advantage of being water-clear and of 'spreading' the readings slightly (Table 3). Contrary to the findings of Hirschfelder & Serles (1934) the sample of gum ghatti used contained no magnesium.

Haury (1937) has said of the direct method of estimation of the magnesium-dye lake 'with this method it is necessary to add an excess of Titan yellow. A definite percentage of the dye combines with or absorbs the magnesium ion to produce the characteristic red magnesium-dye lake. The surplus yellow due remaining in the solution vitiates the red colour to be compared. Consequently unless the concentration of magnesium in the two solutions to be compared be within close range, satisfactory colour matches cannot be obtained.' By choosing a suitable concentration of Titan yellow a very satisfactory adherence to Beer's law can be obtained. The optimum amount was found to be 1.5 ml. of a 0.05% solution for the range over which blood magnesium may be expected to vary. The effect of less or more Titan yellow is shown in Fig. 3.

The amount of sodium hydroxide added must be sufficient to neutralize the excess trichloroacetic acid remaining after precipitation of proteins and to precipitate the magnesium hydroxide. 2 ml. of a 4N-solution was found to provide ample excess.

SUMMARY

1. A rapid and accurate method is described for the estimation of magnesium in plasma or serum depending upon the precipitation of magnesium hydroxide by sodium hydroxide in the presence of Titan yellow.

2. The estimation may be carried out in the presence of calcium or of oxalate.

3. Recoveries of the order of ± 0.05 mg. magnesium/100 ml. are obtained over the normal ranges of plasma or serum magnesium concentration.

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The Initial Stages in the Purification of Pig Liver Esterase

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The early work of Simonds (1919) demonstrated that there was considerable difficulty in obtaining a clear solution of liver esterase, because this enzyme was very firmly bound to the insoluble cell substances. Even autolysis in glycerol solutions for two to three years did not completely liberate it.

Willstätter & Memmen (1924) improved the yield somewhat by introducing the acetone dried powder preparation from which the esterase could be extracted with dilute NH_4OH . Owing to the losses involved in the acetone dried powder procedure and to its inconvenience, Bamann, Mukherjee & Vogel (1934) reinvestigated the extraction from the fresh liver and obtained a greater yield of enzyme more conveniently. They added dilute NH_4OH to the minced tissue and allowed it to autolyse at room temperature for 3–4 days, after which the mixture was made weakly acid with dilute acetic acid and

the supernatant filtered off. Complete extraction of the enzyme was claimed, and the solution was 2–3 times purer than the original fresh liver.

In this paper some properties of the bound esterase are described, and a method is given of increasing the purity of the enzyme by making use of its insoluble state in the fresh liver. By diluting the liver mince with water, collecting the insoluble material in a high-speed centrifuge and washing it twice more in a similar manner with water, it was possible to reduce the concentration of soluble impurities to less than 1% of their initial concentration. Solubility tests of the type used by Butler & Montgomery (1933) and by Roche, Dorier & Samuel (1936) were also carried out both on the esterase content of the extract prepared by the method described in this paper and on that of Bamann *et al.* mentioned above.

EXPERIMENTAL

Esterase estimations. The esterase in 0.5 or 1.0 ml. samples was estimated by measuring the rate of fall of pH (glass electrode) of 20 ml. of half-strength pH 7 phosphate-borate buffer (Kolthoff, 1925) containing 0.5 ml. ethyl acetate at 37°. The relation between the rate of fall of pH and enzyme concentration was linear between pH 7 and 6.2. One enzyme unit was defined as the amount which lowered the pH by 0.001 pH unit/min.

Protein-N estimations. The protein in 1 ml. samples was precipitated with 5 ml. 5% trichloroacetic acid and the mixture heated at 80° for 20 min. (Northrop, 1932). After this the agglutinated precipitate was collected on to a 16 mm. disk of Whatman no. 42 filter paper in the perspex micro-Buchner funnel shown in Fig. 1. It is operated somewhat as follows. The trichloroacetic acid suspension is

Fig. 1. Micro-Buchner filter for preparing ammonia-free protein precipitates.

poured into the perspex tube A and drawn through the filter with very gentle suction. Next, tube A, which is a tight fit in the funnel, is raised 5 mm., half filled with ammonia-free 5% trichloroacetic acid, and any protein adhering to its lower end is detached with a glass rod and caught on the filter paper. Tube A is then removed and four Buchner-funnels of fresh trichloroacetic acid solution are drawn through to wash away any non-protein nitrogen present. The filter paper with the protein on it is removed, dried for an hour at 100° and stored in a desiccator until it is convenient to estimate the protein-N by the usual micro-Kjeldahl method. The method can be used to estimate protein nitrogen in a solution saturated with ammonium sulphate, the quantitative removal of the ammonia taking about 10 min.

Solubility test method. The amounts of solid ammonium sulphate, calculated to produce the salt strength required for each point of the graph, were weighed out into a series of small, dry, clean glass vessels. Samples, 5 ml. each, of the solution under

test were then added to each tube and stirred until all the ammonium sulphate had dissolved. As the addition of the ammonium sulphate lowered the pH of the enzyme solution, the pH's were adjusted to 6.0 by adding small measured quantities of 0.1 N-NaOH to each tube. The solutions, having stood in the refrigerator for 12 hr., were filtered in the refrigerator through 16 mm. disks of filter paper (Whatman no. 42) in the micro-filters shown in Fig. 2. Under these conditions the protein adsorption from the solution by the filter paper was negligible. These filtrates remained clear when kept

Fig. 2. Micro-filter for clarifying solubility test solutions.

in the refrigerator for a further week showing that precipitation had ceased. The filtrates were analyzed for esterase content as soon as possible, and the results corrected for the dilution caused by the addition of the ammonium sulphate. The dilution factor D by which the results were multiplied was calculated for each point by means of the formula $D = (100 + G)/100 S$, where G is the weight of ammonium sulphate added to 100 ml. of solution and S is the specific gravity of the resulting solution. The specific gravity figures, etc., used were those of Schiff as quoted in the *Handbook of Physics and Chemistry* (Hodgman, 1944).

In making the enzyme determinations a small correction had to be applied to allow for the buffering effect of the ammonium sulphate present in the enzyme solutions. This correction was found by making a series of blank measurements with enzyme solutions of known strength containing increasing quantities of ammonium sulphate.

Determination of relation between pH and amount of esterase in solution. Well-minced pig liver was suspended in twice its weight of water, 5 ml. toluene/l. added, and the whole stirred for about an hour. The suspension was next strained through well-washed mutton cloth, and some strands of capsular material and any insufficiently minced liver were removed.

Samples, 20 ml. each, of this suspension were placed in a series of twelve centrifuge tubes and measured quantities of 0.1 N-HCl and NaOH added to bring the pH of each tube to the desired value. Next day when the suspensions had warmed up to room temperature, in each tube the final pH's were redetermined and the total esterase/ml. measured. Most of the insoluble material in the tubes was thrown down by centrifuging at 2000 *g* for 10 min. and the supernatants were filtered through Whatman no. 42 filter paper till clear. The esterase/ml. of each filtrate was determined and plotted against the pH of that filtrate (Fig. 3).

Fig. 3. Relation between esterase eluted from insoluble enzyme complex and pH. ○—○ Esterase/ml. in solution. +—+ Total esterase/ml. uncorrected for added HCl or NaOH. ●—● Total esterase/ml. corrected for added HCl and NaOH.

It is clear from the graph of total esterase present that the enzyme is stable between pH 5 and 10, but that below pH 5 it shows signs of losing its activity. Moreover, the bound esterase can be washed most economically at pH 5.7, since the esterase in solution at this point is minimal. Also the maximum elution is achieved by raising the pH to 9.5.

Washing of bound esterase. The insoluble material from the crude liver suspension described above was collected by adjusting the pH to 5.7 and passing it slowly through a Sharples' 'Super centrifuge'. This process of washing by suspending in water and

centrifuging was repeated twice, then the bound esterase was eluted by raising the pH to 9.5 and leaving the suspension in the refrigerator overnight. Next morning the eluted enzyme was collected by lowering the pH to 6 with 0.1 N-HCl and centrifuging off the insoluble material. Final clarification was effected by passing the solution through a Whatman no. 1 filter paper.

About 25 % of the total enzyme is lost in the washing process. A further 30–40 % is left behind in the elution and clarification. The result is a solution of about 40 % of the original esterase which is 10–15 times purer than the untreated liver. Some increase in the purity of these extracts occurs if they are left in the refrigerator with excess toluene. Under these conditions much unstable protein is precipitated and a purity of 20 times the original may be reached in a few months. Dialysis followed by precipitation of some inactive protein at pH 5 also increases the purity slightly.

All the extracts prepared in the above manner are light brown in colour and undoubtedly contain catalase as shown by quantitative absorption spectroscopy in the visible range and by chemical measurement of destruction of hydrogen peroxide.

Attempts to elute the esterase with 6M-urea or 6M-glycerol were unsuccessful. Drying of the washed bound enzyme at room temperature with acetone and ether resulted in complete loss of activity.

DISCUSSION

Preparations of extracts. The method of Bamann *et al.* (1934) gives an almost theoretical yield of esterase, but the increase in purity is only 2.5–3 times that of fresh liver. If the impurities present in the extract of Bamann *et al.* were easily removed, it would obviously be the method of choice. Unfortunately, the difficulties inherent in enzyme purification are such that the yield has sometimes to be sacrificed to the attainment of increased purity.

There appear to be at least three ways in which the efficiency of the method described in this paper could be increased. First, very thorough and complete homogenization of all the liver tissue would increase the yield of enzyme, as quite a lot of esterase is discarded with the capsular material, etc. It would also assist in the washing away of any soluble substances which might be released when the pH of the suspension is raised to 9.5. Secondly, if the washing of the bound enzyme could be conducted without so much loss, the yield of enzyme and the purity of the final extract would be considerably increased. There is some evidence to show that washing with dilute sodium chloride solutions instead of water has this effect. Finally, if quantitative elution of the enzyme could be achieved, almost 30 % of it could be saved. Bamann *et al.* (1934) recommend alkaline autolysis at room

temperature for 3–4 days without any antiseptic. In our experience this always yields a heavily infected product, and while the enzyme seems to escape we prefer to elute it under more controlled conditions. It is possible that by using digitonin, digitalin or saponin as recommended by Matlack & Tucker (1940) for the elution of muscle esterase, a more efficient elution might be effected.

Esterase content of extracts. Since the method of Bamann *et al.* (1934), referred to above, gives higher yields of esterase than our technique, we have attempted to compare the esterase content of the extracts in order to get a clearer view of the esterases in the whole liver.

both these are continuous processes, no inflexion due to the solution saturating with respect to the second enzyme can occur, unless the solubility of the second enzyme in the precipitated first enzyme is so low that the solution saturates with respect to the second enzyme before it can be removed by its solution in the first. It therefore appears that there are two liver esterases which we will refer to in order of their solubility as liver esterases I and II until a more satisfactory basis is found for their characterization.

The similarity of the two curves in Fig. 4 suggests that the relative amounts of the two esterases present are similar.

Fig. 4. Solubility test of: A, esterases in extract prepared by method of Bamann *et al.* (1934); B, esterases in extract prepared by method described in this paper.

The enzyme precipitation curves in Fig. 4 show the presence of two esterases, one more soluble than the other. The presence of the two inflexions indicates that these two enzymes behave independently of each other in the solid phase. If the second and more soluble enzyme dissolved in the precipitate of the first to form a solid solution, an equilibrium between its solution in the liquid and solid phases would be set up. As ammonium sulphate was added after the solution had saturated with respect to the first enzyme, more and more of the second enzyme would pass out of solution in the liquid phase for two different and independent reasons. First, the amount of solid phase in which the second enzyme could dissolve increases, and secondly, the tendency of the second enzyme to precipitate would increase. Since

SUMMARY

1. A method is described of preparing a solution of pig liver esterase which is 10–15 times purer than whole liver.
2. The existence of two esterases in this extract is demonstrated.
3. An improvement in the technique of estimating small amounts of protein-N in solutions containing large amounts of ammonium sulphate is described.

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