CCLIX. THE EFFECT OF ENZYMES ON THE PATHOGENICITY OF THE ROUS AND FUJINAMI TUMOUR VIRUSES.

By ANTOINETTE PIRIE.

From the Medical Research Council's Field Laboratories, Mill Hill.

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THE effect of various enzymes on the pathological action of viruses has been investigated frequently. Glover [1931] found that trypsin had no effect on the virulence of contagious pustular dermatitis of sheep, Woodruff and Goodpasture [1929] obtained the Bollinger bodies of fowl pox in an active state by tryptic digestion of the tissue, and Jojkin and Vinson [1931] have found the virus of tobacco mosaic resistant to emulsin, pepsin and yeast extract, while it was inactivated by trypsin, pancreatin and papain. Fresh untreated tobacco juice was not affected by any enzyme. Wollmann [1925] found both trypsin-resistant and trypsin-sensitive phages, and Arnold and Weiss [1926] found that a phage was present in commercial pancreas preparations and that one phage was resistant to 4 % trypsin. Hirano [1931] carried out experiments with vaccinia and found that takadiastase and a commercial preparation of trypsin had no effect on the activity of the virus. He found that a fresh glycerol extract of pancreas inactivated it and concluded that lipase was the enzyme responsible. A low p_H of itself inactivated vaccinia so that the action of pepsin could not be investigated. Baker and McIntosh [1927] were able to inactivate the virus of the Rous sarcoma by treatment at p_H 8.0 with a commercial trypsin preparation. At p_H 6.0 they found that the same preparation had an activating effect on the virus. Sugiura [1932] also found that pepsin and trypsin inactivated the virus of the Rous sarcoma while takadiastase, Soy bean urease and castor oil bean lipase did not. He therefore concluded that the proteolytic enzymes were responsible for the inactivation. On the other hand Fränkel [1931] obtained tumours with trypsinised Rous cells and filtrates. In those experiments in which the source of the enzyme used has been stated it has always been a commercial preparation. Since these are mixtures, the results obtained by their use cannot be used to draw conclusions on the chemical nature of the virus in question. It is only by the use of separate enzymes that their true effects on the virus can be studied. In a previous paper [1931] I reported that the virus of the Rous Sarcoma No. 1 could be purified by adsorption until the infective dose contained only a very small amount of nitrogen. Similar results were obtained by Maschmann [1931] and by Lewis [1931]. The latter considered that the Rous virus could be obtained free from protein by this method. In an attempt to decide whether this was really so and if the nitrogen that remained in the purest preparations of virus obtainable by this method was then either extraneous to the virus or else peptide-nitrogen, I have investigated the action of separate proteolytic and other enzymes on the activity of the virus. I considered that if I found that the activity of the virus was not affected by any proteolytic enzymes I could draw no conclusions from the result, since it would be possible for the

virus protein to be quite resistant to their action; if, on the other hand, the virus was inactivated by a proteolytic enzyme, then the conclusion might be drawn that a protein or peptide was a constituent of the virus itself. Since Baker and McIntosh and Sugiura found that the virus was inactivated by trypsin mixtures I first investigated the action of Merck's pancreatin upon it.

Preparation of separate enzymes.

The method of separation of the enzymes in pancreatin has been described by Willstätter and his collaborators. They are conveniently summarised in Grassmann's book [1928] and the technique described there has always been followed. The preparation and estimation of the carboxypeptidase is described by Waldschmidt-Leitz [1929]. The sample of Merck's pancreatin which has been used contains an active protease, carboxypeptidase, amylase and lipase, but no aminopeptidase or dipeptidase. Waldschmidt-Leitz and Schaffner [1926] found that these latter enzymes were destroyed by treatment with acetone and ether. It is possible that these enzymes were destroyed during the preparation of pancreatin since acetone and ether are often used in drying the pancreas.

Estimation of enzymic activity.

Protease. 1.0 cc. of enzyme and 3.0 cc. of 5 % caseinogen (B.D.H.) were incubated at p_H 8.0 for 30 minutes at 37°. The increase in amino-groups was measured by formaldehyde titration with $N/10$ NaOH using phenolphthalein as an indicator. An unincubated mixture was titrated as a control.

Carboxypeptidase. The activity of the carboxypeptidase was tested by measuring the hydrolysis of chloroacetyltyrosine. 1.0 cc. of enzyme plus 1.0 cc. (sometimes 5.0 cc.) of 0.01 M substrate were incubated at p_H 7.2 for 30 minutes at 37°. The increase in formaldehyde titration was measured with $N/10$ NaOH using bromothymol blue as an indicator.

Aminopeptidase. The activity of the aminopeptidase was tested by measuring the hydrolysis of leucylglycylglycine.

Dipeptidase. The activity of the dipeptidase was tested by measuring the hydrolysis of glycylglycine.

Amylase. 1.0 cc. or less of the enzyme was incubated with 2.0 cc. of soluble starch and the achromic point was noted using iodine as an external indicator.

Lipase. 1.0 cc. of enzyme and 5.0 cc. of olive oil emulsion at p_H 8.0 were incubated for 30 minutes at 37°, and the increase in fatty acids was estimated by titration with $N/10$ NaOH in the presence of alcohol. Phenolphthalein was used as an indicator. The control was an incubated mixture of boiled enzyme and oil.

Papain. The time of liquefaction of 5.0 cc. of 6.0 % gelatin by 1.0 cc. of enzyme was found.

The effect of enzymes on the agents of the Rous and Fujinami tumours.

The tumour filtrates were all made by filtration through paper-pulp and sand according to the method of Gye and Andrewes [1926]. In all experiments, unless it is otherwise stated, equal volumes of the enzyme solution and of the tumour filtrate were mixed and incubated at 37° for two hours, and the mixture was then injected into chickens in 1.0 cc. or 0.5 cc. doses. The controls were mixtures of boiled enzyme solution and filtrate, also incubated at 37° for two hours. No buffers were added to the solutions, but the p_H was adjusted by the addition of dilute acid or alkali. No p_H shift occurred during incubation.

Table I shows the effect of fresh extracts of pancreatin on the activity of the agents of the Rous tumour No. 1 and of the Fujinami tumour grown in a hen and transplanted into a duck, and grown in a duck and transplanted into a hen. Gye and Purdy [1931] have shown that the Fujinami virus extracted from a fowl

tumour contains some substance which is neutralised by antisera to hen embryo. This substance is not present in the virus from a duck-grown Fujinami tumour. It seemed possible that an enzyme might destroy the hen substance in such a way that the virus was still able to produce a tumour in ducks though not in hens. The results show that after treatment with pancreatin the activity of the virus is inhibited in both susceptible animals. These experiments confirm the work of Baker and McIntosh and of Sugiura on the virus of the Rous sarcoma. Experiments were done with glycerol extracts of pancreatin since the separate enzymes are obtained in glycerol solution and it is known that glycerol has a stabilising effect on the virus of the Rous sarcoma [Maschmann, 1931].

Table I. *Effect of fresh pancreatin on tumour-producing power.*

In this and all other tables the number of crosses denotes the size of the tumour at the death of the bird. Four crosses represent a tumour of maximum size and one cross in brackets a tumour the size of a pea. All birds were killed one month after injection. The usual age of the hens was between three and six months. Very young ducks were used as the tumour regresses in older birds [Gye and Purdy, 1931].

No. of bird	Control	Pan- creatin	Tumour	Enzyme preparation
(a) Ducks				
654	XX	Neg.	Fujinami	5 % pancreatin in saline
671	XXX	Neg.	Fujinami	5 % pancreatin in saline
746	XXXX	Neg.	Fujinami	5 % pancreatin in saline <i>plus</i> 20 % glycerin
(b) Hens				
655	XX	Neg.	Fujinami	5 % pancreatin in saline
659	XXX	Neg.	Fujinami	5 % pancreatin in saline
656	XXX	X	Fujinami	5 % pancreatin in saline
669	XXX	(X)	Rous	5 % pancreatin in saline
765	XXXX	X	Rous	5 % pancreatin in saline <i>plus</i> 20 % glycerol
763	XXXX	Neg.	Rous	5 % pancreatin in saline <i>plus</i> 20 % glycerol
673	XXXX	Neg.	Fujinami	5 % pancreatin in saline <i>plus</i> 20 % glycerol
672	XXXX	(X)	Fujinami	5 % pancreatin in saline <i>plus</i> 20 % glycerol

A glycerol extract of pancreatin was then made according to the directions of Grassmann and adsorption was carried out at p_H 4.0 with alumina B in order to remove lipase. The neutralised residue contained active protease, carboxypeptidase and amylase. Usually there was also a little lipase. It was found that the enzymes contained in this fraction of pancreatin had no effect on the tumour-producing power of the filtrates. The activity of the enzymes was tested by the methods already outlined earlier in the paper and was found to be almost as great as that of the original pancreatic extract. The effect of another proteolytic enzyme was therefore tested. Papain at p_H 5.0, 6.0 and 7.0 did not inhibit tumour formation. At p_H 6.0 the same result was obtained using papain after activation with 1/1000 HCN. A 10 % extract of B.D.H. papain was so active that it caused haemorrhages in the muscles of the hens and it was necessary to kill them. All tests were then done with 1.0 % extracts.

The action of that fraction of the original pancreatin which was adsorbed at p_H 4.0 by alumina B was next investigated. The alumina was washed with glycerol and eluted with a mixture of ammonium phosphate and ammonia in glycerol and water according to Grassmann's method for the separation of lipase. The alumina was centrifuged off and the supernatant was neutralised. Glycerol to a concentration of 60 % was added to stabilise the lipase. Table II shows that the enzyme which destroys the tumour viruses is in this fraction of the pancreatin. In order to determine whether lipase was the enzyme responsible

Table II. *Effect of second fraction of pancreatin on tumour production.*

No. of bird	Control	Enzyme	Tumour	Activity of enzyme preparation			Conc. of enzyme by volume %
				Caseinogen cc.	Chloro-acetyl-tyrosine cc.	Olive oil (+ CaCl ₂) cc.	
524	XXX	(X)	Fujinami	0.8	0.55	3.9	50
525	XXXX	(X)	Fujinami	0.8	0.55	3.9	50
526	XXX	(X)	Fujinami	0.8	0.55	3.9	50
512	XXXX	X	Rous	0.85	0.3	0.5	50
514	XXXX	X	Rous	0.85	0.3	0.5	50
511	XXXX	XX	Rous	0.85	0.3	0.5	50
	XXX	X	Rous	0.85	0.3	0.5	50
573	XXXX	XX	Fujinami	None	None	0.5	50
	XXX	(X)	Fujinami	None	None	0.5	66
569	(X)	—	Fujinami	None	None	0.5	75
	XXX	XX	Fujinami	None	None	0.5	50
571	XXX	X	Fujinami	None	None	0.5	66
	XXX	—	Fujinami	None	None	0.5	75

for inactivation, the effect of calcium on the action of pancreatin was investigated. The hydrolysis of olive oil by pancreatin was increased about five times by the addition of calcium chloride, but the concentration of pancreatin required just to inactivate a given tumour filtrate was not affected by the addition of this salt. This seemed to point to some other enzyme than lipase as the inactivator of the viruses. A further test of the action of lipase was made with pancreatic juice. Pancreatic juice collected from the duct contains active lipase but very little active protease before treatment with enterokinase. A sample was obtained from a dog and its action on the tumour viruses determined in the usual way. Neither before nor after activation with enterokinase was any inhibition obtained. The concentration of protease, carboxypeptidase and lipase in the activated pancreatic juice, however, was as great as in the 5 % pancreatin extracts which regularly inactivated the viruses. It is probable that the inactivating enzyme is contained in too low a concentration in freshly collected pancreatic juice to be demonstrable using the very active pulp and sand filtrates of the tumours. The experimental results obtained seem definitely against the view that the protease, carboxypeptidase or lipase in pancreatin is responsible for the destruction of the tumour viruses.

In a further study of the inactivating enzyme it was found that it rapidly disappeared from pancreatin extracts kept at 0°. The extracts were usually inactive after 7 days in the ice-chest. The addition of enterokinase does not restore the inactivating power. The proteolytic enzymes in extracts of pancreas are known to be stable whereas Willstätter and Waldschmidt-Leitz [1923] found that pancreatic lipase is unstable in solutions. This was confirmed with the pancreatin extracts used in this work. In view of the parallelism between the loss of inhibiting power and loss of lipase activity of the extracts it would be tempting to consider lipase as the inactivating enzyme, but the fact that pancreatic juice, which contains an active lipase, fails to destroy tumour-producing power is irreconcilable with this idea. It seems that an unknown enzyme is responsible for the destruction of the tumour viruses.

From these results one cannot decide whether the nitrogen remaining in the purest obtainable preparations of the tumour viruses is a constituent of the virus or not. It is possible that virus protein is resistant to the action of protease and carboxypeptidase, or the enzyme present in pancreatin extracts which destroys them may be a proteolytic enzyme of a different type.

SUMMARY.

The inactivating effect of preparations of dried pancreas on the agents of the Rous and Fujinami tumours has been investigated. A separation of the inactivating enzyme has been attempted but its identity has not been established. It is adsorbed by alumina B at p_H 4.0 together with lipase but it does not seem to be lipase. The protease and carboxypeptidase which are present in dried pancreas preparations are also inert. The inactivating enzyme is unstable at 0° in glycerol solutions.

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