The Influence of pH and Temperature on the Properties of Myosin

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1. The rate of denaturation of myosin solutions at temperatures between 32° and 45° and at pH values between 5.3 and 6.2 has been studied, by using adenosine-triphosphatase activity and solubility in *m*-potassium chloride at pH 6.1 as criteria. 2. Myosin, when heated, loses its adenosine-triphosphatase activity before it becomes insoluble. 3. The loss of adenosine-triphosphatase activity and solubility are both first-order and pH-dependent reactions. Myosin, however, becomes insoluble only when heated within a narrow range of pH values. 4. The thermodynamic functions found for the two processes of denaturation are compared and discussed. 5. The possibility is discussed that, in muscle undergoing rigor, conditions may obtain that would denature myosin.

During the transition from the living state to full rigor mortis, muscle undergoes a series of biochemical changes that markedly alter the internal environment of the cell and of its constituent proteins. These have been reviewed in detail elsewhere (Bendall, 1960; Bendall & Lawrie, 1964). The most important reaction is the turnover of ATP, which proceeds for some hours in the dying muscle at about the same rate as in resting living muscle. In the anaerobic conditions prevailing after death, this initiates the resynthesis of ATP by the mechanism of glycolysis, with concomitant production of lactic acid. It is the lactic acid that determines the rate and extent of the pH fall in the muscle, and this in turn influences the quality of the muscle as meat, particularly in the important characteristics of texture and water-holding capacity, owing to the potential denaturing effect of low pH on the muscle proteins.

In normal muscle, with an initially high glycogen content, the pH falls from about pH 7.0 to about pH 5.4, during the course of rigor, but the rate of fall is usually so low that the muscles on a carcass will have cooled well below body temperature (38°) before the ultimate value is attained. In certain cases, however, particularly in pig muscle, the rate of glycolysis and of pH fall can often be three or more times the normal rate, and the muscle will not have had time to cool below 38° before the pH has fallen below 6.0 (Ludvigsen, 1954; Wismer-Pedersen, 1959; Briskey & Wismer-Pedersen, 1961). Such conditions give rise to considerable protein denaturation and the occurrence of so-called 'white' or 'watery' meat, which may exude more than 10% of its weight as drip.

The extractability, in *m*-potassium chloride, of the actomyosin from pale watery meat is less than 50% of that of meat allowed to go into rigor at lower temperatures. It has been suggested that this arises because of the deposition of denatured sarcoplasmic proteins on to the myofilaments, without necessarily involving the denaturation of actomyosin itself (Bendall & Wismer-Pedersen, 1962). Later, Scopes (1964, 1965) showed that creatine kinase was the most easily denatured of the sarcoplasmic proteins under these conditions, whereas most of the other proteins retained their native form.

Attractive as the above hypothesis is, it still leaves open the question whether the actomyosin of watery meat is itself denatured or not. Unfortunately this cannot be directly investigated by heating washed sarcoplasm-free myofibrils under identical conditions, because the fibrils then tend to clump together into ill-defined aggregates of different sizes, and the same problem arises with actomyosin preparations at physiological ionic strength. For this reason, it was decided as a first attempt to study the denaturation of myosin itself, under the conditions of temperature and pH encountered in practice, that is between pH values of 5.3 and 6.1, and between temperatures of 33° and 43°. As tests of the extent of denaturation, both the decrease in the ATPase* activity and in solubility were employed, of which the former is, in general, more sensitive and easier to follow than the latter.

It has been shown by other workers that actomyosin and myosin lose their ATPase activity when heated in the range pH 6.5–10 (Pelletier & Ouellet, 1961; Yasui, Fukazawa, Hashimoto, Kitagawa &

* Abbreviation: ATPase, adenosine triphosphatase.

Sasaki, 1958; Yasui, Hashimoto & Tonomura, 1960; Kofman & Molikova, 1960), but the effect at the lower pH values I have mentioned does not seem to have been investigated. In general, the present results show higher rates of denaturation than any reported previously at similar temperatures, but they also demonstrate that the presence of actin provides a considerable degree of protection to the myosin. Hence they still do not provide an exact picture of what happens in the intact muscle during high temperature rigor, although it can confidently be concluded that the most extreme of the conditions outlined would denature most, if not all, of the actomyosin present.

MATERIALS AND METHODS

ATP was obtained as the disodium salt from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). Other reagents were AnalaR Grade from British Drug Houses Ltd. (Poole, Dorset). The water used was passed through a mixed resin bed (Elgastat; Elga Products, London, S.W. 19).

Preparation of myosin and actin. Myosin was prepared from rabbit muscle by the method of Perry (1955). The myosin, which had been purified by reprecipitation three times by a tenfold dilution from solution in 0.5M-KCl, still contained nucleotides (Perry, 1960) since the E_{280}/E_{260} ratio was usually about 1.5–1.6. (Most of the experiments reported were carried out with this myosin.) Further purification was obtained by eluting the myosin from a DEAE-cellulose (Whatman DE50) column with 0.22M-KCl–20mM-tris-chloride buffer, pH 8. The myosin was not held on the column, but a fraction with an E_{280}/E_{260} ratio 0.55 was firmly retained. The eluted myosin had an E_{280}/E_{260} ratio 1.80. Some of the myosin was stored in M-KCl at 0° for up to 2 weeks and the remainder in 30% glycerol at –10° until required.

Actin was prepared by extracting at 0°, with 0.2mM-ATP–0.2mM-ascorbic acid, pH 7.5, an acetone-dried powder prepared from rabbit muscle, after removal of the myosin, by the method of Feuer, Molnar, Pettko & Straub (1948). The resulting G-actin was transformed into F-actin in 0.1M-KCl–mM-MgSO₄.

Synthetic actomyosin was prepared by mixing myosin and F-actin in the ratio of 3.75:1 and recovering the actomyosin by dilution.

Inactivation of adenosine-triphosphatase activity. Myosin or actomyosin was added to M-KCl in 30mM-sodium glycerophosphate buffers, adjusted with HCl to pH values from 5.3 to 6.5, so that the final protein concentration was 3–4mg./ml. Samples (1ml.) of these solutions were pipetted into a series of tubes, which were placed in a constant-temperature water bath ($\pm 0.1^\circ$), and at regular time-intervals a tube was withdrawn. To stop denaturation and to provide a suitable medium for determining ATPase activity, 3ml. of an ice-cold solution of M-KCl–13mM-CaCl₂–67mM-imidazole-chloride buffer, pH 7.2, was added.

Denaturation of myosin at low ionic strength was measured by pipetting 0.5ml. of myosin in 0.34M-KCl into tubes and adding 1ml. of 30mM-sodium glycerophosphate, adjusted with HCl to the appropriate pH. The final ionic strength therefore was 0.16. The tubes were then heated and the denaturation was stopped as before.

To measure ATPase activity the above solutions were incubated at 20° and the reaction started by the addition of 1ml. of 20mM-ATP. Samples (1ml.) were pipetted at intervals into 10ml. of 12% (w/v) HClO₄ and immediately centrifuged. Then 10ml. of the supernatant was diluted to 20ml. with water and P_i determined by the method of Allen (1940). Blanks containing no enzyme showed that there was no hydrolysis of ATP in 12% HClO₄ for at least 15min., which was in excess of the time taken for the above operations.

Solubility. Samples (2ml.) of myosin solution in M-KCl–30mM-sodium glycerophosphate buffers at different pH values were heated as above, and the reaction was stopped by the addition of 5ml. of ice-cold M-KCl–30mM-sodium glycerophosphate, pH 6.1. The solutions were left overnight at 0° and then centrifuged at 30000g for 15min. The E_{280} and E_{320} values of the solutions were determined, the difference being taken as the measure of the amount of protein in solution.

The loss of solubility at a lower ionic strength was determined by first heating 1ml. of myosin in M-KCl–30mM-sodium glycerophosphate buffers at 35°, then adding to the heated myosin solutions, 1ml. of 0.2M-imidazole, pH 7.2, and 2.7ml. of ice-cold water giving an approximate final pH 7.2 and I 0.25. These conditions were chosen from the results of Johnson & Rowe (1961), who showed that myosin became less soluble at I 0.3 after heating at 25°. To compensate for the absorption of imidazole, a blank containing the same amount was used.

Protein estimations were made by the Kjeldahl method, or by measurements of $E_{280} - E_{320}$, when $E_{1\text{cm}}^{1\%}$ is 5.9 for myosin.

RESULTS

Loss of adenosine-triphosphatase activity. The loss of the Ca²⁺-activated ATPase activity of actomyosin has been shown by Pelletier & Ouellet (1961) and Yasui *et al.* (1958) to be a first-order reaction, although the latter authors found that the order changed to second after about 50% of the activity had been lost. The results in Fig. 1, however, show that the reaction for myosin and actomyosin follows first-order kinetics until at least 90% of the activity has been destroyed.

The rate of inactivation at 35° of myosin was found to vary with pH, ranging from k 1.42×10^{-3} sec.^{–1} at pH 6.0 to k 1.2×10^{-2} sec.^{–1} at pH 5.3.

Normally the initial activity of the ATPase was 5 μ g. of P_i /min./mg. of protein, but a solution of myosin, at room temperature, lost as much as 50% of its original activity after standing for about 10min. at pH 5.3. Since the reaction is of first-order this did not affect the results, but it made it impossible to follow the course of the reaction below pH 5.3.

If the logarithm of the reaction constant is plotted against pH, as shown in Fig. 2, a straight line is obtained. The rate of inactivation was also measured at different temperatures and a series of parallel straight lines of slope –1.3 was obtained. According to the theory first put forward by

Fig. 1. First-order log plots for denaturation, as measured by ATPase activity (μ moles of P_i /min.) of myosin (●) and actomyosin (■) heated at 35° in M-KCl-30mM-sodium glycerophosphate buffer, pH 5.7, and actomyosin (▲) heated at 35° at pH 5.3.

Steinhardt (1937), and since applied to the denaturation of several proteins, the rate of denaturation of protein is dependent on the dissociation of H^+ ions from a small number of key groups. This dissociation can be estimated from the plot of $\log k$ against pH, the negative slope of which is equivalent to the number of H^+ ions. With myosin ATPase, therefore, 1.3 H^+ ions are involved in the denaturation process.

The values of $\log k$ at pH 5.7, at different temperatures, were selected from Fig. 2 and plotted according to the integrated Arrhenius equation:

$$\log k = \frac{-E}{2.3 RT} + \text{constant}$$

Fig. 2. Plot of $\log k$ (sec.^{-1}) against pH for the denaturation of myosin and actomyosin, heated at different temperatures in M-KCl-30mM-sodium glycerophosphate buffer, as measured by ATPase activity. Myosin at 33° (○), 35° (●), 37.5° (□), 39° (■) and 42.5° (△); actomyosin at 35° (▲).

The straight line obtained is shown in Fig. 3. Independent results, obtained from a sample at pH 5.7 and incubated at different temperatures, are also shown in Fig. 3. From the slope, the apparent activation energy E was calculated to be 47.6 kcal./mole. By applying the Eyring theory of absolute reaction rates (Glasstone, 1948), at pH 5.7 and 35° the apparent heat of activation ΔH^* was 47.6 kcal./mole and the apparent entropy change ΔS^* was calculated as 85 cal./deg./mole.

Similar, although less detailed, experiments were carried out on synthetic actomyosin. The results in Figs. 2 and 3 show the same effect of pH and temperature as on myosin, but the rate of inactivation at 35° at pH 5.7 was $k 1 \times 10^{-3} \text{ sec.}^{-1}$, which was about one-quarter of that of myosin. Thus the protective effect of actin against the aggregation of myosin as shown by Connell (1960) also applies to the deactivation of the ATPase.

When myosin was heated at 35° at pH 5.7 and 1.0-16 the rate of denaturation was less than that for myosin at 1.0 under the same conditions, the respective rates of denaturation being $k 1.2 \times 10^{-3} \text{ sec.}^{-1}$ and $3.89 \times 10^{-3} \text{ sec.}^{-1}$. When myosin was heated at different temperatures at 1.0-16 and pH 5.7, and the results plotted according to the Arrhenius equation (Fig. 3), the activation energy was calculated to be 43.5 kcal./mole, from which ΔH^* is 43.5 kcal./mole and ΔS^* is 70 cal./deg./mole.

Fig. 3. Arrhenius plot of the effect of temperature on the rate of denaturation, as measured by ATPase activity, of myosin (○) and actomyosin (■) heated in M-KCl-30mM-sodium glycerophosphate at pH 5.7; the points shown ● are taken from Fig. 2 at pH 5.7. Results for myosin denatured in 0.11 M-KCl-20mM-sodium glycerophosphate buffer, pH 5.7 (□), are also shown.

Loss of solubility in M-potassium chloride-30mM-sodium glycerophosphate at pH 6.1. Quantitative measurements on the effect of pH and temperature on the solubility of myosin in M-potassium chloride at pH 6.1 were more difficult to assess. It was found that the reaction did not go to completion even after heating for 60 min. There was present in solution a fraction that gave an E_{280} reading that was about 10% of the initial E_{280} reading and remained unaltered even after prolonged heating. Nitrogen determinations by the Kjeldahl method also showed that 10% remained in solution. This fraction corresponds to the undenatured P_1 fraction, found by Locker (1959), after heating myosin at 53°, and shown to contain nucleotides and adenylate deaminase. Takahashi, Hashimoto & Tonomura (1963), however, showed that the amount of undenatured material was decreased in myosin that had been passed through DEAE-cellulose. In the present experiments, a decrease to 4% in the undenatured soluble fraction was obtained with myosin treated with DEAE-cellulose.

To evaluate the rate of insolubilization of myosin, the E_{280} reading for the remaining soluble portion

Table 1. Comparison between the remaining adenosine-triphosphatase activity and the time lag before the myosin became insoluble in M-potassium chloride-30mM-sodium glycerophosphate buffer, pH 6.1, after incubation at 42.5° and at 37.5° at various pH values

42.5°			37.5°		
pH	Time lag (sec.)	ATPase (% of initial value)	pH	Time lag (sec.)	ATPase (% of initial value)
5.6	80	10.4	5.4	93	15
5.7	110	9.6	5.5	182	11
5.8	160	8.0	5.55	238	12
5.9	225	4.3	5.60	296	13
6.0	290	3.0	5.65	600	0

was subtracted from the other values. It was then found that the course of reaction could be divided into three stages. There was first a lag phase that varied with temperature and pH, during which at least 85% of the ATPase activity was destroyed, as shown in Table 1. The next stage of the reaction followed first-order kinetics closely until about 70% of the myosin had become insoluble. Thereafter the course of the reaction became indeterminate. This applied also to myosin treated with DEAE-cellulose. First-order rate constants at 35° ranged from 7.4×10^{-3} sec.⁻¹ at pH 5.45 to 1.3×10^{-3} sec.⁻¹ at pH 5.65. It was also found that above a certain pH value, which ranged from pH 5.7 at 35° to 6.5 at 45°, myosin did not come out of solution, even after prolonged heating. Therefore the loss of solubility could be studied only over a narrow range of pH values. The plot of $\log k$ (sec.⁻¹) against pH shown in Fig. 4 indicates that the rate of insolubilization reaches a maximum at pH 5.45. Below this pH value there is a progressive lowering of the rate of denaturation at 35° and 37.5°, but at 40° the rate remains at the maximum. Native myosin, however, is precipitated from M-potassium chloride at pH values lower than 5.45, and the above phenomenon may therefore be explained by a difference in the properties of myosin when it is not in solution.

Calculation of the slopes of the linear portions of the curves at pH values greater than 5.45 showed that at 35° the slope was -4, at 37.5° -3.2 and at 40° -1.35. Thus it would appear that as the temperature increases the number of H⁺ ions involved in the reaction diminishes.

The apparent heat of activation, ΔH^* , calculated from the values at pH 5.55, was found to be 51.6 kcal./mole. This value is meaningful only at this pH because the shape and slope of the curves vary at the other pH values. Similarly the apparent entropy change, ΔS^* , under the same conditions

Fig. 4. Plot of $\log k$ (sec.^{-1}) against pH for the denaturation of myosin heated in M-KCl-30mm-sodium glycerophosphate buffers at different temperatures, namely 35° (●), 37.5° (○) and 40° (■), as measured by the solubility in M-KCl-30mm-sodium glycerophosphate, pH 6.1.

was 98 cal./deg./mole. A comparison of the thermodynamic functions found for the insolubilization process and the loss of ATPase activity is shown in Table 2.

Further investigations were made on the reactions taking place during the lag phase. Myosin in M-potassium chloride at pH 6.2 was heated at 37.5° for 4 hr., during which time all the ATPase activity had been destroyed, but the myosin remained in solution. The pH was then lowered to 5.65 and the myosin heated at 37.5°. Measurement of the loss of solubility in M-potassium chloride in the usual way showed that the lag period had been decreased from 10 min. to 2 min., but the rate of insolubilization remained the same.

Examination of the u.v. spectra of solutions of myosin in M-potassium chloride at pH 6.2, before and after heating for 4 hr. at 37.5°, showed that the absorption maximum at 278 m μ was unaltered and the value at 295 m μ at pH 12 was also the same, indicating that no alteration of tyrosyl groups had occurred. A considerable increase in the absorption minimum at 250 m μ was observed, which is presumably due to the slight opalescence that developed in the solution after heating.

Loss of solubility at low ionic strength. Johnson & Rowe (1961) have described another solubility phenomenon of myosin. They found that, when they heated myosin at 25° at pH 6.7, it became progressively less soluble at ionic strengths below 0.3, although it was still in solution at higher ionic

Table 2. Comparison of the thermodynamic functions found for the denaturation of myosin, that had been heated at 35°, in solution in M-potassium chloride-30mm-sodium glycerophosphate buffers of different pH values

The functions in Expt. A were determined from the rate of loss of ATPase activity and in Expt. B by the loss of solubility in M-KCl at pH 6.1. In Expt. C the denaturation was carried out at 10.16 and measured by loss of ATPase activity.

	pH	$10^3 k$ (sec.^{-1})	ΔH^* (kcal./mole)	ΔS^* (cal./deg./mole)
Expt. A	6.0	1.42		
	5.7	3.89	47.6	+85
	5.45	7.6		
Expt. B	5.65	1.3	82.7	+165
	5.55	3.3	51.6	+98
	5.45	7.4	33.5	+41
Expt. C	5.7	1.2	42.9	+69

Fig. 5. Comparison of the plot of $\log k$ (sec.^{-1}) against pH for the denaturation of myosin heated at 35° in M-KCl-30mm-sodium glycerophosphate buffer as measured by (○) ATPase activity and (●) solubility in 0.23M-KCl-45mm-imidazole, pH 7.2.

strengths. The effect of pH on the rate of formation of myosin insoluble at pH 7.2 and 10.25 has therefore been examined here. The reaction behaved in a similar manner to that described for solubility in M-potassium chloride. There was no lag phase, but a residual soluble fraction, amounting to about 4% of the initial value, was found for DEAE-cellulose-treated myosin. The results best fitted the equation for first-order kinetics until about 70% of the myosin had become insoluble. The plot of $\log k$ (sec.^{-1}) against pH, however, was linear throughout the range of pH studied. A comparison with ATPase

deactivation (Fig. 5) shows that the loss of solubility is faster than loss of enzyme activity at pH values lower than 5.7 and slower above 5.7. The slope of the straight line obtained was -2.5 .

DISCUSSION

Myosin in solution is a very unstable molecule. It was therefore not surprising that, under the conditions described above, the properties of myosin should have been altered. Even at neutral pH values, rabbit myosin at 20° (Lowey & Holtzer, 1959; Johnson & Rowe, 1961) and cod myosin at 0° (Connell, 1960) are known to form insoluble aggregates slowly.

In the present work, the low pH values used caused a very rapid acceleration of the reactions. The first-order rate constant of $1.42 \times 10^{-3} \text{ sec}^{-1}$ at pH 6.0 and 35° compares with values of $0.12 \times 10^{-4} \text{ sec}^{-1}$ found by Yasui *et al.* (1958) and $0.4 \times 10^{-4} \text{ sec}^{-1}$ found by Connell (1960) at pH 7 and 25°. Since the pH range here is such that the myosin usually becomes irreversibly insoluble, it is possible to consider the separate steps involved.

From the evidence presented it would appear that, when myosin is heated in solution at 11, the ATPase activity is destroyed first. This means that the structure of the heavy-meromyosin fragment of the molecule, which contains the enzyme activity, is altered in some way. This stage requires the addition of 1.3 H^+ ions for its initiation, compared with 4.5 H^+ ions found by Pelletier & Ouellet (1961) for the deactivation of actomyosin ATPase at pH 10.4.

It is not clear whether the mechanism that deactivates ATPase is the same as that which produces myosin insoluble at 10.25. There are slight differences in the rates and pH-dependence of the two reactions, but the data are not conclusive. There is also no indication at what stage irreversible aggregates are formed. According to Johnson & Rowe (1961), aggregation of myosin, to give dimers, occurs after the formation of myosin insoluble at 10.25.

It is clear, however, that the reactions described above precede the formation of myosin insoluble in $\text{m-potassium chloride}$. The evidence of the loss of ATPase activity during the lag phase and the decrease of the lag phase by prior heating support this statement. The higher heat and entropy of activation also indicates a much greater degree of uncoiling of the protein structure than is necessary to deactivate the enzyme. However, myosin does not become irreversibly insoluble in $\text{m-potassium chloride}$ unless it is heated within a narrow range of pH values.

If the theory of Steinhardt (1937) is applied, it would appear that at pH values greater than 5.45

the number of H^+ ions involved in the insolubilization process are, 4, 3.2 and 1.3 at temperatures of 35°, 37.5° and 40° respectively. To explain this, one must presume that there are several states for myosin labile to insolubilization. Each of these states, however, requires a different energy of activation, which increases with higher pH values. Thus the ionized states of myosin, which exist at pH 5.7, require such a high energy of activation that the reaction does not proceed at 35°. But, as the pH is lowered, a greater proportion of the states requiring a lower energy of activation are formed and thus, at pH 5.45, the activation energy is low enough for the reaction to proceed even at 32°.

Solubility and ATPase activity are the usual criteria by which the condition of myosin is judged and it is often assumed that the two represent the same state. It is obvious, however, that ATPase activity is a much more sensitive indicator of denaturation than solubility. Nevertheless, it is not possible to say from the present experiments how myosin in itself would react *in situ* in the muscle, although since the addition of actin has been found to give considerable protection one would expect the highly organized actomyosin of the myofibrils to be more stable.

This expectation is borne out by some preliminary results on the ATPase activity of myofibrils during heating, which show the rate of deactivation of the latter is less, but the extent is the same. It is therefore very likely that, in muscle undergoing rigor in conditions of high temperature and low pH, deactivation of even the resistant ATPase of the myofibrils would occur, with the subsequent formation of insoluble denatured actomyosin.

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REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
- Bendall, J. R. (1960). In *Structure and Function of Muscle*, vol. 3, p. 227. Ed. by Bourne, G. H. New York: Academic Press Inc.
- Bendall, J. R. & Lawrie, R. A. (1964). *Anim. Breed Abstr.* **32**, no. 1.
- Bendall, J. R. & Wismer-Pedersen, J. (1962). *J. Food. Sci.* **27**, 144.
- Briskey, E. J. & Wismer-Pedersen, J. (1961). *J. Food. Sci.* **26**, 207.
- Connell, J. J. (1960). *Biochem. J.* **75**, 530.
- Feuer, G., Molnar, F., Pettko, E. & Straub, F. B. (1948). *Hung. acta physiol.* **1**, 150.
- Glasstone, S. (1948). In *Textbook of Physical Chemistry*, 2nd ed., p. 1098. London: Macmillan and Co. Ltd.
- Johnson, P. & Rowe, A. J. (1961). *Biochim. biophys. Acta*, **53**, 343.
- Kofman, E. B. & Molikova, A. N. (1960). *Biokhimiya*, **25**, 242.

- Locker, R. H. (1959). *Biochim. biophys. Acta*, **32**, 189.
- Lowey, S. & Holtzer, A. (1959). *J. Amer. chem. Soc.* **81**, 1378.
- Ludvigsen, J. (1954). *Beretn. Forsøgslab. Kbh.* no. 272.
- Pelletier, G. E. & Ouellet, L. (1961). *Canad. J. Chem.* **39**, 265.
- 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.
- Scopes, R. K. (1964). *Biochem. J.* **91**, 201.
- Scopes, R. K. (1965). *Arch. Biochem. Biophys.* **110**, 320.
- Steinhardt, J. (1937). *K. danske vidensk. Selsk. Mat.-Fys. Medd.* **14**, no. 11.
- Takahashi, K., Hashimoto, Y. & Tonomura, Y. (1963). *J. Biochem., Tokyo*, **54**, 550.
- Wisner-Pedersen, J. (1959). *Food Res.* **24**, 711.
- Yasui, T., Fukazawa, T., Hashimoto, Y., Kitagawa, S. & Sasaki, A. T. (1958). *J. Biochem., Tokyo*, **45**, 717.
- Yasui, T., Hashimoto, Y. & Tonomura, Y. (1960). *Arch. Biochem. Biophys.* **87**, 55.