STUDIES ON ISOLATED CELL COMPONENTS

XIII. THE EFFECT OF HEAT ON RAT LIVER SOLUBLE PROTEINS

N. G. ANDERSON, T. MAKINODAN and C. B. NORRIS

Biology Division, Oak Ridge National Laboratory,[1] *Oak Ridge, Tennessee, U.S.A.*

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THE hypothesis that cytoplasmic sol-gel changes occurring in response to chemical, physical, and electrical stimuli, after fertilization, during cell division, at the first stages of cytolysis, during amoeboid movement, and in the surface precipitation reaction have a common molecular basis [11, 12] cannot be tested until the detailed mechanisms active in each instance are known. Considerable progress has been made in relating changes observed in cell breis containing added calcium [7–10, 15, 16] to intracellular events, but the mechanisms involved are not sufficiently understood to be demonstrated in well-characterized, reconstituted systems. As the first step in the characterization of a relatively simple system–the soluble phase of rat liver—protein precipitation was examined as a function of time at different temperatures.

Previous studies [3] showed that a large fraction of the proteins present in this preparation precipitates *in vitro* at body temperature in a few hours, and a remnant of structure-forming capacity persists [4]. In this and succeeding papers, the roles of proteolytic enzymes, lipid melting point, and protein denaturation and aggregation, all of which have been implicated in sol-gel changes, will be examined and mechanisms for their intracellular regulation discussed.

MATERIALS AND METHODS

Rat liver soluble phase (or supernatant protein solution) was prepared from adult male Sprague Dawley rats by perfusion, homogenization, and ultracentrifugation, as previously described [2]. Two concentrations of protein were used; one contained 4 ml of 0.25 *M* sucrose/g of liver and the other contained 2 ml of sucrose/g of tissue. The clear supernatant solution was incubated in circulating baths in 0.8 × 5-cm Lusteroid tubes or 1.0 × 7.7-cm Pyrex tubes. For nitrogen determinations, the tubes were chilled after withdrawal from the water bath, centrifuged 20 minutes in the International Equipment Co. No. 296 head at 16,560 rpm (9198 × *g* at R_{min}, 20,726 × *g* at R_{max}), and the supernatant solution was analyzed by the method of Ma and

[1] Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

Zuazaga [20]. Results obtained were confirmed with 2-ml tubes equipped with nylon adapters in the No. 40 Spinco preparative rotor operated at 40,000 rpm. Turbidity was measured in a Coleman Junior Spectrophotometer at 650 mμ in matched 1.0 × 7.7-cm tubes. A Beckman Model G meter was used for pH measurements. The effects of adding various substances to the rat liver soluble protein solution were determined by adding the experimental substance dissolved in 0.5 ml of distilled water to 1.5 ml of protein solution.

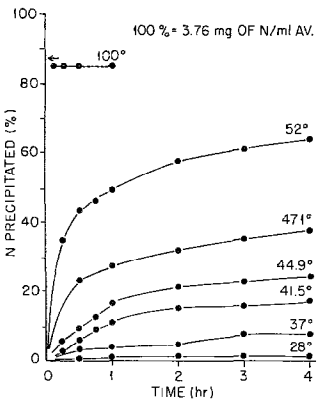

Fig. 1.

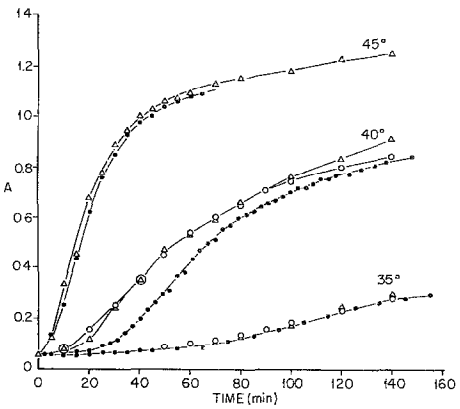

Fig. 2.

Fig. 1.—Percentage of total nitrogen precipitated from soluble liver protein solutions as a function of time and temperature. Arrow along ordinate indicates percentage of total nitrogen not lost during dialysis.

Fig. 2.—Effect of incubation at indicated temperatures on the absorbancy at 650 mμ of soluble rat liver proteins. Differences between frozen and unfrozen preparations were most marked at 40°C. △ frozen 17 hr; ○ frozen 82 hr; ● fresh.

RESULTS

The percentage of total nitrogen precipitated in 20 per cent liver preparations as a function of time and temperature is shown in Fig. 1. An average of 86 per cent of the nitrogen was precipitated by heating to 100°C for 5–60 minutes. At 4°C (not shown in Fig. 1), only 2.3 per cent of the nitrogen in one preparation precipitated in 72 hours, whereas the same amount of precipitate in another extract required 8 days.

Turbidimetric values cannot be directly compared with results obtained by nitrogen analysis, but the method produced curves having similar shapes and allowed greater experimental flexibility. The results of observations at three temperatures are recorded in Fig. 2. As shown, the effect of freezing had a significantly deleterious influence, especially evident at 40°C. Freshly made preparations were therefore used for all subsequent studies.

Since marked precipitation had previously been observed when the pH of the liver soluble phase was lowered toward 5 [3], we thought that part of the result might be caused by such a pH drop. The effect of incubation at 37.5°C was therefore examined with pH electrodes immersed in the protein solution. In the first preparation, the pH dropped from 6.44 to 6.39 in 225 minutes; in a second experiment, the pH rose from 6.45 to 6.51 in 165 minutes. The precipitation of soluble-phase proteins is probably not attributable to a decrease in pH with time. This conclusion was confirmed at other temperatures by measuring the pH after turbidimetric studies.

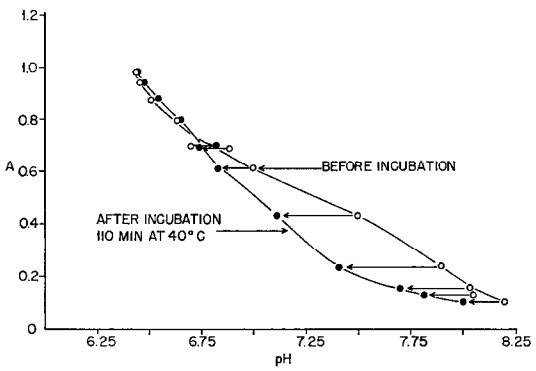

Fig. 3.—Effect of pH on the amount of turbidity formation in soluble rat liver proteins incubated at 40°C. 1.5 ml of rat liver soluble protein solution mixed with 0.5 ml of 0.05 *M* Tris phosphate buffer to give pH values before and after incubation.

We attempted to study the effect of pH on turbidity development by raising the pH of samples with 0.2 *N* NaOH before incubation. In all preparations having a pH below 8.5, however, the pH fell almost to the starting point during the experiment. The results suggest also that the pH for maximum production of turbidity is below 6.5 (where isoelectric precipitation begins) and the rate falls as the pH is raised. This was confirmed in studies in which Tris phosphate (0.0125 *M*) was used as a buffer (Fig. 3). Although the system has a marked tendency to return to the original pH if the pH is raised, the precipitation rate is actually very pH sensitive.

The lag period observed in fresh sol preparations incubated at 40°C (Fig. 2) suggests that turbidity production might be inhibited by a labile substance destroyed during incubation. The effect of adding adenosine triphosphate (ATP) on turbidity production was therefore examined (Fig. 4). All concentrations studied gave evidence of precipitation inhibition, which disappeared during incubation, possibly because of ATP hydrolysis. If observed long

enough, turbidity was seen to rise to or exceed the control value, suggesting that the hydrolysis products may favor turbidity formation.

If ATP is protective simply because of its high negative charge, other highly charged polyanions should give similar effects. The effects of adding RNA, heparin, and other acid polysaccharides were therefore examined. As shown in Fig. 5, addition of RNA had a protective effect, whereas digestion of RNA present in the preparation with RNase increased the rate of turbidity formation. By far the most effective protective agents were heparin (Fig. 6) and Paritol[1] (polymanuronic acid sulfate). Chondroitin sulfate was intermediate in effectiveness.

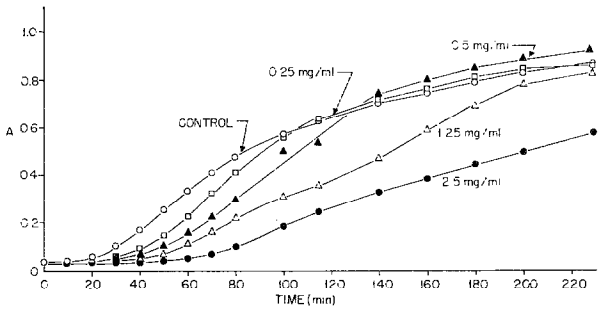

Fig. 4.—Effect of added ATP at different concentrations on the rate and amount of turbidity formed on incubation at 40°C.

The protective effect of heparin suggested that it might reverse the precipitation. It was therefore added to incubating soluble-phase preparations at intervals (Fig. 7). Since the addition involves diluting 1.5 ml of sol with 0.5 ml of heparin solution, two controls were used—one undiluted and the other diluted with 0.5 ml of water per 1.5 ml of sol. If heparin has no reversing effect when it is added, the turbidity should merely drop from the undiluted to the diluted control level. As shown in Fig. 7, only a slight reversing effect was seen, although heparin effectively prevented further precipitation.

Since calcium has been considered to play a key role in cytoplasmic events, the effects of several concentrations of $CaCl_2$ and sodium ethylenediamine tetraacetate were examined. The results (Fig. 8) suggest that very low (0.001 *M*) concentrations of $CaCl_2$ may have a slightly protective effect, but that higher (0.0025–0.5 *M*) concentrations cause a marked increase in rate of precipitation and also abolish the initial lag observed in the controls. No concentration of versene studied showed a protective effect so great as that

[1] Kindly supplied by Wyeth Laboratories, Inc.

observed with heparin. The results obtained with $MgCl_2$ were very similar to those seen with $CaCl_2$.

The incomplete protection by versene suggests that the effect is not so dependent on calcium as is blood coagulation. The possibility must still be considered, however, that activation of a proteolytic enzyme, possible analogous to thrombin, occurs. Experimentally, no evidence of enzyme activation was obtained in studies of mixtures of sol previously incubated at 37.7°C and fresh unincubated material.

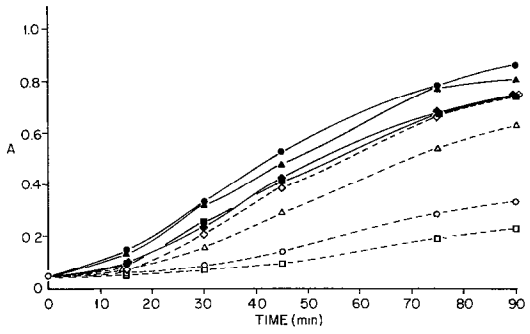

Fig. 5.

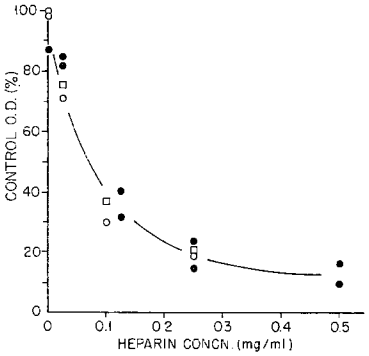

Fig. 6.

Fig. 5.—Effect of added RNA (open symbols) and RNase (closed symbols) on turbidity formation by soluble rat liver proteins. ◆, Control; □, 2.5 mg/ml; ○, 1.25 mg/ml; △, 0.25 mg/ml; ◇, 0.025 mg/ml; ●, 0.25 mg/ml; ▲, 0.025 mg/ml; ■, 0.0025 mg/ml.

Fig. 6.—Effect of heparin, Paritol "C", and Paritol "IM" on turbidity formation. ○ Paritol "C"; □ Paritol "IM"; ● Heparin, 80 min at 40°C.

How the cell either prevents or controls *in vivo* the still obscure reactions leading to protein precipitation *in vitro* seems to depend on maintenance of the anionic polyelectrolyte level. Experiments were therefore undertaken to see whether production of ATP by aerobic glycolysis was important in preventing turbidity formation. The salts and cofactors required for glycolysis suggested by LePage [17, 18] were added and found to have a significant protective effect: however, this was enhanced by omission of $MgCl_2$.

Addition of glucose alone (0.25 *M*) had a marked protective effect. Other sugars examined in similar concentrations were effective in the order glucose > mannose > galactose. Rhamnose, arabinose, ribose, and sorbose had little effect, and xylose slightly accelerated turbidity formation.

Potassium bisulfite and sodium arsenate were examined in concentrations of 0.01 and 0.001 *M*. Bisulfite produced a very marked protective effect; arsenate (0.01 *M*) reduced the amount of turbidity to one-seventh the control value but produced a rise in pH.

Since fatty acids have been reported to increase the thermal stability of proteins [5], the effect of adding various concentrations of linoleic, linolenic, oleic, caprylic, and acetic acids on turbidity formation was studied. In no instance was any protective action observed.

Because the original brei was prepared in sucrose, the possibility must be considered that the precipitation during *in vitro* incubation may be caused simply by a salt concentration lower than that existing within the cell. Adding NaCl to give final concentrations ranging from 0.001 to 1 *M* demonstrated that this was not the case. With 0.001 *M* NaCl no effect was observed; with 0.1 *M* NaCl there was a pronounced increase in the rate of turbidity formation. This effect diminished rapidly as the concentration was increased until,

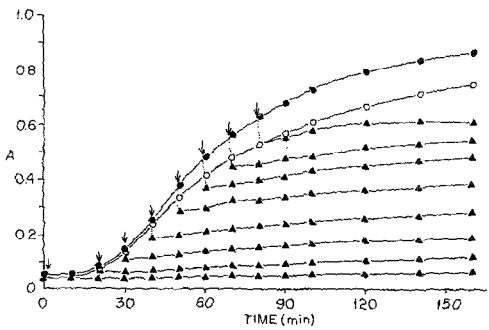

Fig. 7.—Effect of adding heparin (0.5 *mg/ml* final concentration) to soluble rat liver solutions before incubation (40°C) and at various times after starting of incubation. If no effect is produced by heparin addition, absorbancy value *should* fall from undiluted to diluted *control* values. Heparin added at times indicated by arrows. ●, undiluted control; ○, diluted control; ▲, after heparin addition, 0.5 mg/ml 40°C.

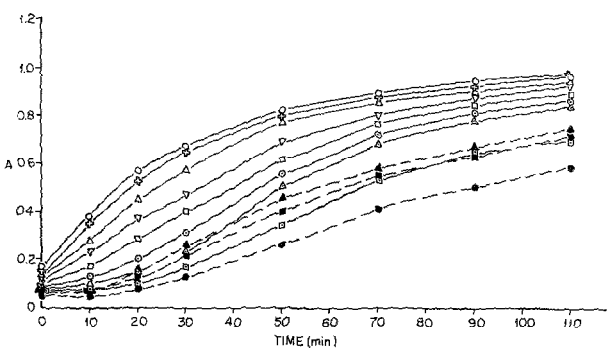

Fig. 8.—Effect on turbidity formation of various concentrations of $CaCl_2$ and versene added before incubation. ▲, Control. $CaCl_2$ concentrations: ○, 0.01 *M*; ◌, 0.0087 *M*; △, 0.0075 *M*; ▽, 0.0062 *M*; □, 0.0052 *M*; ◌, 0.0037 *M*; ⧋, 0.0025 *M*; ⊡, 0.001 *M*. Versene concentrations: ●, 0.0025 *M*; ■, 0.00025 *M*.

after 105 minutes' incubation, the turbidity was diminished to approximately one-third that of the control by 1 *M* NaCl.

Exploratory studies were carried out to determine whether tryptic or chymotryptic activity in the preparation could be involved. Trypsin inhibitors (pancreas, lima bean, and soybean) were without protective effect. Trypsin (0.3 mg/ml) very slightly increased turbidity after 10 minutes at 40°C but caused very little further rise during the next 2 hours, suggesting that trypsin digested the precipitated material as fast as it was formed. Trypsin (0.01 mg/ml), when added 1:3, did not alter the rate from that of the control. Chymotrypsin (0.3 mg/ml) was more protective than trypsin, and the turbidity was below that of the control from the very start.

Since sulfhydryl compounds have been implicated in structural changes occurring during cell division, the effect of adding sulfhydryl reagents is of considerable interest. Freshly prepared AET (S, 2-aminoethylisothiuronium bromide hydrobromide), cysteine, and Tris thioglycolate (all at pH 7.0, 0.0005–0.005 *M*) produced a marked *increase* in the rate of turbidity formation, but dithiodiglycol concentrations up to 0.005 *M* had no effect. Iodoacetate had a marked concentration-dependent turbidity-increasing effect.

Colchicine (0.00025–0.25 mg/ml) produced no change in the rate of turbidity formation.

DISCUSSION

A large fraction of the soluble liver proteins is precipitated *in vitro* by incubation for times and temperatures not lethal for the intact animal. Two general problems arise: first, whether the mechanisms involved are thermal, enzymic, or both; and second, the relation of these mechanisms to intracellular events.

The rate of precipitation observed at 40°C was markedly decreased as the pH was raised, suggesting that the optimal pH was below that of the freshly isolated material (pH 6.5), in the range where isoelectric precipitation occurs in the cold. Marked inhibition of precipitation was observed when ATP, heparin, RNA, and other polyanions were added: whereas Ca^{2+}, Mg^{2+}, fatty acids, and sulfhydryl compounds (cysteine, AET, thioglycolate) increased the rate of precipitate formation.

These results support the view that the stability of cytoplasmic proteins is controlled by the level of polyanions, including ATP. The glycolytic activity of the soluble phase of liver is insufficient to maintain the normal ATP level [17–20]. In the present study we did not find that addition of metabolites and

cofactors in physiological concentrations affords more than 50 per cent protection. Significantly, RNA, which protects the soluble liver protein system, breaks down when the level of ATP drops in liver breis [18]. Runnström [22] emphasized that glycolysis in sea urchin eggs does not supply enough energy to maintain the egg cytoplasm. The ATP-sensitive reactions involved in the egg cytoplasm may well be analogous to those observed *in vitro* here. The solating effect of heparin when injected into amoebae and the importance of Ca^{2+} in the surface precipitation reaction [11] also have their counterparts in the liver soluble protein system. Until details of the reactions involved in each instance are elucidated, however, the validity of such an analogy remains open to question.

For the major portion of the work reported here, a temperature of 40°C was used, which is very close to the thermal death point of the intact rat [13]. Mammalian cells in tissue culture, however, are able to survive at temperatures several degrees higher [14, 21]. It seems therefore that the reactions leading to precipitation *in vitro* may be inhibited *in vivo*, as suggested by Allen [1].

Subsequent studies will be aimed at distinguishing between the physical protection of proteins against thermal denaturation by polyanions [6] and polyanionic inhibition of proteolytic activity.

SUMMARY

The precipitation of rat liver soluble proteins as a function of time and temperature has been studied in terms of total nitrogen precipitated and turbidity developed. At 40°C ~ 10 per cent of the nitrogen precipitated in 2 hours; at 100°C, 86 per cent was flocculated. The amount of precipitation diminished rapidly as the pH was raised from 6.4 to 8.2. The flocculation observed especially at lower temperatures (~ 40°C) is not attributable to a decrease in pH during incubation. Evidence for a pH homeostatic mechanism was found.

Addition of polyanions (ATP, RNA, heparin, polymanuronic acid sulfate) markedly decreased the rate of precipitate formation, but RNase, calcium chloride, and magnesium chloride promoted precipitation. It is suggested that *in vivo* precipitation is largely prevented by maintaining appropriate levels of ATP, RNA, and other polyanions. The relation of these findings to cytoplasmic structure and function and to intracellular viscosity changes is discussed.

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