SLAGTERIERNES FORSKNINGSINSTITUT
8. september 1961

SVIN-KØDKVALITET
Manuskript nr. 173

116

9A

Some properties of the fibrillar proteins of normal and watery pork muscle

by J.R.Bendall*) and J.Wismer-Pedersen

In recent years several reports have appeared in the literature describing a condition in the ham and back muscles of the pig which is characterised by pale colour and excessive loss of fluid from the meat when the carcase is cut up for the market. (Herter & Wilsdorf (1914), Ludvigsen (1954), Wismer-Pedersen (1959), Briskey et al. (1959), Lawrie, Gatherum and Hale (1958) Lawrie (1960) and others). This condition was described by Ludvigsen as muscle degeneration (MD) and by Wismer-Pedersen (1959) as watery pork. Both these authors have shown that it is characterised by very rapid glycolysis post-mortem, so that the pH of the meat reaches values of < 6.00 with $\frac{3}{4}$ hr of slaughter. Thus, the meat is at a low pH and a high temperature ($> 35^{\circ}C$) during the first 1-2 hrs post mortem under commercial factory conditions. In contrast, the fall of pH in meat of normal appearance is much slower, so that the carcase has time to cool well below $25^{\circ}C$ before the pH has fallen to 6.0. Lawrie (1960) describes a similar condition in English Landrace pigs as "white-muscle disease" which is also characterised by rapid glycolysis post mortem, but is often further accentuated by excessively low ultimate pH values of < 5.20, in contrast to the findings in the Danish Landrace breed, where the ultimate pH of the 'watery' muscles lies in the normal range of 5.30 to 5.60 (Briskey and Wismer-Pedersen, 1961a).

...

*) Present address: Low Temperature Research Station, Cambridge

SLAGTERIERNES FORSKNINGSINSTITUT
8. september 1961

SVIN-KØDKVALITET
Manuskript nr. 173

Wismer-Pedersen (1959) and Wismer-Pedersen and Briskey (1961) have shown that watery muscle differs in many ways from normal muscle, but particularly in the reduced extractability of the muscle proteins at high salt concentrations and in a reduced water-holding capacity. They suggested that these changes could be accounted for by denaturation and aggregation of the muscle proteins, particularly of the actomyosin filaments of the myofibrils, and this suggestion seemed to be supported by a characteristic rise in pH which occurred in the watery pork after processing was complete and while the temperature was falling to that of the cooling room.

In the present paper, we have re-examined the proteins of watery pork in rather more detail, with particular emphasis on the proteins of the muscle fibril, actin and myosin. For this purpose, we washed the fibrils free of sarcoplasmic proteins in dilute salt solutions, and then examined their water-binding capacity, protein content and titration curves and their extractability at high salt concentrations. We found that the washed fibrils from watery pork invariably had a higher protein content, but a lower water-binding capacity and much lower extractability than normal fibrils. On the other hand, their isoelectric point, as judged by the pH for minimal swelling, was nearly the same or lower than that of normal meat, whereas their proton-binding capacity per g of protein was reduced. Their buffer capacity curves showed, however, similar maxima and minima to those of normal meat, and differed entirely from those of heat coagulated fibrils, whether watery or normal.

These facts taken together suggest that the watery condition of pork meat is not due to aggregation or denaturation of the fibrillar proteins in the classical sense, but rather to adsorption of denatured sarcoplasmic proteins

...

onto the surface of the fibrils and myofilaments, thus reducing the number of charged groups available for proton-binding and water-binding. This conclusion is supported by the fact that the proton-binding of normal washed fibrils is not changed by keeping them at 37°C for 1½ hours at low pH (5.40), whereas whole meat allowed to pass into rigor at 37°C shows all the characteristic symptoms of wateriness.

Against these arguments there is the finding of Briskey and Wismer-Pedersen (1961a) that the pH of intact meat of the watery type apparently reaches a minimum 1½ hours after slaughter and then rises again slowly as the meat cools. pH increases of this kind could be taken to mean that considerable denaturation of the proteins had occurred (Bull 1938, Bate-Smith & Bendall 1946, Bendall 1947). Examination of the pH changes in washed and unwashed fibrils, as the temperature was raised or lowered, showed, however that the effect was entirely reversible, that is the pH fell as the temperature was raised and regained its original level as the temperature was once more lowered. This suggests that the effect is mainly due to changes in the pK values of charged groups on the fibrillar and sarcoplasmic proteins.

METHODS

Preparation of Pork Samples. To obtain meat of normal or watery type, samples of long. dorsi muscle were cut from the lumbar region of the carcases 24 hrs after slaughter. The pigs were of Danish Landrace breed, reared at the progeny testing station Sjælland and slaughtered at Roskilde bacon factory after electrical stunning. The pH taken ¾ hr after slaughter (pH_1) was above 6.20 in the normal muscles and below 5.60 in the watery muscles. Some samples were obtained 10 mins. after slaughter of the animals and were allowed to go into rigor at ~ 20°C. These served as controls in the titration studies.

...

SLAGTERIERNES FORSKNINGSINSTITUT
8. september 1961

SVIN-KØDKVALITET
Manuskript nr. 173

Preparation of fibrils. Fibrils were prepared from the meat by first passing it through a mincer, and weighing out 5 g samples of the mince into KCl or buffer solution. The mince was then homogenised to fibril level by means of an Ultra- Turrax blendor, which was taken to full speed in 3 bursts of 10 seconds duration, so as to avoid excessive heating. When unwashed fibrils were required, no further preparation was necessary. The preparation of washed fibrils is described later. It should be noted that homogenates prepared in this way contain pieces of connective-tissue not thoroughly disintegrated by the blendor. In titration studies it was necessary to cut these up finely with scissors. Microscopic examination of the homogenates revealed that all the muscular tissue proper was reduced to fibril level by the procedure.

Measurement of fibrillar volume and nitrogen content

For the purpose of comparing the fibrillar volumes and nitrogen contents of watery and normal fibrils (as in table 1), 5 g muscle was homogenised in 40 ml of 0.04 M potassium phosphate (pH 7.00, I = 0.09) and centrifuged for 10 mins at 1500 x g. The supernatant was discarded and the fibrils re-mixed, by means of the Ultra- Turrax blendor, in 40 ml of the phosphate buffer. They were then centrifuged for 10 mins, the supernatant was again discarded, and the fibril layer was weighed and it nitrogen content estimated by the usual Kjeldahl procedure. The results of the nitrogen estimations were expressed as g fibrillar protein per g of meat, taking the nitrogen content of the fibrillar proteins as 16.7 % (Bailey 1937).

Extractability. To measure the extractability of the muscle proteins at high ionic strength, the minced meat was homogenised and washed as in section 3 above, except that the buffer used was 0,04 M potassium phosphate (pH 6.50, I = 0.05). The fibril layer was then re-suspended in 40 mls of a

...

solution of 0.5 M KCl + 0.04 M potassium phosphate (pH 6.5, I = 0.55), left for 30 mins at 0°C and then centrifuged for 30 mins at 1500 x g. The supernatant fluid was decanted and the fibril residue weighed. The nitrogen content of residue and supernatant was estimated.

Preparation of samples for titration studies and the measurement of isoelectric point. 5 g samples were minced as above and homogenised in 20 ml 0.1 M KCl. Washed fibrils were prepared from this homogenate by washing twice in 20 ml of 0.1 M KCl. Finally the fibril layer was suspended in 20 ml of 0.1 M KCl. Measurement of phosphate content showed that such washed fibrils contain less than 1 mol. of acid-soluble phosphate per 10^5 g of protein. For the measurement of IP or point of minimal swelling the pH was adjusted with 0.1 N HCl or NaOH. When the IP of unwashed fibrils was to be measured 0.004 M iodoacetate was included in the KCl solution to prevent any possible glycolysis. In either case, the procedure was to allow the fibrils to equilibrate for 15 mins at 20°C at the desired pH, and then to centrifuge them for 5 mins at 1500 x g. The fibril layer was weighed after decantation of the supernatant.

Titration studies. Fibrils, prepared as above, were titrated stepwise with 0.1 N HCl or NaOH over the range pH 1.8 to pH 11.00, care being taken to allow the samples to equilibrate for 5 to 10 mins after each addition of acid or alkali. The pH was measured with a Radiometer pH meter, type 22.

The titration curves are expressed as the number of protons absorbed or released per 10^5 g protein. This paraméter was estimated from the equations:

...

(1) $$\log \frac{C_b}{C_a} = pH_a - pH_b ;$$

and

(2) $$\text{Mols. } H^+ \text{ bound} = [V(C_a - C_b)/W] \times 10^5$$

where C_a and pH_a represent the concentration of protons and the pH respectively, of an 0.1 M KCl solution to which HCl or NaOH have been added in the absence of fibrils, and C_b and pH_b are the corresponding values in the presence of fibrils; V is the volume of the solution in mls and W is the weight of protein in g. C_b can be exactly computed from pH_b from:

(3) $$C_b = \frac{[H^+]_b}{\gamma_H} = \text{anti-log } (-pH_b - \log \gamma_H)$$

where γ_H is the activity coefficient of the H^+ ion at pH_b (Cohn and Edsall 1943). In practice the necessary correction was estimated from the pH of HCl/KCl or NaOH/KCl solutions of known concentration.

Measurement of the dependence of the pH of the meat on temperature.

Washed or unwashed fibrils were prepared in 0.1 M KCl, as for the titration studies. The pH was measured at 20°C, and then the temperature was raised slowly to 40°C (10 mins) and the pH recorded as it fell back to 20°C. For measurements below 20°C the beaker containing the fibrils was cooled in an ice-water mixture to ~8°C and allowed to warm spontaneously again to 20°C. To simulate the conditions of the experiments of Briskey and Wismer-Pedersen (1961), the pH meter was set to 6.50 at 20°C, using a Sørensen buffer of that pH as standard, and the temperature adjustment of the meter was subsequently kept constant at 20°C regardless of the actual temperature. Corrections were then made by measuring the pH of the Sørensen buffer against temperature in exactly the same manner. To check the accuracy of the measurements, the variation of the pK value of 0.04 M imidazole (pH 7.2)

...

with temperature was measured. The heat of ionisation (ΔH) derived from these data was found to be + 6600 cals/mole from 8 to 40°C. The published value for the imidazole group of histidine is + 6900 cals/mole (Cohn and Edsall 1943). The agreement is seen to be satisfactory.

RESULTS

Water-retention and protein-content of fibrils washed at low ionic strength.

The water-retention and protein-content of fibrils of normal and watery meat prepared by washing in 0.04 phosphate buffer at pH 7.00, I = 0.09, are shown in table 1. It is seen that the fibrils from meat with low pH_1 *) values (<5.60) have a higher protein content (1.23x) but a lower water-retention per g of protein (0.58x) than fibrils from meat with high pH_1 (>6.20). Expressed as extra water retained per g of meat, the fibrils with high pH_1 retain 3 x as much water as those with low. The protein content of the washed normal fibrils is a little higher than the value of 0.115 g/g muscle given by Bendall (1961) for rabbit fibrils, free of stroma. The origin of the extra-protein of watery fibrils will be discussed later.

The table also shows that the differences in water retention between normal and watery fibrils are not due to differences in ultimate pH or total nitrogen content, which are nearly identical in the two types.

Extractability at high ionic strength (I = 0.55) at pH 6.50.

The amounts of protein extracted by a phosphate buffer of I = 0.55, pH 6.50, from fibrils washed free of soluble sarcoplasmic proteins at I = 0.05, pH 6.50, are shown in table 2. It is seen that normal fibrils (pH_1 > 6.20)

---

*) pH_1 is the pH of the long. dorsi muscle in the lumbar region measured by probe electrodes ¾ hr after slaughter.

are almost completely extracted by the procedure, giving a highly viscous solution containing 88.5 % of the fibrillar proteins, whereas in the case of 'watery' fibrils (pH_1 < 5.60) only 11 % of the fibrillar proteins are extracted, and the solution is quite limpid. These extreme differences between the types are best shown up by extraction at pH 6.50, because at higher pH values the normal fibrils yield an extract so viscous that it cannot be centrifuged, and at lower pH values extraction is very incomplete. It would clearly be desirable to study in more detail the relation between extractability and pH.

—

The origin of the extra-fibrillar protein of watery meat. The results in table 1 might be taken to indicate that there is a higher content of true fibrillar protein in watery meat than in normal meat. This, however, is unlikely to be the case, because an exactly similar gain in apparent 'fibrillar' protein can be brought about merely by heating a minced sample of normal muscle at 37°C for 1½ hours, or by allowing a sample of muscle cut from the animal immediately after death to pass into rigor at 37 - 41°C. In one experiment of the first type, for example, the unheated fibrils had a protein content of 0.122 g per g meat, after washing out the sarcoplasmic proteins, whereas the fibrils prepared from the heated mince had a protein content of 0.141 g per g meat. Similarly, in an experiment in which one piece of muscle was allowed to go into rigor at 20°C and the other at 40.5°C, the protein contents of the washed fibrils were 0.128 and 0.150 g per g meat, respectively. A similar, but rather more detailed experiment is given in table 3 to illustrate the differences in water-retention, protein-content and extractability between fibrils from meat allowed to pass into rigor at 20°C and fibrils from the same meat passing into rigor at 37°C. They are again similar in magnitude to the differences between watery and normal fibrils shown in tables 1 and 2.

...

SLAGTERIERNES FORSKNINGSINSTITUT
8. september 1961

SVIN-KØDKVALITET
Manuskript nr. 173

The relation between water-retention and pH in the fibrils from normal and watery pork.

Fig. 1. illustrates the relation between the water-retention per g of protein and the pH for washed fibrils from normal and watery pork at I = ~0.1. In this case, the normal fibrils were prepared from meat allowed to go into rigor at 20°C. It is seen that 'watery' fibrils retain less water per g of protein than those of normal pork at all pH values between 4.5 and 7.5. In spite of this, the zone of minimum swelling, which is probably also the isoelectric region, is about the same in the two cases, although the zone is broader with the 'watery' fibrils and extends to lower pH values. Taking the midpoint of the zone as the isoelectric point, then normal washed fibrils at I = 0.1 are isoelectric at ~pH 5.5 and the watery fibrils at ~pH 5.3. This is exactly the reverse of what happens when the proteins of normal meat are aggregated by heat, as we see from fig. 2, which shows the water retention of fibrils, coagulated at various pH values at 90°C. The minimum of the swelling curve is now between 5.90 and 6.00. It should be noted that the absolute values of water-retention for the coagulated fibrils cannot strictly be compared with those for the native fibrils, because of the very different nature and packing density of the coagulated particles. The isoelectric point measured in this way is very close to the pH of ~6.00 attained by washed normal fibrils after coagulation under isoelectric conditions at pH 5.40. This new pH reached after coagulation must represent the isoelectric point of the coagulum, since no acid or base has been added to the system.

The similarity of the IP's of normal and watery meat can also be shown by measuring the water-retention of unwashed fibrils (see fig. 3). The curves show that the unwashed watery fibrils retain about the same amount of water

...

at all pH values as the washed 'watery' fibrils in fig. 1, whereas the unwashed normal fibrils retain considerably more than the washed. Since the unwashed fibrils contain all the salts, non-protein nitrogenous compounds and sarcoplasmic proteins of the meat, it is difficult to say which of these components is responsible for the difference in the latter case. Certainly, it is not due solely to the presence of Mg or Ca ions, since addition of these to washed fibrils in the concentrations expected to be present in the homogenates, that is ~2 mM and 0.4 mM respectively, diminishes the water-retention rather than increasing it. It seems more likely that the effect is due to interactions between the sarcoplasmic and fibrillar proteins, but more experiments are necessary to confirm this suggestion. It will also be noted from the figure that the IP of the watery fibrils (~5.20) is again lower than that of the normal fibrils (~5.40). Also illustrated in fig. 3 is an experiment comparing the water-retention of unwashed fibrils from meat passing into rigor at 20°C with that of fibrils from the same meat allowed to go into rigor at 37°C. It is seen that the '20°C' fibrils follow the curve for normal fibrils whereas the '37°C' fibrils follow that for watery fibrils.

<u>The reversible effect of temperature on the pH of muscle fibrils and extracts.</u>

The experiments of Briskey and Wismer-Pedersen (1961) on intact pig carcases showed that in those pigs where the pH fell rapidly (watery type 4) it also fell to the very low level of ~5.15 and subsequently rose again to ~5.50 during the cooling of the carcase. Their results are plotted as pH against temperature in fig. 4. It will be noted that the pH tends to increase most steeply as the temperature falls from 20 to 10°C. If the rise of pH is to be taken as a measure of denaturation this feature of their curve is exactly the opposite of what would be expected, since it is well known that the rate of denaturation falls rapidly with falling temperature (Hamm and

Deatherage 1960). For this reason, we have re-examined the phenomenon using washed and unwashed fibrils, coagulated minced meat and the Kochsaft prepared from whole meat, that is the filtered liquid obtained after heat-coagulation at 90°C. The curves in fig. 4 show that large changes in pH occur with all the preparations studied as the temperature is raised or lowered, and that they are all inversely related to the temperature. That the effects with native fibrils are not due to denaturation is shown first by the fact that they are reversible within ± 0.03 pH units at 20°C, and also that coagulated minced meat shows a comparable effect. The curves as shown have not been corrected for temperature effects on the setting of the pH meter, which was standardised to pH 6.5 at 20°C without altering the temperature compensator from 20°C, as in the experiments of Briskey and Wismer-Pedersen. The measured corrections are shown as in inset, and are seen to be quite small.

The pH/temperature curves, after correction, can be converted into numbers of protons (H^+) bound or released by the buffering substances of the meat as the temperature is changed, by employing the measured buffering capacities of each of the respective preparations. The values calculated in this way are shown in table 4. The results of Briskey and Wismer-Pedersen have been similarly recalculated, using a buffering capacity of 54×10^{-6} moles H^+ per pH per g meat. It is seen that the unwashed fibrils show a reversible absorption or release of protons of the same order as that shown by the intact meat on the carcase.

Titration curves. To illustrate the form of the titration curves, we have chosen the curves for normal and watery fibrils in the native state (figs 5 and 6 respectively). The buffering capacities ($\Delta B/\Delta pH$), derived from these curves at intervals of 0.2 to 0.4 pH units, are shown in figs 7 and 8

...

respectively, and also the buffering capacities of normal and watery fibrils after coagulation at ~pH 5.40 at 90°C. Since we have shown above that the washed fibrils of watery meat contain extra protein, probably in the form of denatured sarcoplasmic protein, we have also titrated the coagulated sarcoplasmic proteins of normal meat. Table 1 shows that such protein is present in watery fibrils to the extent of ~0.19 g per g of total protein, the remainder being in the form of true fibrillar protein. The calculated titration curve for the proteins in this proportion is shown in fig. 6, and also the actual curve for 0.19×10^5 g coagulated sarcoplasmic protein alone.

We see from figs 5 and 6 that there are considerable losses of titratable groups (proton-binding) in both the alkaline and acid regions of the curves on passing from the normal to the watery state, although these losses do not result in any appreciable shift in the maxima and minima of the $\Delta B/\Delta pH$ curves in the acid or neutral region (figs 7 and 8). This is in complete contrast to the effect of coagulation, which is characterised not only by a loss of groups, but also by a large shift in the maxima and minima of the $\Delta B/\Delta pH$ curves in the acid and neutral regions.

In connection with the titration curves of watery fibrils, it is of some interest that the fibrils from a sample of meat allowed to go into rigor at 40.5°C showed almost identical proton-binding per g of protein to the samples of watery fibrils in fig. 6, and an almost identical gain of protein of sarcoplasmic origin. By contrast, washed normal fibrils, held at 40°C for 1½ hours, gave a normal titration curve of the type in fig. 5, and showed no loss of titratable groups.

...

SLAGTERIERNES FORSKNINGSINSTITUT
8. september 1961

SVIN-KØDKVALITET
Manuskript nr. 173

To gain further insight into the nature of the changes which have occurred, we have calculated the apparent titration constants (pK') of each curve, by a process of trial and error. These constants are analogous to, but not necessarily identical with, the true pK values of the groups titrating in the various regions of the curves (Cohn & Edsall 1943). We started from the assumption that the peak in the $\Delta B/\Delta pH$ curve of normal meat at pH 4.15 (fig. 7) represented the pK' of a class of similar groups, virtually undisturbed by the presence of other groups in the region pH 3.65 to 4.65. We then applied the general equations:

(4) $$pH = pK'_q + \log \frac{\alpha_q}{1-\alpha_q}$$

(5) $$n\alpha_q = n\left\{\frac{\text{antilog } (pH - pK'_q)}{1 + \text{antilog } (pH - pK'_q)}\right\}$$

where pK'_q is the titration constant of the qth class of groups; α_q is the fraction of the total number of groups in the class which have given up protons to base at any pH; and $n\alpha_q$ represents the number of protons given up by groups of the qth class at any pH. It follows from these equations that 52 % of the groups in the class with pK' = 4.15 should titrate between pH 3.65 and 4.65. We actually find 54.5 protons given up per 10^5 g. protein over this range of pH, so that the total number of groups with pK 4.15 is ~105. Removing these groups from the observed curve, as they would titrate according to equation 5, we found by similar reasoning that there was a class of 48 groups with a pK' of 2.60 in the acid region, and a class of ~26 groups with a pK of 6.40 in the neutral region. In the alkaline region, there appeared to be a class of 72 groups with pK 10.20, and this would mean that the remaining 50 or 60 basic groups should have pK' values greater than 11.00 (Mihályi 1950, Kominz et al. 1954). Unfortunately, this could not be checked with the electrode assembly available, which had a large 'alkaline' error above pH 11.40.

...

From these calculations it is possible to describe the titration of the native fibrils of normal meat from pH 1.8 to 10.2 as follows:

Protons bound = $48\alpha_1 + 105\alpha_2 + 26\alpha_3 + 72\alpha_4$

$$= 48\left\{\frac{\text{antilog (pH-2.6)}}{1 + \text{antilog (pH-2.6)}}\right\} + 105\left\{\frac{\text{antilog (pH-4.15)}}{1 + \text{antilog (pH-4.15)}}\right\} +$$

$$26\left\{\frac{\text{antilog (pH-6.4)}}{1 + \text{antilog (pH-6.4)}}\right\} + 72\left\{\frac{\text{antilog (pH-10.2)}}{1 + \text{antilog (pH-10.2)}}\right\}.$$

The curve calculated from this equation is shown in fig. 5 and is seen to agree well with the observed values for two different fibril preparations up to the limit at pH 10.2.

A similar type of analysis was applied to the curves for watery fibrils and for the coagulated fibrils of normal and watery meat. The results of the analyses are given in table 5 in terms of the classes of pK' values and the number of groups titrating in each class. To demonstrate the general validity of these constants we have plotted the average values of the observed results against the values calculated from table 5 for the particularly complex case of coagulated normal fibrils (fig. 9). It is seen that the agreement is good in the acid range, but small discrepancies occur in the alkaline range after about 30 groups have been titrated (pH 8.2 to 10.0) and a larger discrepancy after 60 groups have been titrated (pH 10.4 and above). The first discrepancy may be due to the constant at pH 6.70 being too low, or to the release of α-amino end-groups, not taken into account in the calculations. The second discrepancy arises because we do not know the pK' values of the groups alkaline to pH 11.00. As a further check, we may note that the calculated curve for watery fibrils (curve 2, fig. 6) also agrees well with the observed points, up to the limit at pH 10.2.

SLAGTERIERNES FORSKNINGSINSTITUT
8. september 1961

SVIN-KØDKVALITET
Manuskript nr. 173

From the above arguments, we may have reasonable confidence in the constants given in table 5. We see from this table that the effect of complete coagulation of fibrils is far greater than the change from normality to wateriness. In the first case, all the pK' values in the acid and neutral range are altered by the coagulation process, and in addition, there is a total loss of about 25 groups, whereas in the second case there is no change in the pK' values calculated from the combined sarcoplasmic and fibrillar components of the watery fibrils, but a comparable loss of titratable groups. Thus, all the 10 groups expected to titrate in the watery fibrils with pK' 2.10 have disappeared, and also 7.5 groups with pK 4.50. In addition 5 groups have been lost from the class of 85 groups at pK 4.15. Similarly in the alkaline region, about 15 groups have been lost with pK' 10.2, compared with about 18 groups after the coagulation of normal fibrils.

Like normal fibrils, coagulation of watery fibrils leads to considerable shifts in the pK' values, but without such a great loss of titratable groups. There is indeed very little change in the total titration in the acid region, because, although 11 groups have disappeared at pK' values between 3.40 and 3.50, 9 new groups have appeared with pK values of 3.00 and below. In the alkaline region about 6 groups have been lost at pK 10.2, compared with about 18 in the case of coagulation of normal meat. Unfortunately we again do not know what losses or gains of groups there may have been in the extreme alkaline range.

It is believed that this study of the titration constants of native, watery and coagulated fibrils is the first of its kind, and it is therefore necessary to enquire whether the data for the native fibrils agree with the amino-acid analysis and titration curves of actin and myosin. Mihályi (1950),

...

for instance, showed that purified myosin contained 165 titratable acid groups, 16 imidazolium groups and 134 basic groups per 10^5 g of protein. There are no comparable data for actin, but the amino-acid analysis of Kominz et al. (1954) shows the presence of 117 free acid groups, 19 imidazolium groups and 109 free basic groups. If the fibrillar proteins contain 1 part of actin to 2 parts of myosin (cf. Hanson and Huxley 1957), there should therefore be ~148 acid groups and ~18 imidazolium groups per 10^5 g. We find a total of ~153 acid groups and ~26 groups titrating with a pK' of 6.40, which are likely to be imidazolium groups (Cohn and Edsall 1943). Similarly we find 72 groups titrating with a pK' of 10.20, close to the expected pK values of tyrosine and lysine (Cohn and Edsall 1943). The analytical data of Kominz et al. (1954) would yield 23 tyrosine and 74 lysine groups for an actin/myosin ratio of 0.5. The overall agreement is, therefore, fairly good, particularly as we have not taken any account of the stroma, connective tissue and tropomyosin undoubtedly present in our preparations. These components, however, probably cancel each other out, because connective tissue has a small number of charged groups and tropomyosin a very large number. According to Lawrie (1960) and Hanson and Huxley (1957) respectively these components are present in the fibrillar proteins in about equal amounts of 10 %. In this connection, we may note that Wismer-Pedersen and Briskey (1961b) found normal whole meat to bind 144.4 equivs of safranin per 10^5 g total protein, whereas watery meat bound 187 equivs per 10^5 g. This measure of the number of acidic groups in normal meat agrees well with the present results, which would show about 146 protons to be bound at pH 1.8. In watery meat, however, the proton-binding is not likely to exceed 130 protons per 10^5 g total protein. It is difficult to account for the large discrepancy in the latter case, although it may be pointed out that it is not certain the dye-binding method is an exact measure of proton-binding particularly

...

where protein-protein interactions may have taken place in the material under study. For example, Hamm and Deatherage (1960), using this method, detected a loss of acidic groups after heat-denaturation of whole beef muscle, but could find no comparable loss of basic groups which would have been expected from the results of the present titration studies.

Discussion

The changes which are responsible for the occurrence of wateriness in pork meat have been variously ascribed to acute degeneration of the muscular tissue in the living animal (Ludvigsen 1954), to excessively low ultimate pH values of 5.20 (Lawrie 1960) and to a rapid fall of pH post-mortem (Briskey and Wismer-Pedersen 1961). Although it is clear that both muscle-degeneration and very low ultimate pH values might give rise to wateriness in the meat, it is most unlikely that either of these changes is responsible for the condition in the Landrace pigs we have studied here. In the first place, the mean ultimate pH values of the normal and the watery muscles were identical in our experiments, as they were in those of Briskey and Wismer-Pedersen (1961a), and secondly we were able to confirm the observation that the watery condition could be produced merely by holding a normal piece of meat at 33°C for 2 hours (Wismer-Pedersen 1959), or by allowing rigor to occur at 37 to 41°C. Moreover, in detailed studies of the rigor process at 37°C, to be reported elsewhere, it was observed that the muscles began to weep and their colour to change only when they were about to pass into rigor, that is when the pH had fallen to 6.00 or below. It is, therefore, obvious that the immediate cause of wateriness in the Landrace pigs studied here is the combined effect of high temperature and low pH on the muscle proteins, as Wismer-Pedersen and Briskey (1961a) have shown. In fact, wateriness appears to be a general phenomenon, not confined solely to Landrace pigs,

...

but characteristic of all muscles when rigor is allowed to take place at 37°C at low pH values, because it is then also found in a more or less severe form in the longissimus dorsi and psoas muscles of the rabbit and the ox (Bendall - unpublished observations) and to a very marked degree in whale muscle (Marsh, 1952).

Besides their decreased extractability and water-retention, the main feature which distinguishes watery fibrils from normal fibrils is their higher protein content. This extra protein is derived from the sarcoplasm, and is evidently present either in an aggregated form or firmly combined in some other way with the fibrillar proteins, actin and myosin. The problem is therefore to decide whether the reduced water-retention and the greatly reduced extractability of watery fibrils is due simply to the deposition of this layer of sarcoplasmic protein onto the surface of the myofilaments or whether it is due to denaturation and aggregation of the fibrillar proteins themselves. Two criteria may be used to distinguish between these possibilities: first, to enquire whether a rise of pH and of isoelectric point, characteristic of the phenomenon of denaturation, can be detected as the muscles pass into the watery state (cf. Bull, 1938; Bate-Smith and Bendall, 1946; Bendall 1947; Putnam 1953); and secondly whether the titration curves of watery fibrils show any of the changes which characterise denatured proteins(Bendall 1947).

As we have already pointed out, Briskey and Wismer-Pedersen (1961) have described a rise of pH in muscles of watery type, but this cannot necessarily be taken as an indication of denaturation, first because it occurs as the temperature of the meat is falling, secondly because it is larger than the actual rise of 0.25 to 0.30 units observed after the complete heat-coagulation of whole meat, and thirdly because a similar but

...

reversible effect of temperature on pH can be demonstrated both in native fibrils and heat-coagulated whole meat (fig. 4). The number of protons reversibly bound or released as the temperature of the unwashed native fibrils and of the heat-coagulated whole meat is raised or lowered is seen to be nearly the same in the unwashed native fibrils and in the heat-coagulated whole meat as it is in the intact carcase (table 4). It is therefore reasonable to suppose that the effect is due to changes in the pK values of the various buffering substances of the meat with temperature. The main groups likely to be involved in this range of pH are the imidazole and carboxyl groups of the proteins, and those of carnosine and anserine. The pK of the imidazolium groups has a negative temperature coefficient and that of the carboxyl groups a positive one, so that in both cases protons will be released on raising the temperature and bound again on lowering it (Cohn and Edsall 1943). Unfortunately, we do not know the exact numbers or pK values of these groups in whole meat, so that we cannot accurately predict their effect on the proton-binding. We can, however, make the following assumption (1) that the 26 groups per 10^5 g of protein, which titrate in the washed fibrils with a pK' of 6.40 are imidazolium groups and that the 105 groups titrating with pK' = 4.15 are carboxyl groups (see table 5); (2) that a similar number of imidazolium and carboxyl groups are present in the sarcoplasmic proteins; (3) that the total protein content is about 0.20 g per g of meat; and (4) that the heat of ionisation (ΔH) of the imidazolium groups is + 6900 cals per mole and that of the carboxyl groups $\sim$ - 1500 cals per mole (Cohn and Edsall 1943). Calculating on this basis for the effect of changing the temperature of the unwashed fibrils in table 4 from 20 to 37°C, we would expect a release of $\sim 5.2 \times 10^{-6}$ moles of H^+ against the found value of 7.3×10^{-6} moles. The discrepancy is not large, when we consider that no account has been taken of the possible effect of carnosine or anserine nor of the other buffering substances known to be present. We may conclude that we can more easily account for the

...

apparent rise of pH of watery meat in terms of the effect of temperature on the pK values than by invoking a general denaturation phenomenon. Nevertheless a small rise of pH would be expected in the watery muscles as the sarcoplasmic proteins were denatured and became deposited on the fibrils. Calculating for the known amount of protein deposited in this way (table 1), we would expect the rise of pH not to exceed 0.05 units in the whole meat, whereas complete heat-coagulation results in a rise of 0.25 to 0.30 units at pH 5.40. Such a small rise would probably go undetected in the intact carcase.

The criterion of the isoelectric point (IP) similarly indicates that denaturation in the classical sense can have occurred only to a minor degree, if at all, in the watery fibrils, because their IP tends to be lower than that of normal fibrils, whereas the IP of fully coagulated fibrils is much higher (figs 1-3). It is also of some interest that the swelling of unwashed normal fibrils is higher at all pH values than that of washed fibrils, whereas watery fibrils show about the same swelling in both cases. This suggests that the sarcoplasmic and fibrillar proteins are mutually active in the swelling process in the normal meat but not in the watery meat.

The other criterion we may apply to watery meat is that of proton-binding. We have already seen that the proton-binding of the washed watery fibrils is lower than normal in both the acid and alkaline regions, but that this decrease is not accompanied by the characteristic shifts in the titration constants, observed after complete heat coagulation (see table 5). On the other hand, the titration constants of watery fibrils are shifted by heat coagulation in much the same manner as those of normal fibrils, particularly in the acid and neutral regions. This again suggests that the bulk of the protein of watery fibrils is not in a denatured or coagulated form. If this

...

is so, however, we must seek some other explanation of the decreased water-retention and extractability of watery meat. A possible explanation can be suggested from the titration studies. We have shown that the extra protein of watery fibrils must arise from the sarcoplasm, and yet when we calculate the expected contribution of this protein to the total proton-binding we find that nearly all of it has disappeared from the actual titration curves of the watery fibrils (table 5). The only way in which this could happen would be for the missing groups to have become linked to groups on the fibrillar proteins. We may assume, for instance, that the carboxyl groups titrating with pK' values of 2.60 and 4.15 belong to the fibrillar proteins, whereas the groups with pK' 3.40 belong to denatured sarcoplasmic proteins. This would mean that all the 11 groups with pK' 2.10 and the 7.5 groups with pK' 4.50 contributed by the sarcoplasmic proteins have become linked to the fibrillar proteins. This could occur by the formation of bonds between these groups and the hydroxyl groups of tyrosine or even the ϵ - amino groups of lysine, or the guanidino groups of arginine of the fibrillar proteins. In addition, 5 carboxyl groups with pK 4.15 have been lost from the fibrillar contribution, possibly by linkage to basic groups on the sarcoplasmic proteins. The overall effect should be a loss of 23 or 24 groups titrating in the alkaline range. We actually observe a loss of ~15 groups with pK' 10.20, and it is possible that the remainder are lost at more alkaline pH values. These losses are, indeed, similar in magnitude to those observed after the complete coagulation of normal fibrils, which in the acid region amount to ~25 groups and in the alkaline region to ~18 groups, although in the latter case the missing carboxyl groups must have become linked to hydroxyl basic groups either on the same molecule or between molecules of the same protein type, which might account for the greater shift in the pK' values of neighbouring groups. Similarly, complete heat coagulation of watery fibrils must result in the breaking of some of the postulated

...

bonds between the fibrillar proteins and the layer of denatured sarcoplasmic proteins on their surface, because we find considerable shifts in the pK values after coagulation which are similar in kind, but not in degree, to those observed in coagulated normal fibrils.

We may conclude that we have an example in watery meat of a unique type of protein-protein interaction, where the main fibrillar protein, actomyosin, is in the native form, but has become covered with a layer of denatured sarcoplasmic protein, which is bound to it sufficiently strongly to make it resistant to extraction at high, and to hydration at low ionic strengths. This change in the structure of the muscle, unlike the effect of coagulation, leaves the titration constants of the combined proteins more or less unaltered, with the result that the isoelectric point of watery fibrils is the same or even slightly lower than that of normal fibrils.

Acknowledgement

We wish to thank Miss L. Jensen and Miss B. Mogensen for laboratory assistance in this work.

Summary

1) It is shown that the washed muscle-fibrils obtained from watery pork have a lower water-retention at low ionic strength, and much lower extractability at high ionic strength than the fibrils from normal pork. These changes are accompanied by a gain of protein by the washed watery fibrils, and this protein originates from the soluble sarcoplasmic proteins.

...

SLAGTERIERNES FORSKNINGSINSTITUT
8. september 1961

SVIN-KØDKVALITET
Manuskript nr. 173

2) All the changes, including the characteristic gain of protein, can be artificially induced in normal meat by allowing it to pass into rigor at 37°C.

3) The IP, or point of minimum swelling of watery fibrils, whether washed or unwashed, is similar to or slightly lower than that of normal fibrils. There is a broad isoelectric zone in all cases, the midpoint of which lies at ~pH 5.40 for normal fibrils and at 5.2 - 5.3 for watery fibrils. On the other hand, the IP of fully coagulated fibrils lies between 5.6 and 6.1.

4) Washed and unwashed fibrils of watery meat show about the same degree of swelling at all pH values. Normal fibrils, however, show a higher water-retention in the unwashed state than the washed. This effect is not due to the Mg or Ca ions included in the unwashed samples, but may result from interaction between the sarcoplasmic and fibrillar proteins. In the unwashed state, the swelling of normal fibrils is nearly double that of the watery fibrils at all pH values.

5) It is shown that the rise of pH found by Briskey and Wismer-Pedersen (1961) in intact carcases of watery meat as they cooled from 37 to 10°C, was probably due to the effect of temperature on the pK of ionisable groups of the proteins and buffering substances. It can be reproduced artificially and reversibly in native and coagulated minced meat, merely by raising or lowering the temperature.

6) The titration curves of watery fibrils show similar titration constants (pK') to those of normal fibrils, but a loss of titratable groups. Heat coagulation on the other hand, not only results in a bigger loss of titratable groups, but in a much larger shift in the titration constants. These results

...

SLAGTERIERNES FORSKNINGSINSTITUT
8. september 1961

SVIN-KØDKVALITET
Manuskript nr. 173

can be interpreted to show that the fibrillar proteins of the watery fibrils are not denatured or aggregated in the usual sense, but are probably covered by a layer of denatured sarcoplasmic protein, which is firmly bound to the surface of the myofilaments.

References

Bailey, K. (1937) Biochem. J. 31 1406

Bate-Smith, E.C. and Bendall, J.R. (1946) Nature 156 632

Bendall, J.R. (1947) Proc. Roy. Soc. B 134 272

Bendall, J.R. (1961) Biochem. J. In press.

Briskey, E.J. et al. (1959) J. Animal Sci. 18 146

Briskey, E.J. and Wismer-Pedersen, J. (1961) Food Science 26 297

Bull, H.B. (1938) Cold Spring Harbor Symp. Quant. Biol. 6 140

Cohn, E.J. and Edsall, J.T. (1943) Proteins, Amino Acids and Peptides. Reinhold Publishing Corp. New York. p.p. 89, 449, 454

Hamm, R. and Deatherage, F.E. (1960) Food Research 25 587

Hanson, J. and Huxley, H.E. (1957) Biochem. et biophys acta 23 250

Herter, M. and Wilsdorf, G. (1914) Deutsche Landwirtschafts-Gesellschaft. Berlin. Heft 270.

Kominz, D.R., Hough, A., Symonds, P. and Laki, K. (1954) Arch. Biophys. Biochem. 50 148

Lawrie, R.A., Gatherum, D.P. and Hale, H.P. (1958) Nature 182 807

Lawrie, R.A. (1960) J. Comp. Path. 70 273

Ludvigsen, J. (1954) Beretning fra forsøgslaboratoriet, København, I 272

Marsh, B.B. (1952) Biochem. et biophys. acta 9 127

Mihályi, E. (1950) Arch. Biochem. Biophys. 41 104

Putnam, F.W. (1953) in the Proteins. Vol. 1. Part B. pp. 850-853. Ed. Neurath, H. and Bailey, K. Academic Press Inc. New York. 1953.

Wismer-Pedersen, J. (1959) Food Research 24 711

Wismer-Pedersen, J. and Briskey, E.J. (1961a) Food Technology 15, 232

Wismer-Pedersen, J. and Briskey, E.J. (1961b) Nature 189 318.

JRB/JWP/AT

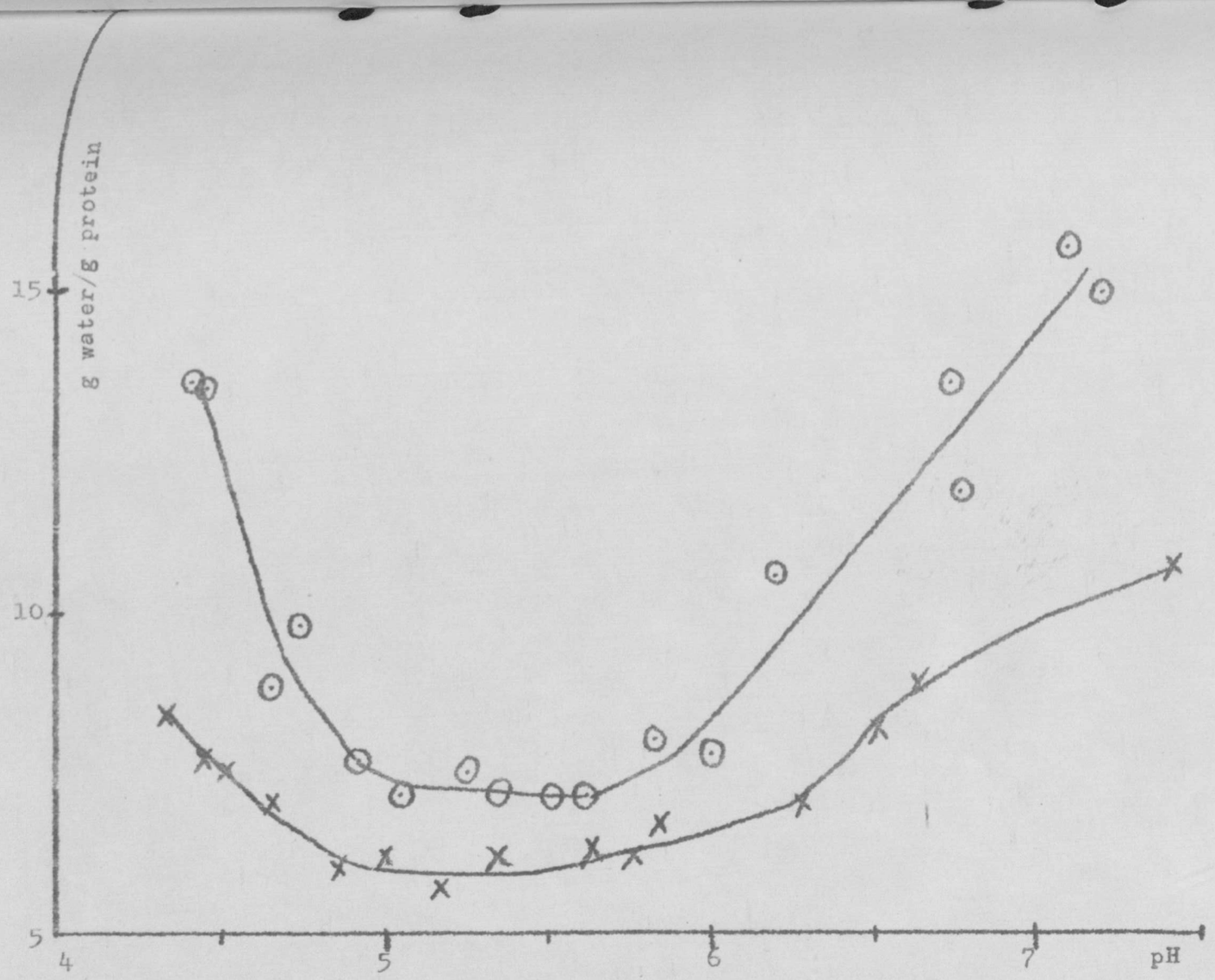

Fig. 1. Swelling curves of the washed fibrils of normal and watery pork, in 0.1 M KCl at various pH values. Swelling expressed as g water retained per g protein, after centrifuging at 1500 x g for 5 mins.

⊙ Samples of normal pork, allowed to go into rigor at 20°C

× Samples of watery pork (pH_1 = 5.60)

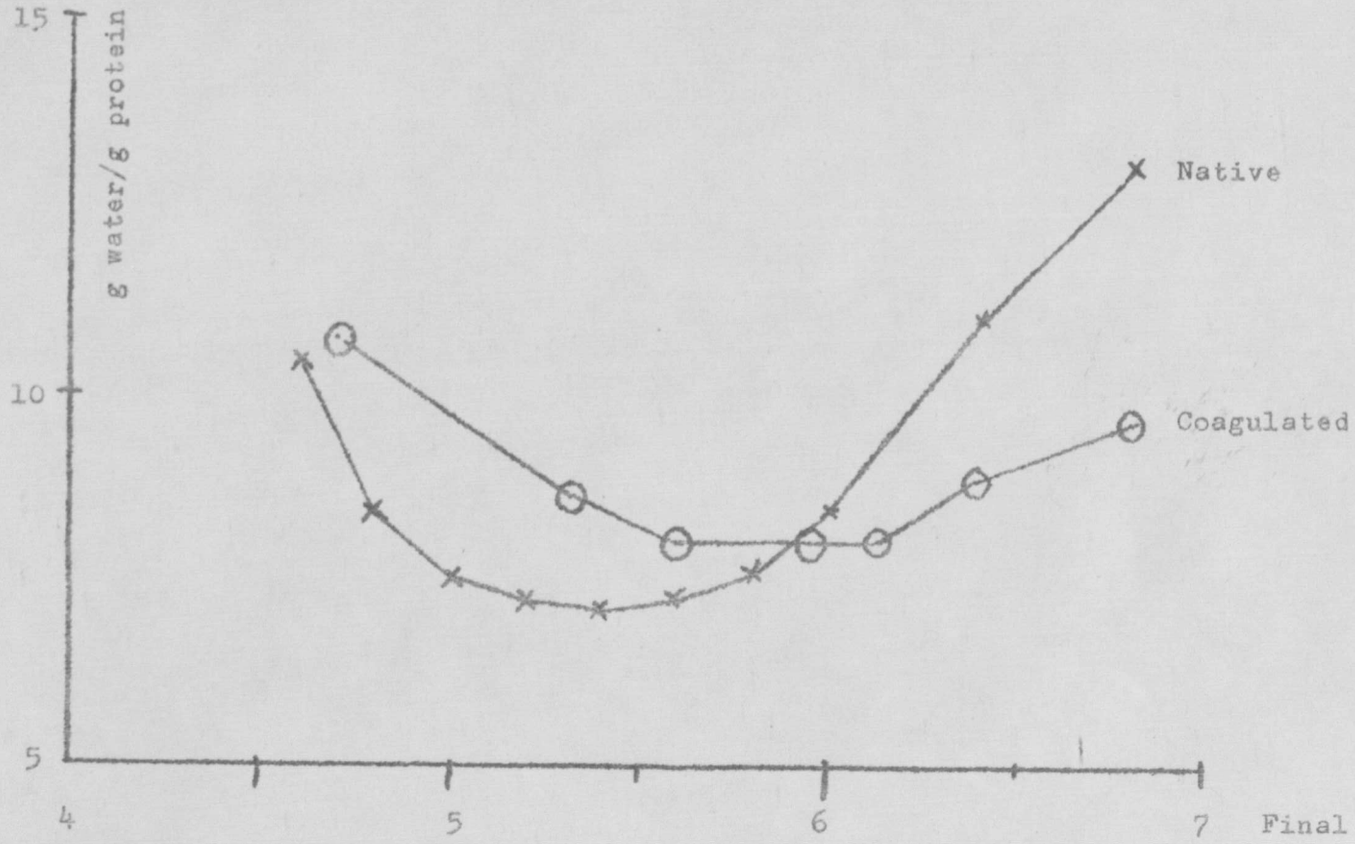

Fig. 2. Swelling curves of normal washed fibrils coagulated at various pH values at 90°C, in 0.1 M KCl. The average curve for washed fibrils from fig. 1. is superimposed. Results expressed as in fig. 1.

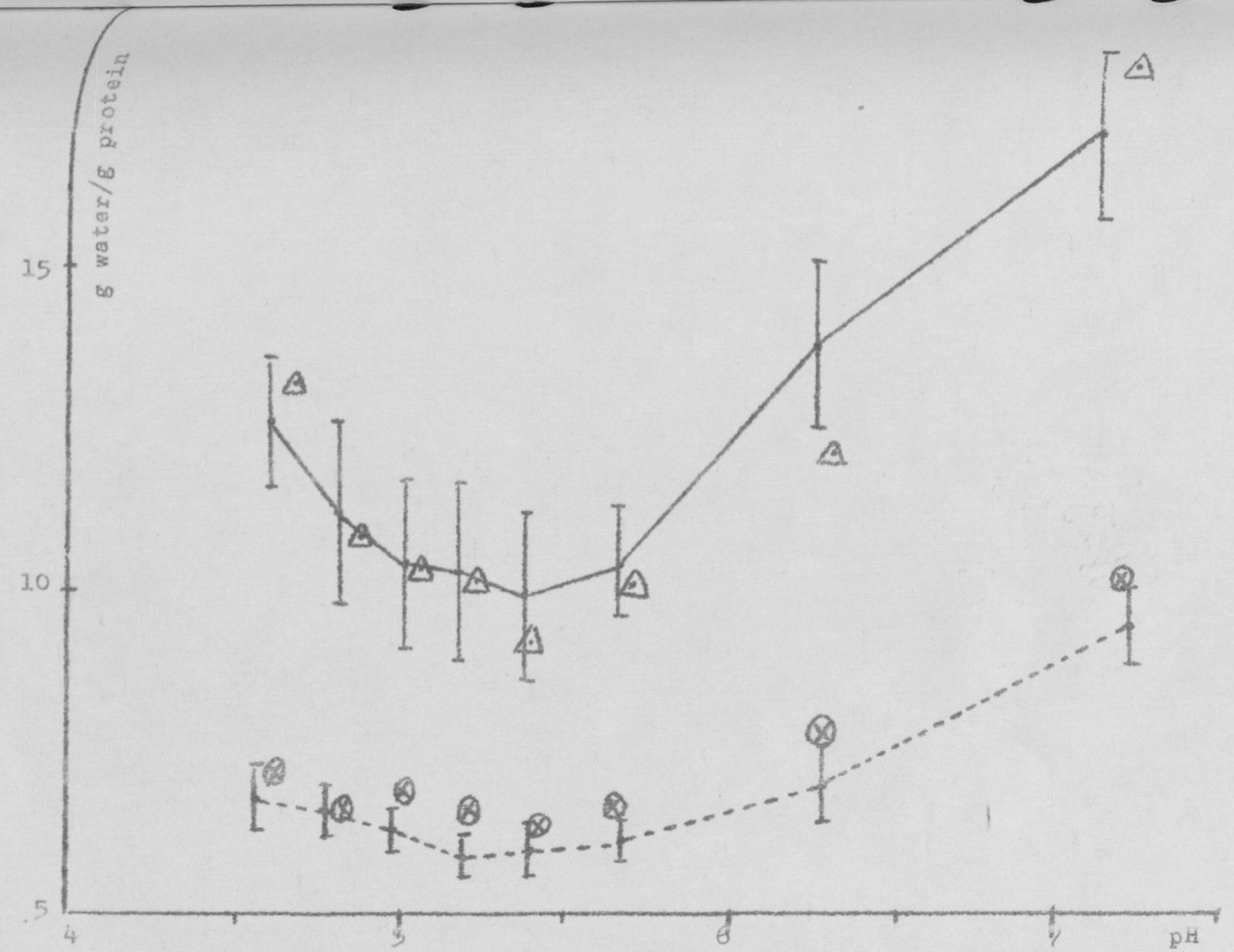

Fig. 3. Swelling curves of unwashed fibrils of normal meat (pH_1 6.30) and of watery meat (pH_1 5.60). Results expressed as g water retained per g fibrillar protein. Mean values for 3 experiments are given with the S.E's of the means. ———— Normal; - - - - - - - - Watery.

△ indicates an experiment with meat passing into rigor at 20°C.

⊗ indicates an experiment with the same meat passing into rigor at 37°C.

SLAGTERIERNES FORSKNINGSINSTITUT
3. august 1961

SVIN-KØDKVALITET
Manuskript nr. 173

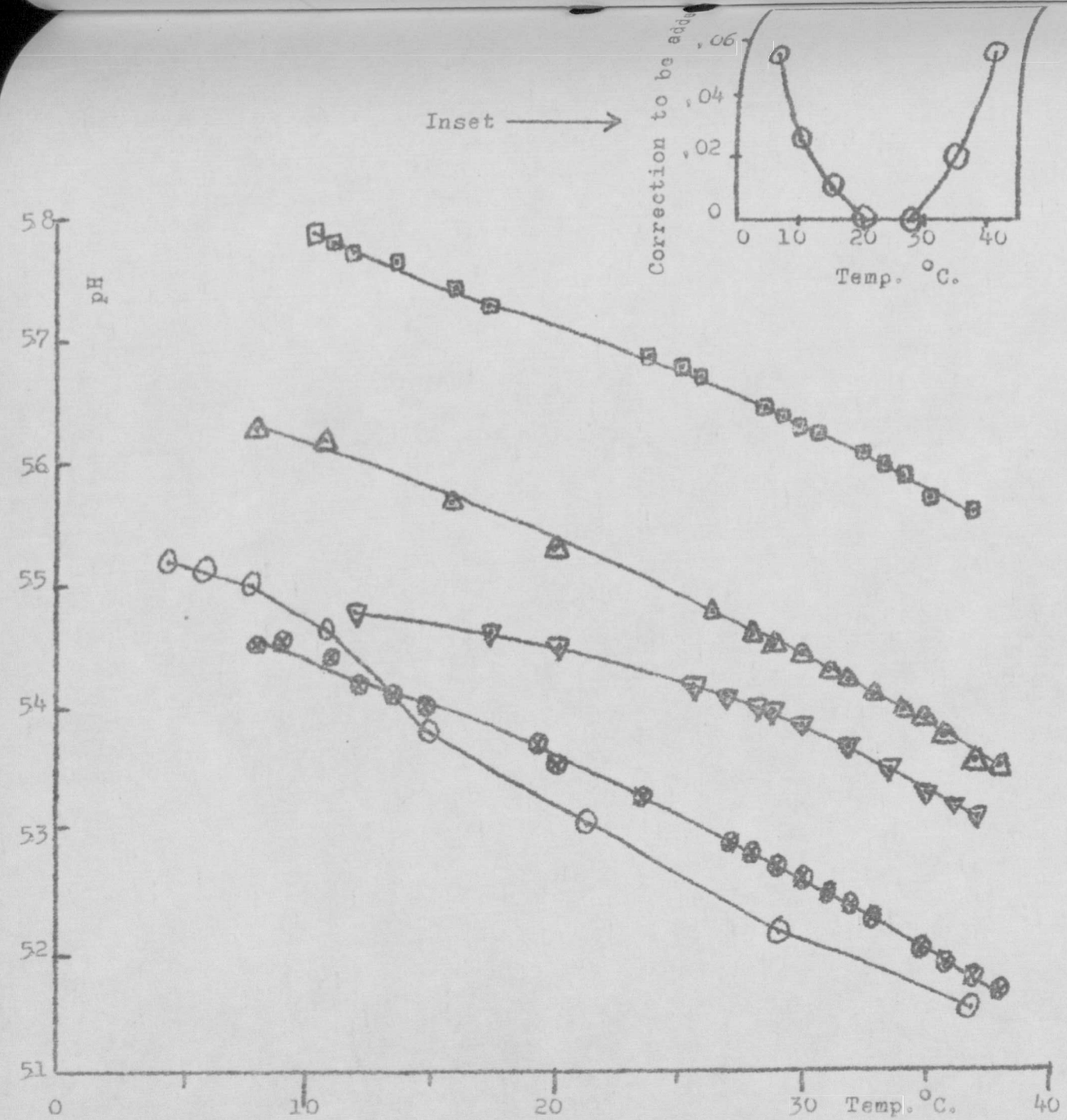

Fig. 4. The reversible effect of temperature on the pH of whole meat and varicus preparations of fibrils.

- ⊙ Intact carcase (Briskey and Wismer-Pedersen, 1961)
- ⊗ Washed fibrils (5 g meat in 10 ml 0.1 M KCl)
- ▽ Kochsaft prepared from 10 g meat, heat coagulated in 20 mls 0.1 M KCl
- △ Unwashed fibrils (5 g meat in 10 ml 0.1 M KCl)
- ⊡ Coagulated mince (10 g meat + 3 ml 0.1 M KCl)

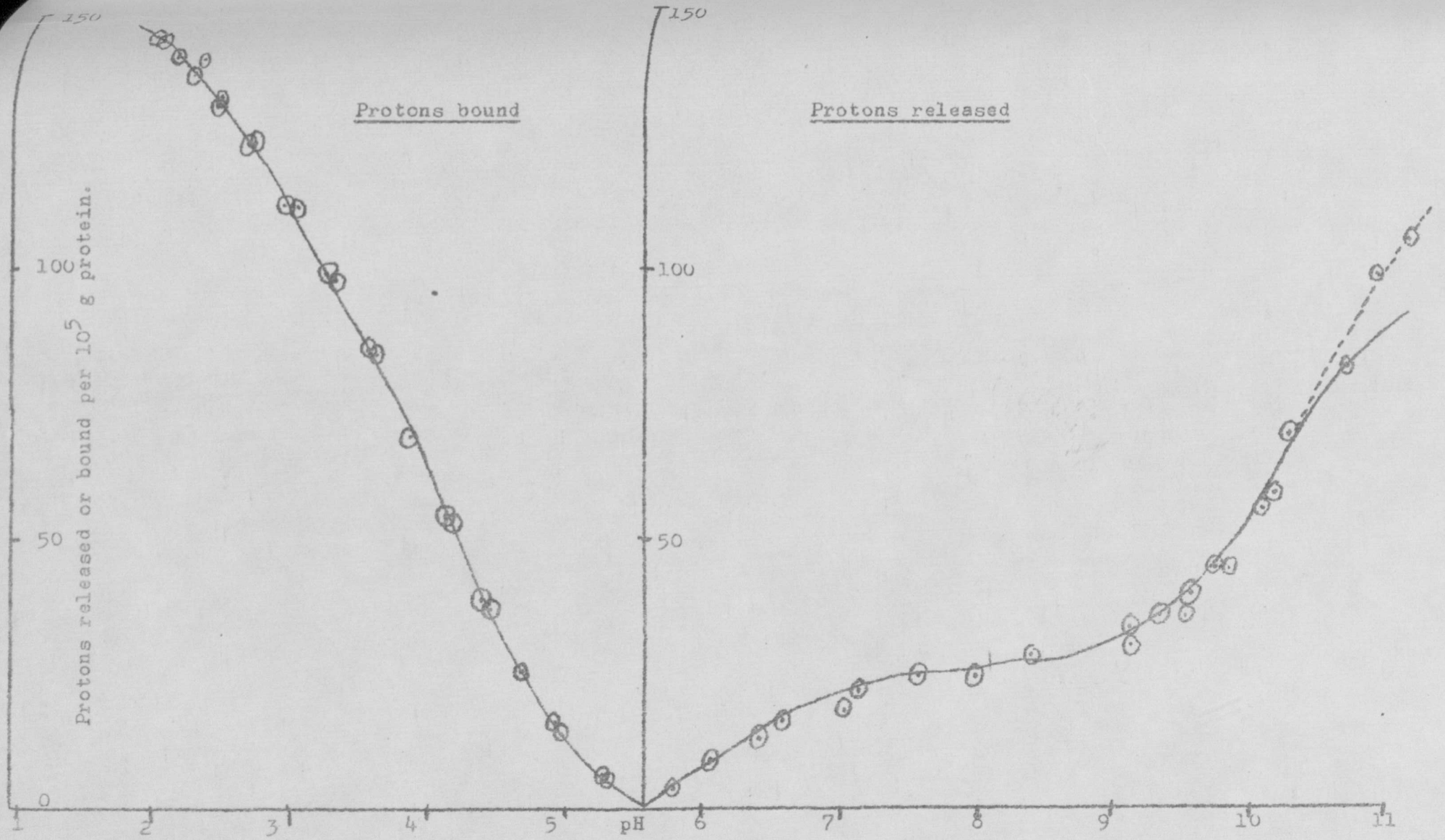

Fig. 5. Titration curves of normal washed fibrils in the acid and alkaline range, expressed as protons released or bound per 10^5 g protein. Average fibrillar protein content = 0.128 g per g meat. Full line calculated from the constants in Table 5. ⊙ indicates the observed points.

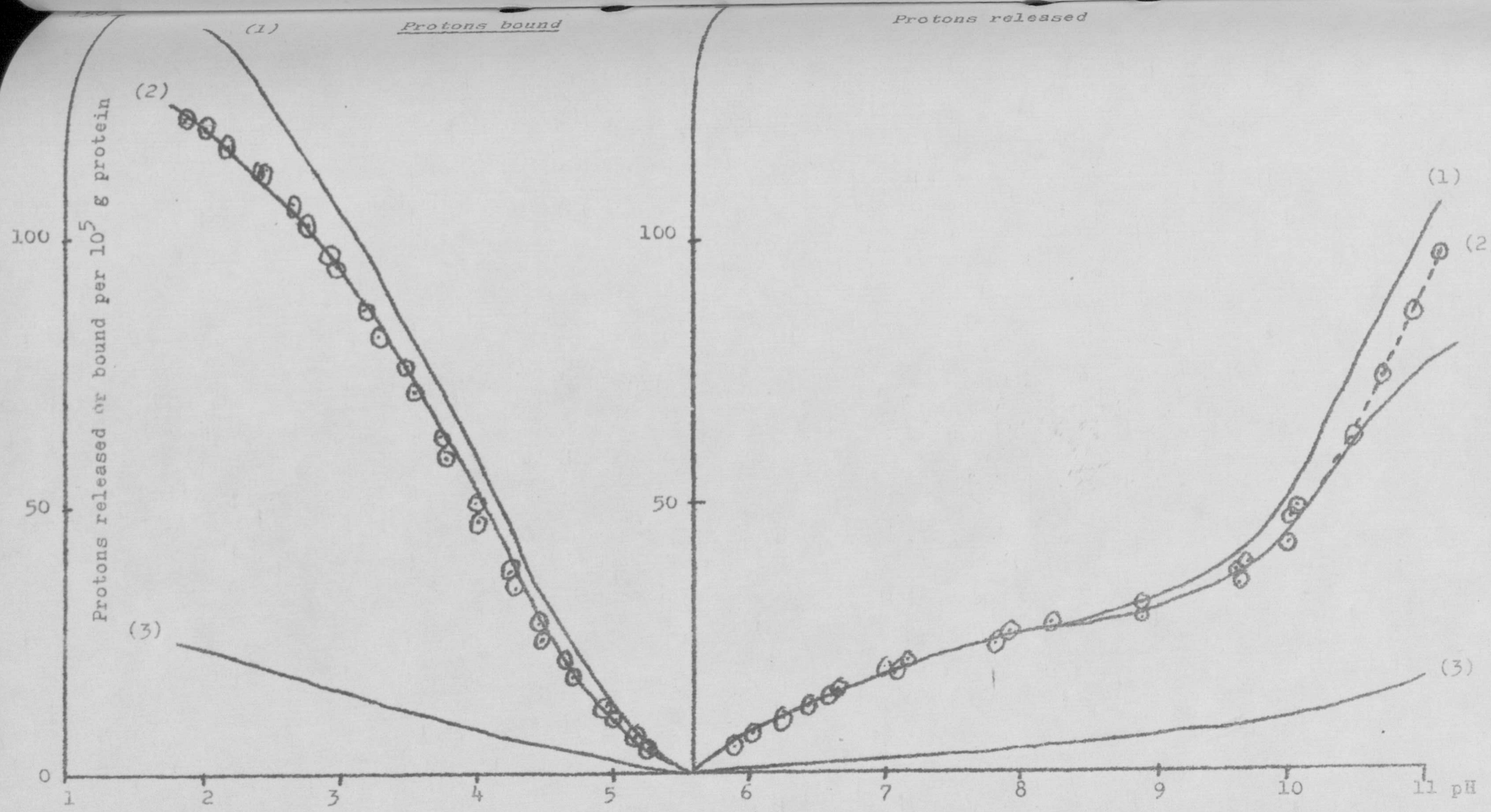

Fig. 6. Titration curves of fibrils from watery meat, expressed as in fig. 5. Average fibrillar protein content = 0.158 g per g meat. Curve (1) is calculated from the known proton binding of 0.19×10^5 g denatured sarcoplasmic protein + 0.81×10^5 g fibrillar protein. Curve (2) is the curve calculated for watery meat from the constants in table 5. Curve (3) is the titration curve for 0.19×10^5 g denatured sarcoplasmic protein. ⊙ indicates the observed points.

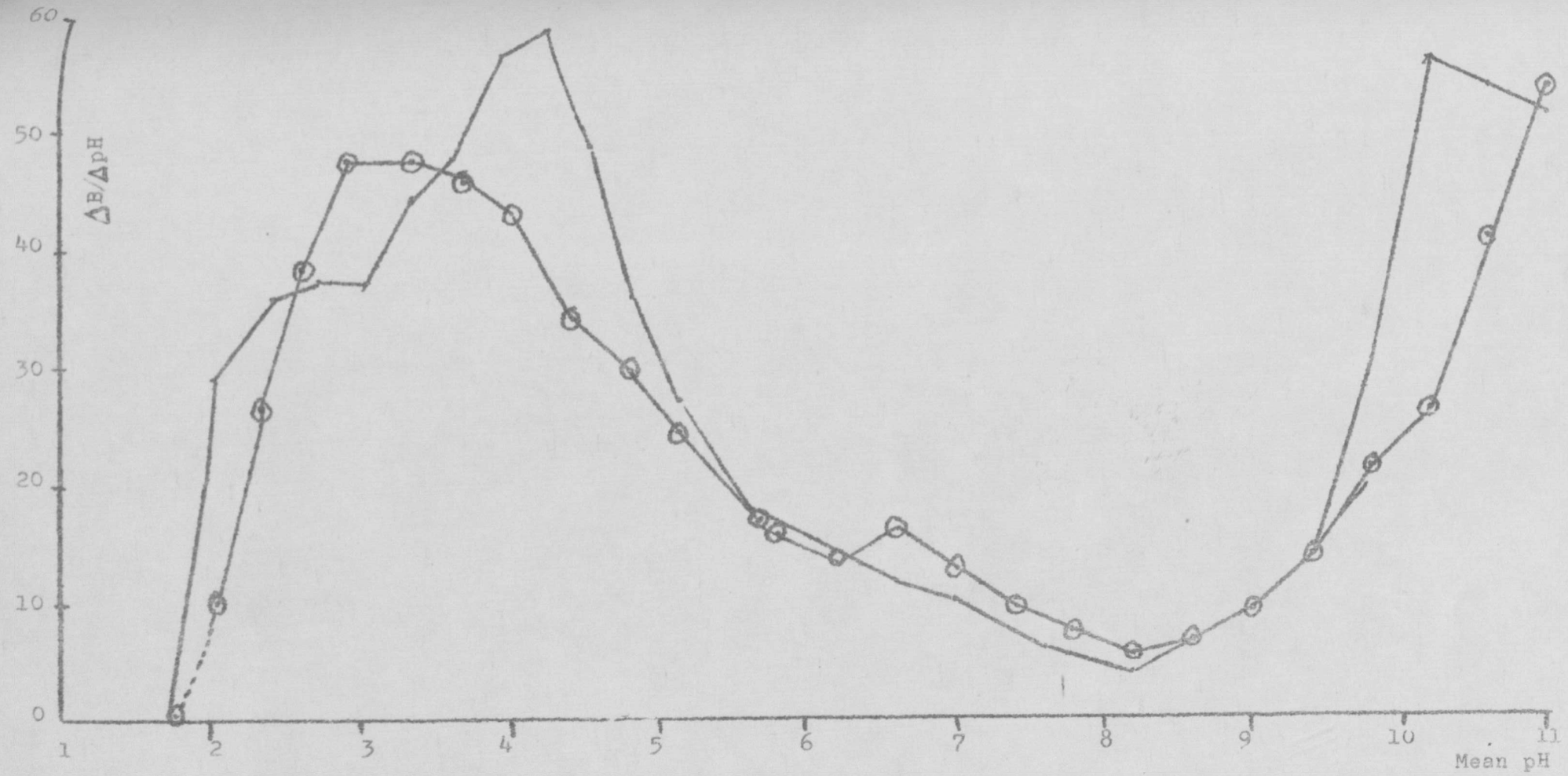

Fig. 7. Mean buffering capacity curves for normal native and coagulated fibrils. $\Delta B/\Delta pH$ = number of protons bound per pH unit per 10^5 g protein. Plain line = native fibrils; ⊙ = coagulated fibrils

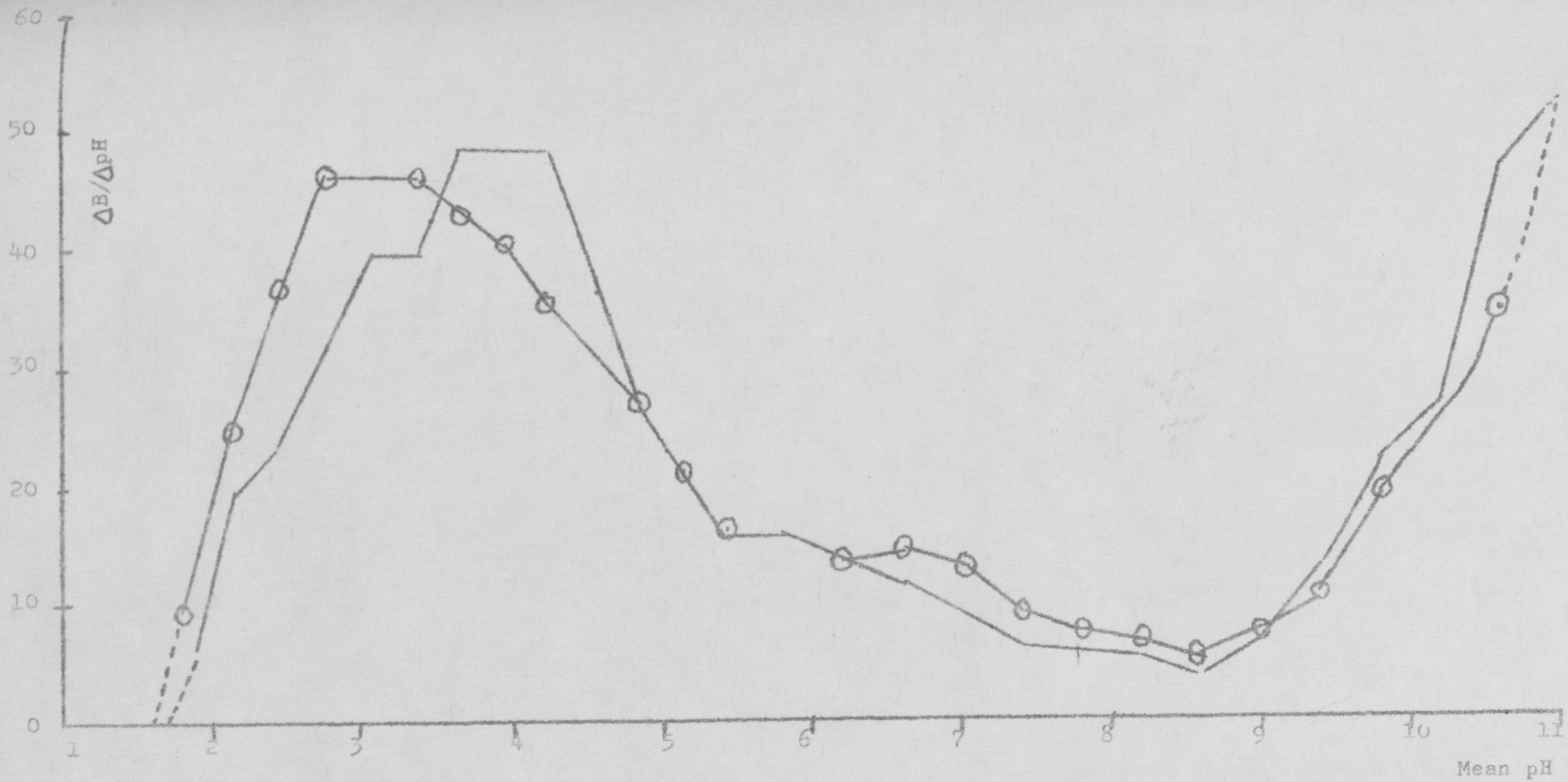

Fig. 8. Mean buffering capacity curves for the native and coagulated fibrils of watery pork, expressed as in fig. 7.

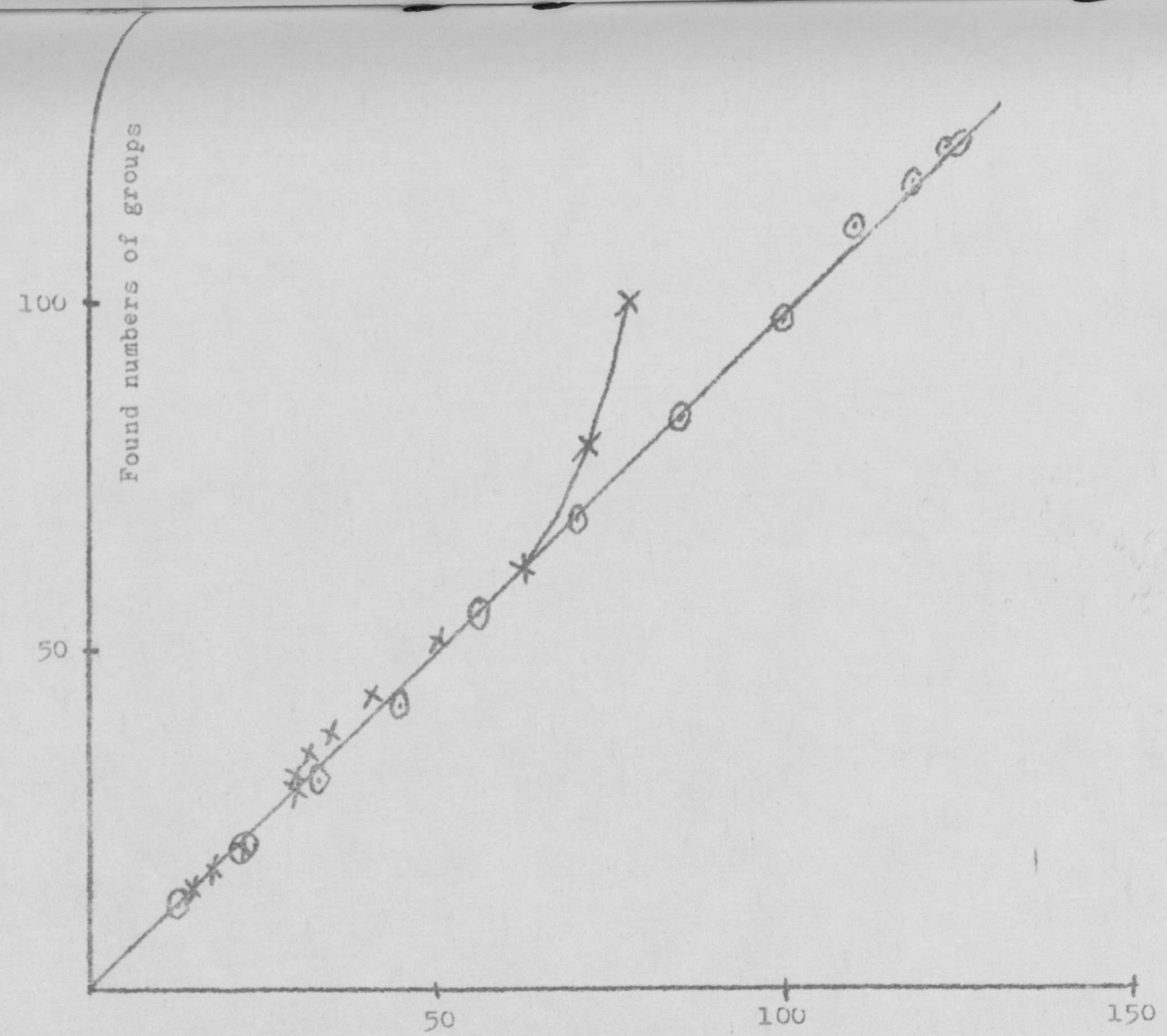

Fig. 9. Plot of calculated numbers of titratable groups in normal coagulated fibrils against the average number actually found at any given pH value (see table 5).

⊙ Values from pH 5.60 to 1.80, taking pH 5.60 as the starting point

× Values from pH 5.60 to 11.20, taking pH 5.60 as the starting point

Table 1. Fibrillar protein-content and water-binding of normal and watery pork after washing the fibrils in 0,04 M K phosphate (pH 7.00, I = ~0.09)

High pH_1			Low pH_1		
pH_1-	Fibrillar protein (g per g meat)	g water retained per g protein	pH_1-	Fibrillar protein (g per g meat)	g water retained per g protein
6.59	0,131	10.7	5.41	0,157	6.0
6.42	0,129	11.2	5.51	0,145	7.2
6.51	0,133	10.9	5.36	0.154	5.8
6.25	0,116	12.9	5.38	0,139	7.6
6.81	0,116	12.0	5.39	0,167	6.5
6.46	0,132	11.0	5.40	0,164	7.0
6.50	0,132	12.1	5.50	0,173	6.5
6.52	0,135	11.0	5.41	0,163	5.8
6.26	0,138	11.4	5.50	0,172	6.1
6.26	0,133	9.1	5.60	0,166	5.9
6.35	0,114	11.1	5.60	0,143	6.8
Mean 6.45	0.128	11,22	5.46	0,158	6.47
Extra water retained per g meat *)		0.56 g			0.18 g
Ultimate pH		5.44 ± 0.036 (9)			5.39 ± 0.054 (9)
Total N %		3.77			3.78

*) Extra water $= \left(\frac{\text{Wt of meat after treatment}}{\text{Wt of meat before treatment}}\right) - 1$

Table 2. Extraction of actomyosin at pH 6.50 (I = 0.55) from washed fibrils of normal and watery pork.

	High pH_1				Low pH_1			
	pH_1-	(1) Protein extracted g/g meat	(2) Residue g/g meat	(1)+(2) = Total fibrillar protein	pH_1-	(1) Protein extracted g/g meat	(2) Residue g/g meat	(1)+(2) =Total fibrilla protein
	6.59	0,118	0,0146	0,133	5.41	0,0156	0,138	0,153
	6.42	0,121	0,0180	0,138	5.51	0,0189	0,113	0,132
	6.51	0,122	0,0156	0,138	5.36	0,0146	0,133	0,147
	6.25	0,120	0,0180	0,138	5.38	0,0162	0,125	0,141
	6.81	0,119	0,0118	0,131	5.39	0,0144	0,147	0,162
	6.46	0,120	0,0273	0,147	5.40	0,0192	0,140	0,159
	6.50	0,127	0,0135	0,140	5.50	0,0210	0,164	0,186
	6.52	0,136	0,0135	0,149	5.41	0,0176	0,145	0,162
	6.26	0,133	0,0125	0,146	5.50	0,0214	0,164	0,185
Mean	6.48	0,124	0,0161	0,140	5.43	0,0177	0,141	0,159
Values as % total protein		88.5	11.5			11.1	88.9	

151

SLAGTERIERNES FORSKNINGSINSTITUT
3. august 1961

SVIN-KØDKVALITET
Manuskript nr. 173

Table 3. Effect of temperature of rigor on the water-retention and extractability of fibrils

Treatment	Fibrils washed at pH 7.00, I = 0.09		Fibrils extracted at pH 6.5, I = 0.55
	g H_2O retained per g protein	g fibrillar protein per g meat	% Fibrillar protein extracted
Rigor at 20°C	11.8	0.119	81.0
Rigor at 37°C	7.7	0.137	9.4

Table 4. Reversible effect of temperature on pH and proton-binding of various preparations of pork muscle

Preparation	pH at 10°C	pH at 20°C	pH at 30°C	Change in H^+ bound (in 10^{-6} mols per g of meat) 10°C	20°C	30°C
Whole meat [Briskey and Wismer-Pedersen(1961)]	5.495	5.32	5.18	+9.75	0	-7.50
Unwashed fibrils	5.66	5.53	5.395	+7.50	0	-7.60
Coagulated mince	5.82	5.71	5.60	+6.40	0	-6.40
Washed fibrils	5.47	5.35	5.21	+3.20	0	-3.70
Expected value for sarcoplasmic proteins	"	"	"	~+1.60	0	~ -1.90
Kochsaft	5.51	5.45	5.35	+1.30	0	-2.10

Table 5. Summary of titration constants and number of groups titrating, in various fibrillar preparations

Type of preparation	Number of groups titrating per 10^5 g protein at pK' =										
	2.10	2.60	3.00	3.40	3.50	4.15	4.50	4.70	6.40	6.70	10.2
Normal - native	-	48	-	-	-	105	-	-	26	-	72
Normal - coagulated	-	-	48	-	40	-	-	44	-	26	54
Watery - native	-	39	-	11	-	80	-	-	26	-	57
Watery - coagulated	-	48		-	41	-	-	40	-	26	51
Sarcoplasm - coagulated	52	-	-	58	-	-	40	-	?*	?*	?*
Calc^{d} for 0.19 parts sarcoplasm to 0.81 parts fibrillar protein	10	39	-	11	-	85	7.5	-	25	-	72

* **Footnote:** Protons bound per 10^5 protein = 29 from pH 8.4 to pH 5.60 and 63.5 from pH 11 to 8.4.