The Effect of Actin on the Magnesium-Activated Adenosine Triphosphatase of Heavy Meromyosin

By L. LEADBEATER* and S. V. PERRY
Department of Biochemistry, University of Birmingham

The evidence is now strong that magnesium plays an important role in the interactions between myosin, actin and ATP which are associated with the physiological activity of muscle. Although calcium activates the adenosine triphosphatase of both L-myosin and isolated myofibrils, in the presence of this cation alone neither contraction (Ashley, Arasimavicius & Hass, 1956) nor relaxation (Bendall, 1953; Bozler, 1952) of myofibrillar systems can occur. On the other hand magnesium, which is essential for the contraction–relaxation cycle, either inhibits or has little effect on L-myosin adenosine triphosphatase, depending on the ionic strength. Nevertheless, this cation strongly activates the hydrolysis of ATP by actomyosin systems at low ionic strength. Hence the mechanism by which actin modifies the role of magnesium in the myosin–adenosine-triphosphatase system is of great interest both on general enzymological grounds and for the understanding of the mechanicochemical process associated with contraction in muscle.

An important feature of the magnesium-activation of actomyosin adenosine triphosphatase is that it does not occur at ionic strengths greater than 0·15–0·20. To explain this effect it has been suggested that at low ionic strength the enzyme is in the form of the actomyosin complex which is magnesium-activated, whereas as the ionic strength increases the complex is dissociated and the system assumes the enzymic characteristics of free L-myosin, i.e. it is magnesium-inhibited (Szent-Gyorgyi, 1951; Hasselbach, 1952). A direct test of this hypothesis is difficult because actomyosin is insoluble under those ionic conditions at which magnesium-activation occurs, and is therefore not very amenable to physical studies that enable the physical state of the complex to be determined. The heavy meromyosins, however, offer advantages for studies of this kind, as they retain the biological activity of the original myosin and form a complex with actin which is soluble at low ionic strength (Szent-Gyorgyi, 1953; Gergely, Gouvea & Karibian, 1955).

The present investigation is a study of the effects of magnesium on the enzymic activity of the heavy

meromyosins and their complexes with actin. It provides evidence that actin can influence the enzymic activity of the heavy meromyosins even when viscometric studies suggest dissociation of the complexes formed by actin with these sub-units of the myosin molecule. Some of these findings have been briefly reported (Perry & Leadbeater, 1963).

METHODS

Preparation of muscle proteins. The L-myosin was obtained from back and leg muscles of the rabbit as described by Perry (1955), and the method of Straub (1943) was used to prepare acetone-dried muscle fibre for actin preparations. In some cases the acetone-dried fibre was prepared from the residue of the muscle centrifuged down in the first stage of the myosin preparation. This was carried out by extracting the residue with 10 vol. of 0·4% $NaHCO_3$ for 10 min. and filtering the suspension through muslin. As much liquid was squeezed out as possible, and the residue was minced again and stored at $-10°$ wrapped in aluminium foil. When required, acetone-dried fibre was prepared from it by Straub's (1943) procedure. The dry fibre was stored at $0°$ and F-actin solutions were made up as follows. The G-actin solutions obtained by extracting the fibre with 20 vol. of CO_2-free water were precipitated with 10 mM-sodium acetate buffer, pH 4·7. The precipitate was quickly centrifuged down and dissolved in a minimal amount of saturated $NaHCO_3$ solution to give a viscous solution of F-actin, at pH 7·4, containing 6–10 mg. of protein/ml. These and all other preparative manipulations were carried out at about 1° unless stated otherwise.

Preparation of heavy meromyosins. Heavy meromyosin was prepared by the tryptic digestion of myosin by the method of Szent-Gyorgyi (1953) as described by Mueller & Perry (1961). The preparation was then purified by collecting the protein precipitated in the 40–55% saturated $(NH_4)_2SO_4$ fraction and reprecipitating it twice with 55% saturated $(NH_4)_2SO_4$. Finally the preparation was dialysed against 25 mM-tris–HCl buffer, pH 7·6, for 48 hr. with frequent changes of buffer until the last traces of $(NH_4)_2SO_4$ had been removed. This preparation is referred to below as 'heavy meromyosin-T'.

'Heavy meromyosin-CT', which was used in most of the experiments, was prepared by the chymotryptic digestion of myosin by a procedure similar to that described for the preparation of heavy meromyosin-T. To an L-myosin solution in 0·5M-KCl (E_{280} about 6·0, i.e. approx. 10·0 mg. of protein/ml.) one-tenth of its volume of 0·1M-boric acid–sodium tetraborate buffer, pH 8·6, was added, the solution was warmed to 23° in a bath, and then one-tenth of its

* Present address: Chemical Defence Experimental Establishment, Porton Down, nr Salisbury, Wilts.

volume of chymotrypsin (0·5 mg./ml., containing 0·05 mg./ml. of trypsin inhibitor) was added. After 10 min. the proteolysis was stopped by the addition of sufficient 10 mM-di-iso-propyl phosphorofluoridate to bring its final concentration to 1 mM. The digest was dialysed against 10 vol. of 6·7 mM-Sørensen phosphate buffer, pH 6·8, the light meromyosin was removed and the heavy meromyosin was precipitated three times with $(NH_4)_2SO_4$ as in the preparation of heavy meromyosin-T.

Viscometry. Relative viscosities were determined on 3 ml. samples at 0° and 25° with Ostwald viscometers of flow-times for water at 0° of 55–60 sec. After determination of the viscosity in the absence of ATP, not more than 0·076 ml. of ATP of suitable concentration was added to the solution in the viscometer, rapidly mixed and the viscosity immediately redetermined.

Enzymic assays. Adenosine-triphosphatase assays were carried out in general as described by Perry & Grey (1956) and Perry (1960), the precise ionic conditions being as indicated in the text. The measurement of pH was carried out with the glass electrode as described by Mueller & Perry (1961).

Materials. Salt-free crystalline bovine chymotrypsin and ATP (disodium salt) were supplied by the Sigma Chemical Co., St Louis, U.S.A. The latter reagent was converted into the tetrasodium salt at pH 7·0 and stored frozen as a 50 mM-solution. Soya-bean trypsin inhibitor and salt-free twice-recrystallized trypsin were obtained from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

RESULTS

Effect of potassium chloride and magnesium chloride on the adenosine triphosphatase of heavy-meromyosin preparations. In general the effect of potassium chloride, either in the presence of magnesium chloride or in the absence of bivalent cations, on the adenosine-triphosphatase activities of the heavy meromyosins prepared by tryptic or chymotryptic digestion was very similar to that of the original L-myosin. When measured with buffer and substrate only present, the adenosine-triphosphatase activities of both the heavy-meromyosin preparations used were very low but increased sharply as the ionic strength was raised by the addition of potassium chloride. The behaviour of heavy meromyosin-CT is illustrated in Fig. 1. In the presence of 2·5 mM-magnesium chloride the adenosine-triphosphatase activity of each of the meromyosins was no longer stimulated by increasing the ionic strength, but either did not rise, or fell off slightly, as the potassium chloride concentration was increased. In the absence of added potassium chloride a very slight activation of the adenosine triphosphatase of both heavy-meromyosin preparations was obtained on the addition of magnesium chloride (2·5 mM) (Fig. 1). This activation, although consistently observed, was hardly significant compared with that produced by higher concentrations of potassium chloride in the absence of

magnesium chloride and was not observed with L-myosin preparations.

Magnesium-activated adenosine triphosphatase of acto-heavy-meromyosins. When two parts (related to total N) of heavy meromyosin-CT, carefully equilibrated against 25 mM-tris–hydrochloric acid, pH 7·6, by dialysis, were added to approximately one part of actin freshly prepared by isoelectric precipitation, and the system was well mixed, the resulting complex possessed high magnesium-stimulated adenosine-triphosphatase activity. This activity was sensitive to increasing ionic strength (Fig. 2) and was comparable with that obtained with the complex produced from the original L-myosin and actin.

The proportions of heavy meromyosin and actin used in the experiment illustrated in Fig. 2 are similar to those found in the myofibril, but magnesium-activation could also be demonstrated in the presence of lower relative amounts of actin. Although the absolute amount of activation obtained under standard conditions decreased as the relative proportion of actin fell, the ratio of adenosine-triphosphatase activity with and without magnesium chloride was approximately the same irrespective of the actin:myosin ratio (Fig. 3).

Parallel studies with heavy meromyosin-T indicated that at low ionic strength activation by magnesium was usually obtained, but often the percentage increase over the basal level obtained in the absence of bivalent cation was less than that achieved with heavy meromyosin-CT.

The indications that tryptic digestion of myosin modified the response of the enzyme to magnesium in the presence of actin more than did digestion under similar conditions with chymotrypsin were
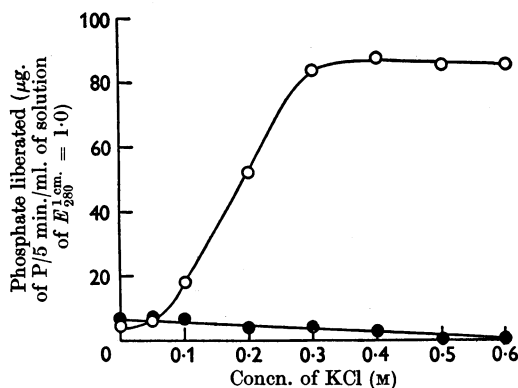

Fig. 1. Effect of potassium chloride on the adenosine-triphosphatase activity of chymotrypsin-prepared heavy meromyosin. The incubations were carried out for 5 min. at 25° in 2 ml. containing 0·3 ml. of heavy meromyosin-CT (0·83 mg./ml.), tris–HCl, pH 7·6 (50 mM), and ATP (2·5 mM). ○, MgCl₂ absent; ●, MgCl₂ (2·5 mM) present.
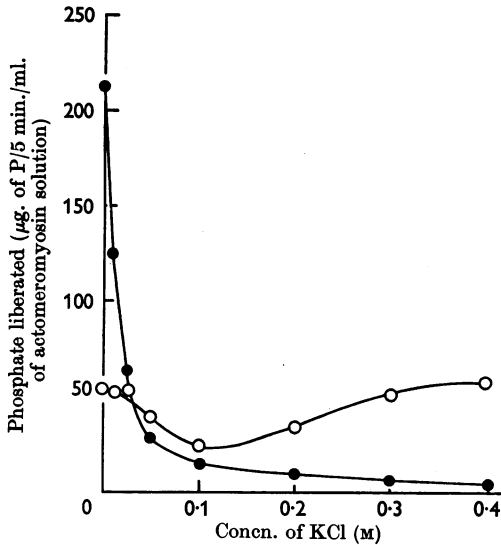
Fig. 2. Effect of potassium chloride on the adenosine-triphosphatase activity of acetomeromyosin. The incubations were carried out for 5 min. at 25° in 2 ml. containing tris–HCl, pH 7·6 (25 mM), ATP (2·5 mM) and 0·4 ml. of acto-H-meromyosin solution in 25 mM-tris–HCl, pH 7·6, containing approx. 2·5 mg. of heavy meromyosin-CT/ml. and 1·25 mg./ of F-actin/ml. O, MgCl₂ absent; ●, MgCl₂ (2·5 mM) present.

confirmed by studying the enzymic response during the formation of the heavy meromyosins. In the experiments illustrated in Figs. 4 and 5, L-myosin was digested for different periods with the proteolytic enzymes, and excess of the latter inactivated either by di-isopropyl phosphorofluoridate or by trypsin inhibitor as was appropriate. The whole digests were combined with fixed amounts of actin and the extent of magnesium-activation was determined in the absence and presence of 0·1 M-potassium chloride. The fall in activity produced by the 0·1 M-potassium chloride is a measure of the magnesium-activation, and the potassium-sensitive magnesium-activated adenosine triphosphatase survives digestion by chymotrypsin better than that by trypsin under otherwise identical conditions at pH 8·6.

Assay of the adenosine-triphosphatase activity of the digest alone in the presence of calcium under the standard conditions used in this Laboratory, i.e. calcium chloride (5 mM), ATP (5 mM), potassium chloride (0·2 M) and tris–hydrochloric acid (50 mM), pH 7·6, indicated that, as reported by Perry (1951), the enzymic activity rose slightly
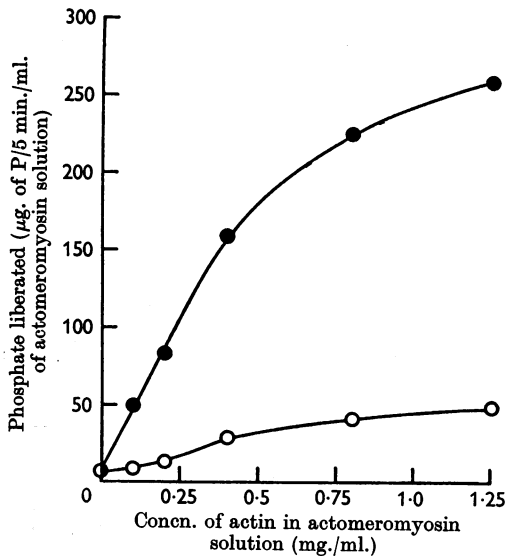

Fig. 3. Effect of actin concentration on the magnesium-activation of heavy-meromyosin adenosine triphosphatase. The conditions of the assay were similar to those of Fig. 2 except that no KCl was added and the acto-H-meromyosin used contained 2·5 mg. of heavy meromyosin-CT/ml. and various amounts of actin. O, MgCl₂ absent; ●, MgCl₂ (2·5 mM) present.
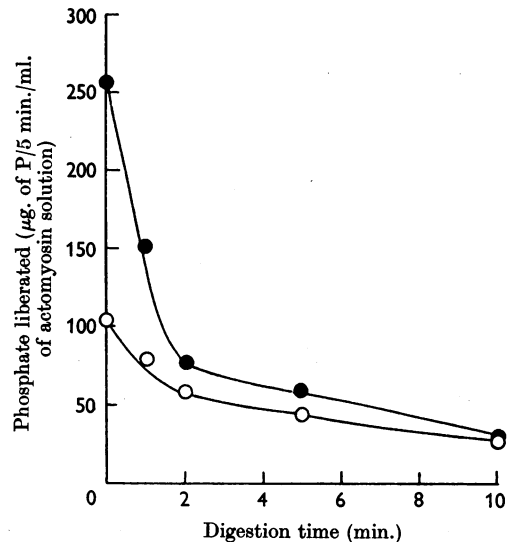

Fig. 4. Effect of potassium chloride on the magnesium-activation of the adenosine triphosphatase of the complex formed with actin by the products of tryptic digestion of L-myosin. The digestion was carried out as for the heavy meromyosin-T preparation (see the Methods section). The digestion was stopped with trypsin inhibitor at the times indicated, and 'actomyosin' containing 2·5 mg. of myosin digest/ml. and 1·25 mg. of F-actin/ml. was formed. The incubations were carried out for 5 min. at 25° in 2 ml. of medium containing 0·4 ml. of 'actomyosin', tris–HCl, pH 7·6 (25 mM), ATP (2·5 mM) and MgCl₂ (2·5 mM). ●, KCl absent; O, KCl (0·1 M) present.

during the digestion with trypsin and chymotrypsin (Figs. 4 and 5).

When assayed in tris–hydrochloric acid, pH 7·6 (25 mM), magnesium chloride (2·5 mM) and ATP (2·5 mM), acto-heavy-meromyosin-T preparations, e.g. that used in the experiment illustrated in Fig. 2, liberated inorganic phosphate at 60–70 % of the rate obtained with heavy meromyosin-T alone activated by calcium under the standard conditions described above. The activity obtained with acto-heavy-meromyosin (acto-H-meromyosin) when magnesium was the activator was similar to that obtained with the original myosin preparation under the standard conditions with calcium as activator.

Viscometric and sedimentation studies. The meromyosin preparations used in this study were clearly
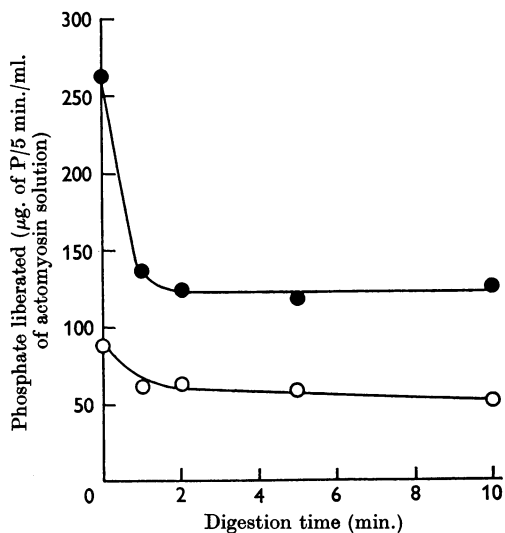

Fig. 5. Effect of 0·1 M-potassium chloride on the magnesium-activation of adenosine triphosphatase of the complex formed with actin by the products of the chymotryptic digestion of L-myosin. The digestion was carried out as for heavy meromyosin-CT preparations (see the Methods section). The conditions otherwise were as for Fig. 4. ●, KCl absent; ○, KCl (0·1 M) present.

soluble at low ionic strength, for this property was used in their preparation. On the addition of F-actin up to a concentration of about 0·5 mg./ml. to solutions containing approximately 2·25 mg. of heavy meromyosin/ml., acto-H-meromyosin complexes were obtained as clear solutions. As the actin concentration was increased still further the solutions of the complex became slightly more turbid than would be expected from the appearance of the constituent actin and heavy meromyosin-CT solutions. A similar phenomenon is observed with actomyosin sols at higher salt concentrations and is considered to indicate complex-formation. No protein was sedimented by centrifuging the acto-H-meromyosins for 30 min. at 5000g, but some sedimentation of very hydrated material sometimes occurred when the complex containing the higher concentrations of actin used (about 1 mg./ml.) was centrifuged for 30 min. at 20000g. The adenosine triphosphatase of the acto-H-meromyosin remaining in the supernatant after centrifuging was magnesium-activated. On the addition of ATP any turbidity apparent in the acto-H-meromyosin solutions was much decreased and no material could be sedimented on subsequent centrifuging for 30 min. at 20000g at 0°. Under similar ionic conditions actomyosin is almost completely insoluble and the effect of ATP is to induce superprecipitation.

The behaviour of the acto-H-meromyosin in tris–hydrochloric acid (25 mM) and magnesium chloride (2·5 mM) is very similar to that of actomyosin sols at ionic strength greater than 0·3. Under the conditions of the enzymic assay, by analogy with actomyosin, the acto-H-meromyosin would be expected to be completely dissociated into its component proteins by ATP. That this was indeed the case was suggested by the fact that the addition of ATP to acto-H-meromyosin systems under ionic conditions identical with those used for enzymic studies brought about a marked drop in relative viscosity (Table 1).

This effect could be more conveniently studied at 0° when the adenosine-triphosphatase activity of the system was sufficiently decreased to enable several consecutive viscosity measurements to be

Table 1. *Effect of adenosine triphosphate on the viscosity of acto-H-meromyosin solutions*

Viscosities were determined on acto-H-meromyosin (1·55 mg. of heavy meromyosin-CT/ml., and 0·75 mg. of F-actin/ml.) in 25 mM-tris–HCl, pH 7·6, containing $MgCl_2$ (2·5 mM). Experimental details are given in the text.

Expt. no.	Temperature	Concn. of ATP (mM)	Relative viscosity	
			ATP absent	ATP present
1	0°	2·5	3·09	1·35
2	25	5·0	3·89	1·40
3	0	5·0	3·89	1·34
	25	5·0	4·34	1·44

made before the concentration of the ATP in the system fell sufficiently to enable the complex to re-form. Likewise at 25°, the temperature at which the enzymic assays were carried out, there was clear evidence of dissociation, but enzymic activity was so high that the viscosity of the system often had begun to rise before a single viscometric determination in the presence of ATP could be carried out. At this temperature an increase of the ATP concentration to 5 mM with the consequent decrease in enzymic activity by substrate-inhibition enabled satisfactory determinations of viscosity to be made (Table 1).

DISCUSSION

Nagai, Konishi, Yutasaka, Takahashi & Makinose (1957) reported that acto-H-meromyosin differs from actomyosin in not being activated by magnesium at low ionic strength. This is in contrast with our findings, and the reason why the Japanese workers were unable to demonstrate activation of the acto-H-meromyosin is not clear. However, they used heavy meromyosin prepared by tryptic digestion which we have shown to be somewhat variable in behaviour and whose magnesium-activated adenosine triphosphatase appears to be particularly sensitive to ionic strength. It is apparent from the results in the present paper that, although at low ionic strength the adenosine triphosphatase of the acto-H-meromyosin is strongly activated by magnesium, its physical properties are comparable with those of actomyosin in solution, an enzymic system not activated by this cation. Further, the effects of ATP in reversibly diminishing both the viscosity and turbidity of acto-H-meromyosin solutions are also strictly analogous to those obtained with actomyosin sols.

Although Blum & Morales (1953) have questioned the original hypothesis of Szent-Gyorgyi (1945) that the fall in viscosity of actomyosin solutions which occurs in the presence of ATP is due to dissociation into the constituent proteins, the newer evidence available has suggested that dissociation indeed occurs (Weber, 1956; Gergely & Martonosi, 1958; Gergely, 1959; Gellert, Hippel, Schachman & Morales, 1959). It is logical to expect therefore that the viscometric changes produced by ATP on acto-H-meromyosin in solution arise from a similar change in the physical state of the system. It follows that, under conditions where the viscometric data suggest that actin and meromyosin are dissociated, strong magnesium-activation of the enzyme occurs. The control experiments with meromyosin alone demonstrate that its magnesium-activation is not simply a phenomenon found only at low ionic strength but is a specific effect requiring the presence of actin. This implies that, in

the presence of ATP when the normal type of interaction responsible for the original high viscosity of the complex has been broken, the actin can still interact either with the meromyosin or with some other component of the enzymic system so that magnesium can activate the hydrolysis of ATP. It may be that the viscometric change is a somewhat insensitive index of the extent of interaction between the actin and meromyosin and that two types of interaction can occur: one that gives rise to the viscometric effects, and a more subtle effect not accompanied by any obvious change of state in the system but such as to enable the enzymic behaviour of myosin to be modified. The findings of Bárány & Bárány (1959), suggesting that different centres on the myosin molecule are associated with adenosine-triphosphatase and actin-binding activities, may also be of significance in this respect.

Ultracentrifuge studies should be valuable in investigating the more subtle aspects of the actin–heavy meromyosin interaction, although some difficulties will arise in interpretation owing to the increased electropotential effects at the low ionic strength at which such a study must be carried out. Owing to these problems and the special difficulties involved in the sedimentation of the viscous acto-H-meromyosin system, these aspects have not been dealt with here.

Because of the similar characteristics of the magnesium-activation of the meromyosins compared with those of the parent molecule, it seems reasonable to conclude that actin can influence the enzymic activity of myosin itself in the gel state under conditions in which ATP might be presumed to have broken the usual type of interaction between these proteins. Caution should therefore be exercised in concluding that magnesium-activation is only a feature of actomyosin undissociated in the accepted classical sense.

By extrapolating from these results, it is likely that in resting muscle, when the usual type of interaction between actin and myosin may be presumed to be broken by the ATP present, the myosin will still retain the characteristics of the magnesium-activated enzyme system, the special features of which are of great significance for our present views on the enzymic processes underlying contraction and relaxation.

SUMMARY

1. The effect of magnesium on the adenosine-triphosphatase activities of meromyosins prepared by tryptic and chymotryptic digestion has been investigated.

2. At low ionic strength in the presence of actin enzymic activities of both meromyosins show strong activation by magnesium. There was some

evidence to suggest that this property was less sensitive to chymotryptic than to tryptic digestion of myosin under the conditions employed.

3. Under the conditions of low ionic strength at which magnesium-activation of the adenosine triphosphatases occurred the acto-H-meromyosin systems were soluble and viscometric measurements suggested dissociation into actin and meromyosin.

REFERENCES

Ashley, C. A., Arasimavicius, A. & Hass, G. M. (1956). *Exp. Cell Res.* **10**, 1.
Bárány, M. & Bárány, K. (1959). *Biochim. biophys. Acta,* **35**, 293.
Bendall, J. R. (1953). *J. Physiol.* **121**, 232.
Blum, J. J. & Morales, M. F. (1953). *Arch. Biochem. Biophys.* **43**, 208.
Bozler, E. (1952). *Amer. J. Physiol.* **168**, 760.

Gellert, M. F., Hippel, P. H. von, Schachman, H. K. & Morales, M. F. (1959). *J. Amer. chem. Soc.* **81**, 1384.
Gergely, J. (1959). *Ann. N.Y. Acad. Sci.* **72**, 538.
Gergely, J., Gouvea, M. A. & Karibian, D. (1955). *J. biol. Chem.* **212**, 165.
Gergely, J. & Martonosi, A. (1958). *Fed. Proc.* **17**, 227.
Hasselbach, W. (1952). *Z. Naturf.* **7b**, 163.
Mueller, H. & Perry, S. V. (1961). *Biochem. J.* **80**, 217.
Nagai, T., Konishi, K., Yutasaka, Y., Takahashi, H. & Makinose, M. (1957). *Biokhimiya*, **22**, 37.
Perry, S. V. (1951). *Biochem. J.* **48**, 257.
Perry, S. V. (1955). In *Methods in Enzymology*, vol. 2, p. 582. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Perry, S. V. (1960). *Biochem. J.* **74**, 94.
Perry, S. V. & Grey, T. C. (1956). *Biochem. J.* **64**, 184.
Perry, S. V. & Leadbeater, L. (1963). *Abstr. Conf. on Muscular Contraction, Boston* (in the Press).
Straub, F. B. (1943). *Stud. Inst. med. Chem. Univ. Szeged,* **3**, 23.
Szent-Gyorgyi, A. (1945). *Acta physiol. scand.* **9**, suppl. no. 25.
Szent-Gyorgyi, A. (1951). *Chemistry of Muscular Contraction*, 2nd ed. New York: Academic Press Inc.
Szent-Gyorgyi, A. G. (1953). *Arch. Biochem. Biophys.* **42**, 305.
Weber, A. (1956). *Biochim. biophys. Acta*, **19**, 345.

Rapidity of Carotene Biosynthesis in *Narcissus*

By V. H. BOOTH*
Dunn Nutritional Laboratory, University of Cambridge

The highest concentration of β-carotene recorded in biological material (Booth, 1957a) is believed to be that in the deep-red fringe of the corona of *Narcissus majalis* Curtis (pheasant's-eye narcissus). Evidence is presented in this paper that the carotene is produced in a few days during the development of the flower.

MATERIAL AND METHODS

N. majalis plants were grown from bulbs out-of-doors at Cambridge.

A criterion was needed for recognizing and numerically recording the stage of development at which flowers were taken for analysis. The most precise single event was the opening of the bud. But analyses were necessarily done before this event occurred, and different plants reached this stage of maturity on different dates. The dates of

analysis were therefore converted into a system of day numbers. Zero day for each flower was that on which the perianth opened to display the corona. The development of several buds was observed until they opened. Measurements were recorded and sketches were made daily: the angular position of the bud on the stem, and the distance between the middle of the ovary and the base of the corona, were especially characteristic. These observations were used to estimate the number of days to blooming of other buds that were analysed, and hence destroyed, before opening.

β-Carotene was extracted from individual coronas with acetone and light petroleum (Booth, 1959a), chromatographed on aluminium oxide plus sodium sulphate, and determined spectrophotometrically (Booth, 1957b).

RESULTS

Observations were made in 4 successive years, and 32 coronas were analysed. The results are summarized in Fig. 1, on which each spot represents an

* Member of the scientific staff of the Agricultural Research Council.