ON THE EXISTENCE OF A REDUCING ENDO-ENZYME IN LIVER AND KIDNEY

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HISTORICAL

Hoppe-Seyler¹³ was the first to draw attention to strong reducing processes in living tissues. He suggested that by means of reduction molecular oxygen was rendered active by conversion into nascent oxygen and thus oxidised tissue somewhat after the manner of hydrogen-saturated palladium-foil which can oxidise indigo.

Paul Ehrlich⁵ two years later, in 1885, published his researches on the reducing-power of tissues *intra vitam* and at the moment of death. He classified tissues as regards their oxygen-avidity as follows:—

1. Those in which indo-phenol-blue remained 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, those with greatest oxygen avidity. Examples: lung, liver, fat-cells and the gastric mucosa.

Ehrlich injected the pigments subcutaneously *intra vitam*. He pointed out that in the case of naturally *red* organs—e.g., heart and spleen—it is difficult to appreciate degrees of blueness, so he plunged them into hot water; this makes them paler. Ehrlich noticed that heat arrested the reducing power. He did not suggest that tissue-reduction was due to an enzyme.

J. de Rey-Phailhade^{25, 26} wrote extensively between 1888 and 1909 on a substance which he called 'philothion,' which he regarded as one of the mercaptans and indistinguishable from cysteine. To this substance he attributed extreme importance in the fixation of oxygen by the tissues.

He thus represented the reactions involved :—

Rey-Pailhade further remarks :—‘ L’hydrogene philothionique que se transporte facilement sur certaines mati\`eres colorantes explique clairement les ph\`enomen\`es de r\`eduction observ\`es par Ehrlich *in vivo*.’

Spitzer²⁷ noticed that for a time after the death of the animal, while the reducing-power of the tissues increased, the oxidising capacity rapidly disappeared. He also noticed that the temperature of 100° C. might not always destroy the reducing-power, whereas it always destroyed the oxidising.

Victor Horsley and A. Butler Harris¹⁴ made a report to the Scientific Grants Committee of the British Medical Association on the appearance of tissue of animals injected subcutaneously *intra vitam* with methylene blue. The gastric mucosa at first secreted it blue, later greenish. In the milk and in the urine a leuco form was found. In the saliva, at first neither the blue nor the leuco form was found, later the blue appeared.

On faradisation of the living *cortex cerebri* these workers found a state of reduction at the stimulated spot at a time when the blue coloration was at its height. The resting warm tissue became blue. The decoloration was not due to ionised hydrogen at the kathode, for when the cortical excitability had disappeared the reduction of the pigment could no longer be obtained.

Frequently they found that oxidation prevailed over reduction; thus the pylorus was blue when active digestion was in progress, and the voluntary muscles were very blue whilst active, maximal oxidation being thereby indicated. Muscles with cut nerves showed a more intense blue than did normal muscles, minimal reduction and minimal oxidation being indicated as simultaneously existing. In 1896 I^{6,7} found that living tissues from the cat and rabbit—such as kidney, liver, heart, the 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 the air or by treatment with hydrogen peroxide at once 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 term 'reductase' as indicating a tissue-ferment capable of effecting reduction-processes was first used by Abelous and Gerard¹ in 1899.

Pozzi-Escot²³ published the results of work on the reducing action of vegetable and animal tissues on solutions of indigo, litmus and Prussian blue out of contact with the air. He held that a reductase may be suspected when living tissue decomposes hydrogen peroxide but does not affect a mixture of guaiacum and hydrogen peroxide. He confirmed Rey-Pailhade in finding that tissues could form di-hydrogen sulphide from sulphur, and could reduce potassium iodide when out of contact with the air. Oxidases, he said, are to be recognised by the blue coloration with guaiacum and by the fact that they re-oxidise phenol-phthalin to phenol-phthalëin 'so easily recognised by alkalies.'

C. A. Herter^{15, 16} published two papers on the reducing powers of the living tissues. He used Grübler's methylene blue dissolved in 0.85 per cent. sodium chloride and injected it into the jugular vein of the rabbit *intra vitam* under chloroform anaesthesia. He stated that 'the liver usually retains a high grade of reducing activity for several hours after death.'

During life the lungs, supra-renals and grey matter reduce methylene blue to the 'leuco base.' These are more powerful reducers than muscles, spleen, kidneys and connective-tissue. An animal which was chilled by wet cloths or by ice 'exhibited the power of reduction much diminished by cold.'

Herter showed that, on the other hand, increased tissue metabolism produced by experimentally induced fever (hog-cholera) was accompanied by a greatly increased reducing action towards methylene blue and indophenol. He found the post-mortem decline of reducing power in rabbit's muscle was very marked. He believed chloroform retarded the rate of reduction.¹⁸

F. P. Underhill and O. E. Closson²⁹ published the results of a research into the behaviour of methylene blue and methylene azure in the living organism. Their paper, which is mainly of chemical interest, confirms Herter in that 'the experiments demonstrate the simultaneous action of both oxidation and reduction processes in the animal organism.'

In conjunction with J. C. Irvine, of the Chemical Department, the University, St. Andrews, I⁹ showed that the *intra vitam* reduction of Prussian blue was not a deoxidation but the removal of an ionic charge. We insisted that the reduction of methylene blue to methylene white was

not a deoxidation, in spite of the statements of several authors, recently, for instance, that of Hill in 'The Body at Work'¹²:—'Methylene blue contains some easily removable oxygen.'

By perfusing the surviving washed-out kidney of the sheep with the Prussian blue mixture, I obtained from the cannulated ureter an absolutely colourless, gelatinous, artificial 'urine,' which was blued immediately on the addition of hydrogen peroxide. At the time these experiments were done I had not seen any of the papers by Herter or his pupils.

A. Heffter^{10, 11} has recently published papers on work done on the reducing power of tissue-extracts. He opposes the notion that the existence of a reductase is needed to carry out any of the reductions performed by living tissues or tissue-juices. He holds that the labile hydrogen of colloids in such a grouping as cysteine is able to effect all the reductions observed. Autoxidation is the more important phenomenon of tissue vitality, reduction an unavoidable concomitant. Crystallised egg-albumin can bring about many reductions.

G. P. Mudge²² has made a communication on the pigmentation of hairs as due to the interaction of a chromogen and a ferment. He thinks that the albino, for instance, has only the chromogen. Regarding fermentation as a process either of oxidation or reduction he thus writes: 'If an albino does carry a chromogenous body which only needs the influence of an oxidising or reducing ferment to cause it to produce pigment,' etc.; that is to say, this author recognises the functional existence of a 'reductase.'

EXPERIMENTAL METHODS

Preparation of the press-juices. I employed press-juice from perfectly fresh livers of the sheep and frog and from kidneys of the ox.

A Klein's press for tissue-juice was used.

The following will indicate the typical procedure followed to obtain fairly large quantities of the juice:—

The entire liver was without delay perfused with 0.9 per cent. sodium chloride solution in tap-water at a temperature of 40° C. through the portal vein until the fluid from the hepatic vein was bloodless. The mass was then allowed to drip suspended in a piece of muslin previously washed free from starch and was then cut up into small pieces which were pressed over thick flannel in the Klein's press. From a liver weighing about 1,000 grammes, 200 c.c. of press-juice could be obtained by this method,

The procedure for the ox kidney was similar.

The keeping of the juice. Obviously it is of the utmost importance to prevent the press-juice decomposing, as even traces of 'products of putrefaction' have intense reducing powers. Toluene and benzene were the only antiseptics I used. Battelli² has emphasised the inhibitory effect of antiseptics on the enzymic and respiratory powers of tissues. It may be assumed that antiseptics do, to some extent, inhibit enzymic action. Fresh juice well shaken with toluene and left under a layer of toluene will remain perfectly undecomposed and active for upwards of six weeks; but there is a progressive weakening of reducing power, until at the end of three months the juice is quite inoperative.

In all routine work I purposely refrained from using any saline or acid substance in case such might prove to be an inhibitor or an activator of the enzyme investigated. Sodium fluoride, for instance, I rejected on these grounds.

It is clear that the reductions observed in the following instances were not due to putrefactive or autolytic products, for had that been so, the juice would have acquired more and more reducing-power instead of less as time went on. After six weeks there is a falling off in the energy of reduction, and by ten to twelve weeks it has practically disappeared.

Hepatic juice three months under toluene was pronounced sterile after bacteriological examination by Dr. Sholto Douglas, to whom my thanks are due for having examined the material. At his suggestion I kept all juice expressed after December, 1909, in paraffin-lined flasks, as the alkalinity of glass is known to cause the activity of certain enzymes to diminish.

Pigments used in judging of reduction. Unless when otherwise stated, I judged of reduction, and therefore presumably of the presence of a reductase, by the change of colour or fading of the following three solutions kept at 41° C., to which press-juice had been added:—

1. Potasio-ferric-ferrocyanide (soluble Prussian blue) of 0.062 per cent. strength in distilled water.
2. Sodium-indigo-disulphonate (indigo blue) of 0.08 per cent. strength in distilled water.
3. Methylene blue of 0.0125 per cent. strength in distilled water.

Usually 10 c.c. of one or other of these solutions were mixed with 3 to 5 c.c. of the juice being investigated.

The above concentrations were arrived at after a number of trials to discover the most suitable strength for the coloured solutions, so that they would show within a few hours some degree of fading or change of colour in presence of 3 to 5 c.c. of juice. *Quite fresh* hepatic or renal juice will cause Prussian blue to fade within a minute or two at room temperature.

Certain characters of the press-juice. Examined under the microscope, no intact cells could be seen in either hepatic or renal juice. The juice was a highly granular, pinkish, opaque liquid filterable with extreme slowness through flannel; not filterable through paper.

It had at first a neutral reaction, but liver juice acquired an acid reaction (to litmus) on being kept for a few days.

On boiling, a massive heat-coagulum was obtained.

By saturating with magnesium sulphate it was possible to separate a cell-globulin from a cell-albumin.

The first thing to ascertain was whether 'raw' liver press-juice reduced any pigment to the leuco condition.

Five c.c. of the fresh liver juice were shaken up with toluene and added to 10 c.c. of dilute solutions—at that time of unknown strengths—of soluble Prussian blue, sodium indigo-disulphonate and methylene blue, respectively, and were thereafter placed in the thermostat at 41° C. A control in each case consisted of the filtrate from an equal volume of juice boiled for five minutes. In four hours the Prussian blue was seen to be of a very pale green colour, which was immediately restored to a deep blue by the addition of hydrogen peroxide. The indigo blue was entirely bleached within twenty-four hours, the methylene blue within the same time was rendered considerably paler. The filtrate from the heat-coagulated juice failed in each case to effect any degree of reduction whatever. The inference seemed justified that we were here dealing with an endo-enzyme not indeed isolated but dissevered from the intact hepatic cells and associated with the cell-proteins. It seemed capable (1) of reducing a pigment that contained oxygen, the indigo blue, (2) of reducing pigments that contained no oxygen (Prussian blue and methylene blue), and (3) it was destroyed at the temperature of 100° C.

The criticism at once occurred to me; might this change of colour not be 'bleaching due to the true presence of organic material.' If, for instance, white-of-egg be mixed with Prussian blue, the colour is discharged within twenty-four hours, and in a less degree indigo blue is bleached by egg-white. Similarly potassium permanganate is 'reduced'

to a colourless condition by organic matter presumably containing no enzyme whatever.

I therefore used for the experiment on this point fresh liver-juice in presence of toluene, and for the control an equal amount of heat-coagulated liver-juice (100° C.). Thus if the bleaching were due to the presence of organic matter, the control tubes ought to have shown as much or nearly as much reduction as the tubes with the unaltered juice. The result was that the tubes containing the boiled juice were scarcely affected. The mere presence of organic material was therefore not the active agent in bringing about the reduction, but this was presumably due to an endo-enzyme destroyed at the temperature of 100° C.

On the spontaneous re-blueing of the chromogen. At an early stage I 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 third or fourth day onwards. A mixture of fresh liver-juice shaken up with pigment of suitable strength would begin to become blue again, always beginning at the top, in spite of the fact that the mixture was covered by a layer of the lighter toluene to the depth of an inch. This I provisionally explained by assuming that the restoration of colour was due to the activity of an oxidase utilising atmospheric oxygen which had diffused through the supernatant toluene. Later I got quite independent evidence of the presence of an oxidase in the press-juice of both liver and kidney. To test this point, I arranged to keep fresh liver-juice in toluene in presence of some Prussian blue under an atmosphere of pure hydrogen, with the object of seeing whether one might thus permanently avoid atmospheric oxidation. The experiment was quite successful; the pigment remained in the leuco state for three and a half months. On breaking open the vessel and adding hydrogen peroxide, the contents immediately became bright blue. Exposure to the air produced the same result but more slowly.

Oxidative restoration of the pigment by a co-existing oxidase is therefore a factor to be borne in mind in these experiments on endo-enzymic reduction.

THE VARIATIONS OF REDUCING ACTIVITY RELATED TO VARIATIONS OF TEMPERATURE

These are characteristic of enzymic action and are well shown in the case of hepatic or renal reductase.

A typical experiment may be quoted:—

Three water-baths were brought to (*a*) between 40° C. and 41° C., (*b*) 42° C. and 43° C., and (*c*) 44° C. and 46° C. respectively. In each a tube was placed containing 3 c.c. of raw hepatic juice shaken up with 12 c.c. of Prussian blue under toluene. In six hours the tube in (*a*) was blue-green, that in (*b*) was white-green, the one in (*c*) was quite white; twenty-four hours later the tube in (*b*) was white. I frequently observed the acceleration in bleaching at 40° C. as compared with room-temperature (15° C.). The inference here is that we are dealing with an endo-enzyme the velocity of whose action is distinctly accelerated by a rise of temperature from 15° C. to 46° C.

Herter, using 'triturerated tissues' in presence of an aqueous solution of methylene blue, found a marked acceleration of reduction at 43° C.

The effects of cold. Taking the degree of reduction exhibited in a tube containing liver-juice and Prussian blue at 15° C. as the standard, I compared this with a similar mixture kept in an ice and salt freezing mixture for several hours. The contrast was very marked; there was no appearance of fading in any of the chilled tubes at temperatures varying from zero to — 14° C.

This marked inhibition by cold corroborates Herter's observation with methylene blue in the intact animal, 'the power of reduction . . . is much diminished by cold,' and 'a considerable fall in body-temperature is attended by a diminished reduction of methylene blue to leuco methylene blue, and this result is particularly striking and unequivocal in the case of the muscles including the heart and diaphragm and the grey substance of the central nervous system.'

ATTEMPTS TO DETERMINE THE 'DEATH' TEMPERATURE

The determination of the exact temperature at which reductase ceases to act is for many reasons a very difficult matter.

The volume of juice taken is a factor to be reckoned with. I took as standard the behaviour of 5 c.c. of raw hepatic juice kept in contact with 12 c.c. of Prussian blue for twenty-four hours at 41° C. In order to judge of the colour of the pigment unobscured by the presence of precipitates, heat-coagula, etc., the contents of the tubes were filtered and the depth of colour of the filtrate carefully scrutinised. A series of

these tubes was kept under observation, thus:—

- (a) for twenty-four hours at 41° C.,
- (b) for twenty-four hours at 70° C. to 73° C.,
- (c) for twenty-four hours at 80° C. to 83° C.

On filtering off the liquid present in each case, that from (a) was seen to be of a very pale green, that from (b) was blue-green, while that from (c) was almost a pure blue colour.

I had evidence that above 60° C. there was progressive inhibition of the energy of reduction. The destruction-temperature seems to lie between 75° C. and 80° C. Juice heated to temperatures above 80° C. did not adsorb the pigment so thoroughly as juice less highly heated. The general inference to be drawn from a large number of observations on the effect of heat on the enzyme is that the cell-proteins protect the reductase from the destroying effects of heat.

EFFORTS TO OBTAIN THE ENZYME IN SOLUTION

The typical method of extraction with alcohol was first carried out with a view to induce the ferment to pass into solution in distilled water. The results of this procedure were entirely negative. The liquid contained protein and yielded flocculi at 41° C., but it possessed no reducing power. The inference was that the alcohol had either destroyed the reductase in the raw juice or had rendered it insoluble in water, if it was originally soluble. To test the point as to possibility of the alcohol having destroyed the reductase, I omitted alcohol in preparing the aqueous extract thus—Some fresh liver juice under toluene was dried in vacuo at 17° C., while an equal quantity was dried at 100° C. over the water-bath. Both portions were extracted by prolonged grinding with sand in distilled water. To a series of the three pigments a few cubic centimetres of the vacuo-dried extract were added and the tubes kept for eighteen hours at 41° C. A control series was made with the extract of the material dried at 100° C. In no tube of the six was there any reduction whatever. The inference here is that drying the fresh juice in vacuo at room temperature (and a fortiori at 100° C.) destroys the reductase and renders it insoluble in water or otherwise inactivates it. That it is destroyed is probable from the further fact that subsequent addition of some of the powdered vacuo-dried juice caused no reduction in any of the tubes within so long a time as three to four days.

Professor Adrian Brown kindly suggested to me the possibility of the endo-enzyme being originally insoluble, but capable of becoming

active on its being moistened in its inhibited condition in vacuo-dried juice. Accordingly I moistened some vacuo-dried liver juice with some distilled water and added the paste to Prussian blue kept at 40° C. There was no reduction until putrefaction set in. The inference seems to be that whether the reductase is originally soluble or insoluble, it is rendered profoundly inactive by desiccation even at ordinary temperatures.

The following conditions then seemed possible:—Either the reductase existed in the liver-cells and was subsequently in the press-juice in an insoluble state, but capable of reducing pigments in solution (treatment with alcohol or desiccation being highly injurious to it), or the reductase was originally soluble but so sensitive to alcohol or desiccation at any temperature—ordinary, medium or high—that it was rendered insoluble or inhibited or destroyed.

Proceeding on the assumption that the process of drying was injurious to the ferment owing to its being in some artificial condition in press-juice as compared with its natural state in intact hepatic cells, I dried small pieces of perfectly fresh liver on the water-bath at 40° C., while an equal weight was simultaneously dried at 100° C. Watery extracts of neither of these substances effected any reduction. The inference from these observations seemed to be that the reductase was originally insoluble in the liver cells and non-extractable by water or so bound to the cell-proteins that desiccation at a temperature of 41° C. rendered it insoluble or non-extractable by water. Accepting then the position that treatment with alcohol or by desiccation was equally inadmissible, I proceeded to study the behaviour of a relatively purer form of liver-juice than the raw juice as it comes from the tissue-press.

A sheep's liver was perfused as usual until bloodless, allowed to drip, pressed through flannel, and the juice thoroughly ground up in a mortar with fine sand and toluene. The fluid was then centrifugalised and filtered through two layers of a Schleicher and Schüll's filter-paper. A non-granular liquid came through with extreme slowness. A little of this liquid added to the pigments and kept at 41° C. caused them to assume a curious and characteristic appearance which I have since noticed on other occasions; I call it 'the white and blue appearance.' The whole interior of the tube is lined by the flocculent precipitate which has adsorbed the whole of the pigment, but on the side towards the lumen of the tube the precipitate is white, on the side next the glass it is blue. The inference from this seems to be that where the juice is in free contact with the maximum of water, there the reductase is under the maximum condition

for its activity, where the water is at a minimum the ferment is inactive. The reductase seems to cling to the cell-proteins and to be entangled and adsorbed by them when they are precipitated at 41° C.

To the extent that the proteins are soluble in normal saline the reductase is soluble along with them, but so far, I have not succeeded in obtaining a solution of the endo-enzyme apart from the cell-proteins.

Still avoiding both alcohol and drying, I wished to submit the press-juice to exceedingly thorough stirring with normal saline to induce the endo-enzyme to pass into solution if that was by any means possible. Two hundred c.c. of hepatic juice (sheep) were mixed with 200 c.c. of 0.75 per cent. sodium chloride and some toluene, and the whole was stirred by a glass stirrer driven by a water-motor for sixty hours. The stirred liquid was then filtered through a layer of paper over a porous porcelain vessel by an exhaustion-pump. The filtrate, which was yielded very slowly, was a rather viscous, clear brown liquid with the following characters:— It gave the xanthoproteic reaction at once; on saturation with magnesium sulphate a precipitate of cell-globulin was obtained; it reduced Fehling's solution very slightly; it gave no blue with guaiacum alone nor with hydrogen peroxide subsequently added. I allude to it as the 'exhaust filtrate.' Five c.c. of the exhaust filtrate gave with Prussian blue at 41° C. in a few hours the white and blue appearance. A similar mixture kept at air temperature presented the same appearance after a longer interval. The inference again is that the reductase was in intimate association with the cell-proteins which adsorbed the pigment.

I next tried to discover whether the ferment was associated with one cell-protein more than with another. On saturating with ammonium sulphate and filtering, the filtrate did not reduce Prussian blue at all, whereas the precipitated material did, the inference being that the reductase was entangled with all the proteins. This was corroborated by finding that on saturation with magnesium sulphate both the filtrate and the original saline precipitate reduced Prussian blue (with the white and blue appearance).

Thus the endo-enzyme seems to be able to associate itself either with the globulins or with certain other cell-proteins, but not to be able to free itself from some one or more of these cell-constituents. The degree of reduction as compared with that effected by the raw juice was less intense in the case of the various preparations referred to—filtrates, exhaust juice and saline precipitates and filtrates therefrom.

· ATTEMPT TO OBTAIN THE ENDO-ENZYME IN SOLUTION BY DIALYSIS

These laborious and numerous experiments were negative. I completely failed by dialysis through parchment tubes in tap water to induce any ferment to enter the water outside.

The inference from this is confirmatory of the finding as to the insolubility of reductase. This insolubility is in striking contrast with the high solubility in water of the catalase separated by Usher and Priestley³⁰ from the sheep's liver. A catalase capable of liberating oxygen from hydrogen peroxide was obtained from dried liver powdered and extracted with water, the solution being precipitated with excess of absolute alcohol. This catalase as compared with hepatic reductase is either very stable or insensitive to physico-chemical reagents.

REDUCTION WITH SUBSTANCES OTHER THAN THE THREE BLUE PIGMENTS

Fehling's solution is slowly but slightly reduced, as is also osmium peroxide: neither is a suitable substance for routine work.

To the use of ferric thiocyanate, ferric chloride, ferric sulphate and potassium permanganate there are objections of various kinds. They have all been used, but have not yielded results worthy of being recorded. As to organic substances: alkaline pyrogallol is unsuitable owing to the speed with which it becomes reoxidised in contact with the atmosphere.

Quinone. By both liver and kidney juice this substance is reduced to quin-hydrone, although not to the colourless hydroquinone. Probably the simultaneous presence of the oxidase prevented this.

I could obtain no evidence that either hepatic or renal juice could reduce sulphur to hydrogen sulphide after the manner of a ferment produced by yeast-cells.*

OBSERVATIONS WITH GLYCERINE EXTRACTS OF TISSUES

Under the impression that the reductase would more readily or perfectly cling to material of comparatively large molecule such as glycerine than to dilute saline solutions, I crushed a fresh blood-free liver (sheep) in glycerine and water (equal parts) and left the crushed material to soak for three days. Three c.c. of the filtered glycerine extract added to ten c.c. of dilute Prussian blue almost entirely bleached the

*According to unpublished information kindly communicated to me by Professor Adrian Brown.

pigment in eighteen hours: the surface layer remained blue. A few cubic centimetres of the reduced material were at once restored by hydrogen peroxide. From this I inferred that the glycerine had extracted both the endo-enzymes, the oxidase and the reductase. This was confirmed by my finding that on boiling the glycerine extract, it completely failed to reduce Prussian blue under the usual conditions, even after a few days' contact.

Methylene blue was reduced to a pale green by the glycerine extract of liver. I noted that the boiled, filtered, glycerine extract used as controls in these experiments had a great tendency to adsorb the respective pigments, though it did not reduce them.

The hepatic glycerine extract in a day or two became acid and smelt sour. Suspecting lactic acid, I neutralised the extract with sodium carbonate, and found that the neutral liquid reduced Prussian blue at 40° C. with the typical white and blue appearance. The previous reduction was thus not due to the presence of acid; and I found that at any rate fermentation lactic acid does not reduce Prussian blue.

Very similar results were obtained by using glycerine extract of crushed blood-free kidney (sheep). This extract reduced Prussian blue to a white, methylene blue to a pale green, indigo blue to a paler blue: this last-mentioned salt was the least affected. The behaviour of the boiled glycerine extract of kidney juice is rather interesting. Neither methylene blue nor indigo blue was reduced by it, but in each case the pigment was adsorbed. When, however, *boiled* glycerine extract was added to Prussian blue the colour was bleached by the third day: it was revived by hydrogen peroxide. Enough reductase had survived the action of the heat to effect a slow reduction but of Prussian blue only—the most easily reduced of the three pigments. To some extent therefore the glycerine shielded the endo-enzyme from destruction by heat.

The superiority of a glycerine over a saline extract was shown in the case of the sheep's liver. This had been cut into pieces and dried at 40° C. six months previously. It was then extracted with ether at least twelve times. [The ethereal extract does not reduce.] The dried, fat-free residue ground up with one per cent. sodium chloride and left in contact with Prussian blue for two days reduced it to a blue-green within six hours, whereas the glycerine and water extract of the dried residue (after two days' contact) made the Prussian blue of a very pale green colour after twenty-four hours' incubation. The boiled glycerine extract had no effect whatever. Some of the fat-free residue itself on being added to

the glycerine extract adsorbed much of the pigment and presented the white-and-blue appearance. The subsequent addition of the original solid matter itself did not seem to increase the velocity of the reduction, so that there was no such relationship as that of ferment and kinase.

Within three months the reducing power of the glycerine extracts had almost disappeared, but they still gave the xanthoproteic reaction. There is evidence that glycerine extracts, especially of kidney, contain more oxidase than saline extracts: boiling destroys the oxidase in all cases. Oxidase was assumed when guaiacum and hydrogen peroxide gave a blue colour with the juice or extract.

In reviewing the experimental work done to demonstrate the existence of a reducing endo-enzyme in animal tissues, one must conclude that up to the present the verdict is 'not proven.' But the evidence for the existence of a reductase is just as good as, and is in some respects better than, that for the existence of certain ferments now recognised, e.g. xanthase, adenase, carbonase, hydrogenase, or the autolytic ferment.

If we follow A. Heffter, protein even in the form of crystallised ovalbumin is autoxidisable by reason of the existence in it of one or more S·H groups, the mobile H of which performs strong reductions. Thus for Heffter neither an oxidase nor a reductase is necessary. Colloids can do all that is attributed to 'respiratory enzymes.' While this may be excellent criticism chemically, it is a protest against the prevailing conception of the distribution of the various vital activities amongst distinct enzymes, some extra- some intra-cellular. Protests of this kind have been made before.* To Heffter, respiratory enzymes are quite superfluous.

Now there is gradually being accumulated a good deal of evidence for the existence of respiratory enzymes. Vernon³¹ wrote in 1908 that although 'we are supremely ignorant as to the connection of the oxidases and peroxidases with tissue respiration,' yet we are to hope 'that the connecting-links may be discovered in course of time.' To explain everything capable of being effected by protoplasm in terms of chemical activity alone is not to assist in the elucidation to which Vernon refers, nor to give us any more detailed view of the *modus operandi* of vital procedures. On Heffter's view the mobile H freed from an S·H grouping

*A. MacKendrick, 'Dissociation in certain vital phenomena,' *Journ. Anat. and Phys.*, Vol. XXXIX, p. 285, 1905.

is all that is needed to effect reductions and convey oxygen thus—

Heffter attributes these powers to cysteine or some similar compound mercaptan, and Rey-Pailhade freely admits that cysteine possesses all the characteristics of 'philothion,' an alleged ferment on which he has written extensively.

While Rey-Pailhade by no means disregards oxidase in his speculations regarding tissue-respiration, oxidase is as unnecessary to Heffter as is a reducing ferment. One would expect blood-serum to have active reducing powers if, as Heffter insists, pure proteins can perform all the reductions attributed to endo-enzymes.

As judged by the same pigments used to test press-juices—Prussian blue and methylene blue—blood-serum has a most trifling power of reduction, probably sufficiently accounted for by the dextrose and other reducing substances in solution. Thus serum (aseptic) dialysed against distilled water does not reduce either pigment in the slightest degree.

Neither serumalbumin alone nor serumglobulin alone has any reducing power on these two pigments. It has been long known that these and other pigments injected into the blood are not reduced in it, though in and by the tissues they are.* This seems against Heffter's view.

A THEORY OF THE ACTION OF REDUCTASE

I venture to identify reductase with the elusive 'oxygenase of protein character which takes up molecular oxygen with formation of peroxides.' According to Moore and Whitley,²¹ the evidence for an 'oxygenase' producing a peroxide is flimsy. But if we assume the endo-enzyme reductase is none other than the so-called oxygenase which becomes the

*I have noticed that normal serum heated for two to five minutes at 100° C. does acquire some reducing power as also the liquid filterable from the heat-coagulum. I have as yet arrived at no satisfactory explanation of this. If heated for several hours, the serum on the other hand has no reducing power.

organic peroxide on assuming its charge of oxygen, then we account for—

- (1) The existence of an enzyme which carries oxygen and yet is not itself a 'peroxidase';
- (2) The existence of an 'organic peroxide';
- (3) A tissue-oxidase or peroxidase simultaneously present along with a reductase.

Vernon holds that the tissues contain a substance which can absorb oxygen from their surroundings to form an organic peroxide. There seems a high probability that this 'substance' and tissue-reductase are identical. Number one in the above classification is a protein or closely allied to such, while all workers seem agreed that number three is not. Provisionally, I suggest that the organic peroxide is the reducing ferment with its burden of oxygen in the act of handing it over to the peroxidase to apply it to the oxidation of the carbon and hydrogen in or in the immediate vicinity of the living bioplasm.

SUMMARY

1. The reductions effected by the press-juice of liver and of kidney are not due to products of putrefaction or autolysis. The reductions are most energetic when the juice is recently pressed: these juices kept aseptic in glass under toluene gradually lose their reducing power: by the end of three months they are inactive.

2. The press-juice is much more active as regards reduction than pieces of the organ itself: in this respect reductase resembles glycogenase,²⁰ an undoubted endo-enzyme.

3. The reducing power is completely inhibited but not destroyed by temperatures from 0° C. to -14° C.; its optimum is from about 42° C. to 46° C.; at about 70° C. it begins to be destroyed; it is completely destroyed at 100° C.

4. Reductase is inactivated by treatment with alcohol and by drying, and more so in press-juice than in the intact cells.

5. It is relatively insoluble in that it will not dialyse away from the cell-proteins; it is precipitable along with them by ammonium sulphate.

6. The simultaneous presence of oxidase or a peroxidase must be remembered. It accounts for the re-blueing of the surface of the leuco mixtures of press-juice and pigments. Surface oxidation may be indefinitely postponed by an atmosphere of hydrogen, and almost as perfectly by a layer of olive oil at least one inch thick,

7. There is some evidence that the heat-destruction temperatures are not the same for the two enzymes; for certain glycerine extracts, after being boiled for three to five minutes, produced a leuco condition without any subsequent surface re-blueing. This is in agreement with what Spitzer found as to reducing ferments being still active after boiling, and with the difficulty of Moore and Whitley in destroying certain peroxides by boiling.*

8. Glycerine extracts, both of liver and of kidney, seem to have a greater reducing-power than normal saline extracts, but to undergo a similar deterioration in the same interval of time.

9. Judged by the criterion of solubility, the reducing endo-enzyme has not been isolated. In the opinion of certain biological chemists insoluble endo-enzymes do exist, and that therefore the condition of solubility is too rigorous a test. Reductase would belong to this class. Certainly 'extracts' (if they are not solutions) of some active constituent of liver and kidney possess a power of reduction which is diminished with lapse of time and is injuriously affected by certain reagents and agencies in a manner wholly comparable with that of substances long recognised as enzymes.

10. The deteriorated substance is still a protein.

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**Loc. cit.*, p. 139.

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