VIII. THE DIFFERENTIAL BEHAVIOUR OF THE ANTINEURITIC AND ANTISCORBUTIC FACTORS TOWARDS ADSORBENTS.

BY ARTHUR HARDEN AND SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

(Received February 7th, 1918.)

ALTHOUGH we have as yet no definite information about the chemical identity of the antineuritic principle, several of its important properties have been disclosed by the researches of the last decade. Its behaviour towards certain adsorbents is an interesting feature. Chamberlain and Vedder [1911] observed that the antineuritic substance was removed by filtering through bone black. More recently Seidell [1916] has found that a pure preparation of fuller's earth, known as Lloyd's reagent, will also adsorb it and if used in suitable quantity will remove the active principle quantitatively, becoming at the same time activated itself. Williams and Seidell [1916] employed this procedure in order to isolate the active principle from autolysed yeast. They obtained a substance which was identical with adenine and which retained a certain degree of antineuritic activity for some time. Pure adenine from yeast has been shown however by Voegtlin and White [1916] and ourselves [1917] to be inactive. It is therefore probable that the adenine obtained by Williams and Seidell was only associated with the antineuritic factor in yeast in the same way as nicotinic acid has been shown to be associated with it in the extract of rice polishings [Barger and Ewins, quoted by Barger, 1914, p. 112; Drummond and Funk, 1914]. Both when purified become inactive. It is seen therefore that the adsorption procedure has not yet enabled us to isolate the looked for substance.

Very little is known about the chemical and physical nature of the antiscorbutic substance present in many plants and fruit juices, which is capable of preventing and curing scurvy. We know that it is not identical with the antineuritic factor, since substances showing marked antineuritic activity are antiscorbutically inactive and vice versa. There is also evidence that

the antiscorbutic factor is more thermolabile than the antineuritic. It was our original intention to study the behaviour of the antiscorbutic towards adsorbents and in the event of adsorption to study its chemical nature. We have however found that unlike the antineuritic substance it showed under the conditions which have hitherto been employed no tendency to be adsorbed by the materials which removed the former. This differential behaviour of the two active principles towards adsorbents suggested to us the possibility of separating the two when present in the same solution. For want of a natural substance which would contain both in marked quantities we have mixed equal quantities of autolysed yeast and orange juice, the former being antineuritically very active, the latter rich in the antiscorbutic. We then found that we were able by means of fuller's earth quantitatively to remove the antineuritic substance, leaving the antiscorbutic unchanged. The description of the above experiments forms the object of this communication.

EXPERIMENTAL.

Mention was made above of Seidell's work on the adsorption of the antineuritic factor by fuller's earth and we have taken the opportunity of repeating the experiments. Being unable to procure Lloyd's reagent we made use of ordinary fuller's earth. Brewer's yeast after having the wort pressed out was autolysed at 37° and filtered through a Buchner funnel. The filtrate was then shaken for 15 minutes with fuller's earth (50 gms. of fuller's earth to 1 litre of the autolysed yeast), centrifuged, and the supernatant liquid decanted, this treatment being carried out three times. The resulting autolysed yeast fluid was tested curatively and protectively.

Exp. 1. The following experiment describes the action of the autolysed yeast after treatment with the adsorbent on a polynuritic bird.

Pigeon E showed retracted neck and general paralysis. Received 5 cc. of the treated autolysed yeast. Next day very slight improvement noticeable. On the third day the bird had a relapse and a further 15 cc. was administered. As no improvement was noticeable during the day another 10 cc. was given at 3 P.M. on the same day. The bird was found dead next morning. All the doses were delivered direct into the crop by means of a stomach pump.

The above experiment shows that the autolysed yeast had been entirely inactivated by treatment with fuller's earth.

Exp. 2. For the protective experiment a batch of autolysed yeast was divided into two portions. One portion was treated with fuller's earth as described above while the other was left untreated. Four pigeons, H, I, K,

and *L*, were used. All the birds were fed artificially with 80 gms. of rice a day in two portions of 40 gms. each. In addition the birds *H* and *I* received daily by means of the stomach pump 3 cc. of untreated autolysed yeast, while *K* and *L* were dosed with 3 cc. of the treated autolysed yeast. Fig. 1 gives a graphic representation of the changes of weight of the individual birds.

Fig. 1.

It is seen that while *H* and *I* did well on the treatment, even putting on weight, *K* and *L* gradually lost weight, the former succumbing after 46 and the latter after 21 days to polyneuritis. The bird *H* having injured itself had to be killed, while *I* was alive and well at the time the experiment was stopped.

The above experiments fully confirm Seidell's observations.

We have further observed that the active principle is also adsorbed when autolysed yeast is treated with dialysed iron.

Exp. 3. Our procedure was as follows. 200 cc. of autolysed yeast was shaken with 200 cc. of dialysed iron, and the precipitated iron compound was filtered and pressed with a hand-press almost to dryness. The mass was then shaken with a litre of 95 % alcohol for 3 hours, the alcoholic extract

filtered and evaporated in vacuo at 45–50° and the residue taken up in 30 cc. of water. 10 cc. of the solution cured a polyneuritic pigeon. The following are the particulars of the case.

Pigeon 188 showed very grave symptoms of polyneuritis, convulsions and retracted neck. 10 cc. of the solution was given per os in the morning; gradual improvement during the day. Next day the bird was almost normal and received another 10 cc. of the extract. The following day the pigeon was quite normal and walked and flew freely. It was then put on a rice diet and developed another attack of polyneuritis seven days after the cure.

We have also observed that when autolysed yeast is treated as described above the filtrate still retains some activity. There is however little doubt that by treating the autolysed yeast with a suitable quantity of dialysed iron the entire content of the antineuritic principle could be removed as in the case of fuller's earth. We have not however as yet pursued the subject any further on these lines.

It has already been mentioned that our adsorption experiments with antiscorbutic solutions were primarily undertaken with the object of isolating or concentrating the active principle. The adsorbents that were able to remove the antineuritic factor from active solutions suggested themselves as suitable substances for the removal of the antiscorbutic. We therefore employed dialysed iron and fuller's earth for the purpose.

Exps. 4 and 5. 120 cc. of dialysed iron was added to 60 cc. of orange juice, which as is well known is a strong antiscorbutic, and shaken for some time. The precipitated iron was then filtered, pressed almost to dryness and shaken with 95 % alcohol for about 2 hours, the alcoholic extract being then filtered and concentrated in vacuo at a temperature of 30–40°. One third of the extract, which was equivalent to 20 cc. of the original orange juice, was used as a daily dose for an animal. The treatment with the fuller's earth was very similar. 10 g. of fuller's earth was employed for 60 cc. of orange juice, and after being shaken, filtered, and pressed out, was extracted with one litre of 95 % alcohol containing 0.5 % of citric acid for 2 hours. The alcoholic extract was concentrated in vacuo at 30–40° and, as in the case of the dialysed iron, one third of the quantity was used as a daily dose. Although 5 cc. of orange juice is known to be ample as a preventive dose in a guinea-pig we thought it advisable to employ the equivalent of 20 cc. in case the adsorption should turn out to have been incomplete.

We tested the antiscorbutic potency of our extracts on guinea-pigs. Holst and Frölich [1912] in their classical work have shown that if guinea-pigs

are fed on certain diets they develop a condition which is pathologically identical with human scurvy. Oats, bran and water prove to be a very suitable diet for producing experimental scurvy in guinea-pigs and we have accordingly adopted it. In some of our later experiments, in view of the results obtained by Chick and Hume [1917], autoclaved milk was substituted for the water. We found that a daily ration of 50 cc. of autoclaved milk kept the pigs in a better condition but at the same time did not prevent the onset of

Fig. 2.

scurvy. If guinea-pigs are fed on this diet they commence to drop in weight about 14 to 21 days from the commencement of the experiment and eventually die within a month. At the post mortem a pathological picture very much the same as that of human scurvy is disclosed. Subcutaneous and intramuscular haemorrhages, the latter specially pronounced in the thigh region, are well in evidence. Subperiosteal haemorrhages are also to be observed. The costochondral junctions in the ribs are abnormally enlarged

and haemorrhaged. The bones are very brittle and the teeth are loose. If the scorbutic diet is supplemented with an antiscorbutic, scurvy does not develop and the animal continues its growth in a normal way. For instance, a few cc. of orange juice given in addition to the oats, bran and autoclaved milk will keep a guinea-pig in excellent condition for months.

Our extracts were administered daily per os by means of a syringe, thus insuring quantitative consumption of the dose.

Fig. 8.

The vertical dotted line indicates the date at which the experimental treatment commenced.

Figs. 2 and 3 represent the experiments with dialysed iron and fuller's earth extracts of orange juice.

These curves show the weights of the guinea-pigs employed, two for each experiment. In both cases the animals dropped in weight as they do on a purely scorbutic diet and eventually died within the usual period. At the post mortem they all showed a condition of acute scurvy. The administered extracts neither prevented nor even delayed the onset of scurvy and we are bound to conclude that they were inactive.

The above experiments do not however entirely exclude the possibility of total or partial adsorption of the antiscorbutic by the adsorbents, as the solvents used, i.e. 95 % alcohol and acidified alcohol, may not have been suitable for extraction or the adsorption may have been of an irreversible character. To settle this point it was necessary to determine the activity of the solutions after being treated with the dialysed iron and fuller's earth.

For this purpose filtrates of orange juice treated with dialysed iron and fuller's earth respectively, according to the method already described, were tested. The method for the determination of the antiscorbutic potency of a substance is purely biological, and quantitative estimations cannot be carried out with the same degree of accuracy as are chemical estimations. There is very little doubt however that marked differences in the antiscorbutic content of solutions can be ascertained. For instance, we know that in the case of orange juice 3 cc. per day is about the minimum dose [Chick, Hume and Skelton, 1918] that will prevent the onset of scurvy in a guinea-pig and if an extract which is equivalent to 3 cc. of the original juice serves the same purpose we are safe in assuming that the loss of the antiscorbutic potency in the extract, if loss there be, is not very considerable.

Exps. 6 and 7. It was on these lines that we examined our filtrates. Figs. 4 and 5 show the weight curves of the animals employed.

Guinea-pigs 50 and 52 (Fig. 4) received orange juice treated with dialysed iron. Nos. 65 and 63 (Fig. 5) were dosed with orange juice extracted with fuller's earth, while No. 59 A (Fig. 5) served as a control for 65 and 63, receiving 3 cc. of untreated orange juice. None of the animals developed scurvy, showing that the solutions were active. It is further seen from Fig. 4 that guinea-pig No. 50, receiving an equivalent of 3 cc. of dialysed iron extract, did almost as well as No. 52, receiving 8 cc. As was previously mentioned, 3 cc. of ordinary orange juice is about the minimum preventive dose for a guinea-pig; consequently the antiscorbutic in the juice, if adsorbed at all, could not have been removed by the dialysed iron to any considerable extent.

The experiments with orange juice treated with fuller's earth gave similar results. No. 63, which received 3 cc. of the extracted juice, shows a weight curve almost identical with that of No. 59 A which received the same quantity of untreated orange juice. The juice in both cases emanated from the same batch. No. 65 received 8 cc. of the treated orange juice and developed somewhat better than the others. It must be mentioned here that all these animals were chloroformed at the close of the experiment and at the post mortem examination showed no signs of scurvy.

The above experiments show that the antineuritic and antiscorbutic factors behave differently towards the same adsorbents in their original media. It was of interest to see whether this differential behaviour held also true when the two principles were present in the same medium. We have so far not come across a natural product which contained both in quantities suitable for investigation and we therefore decided to make up a solution which would contain both in ample quantities and test it before and after adsorption.

Fig. 4. The first vertical dotted line indicates the date on which the animals were put on a scorbutic diet; the second the date on which the experimental solutions were first given.

Exp. 8. A mixture of autolysed yeast and orange juice was therefore employed.

The yeast solution and the orange juice were mixed in equal quantities. One portion of the mixture was treated with fuller's earth three times; the other portion was employed untreated. Each portion was tested curatively and protectively for the antineuritic on pigeons and protectively for the antiscorbutic on guinea-pigs.

As would be expected, the untreated mixture proved effective in curing an acute case of polyneuritis; the mixture treated with the adsorbent however

failed to show any antineuritic potency. The following protocol describes two cases of polyneuritic birds dosed with the treated mixture.

Pigeon C_2 showed paralysis and forward head. Received 10 cc. of treated mixture at 4.30 P.M. per os. Next day the bird was worse and another 10 cc. was administered. On the third day the bird was in a much worse condition and died at 2.30 P.M.

Fig. 5.

Pigeon D_2 was found in convulsions showing general paralysis with its head well forward. It received 10 cc. of the treated mixture at 10 A.M. No improvement during the day. Found dead the next morning.

For the protective experiment 10 cc. of the mixture, which was equivalent to 5 cc. of the original autolysed yeast, was administered daily to pigeons fed

on an exclusive diet of polished rice. Four pigeons were employed. Pigeons A_2 and B_2 received the untreated, while pigeons A_1 and B_1 were dosed with the treated, mixture. A_1 and B_1 developed polyneuritis within 33 and 45 days respectively. A_2 and B_2 on the other hand showed no signs after 50 days. Fig. 6 represents the weight curves of these birds.

It will be seen that the birds in this experiment behaved very similarly to those in the experiment with autolysed yeast alone. Both from the curative and protective experiment it is evident that the treatment with the fuller's earth has inactivated the mixture as it did the unmixed autolysed yeast.

Fig. 6.

To test the antiscorbutic potency of the mixtures four guinea-pigs were employed. One received 6 cc. (equivalent to 3 cc. of orange juice) of the untreated mixture, the other three received 6 cc., 10 cc. and 12 cc. (equivalent to 3 cc., 5 cc., and 6 cc. of orange juice) respectively of the treated mixture. As will be seen from Fig. 7, all the animals developed normally. At the end of two months the guinea-pigs were chloroformed and at the post mortem they were all found in excellent condition without revealing any signs of scurvy. The treatment of the mixture then with fuller's earth has not affected its antiscorbutic potency to an appreciable extent.

We have performed one more set of experiments which had some bearing on the behaviour of the antiscorbutic principle towards adsorbents, namely the effect of filtration of an antiscorbutic through a porous Berkefeld filter. Expressed orange juice contains much suspended matter and is not a solution easily amenable to chemical manipulation. Filtration through filter paper is slow and the resulting filtrate is not very clear. When the juice however is passed through a Berkefeld filter by means of suction a brilliant, clear filtrate is obtained. It was of interest to ascertain whether any of the antiscorbutic had been adsorbed during the process of filtration.

Exp. 9. An experiment was instituted in which three sets of two guinea-pigs each received 3 cc., 5 cc., and 8 cc. of filtered and unfiltered juice respectively. Unfortunately the animal receiving the 3 cc. of the filtered juice succumbed to an unknown complaint 40 days after the experiment commenced. The post mortem did not reveal any signs of scurvy. The animals receiving 5 cc. and 8 cc. of filtered juice failed to develop scurvy and behaved

very much in the same manner as the controls which were given the same quantities of the unfiltered juice. They were all chloroformed and no signs of scurvy were found at the post mortem. Fig. 8 gives a graphic representation of the experiment. These experiments show then that filtration through a porous medium like a Berkefeld filter does not affect the antiscorbutic potency of orange juice.

Fig. 8.
U, unfiltered F, filtered

CONCLUSIONS.

The experiments we have described show that under certain conditions at least the antineuritic factor behaves differently towards adsorbents from the antiscorbutic factor; the latter showing no tendency to be adsorbed by substances which remove the former from solution. We have shown that it is possible to separate the antineuritic principle from our artificial mixture containing both factors. Whether this is possible under all conditions we are not at present in a position to say. It is well known that adsorption is regulated by many factors and the H ion concentration of the medium is a condition to be considered. To work this problem out in detail is fraught with many technical difficulties. However it is quite within the limits of our present technique to investigate the behaviour of these accessory factors towards adsorbents in media differing in reaction in a marked degree and we are at present engaged in an investigation on these lines.

SUMMARY.

1. The authors have confirmed the fact that fuller's earth adsorbs almost quantitatively the antineuritic factor out of autolysed yeast.
2. Dialysed iron also adsorbs the antineuritic factor present in autolysed yeast.
3. Treatment with fuller's earth or dialysed iron does not affect appreciably the antiscorbutic potency of orange juice.
4. On treating a mixture of equal volumes of autolysed yeast and orange juice with fuller's earth the antineuritic factor is removed while the antiscorbutic activity remains unaltered.
5. Orange juice can be filtered through a Berkefeld candle without having its antiscorbutic activity vitiated to any appreciable extent.

REFERENCES.

- Barger (1914), *The simpler natural bases* (Longman's).
Chamberlain and Vedder (1911), *Philippine J. Sc.* (B), 6, 399.
Chick and Hume (1917), *J.R.A.M.C.*, 29, 121.
Chick, Hume and Skelton (1918), *Lancet*, 1, 1.
Drummond and Funk (1914), *Biochem. J.*, 8, 598.
Harden and Zilva (1917), *Biochem. J.*, 11, 172.
Holst and Frölich (1912), *Zeitsch. Hyg.*, 72, 1.
Seidell (1916), *U.S.A. Public Health Reports*, 31, 366.
Voegtlin and White (1916), *J. Pharm. Exp. Ther.*, 9, 155.
Williams and Seidell (1916), *J. Biol. Chem.*, 26, 431.