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Invertase Activity in Hevea *Latex Serum: Interaction between pH and Serum Concentration*

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When C-serum was diluted from its natural (undiluted) concentration, there was a shift of pH optimum in its invertase activity from basic (about 8.8) to near neutral (about 7.4). This effect was especially obvious when the dilution was 50% or greater. There was, at the same time, a marked apparent increase in enzyme activity at pH below 8.4 when the serum was diluted. The shift in the pH optimum in undiluted serum towards neutral could be partially effected by Trisacryl GF05 filtration of the serum. Addition of boiled C-serum to dilute unboiled serum tended to shift the optimum towards the basic pH values. Besides the known effect of the pH of latex on its invertase activity, the factors concerned with the phenomena observed might also serve as a means of fine adjustment of this activity.

Invertase is a key enzyme in *Hevea* latex that activates the initial step of the glycolytic pathway. This itself initiates a chain of biochemical reactions both in respect of respiration and, essentially, rubber biosynthesis. Invertase activity in C-serum has been shown by Tupy[1,2] to be highly sensitive to pH. Activity of this enzyme was found to be negligible at pH lower than about 6.5. At pH above this, there was a rapid rise in invertase activity[1,2,3] reaching an optimum at pH 7.2 - 7.5. It was this abrupt increase in enzyme activity that prompted the ascription of serum pH as a major factor regulating the activity of this enzyme in the tree. As the distinct invertase peak in the region of pH 7.4 had always been readily perceived, activity of the enzyme was rarely carried out beyond pH 8 (where, moreover, the normally used buffers such as phosphate were inadequate in buffering capacity and a second buffer would have been obligatory). Using a glycine–NaOH buffer to adjust for the pH range 8 - 10, Chong[4] noted a pH optimum of 8.9 for invertase in the latex serum from unstimulated trees. In view of the discrepancy in the reported pH optima, a study was initiated to re-examine the pH dependency of *Hevea* C-serum invertase with special emphasis on its activity above pH 8. The interaction between pH and serum concentration was also investigated.

MATERIALS AND METHODS

The first half-hour flow of latex from RRIM 501, RRIM 600, RRIM 701, GT 1, PR 107 and Tjir 1 trees (all tapped on *Panel BI–1)* grown in the RRIM Experiment Station, Sungei Buluh, was collected into chilled containers. C-serum was obtained from the latex by centrifugation for 1 h in a Sorvall RC-2B centrifuge at 19 000 r.p.m. (44 000 g max.) at 3°C - 4°C. Sera from all the clones were used fresh in the first experiment (represented by the results from RRIM 701 in *Figure 1*). In all subsequent experiments, sera from RRIM 600 and GT 1 were freeze-dried and then re-constituted before use by the addition of water and/or buffer-substrate to the appropriate concentration. The natural concentration of C-serum was taken as 50 mg freeze-dried solids per millilitre.

The invertase assay for fresh sera was carried out as previously described[5] by incubating the serum with sucrose. The products of the enzymatic reaction (glucose and fructose) were assayed as reducing sugar by the Nelson-Somogyi reaction[6,7]. With the freeze-dried sera, a generally similar incubation procedure[8] was employed but an enzymatic method[9] was adopted for the quantification of glucose and fructose separately. Sodium fluoride (60 mM) was added to the incubation mixtures containing

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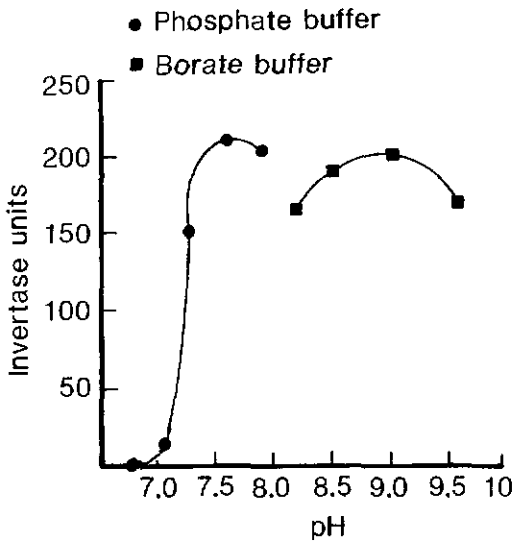

Figure 1. Invertase activity in fresh RRIM 701 C-serum at various pH adjusted with phosphate and borate buffers. The proportion of serum in the incubation mixture was 50%.

fresh serum and to some of the incubation mixtures containing freeze-dried serum (in experiments where high serum concentrations were used). This inhibited regeneration of uridine diphosphate glucose and so obviated interference in the sucrose levels by the action of sucrose synthetase present in the serum[10]. Three buffer systems were used in the invertase assays. Phosphate buffer (0.1*M*) was used in assays carried out at pH 6.0 - 7.8 while borate-KCl-NaOH buffer (0.1*M*) was used at pH 8.1 - 9.9. A third buffer, Prideaux's universal buffer[11] (consisting of a 50 mM solution with respect to boric, acetic and phosphoric acids adjusted with NaOH), was used in assays over the entire pH range from pH 6.0 to 9.9. Invertase activity was expressed in invertase units where 1 unit = 1 μmole sucrose hydrolysed per 10 ml undiluted C-serum in 30 min.

To remove low molecular weight molecules from the serum, concentrated serum (517 mg per millilitre) was filtered through a 30 cm Trisacryl GF05 gel column equilibrated with 50 mM phosphate buffer at pH 7. The pooled filtrate containing all the serum proteins (as determined by adsorption at 280 nm) was concentrated using an Amicon filter with a cut-off at molecular weight 10 000. The resultant filtrate was essentially free of salts and other small molecules.

Inactivated C-serum was prepared by boiling freeze-dried GT 1 serum that had been reconstituted to the required concentrations. The precipitate formed on boiling was removed by centrifugation.

RESULTS

Invertase activities in fresh C-serum from RRIM 501, RRIM 600, RRIM 701, GT 1, PR 107 and Tjir 1 trees were assayed over a range of pH using two buffer systems: phosphate buffer for pH 6.8 to 7.9 and borate buffer for pH 8.2 to 9.6. The proportion of serum in the incubation mixtures was 50%. The results for RRIM 701 are given in *Figure 1*. Two pH optima were observed — pH 7.5 and pH 8.9 — one for each buffer system. Essentially similar trends were observed with the other clones (data not presented). It was not clear from these experiments, however, if the two pH optima observed had resulted from interactions of the enzyme with the two dissimilar buffer systems used. To resolve this ambiguity, it was therefore desirable to have a single buffer covering the whole pH range being investigated. Prideaux's universal buffer was adopted for this purpose.

Invertase assays were carried out with freeze-dried RRIM 600 C-serum with the proportion of serum in the incubation mixtures being adjusted to be equivalent to 4.4%, 50% and 100% that of fresh serum. For the purpose of comparison with the universal buffer, phosphate and borate buffers were also used in parallel assays within their effective ranges. When the serum concentration in the incubation mixture was dilute (4.4%) the change in invertase activity with pH change took the form of a single smooth curve peaking at pH 7.5 *(Figure 2)*. With increased serum concentration (50% and 100%), two curves — somewhat discontinuous with one another — were seen in

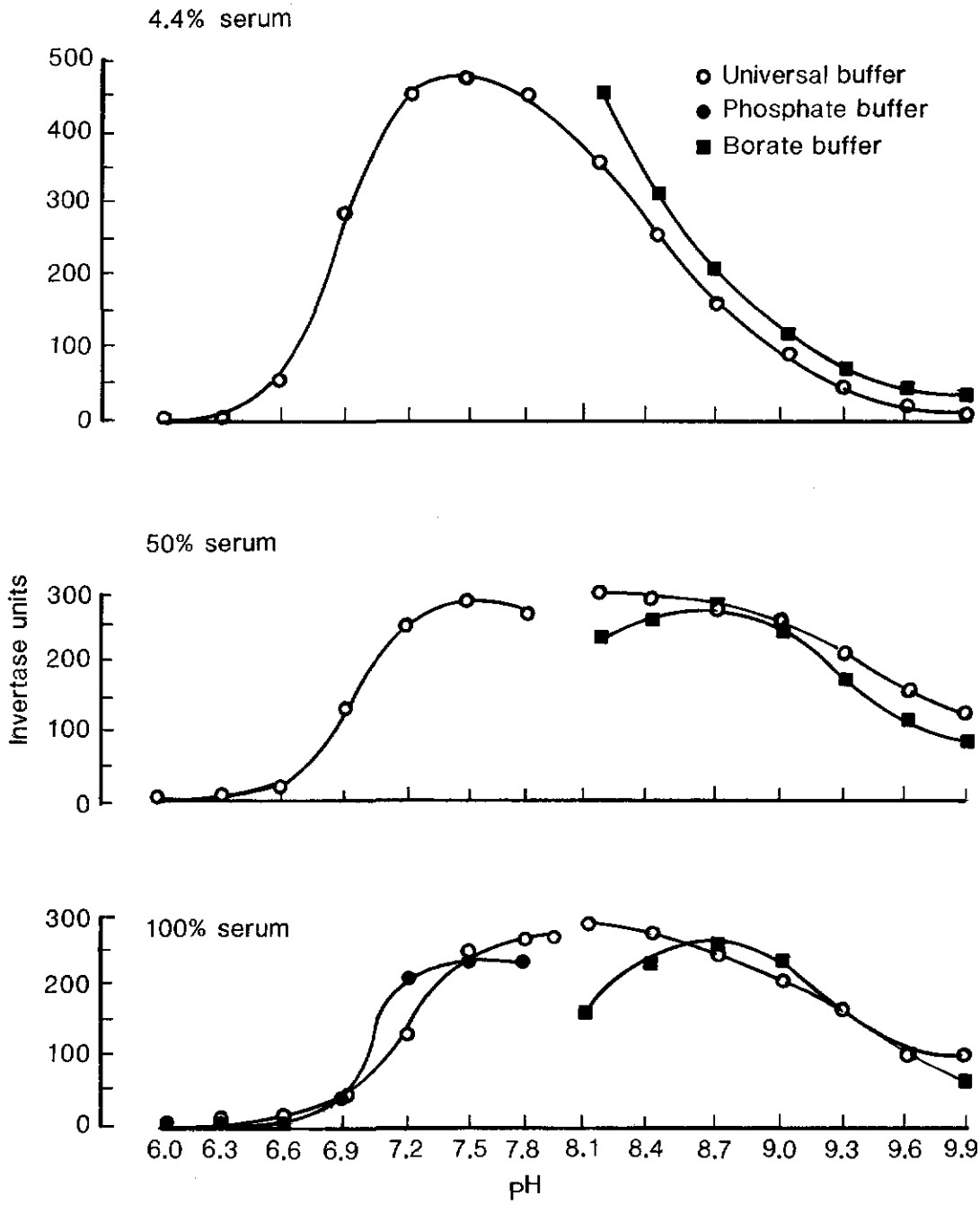

Figure 2. Invertase activity in freeze-dried RRIM 600 C-serum at various serum concentrations and at various pH adjusted with universal, phosphate and borate buffers.

assays using the universal buffer *(Figure 2)*. At 50% serum concentration, the pH optimum at 7.5 was still evident while at 100% concentration, invertase activity continued to rise beyond pH 7.5. The net effect was that the pH optimum in universal buffer tended to shift to the right (*i.e.* towards basic pH) with increasing serum concentration. A second distinctive feature of the results of this experiment was the very significant apparent decrease in activity as the proportion of serum in the incubation mixture rose from 4.4% to its undiluted state, when the pH was below 8.4 *(Figure 2)*. Beyond this pH, there was an increase in enzyme activity as concentration of the serum rose. When phosphate and bórate buffers were used in the assays, the curves obtained were generally similar to those for the universal buffer. With borate buffer especially, a distinct peak in the region of pH 8.8 was observed when serum concentration in the incubation mixture was 50% or 100% *(Figure 2)*.

To verify further the results obtained with RRIM 600 serum, a similar series of assays were carried out in universal buffer using freeze-dried GT 1 serum. A total of five dilutions of serum in the incubation mixture were tested: 5%, 25%, 50%, 75% and 100%. The results *(Figure 3)* were generally similar to those for RRIM 600 serum. More so than in RRIM 600, GT 1 serum invertase showed a gradual increase in activity from pH 6.9 to its peak at pH 8.7 when the serum was in its undiluted state. This contrasted with the behavior of the enzyme in highly dilute (5%) serum when its activity rose abruptly from pH 6.9 to pH 7.2 and declined rapidly thereafter. There was a marked increase in invertase activity as the serum was diluted when assays were carried out at pH below 9. This increase was not seen between undiluted serum and serum diluted to 75% its natural concentration. Some increase occurred at 50% serum while at the higher dilutions (25% and 5% serum) a sharp increase in activity was observed. This increase was especially marked at pH 7.2, the optimum pH for invertase activity in dilute serum. At pH greater than 9.0, dilution did not result to an increase in invertase activity *(Figure 3)*. The effects of serum dilution on the pH optima of invertase and its level of activity were therefore quite similar in the two clones, RRIM 600 and GT 1.

Reconstituted freeze-dried GT 1 C-serum was filtered through a Trisacryl GF05 gel column to remove salts and other low molecular weight molecules. The eluted fractions containing proteins were subsequently concentrated using an Amicon filter. Invertase assays in universal buffer were then carried out with filtered and unfiltered sera, their concentrations in the incubation mixtures having been adjusted to 100% (resembling natural, undiluted serum) or 9.5%. The results, presented in *Figure 4*, showed the typical curves (resembling those in *Figure 3*) of invertase activity for dilute and undiluted *unfiltered* sera over a range of pH. With *filtered* sera, it was found that filtration had the effect of lowering the invertase activity in the dilute serum. The shape of the pH–activity curve was unaltered as compared to the unfiltered serum. On the other hand, there was considerable change in the response of invertase activity to pH when the filtered serum was used in its undiluted form. The peak around pH 8.8 was no longer evident while there was a significant increase in activity at the lower pH values (pH 6.9 – 8.1) as compared with concentrated serum that was unfiltered *(Figure 4)*. The net effect was that, with filtration, the pH optimum of invertase shifted towards the left (neutral pH).

Boiled C-serum was added to GT 1 freeze-dried C-serum that had been reconstituted to 10% of its natural concentration. The final content of boiled serum in the invertase incubation mixture was equivalent to 28 mg freeze-dried serum solids per millilitre, this being equatable to 56% of natural serum (assumed to be 50 mg per millilitre). The results revealed a loss of activity at the lower pH range (pH 6.9 – 7.8), but an increase in activity at pH greater than 7.8 *(Figure 5)*. In effect, there was an apparent shift of the pH optimum to the right from near neutral towards basic pH. When a higher concentration of boiled C-serum (150 mg per millilitre, equivalent to 300% of serum) was added to the dilute serum, the results were generally similar to those when less boiled serum was used, other than that, activities at the basic pH values were higher.

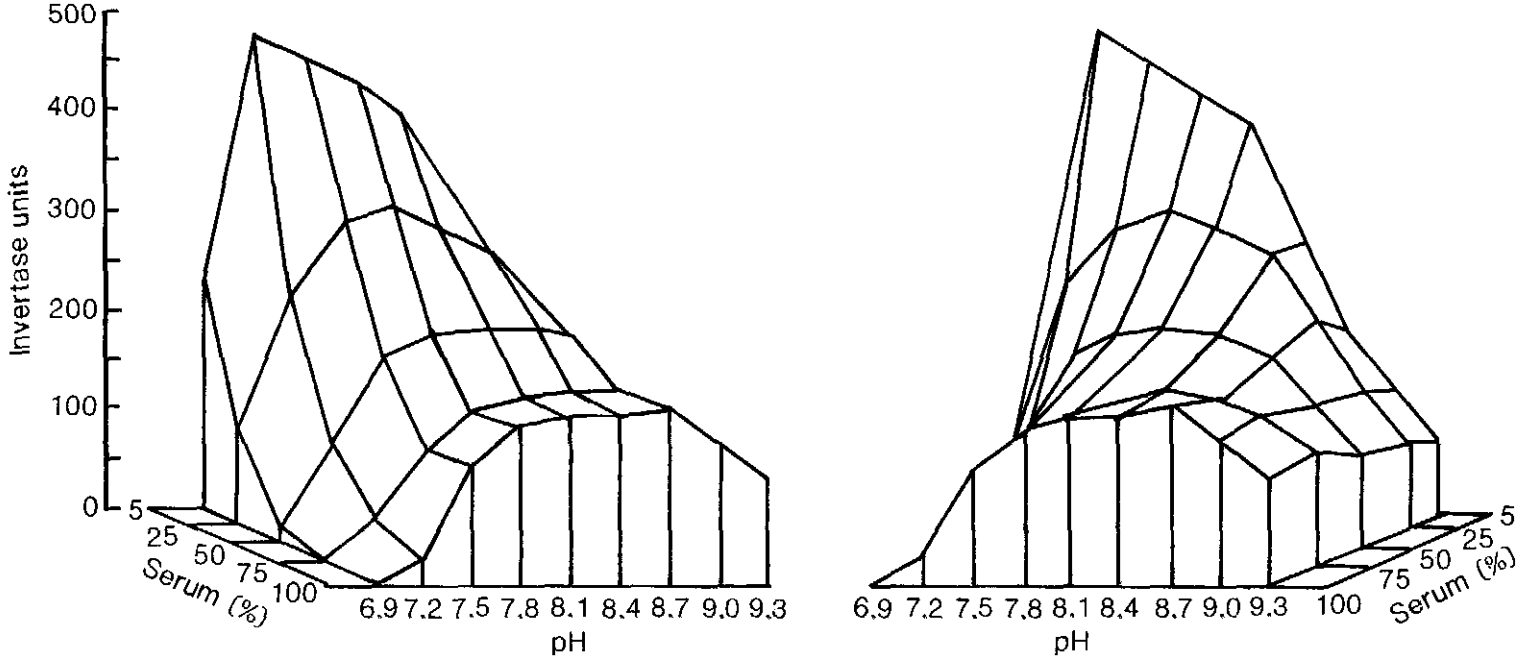

Figure 3. Invertase activity in freeze-dried GT 1 C-serum at various serum concentrations and at various pH adjusted with universal buffer (two perspectives).

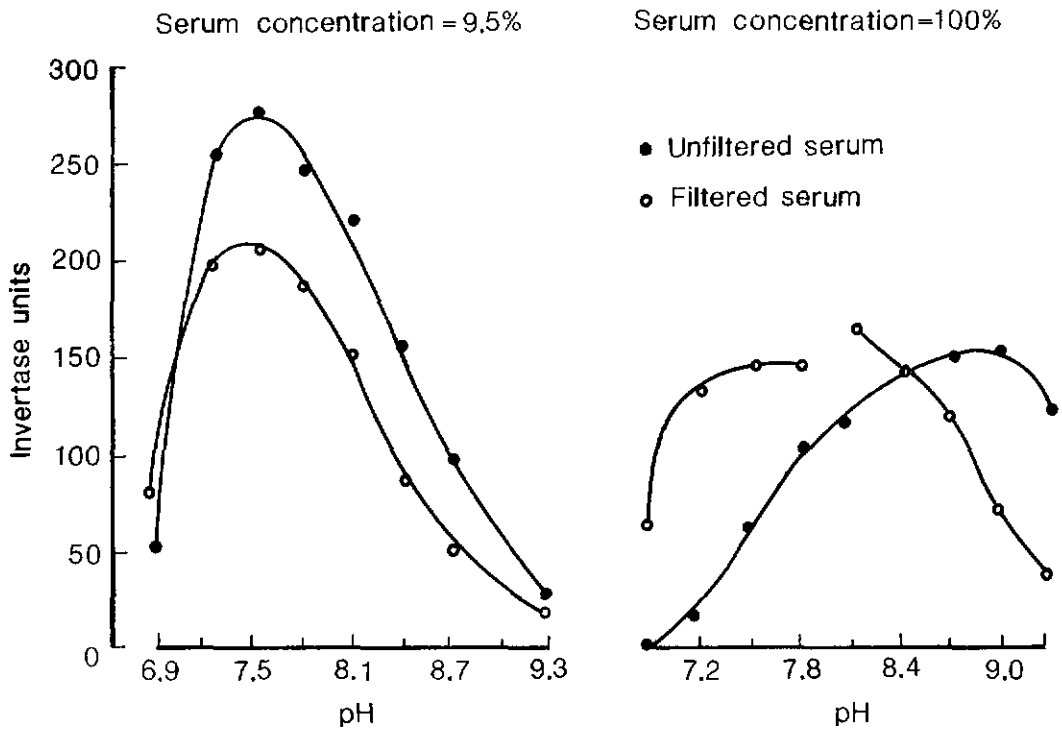

Figure 4. Effect of Trisacryl GF05 filtration of freeze-dried GT 1 C-serum on its invertase activity. Universal buffer was used to adjust the pH.

DISCUSSION

Two important characteristics of invertase have been repeatedly observed when serum in the enzyme incubation mixture was diluted from its natural concentration in serum.

Firstly, there was generally an apparent increase in invertase activity with serum dilution, a property already noted by Tupy[2]. This increment did not extend over the entire pH range investigated but was apparent only at pH below 8.4 (RRIM 600) and 9.0 (GT 1). Beyond these pH values, dilution of the serum decreased activity of the enzyme. Nevertheless, at the physiological pH of the latex (about 7), the tendency would be towards the increment of invertase activity with dilution.

Secondly, there was a shift in pH optimum from about 8.8 to about 7.4 with dilution. That two pH optima were also found in experiments using fresh serum indicated that the process of freeze-drying did not affect this enzyme characteristic. The use of a single buffer over the entire pH range investigated showed that these observations were not an effect of interactions with dissimilar buffers.

Two hypothetical mechanisms which can explain most of the observations from the experiments are proposed.

One possible mechanism is as follows. Invertase is subject to both the inhibiting and activating actions of one or more pH sensitive effectors which are small molecules. The putative effectors activate invertase at basic pH but inhibit it at pH closer to neutral. At normal (*i.e.* high) serum concentrations, there is a high concentration of the effectors; thus, activity in the vicinity of neutral pH is suppressed while that towards the basic end is enhanced. On diluting the serum (to 10% and below), there

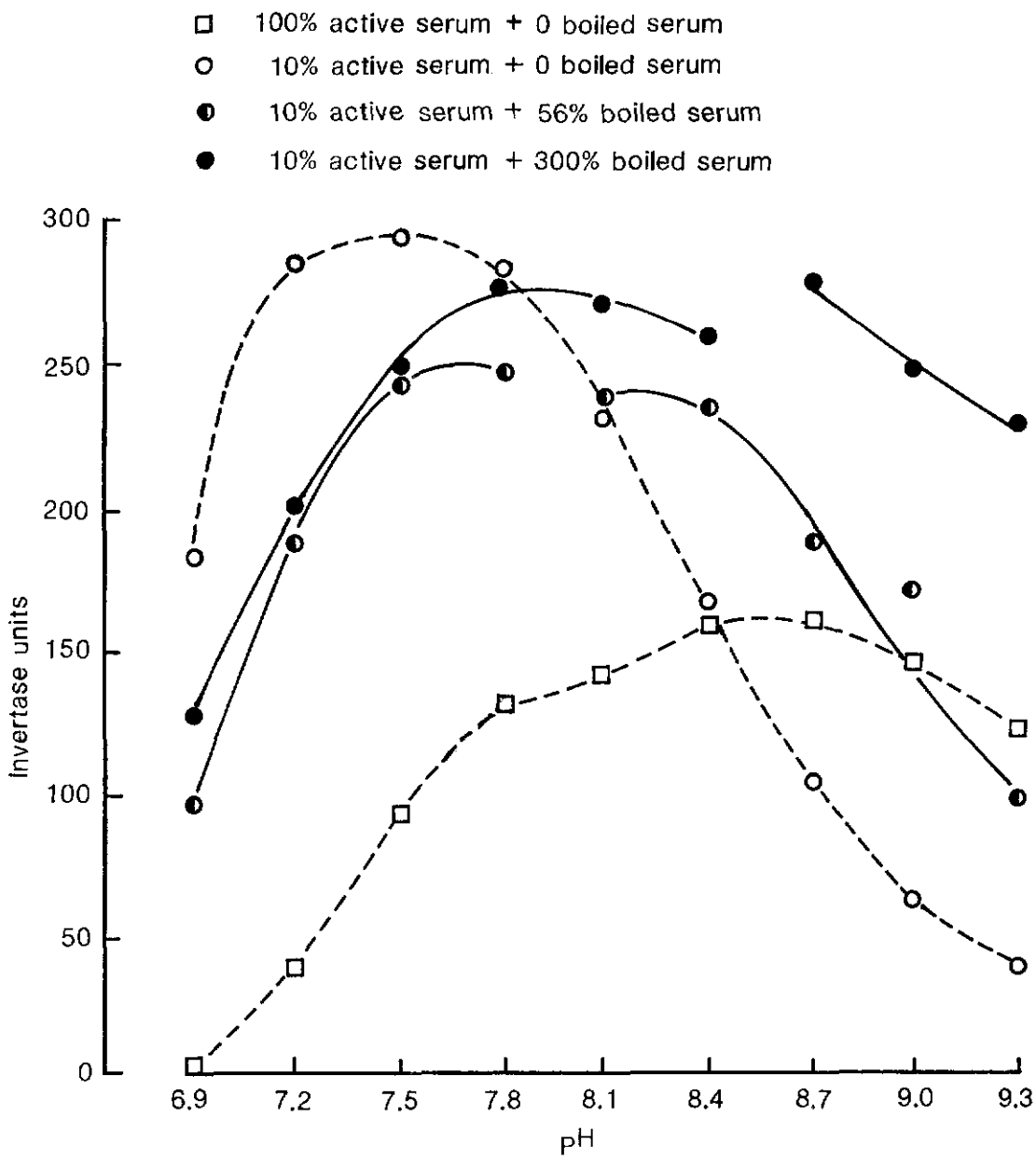

Figure 5. Effect of adding boiled C-serum to freeze-dried GT 1 C-serum on its invertase activity. Universal buffer was used to adjust the pH.

is a rise in activity around and just above neutral pH as the inhibition by the effectors is removed. At the same time, activation by the effectors at the high pH range is diminished resulting in a decrease in activity at this end of the pH scale.

On removing small molecules by gel filtration, the concentrated serum experiences a loss in activity at basic pH. This may be attributed to the loss of the small molecule effectors from the serum. For the same reason, there is a concomitant increase in activity towards the neutral pH values. To some extent, therefore, filtration has the same general effect as the dilution of the serum in shifting the pH optimum from basic towards the neutral values.

On addition of boiled serum to the incubation mixture, invertase activity at basic pH is elevated because of the action of additional effectors present in the boiled serum. The effectors' inhibitory property, on the other hand, suppresses activity when pH tends towards neutral. Hence, the addition of boiled serum partially simulates the effect of increasing the C-serum concentration.

An alternative explanation is as follows. There are two interchangeable forms of invertase having pH optima around 7.4 *(Type A)* and 8.8 *(Type B)*. Of the two, *Type B* is the less active. A small molecule acts as a transformer to change the invertase molecules from *Type A* to *Type B* and to maintain it in the latter state. Thus, at the natural (*i.e.* high) concentration of the serum, the transformer molecule is in abundance, and accordingly, the basic type *(Type B)* of invertase predominates. On dilution, the transformer molecule is scarce and consequently, *Type B* invertase is transformed into *Type A* invertase, this being accompanied by the shift of pH optimum from 8.8 to 7.4.

With filtration, some of the transformer molecules in the concentrated serum is lost; hence, a partial shift of the pH optimum towards the neutral values. Why there should be a reduction in activity in the dilute serum after filtration is not clear but various promoters of invertase present in the serum would have been lost in the course of filtration.

On addition of boiled serum to dilute serum in the enzyme incubation mixture, the transformer contained in the boiled serum converts *Type A* invertase to *Type B*, shifting the pH optimum towards the basic in the process.

In summarising the proposed regulatory mechanisms, therefore, there could exist in C-serum small molecule effectors that inhibit invertase activity in the vicinity of neutral pH but activate it at higher pH. Alternatively, a small molecule transformer might transform the enzyme molecule from one form (with pH optimum about 7.4) to another (with pH optimum about 8.8). A polymeric form of invertase which can dissociate into and reassociate from active sub-units has been reported in *Neurospora*[12].

A critical role of serum pH in the regulation of invertase activity has repeatedly been emphasised. This line of thought stemmed from the previous observation in sera, diluted 50% or more, that a very steep increase in activity accompanied the increase of pH from about 7 to 7.5. The findings in the present investigation, especially in GT 1 serum *(Figures 3 – 5)*, showed that at its natural concentration the increase in activity with pH over this range was considerably more gradual than had been thought; the enzyme did not have as narrow a pH optimal as supposed. As such, the regulation of invertase activity by latex pH in the tree, while substantiated, is probably of lesser importance than has been generally believed.

Although serum dilution can lead to a drastic change in pH optimum and relative activity, it is doubtful if this mechanism gives rise to extreme fluxes in invertase activity in the tree. The effects of dilution was marked only when it reached 50% or greater *(Figure 3)*, this extent of dilution not being normally encountered *in vivo*. On the other hand, changes in serum concentration within the physiological range might serve as a means of fine adjustment to latex invertase activity.

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