LVIII. THE ACTION OF POISONS ON REDUCTASE AND ATTEMPTS TO ISOLATE THIS ENZYME.

BY DAVID FRASER HARRIS
AND HENRY JERMAIN MAUDE CREIGHTON.

*From Dalhousie University, Halifax, N.S., and
Swarthmore College, Swarthmore, Pennsylvania, U.S.A.*

(Received Sept. 28th, 1914.)

Although it has been shown [Harris, 1910] that tissue-reductase is comparatively insoluble, the following attempts at its extraction have been made; they led not to its being isolated but to a study of the action of poisons on it.

Extracts or solutions of reductase were made thus: 1. A liver extract; the livers of four pigeons, before the animal heat had left them, were crushed in the juice-press, ground up with sand in a mortar in the presence of 200 cc. of 0.75 per cent. sodium chloride solution and the mixture allowed to remain under toluene for 24 hours. 2. The pectoral muscles of two pigeons, still warm, were crushed in presence of glycerol and allowed to remain under this liquid for 24 hours. 3. The pectoral muscles of two other pigeons were crushed with glycerol and 0.75 per cent. sodium chloride solution and the mass allowed to stand for 24 hours. There were, then, three extracts or solutions of reductase; a saline extract of liver, a pure glycerol extract of muscle, and a glycerol and saline extract of muscle.

The presence of reductase in the extract was detected by determining whether it effected the reduction of the two-banded oxyhaemoglobin to the one-banded haemoglobin. In the reduction experiments, one part of extract was mixed with two parts of a blood solution prepared by diluting 10 cc. of freshly defibrinated cat's blood to 250 cc. with water. The reduction was carried out at 40°.

The progress of the reduction of oxyhaemoglobin to the fully reduced pigment was followed spectroscopically, a small direct vision spectroscope being employed. The replacing of the two bands of oxyhaemoglobin by the

one band of fully reduced haemoglobin gives an end-point which is not affected by any personal factor. But the disappearance of the two bands is from a very early period accompanied by the appearance of a fainter band between them in the intralinear green, so that at a certain stage of the reduction the single band of the reduced haemoglobin is flanked by the remains of the two bands of the oxyhaemoglobin. The pigment was never considered fully reduced until the remains of these margins of the single band had completely disappeared. In this way it was possible to obtain an end-point of considerable definiteness. It was evident that owing to the opacity of the mixture of extract and diluted blood in even a narrow test tube, the spectrum of the oxyhaemoglobin could not be seen sufficiently distinctly to be certain of the end-point. It was found that glass vessels made by drawing out short lengths of glass tubing (6 mm. diam.) to a conical end answered the purpose very well, for if we were in doubt as to the persistence of the two bands, when the spectroscope was directed across the tube itself, it was only necessary to look through the conical part of the tube where the mixture was sufficiently transparent to obtain a distinct spectrum. Complete reduction was considered to have taken place at the first moment the two bands had vanished, and when they were no longer distinguishable as ribbed margins to the single band.

It was previously ascertained that neither the glycerol, the saline solution nor a mixture of these effected the reduction of oxyhaemoglobin at 40°.

A mixture of the glycerol and saline extract of the pigeon's muscle with the blood solution had its oxyhaemoglobin completely reduced in two minutes in one experiment, and in three in another. Indeed the small quantity of residual blood in this extract of muscle was found to be in the reduced condition. The pure glycerol extract was incapable of effecting reduction. The saline extract of the liver completely reduced the oxyhaemoglobin in 16 minutes. There was evidence, therefore, that a certain amount of reductase passed into solution in a menstruum of glycerol and normal saline both from disintegrated liver and from muscle, but that evidently it did not pass into solution in the pure glycerol.

Efforts were next made to precipitate the reductase from these extracts or solutions. A quantity of the saline glycerol solution of hepatic reductase was thrown into an excess of absolute alcohol, and the precipitate allowed to stand for some time. The alcohol was then decanted, the precipitate washed with water and dried in a current of air at room temperature. This precipitate was shaken with glycerol and normal saline and some of the

resulting cloudy liquid mixed with dilute blood in a conical observation tube. No appreciable reduction could be detected at 40° even at the end of an hour. The filtrate from the alcohol precipitate had no reducing power. The toxicity of alcohol towards reductase was thus once more demonstrated, it having been previously found [Harris, 1911] that alcohol destroyed the power of reductase to reduce soluble Prussian blue. The glycerol and saline extract of the pectoral muscles was saturated with ammonium sulphate, but it was found that neither the precipitate itself nor the filtrate from it was able to effect any appreciable reduction of oxyhaemoglobin. In other words, ammonium sulphate inactivates or poisons reductase.

The poisonous influence of many substances towards enzymes has long been recognised, and has been the subject of considerable investigation. For example, the poisonous action of a number of substances on the catalase of the blood has been investigated by Senter [1903]. Indeed, many of the enzymic poisons are the same substances which inhibit the action of certain inorganic catalysts, as has been shown by the investigations of Bredig and his pupils during the last few years. The effect of different poisons on inorganic catalysts and enzymes is illustrated in the accompanying table, in which is shown the concentration of different poisons that is necessary entirely to destroy the catalytic action of colloidal platinum [Bredig and v. Berneck, 1899] and of catalase [Senter, 1903] on hydrogen peroxide.

TABLE I.

Poison	Colloidal platinum	Catalase
H ₂ S	1: 300000 molar	1: 1000000 molar
HCN	1: 20000000 "	1: 1000000 "
HgCl ₂	1: 2000000 "	1: 2000000 "
Hg (CN) ₂	1: 200000 "	1: 300 "
I in KI	1: 5000000 "	1: 50000 "
NH ₂ (OH) HCl	1: 25000 "	1: 80000 "
Aniline	1: 5000 "	1: 40000 "
As ₂ O ₃	1: 50 "	1: 2000 "
CO	very poisonous	no paralysis
HCl	1: 3000 molar	1: 100000 molar
NH ₄ Cl	1: 2000 "	1: 1000 "
HNO ₃	no paralysis	1: 250000 "

As our previous work has shown [Harris and Creighton, 1912] that the liver and other organs contain a reducing enzyme, reductase, and that attempts to isolate this enzyme have indicated that its activity is greatly reduced by contact with foreign substances, it seemed desirable to investigate the action of small quantities of a number of substances towards it. For

this purpose the reductase contained in cat's liver-juice was employed. The action of the foreign substances on the reductase was measured by determining the difference between the time required for the reduction of oxyhaemoglobin to the one-banded condition, by pure liver-juice, and by liver-juice containing some of the foreign substance with which it had been in contact for a definite time. The progress of the reduction was followed spectroscopically. In the experiments, a mixture of 1 cc. of fresh liver-juice and 1 cc. of an aqueous solution of the poison was allowed to remain in contact for ten minutes, at the end of which time 2 cc. of the blood solution (dilution 1 : 25) were added and the mixture introduced into observing tubes which were then placed in a thermostat at 40°. The spectrum of these mixtures was examined every half-minute. The times required for the reduction at 40° by liver-juice which contained foreign substances were compared with the time required for the reduction, at the same temperature, of a *normal* mixture containing 1 cc. of liver-juice, 1 cc. of distilled water and 2 cc. of the blood solution. Only such substances were employed as were found to produce no change in the spectrum of oxyhaemoglobin. It was not possible, therefore, to use a number of desirable substances such as copper sulphate, iodine, acids, alkalies, etc. The results of the experiments are given in Table II. The times given in this table represent the means of several experiments which seldom differed by more than a few per cent. and which, in most cases, were identical.

TABLE II.

Ten minutes required for reduction of normal mixture.

Poison	Time required for the reduction of mixtures containing 1 cc. of liver-juice, 2 cc. of blood solution and 1 cc. of an aqueous solution of the poison having a concentration of	
	0·01 molar	0·1 molar
As ₂ O ₃	33 minutes	
KCN	30 "	34 minutes
HgCl ₂	17 "	blood destroyed
Na ₃ AsO ₃	17 "	" "
AuCl ₃	15 "	16 minutes
OsO ₄	13 "	19 "
MnCl ₂	11 "	25 "
NH ₄ Br	11 "	13 "
HCHO	10 "	48 "
NH ₄ Cl	9 "	9 "

The solution of As₂O₃ employed was somewhat more dilute than 0·01 molar.

In Table II the different substances are arranged in the order of their poisoning action with respect to the 0.01 molar concentration. The order is quite different with respect to the higher concentration. At the higher concentration some of the substances are relatively much more poisonous than at the lower. Thus formaldehyde, which exhibits no toxic action in the smaller concentration, is the most powerful poison in the list when its concentration is increased to 0.1 molar.

The poisonous action of the foregoing foreign substances is of physiological interest. A new method of studying the action of poisons on one phase of tissue-respiration has been used. It is possible that it may be found a convenient one where injection of poison into the entire animal is not desirable. One substance only of those examined exerted no retarding action on reductase, namely, ammonium chloride. This is in accordance with what is known of ammonium chloride therapeutically; it is a mild expectorant and a ciliary excitant and is not a toxic substance at all in the ordinary meaning of the word. It may be said that the more poisonous a substance is, the longer is the time required for reductase to effect complete reduction in the presence of the weaker concentration of the substance. Judged by this, neither ammonium bromide nor manganese chloride is a poison, and this, at least as regards the former salt, is quite in agreement with the experience of therapeutics. Ammonium bromide, except in exceedingly high doses, is not a poison. It is, however, exceedingly interesting that mercuric chloride and potassium cyanide, for instance, substances well known to be most energetic in destroying the activity of the inorganic ferment colloidal platinum and of catalase should be among the most toxic of the substances which were examined. In concentrations so dilute as 0.01 molar they interfered with the activity of reductase, causing the reduction to be lengthened one and a half times in one case and three times in the other. Quite distinctly toxic, even in such dilution, is sodium arsenite. Not far behind it come gold chloride and osmic acid, the latter a well-known protoplasmic poison. It need be a matter of no surprise that eight of the ten substances examined, more or less at random, should be toxic for reductase. None of these substances is a constituent of the normal cell-lymph in which reductase must act. The slightest trace of any of these salts or acids is foreign to the environment of living cells, and as such cannot but be highly injurious to them. Nevertheless reductase is found within limits to be fairly resistant, for concentrations of these poisons ten times as high as 0.1 molar did not entirely prevent it exerting its reducing action. In the case of formaldehyde

the time taken to reduce at the higher concentration, 0.1 molar, was more than quadrupled, being prolonged to 48 minutes.

That dilute formaldehyde should not exert a poisonous action on tissues is in keeping with what is believed to be its rôle in the anabolic processes carried out by vegetable protoplasm. Formaldehyde has been proved to be the first organic substance produced in the synthesis of carbohydrate in the plant, under the influence of sunlight on chlorophyll. This function of formaldehyde is incompatible with its being in high dilutions a protoplasmic or enzymic poison.

The fact that the poisons studied behave towards some substance in fresh tissue exactly as they behave towards catalysts in general, is an additional indication that that substance in tissue-juice is itself catalytic.

The physiological significance of the relative insolubility of reductase is undoubtedly this, that its sphere of action is *within* the cell. Inside the cell but separated from the oxygen-containing lymph by only the more or less permeable membrane or wall of the cell, the reducing enzyme extracts oxygen from the circumambient lymph. Reductase could not perform this particular service in tissue-respiration if it could dissociate from cell proteins and become dissolved in the lymph and blood. It would then circulate and perform a rôle wholly different from that which it evidently does perform at the surface of, but still inside, the living cells. Reductase is not a secretion, it is an *endo*-enzyme.

SUMMARY.

1. It has been found that the reductase contained in pigeon's liver and muscle is soluble to some extent in normal saline, and in a mixture of normal saline and glycerol but not in pure glycerol. All attempts to isolate the active enzyme have failed.
2. The poisonous action of ten substances, chosen more or less at random, towards reductase has been investigated.
3. In conclusion, the action of the poisons on reductase and the insolubility of the latter have been discussed from the physiological standpoint.

REFERENCES.

- Bredig, G., and Muller v. Berneck (1899). *Zeitsch. physikal. Chem.*, **31**, 258.
Harris, D. Fraser (1910). *Biochem. J.*, **5**, 143.
— (1911). *Biochem. J.*, **6**, 151.
— and Creighton, H. J. M. (1912). *Proc. Roy. Soc., B*, **85**, 487.
Senter, G. (1903). *Zeitsch. physikal. Chem.*, **44**, 257.