DISCUSSION

Recent advances in molecular biology such as the recombinant DNA technology, the demonstration of intervening sequences in eukaryotic genes etc., have been possible through a continuous development of new techniques in nucleic acid enzymology. Among these are included the identification of various restriction endonucleases, single strand specific nucleases, nucleases cleaving DNA - RNA hybrids and DNA - RNA covalent conjugates etc. (145). In this context nucleases having unusual reaction requirements such as a relatively high optimum pH may also be important in that they may find use as biochemical tools in molecular biology research. The study of such enzymes is also interesting in view of the fact that the secondary structure of DNA is partially destabilised above pH 10.0 (130). It is therefore of further interest to identify the exact nature of the substrate (e.g. denatured or partially denatured) for such nucleases.

The experiments described in results under "Studies with crude midgut contents" demonstrate the presence of a nuclease activity in the larvae of *Spodoptera litura*, with a pH optimum of 10.5 as seen by the hydrolysis of native and denatured DNA.

Proteolytic enzymes of insect gut usually have a pH optimum in the neutral and alkaline region (146, 147, 75, 10, 125). Since the pH of the midgut fluid is also in the alkaline region it was reasonable to assume that most digestive enzymes in the insect may be active in the high alkaline range. The nuclease of Galleria larvae appears to be no exception. It is possible that a variety of nucleases ranging from pH optimum of 8.0 to 11.0 may be present in the gut as in the case with protease activity (87). The shoulder at pH 8.0 in the pH activity profile (Figure 2) may be indicative of at least one more nuclease activity. However, the nuclease activity with pH optimum of 10.5 must predominate in the midgut contents. The hydrolysis of denatured DNA was consistently found to be faster than native DNA. As already discussed in results, one reason for this could be the generation of a larger number of termini on denaturation due to the presence of single stranded nicks in the double stranded molecule. However, the natural preference of the enzyme for single stranded DNA cannot be ruled out. It may be mentioned that the pupal nuclease of the silk worm Bombyx mori prefers native DNA to denatured DNA (148). At pH 10.5 any nuclease as well as the DNA

substrate can be expected to be at least partially destabilised in their secondary and tertiary structures. It was therefore of interest to study the structural features of the nuclease and to define the nature of the substrate with respect to its secondary structure. Purification of the nuclease activity active at 10.5 was therefore undertaken.

Purification and Characterization of Larval Alkaline Nuclease

The larval nuclease was purified using affinity chromatography on Heparin-agarose. Heparin is a polyanion and many proteins which bind to DNA, possibly through electrostatic interactions, also bind to heparin. The procedure involved a preceding step of ammonium sulphate fractionation. The protein fraction precipitated between 30-40% ammonium sulphate saturation was used for affinity chromatography. On the heparin-sepharose column most of the proteins and a considerable amount of nuclease activity did not bind to heparin and was therefore eluted in the wash. Almost all the protein bound to the column was a nuclease active at pH 10.5. The nature of the unbound nuclease activity is known and was not explored any further. Presumably it contains additional nucleases or degradation products

of the purified nuclease, which might still have retained enzyme activity. The latter possibility can be reasonably ruled out by the fact that the purified enzyme was found to be refractory to the action of trypsin (to be discussed later). The purified enzyme binds to heparin fairly tightly since it was eluted with a mean sodium chloride concentration of 0.4 M. This fraction was concentrated and used for the characterisation of the enzyme. On polyacrylamide gel electrophoresis on native gels a single band was observed which also corresponded to ribonuclease and deoxyribonuclease activity. SDS gel electrophoresis of the purified nuclease, in order to determine its sub-unit structure if any, was not successful. 5-15% SDS polyacrylamide gels were tried. Presumably the high carbohydrate content of the enzyme drastically impairs SDS binding with protein, resulting in an insufficient negative charge per unit mass of protein, thereby not allowing the protein to enter the gel (129). Influence of the primary structure of proteins on SDS binding has also been reported and highly charged protein molecules bind insufficient amounts of SDS per gram of polypeptide for it to be electrophoresed.

In agreement with the results obtained with crude extracts, the purified enzyme showed an optimum pH of 10.5; however, the purified enzyme showed no activity below pH 8.0. This is in contrast with the observations made with crude extracts and provides further evidence that the digestive juice may contain other nucleases active at lower pH values. Activity versus enzyme concentration and incubation time profile again suggested the preference of the nuclease for denatured DNA (Figures 11 and 12). The effect of temperature on enzyme reaction also shows this effect (Figure 13), where optimum temperature with both substances was found to be 55°C. However at temperatures above 55°C the rate of hydrolysis of both native and denatured DNA was about equal. That the enzyme actually prefers denatured DNA is suggested by this observation, since presumably at pH 10.5 and at 55°C the secondary structure of native DNA is sufficiently disrupted to enable the enzyme to hydrolyse both substrates with an approximately equal rate. This interpretation is further substantiated by the experiments of figures 15 and 16, where the strand separation of native DNA has been suppressed by covalent cross-linking of the strands and high ionic

strength. The extent of denaturation caused at 60°C and at pH 10.5, however is not understood. The melting temperature of RNA at pH 10.5 did not show significant lowering. Therefore one possibility could be the denaturation at the ends of the molecules under these conditions. This could make available to the enzyme a similar number of termini as denatured RNA and can therefore account for similar rates of hydrolysis at 60°C.

The glycoprotein nature of the nuclease and its high carbohydrate contents also account for its stability at high temperatures. The nuclease also hydrolyses RNA with a rate approximately equal to that of native RNA. The RNase activity is also stimulated by Mg^{++} and has an approximate Km similar to that with RNA. The molecular weight of the nuclease is 18,200, as determined by gel filtration and is quite close to that of the larval nuclease of Bombyx mori which was found to be 22,000 (102). In this latter insect the pupal nuclease which is active at pH 9.0 has a molecular weight of 86,000 and is immunologically similar to the larval nuclease.

Mode of degradation of denatured DNA by the nuclease suggested the production of oligonucleotides as the major final products of hydrolysis. Further the enzyme was characterised as an endonuclease. The production of a small amount of mononucleotides was also indicated. These observations are in agreement with those recorded for the B.mori larval nuclease (149, 150). Further characterisation of the oligonucleotide products was not made. However the silk worm larval nuclease has been reported to produce di - and trinucleotides terminating in 5'-phosphates.

Among the many purified and well characterised nucleolytic enzymes, quite a few have been identified as glycoproteins. Among these are the bovine and ovine pancreatic deoxyribonucleases (151, 152). Single strand specific nuclease from mung bean (153) and Aspergillus oryzae nuclease (154). Since the Spodoptera litura larval nuclease exists in an environment which contains a number of proteolytic enzymes with high activity, it was suspected that the nuclease must possess some special structural features which prevent it from proteolysis. This idea is confirmed by the refractory nature of this nuclease to proteolysis by trypsin. The glycoprotein nature of the enzyme is

demonstrated by its binding to Con-A sepharose and the superimposition of the elution profile of the enzyme, with that of carbohydrate from Sephadex G-150 column. Therefore, to our knowledge the larval nuclease is the first nuclease from insect sources to be demonstrated as glycoprotein. In addition the most striking feature of this aspect is the high carbohydrate content which was estimated to be upto 50%. The mung bean and S_1 single stranded specific nucleases on the other hand contain 2% and 10% carbohydrate respectively. The high carbohydrate content possibly affords the nuclease protection in an highly proteolytic environment. Resistance to proteolysis in vitro of carbohydrate protein conjugates has been noted (141). An important prediction of this line of reasoning would be that all digestive enzymes in the insect gut should be glycoproteins. This remains to be tested. It should be noted that most extra cellular enzymes are considered to be glycoproteins (155).

Further studies on the larval nuclease, such as changes during the development of the larvae, its relationship with the mid gut tissue nuclease of the

larvae and the pupal nucleases, and the transport of the enzyme from the midgut tissues to the lumen are of interest. Because of its unusual properties such as high alkaline pH range and its glycoprotein nature, this enzyme is not only of fundamental interest but has potential as a tool in DNA sequencing and other structural work on nucleic acids. This latter aspect has already been demonstrated with the silk worm larval nuclease (156).