Carp Myogens of White and Red Muscles

GROSS ISOLATION ON SEPHADEX COLUMNS OF THE LOW-MOLECULAR-WEIGHT COMPONENTS AND EXAMINATION OF THEIR PARTICIPATION IN ANAEROBIC GLYCOGENOLYSIS

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1. The combined low-molecular-weight protein components of the myogens from carp white and red muscles [about 30% (w/w) of the myogen proteins] have been isolated by gel filtration on Sephadex G-75 columns. 2. The presence in this fraction from myogen of white muscle of the three main electrophoretic components previously isolated has been confirmed, and the low molecular weight of the fourth component has been definitely established. 3. The exclusive presence of this fourth component in the myogen of red muscle, apart from myoglobin, has also been demonstrated. 4. Glycogenolysis experiments *in vitro* have shown that the low-molecular-weight protein fraction from carp myogen does not contain enzymes from the Embden-Meyerhof chain.

The investigations described in the two preceding papers (Hamoir & Konosu, 1965; Konosu, Hamoir & Pechère, 1965) have led to the isolation of three individual low-molecular-weight proteins from carp myogen that amount together to about 30% (w/w) of the myogen protein. The procedure involved ammonium sulphate fractionation and zone electrophoresis. Methods are available at present (Porath & Flodin, 1959), however, that also allow the fractionation of protein components according to their molecular size. This method of protein separation has now been applied to whole carp myogen, and the results so obtained have been compared with those described by Hamoir & Konosu (1965) and Konosu *et al.* (1965). The possibility, also, of the simultaneous isolation of all the low-molecular-weight protein components that are so characteristic of the myogen of fishes (Hamoir, 1955b; Connell, 1958; Henrotte, 1960) and other lower vertebrates (Deuticke, 1934) seemed to provide a favourable way to approach the examination of their biochemical and physiological significance.

As myogen is mainly made up of those enzymes that catalyse the chain of reactions involved in anaerobic glycogenolysis, their contribution to the small proteins was examined first. Most of these enzymes have been isolated in crystalline form and well characterized from the tissues of higher vertebrates (Czok & Bücher, 1960), and their relatively high sedimentation coefficients and low negative electrophoretic mobilities have been well estab-

lished. But little is known of the corresponding enzymes of lower vertebrates, and detailed investigations on fish myogen, in particular, refer only to glucosan phosphorylase (Cordier & Cordier, 1957; Ono & Nagayama, 1957), phosphoglucomutase and phosphoglucose isomerase (Martin & Tarr, 1961), aldolase (Shibata, 1958; Grandjean, 1959) and glyceraldehyde phosphate dehydrogenase (Ludovicy-Bungert, 1961). In addition, as the catalytic behaviour of these few enzymes has been found to be similar to that of the corresponding enzymes of higher vertebrates, it would be of interest to obtain electrophoretic and ultracentrifugal data to supplement that already obtained by Grandjean (1959) and Ludovicy-Bungert (1961), and thus allow comparisons of the enzymes at the molecular level. The present evidence is not sufficient to exclude the possibility that some of the other enzymes that also participate in the Embden-Meyerhof pathway might be encountered in the low-molecular-weight fraction from carp myogen, especially in view of the very high proportion of this fraction in the total myogen. Preliminary observations (G. Hamoir & G. Siebert, unpublished work) suggested that individual tests for the presence of the different enzymes in the three isolated components (Hamoir & Konosu, 1965) would be cumbersome. On the other hand, the availability of the low-molecular-weight fraction as a whole provides an excellent way to decide its possible involvement in glycogenolysis. Its incubation in the presence of

glycogen and the necessary cofactors would not lead to the appearance of the final product, lactic acid, if even one enzyme of the chain were missing.

The gross isolation of the low-molecular-weight components of carp myogen on columns of Sephadex G-75, allowing their further characterization, is described below, and also glycogenolysis experiments *in vitro* that lead to the conclusion that these components do not participate in the normal course of the Embden-Meyerhof metabolic pathway.

METHODS

Materials. Mirror carp (*Cyprinus carpio* L.) were used. The killing of the animals and the isolation of the muscles were as described by Hamoir & Konosu (1965).

Sephadex G-25 and G-75 were obtained from Pharmacia A.B., Uppsala, Sweden.

Glycogen (E. Merck A.-G., Darmstadt, Germany), glucose 6-phosphate (barium salt) (Sigma Chemical Co., St Louis, Mo., U.S.A.), glucose 1-phosphate (potassium salt) (Schwarz BioResearch Inc., Mount Vernon, N.Y., U.S.A.), fructose 6-phosphate (barium salt) (Schwarz), fructose 1,6-diphosphate (dibarium salt) (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), lithium lactate (Th. Schuchardt, München, Germany), ATP (disodium salt) (Sigma), NAD (Sigma), creatine (British Drug Houses Ltd., Poole, Dorset) and cysteine hydrochloride (Merck) were used. The insoluble barium salts were transformed into the corresponding soluble sodium salts by precipitation of Ba^{2+} from their acid (0.1 N-HCl) solution with Na_2SO_4 and subsequent neutralization.

All other chemicals used were A.R. grade.

Preparation of the extracts. The isolated muscles were minced at room temperature in a domestic meat grinder, or, if stored frozen, were sliced in 40μ -thick slices with a freezing-microtome. The pulp so obtained was then extracted by stirring for 1 hr. in the cold ($3-4^\circ$) with 0.8 vol. (v/w) of 0.3 M-sucrose-10 mM-triethylamine-3 mM-EDTA (disodium salt) (Czok & Bücher, 1960). The pH obtained was between 7.0 and 7.4. After extraction, the suspension was centrifuged for 15 min. in the cold at 12000 rev./min. and further clarified for 25 min. at 25000 rev./min. in a pre-cooled Spinco model L ultracentrifuge. The extract was then used directly or stored frozen. No difference was noticed between the extracts obtained by these two ways of handling.

Gel filtration. Columns of 2.2 cm. $\times$ 40.5 cm. (Sephadex G-25) and 1.3 cm. $\times$ 46 cm. or 2.2 cm. $\times$ 39 cm. (Sephadex G-75) were run in the cold room ($3-4^\circ$) at moderate speed (about 25 ml./hr. for the Sephadex G-25 column and about 5 and 10 ml./hr. for the small and large Sephadex G-75 columns respectively), with 0.1 M-KCl-1 mM- KH_2PO_4 , adjusted to pH 7.3 with 1 N-KOH, as eluent, after equilibration with the same liquid. Samples (5 ml.) containing about 20 mg. of protein/ml. were normally applied to the Sephadex G-25 column, and 3 ml. or 6 ml. samples containing between 13 and 20 mg. of protein/ml. to the small or large Sephadex G-75 column respectively. The separations were followed by continuous ultraviolet monitoring of the effluents (Uvicord Ultraviolet Absorptiometer; LKB, Stockholm, Sweden) and by performing a biuret reaction on 0.3 ml. samples of each fraction.

Protein concentrations. These were estimated with the biuret reagent by using the procedure of Gornall, Bardawill & David (1949) and with reference to a standard curve established on a carp myogen solution of which the protein content had been measured by refractometry assuming a refractive index increment of 0.000182 ml./mg. (Hamoir, 1955a).

Glycogenolysis. A modification of the conditions described by Curtius & Ohlmeyer (1938) was used. To 9 vol. of glycogenolytic medium [0.4% (w/v) glycogen in 0.22 M-potassium phosphate buffer (pH 7.4)-28 mM- KHCO_3 -4.4 mM- MgSO_4 -1.1 mM-ATP-0.2 mM-NAD-0.55 mM-cysteine hydrochloride-16 mM-creatine, final pH 7.48] 1 vol. of fractionated (or unfractionated) muscle extract was added, at 25° , both mixtures having been pre-equilibrated at that temperature. At subsequent definite time-intervals (usually multiples of 10 min.), 2.5 ml. samples were withdrawn and added to 0.5 ml. of 32% (w/v) trichloroacetic acid. After centrifugation, the amount of the metabolite under examination was estimated in the supernatant.

When the above procedure was first applied to whole carp myogen after it had been submitted to filtration through a Sephadex G-25 column to separate the proteins from native cofactors and metabolites, no lactic acid formation was observed. Further investigation (see the Results section) definitely suggested that the process was interrupted at the level of transformation of fructose 6-phosphate into fructose 1,6-diphosphate, as if the enzyme 6-phosphofructokinase was absent from the protein mixture used for incubation. Control experiments indicated that neither high-speed clarification of the extracts nor passage through Sephadex G-25 was responsible for the lack of activity, this deficiency being already detectable in extracts that had not been submitted to these treatments. Actually, it is known (see the Discussion section) that 6-phosphofructokinase is comparatively difficult to extract from muscle and that it is fairly labile. In spite of considerable efforts, however, involving modifications of the extraction medium [addition of cysteine in variable amounts, extraction with 10 vol. (v/w) of sucrose medium or with 30 mM-KOH (Ling, Byrne & Lardy, 1955), use of an homogenizer] or of the glycogenolytic medium (addition of cysteine, changes in Mg^{2+} and ATP concentrations), it was found impossible to observe lactic acid formation from glycogen without the addition of fructose 1,6-diphosphate. Inclusion of this metabolite in the glycogenolytic mixture above is of course equivalent to a disregard for the presence of all the enzymes that are necessary for its formation from glycogen. On the other hand, the addition of a commercial 6-phosphofructokinase preparation might also lead to the involuntary introduction of some other glycogenolytic enzyme. To avoid these drawbacks, it was therefore decided to split the examination of the participation of the low-molecular-weight protein components in glycogenolysis into two parts: from glycogen to fructose 6-phosphate (as followed by fructose 6-phosphate determinations) and from fructose 1,6-diphosphate to lactic acid (as followed by lactic acid determinations). Except for the additional presence of 0.33 mM-fructose 6-phosphate in the second case and the occasional presence of 0.045 mM- Ca^{2+} in the first one (see the Results section), the same medium as described above was used in both sets of incubations.

Fructose 6-phosphate. This was determined by the method of Dische (1953).

Fig. 1. Elution profile of the proteins of the myogen from white muscle after passage through a Sephadex G-75 column (1.3 cm. $\times$ 46 cm.) in 0.1 M-KCl-1 mM-KH₂PO₄ buffer, pH 7.3 (see the text). The volume of the sample (39.6 mg. of protein) was 3 ml., and that of the collected fractions 1.2 ml. The flow rate was 8.7 ml./hr. ●, Biuret colour of 0.3 ml. samples from each fraction; ○, ultraviolet absorption (3 mm. light-path) of each fraction as estimated from the tracing of the effluent monitor.

Lactic acid. This was determined by the procedure of Barker (1957).
Electrophoresis. This was conducted as described by Hamoir & Konosu (1965).

RESULTS

Fractionation of carp myogen on Sephadex columns. If an extract from carp white muscle is passed through a Sephadex G-25 column, the usual separation into proteins (non-retarded) and diffusible components (retarded) occurs. The cofactors and metabolic sfumronnces present in the original muscle can thus be removed before doing the glycogenolytic experiments. When a sample of the pooled fractions of the protein peak from this first column is now passed through a Sephadex G-75 column, the elution profile depicted in Fig. 1 is obtained: the mixture is resolved into two peaks. The retarded one corresponds to proteins having a molecular weight below 30 000 and this is subsequently referred to as the total low-molecular-weight fraction. It amounts to 28% (w/w) of the total proteins, as estimated from the ratio of the biuret-positive areas of the two peaks. This value is in excellent agreement with the ultracentrifugal data described by Hamoir & Konosu (1965). Also, the specific ultraviolet absorption of the total low-molecular-weight fraction is much smaller than that of the first, non-retarded, peak, again in agreement with previous findings (Konosu *et al.* 1965).

When whole carp myogen from red muscle is submitted to the same treatment, two peaks are also obtained (Fig. 2). However, the low-molecular-weight fraction, which in this case amounts to 25% (w/w) of the total proteins, appears to consist almost exclusively of myoglobin. Indeed, when the

Fig. 2. Elution profile of the proteins of the myogen from red muscle after passage through a Sephadex G-75 column (1.3 cm. $\times$ 46 cm.) in 0.1 M-KCl-1 mM-KH₂PO₄ buffer, pH 7.3 (see the text). The volume of the sample (39.3 mg. of protein) was 3 ml., and that of the collected fractions 1.2 ml. The flow rate was 8.7 ml./hr. ●, Biuret colour of 0.3 ml. samples from each fraction; ○, ultraviolet absorption (3 mm. light-path) of each fraction as estimated from the tracing of the effluent monitor.

percentage of this pigment in the sample applied to the column is estimated from the extinction coefficients at 410 and 495 mμ given in the literature (Theorell, 1934), a value of 24% (w/w) of the total proteins is found. Only about 1% of the total proteins of the myogen from red muscle is thus left for low-molecular-weight constituents other than myoglobin, whereas a similar calculation made for the myogen from white muscle indicates that 26% (w/w) of the total proteins are low-molecular-weight components different from myoglobin, this last protein then amounting to only about 2% (w/w) of the total. The considerable difference between the composition of the myogens from the two types of muscle, already noticed in electrophoretic experiments (Hamoir & Konosu, 1965), is thus well supported by the technique of gel filtration.

Electrophoresis of the total low-molecular-weight components of carp myogen. This comparison was further substantiated by examining the electrophoretic behaviour, in the Tiselius apparatus, of the total low-molecular-weight components of both types of muscle under the same conditions as were used previously (Hamoir & Konosu, 1965; Konosu *et al.* 1965). With white muscle, the diagrams obtained show the presence of only four components, in proportions very similar to those that are found, during electrophoresis of total myogen, for components 1, 2, 3 and 5 (Hamoir & Konosu, 1965). With red muscle, the same holds true for components 1, 6 and 7 of this total myogen. The mobilities of the two sets of components are compared in Table 1 with those of the individual proteins found in the ex-

Table 1. *Electrophoretic mobilities (ascending) of low-molecular-weight components of carp myogen*

Component	$10^5 \times \text{Mobility (cm.}^2\text{v}^{-1}\text{sec.}^{-1})$	
	Present study	Hamoir & Konosu (1965)
White muscle:		
1	-5.73	-5.80
2	-4.87	-4.74
3	-3.91	-3.86
5	-3.06	-2.88
Red muscle:		
1	-5.84	-5.81
6	-2.52	-2.43
7	-1.60	-1.63

periments on the ammonium sulphate-fractionated myogens (Hamoir & Konosu, 1965). The agreement is very good.

Participation of the low-molecular-weight components in anaerobic glycogenolysis. (a) From glycogen to fructose 1,6-diphosphate. The total proteins of the myogen from carp white muscle, as separated on Sephadex G-25, catalyse the conversion of glycogen into fructose 6-phosphate in the presence of the necessary cofactors (see the Methods section). However, after some time, the production of phosphoric ester decreases before resuming again (Table 2). In control experiments, where glycogen was replaced in the glycogenolytic medium by glucose 1-phosphate (18 mM) or glucose 6-phosphate (18 mM), the production of fructose 6-phosphate followed a normal progress curve, without transitory decrease. It was therefore inferred that the apparently abnormal curve with glycogen as substrate is probably in some way related to the behaviour of the α -glucan-phosphorylase system. It is known (Krebs, Graves & Fischer, 1959) that the activation of this system is a very complicated process, which can be partially favoured by Ca^{2+} . The myogen protein mixture was therefore also preincubated for 20 min. at 25° in the presence of 0.045 mM-calcium acetate. This maintained the rapid initial production of fructose 6-phosphate for a longer period (Table 2), but without leading to a complete disappearance of the transitory decrease. It was decided, nevertheless, to preincubate the protein mixtures with calcium acetate (0.045 mM), as described above, when dealing with the first part of the glycogenolytic chain.

To compare the glycogenolytic activities of the two protein peaks obtained after passage through the Sephadex G-75 column, the appearance of fructose 6-phosphate was followed in three incubation mixtures: the first contained proteins from peak 1 (high-molecular-weight proteins) alone (0.068 mg. of protein/ml.); the second contained proteins from

Table 2. *Production of fructose 6-phosphate from glycogen by the total proteins from carp white muscle*

The conditions of the experiments are given in the text. Protein concentrations in the reaction mixture were 0.162 mg./ml. (without Ca^{2+}) and 0.135 mg./ml. (with Ca^{2+})

Time (min.)	Fructose 6-phosphate produced ($\mu\text{mole/ml. of reaction mixture}$)	
	Without preincubation with Ca^{2+}	After preincubation with Ca^{2+}
0	0.000	0.000
10	0.031	0.033
20	0.006	0.052
30	0.011	0.042
40	0.045	0.045
50	0.068	0.058
60	0.066	0.057

peak 1 (0.068 mg. of protein/ml.) and peak 2 (low-molecular-weight proteins) (0.068 mg. of protein/ml.) together, their relative proportions thus being 1:1 (w/w) and the final concentration of peak-1 proteins being the same as in the first incubation mixture; finally, the third incubation mixture contained peak-2 proteins alone at about the same concentration (0.084 mg. of protein/ml.) as that of peak-1 proteins in the first incubation mixture. In spite of the irregular shape of the curve for fructose 6-phosphate production, as discussed above, Fig. 3 shows that the proteins of peak 1 have very much the same glycogenolytic activity alone or together with the proteins from peak 2, which indicates that the latter do not contribute to the activity. This is completely corroborated by the total lack of activity of the proteins of peak 2 alone. Further, entirely similar results were obtained in parallel experiments with glucose 1-phosphate as substrate instead of glycogen, when normal progress curves were found. It is therefore clear that the low-molecular-weight proteins of carp myogen from white muscle do not take part in the glycogenolytic activity of this mixture, up to the level of fructose 6-phosphate.

(b) From fructose 1,6-diphosphate to lactic acid. The participation of the low-molecular-weight components in the second part of the glycogenolytic pathway was examined in exactly the same way as for the first part. No difficulty was experienced, in this instance, with the appearance of the metabolite under observation, lactic acid, the progress curve having a much more normal shape. The power of the proteins from the first peak (0.019 mg. of protein/ml.) to transform fructose 1,6-diphosphate into lactic acid (Fig. 4) is again remarkably similar whether they be alone or mixed with the proteins from the second peak (0.019 mg. of protein/ml.) Here also, proteins from the second peak alone

Fig. 3. Production of fructose 1-phosphate from glycogen (Ca^{2+} present) by the proteins from peak 1 (●), peak 1 plus peak 2 (○) and peak 2 (□) under the conditions described in the text.

Fig. 4. Production of lactic acid from fructose 1,6-diphosphate by the proteins from peak 1 (●), peak 1 plus peak 2 (○) and peak 2 (□) under the conditions described in the text.

(0.033 mg. of protein/ml.) were completely devoid of activity. It is therefore concluded that there is no participation of the low-molecular-weight proteins of carp myogen from white muscle in glycogenolysis.

DISCUSSION

The use of Sephadex G-75 columns has permitted the rapid isolation of the combined low-molecular-weight protein components present in the myogen

of both carp white and red muscle, and the results obtained by this method agree very well with those obtained by ultracentrifugation (Henrotte, 1960; Hamoir & Konosu, 1965). The percentage of low-molecular-weight proteins in the total myogen deduced from biuret-positive areas of the elution profile after gel filtration are at slight variance with those estimated from refractive-index tracings in the ultracentrifuge [e.g. 28% (w/w) and 32% (w/w) respectively, in white muscle]. Such a small discrepancy is in the direction that is to be expected, since the results from the ultracentrifugal examination are slightly increased owing to the Johnston-Ogston effect (Johnston & Ogston, 1946). Sufficiently large amounts of the total low-molecular-weight proteins from the myogen of both white and red muscles have thus been conveniently isolated so that they could be submitted to electrophoretic and biochemical investigations.

The composition of this fraction in both types of muscle could then be examined. In white muscle, electrophoresis confirmed the presence of the three main components 2, 3 and 5 described by Konosu *et al.* (1965), and, further, showed unequivocally that component 1 (Hamoir & Konosu, 1965) is also a low-molecular-weight protein. No other low-molecular-weight component could be detected. Component 1 is also present in the myogen of red muscle, together with myoglobin, and is the only low-molecular-weight component that occurs in the myogen from both types of muscle. The difference between the carp myogens from the two types of muscle is thus much greater than in the rabbit (Weber & Meyer, 1933; Amberson, Erdos, Chinn & Ludes, 1949; Czok & Bücher, 1960), where the presence or absence of myoglobin is the main variable.

The use of Sephadex columns also allowed an examination of the possible participation in anaerobic glycogenolysis of the low-molecular-weight components from white muscle. Difficulties, however, were experienced in the development of a complete glycogenolysis system functioning *in vitro*. Very few descriptions of similar attempts were found in the recent literature: glucose has generally been the substrate used and not glycogen. Lactic acid was not formed from the latter substrate under conditions that are theoretically adequate for such a reaction. The control experiments established that this was due to a lack of 6-phosphofructokinase activity in the extracts; but it was not possible to decide between the absence or the inactivation of the enzyme during the extraction procedure. It is known that this enzyme is particularly hard to extract from animal tissues (Czok & Bücher, 1960), and there are many recent reports of its scarcity in fish muscle extracts (Shibata & Yoshimura, 1960; McLeod, 1961; McLeod, Jonas &

Roberts, 1963) and frog muscle extracts (Margreth, 1963; Margreth, Muscatello & Andersson-Cedergren, 1963). It also appears (Reuter, 1960; Dingle & Hines, 1960; McLeod *et al.* 1963) that extracts of fish muscle at low ionic strength have a fairly high adenosine-triphosphatase activity that, under the conditions of the experiments *in vitro*, could destroy the ATP needed by 6-phosphofructokinase. Finally, the enzyme has rather exacting requirements for its proper functioning (Lardy, 1962), and these might not have been satisfied. Deficiencies in its activity are usually compensated by the addition of either fructose 1,6-diphosphate or yeast 6-phosphofructokinase. It was found preferable in the present study to split the tests for activity of the glycogenolytic chain into two parts, from glycogen to fructose 6-phosphate and from fructose 1,6-diphosphate to lactic acid. It is true that 6-phosphofructokinase, if it existed as a low-molecular-weight species in carp muscle, would then escape detection, but since this enzyme is not extracted at all the objection is of no consequence in this connexion. There are, further, other reasons to believe that 6-phosphofructokinase, in carp muscle, is not a low-molecular-weight protein.

The comparison of the glycogenolytic powers of the high- and low-molecular-weight protein species (in terms of gel filtration on a Sephadex G-75 column) from carp myogen thus definitely excludes the presence of all but one of the enzymes of the Embden-Meyerhof chain in the low-molecular-weight fraction. This conclusion is further reinforced by the fact that, when myoglobin is disregarded, only a negligible amount of low-molecular-weight protein is found in the myogen from red muscle, a tissue that nevertheless metabolizes glycogen as well as white muscle. It would therefore be very surprising if 6-phosphofructokinase behaved as an isolated exception and was a low-molecular-weight protein in carp muscle corresponding to the very acidic component 1 of the total myogen (Hamoir & Konosu, 1965).

It thus seems that all the glycogenolytic enzymes from the muscles of this fish, and probably of other lower vertebrates, belong to those proteins that are not retarded during gel filtration on a Sephadex G-75 column, and that have high sedimentation coefficients (5–7s) and low negative mobilities at neutral pH. This makes them very similar in size and charge to the corresponding enzymes from higher vertebrates.

The biochemical and physiological significance of the large amounts of low-molecular-weight protein present in the myogen of many lower vertebrates therefore remains unknown.

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REFERENCES

- Amberson, W. R., Erdos, T., Chinn, B. & Ludes, H. (1949). *J. biol. Chem.* **181**, 405.
- Barker, S. B. (1957). In *Methods in Enzymology*, vol. 3, p. 241. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Connell, J. J. (1958). *Biochem. J.* **69**, 5.
- Cordier, D. & Cordier, M. (1957). *C.R.Soc. Biol., Paris*, **151**, 1909.
- Curtius, L. & Ohlmeyer, P. (1938). *Biochem. Z.* **298**, 412.
- Czok, R. & Bücher, Th. (1960). *Advanc. Protein Chem.* **15**, 315.
- Deuticke, H. J. (1934). *Hoppe-Seyl. Z.* **224**, 216.
- Dingle, J. R. & Hines, J. A. (1960). *Canad. J. Biochem. Physiol.* **38**, 1437.
- Dische, Z. (1953). *J. biol. Chem.* **204**, 983.
- Gornall, A. C., Bardawill, C. S. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
- Grandjean, Th. (1959). Thèse de Licence: Faculté des Sciences, Liège.
- Hamoir, G. (1955a). *Arch. int. Physiol. Biochim.* **63** (suppl.), 1.
- Hamoir, G. (1955b). *Advanc. Protein Chem.* **10**, 227.
- Hamoir, G. & Konosu, S. (1965). *Biochem. J.* **96**, 85.
- Henrotte, J. G. (1960). *Biochim. biophys. Acta*, **39**, 103.
- Johnston, J. P. & Ogston, A. G. (1946). *Trans. Faraday Soc.* **42**, 789.
- Konosu, S., Hamoir, G. & Pechère, J.-F. (1965). *Biochem. J.* **96**, 98.
- Krebs, E. G., Graves, D. J. & Fischer, E. H. (1959). *J. biol. Chem.* **234**, 2867.
- Lardy, H. (1962). In *The Enzymes*, vol. 6, p. 67. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.
- Ling, K. H., Byrne, W. L. & Lardy, H. (1955). In *Methods in Enzymology*, vol. 1, p. 307. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Ludovicy-Bungert, L. (1961). *Arch. int. Physiol. Biochim.* **69**, 265.
- McLeod, R. A. (1961). In *Comparative Physiology of Carbohydrate Metabolism in Heterothermic Animals*, p. 115. Ed. by Martin, A. W. Seattle: University of Washington Press.
- McLeod, R. A., Jonas, R. E. E. & Roberts, E. (1963). *Canad. J. Biochem. Physiol.* **41**, 1971.
- Margreth, A. (1963). *Biochim. biophys. Acta*, **77**, 337.
- Margreth, A., Muscatello, U. & Andersson-Cedergren, E. A. (1963). *Exp. Cell Res.* **32**, 484.
- Martin, O. & Tarr, H. L. A. (1961). *Canad. J. Biochem. Physiol.* **39**, 297.
- Ono, T. & Nagayama, F. (1957). *Bull. Jap. Soc. sci. Fish.* **23**, 260.
- Porath, J. & Flodin, P. (1959). *Nature, Lond.*, **183**, 1657.
- Reuter, A. (1960). *Arch. int. Physiol. Biochim.* **68**, 339.
- Shibata, T. (1958). *Bull. Fac. Sci. Hokkaido Univ.* **9**, 218.
- Shibata, T. & Yoshimura, K. (1960). *Bull. Jap. Soc. sci. Fish.* **26**, 294.
- Theorell, H. (1934). *Biochem. Z.* **268**, 55.
- Weber, H. H. & Meyer, K. (1933). *Biochem. Z.* **266**, 137.