CLXXI. STUDIES ON THE ANTINEURITIC VITAMIN.

II. THE PROPERTIES OF THE "CURATIVE" SUBSTANCE.

By JOHN LEWIS ROSEDALE
AND CHRISTOPHER JOSEPH OLIVEIRO.

*From the Biochemical Department, King Edward VII College
of Medicine, Singapore.*

(Received September 3rd, 1928.)

IN preparing extracts of the water-soluble vitamin from rice polishings, we noticed that, unless precautions were taken, alcoholic fermentation readily occurred at laboratory temperature. This fermentation was due to the presence of yeasts, and while yeast has the power of synthesising at least one of the water-soluble vitamins, it has been shown that its growth in wort, or in cultures containing substances which are possibly akin to these vitamins, is superior to that obtained on purely synthetic media [Nelson, Fulmer and Cessna, 1921; Harden and Zilva, 1921].

It was decided, therefore, to ascertain whether fermentation had any deleterious effect upon our extracts.

When our original extract was fractionated by lead acetate, we observed that the filtrate, which contained the curative factor, alone underwent fermentation. The precipitate portion could not be induced to grow yeasts. During the progress of our experiments a paper by Kon and Drummond [1927] appeared, in which they show that the activity of sacroclastic enzymes is not diminished in the polyneuritic pigeon, which, these authors state, is not in accord with the experiments of Farmer and Redenbaugh [1925]. The inference drawn by Kon and Drummond that vitamin B is associated only with protein metabolism, and bears no relationship to the metabolism of fat and carbohydrate, appears at variance with the evidence of Rando and Simmonet [1924], and of Plimmer, Rosedale and Raymond [1927].

At the time of the arrival of Kon and Drummond's paper, our first series of experiments was giving evidence of deterioration of our extract by fermentation, so that it became of interest to ascertain whether any reason for metabolic disturbance could be obtained from our birds.

Although Plimmer and Rosedale [1922] investigated the distribution of enzymes in the alimentary canal of the normal chicken, we could find no such data for the normal pigeon, so that we were obliged to secure this in order to

compare with pathological cases. Our investigations have been extended to human cases. Our data seem to indicate a difference between normal and beriberi cases as regards the pancreas where we have noticed the absence of trypsin and lipase. Our extract has been similarly examined for enzymes, and while our active (unfermented) preparation invariably shows marked lipoclastic action, we have been unable to establish the presence of proteoclastic enzymes.

EXPERIMENTAL.

Our extracts were prepared according to the procedure of Rosedale [1927], by extracting rice polishings in 1 % acetic acid at 40° for 8 hours, filtering, and concentrating *in vacuo* at 40° to such a volume that 1 cc. corresponds to 1 g. of the original polishings. This extract was then treated with lead acetate until no further precipitate could be obtained, when it was filtered. This filtrate was subsequently concentrated to the original volume and constitutes the extract which is the subject of the experiments reported in this paper. The extract can be kept from fermenting for some time in our animal house, which is a cool building, shaded from the sun for the greater part of the day. In the laboratory, where the average temperature is 30°, fermentation usually sets in after standing for 24–36 hours.

1st series. On June 22nd four pigeons were started on a white rice diet which was given *ad lib.* and in addition received daily 5 cc. of our extract. On the same day three pigeons suffering from typical polyneuritis were cured by a 5 cc. dose of this batch of extract. This extract was kept in the laboratory and fermentation was evident on the following day. On July 15th it was clear that the appetite of the birds had fallen, and their food was scattered about the cage. On July 25th all the birds showed signs of weakness and only one could mount to the perches. One bird died on July 30th, a second and a third on Aug. 8th, while the fourth was chloroformed on Aug. 19th.

2nd series. On Aug. 1st two birds suffering from polyneuritis were cured by 5 cc. each of a fresh batch of extract, 500 cc. of which were set aside in the laboratory and allowed to ferment. On Aug. 8th four pigeons were placed on a diet of white rice. Plimmer, Rosedale and Raymond [1927] have shown that results similar to those obtained in the 1st series may occur when feeding vitamin extracts in too small a proportion in the diet. In view of this and also of the fact that three of the birds in our 1st series had already died, it was decided to give 10 cc. of the fermented extract daily to each bird. The scattering of food was first recorded on Aug. 15th and food consumption was greatly reduced after Aug. 20th. The first death occurred on Sept. 2nd and the succeeding ones on Sept. 4th, 5th and 10th. *Post mortem* examination showed the dropsical condition in the third bird, while the others exhibited the dry condition. The organs of these birds as well as of the last bird in the 1st series were used for the study of enzymes.

These series indicate a loss of vitamin from the fermented extract.

The subsequent series were planned to ascertain whether sterilisation of the extract, which prevented fermentation, would have any effect on the vitamin content. Fresh extracts were first tested in 5 cc. doses upon birds suffering from typical polyneuritis. These proved the extracts to be capable of curing. The extracts were then sterilised either by filtration through a Berkefeld filter or by heating at 95° for 5 minutes. The sterilisation was kindly carried out for us by Prof. W. A. Young in the following way. The filtered extracts were prepared by filtration through previously tested Berkefeld filter candles into heat-sterilised flasks, which were subsequently plugged with sterile cotton wool. The heat-sterilised extracts were sterilised in previously heat-sterilised flasks plugged in the usual way.

Both extracts were distributed into flasks in quantities sufficient for one dose so that a fresh and sterile dose was given on each occasion and any danger of aerial contamination from using a previously opened flask was avoided. Before using, sterility tests were carried out on each batch of extract.

(a) Aerobic cultures. Samples were inoculated into nutrient broth tubes, into glucose broth and litmus milk, and on to agar and Sabouraud's agar slants.

(b) Anaerobic cultures. Samples were inoculated into Robertson's meat medium.

Extracts were used if all test media were sterile at the end of 48 hours, but the tests were kept under observation up to 14 days. No growth was found to occur in any test medium which had not appeared at the end of 48 hours. One batch only was found to be contaminated with aerobic sporing organisms, and was discarded.

3rd series. On Sept. 1st eight pigeons were placed upon white rice, four receiving daily 10 cc. of the filtered extract, and the remaining four 10 cc. of the heated extract per bird. All these birds died within 34 days of the commencement of the experiment, and the results are shown in Table I.

Table I.

Date of death	Nature of extract fed	Results of <i>post mortem</i> examination
Sept. 19	Filtered	Dropsy
23	Filtered	Dropsy
24	Heated	Dry, enlarged heart
24	Heated	Dropsy
26	Heated	Dry, enlarged heart, yellow liver
30	Filtered	Dry, enlarged heart, yellow liver
Oct. 2	Filtered	Dropsy
4	Heated	Dropsy

The only case of typical head retraction occurred in the last bird on Oct. 2nd. A dose of 5 cc. of a fresh extract effected a temporary cure of the typical condition, but the bird died on Oct. 4th.

4th series. Opportunity was taken of repeating the 3rd series. On June 27th, 1928, eight birds were again placed on a white rice diet. Four each received daily 10 cc. of the filtered extract, and four 10 cc. of the heated extract.

These doses were from batches of extract sterilised by Prof. Young in Aug. 1927, but although they had stood (plugged) at laboratory temperature for many months they showed no visible fermentation or deterioration. The first bird died on July 17th and the last on July 29th. No case of the typical head retraction was observed in this series.

Sterilisation of the extract by filtration and by heat destroys the vitamin.

Experiments with the enzymes of the alimentary canal.

The methods employed followed closely those of Plimmer and Rosedale [1922] and are consequently limited to qualitative detection. The various organs were taken from birds as soon after death as possible and several experiments were made with both normal and pathological pigeons. The crop and intestines were cut open and well washed. The mucous membrane was scraped off, ground with sand and extracted with water. The secretion from the proventriculus was placed directly into water and filtered. The pancreas was ground up with sand and extracted with glycerol. After extraction, water was added and the sand filtered off.

As substrates we used for diastase and invertase 10 cc. of a 1 % starch solution and a 1 % sucrose solution respectively. Two portions of each were measured into separate flasks, a known volume of tissue extract being added to one and an equal volume after boiling and cooling to the other to serve as control. After incubation, tests were made for starch with iodine and for glucose in the sucrose series.

Lipase. Two equal portions of olive oil were placed in separate flasks and made just alkaline to phenolphthalein. Tissue extract was added to one and an equal volume of boiled extract to the other. The discharge of the colour from the flask containing unboiled extract, in distinction to no change of colour in the boiled, was taken as an indication of the presence of lipase.

Proteoclastic enzymes were detected by their action on Congo red fibrin in acid, alkaline and neutral media. Similar volumes of extract and weighed quantities of fibrin were used in the experimental and control tubes. An equal volume of dilute acid or alkali or distilled water was added to each tube. Toluene was employed as preservative. Enzyme action was judged after incubation by the solution of the fibrin. The controls did not show any solution of the fibrin.

Table II.

	Crop		Proventriculus		Pancreas		Intestine	
	Normal pigeon	Poly-neuritic pigeon	Normal pigeon	Poly-neuritic pigeon	Normal pigeon	Poly-neuritic pigeon	Normal pigeon	Poly-neuritic pigeon
Diastase	+	+slow	0	+slow	++	++	+	+
Invertase	0	0	0	0	+	+slight	+	+slight
Lactase	+slight	0	0	0	0	0	0	0
Lipase	.	.	.	.	+	0	.	.
Proteoclastic in neutral media	0	0	0	0	+slight	0	+slight	0
Proteoclastic in acid media ...	0	0	++	++	+slight	0	0	+slight
Proteoclastic in alkaline media	0	0	0	0	++	0	+	0

The results (Table II) with the normal pigeon agree closely with those of Plimmer and Rosedale for the chicken. The polyneuritic pigeons, which were taken from the series of feeding experiments described above, appear to show differences. In each case sucroclastic enzymes were present. In no case have we been able to observe any tryptic action in the pancreas. In the intestine no proteoclastic enzyme has been detected except in the case of the bird from our 2nd series which died on Sept. 5th. In this case we found a slight hydrolysis of the fibrin in acid medium. The pancreas of this bird also exhibited lipoclastic action, though in no other case were we able to demonstrate the action of lipase in the pancreatic extracts. This bird on *post mortem* examination showed the wet or dropsical condition.

We had the opportunity of carrying out similar experiments with the human pancreas, from both normal and beriberi cases. While trypsin and lipase were readily demonstrated by our methods in the normal cases, neither was observed in the beriberi cases which were reported to us as being of the dry type.

Enzymes of the extract of rice polishings.

Owing to the observation of alcoholic fermentation and the presence of yeasts we have made no further experiment with sucroclastic enzymes, but in view of the results with beriberi animals we have attempted to ascertain the presence of proteoclastic enzymes and of lipase. As already stated, we have not been able to show the presence of the former, but lipase has been found to be present. The technique was similar to that employed for tissue extract and we used 20 cc. in each experiment as enzyme solution, each experiment being controlled by an equal volume of boiled extract. In no case did any of our controls show any sign of action.

DISCUSSION.

Our results indicate that during polyneuritis, metabolic disturbances occur involving a certain amount of inactivation of the pancreas. In the "dry" cases we have found failure of both proteoclastic and lipoclastic enzymes, although our methods are unable to show any deterioration of sucroclastic enzymes. Since lipoclastic enzymes appear to be inactive, and as our diet of white rice alone contains but little protein, we cannot conclude with Kon and Drummond that vitamin B bears relationship only to protein metabolism. Plimmer, Rosedale and Raymond [1927] found that the balance between vitamin B and protein was more difficult to demonstrate than that between carbohydrate or fat and vitamin B, but the experiments of Reader and Drummond [1926] leave no doubt that protein is similarly affected.

From our general results we consider that the curative substance has at least some control over the action of pancreatic enzymes. Work in progress by one of us on the bile of polyneuritic animals prevents the conclusion at the moment, however, that the curative substance itself is enzymic.

SUMMARY.

1. The antineuritic vitamin of an extract of rice polishings is destroyed by fermentation and by sterilisation.
2. The potent curative extract of rice polishings contains sucroclastic and lipoclastic enzymes, but it has not been possible to show the presence of proteoclastic enzymes.
3. In cases of "dry" beriberi, the pancreas has been found incapable of lipoclastic and tryptic digestion.

We should like to take this opportunity of thanking Prof. Young for so kindly carrying out the sterilisation of the extracts for us.

REFERENCES.

- Farmer and Redenbaugh (1925). *Amer. J. Physiol.* **75**, 27, 45.
Harden and Zilva (1921). *Biochem. J.* **15**, 438.
Kon and Drummond (1927). *Biochem. J.* **21**, 632.
Nelson, Fulmer and Cessna (1921). *J. Biol. Chem.* **46**, 77.
Plimmer and Rosedale (1922). *Biochem. J.* **16**, 23.
Plimmer, Rosedale and Raymond (1927). *Biochem. J.* **21**, 913.
Randoïn and Simmonet (1924). *Bull. Soc. Chim. Biol.* **6**, 601.
Reader and Drummond (1926). *Biochem. J.* **20**, 1256.
Rosedale (1927). *Biochem. J.* **21**, 1266.