TRANSACTIONS

OF THE

Nova Scotian Institute of Science,

SESSION OF 1913-1914.

On the Existence of a Reducing Endo-Enzyme in Animal Tissues.

—By D. Fraser Harris, M. B., C. M., M. D., B. Sc., (Lond.); D. Sc., (Birm.); F. R. S. E., *Professor of Physiology and Histology* in the Dalhousie University, Halifax, Nova Scotia.

(Read 10 November 1913)

I. Historical.

It has for many years been recognized that both living and "surviving" animal tissues possess deoxidizing or reducing powers.

Hoppe-Seyler(1)in 1883 was the first to draw attention to the presence of powerful reducing processes in living tissues. He suggested that, through reduction, molecular oxygen was rendered active by conversion into nascent oxygen and thus enabled to oxidize certain constituents of tissues after the manner in which hydrogen-saturated palladium-foil can oxidize indigo.

Paul Ehrlich(2)two years later published his researches on the reducing powers of tissues during life and at the moment of death.

He classified tissues as regards their oxygen-avidity as follow:—

1. Those in which indo-phenol blue remains unchanged: these he regarded as saturated with oxygen. Examples; heart, renal cortex and the grey matter of the central nervous system.

2. Those which reduce indo-phenol blue to indo-phenol white, but not alizarine blue to alizarine white; Examples: striated and non-striated muscle, gland parenchyma.

3. Those which reduce alizarine blue to alizarine white, that is those with the greatest oxygen-avidity. Examples; lung, liver, fat-cells and the gastric mucosa.

Ehrlich injected the pigments subcutaneously *intra vitam*; he noticed that a certain degree of heat arrested the reducing-power, but he did not suggest that tissue-reduction was due to an enzyme.

Between 1888 and 1909 J. de Rey-Pailhade[3] wrote on a substance he called philothion which he regarded as one of the mercaptans and indistinguishable from cystëine. To this substance he attributed great importance in the fixation of oxygen by tissues.

Spitzer [4] in 1894 noticed that after the death of the animal, while the reducing powers of the tissues increased, the oxidizing capacity rapidly disappeared. He also noticed that the temperature of 100° C might not always destroy the reducing power, whereas it always destroyed the oxidizing.

In 1895 Sir Victor Horsley and A. Butler Harris[5] made a report to the Scientific Grants Committee of the British Medical Association on the appearance of tissues of animals injected subcutaneously *intra vitam* with methylene blue. In the milk and in the urine a leuco form was found. On faradization of the living cortex cerebri these workers demonstrated a state of reduction around the stimulated spot at a time when the blue coloration elsewhere was at its height. The

decoloration was not due to ionized hydrogen at the kathode, for when the cortical excitability had disappeared, the reduction of the pigment at a stimulated spot could no longer be obtained.

These workers therefore recognized the simultaneous activity of two processes oxidation and reduction, the precise colour at any moment being the result of the relative predominance of the one process over the other. Frequently they found that oxidation prevailed over reduction.

In 1896 I(5)*found that living tissues of cat and rabbit, —kidney, liver, heart, glands—reduced the blue potassio-ferric ferrocyanide in the Prussian blue and gelatine injection mixture to the green or white leuco state of the dipotassio-ferrous ferrocyanide which on exposure to air slowly, or by treatment with hydrogen dioxide rapidly, became blue again.

The pigment was reduced only in the washed out smaller vessels and capillaries; in presence of blood not washed out of the larger vessels, the Prussian blue remained unreduced. The colour of the blood was therefore a purple.

In 1899 the term "reductase" as indicating a tissue-ferment, capable of effecting reduction processes seems to have been first used by Abelous and Gerard.(7)

Pozzi-Escot(8)in 1902 published the results of work onthe reducing action of vegetable and animal tissues on solutions of indigo, litmus and Prussian blue out of contact with air. He confirmed Rey-Pailhade in finding that the tissues could form hydrogen sulphide from sulphur and could reduce potassium iodide when out of contact with air.

He held that a reductase might be suspected when a living tissue decomposes $H_2 O_2$, but does not affect a mixture of guaiacum and $H_2 O_2$.

---

*At this da te I had seen only Ehrlich's paper on oxygen avidity.

C. A. HERTER[9] in 1904 and 1905 published two papers on the reducing powers of living tissues. He injected methylene blue *intra vitam*. He stated that "the liver usually retains a high grade of reducing activity for several hours after death." He found lung, suprarenal capsule and grey matter of central nervous system all reduced the blue to the leuco state. An animal which was chilled by wet cloths or ice "exhibited the powers of reduction much diminished by cold". Herter showed that, conversely, the reducing power of the tissues of an animal injected with the micro-organisms of a specific fever was increased.

Underhill and Closson [10] in 1905 confirmed Herter's views and came to the conclusion that their experiments demonstrated the simultaneous action of both oxidative and reducing processes in the animal organism.

In 1906 Professor J. C. Irvine and I[11] showed that the *intra vitam* reduction of Prussian blue was not a deoxidation, but the removal of an ionic charge.

By perfusing the surviving kidney of a sheep with the Prussian blue mixture, I obtained from the ureter an absolutely colourless artificial urine which was blued immediately on treatment with $H_2 O_2$.

Authors with increasing frequency are recognizing the existence of reductase.

Oppenheimer [12], for instance in his large work on "Ferments" does so: most of the authors of text-books mention the reducing power of tissues even when they do not recognize "reductase".

Some, however, frankly postulate a reducing ferment; thus, G. P. Mudge[13] writes, "If an albino does carry a chromogenous body which only needs the influence of an oxidizing or reducing ferment to cause it to produce pigment", etc.

II. Materials used in Judging of Reduction by Tissues.

These may be classified as:—

1.(a)Those containing,and(b)those not containing oxygen.

II.(c)Those which are and (d)those which are not pigments.

A. *Pigments*: 1. Containing oxygen: haemoglobin; methaemoglobin; sodium-indigo-disulphonate.

2. Not containing oxygen: methylene blue; Prussian blue.

B. *Non-pigments*: 1. Those with oxygen, e. g., sodium nitrate.

2. Those without oxygen, e. g., ferric chloride.

III. Methods of Studying the Reducing Powers of Tissues.

All the following methods of bringing the pigments and other substances into contact with the tissues or tissue-juices, or other preparations of tissues have been tried: (a) immersing pieces of surviving organs in the test substances; (b) mixing the liquids with aqueous, saline or dilute glycerol so-called "solutions" of reductase; (d) injecting surviving organs with the Prussian blue and gelatine mixture, (e) perfusing this injection mass or, for instance, ferric chloride, through the vascular system of a surviving organ; (f) perfusing the blood-vessels, and obtaining in the case of the kidney, artificial urine, in the case of the liver, artificial bile.

As might be expected, the method merely of immersing pieces of tissue was by far the least satisfactory. No good results comparable with those got by Dr. Vernon ([14]) in the case of oxidase were obtained, but in this respect reductase resembles glycogenase, an undoubted endo-enzyme.

The routine method followed was to use the press-juice from a Klein's press. This was kept sterile under toluene. Its reducing power gradually declined in energy, until at the end of three months it had vanished.

Various extracts of organs were made—aqueous, saline and glycerol—but as their reducing power was considerably weaker than that of press-juice, these were not extensively used in examining the properties of reductase.

Injection of the Prussian blue and gelatine mixture into the blood-vessels of organs was not used on many occasions. It was, however, originally by this method that my attention was drawn to tissue reduction, as I suspected that the "fading" of the mixture in the capillaries of the parenchyma of liver and kidney was chemically of the nature of a reduction. This does not constitute a convenient method owing to the liability of the gelatine to "set" if the proper temperature is not maintained.

The revival of the blue colour in an injected and almost colorless kidney or liver cut open and exposed to the air or to the action of $H_2 O_2$, is striking when seen for the first time. The vessels on the cut surface begin to show up like letters written in "sympathetic" ink.

It was by this method that I obtained an artificial, gelatinous, leuco urine from the sheep's ureter: it became blue on treatment with $H_2 O_2$.

The method of injecting ferric chloride through the portal system and examining both the hepatic emergent fluid and the contents of the gall-bladder for ferrous chloride, in both of which it was found, proved a satisfactory method.

IV. Preparation of the Juice.

The following may be taken as typical of the technique. A liver removed from the animal (rabbit, cat, dog, pig) before the heat has left it, is perfused through the portal vein with tap water at 40° C or with 0.75% NaCl until the water from the hepatic vein is colorless. The organ is then rapidly cut into largish pieces from which a good deal of water is allowed to drain away. The pieces are then cut up into much smaller bits and forced into the juice-press in which they are crushed

under considerable pressure. A fawn coloured, viscid liquid drips out and is received under toluene. This juice is subsequently ground up with powered glass and filtered through two layers of cheese cloth to free it from connective-tissue and the debris of blood-vessels, etc. Some preventative of putrefaction must be used although any such substance reduces the energy of tissue-respiration.

V. Description of a Typical Observation.

Three cubic centimeters of *absolutely fresh* press-juice prepared as just described, were shaken in a test-tube with 10 c.c. of 0.05% solution of soluble Prussian blue at room temperature (about 17° C). The blue colour began to disappear immediately, and in less than a minute after passing through light blue, light green and greenish grey, the mixture became light grey in colour. No trace of pigment remained.

When the same volume of *boiled juice* was used, no decrease in the intensity of the blue colour of the solution was observed at the end of several hours. The reducing activity of the juice was found to diminish somewhat rapidly with time. With a mixture containing 3 c.c. of the press-juice 24 hours old, and 10 c.c. of 0.05% Prussian blue solution, it was found that ten minutes elapsed before its colour became green grey, and two hours before it became completely colorless, (grey).

VI. Examination of Possible Fallacies.

Since the change from the coloured to the leuco condition is the sign of reduction having taken place, one must guard against confusing the fading of pigments through reduction with fading from causes other than bio-chemical reduction.

(a) The earliest criticism offered was that the fading of the Prussian blue was due to the presence of "alkaline salts". Now free alkali, which undoubtedly fades Prussian blue, does not exist in the tissues or their juices. The inorganic salts of tissues and tissue-juices do not bring about any fading of soluble Prussian blue.

Ringer's solution added warm to Prussian blue produces no change of colour beyond that due to a corresponding dilution with water.

None of the salts of the tissues, NaCl, KCl, Na_2CO_3, $Ca_3\,2\,(PO_4)$, Na_3PO_4 in strengths under 1% solution added warm singly or in any kind of combination, caused any fading to the green or to the leuco condition, whereas the subsequent addition of such a reducer as pyrogallol at once caused fading through green to white.

When the gelatine and Prussian blue mixture is used to inject organs still living, the pigment is reduced, as I believe, by the agency of the living tissues; and histologists aware of this fading, attribute it to "contact with the alkaline salts of the tissues."

Thus Rawitz[16] recommends that a little acetic acid be added to the injection-mass to prevent the "fading" by alkaline tissues.

Naturally, this criticism applies only to pigmentary substances, and has no applicability to non-pigmentary salts used to demonstrate bio-chemical reduction.

(b) The next source of fallacy one must bear in mind is the possible putrefaction of the proteins of press-juice in in specimens of juice kept for more than a few days.

Toluene was the antiseptic used for all press-juices; some kind of antiseptic is absolutely necessary, although Battelli[23] has emphasized the inhibitory effect of antiseptics on the enzymic and respiratory powers of tissues. The antiseptic used had obviously to be one which would not of itself bleach or reduce the pigments or other substances and would not act as an activator or inhibitant of the enzyme. Sodium fluoride and many other substances had to be rejected on some of those grounds. Toluene apparently prevented putrefaction in the press-juices used. Had the reductions in old juice (two to six weeks old) been due to putrefaction or autolytic substances, then the reducing power should have

steadily increased with the age of the juice. But exactly the opposite was found, the longer the juice was kept under toluene the *less* it reduced until after ten weeks or so it did not reduce at all. But putrefaction would have been progressive, and therefore reduction due to putrefaction would have been *more* marked as time went on. I had, however, positive evidence of the absence of putrefactive micro-organisms in a specimen of liver juice three months under toluene, which was examined for me by Dr. Sholto Douglas of the University of Birmingham and pronounced sterile.

It seems clear, then, that the reductions studied were not brought about by the products of putrefaction or autolysis.

(c) As regards fallacies, another point to be remembered is that the substances employed—Prussian blue, ferric chloride, etc., are all more or less poisonous. We cannot, therefore, expect the living tissue to reduce unlimited quantities of such substances whether pigmentary or not.

Thus only the earlier portions of liquids emerging from perfused organs or being excreted into the gall-bladder or ureter should be examined for reduced material. Because a kidney perfused indefinitely long with ferric chloride does not continue to produce unlimited quantities of ferrous chloride is no evidence that it was not originally able to reduce some of it, for such substances, even in dilute solution, are more or less toxic to living protoplasm, especially in experiments in which that protoplasm is receiving no blood.

(d) The last criticism is that of A. Heffter ([17]) which is directed not so much against the methods of judging of reduction by the fading of pigments, as against the whole conception of tissue-reduction being enzymic in nature. Heffter holds that the labile H of colloids in such a grouping as cystëine is able to effect all the reductions observed. He says that crystallized egg-albumen can bring about many reduc-

tions. Heffter's contention is that proteins apart from life can actively reduce.

Confining ourselves first of all to Prussian blue, it is certain that all proteins do not cause this pigment to fade, at least within times measured by hours and at room-temperature. For one thing, gelatine itself without acid does not cause soluble Prussian blue to fade even before it is injected into an organ and even when heated.

It is well known that this injection-mass mixed with the blood-proteins in the large vessels of mammals at body temperature is not reduced or caused to fade. Neither is methylene blue; those pigments remaining blue produce along with the red of the blood *a purple* colour. If Heffter be correct, we should expect the blood-proteins to reduce these pigments to a pale green or leuco condition, this they certainly do not do.

If one mixes a saline solution of *pure* serum-albumen or serum-globulin with Prussian blue, no fading takes place at room temperature within 24 hours.

In 1912 my co-worker at that time, Dr. H. J. M.Creighton([18])of the Dalhousie University, Halifax, N. S., investigated this subject with very great care and published his results in the Transactions of the Nova Scotian Institute of Science.

Dr. Creighton showed that if one mixes 10 c.c. of a 15% solution of egg-white in dilute NaCl with 10 c.c. of a 0.05% solution of soluble Prussian blue (potassium ferric ferrocyanide) and keeps the mixture at 60° C the colour will have faded at the end of an hour. The fading is gradual. Dr. Creighton writes, "With pure white-of-egg at a higher temperature, the decoloration of the soluble Prussian blue was found to proceed with greater rapidity". On the other hand, white-of-egg solution and 0.05% Prussian blue mixed and kept at *room temperature*, showed no fading or change of colour at the end of six hours.

Dr. Creighton further showed that the iron ion originally trivalent in the soluble Prussian blue is divalent in the

colloidal complex of albumen and the pigment. There has therefore been reduction. Further, this colorless colloidal complex can be boiled for a short time without its coagulating. For convenience, I call these phenomena, "the Creighton effects". Now there is one significant difference as regards the interaction between proteins and soluble Prussian blue and the interaction between press-juice and that pigment, namely, that whereas there is no fading of the blue in the presence of protein at the end of many hours, the blue in contact with fresh juice fades *at once*. These are clearly not the same phenomenon; for one thing, in the case of the protein mixture the concentration of protein is very much greater than it is in press-juice, but its effect is very much slower.

Further, if the fading of the pigments is due to protein, then the juice kept for three months, in which the protein is well preserved and is sterile, should reduce as well or almost as well as fresh juice; but this is noticeably not so.

Again, the rapid falling off in potency as regards reduction within the first day would have no meaning as a phenomenon due to molecular groupings and labile hydrogen, whereas it has a meaning with reference to the deterioration of the biochemical activity of a ferment.

The fact that glycerol extracts of dried liver and of dried kidney possess some reducing power, is more in accord with the conception of that reduction being due to an enzyme than to a protein, for the glycerol extract of *dried* liver had some cognizable reducing power, and it could have taken up very little protein in "solution". Glycerol by itself has no reducing power.

Again, glycerol extracts deteriorate in potency with time for which there is no particular reason, if protein be the active substance. Blood at 40° C does not reduce ferric chloride, but liver-juice at this temperature reduces it to ferrous chloride. There are proteins in both. While giving due weight to Heffter's contentions, and indeed recognizing certain

phenomena of the fading of pigments in contact with proteins which I have called "the Creighton effects," I still believe that vital reduction is something distinct from these and is probably enzymic.

VII. Indications That a Tissue Endo-Enzyme exists

1. The first consideration regarding reduction being due to an enzyme is that, whereas quite fresh juice vigorously and older juice more gradually reduces several different kinds of chemical substances, boiled controls do not do so at all.

2. The behaviour of the juice in regard to temperature is the next point indicating the presence of an enzyme.

Its optimum is between 42° C and 46° C. Thus Herter found reduction processes were accelerated in the experimentally induced fever of hog cholera. As the temperature falls, the rate of reduction is diminished until *at zero* reduction is entirely *inhibited.* But at a temperature as low as minus 14°C, the reducing power is not destroyed; it is merely kept in check.

I have kept under observation a mixture of absolutely fresh liver-juice and Prussian blue, surrounded by a freezing mixture for 24 hours, without noticing the least degree of fading of the deep blue colour. On removing the tube from the freezing mixture, the colour was completely discharged by the time the juice had reached room-temperature (17°C).

Herter found in the intact animal that "the power of reduction was much diminished by cold."

A typical experiment may be quoted in connexion with temperatures.

Three water baths were brought to (a) between 40° and 41°C; (b) between 42°C and 43°C; and (c) between 44° and 45° C respectively. In each bath a tube was placed containing 3 c.c. of 24 hours old hepatic juice shaken up with 20 c.c. of Prussian blue all under toluene. In 6 hours the tube in (a) was green, that in (b) was green-white, the one in (c) was

quite white; twenty four hours later the tube in (b) was white. The behaviour of tissue-juice is compatible with its active constituent being an enzyme.

3. As judged by the Pozzi-Escot test, a reducing ferment is present in certain tissues; for pieces of tissue, but better their juices, decompose pure H_2O_2without affecting a mixture of guaiacum and H_2O_2.

That the press juice, for instance of liver, is more active than pieces of liver is in accordance with the findings of other workers on ferments. J. J. R. MacLeod([20])noticed this in the case of glycogenase, an undoubted endo-enzyme.

4. The reducing action is accelerated or augumented by the presence of alkaline salts of the tissues, which behave as adjuvants. Professor Irvine and I([11])concluded that reductase acted after the manner of pyrogallol, an organic reducer, in an alkaline medium.

5. In my recent work([21])on the action of protoplasmic poisons on reductase, I found that the acidity (concentration of H ions) was a more profound inhibitant of the reducing power than was toxicity. Concentration of H ions is well known as an inhibitant of the activity of certain enzymes; to this reductase would not form any exception.

The fact that reductase is not totally inactivated by certain virulent protoplasmic poisons—chloroform, sodium fluoride, nitrobenzene, formalin—makes reductase comparable with the ferment in the laurel leaf studied by Dr. Waller([24]). Chloroform was found to kill the leaf, but to set free an enzyme which liberated HCN.

6. As a ferment, reductase is pretty easily inactivated by drying the juice in vacuo at 15°C and by precipitation from juice by alsolute alcohol. As might be expected, drying and alcohol injure it less in tissues than in press-juice.

It clings with considerable tenacity to the cell-proteins, which evidently guard it from inactivation by heat, by drying and by alcohol.

In regard to its sensitiveness towards alcohol, reductase is in marked contrast with glycogenase, which can be obtained in an active state even from livers which have been for months under alcohol. This power that colloids have of protecting enzymes is a well-known property of the relationship between these two classes of bodies.

As judged by the criterion of solubility, reductase is comparatively insoluble; it will not, for instance, dialyze away from the cell-proteins. But in that it can in some measure pass into solution in dilute glycerol, it cannot be regarded as entirely of an insoluble nature

The insoluble endo-enzyme is now fully recognized. Professor Adrian Brown tells me that phyto-enzymes of a non-soluble order exist, and according to Vernon[22] the oxidase of the liver is insoluble. He adds that its insolubility does not preclude its enzymic nature, as there is a good deal of evidence pointing to a similar property in some lipolytic enzymes.

VIII. Remarks on Tissue Respiratory Ferments.

Besides reductase, at least two other types of respiratory enzyme exist in the liver, to confine our attention only to the liver in the meantime, namely a catalase and an oxidase or a number of oxidases. A catalase has long been recognised in the blood and tissues; Creighton and I[25] recently wrote:—

"The existence of a catalytic enzyme in the mammalian liver is fully confirmed. The decomposition of H_2O_2 is effected by this enzyme, and is not due to the presence of proteins or other organic matter in the press-juice."

Boiled juice gives rise to no decomposition of H_2O_2; and the amount of H_2O_2 decomposed bears no relation at all to the amount of protein in the juice, for a few drops of a very dilute juice reduced 97.2% of H_2O_2 in the first five minutes. No doubt it is possible that the two enzymes, catalase and reductase, may co-operate in hepatic reductions.

The presence of an oxidase, more probably of oxidases, must be remembered when one is working with the reducing ferment. As Dr. Vernon has shown, there are oxidases in the liver which must of necessity work in the direction opposite to that taken by the reductase.

Hence when we obtain a less distinct reduction than we expect, we have to remember that the oxidase may have been active. We have, in fact, the converse of the difficulty to which Dr. Vernon([14]) alluded when, investigating "The quantitative estimation of the indophenol oxidase of animal tissues", he wrote: "The unavoidable presence of reducing substances, some of which are possibly enzymes or reductases which act in direct antagonism to the oxidases, and under certain conditions entirely overpower them. Hence the absence of an oxidizing action cannot be held to indicate the absence of oxidase unless the conditions are so chosen to give the oxidase the best possible chance of exerting its activity."

At an early stage I had noticed that in a tube in which the Prussian blue had been completely reduced to the leuco state, a re-establishing of the colour was evident from about the end of the first week onwards. A mixture of fresh liver-juice shaken up with pigment of suitable strength would begin to become blue again in spite of the fact that the mixture was covered by a layer either of toluene or of oil to the depth of an inch.

In the routine observations, I made no attempt to eliminate the oxidase of press juice, but in one experiment Dr. Lovatt Evans and I definitely arranged to exclude the physiological activity of that ferment. Accordingly we kept a sealed up mixture of liver-juice and Prussian blue at room temperature under an atmosphere of pure hydrogen in a completely reduced state for three and a half months. It never showed the slightest re-blueing; on breaking open the tube and adding H_2O_2 the contents immediately became bright blue. Exposure to the air produced the same result more slowly. Evidently the activity of the oxidase was prevented expressing itself owing to there being no oxygen for it to deal with.

According to Spitzer, the vigour of oxidase declines post mortem, whereas that of reductase increases for a time, but it is possible that the former phenomenon is the cause of the latter, the increase in the energy of the reductase being only apparent and due to the diminution of that of the oxidase working in the opposite direction.

Dr. Vernon, ([14]) fixing his attention on the tissue-oxidases, regards reducing ferments as troublesome intruders into his experiments. I, however, am forced to recognzie oxidases as forming as much a part of the cellular, respiratory, enzymic mechanism as are reductases.

It is in this connexion significant that the Cannizzaro reaction—the simultaneous oxidation and reduction of aldehydes—has been observed most frequently with liver tissue in the presence of dilute sodium bicarbonate and oxygen.

Possibly the "aldehydemutase" of Parnas is not one enzyme but a mixture of oxidase and reductase([23]).

In some manner with which we are far from being fully acquainted, catalase, oxidase and reductase are all acting simultaneously in the living cells, carrying on the work of tissue-respiration. I have eliminated the activity of the oxidase for a sufficiently long time to allow the reductase untramelled activity; and conversely Dr. Vernon in his studies on oxidase has had to make due allowance for the presence of reducing substances.

Dr. Vernon and also Prof. B. Moore([25])have pointed out several respects in which oxidase differs from reductase.

It is perhaps too soon to formulate any theory of tissue respiration, but when the scheme is outlined it must be one taking cognizance of all the three respiratory types of enzymes and not a scheme framed in terms of oxidase alone.

Provisionally one might say that by reductase, oxygen is abstracted from tissue-lymph (more remotely from oxyhaemoglobin) and brought within the sphere of the activity of the oxidase which applies to it the oxidation of the carbon, hydro-

gen, sulphur, phosphorus, etc., in, or in the neighbourhood of, the living protoplasm.

With regard to haemoglobin, I have direct evidence that liver-juice can reduce this pigment from the fully oxidized two-banded condition to the fully reduced one-banded within a few minutes at 41°C. The quantities used were a test-tube full of solution of oxy-haemoglobin from freshly drawn defibrinated rabbit's blood, and three grams of freshly disintegrated liver squeezed before the animal heat had left it. This mixture was shaken up from time to time to distribute the juice, and within a minute or two, the solution had begun to lose its brightness which it steadily continued to do. The two bands in the spectrum became progressively hazier until at within ten minutes they had disappeared and been replaced by the single band of haemoglobin; shaking this pigment at once made the two bands re-appear; it was, therefore, reduced but still oxidizable.

A control, similar in all respects except that the juice was *boiled* for five minutes, showed no signs of being reduced at the end of 72 hours. This solution never did become reduced, but passed normally into the state of methaemoglobin.

A period of ten minutes may seem a long one in which to have to wait for reduction to haemoglobin, but we must remember that in vitro we have the entire mass of the solution finally fully reduced, while in vivo we never have the oxyhaemoglobin fully reduced in consequence of contact with the living tissues during only one transit of the blood. The blood is fully reduced only after the many transits in asphyxia.

I think, then, that we are justified in regarding the reductase as the respiratory ferment of the living tissues, the endoenzyme, through whose reducing power oxygen is split off from the oxyhaemoglobin in the several capillary districts

It would seem to be the ferment which starts the process of internal respiration, oxidase that which continues and completes it.

IX. The Chemical Powers of Reductase.

In conclusion I should like to point out the true reducing character of the reductase of animal tissues.

(a) In the first place it is a typical deoxidizer in that it removes oxygen from osmium tetroxide and from such substances as oxyhaemoglobin, which is fully reduced, and methaemoglobin, which is reduced to the oxy condition.

(b) Substances containing oxygen, but not in a form wholly removable, can be reduced from the higher to the lower state, as when sodium nitrate is reduced to sodium nitrite,[25] or when sodium indigo-disulphonate and sodium alizarine-sulphonate are respectively reduced to their pale chromogens.

(c) The reductase can also reduce metallic salts containing no oxygen from their higher to their lower forms, as when ferric chloride is reduced to ferrous chloride[15]. Here the change involved is the removal of an ionic charge from the trivalent ferri-ion which becomes the di-valent ferro-ion.

(d) Finally, certain pigments containing no oxygen such as soluble Prussian blue and methylene blue are reduced to the pale or white chromogenic conditions of the di-potassio-ferrous-ferrocyanide and methylene white respectively.

In all these reductions, the endo-enzyme is behaving after the manner of an inorganic reducing agent in an alkaline medium.

[The expenses of this research were met by a grant from the Government Grants Committee of the Royal Society, which is hereby gratefully acknowledged.]

BIBLIOGRAPHY.

7. Abelous and Gerard, Comp. Rend. Vol. CXXIX, p. 164, 1899.
23. Battelli Comp. Rend. Vol. CXXXVII, p. 1079, 1903. Do. Journ. de Phys., Vol. IX pp. 1,34,328,410,1907.
10. Closson, O. E. and Underhill F. P. Amer. Journ. Phys. Vol. XIII No. V., p. 358, 1905.
18. Creighton, H. J. M., Trans. Nova Scotia. Instit. Sc. Vol. XIII Pt. 2, 1912.
23. Dakin, H. D. Oxidations and reductions in the animal body. London Longmans, 1912.
2. Ehrlich, Paul. Das Sauerstoff-Bedürfniss des Orgniamus, Berlin, 1885.
6. Harris, D. Fraser, Proc. Roy. Soc. Edin. Vol. XXI p. 383.
6. Harris, D. Fraser, Jrl. Anat. and Phys. Vol. XXXI, p 381.
19. Harris, D. Fraser, Science Progress, No. 4, April, 1907.
21. do. Bio-Chem. Jrl. Vol. VI Pt. 2, p 200. 1912.
11. Harris, D. Fraser and Irvine, J. C., Bio-Chem. Jrl. Vol. I nos. 8 and 9, p. 355.
15. Harris, D. Fraser and H. J. M. Creighton, Bio-Chem. Jrl. Vol. VI, Pt. 4.
25. Harris, D. Fraser and H. J. M. Creighton, Proc. Roy. Soc. B. Vol. 85, 1912.
17. Heffter, A. Medizinisch-Naturwissenschaftliches Archiv. Bd. I. Heft. I. p. 81.
do. Archiv f. expr. Path. and Pharm. Festchrift. f. O, Schmiedeberg, p. 253, 1908.
1. Hoppe-Seyler, Berichte. d. deut. chem. Ges. Vol. CXVII, p. 1917, 1883.
5. Horsley, V and Harris, A. B. Brit. Med. Journ. Vol. II. p. 205, 1895.
9. Herter, C.A. Amer.Journ. Phys. Vol. XII.p. 128, 1904.
do. do. Amer. Journ. Phys. Vol. XII.p. 457,1905.
do. do. Proc. Soc. Exp. Biol. Med. Vol. II, 1905.

9 Herter, C. A. and Richards, A. N., Amer. Journ. Phys. Vol. XII, p. 207, 1904.

11. Irvine J. C. and Harris D. Fraser, Bio-Chem. Jrl. Vol. I Nos. 8 and 9, p, 355.

20. MacLeod, J. J. R. Amer. Journ. Phys. Vol. XXV. No. 5., 1910.

26. Moore, B and Whitely, E., Bio-Chem. Jrl., Vol. IV, nos. 3 and 4, p, 136, 1909.

13. Mudge, G.P. Proc. Phys. Soc. Vol. XXXVIII. p, 67.

12. Oppenheimer, C. Die Fermente und ihre Wirkung. Leipzig. Vogel. 1909.

8. Pozzi-Escot, Ann. Chem. Anal. Vol. VII, p. 260, 1902.

3 Rey-Pailhade J. de, Comp. Rend. Feb. June and July 1889.

Rey-Pailhade J. de, Bull. de la Soc; fr. de chim; pp. 165, 523, 1051, 1907.

Rey-Pailhade J. de, pp. 159, 568, 1908; p. 133, 1909.

16. Rawitz quoted in Physiological Histology, G. Mann, Oxford, p. 160, 1902.

4. Spitzer, Berlin, Klin. Woch. p, 949, 1894.

23. Stern and Battelli, Journ. de Phys., Vol. LX, pp., 1, 34, 228, 410, 1907.

10. Underhill, F. P. and Closson O. E. Amer. Journ. Phys. Vol. XIII No. 5, p. 358, 1905.

14. Vernon, H. M. Intracellular enzymes, pp. 135-144, London, Murray, 1908.

Vernon, H. M. Jrl. phys. Vol. XLII. Nos. 5 and 6, 1911.

24. Waller A. D. Proc. Phys., Soc. June 18, 1910. Jrl. Phys. Vol. XL.

22. Vernon H. M. Jrl.Phys. Vol. XLIV. No. 3, 1912.