ON THE USE OF SOLUBLE PRUSSIAN BLUE FOR INVESTIGATING THE REDUCING POWER OF ANIMAL BIOPLASM

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The reducing-power of living animal tissues, of bioplasm, is a property of the first importance from the bio-chemical standpoint. Normally it is exerted upon oxyhaemoglobin, which yields some, not all, of its dissociable oxygen to the living cells through the intermediation of the lymph. This is a case of direct de-oxidation : on its efficiency depends the continued existence of the bioplasm.

Living tissue can, however, act as a chemical reducer under circumstances which do not admit of de-oxidation. This is shown in Paul Ehrlich's (1) well-known experiment in which methylene blue injected *intra vitam* into the circulation is reduced to a green or even colourless condition in the more active organs.

Some confusion seems to exist as to the precise nature of this change, as, despite the fact that methylene blue contains no oxygen, the reaction is frequently referred to as one of de-oxidation (2 and 3). In this particular instance the reduction is due, not to the abstraction of oxygen, but to the addition of two atoms of hydrogen ; thus—

and when subsequently the leuco compound (methylene white) is oxidised, these two hydrogen atoms are removed in the form of water, and the colour is regenerated. A reduction of this type differs from such reactions as conversion of Acetic Acid into Acetaldehyde, or of Phenol into Benzene.

In the former example, the oxygen is *partially* removed by the reducer; in the second case it is *entirely* eliminated. With inorganic substances the mechanism of reduction is frequently complicated, the simplest example being the reduction of a metallic oxide to a metal, but other well-defined types of reducing actions are known, such as the conversion of a Ferric salt to the Ferrous condition.

It will be shown in the present paper that living tissue is capable of effecting reducing actions of this indirect nature, and readily converts soluble Prussian blue to a leuco compound which agrees in its reactions with those of Dipotassio-Ferrous Ferrocyanide.

We consider the reducing-power of bioplasm thus exhibited to be particularly interesting in that not only can living animal matter extract oxygen from such a loose combination as HbO_2 , and so exhibit the inspiratory phase of the 'internal respiration,' but it can perform such a chemical change as is involved in the transformation of methylene blue to its leuco state (or chromogen), or, as one of us (D.F.H.) (4) pointed out in 1897, the reduction of an appropriate Ferric salt to the Ferrous condition.

As is well known, the *intra vitam* injection of the Prussian blue and gelatine 'mass' often appears from the histological point of view to have been a failure, so pale is the mixture, especially in the capillaries and small vessels. This, we believe, is due to the fact that under such conditions the Potassio-Ferric-Ferrocyanide would be transformed into the Dipotassio-Ferrous-Ferrocyanide; and the reduction of the Ferric to Ferrous condition would be accompanied by a corresponding colour change in which the intense blue of the former compound would become pale green, and ultimately white.

The structural change involved is shown by the following formulae :—

Soluble Prussian blue undergoes the same chemical change when treated with alkaline solutions of organic reducers, *e.g.*, hydroquinone or pyrogallol, so that the tissues may be regarded from the biochemical standpoint as alkaline reducers.

The pale green or colourless compound in the vessels of an injected organ may be restored very rapidly to the vivid blue condition by irrigation with H_2O_2 , just as a piece of Prussian blue gelatine 'bleached' to the leuco state by pyrogallol can be completely 'revived' by the same treatment. This revival can be brought about more slowly by exposure to the air; the colourless condition becoming pale green, the pale green becoming blueish.

In the capillaries of the most active organs—liver and kidney—the reduction from the blue to the colourless condition is so complete that anyone slicing up these organs for the first time invariably says: 'The injection mass has not entered here.' One is often only convinced that it has entered and is still in the organ, on the slices being exposed to the air or irrigated with H_2O_2 , when, as one watches the cut surface, the streaks of injected vessels emerge into visibility like letters developing in 'sympathetic' ink. Seen for the first time, this is very striking—more so, we think, than the corresponding methylene blue revival.

One of the most convincing demonstrations of the reducing power of the tissues as exerted on soluble Prussian blue was carried out in the isolated kidney of the pig which was actually induced to excrete an artificial, gelatinous, colourless 'urine.' A pig's kidney, an hour or two after the death of the animal, was first of all washed out with 75% saline at 42°C ., so that all traces of blood were removed, and colourless saline was running from the renal vein and from the

ureter, 6 cm. of which had been preserved. Gelatine and Prussian blue was then substituted for salt solution; when the pressure of injection approached 210 mm. of mercury we noticed a colourless viscid liquid dropping from the ureter, and, subsequently, on chilling the organ and bisecting it, we found the pelvis and calices of the kidney full of colourless gelatine, portions of which, on irrigation with H_2O_2 , immediately became of a bright blue colour.

It was not until we had thus restored the colour with an oxidiser that we assured ourselves that the colourless excretion contained none other than the Dipotassio-Ferrous-Ferrocyanide, which we were now able to change back to the Potassio-Ferric-Ferrocyanide; the reduction had been carried by bio-chemical activity to a more perfect leuco state than we have ever seen effected by pure chemical reducers, *e.g.*, by nascent H ., hydroquinone or pyrogallol. No doubt, the rapid browning which the last two undergo would effect a partial 'masking' of the colourless condition.

Thus gelatine and some soluble Prussian blue had passed through the epithelium either of the Malpighian corpuscles or of the cortical labyrinth, or both; and, having been in this manner brought into such intimate contact with the living bioplasm, it was reduced with the maximum of bio-chemical vigour from the intensely blue of the Ferric salt to the perfectly leuco condition of the Ferrous salt—a change which involves the reduction of trivalent iron to the divalent condition.

We perfused kidneys with strong solutions of soluble Prussian blue without gelatine, and obtained results similar to those just described as regards the green condition in the capillaries, but were led to believe that such strong solutions (half saturated) of this salt are rather toxic.

The toxicity of the soluble Prussian blue was well shown in one experiment with a sheep's kidney which was being perfused with the gelatine mixture; in nine minutes after beginning the perfusion, colourless drops fell from the ureter and continued forming for some minutes longer, but on our maintaining the pressure (250 mm. of Hg) the drops began to be greenish, and finally were quite green.

We take this as indicating that as long as the leuco salt came from the ureter, vital reduction was still being effected by the renal parenchyma, but as the cells become poisoned by the Prussian blue they reduced less perfectly, and so allowed the imperfectly reduced form of the salt to be excreted.

In a subsequent experiment we used lamb's or small sheep's kidneys immersed in a shallow tray of NaCl .75% at 50° C. Having washed out the vessels with NaCl .75% (at low pressures of from 40-110 mm. Hg), we injected the gelatine and Prussian blue mixture at first at a pressure of 50 mm. Hg. No flow from ureter occurred till the pressure reached 100 mm., when a drop of a viscid, colourless material was seen forming at the cut end of the ureter; very slowly it fell, and drop after drop slowly formed—all were colourless. After about a quarter of an hour the drops began to appear greenish, showing that the reduction was becoming diminished in vigour.

We finished with a pressure of 300 mm. Hg., maintained for five minutes, during which time the greenish gelatine continued to flow from the ureter: on disconnecting the injection apparatus and clamping the renal artery of the now very turgid kidney, a greenish material continued to drop from the ureter for the next ten minutes. This greenish substance, on treatment with H_2O_2 , became blue.

Even if this result is largely due to filtration through the glomeruli, it is not filtration of unaltered soluble Prussian blue, but of the more or less perfectly reduced form of the pigment—the renal cells having acted as the reducers.

The production of this absolutely colourless Ferrous salt disposes of the view that the change in colour undergone by the Prussian blue in the tissues is merely due to 'fading' brought about by the alkalinity of the tissues (5).

It is, of course, true that such 'free' alkali as the Sodium or Potassium Hydroxide will 'bleach' the gelatine Prussian blue from a deep blue to a pale blue, and then to a colourless condition; and as a precaution against the effect of 'alkalinity' of tissues, acetic acid is added to the injection mass when prepared by the methods of Paul

Mayer and Ravitz (5). As a matter of fact, the mass that we have always used in these experiments *is* slightly acid to litmus paper.

Now, in the first place, the alkalinity of tissue is not due to the hydrates of Na or K, but to certain alkaline salts, *e.g.*, sodium carbonate, sodium phosphate, and calcium phosphate.

To imitate the alkalinity of tissues, then, one must use such a solution as Ringer's fluid:¹ this, if added warm to a solution of the gelatine Prussian blue, produces in it no change of colour beyond what would be accounted for by dilution.

As Ringer's fluid is neutral to litmus paper, it was necessary to add sodium carbonate to render it distinctly alkaline, and this mixture behaves with regard to the Prussian blue exactly like the neutral solution.

In addition, the following experiment seems to exclude the possibility of the colour change being due merely to the alkalinity of the tissue. A section of an injected kidney to which the blue colour had been restored by means of H_2O_2 was immersed in very dilute caustic soda solution. The blue colour gradually disappeared, and, as subsequent treatment with oxidisers failed to restore it, the Potassio-Ferric-Ferrocyanide had evidently passed into solution. In this way it is shown that when an injected tissue is oxidised by exposure to air or other means the blue colour then formed is due to the regeneration of Prussian blue. As the leuco compound therefore yields Potassio-Ferric-Ferrocyanide by a process of oxidation, it must in turn have been formed by reduction.

But there is another difference between fading due to alkalinity and to bio-chemical reduction, *viz.*, that whereas the first colour change in alkaline fading is to a lighter blue, the first with reduction is to a green colour. We have never seen the blue of the Ferric salt change to the white condition except through a green stage.

The green intermediate stage is a very common one in cases where the tissue is not sufficiently active to effect complete reduction

1. .75 NaCl saturated with calcium phosphate, and 2 c.c. of one per cent. solution of KCl added for each 100 c.c.

to the leuco stage; and the leuco stage on exposure to the air often goes back no further than the green stage.

The green stage is only obtained in vitro with alkalies used alone when they are in concentration far beyond what they are in blood or lymph, but the green stage is always passed through when the blue gelatine is acted on by an alkaline reducer whether in vitro or in blood-vessels.

Injected tissues that have been left blue for at least ten years under alcohol can with great rapidity be turned green and then colourless by alkaline pyrogallol or hydroquinone; and conversely, tissues in a pale green state for ten years can in a few minutes be restored by H_2O_2 to the blue condition.

Although neither Ringer's fluid alone nor its alkaline modification produces any fading of the colour of the blue gelatine, the addition of a trace of pyrogallol at once bleaches the mixture to a pale green tint.

We therefore conclude that the alkaline salts of blood, lymph or tissues are by themselves not the cause of the so-called 'fading' of the Prussian blue injection—the change of colour in the smaller vessels is due to the pigment having been reduced by the surrounding living bioplasm, which has exerted its reducing influence across the lymph-space and across the capillary wall. That the alkalinity of the blood itself is not a cause of the fading of the Prussian blue is interestingly shown as follows:—In those cases where the Prussian blue has been injected into vessels which still contain some blood the mixture is of a dark *green* colour; and the stirring up of a little melted blue gelatine with freshly-shed blood leads to the production of *greenish* masses distributed throughout the blood; or, again, blood added to a dilute solution of blue gelatine turns it pale *green*.

Here we have not the fading to a paler blue due to the alkalinity of the blood, but a certain amount of reduction, small but recognisable, on the part of the blood whereby the blue is changed to green. Compared with the tissues, the reduction in and by blood is, as far as our observations go, minimal. Whether this reduction by blood is due to the 'reducing substances' that are known to exist in it, or to

its leucocytes, is for the present of no moment—the change from blue to green is due to reduction and not to ‘fading.’

Physical factors alone cannot account for this change of colour : the addition of blue to red would produce a pure purple, a blue purple or a red purple, as the case might be, but not a green ; and so similarly would the addition of red to blue : bio-chemical interaction must here play a part.

In large blood-vessels such as the Portal vein, Inferior Vena Cava, &c., from which all blood has been previously washed out, the gelatine mass shows no alteration in colour whatever ; that is, vessels not surrounded by living parenchyma but existing virtually as tubes isolated from tissues, are *not* the seats of reduction—they are not the nutritive centres of oxygen-craving cells, whereas those (capillary) vessels which are divided from the living oxygen-craving tissues only by a lymph-space are the seats of the most energetic reduction.

The relative vigour of the reducing-power of the different living animal tissues can be very well shown by the Prussian blue method. We should say that liver and kidney were the most energetic reducers, the pigment in their capillaries always being very pale green or white ; less energetic we should call alimentary mucosa, glands, brain and skin ; the spleen and lung are either feeble reducers or are easily poisoned. Capsules of organs have little power of reduction compared with the parenchyma they envelope. We are in doubt as to where to place muscular tissue, and hope shortly to make its reducing-power the object of more detailed study.

SUMMARY

1. Potassio-Ferric-Ferrocyanide injected into blood-vessels *intra vitam*, and retained in them *post-mortem*, by being transformed to the green or white Di-Potassio-Ferrous-Ferrocyanide, demonstrates the bio-chemical reducing-power which living tissues possess.

2. This change is not due to the alkalinity of the tissues, although that condition provides for reduction in an alkaline medium.

3. The reducing-power of the renal tissue in particular can also be strikingly demonstrated by perfusing the soluble Prussian blue and gelatine through a surviving kidney until a flow from the ureter of the leuco or pale green salt has been established.

4. Whereas filtration under pressure is undoubtedly *the* factor producing this last-mentioned result, there is present in addition the factor of bioplasmic reduction—the chemical basis of the respiratory phase of tissue respiration.

REFERENCES

1. Paul Ehrlich, *Das Sauerstoff Bedürfniss des Organismus*, Berlin (1885).
2. Starling, in *Text-Book of Physiology, by British authors*. Young J. Pentland, Edinburgh, 1st edit., Vol. II, p. 290.
3. Halliburton, *Text-Book of Physiology*, Murray (1905), p. 379.
4. D. F. Harris, *Proc. Roy. Soc. Edin.* (Session 1896-7), Vol. XXI, p. 383.
5. Mann, *Physiological Histology*, Oxford (1902), p. 160.