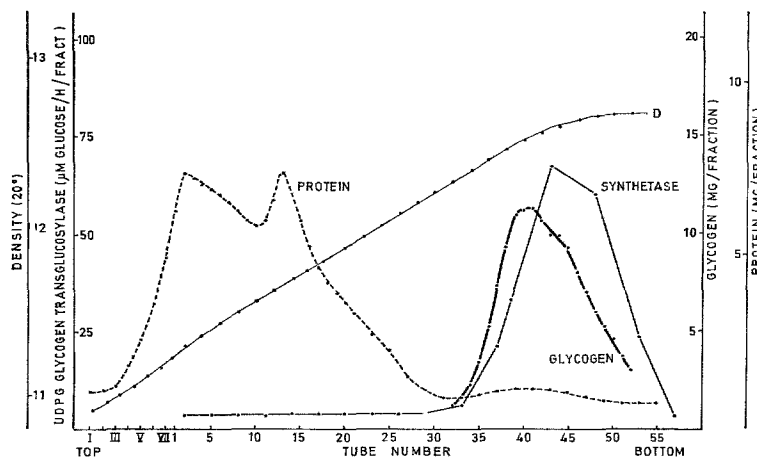


FIGURE 3 Zonal profile of protein, glycogen, UDPG-glycogen transferase, and density at 20°C obtained in the same conditions as described for Fig. 2. Glycogen-synthetase is essentially localized at the level where glycogen particles have sedimented (fractionation tubes 35–55).

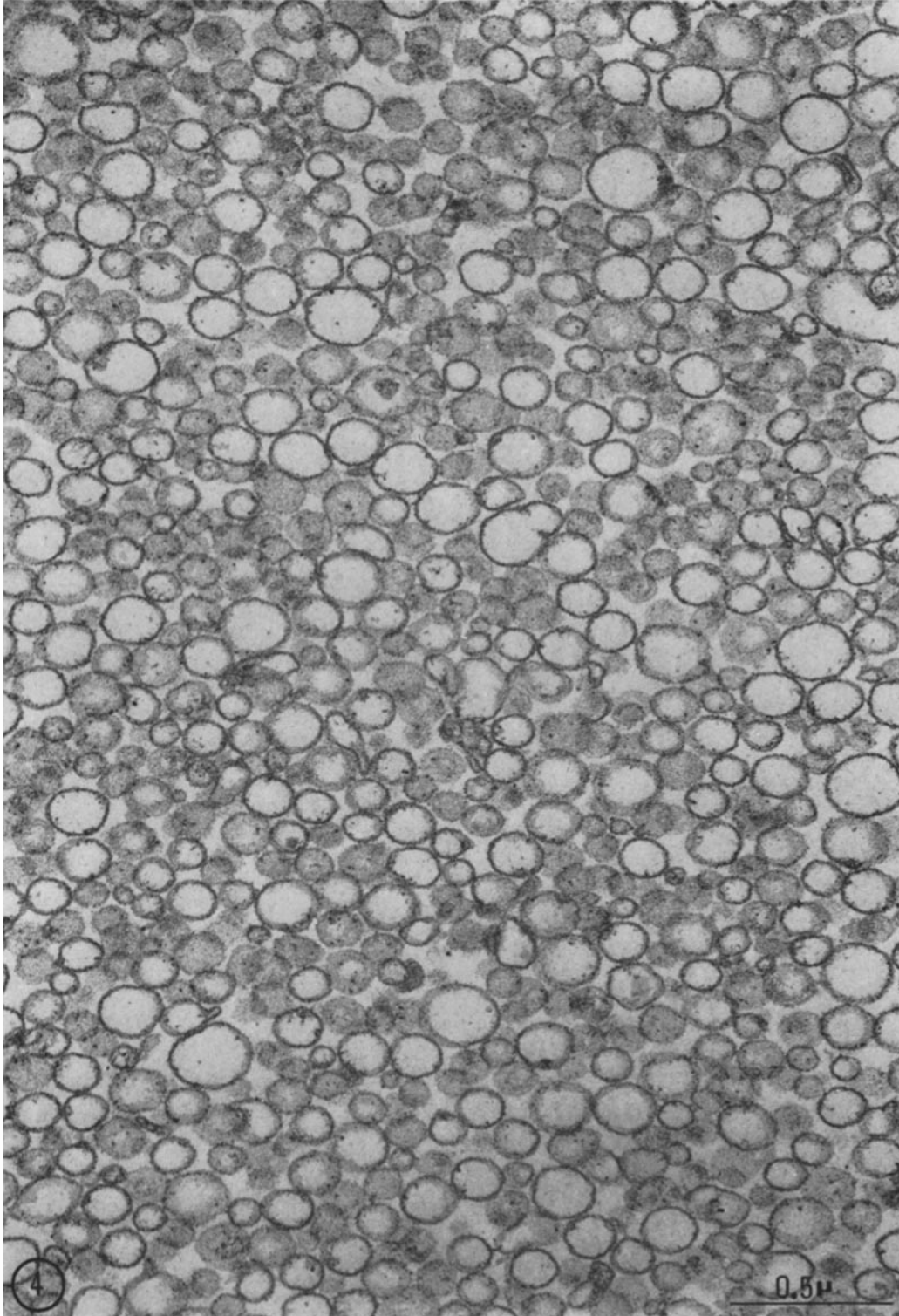


FIGURE 4 Purified sarcovesicles (SV), isolated by zonal centrifugation (fractionation tubes 10-25) and concentrated in a pellet. This view is representative of the totality of the pellet. Notice the high degree of purity and homogeneity of the fraction. No β -glycogen particles contaminate this fraction. $\times 50,000$,

sample to a zonal centrifugation in conditions similar to those described for the usual separations. In these experimental conditions, the sarcoplasmic vesicles sedimented at a slightly lower rate than usual, the sedimentable glycogen disappeared, and the phosphorylase activity was entirely recovered at the top of the gradient, no trace being found linked to the vesicles

MORPHOLOGY OF THE SARCOPLASMIC VESICLES ASSOCIATED WITH PARTICULATE MATERIAL: Special attention was devoted to visualizing eventual particulate material bound to the sarcoplasmic vesicles which would represent the morphological expression of some relationships that may exist between glycogen (primer), phosphorylase, and vesicles

Pellets of the sarcoplasmic vesicles removed from the gradient after zonal centrifugation were examined in the electron microscope. A representative picture of this purified fraction is shown in Fig 4. At higher magnification (Fig. 5), small, densely stained particles, 10–20 μ in diameter, are detected at the surface of the vesicles (arrows). Most of them lay at the outer surface but some seem to be projected at the inner side, namely when the wall of the vesicle is sectioned obliquely. This has to be placed in relation to previous observations (Wanson and Drochmans, 1968 *a, b*) of negatively stained preparations where particles of similar size were described.

This same vesicular material treated with α -amylase, as described in the preceding paragraphs on the degree of binding between phosphorylase and vesicles, lost its particles (Fig 6)

Further Isotopic and Morphological Detection of a Phosphorylase Activity Bound to the Purified Sarcoplasmic Vesicles (SV)

The observations reported above on the enzymatic activities present in the sarcovesicular sub-fractions and the coincidental demonstration on

the vesicle membrane of particles that consist at least partly of a glycogen moiety, were confirmed and extended by incorporation studies of labeled substrates and by a precise localization of the active enzymatic sites on the membranes.

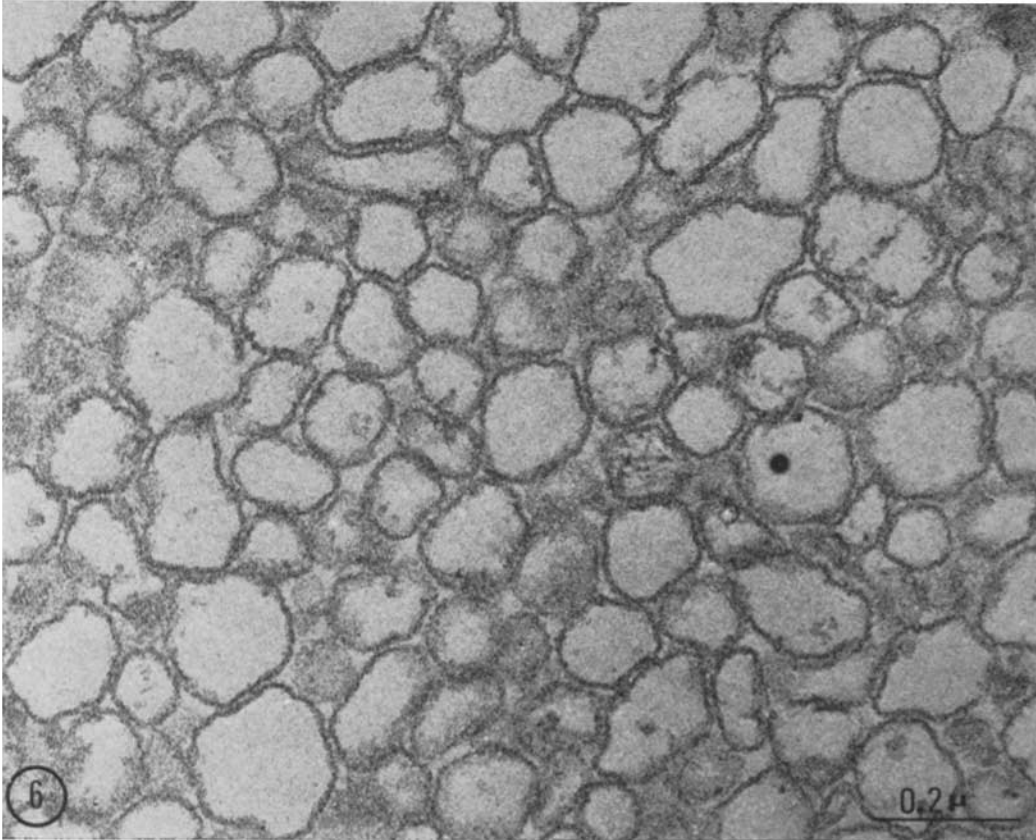
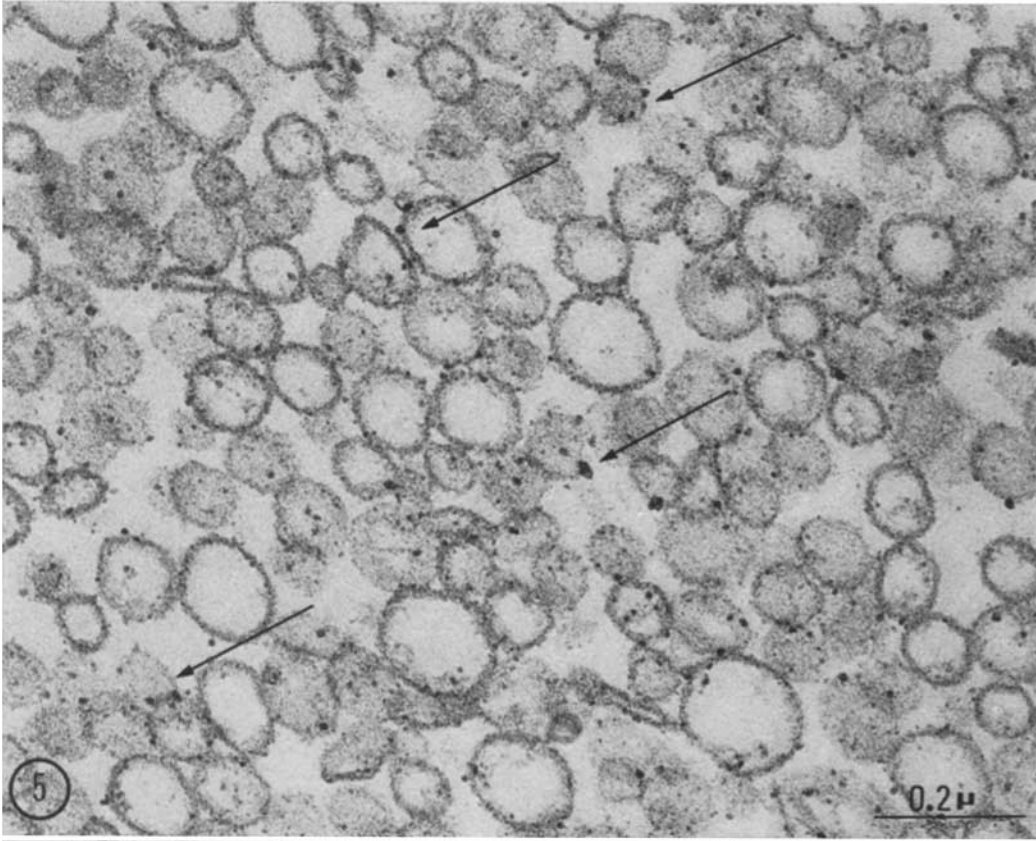
ISOTOPIC ASSAYS: A sample of SV incubated in the presence of ^{14}C -labeled glucose-1-phosphate was centrifuged on a sucrose gradient in a zonal rotor. Radioactive polysaccharide, enriched in labeled glucose by the phosphorylase, is precisely superposed on the sarcoplasmic vesicles (Fig 7). With increasing incubation times, the radioactivity which sediments with the vesicles increased and the peaks of radioactive polysaccharide remain superposed on the profile of proteins (Fig 8, curve 1). A gradual shift of the labeled material to higher gradient densities was also noticed. Iodine reactions carried out simultaneously on the different incubated samples show that iodine forms a complex evolving from a brown-red to a purple-red color with increasing optical density. In parallel, 5–10 μ moles of phosphate were released from glucose-1-phosphate (100 μ moles) during these incubation assays.

The following controls were executed: ^{14}C -labeled glucose-1-phosphate mixed but not incubated with the SV did not adsorb onto the membrane structures (Fig 8, curve 2); ^{14}C -labeled glycogen, derived from a separate incubation experiment, placed in contact with purified SV, and recentrifuged on a gradient, remained in the sample zone fraction and did not sediment with the vesicles (Fig 8, curve 3)

The binding between the newly synthesized polysaccharide and the sarcoplasmic vesicles was affected by α -amylase: part of the labeled material which was collected from fractionation tubes 16–22 (Fig 8, curve 4) was treated with α -amylase, 1%, for 60 min; part was used as control. Both samples, recentrifuged on sucrose gradients (Fig. 9), behaved differently, the amylase-treated vesicles sedimented at the usual level but had lost

FIGURE 5 Purified sarcovesicles (SV). This high magnification allows one to visualize at the level of the sarcoplasmic vesicles, in close contact with the outer membrane surface, small, densely lead-stained particles (arrows), 10–20 μ in diameter. These particles correspond to polysaccharide-enzyme units related to the sarcoplasmic reticulum. $\times 100,000$.

FIGURE 6 Purified sarcovesicles (SV), treated with α -amylase. The polysaccharide nature of the small, dense particles, associated with the sarcoplasmic membranes, is demonstrated by their disappearance after 1 hr of treatment with 0.1% crystallized α -amylase at 37°C. $\times 100,000$.



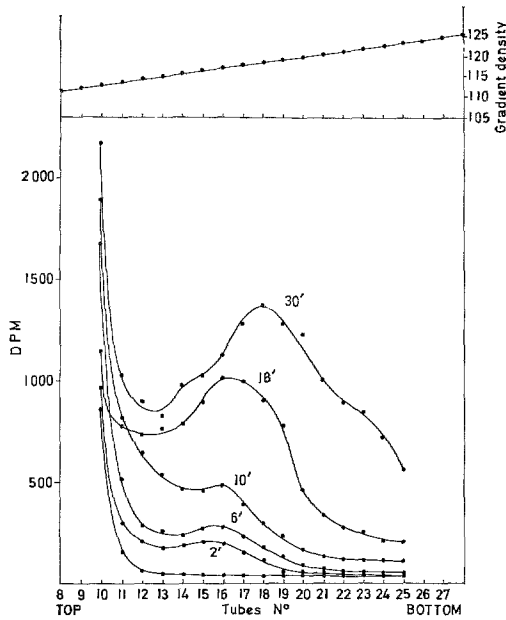


FIGURE 7 Purified sarcovesicular fraction incubated for different time intervals (0, 2, 6, 10, 18, and 30 min) at 37°C in the presence of 0.1 M glucose-1-phosphate-¹⁴C, 0.02 M NaF, and 0.003 M AMP and centrifuged at 24,000 rpm for 5 hr on linear imidazole-buffered sucrose density gradients. Part of the newly synthesized and ¹⁴C-labeled polysaccharide, produced by the phosphorylase, is superposed on the sarcoplasmic vesicles. With increasing incubation times, the radioactivity increases and shifts gradually to higher densities in association with the protein peaks (not indicated here).

their labeled polysaccharide (curve II), and the control vesicles not subjected to amylase digestion sedimented but were loaded with labeled polysaccharide (curve I).

MORPHOLOGY: The sites of synthesis of polysaccharide on the membranes were detected by examining sarcoplasmic vesicles incubated in conditions similar to those used for the isotopic assays. Densely stained particles, irregular in shape and size, were observed in contact with or superposed on the wall of the vesicles. In Fig. 10, an example is shown of a subfraction incubated for 30 min; the dense, newly formed structures sometimes appear as thickenings of the membranes (arrows); others (encircled) form particles which yield fine attachments to the membrane surface. The polysaccharide nature of these structures under synthesis was confirmed by two complementary techniques: the specific histochemical

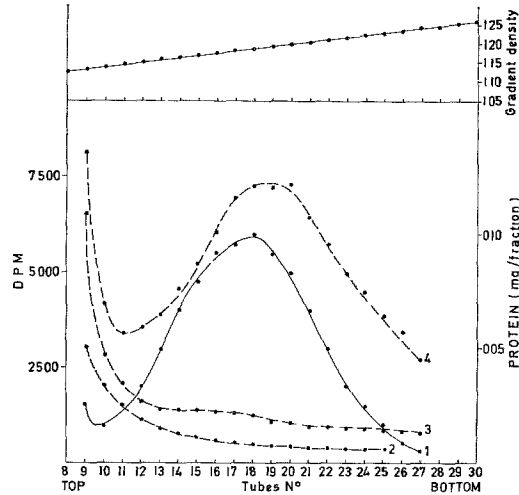


FIGURE 8 Purified sarcovesicular fraction incubated for 15 min with 4 μ Ci of glucose-1-phosphate-¹⁴C and centrifuged at 24,000 rpm for 5 hr on an imidazole-buffered sucrose gradient. curve 1, protein profile; curve 4, radioactivity profile of the incorporated glucose-¹⁴C; curves 2 and 3, controls of sarcovesicles which were not incubated but mixed respectively with glucose-1-phosphate-¹⁴C and glucose-¹⁴C-labeled glycogen.

staining technique for glycogen proposed by Perry (1967) revealed particles (Fig. 11, circles) which bear the same morphological characteristics as the uranyl-lead-stained particles of the classical preparations, the α -amylase treatment of the ultrathin sections according to Monneron and Bernhard (1966) digests the particles. Longer incubation times induce the formation of densely stained filaments (Fig. 12) radiating from a common growing center.

Localization of a Phosphorylase- Phosphorylase Kinase Complex by Phosphorylase Kinase Flash Activation

The flash activation of the phosphorylase system was obtained by adding Ca^{2+} and ATP-Mg^{2+} to the total G.Sv.F and also to the concentrated purified sarcoplasmic vesicles. A very fast but transient conversion of phosphorylase b to a is achieved (Fig. 13) in the total microsomal fraction (curve 1) within 3 min. In the purified sarcoplasmic vesicles a similar conversion occurred, but was less intense (curve 2).

The morphological demonstration of this tran-

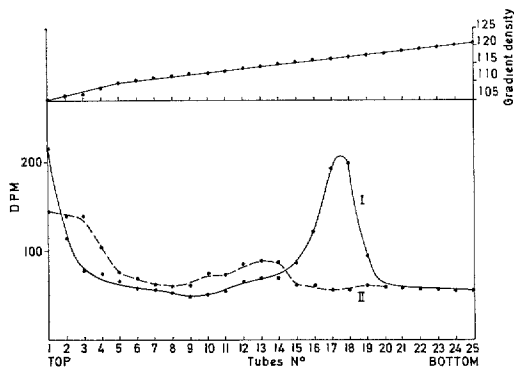


FIGURE 9 Effect of amylase digestion on the sarcovesicles which have incorporated labeled glucose. Treated and untreated samples were centrifuged at 24,000 rpm for 5 hr on imidazole-buffered sucrose gradients. Curve I, untreated sarcovesicles used as control; curve II, sarcovesicles treated with 0.1% α -amylase for 1 hr at 37°C.

sitory activation entailed a slight adaptation of the original method of Heilmeyer *et al.* (1970): the two successive steps of kinase and phosphorylase activation were carried out simultaneously in a single manipulation, and the reaction was prolonged by adding sodium fluoride to inhibit the phosphorylase-phosphatase. In these conditions, the synthesis of polysaccharides at the level of the purified sarcovesicular fraction gave clear pictures. The densely stained particles (Fig. 14) reach 50 μ in diameter. Their structure consists of a dense packing of entangled filaments which constitute a more or less round-shaped granule. Their relation with the vesicular structures appears clearly detectable (arrows). At the periphery of the granules, the filaments stick out and sometimes form a chain with different adjacent particles (star)

DISCUSSION

Localization of Synthetase, Phosphorylase b, and Phosphorylase b Kinase in the Subcellular Fractions Extracted from Skeletal Muscle

Many studies in the past few years have been concerned with the distribution of enzymes related to glycogen breakdown and synthesis in cell fractions isolated from tissue homogenates. The situation is very clear for the UDPG-glycogen transferase which was found intimately bound to particulate glycogen isolated from liver (Leloir and Goldemberg, 1960; Luck, 1961). We have

confirmed these well-established findings in muscle tissue by submitting a muscle homogenate to the precipitation-centrifugation method: the UDPG-glycogen synthetase sediments with the β -glycogen particles, independently of the sedimentation time. Furthermore, the purified sarcovesicular fraction, reconcentrated in a pellet, contains only traces of the enzyme, possibly related to the small amount of glycogen remaining in this fraction. The significance of this latter association is not yet clear and will have to be studied in more detail.

Phosphorylase and phosphorylase b kinase present also a great affinity for glycogen (Madsen and Cori, 1958; Delange *et al.*, 1968), but in a less exclusive manner than synthetase. Phosphorylase mainly bound to glycogen is also found in the postmicrosomal supernatant of a liver homogenate in a proportion which depends upon the degree of depletion in glycogen (Tata, 1964) of the tissue. Phosphorylase b kinase is even less bound and appears, for some authors, as a "soluble enzyme," since 80% is recovered in the postmicrosomal supernatant (Meyer *et al.*, 1970).

Zonal centrifugation of the glycogen-sarcovesicular fraction in a B XIV rotor results in the separation of both constituents, the glycogen particles and the sarcovesicles. The glycogen in its particulate β -form is associated with 11% of the phosphorylase present in the total G Sv F, but 40% of this enzyme and more than 50% of the kinase are bound to the vesicles. At the same level, glycogen detectable with the Krisman procedure is present. It was ascertained by centrifugation tests that the polysaccharide once isolated from the membranes did sediment at a much lower rate than the β -particles and that, under the electron microscope, small particles presenting the staining properties of glycogen were attached to the outer surface of the membranes.

Treatment of the membrane-polysaccharide-enzyme complex with α -amylase led to the digestion of the glycogen moiety and to the release of the enzymes. Fortuitous relocations or bindings were discarded by numerous tests reported in the Results section. Free phosphorylase b kinase (mol wt 1,245,100) and free phosphorylase b (mol wt 185,000) (Secry *et al.*, 1967) would not sediment at the same rate as the vesicles under the conditions of centrifugation used. It appeared, thus, that both enzymes are linked to the sarcovesicles. This relatively stable association was

confirmed by recovery of 40% of the original enzymatic activities in pellets of purified sarco-vesicles obtained by overnight centrifugation. It seems unlikely that enzymes originally bound to the glycogen would have left these particles and finally contaminate the vesicles. This hazardous relocation of two enzymes which differ in molecular weight and affinity for substrates is in contradiction with the reproducibility and clear association between these enzymes and the vesicles.

In order to analyze further the association of the polysaccharide-enzyme complex to sarco-vesicles, polysaccharide synthesis induced by phosphorylase b was performed by incubating the sarco-vesicles at 37°C in the presence of glucose-1-phosphate, NaF, and AMP. The newly synthesized polysaccharide molecules were specifically linked to the sarco-vesicular fraction and remained bound to the vesicles despite recentrifugation. Here, also, the polysaccharide was digested by an incubation with α -amylase. These incubation experiments were confirmed and sustained by electron microscopic examination of the incubated material where the sites of polysaccharide synthesis could be demonstrated. Flash-activation experiments involving a stepwise stimulation of phosphorylase kinase and phosphorylase b led to the synthesis of polysaccharide which was also demonstrated to be confined to the membranes of the sarco-vesicles. Caffeine was added to the reaction medium to prevent activation of phosphorylase b by AMP which might be present in the vesicular fraction or produced from ATP by apyrase ATPase activity.

Nature and Physiological Significance of the Membrane-Glycogen-Enzyme Complex

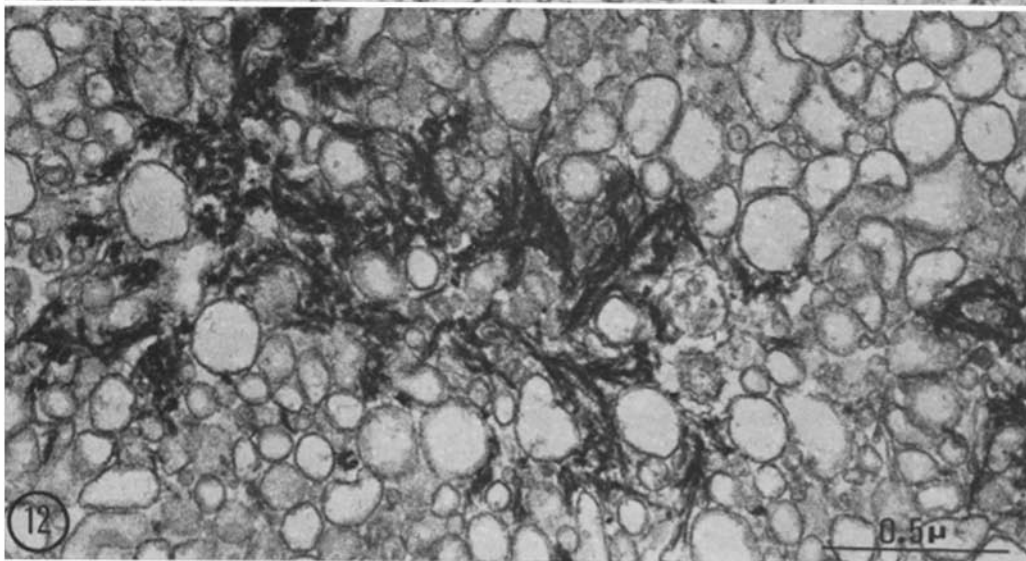
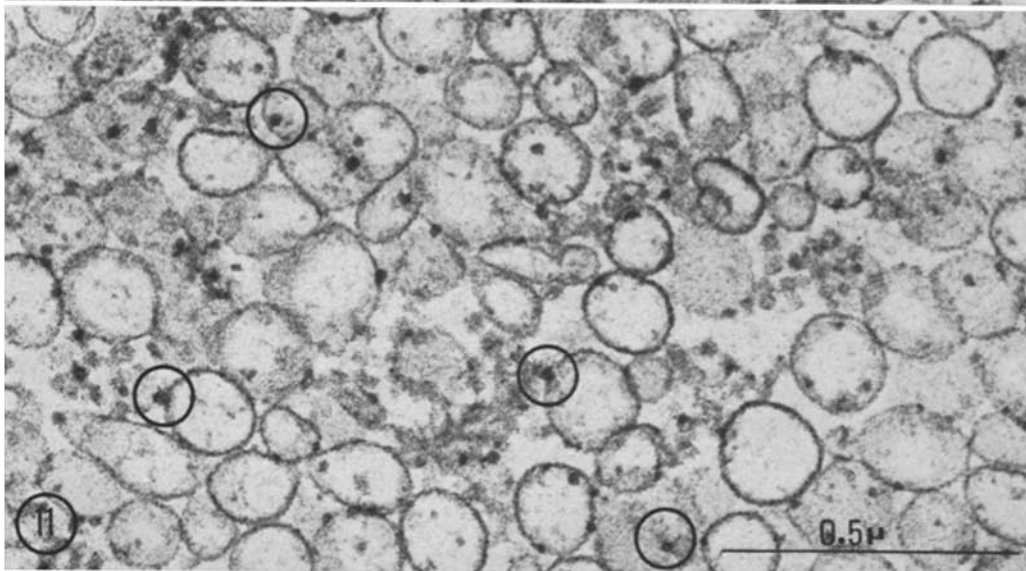
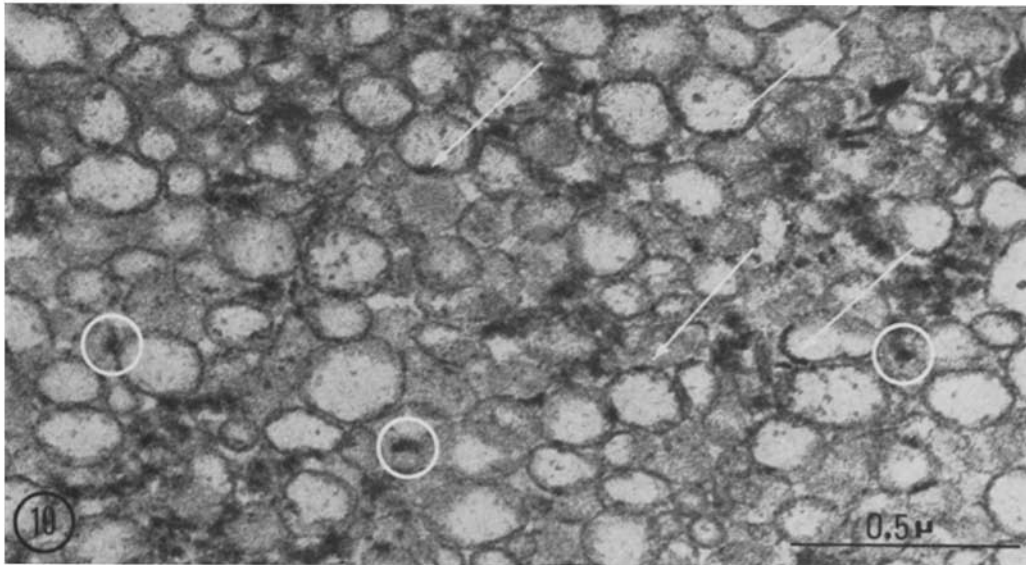
The existence of a glycogen-enzyme complex is now well established. It is not only based on the demonstration of a strong affinity of the enzyme molecules for the glycogen particle but also on morphological and biochemical findings which suggest that glycogen with its metabolizing enzymes constitutes a functional entity (Drochmans, 1964). This concept has been extended recently by a group of workers (Heilmeyer et al., 1970) who have demonstrated that enzymes which control the metabolizing enzymes of glycogen participate in the glycogen-enzyme association. This type of functional unit seems to occur in muscle tissue, when the glycogen is in its particulate β -form, but the situation may be different when the polymer (mol wt 20×10^6) reduces its size. As a result, the enzyme-glycogen balance being modified, the affinity of this complex for other structures and namely for the membranes of the sarcoplasmic reticulum may be enhanced.

The nature of such a binding remains unknown but it seems reasonable to postulate that the complex with its predominant enzyme moiety does not remain free in the sarcoplasm but has a tendency to join a membrane structure. It is not excluded that —SH groups from the phosphorylase, not concerned with enzymatic activity and fully exposed on the enzyme surface (Battell et al., 1968), bind with equivalent groups which are known to exist at the outer surface of the sarcoplasmic vesicles (Hasselbach and Elfvin, 1967). The molecular composition in enzymes and poly-

FIGURE 10 Purified sarco-vesicles, incubated without the addition of polysaccharide for 30 min at 37°C in the presence of 0.1 M glucose-1-phosphate, 0.02 M NaF, and 0.003 M AMP. Sections of these pellets, fixed with 0.1 M PO_4 -buffered distilled glutaraldehyde, 2.5%, were stained with uranyl acetate and lead citrate. The sites of polysaccharide synthesis appear clearly detectable in the form of dense thickenings of the membranes (arrows) and of particles which yield fine attachments to the membrane surface (circles). $\times 60,000$.

FIGURE 11 Purified sarco-vesicles incubated as described in the legend of Fig. 10, treated with 1% periodic acid for 30 min and stained with lead citrate according to the histochemical method of Perry (1967) for polysaccharide detection. The dense staining of the thickenings of the membrane surfaces and of the particles (circles), 20–50 μ in diameter, closely related to the membranes, confirms the polysaccharide nature of these newly synthesized structures. $\times 80,000$.

FIGURE 12 Purified sarco-vesicles incubated for 60 min in the same conditions as those described for Fig. 10. Densely stained filaments radiate from sites located on the membranes. $\times 50,000$.



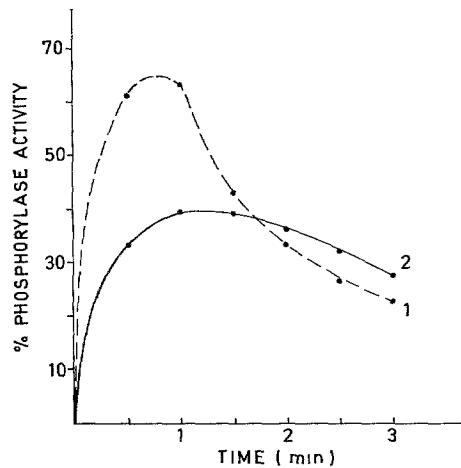


FIGURE 13 Flash activation of phosphorylase in the concentrated G.Sv.F. (curve 1) and in the concentrated purified sarcovesicular fraction (SV, curve 2). Activation was obtained in the presence of 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM ATP.

saccharide also remains hypothetical. On a weight basis, the ratio of phosphorylase b to its kinase would be around 2:1 (Krebs et al, 1964; Delange et al, 1968) if all the phosphorylase was present in the b form, i.e., in the resting muscle. On a molar basis, the ratio would be around 12:1. Further studies will have to define which ratio exists at the level of the units which are linked to the vesicles

The integration of metabolizing enzymes and of their control mechanisms at particular sites in the sarcoplasm parallels the remarkable organization of striated muscle tissue. During muscle contraction, at the beginning of the contraction-relaxation cycle, calcium ions are released from the sarcoplasmic reticulum (Ebashi, 1965; Ebashi et al., 1969). Once released, they would simultaneously trigger the contractile system and activate the glycogenolysis. This latter activation results from the phosphorylase b-to-a conversion

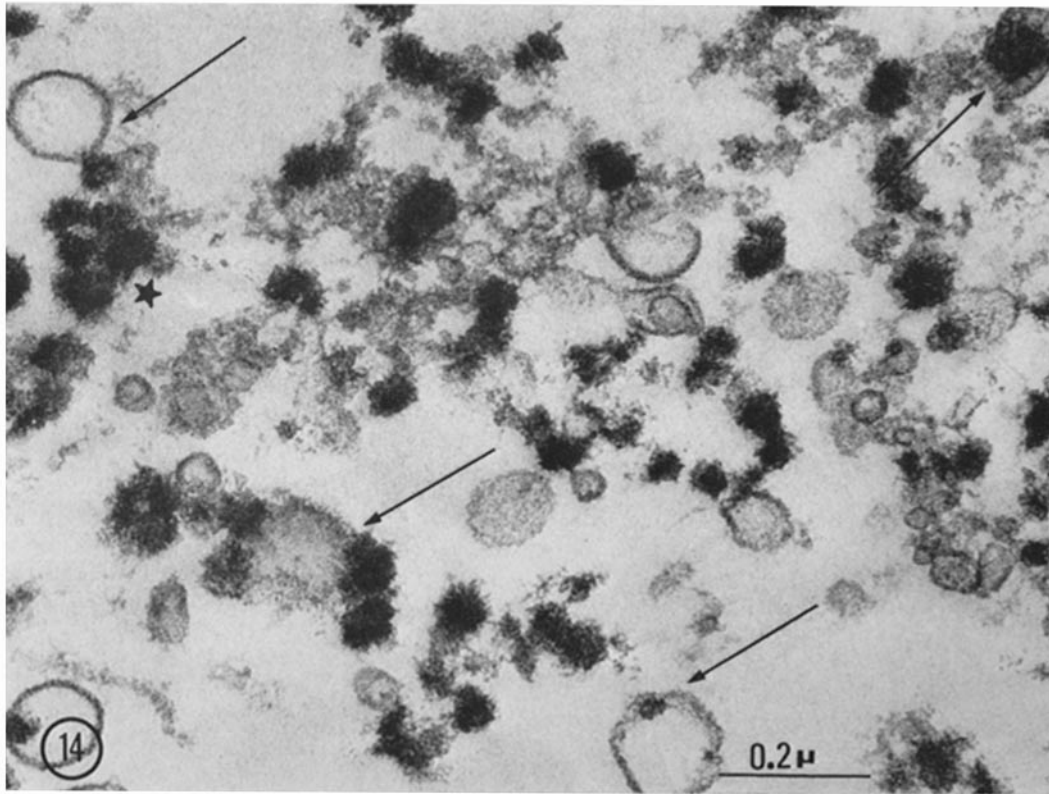


FIGURE 14 Purified sarcovesicles, incubated for 30 min at 37°C in the presence of 5 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM glucose-1-phosphate, 20 mM NaF, 1 mM caffeine, 1 mM ATP, and 0.2 M Tris, pH 6.8. Newly formed polysaccharide material, synthesized under the influence of phosphorylase a, appears closely related to the sarcovesicles (arrows). They constitute densely lead-stained particles, 50-70 m μ in diameter. The particles consist of entangled filamentous structures sticking out in all directions. $\times 100,000$.

(Krebs and Fisher, 1955; Cori, 1956) induced by phosphorylase b kinase (Danforth and Helmreich, 1964, Posner et al, 1965) Calcium activation of phosphorylase b kinase was demonstrated in pure enzyme preparations (Meyer et al., 1964) and at the level of a protein-glycogen complex isolated from striated muscle (Heilmeyer et al, 1970). Moreover, this enzyme activation at physiological Ca^{++} ion concentrations was found to be reversible (Ozawa et al., 1967) The demonstration of flash activations at the level of the purified reconstituted sarcoplasmic vesicles argues in favor of the physiological role of the polysaccharide-enzyme-membrane complex in glycogenolysis accompanying muscle contraction The presence all along the sarcoplasmic reticulum of enzymes which play a role in glycogen breakdown, as phosphorylase kinase and phosphorylase b, associated with polysaccharide molecules, would provide a good model for energy supply at the onset of the contraction-relaxation cycle

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REFERENCES

- BATTELL, M L., C. G. ZARKADAS, L. B. SMILLIE, and N. B. MADSEN. 1968 The sulfhydryl groups of muscle phosphorylase. III. Identification of cysteinyl peptides related to function. *J. Biol. Chem.* **243**:6202.
- BRAY, G. A. 1960 A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1**:279
- CORI, C F. 1956. Regulation of enzyme activity in muscle during work. In *Enzymes: Units of Biological Structure and Function* O. H. Gaebler, editor. Academic Press, Inc., New York.
- DANFORTH, W H., and E HELMREICH. 1964 Regulation of glycolysis in muscle I. The conversion of phosphorylase b to phosphorylase a in frog sartorius muscle *J. Biol. Chem.* **239**:3133.
- DELANGE, R. J., R. G. KEMP, W. D. RILEY, R. A. COOPER, and E. G. KREBS. 1968. Activation of skeletal muscle phosphorylase kinase by adenosine triphosphate and adenosine 3',5'-monophosphate. *J. Biol. Chem.* **243**:2200.
- DROCHMANS, P. 1964. Les macromolécules de la cellule. La structure du glycogène particulaire. VII Congrès Gastro-Entérologie, Acta Gastroenterol. Belgica, Bruxelles.
- EBASHI, S. 1965 The sarcoplasmic reticulum and excitation-contraction coupling. In *Molecular Biology of Muscular Contraction*. S. Ebashi et al, editors Igaku Shoin Ltd., Tokyo 197
- EBASHI, S., M. ENDO, and I. OHTSUKI. 1969. Control of muscle contraction. *Quart. Rev. Biophys.* **2**:351.
- FISKE, C. H., and Y. SUBBAROW. 1925. Colorimetric determination of phosphorus. *J. Biol. Chem.* **66**:375.
- HASSELBACH, W., and L. G. ELFVIN. 1967. Structural and chemical asymmetry of the calcium transporting membranes of the sarcotubular system as revealed by electron microscopy. *J. Ultrastruct. Res.* **17**:598
- HEILMEYER, L. M. G., JR., F. MEYER, R. H. HASCHKE, and E. H. FISCHER. 1970. Control of phosphorylase activity in a muscle glycogen particle. II. Activation by calcium. *J. Biol. Chem.* **245**:6649.
- HERS, H. G. 1964. Glycogen storage disease *Advan. Metab. Disord.* **2**.
- KREBS, E. G. 1966. Phosphorylase b kinase from rabbit muscle *Methods Enzymol.* **8**:543.
- KREBS, E. G., and E. H. FISCHER. 1955. Phosphorylase activity of skeletal muscle extracts. *J. Biol. Chem.* **216**:113
- KREBS, E. G., D. S. LOVE, G. E. BRATVOLD, K. A. TRAYSER, W. L. MEYER, and E. H. FISCHER. 1964 Purification and properties of rabbit skeletal muscle phosphorylase b kinase *Biochemistry* **3**:1022.
- KRISMAN, C. A. 1962. A method for the colorimetric estimation of glycogen with iodine *Anal. Biochem.* **4**:17.
- LELOIR, L. F., and S. H. GOLDBERG. 1960. Synthesis of glycogen from uridine diphosphate glucose in liver *J. Biol. Chem.* **235**:919.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265
- LUCK, D. J. L. 1961. Glycogen synthesis from uridine diphosphate glucose. The distribution of the enzyme in liver cell fractions *J. Biophys. Biochem. Cytol.* **10**:195.
- MADSEN, N. B., and C. F. CORI. 1958 The binding of glycogen and phosphorylase. *J. Biol. Chem.* **233**:1251.
- MEYER, F., L. M. G. HEILMEYER, JR., R. H. HASCHKE, and E. H. FISCHER. 1970. Control of phosphorylase activity in a muscle glycogen particle. I. Isolation and characterization of the protein-glycogen complex. *J. Biol. Chem.* **245**:6642.

- MEYER, W. L., E. H. FISCHER, and E. G. KREBS. 1964. Activation of skeletal muscle phosphorylase b kinase by Ca^{2+} . *Biochemistry*. **3**:1033.
- MONNERON, A., et W. BERNHARD. 1966. Action de certaines enzymes sur des tissus inclus en epon. *J. Microsc. (Paris)*. **5**:697.
- NELSON, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* **153**:375.
- OZAWA, E., K. HOSOI, and S. EBASHI. 1967. Reversible stimulation of muscle phosphorylase b kinase by low concentrations of calcium ions. *J. Biochem.* **61**:4, 531.
- PERRY, M. M. 1967. Identification of glycogen in thin sections of amphibian embryos. *J. Cell Sci.* **2**:257.
- PORTER, K. R., and C. BRUNI. 1959. An electron microscope study of the early effects of 3'-Me-DAB on rat liver cells. *Cancer Res.* **19**:997.
- POSNER, J. B., R. STERN, and E. G. KREBS. 1965. Effects of electrical stimulation and epinephrine on muscle phosphorylase, phosphorylase b kinase, and adenosine 3',5'-phosphate. *J. Biol. Chem.* **240**:982.
- SEERY, V. L., E. H. FISCHER, and D. C. TELLER. 1967. A reinvestigation of the molecular weight of glycogen phosphorylase. *Biochemistry*. **6**:3315.
- TATA, J. R. 1964. Subcellular redistribution of liver α -glucan phosphorylase during alterations in glycogen content. *Biochem. J.* **90**:284.
- VILLAR-PALASI, C., and J. LARNER. 1961. Insulin treatment and increased UDPG-glycogen transglucosylase activity in muscle. *Arch. Biochem. Biophys.* **94**:436.
- WANSON, J. C., and P. DROCHMANS. 1968 *a*. Rabbit skeletal muscle glycogen. A morphological and biochemical study of glycogen β -particles isolated by the precipitation-centrifugation method. *J. Cell Biol.* **38**:130.
- WANSON, J. C., and P. DROCHMANS. 1968 *b*. Detection of phosphorylase with the electron microscope. *Contr. Glycogen Metab. Fed. Eur. Biochem. Soc. Symp.* **4th**. 169.
- WANSON, J. C., and L. TIELEMANS. 1971. Morphological and biochemical characteristics of glycogen particles isolated from rabbit polymorphonuclear leukocytes. *J. Cell Biol.* **49**:316.