the latter method only was a positive result obtained, *T. vivax* appearing in the goat 13 days after the first inoculation of blood. The two monkeys used in these experiments remained negative to daily examinations for 30 days.

With regard to the appearance of trypanosomes in the goat it must be stated that the animal was sent to Sese direct from Entebbe. I was, therefore, only able to make a few preliminary blood examinations before using it for experiment. In the face of former results with *T. vivax* and the fact that the goat was to all appearance quite healthy, there is every reason to conclude that the trypanosomes came from the situtunga.

Studies on the Reductase of Liver and Kidney.—Part I.

By David Fraser Harris, M.D., D.Sc., F.R.S.E., Professor of Physiology and Histology, and Henry Jermain Maude Creighton, M.A., M.Sc., Dr.Sc., Lecturer on Physical Chemistry, Dalhousie University, Halifax, N.S.

(Communicated by Prof. A. J. Brown, F.R.S. Received May 10,—Read June 27, 1912.)

I. *Introduction.*

The notion has been steadily gaining ground that the reducing powers of animal tissues are due to enzymic action. In March, 1910, one of us* adduced evidence that this so-called "reductase" was active in the press-juice of liver and kidney of sheep, ox, horse, and frog. Soluble Prussian blue, methylene blue, and sodium indigo-disulphonate are all reduced more or less perfectly to leuco-compounds by press juice, whereas by a boiled control they are not.

It seemed very desirable to conduct several lines of investigation arising out of the main contention that the tissues were capable of carrying out reduction-processes because they contained an endo-enzyme, viz., How rapidly does the press juice deteriorate in reducing activity; how does it behave in respect of such comparatively stable but reducible substances as $NaNO_3$; in what way, exactly, does its activity vary with temperature; in what way, if any, is the catalase of the liver related to the reductase? It seemed, in particular, highly desirable that a method capable of following the reduction changes quantitatively should be devised in order to enable us to follow the velocity of any given reaction being studied.

* Harris, D. Fraser, 'Bio-Chem. Journ.,' 1910, vol. 5, p. 143.

II. *Technique.*

As previous experience had shown the high probability of the enzyme being comparatively insoluble, we decided in some instances to perfuse the liver or kidney, as the case might be, with tap-water rather than with physiological saline. We desired not to have to deal with a saline press juice, because it was hoped that we might be able to employ the method of following changes in electrical conductivity as quantitative in investigating the progress of reduction. Our hopes in this connection were not realised, so that the liver used on March 18, 1912, was perfused with physiological saline.

The preparation of the juice on March 27 may be taken as typical. We were supplied with the half of an ox liver from an animal which had just been killed, and this we perfused with tap-water heated to 40° C. until the emergent water was bloodless. The liver was then cut into large pieces, and a good deal of the water allowed to flow out of it. The pieces were then cut up into much smaller portions and forced into the juice-press, in which they were crushed under very considerable pressure. A fawn coloured, viscid liquid dripped out and was received under toluene. This juice was subsequently filtered through cheese-cloth to free it of connective tissue and the debris of blood-vessels. Juices were similarly prepared on January 16 from a frozen ox-kidney, on February 5 from perfectly fresh pig's liver, and on March 18 from pig's liver.

III. *Experimental.*

(a) *Observations with Methæmoglobin.*—We were very anxious to make observations on the action of the juice on hæmoglobin. It was not possible at the time of our experiments to get a sufficient quantity of fresh mammalian hæmoglobin, so that we used dried "hæmoglobin scales, soluble, Merck." On dissolving some of these dried scales, and filtering the solution, we found that the pigment gave the spectrum of methæmoglobin, that is, had the band in the red along with the two bands of oxyhæmoglobin. The solution was stable, and was unaltered by bubbling air (freed from CO_2) through it for four hours; the colour was brown, and never became crimson, so that it was still methæmoglobin. Two equal volumes of the brown solution were now taken, and to one of them 6 c.c. of ox-liver juice, two days old, were added, and to the other 6 c.c. of the same juice heated for 10 minutes at 100° C. The juice and the methæmoglobin were shaken together and placed in a thermostat at 40° C. In less than 10 minutes the unboiled tube was of a bright pink colour, the boiled control being of the original rusty brown. On filtering off the clear, pink liquid from the opaque juice, and examining it with the spectroscope, the two bands of oxyhæmoglobin were very distinct,

while the liquid filtered from the control was still methæmoglobin. We concluded that we had here reduction effected by the active juice, while the heat-inactivated juice was quite unable to effect any reduction. We had, in fact, an illustration of the action thus described by the late Dr. Gamgee in writing of methæmoglobin.* "The action of reducing agents reveals . . . . that the molecule of loose oxygen of hæmoglobin is still present in blood which has been acted upon by nitrates for, in the absence of all traces of oxygen, reducing agents first of all and instantaneously liberate oxyhæmoglobin, which is only afterwards reduced." In other words, the liver press juice acted towards a solution of methæmoglobin exactly as the "reducing agents" to which Dr. Gamgee alluded in the paragraph just quoted.

(b) *The Action of Press Juice on Hydrogen Peroxide.*—It was found that hydrogen peroxide was rapidly decomposed by small quantities of press juice, a few drops of the juice causing relatively large quantities of 3-per-cent. hydrogen peroxide to froth violently at room temperature.

A few preliminary experiments on the rate of decomposition of hydrogen peroxide by press juice were carried out. In these experiments the solutions of the peroxide and the press juice were separately brought to the required temperature before mixing. The mixture was then placed in a thermostat at this temperature, and kept agitated by a mechanical device. From time to time definite amounts of the mixture were removed with a pipette, acidified with sulphuric acid, and the concentration of the hydrogen peroxide estimated by titrating with standard potassium permanganate solution. As potassium permanganate is slowly reduced by organic matter, the titrations were made very rapidly, so as to lessen any error due to the presence of the proteins contained in the juice. The amount of organic matter contained in the mixture pipetted off for titration was always small, and, as very small quantities of press juice were used, was practically negligible.

A mixture containing 190 c.c. of 0·0950 molar hydrogen peroxide and 5 c.c. of press juice (one week old) was prepared and kept at 25° C. It was found that at the end of five minutes, 97·2 per cent. of the hydrogen peroxide had decomposed, and at the end of 15 minutes 97·7 per cent. Another experiment was carried out at 25° C. with a mixture containing 190 c.c. of 0·0950 molar hydrogen peroxide solution, 4 c.c. of distilled water, and 1 c.c. of a press juice solution made by diluting one volume juice with nine volumes of water (*i.e.* the concentration of the press juice in the mixture was 1/50 that in the previous experiment). The following table shows the change in hydrogen peroxide concentration with time :—

* Gamgee, 'Schäfer's Text-book of Physiology,' 1908, vol. 1, p. 245.

Time.	0·0332 N. $KMnO_4$ required by 10 c.c. of the reaction mixture.	Amount of hydrogen peroxide decomposed.
mins.	c.c.	per cent.
0	55·72	0·0
5	23·91	57·1
10	12·52	77·6
15	6·94	87·6
20	4·32	92·3
25	2·70	95·2
35	1·33	97·6
45	0·67	98·8
70	0·25	99·5

With boiled press juice no decomposition of hydrogen peroxide occurred.

In order to determine the action of proteins on hydrogen peroxide the rate of decomposition of hydrogen peroxide, at 25° C., in a mixture containing 190 c.c. of 0·0950 molar peroxide solution and 5 c.c. of 15-per-cent. white-of-egg solution was determined. The following table gives results obtained:—

Time.	0·0332 N. $KMnO_4$ required by 10 c.c. of the reaction mixture.	Amount of hydrogen peroxide decomposed.
mins.	c.c.	per cent.
0	55·72	0·0
5	54·72	1·8
10	53·84	3·4
15	53·84	3·4
30	53·80	3·4
60	53·71	3·6

It will be seen from the results given in the foregoing tables that the decomposition of hydrogen peroxide by the press juice of liver cannot be attributed to the organic matter contained in the juice; for in the first experiment the amount of organic matter is extremely small, and in the second, where the amount of organic matter is relatively large, only a small amount of the hydrogen peroxide was found to have been decomposed, even at the end of an hour. Since it has been observed by Spitzer* that the liver is rich in catalase, it is most probable that the decomposition of hydrogen peroxide by liver press juice is due to the presence of this enzyme. If, however, the press juice contains a reducing endo-enzyme, as we suppose, it is not improbable that the decomposition of the hydrogen peroxide may be due to the combined action of the catalase and the reductase.

(c) *Experiments with Soluble Prussian Blue.*—It has already been shown

* Spitzer, 'Pflüger's Archiv,' 1897, vol. 67.

by one of us* that soluble Prussian blue is readily reduced to a leuco-compound by small quantities of press juice from liver and kidney. This action has been confirmed and further studied in the present investigation.

Some experiments carried out with the press juice prepared on March 27 are described below.

Three cubic centimetres of the absolutely fresh press juice were shaken up in a test-tube with 10 c.c. of 0·05-per-cent. solution of soluble Prussian blue at room temperature. 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 pure light grey in colour. When the same volume of boiled press 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 rapidly with time. With a mixture containing 3 c.c. of the press juice 24 hours old, and 10 c.c. of 0·05-per-cent. soluble Prussian blue solution, it was found that 10 minutes elapsed before its colour became greenish-grey, and two hours before it became completely grey; and when the juice was four days old it was necessary to allow the mixture to stand under toluene for seven or eight hours in order to obtain a greenish-grey colour. On adding a few drops of hydrogen peroxide to some of the light grey mixture, the blue colour was immediately restored. The blue colour of the mixture was also slowly re-established by allowing it to stand exposed to the air. This re-blueing commenced at the surface of the mixture and slowly progressed downwards. If the decolorised mixture was spread out in a thin layer on a watch glass, the colour quickly returned. It has been found on allowing the Prussian blue press juice mixtures to stand, that the fawn-coloured protein matter of the juice usually settled to the bottom of the test-tube, and that a white grey substance remained suspended above it. Some of this grey suspension was pipetted off. It was found that it rapidly turned bluish-green on exposure to the air or on being treated with hydrogen peroxide. This blueing of the whitish grey suspended material supports the suggestion of Harris and Irvine† that potassium ferrous ferrocyanide is formed when soluble Prussian blue is acted upon by liver press juice. As has been already mentioned, the colour of soluble Prussian blue, when mixed with small quantities of press juice, gradually fades to a green grey, and after further time has elapsed all trace of green colour disappears, leaving only a pure grey. Further, it has been found that the first colour change takes place more quickly than the second. This phenomenon suggests the forma-

* Harris, D. Fraser, *loc. cit.*

† Harris, D. Fraser, and J. C. Irvine, 'Bio-Chem. Journ.,' 1906, vol. 1, p. 357.

tion of intermediate compounds; and, indeed, it would appear that the first stage of the reduction is more readily effected by the reductase of the press juice than the second stage. We intend to investigate the chemical nature of such intermediate compounds.

A number of experiments was carried out with a view to determining the relation of the reducing activity of the press juice to temperature. In these experiments, the results of which are given below, 3 c.c. of a 24-hour-old juice were mixed with 10 c.c. of 0·05-per-cent. solution of soluble Prussian blue. Both the soluble Prussian blue solution and the press juice were brought to the temperature of the experiment before mixing.

Temperature.	Time required for the mixture to become light green-grey.
° C.	mins.
0	40
14	10
25	6
40	2·5 to 3
55	1·25
67	less than 1
72	2

Press juice and a solution of soluble Prussian blue that had been cooled to −2° C. were mixed and placed in a freezing mixture at −14° C. It was found at the end of two hours, although the mixture quickly became solid after immersion in the freezing mixture, that the colour had turned from blue to green. That reductase is not permanently inhibited by a temperature of −14° C. is shown by the fact that when this mixture was melted and warmed, the green colour faded rapidly to light green-grey. It is most probable that, at −14° C., the extremely weak action of the reductase is due not so much to inhibition of the enzyme as to the formation of the solid phase.

Experiments were also carried out at 100° C. The press juice and the solution of soluble Prussian blue were separately brought to this temperature and then mixed. It was invariably observed that the blue colour of the mixture faded to the light green-grey in about one minute. The blue colour could be re-established, however, by shaking up the cooled colourless mixture with a few cubic centimetres of dilute hydrochloric acid. Since it has been shown that the strong activity of a fresh juice is completely destroyed by heating to 100° C. for two or three minutes, it is evident that at this temperature the decolorisation of soluble Prussian blue cannot be due to the action of reductase, but that it must be ascribed to some other cause. In a recent

investigation it has been shown* that soluble Prussian blue is decolorised by native and derived proteins, and although this action is inappreciable at room temperature at the end of several hours, and even at 60° C. requires an hour or more for completion, at 100° C. the decolorisation takes place very rapidly. The decolorisation of the blue is due to the formation of a complex with the protein, which is broken down by hydrochloric acid with return of the blue colour. In the light of this investigation, the fading of the colour from mixtures of press juice and soluble Prussian blue at 100° C. must be attributed to some action due to the presence of protein, and not to the action of the reductase.

(d) *The Reduction of Iron Salts by the Press Juice of Liver.*—A few experiments were undertaken to see whether the press juice from liver was capable of bringing about the reduction of ferric salts.

To a dilute solution of ferric chloride, 3 c.c. of two-day-old press juice were added, and the mixture kept at 40° C. for 20 minutes. The protein substances were then filtered off, and a few drops of potassium ferricyanide added to the brown coloured filtrate. On the addition of the potassium ferricyanide, a green-blue precipitate which rapidly turned deep blue was formed, indicating the presence of iron in the ferrous condition. Pure press juice gave no coloration with potassium ferricyanide. In a control experiment, where a ferric chloride solution was warmed with 3 c.c. of press juice which had been boiled, no blue coloration was observed on the addition of potassium ferricyanide.

It will be seen from these experiments that ferric salts are readily reduced to the ferrous condition by the reductase of liver.

(e) *The Reduction of Nitrates.*—In a recent investigation, Kastle and Elvove† have shown that various inorganic nitrates are reduced to nitrites by the action of aqueous extracts of certain plants. This suggested the possibility of the reduction of nitrates being brought about by the reductase in liver press juice. Accordingly, a number of experiments was undertaken with this end in view.

In each of two test-tubes, 20 c.c. of two-day-old press juice (prepared March 27) were placed. One of these tubes was placed in boiling water for five minutes. It was then cooled and diluted to its original volume with distilled water. To each tube 20 c.c. of a pure solution of sodium nitrate, containing 5 grm. of the salt, were simultaneously added, and both tubes tightly corked and shaken. The tubes were then placed in a thermostat at 50° C. for an hour and a half, during which time they were frequently

* Creighton, H. J. M., 'Trans. Nova Scotia Inst. Sci.,' 1911—1912, vol. 13 (2), p. 61.

† Kastle, J. H., and E. Elvove, 'Amer. Chem. Journ.,' 1904, vol. 31, p. 606.

shaken. At the end of this time 5 c.c. were pipetted off from each tube, diluted with distilled water, and rapidly filtered from protein material. The clear, light brown filtrates were then decolorised by shaking with powdered animal charcoal, which was subsequently removed by filtration. The colourless filtrates were finally tested for nitrite with Griess' sulphanilic acid reagent, and the amount of nitrite determined colorimetrically. The following are the results obtained :

The nitrite contained in 5 c.c. of the active liquid was found to correspond to 7·65 c.c. of the standard* nitrite solution. Therefore the nitrite contained in the whole solution was equivalent to 1·47186 mgrm. The nitrite contained in the total volume of boiled liquid was less than 0·04 mgrm.

Another experiment similar in every respect to the above, except that the 20 c.c. of the sodium nitrate solution added to each tube contained 2·5 grm. of the salt, gave the following results :

The nitrite contained in 5 c.c. of the active liquid was found to correspond to 5·90 c.c. of the standard nitrite solution. Therefore the nitrite contained in the whole active solution was equivalent to 1·13516 mgrm. The nitrite contained in the total volume of boiled liquid was less than 0·03 mgrm.

From these experiments it will be observed that the amount of reduction brought about by the active press juice is not inconsiderable, while, on the other hand, boiled press juice reduces little or no nitrate. This behaviour affords additional support to the enzymic character of tissue reduction.

As it seemed probable that many important and interesting results regarding the nature of reductase might be obtained by studying the velocity of reduction, we attempted to follow the change in concentration of the soluble Prussian blue solutions by means of electrical conductivity measurements. This method, however, had to be abandoned owing to the extremely small changes in conductivity that were found to occur in the reactions investigated.

Owing to the ease and accuracy with which measurements on the reduction of nitrates can be carried out colorimetrically, we intend, in the immediate future, to employ this reaction for the quantitative study of reductase.

IV. *Summary of Conclusions.*

1. The existence of a catalytic enzyme in the mammalian liver is fully confirmed. The decomposition of hydrogen peroxide is effected by this enzyme and is not due to the presence of proteins or other organic matter in the press juice. We intend to study further the behaviour of this hepatic catalase.

2. We find the existence of a reducing endo-enzyme (reductase) confirmed.

* 1 c.c. of the standard solution employed contained 0·02405 mgrm. of sodium nitrite.

Methæmoglobin is by press juice reduced at body temperature to hæmoglobin after the manner of non-living, reducing agents. Such a relatively stable compound as sodium nitrate is reduced to nitrite, and ferric chloride to the ferrous condition. These reductions are not due to the proteins of the juices, since a control of boiled juice alters none of the substances hitherto used to demonstrate reduction. We intend to use the nitrate reaction as a basis of a method to follow these reduction changes quantitatively.

3. The probability of the enzymic character of tissue reduction is further confirmed by the effect of certain protoplasmic poisons lately investigated by one of us.* Certain virulent protoplasmic poisons inhibit reductase in virtue of their acidity rather than through their toxicity; this finding is in accord with the well established fact that acidity (concentration of H-ions) inhibits the activity of many enzymes.

4. Though the presence of proteins in press juice is not responsible for such a reduction as that of soluble Prussian blue to the colourless condition, yet the proteins of the juice form with the pigment a colourless chemical or physical compound. This change takes place rapidly at 100° C. and exceedingly slowly at room temperature.

One of us† has shown that such proteins as egg-albumin and gelatine readily form with soluble Prussian blue such compounds at higher temperatures. It would be convenient to allude to these phenomena as the "Creighton effect." We desire to distinguish this fading of pigments through combination with proteins from true vital reduction, and we venture to suggest that the so-called reduction effected by colloids and studied by Heffter‡ may be of the nature of the fading of pigments; it is not the same phenomenon as the reduction which we are studying.

5. We lay a considerable degree of stress on the fact that reductase is able to reduce chemical substances differing very widely in structure, propensities and stability. Not only can it reduce compounds containing oxygen such as methæmoglobin and sodium nitrate, but with equal potency substances which contain no oxygen and are of a relatively stable nature, such as ferric chloride and soluble Prussian blue.

The expenses of this work were met by a grant to one of us (D. F. H.) from the Government Grant Committee, which is hereby gratefully acknowledged.

* Harris, D. Fraser, 'Bio-Chem. Journ.,' vol. 6, p. 2.

† Creighton, H. J. M., 'Trans. Nova Scotia Inst. Sci.,' 1911—1912, vol. 13 (2), pp. 61—75.

‡ Heffter, A., 'Medizinisch-Naturwissenschaftliches Archiv,' vol. 1, Part 1, p. 81; also 'Archiv f. expr. Path. und Pharm.—Festschrift f. O. Schmiedeberg,' 1908, p. 253.