ON THE PRESENCE OF OXYDASES IN INDIA-RUBBER, WITH A THEORY IN REGARD TO THEIR FUNCTION IN THE LATEX

By D. SPENCE, Ph.D., A.I.C., *Research Chemist to the Liverpool Institute of Commercial Research in the Tropics.*

From the Bio-Chemical Laboratory, University of Liverpool

(Received April 10th, 1908)

In a previous paper¹ on the distribution of the protein in Para rubber, I have shown that Weber's insoluble oxygen-addition compound of india-rubber is in reality of the nature of vegetable protein and have attempted to account for the singular behaviour of this insoluble protein towards solvents by a physical theory based on its peculiar distribution throughout the rubber in the form of a fibrous network.

In this same paper I drew attention to the rather remarkable striped appearance which moderately dry sections of raw Para rubber exhibit, and as the peculiar shading of colour in the form of dark and light brown layers alternately throughout the rubber seemed to correspond with the distribution of the protein in the latter as evinced by the microscopical examination of stained sections, I suggested that the protein had probably a not unimportant function in the raw product.

These incidental observations in regard to the properties of the insoluble constituent of Para rubber led me, however, to investigate still further the nature of the nitrogenous products which I have since found to be present not only in Para rubber, but in greater or less amount in some forty different brands of raw rubber obtained from the various rubber-producing plants throughout the world.²

1. *Quart. Journ. Liverpool Institute of Commercial Research*, Vol. III, No. 6, p. 47, 1908.

2. There seems indeed to be little doubt that nitrogenous products of a similar character are to be found in greater or less amount in the latex of all the rubber-producing trees or vines. Furthermore in the processes for the coagulation of the latex, unless special steps be taken, the protein invariably becomes coagulated and bound up to a great extent in the rubber clot, giving rise to putrefaction and deterioration in badly-cured samples.

Some time ago my attention was directed to a short note by Tschirch and Stevens¹ on oxidizing enzymes in gums. The singular resemblance in solubility and in general behaviour of these enzymes to the insoluble constituent of Para rubber led me at once to examine this rubber for oxidizing enzymes, and to attempt to determine in how far the rapid darkening in colour and the oxidation of raw rubber in general might not be accounted for by the presence of such an enzyme in the insoluble constituent of india-rubber.

Before proceeding, however, to describe the experimental work which has led to the isolation and identification of such an enzyme, let me briefly outline the literature on this subject.

As the result of numerous investigations in the field of plant physiology, our knowledge of the nature of oxidizing ferments and of their function in the plant has been considerably increased within the last few years, and it must be looked upon as a great step in advance that experimentation, in particular the work of Bach and Chodat, has shown us that it is necessary to distinguish sharply between the 'direct oxydases' and the peroxydases (indirect oxydases) which act only in presence of hydrogen peroxide or another peroxide body.²

Nor can the function of these enzymes in the vital oxidation processes be overlooked, since many oxidation phenomena have been shewn to be due to the intermediate formation of peroxides and the action of oxidizing enzymes, while other important oxidation processes taking place in the plant have been attributed to the action of specific oxidizing enzymes.

The colour changes observed in freshly-cut vegetable tissues exposed to the air have been generally ascribed to the action of oxydases, in many cases without even experimental proof of the existence

1. Separatabdruck aus der *Schweiz. Wochenschrift für Chemie u. Pharmacie*, No. 31, 1905.

2. A review of the present day theories in regard to the nature and the function of oxidizing enzymes in plants is given by Bach and Chodat (*Biochem. Zentralblt.*, Vol. I, p. 416, 1903); a very complete account of all the literature on this subject is to be found in Czapek, '*Biochemie der Pflanzen*,' Vol. II, pp. 464-481, 1905. According to Bach and Chodat the oxydases are a mixture of two enzymes of distinct types; the oxygenases or protein-like bodies, exceedingly unstable towards heat, chemical agents, etc., which take up molecular oxygen from the air with peroxide formation; the peroxydases or enzymes of nitrogenous but non-protein character which are much more stable towards heat, etc., than the oxygenases and activate the oxygenase, causing it to give up oxygen to the auto-oxidisable substrate. That the oxygenase fails in the extracts of some plants is due to its great instability.

of such enzymes in the tissues in question. It is not, therefore, surprising that the gradual darkening in colour which has long been observed to take place in freshly prepared samples of raw rubber, became associated in the minds of observers at a very early stage with the presence of an oxydase in the raw product. Czapek,¹ indeed, states that the latex of rubber-producing plants contains an oxydase which is partially responsible for the darkening in colour of the coagulated product. Weber² attributed the intense darkening in colour of Castilla latex to the presence of an oxydase, without, however, investigating this point, while Tschirch³ states that the darkening in colour of raw rubber is due to the action of enzymes (oxydases). I have not, however, been able to find any reference to experimental evidence supporting these views, and on the other hand Schidrowitz and Kaye⁴ have reported that they examined a sample of Hevea latex but failed to get any indication of the presence of oxydases. The latex, however, which they examined could not be regarded as a normal one seeing that it contained barely 4 per cent. of dry rubber. That oxydases occur in the latex tubes of certain plants Raciborski⁵ has shewn, and the presence of a similar enzyme (laccase) in the gum of Japanese lacqueur has been shewn by Bertrand⁶ and others. Furthermore, the presence of oxydases in gum arabic and indeed in almost all gums has been determined by Struve,⁷ Bertrand and Bourquelot,⁸ Wiesner,⁹ Tschirch and Stevens,¹⁰ and important biological functions have been ascribed to these enzymes by certain authorities.¹¹

Numerous reagents have been suggested and employed successfully

1. Czapek, 'Biocemie der Pflanzen,' Vol. II, p. 705, 1905.
2. Weber, *Ber. chem. Ges.*, Vol. XXXVI, p. 3110, 1903.
3. Tschirch, 'Die Harze und die Harzbehälter,' 2nd edit., Vol. I, p. 992, 1906.
4. *India Rubber Journal*, Vol. XXXIV, No. 1, p. 24, 1907.
5. *Ber. d. deutsch. bot. Ges.*, p. 52, 1908.
6. *Compt. rend.*, Vol. CXVIII, p. 1215, 1894; Vol. CXX, p. 266, 1895; Vol. CXXI, p. 166, 1895; Vol. CXXII, p. 1132, 1896.
7. *Lieb. Ann.*, Vol. CLXIII, p. 160.
8. *Compt. rend. soc. biol.*, Vol. XLIX, 1897.
9. *Sitzb. d. Wien. Akad.*, 1885, and *Monatsb. d. Chem.*, Vol VI, p. 592, 1885.
10. *Archiv. d. Pharm.*, p. 535, 1905, and *Pharm. Zentralb.*, Nr. 26, 1905.
11. See Tschirch, 'Die Harze und die Harzbehälter,' 2nd edit., Vol. I, p. 883, 1907.

from time to time for the detection of oxydases, and it has been shewn that the behaviour of these enzymes towards reagents may vary with the source of the oxydases (certain oxydases giving a negative reaction to one class of indicator and a positive reaction with another). One cannot, therefore, rely on the tincture of guaiacum reaction alone in studying the action of oxydases. For this reason, in the work of isolating the peroxydase from Para rubber which I now propose to describe, I have been led to study the behaviour of the enzyme towards some ten well known reagents for oxydases which will be described in due course, and have only used the reaction with guaiacum as a rough and ready test in preliminary experiments. Further, my original intention in commencing this work being to clear up certain points in regard to the so-called insoluble constituent of Para rubber which has been surrounded by mystery in the past, my attention has been confined in the following communication to Para rubber only.¹

EXPERIMENTAL

Preliminary experiments to isolate an oxydase from raw Para rubber were carried out as follows :—

A large quantity of unwashed raw Para rubber, cut from the interior layers of a very large block, was cut up into very fine sections by means of a sharp knife. These sections, which were as thin as it was possible to cut them, were allowed to soak in a quantity of distilled water sufficient to cover them, for a period of seven to ten days, after which time the aqueous extract was filtered off. About 100 grammes of the finely minced rubber were treated in this way each time, and the extract was tested from time to time by means of tincture of guaiacum with and without hydrogen peroxide. The first experiment showed that it was possible in this way to extract a ferment which had the property of rapidly turning tincture of guaiacum in presence of hydrogen peroxide a fine deep blue, and which was destroyed by boiling (peroxydase). The watery extract was alkaline to litmus, and appeared to give marked indications of the presence of protein by the Millon's and other tests. In order, therefore, to purify the extracts as far as possible they were dialysed for twenty-four hours against running water, preliminary experiments having shewn that in this way the peroxydase activity towards guaiacum was not destroyed.

1. It is hardly to be doubted, however, that what has been shewn to hold for the insoluble nitrogenous body in Para rubber, will also be shewn in time to be equally true for the insoluble nitrogenous product to be found in all varieties of raw india-rubber. Tschirch's work on the insoluble nitrogenous body in the various gums, and the gradual darkening in colour of all the ordinary varieties of india-rubber on the market only serve to support this view.

The dialysed aqueous extract was found to have all the properties of a powerful peroxydase (indirect oxydase) but to be practically devoid of activity in absence of hydrogen peroxide. It was tested as follows :—

Three test tubes were taken and into each was pipetted 10 c.c. of the dialysed extract. To serve as a control one of these was then heated to boiling for a few minutes and cooled, after which a measured quantity (1 c.c.) of a dilute standard solution of hydrogen peroxide was added to it and also to one of the other two quantities of active extract. The three tubes were then set side by side while the same quantity of a standard solution of the indicator to be used was added to each.¹ The activity of the dialysed extract was tested in this manner with reference to the following reagents :—Tinct. guaiacum, p-phenylenediamine, o-phenylenediamine, α -naphthol, phenol-phthalin, hydrochinon, amidol, pyrogallol, p-phenylenediamine + α -naphthol (indophenol reaction), tyrosin. The results observed are given in the following table :—

Reagent	Ten c.c. of the extract boiled, and hydrogen peroxide added	Ten c.c. of the extract without hydrogen peroxide	Ten c.c. of the extract with hydrogen peroxide added
Tinct. guaiacum ³	... Pure white	... Pure white, unchanged	Deep blue in five mins.
p-phenylene-diamine ³	Colourless	Colourless	Dark brownish red
o-phenylene-diamine ³	Faint yellow	Colourless	Yellow, finally red
α -naphthol ⁴	... Colourless	... Colourless, after 2 hours	Marked brown
Phenol-phthalin ⁵	... Faint pink	... „	Deep pink
Hydrochinon ⁶	... Colourless	... „	Intense port red
Amidol ⁷	... Faint pink	... Faint pink	Deep cherry red
Pyrogallol ⁸	... No change after 24 hours	... No change	Yellow coloration and brown deposit
'Indophenol' Mixt. ⁹	... Faint colour	... No change	Deep violet
Tyrosin ¹⁰	... No action	... No action	No action

1. It is hardly necessary to point out that the solutions of these reagents were prepared fresh immediately before use. Peroxide formation in the process of auto-oxidation of these reagents may lead to erroneous results. See 'Die Peroxydasereaktion der Kuhmilch, etc.,' Dr. P. Waentig, *Arbeiten aus dem kaiserlichen Gesundheitsamte*, Vol. XXVI, No. 3, 1907.

2. Van den Broek, *Fabresber. Chem.*, p. 455, 1849-50; and Schonbein, *Zeit. f. Biol.*, Vol. IV, p. 367, 1868. An alcoholic extract of the powdered resin.

3. Aqueous solutions of the hydrochlorides of the bases.

4. Solution in 50 % alcohol containing trace of alkali.

5. Kastle and Shedd, *Amer. Chem. Jour.*, Vol. XXVI, p. 527, 1901.

6. Dilute solution in water.

7. The developer Amidol has not been previously used. As a rapid indicator of oxydase activity it was found to be very useful, giving an intense dark red coloration within a few minutes. An aqueous solution acidified with a few drops of 5 % acetic acid was used.

8. A solution in water. The oxidation proceeds slowly, an insoluble brown precipitate of purpurogallin separating out in the course of 24 hours in the presence of oxydase.

9. Röhmann and Spitzer, *Ber. chem. Ges.*, Vol. XXVIII, p. 567, 1895. The reagent contains 1 mol. α -naphthol, 1 mol. p-phenylenediamine, and 3 mol. Na_2CO_3 in water.

10. A dilute solution in water containing a trace of Na_2CO_3 .

These tests suffice, I think, to shew beyond all doubt that the dialysed aqueous extract from Para rubber contains a peroxydase enzyme or enzymes of the general type, readily destroyed by boiling. That the extract had no action on tyrosin points to the absence of a tyrosinase. Extracts from several different samples of Para rubber were examined in this way under varied conditions and always with the same result. The extract had also the property of a catalase, 20 c.c. of this extract in one experiment in an eudiometer tube over mercury liberating 12 c.c. of oxygen from hydrogen peroxide within a few hours; there was also a very slight indication of proteolytic activity.¹

The dialysed extract was neutral to litmus, and gave neither the xanthoproteic reaction or the familiar coloration for protein on warming with Millon's reagent. No definite biuret test could be got with the dialysed extract. Boiled with a trace of acetic acid the extract became opalescent, and a small quantity of coagulated matter separated.

Very small quantities of acid or alkali did not appear to influence the peroxydase activity to any extent although large quantities of either of these reagents stopped its action completely.

When the dialysed extract was concentrated in vacuo over sulphuric acid it appeared to lose in activity gradually and became finally inert, the sulphuric acid colouring at the same time due to absorption of ammoniacal products. This method, then, obviously could not be used for the isolation of the peroxydase, nor was the method of saturating the extract with ammonium sulphate of much value in this connection.

For the isolation and purification of the peroxydase the following method was found to be most satisfactory :—

The finely cut raw Para rubber was digested for about a week with 40 per cent. alcohol, after which the alcoholic extract, which became intensely yellow during the process due to the extraction of resins, etc., from the rubber, was filtered off and precipitated with several times its own volume of absolute alcohol. On standing, a gummy mass settled out which was filtered off, redissolved in 40 per cent. alcohol, and again precipitated with absolute alcohol. When this process was repeated three times a product was obtained which was infinitely more active than the original precipitate, blueing tincture of guaiacum in presence of peroxide practically instantaneously.

1. Metts digestion tubes.

PROPERTIES OF THE PEROXYDASE FROM PARA RUBBER

The product obtained in this way no longer appeared to possess either catalytic or proteolytic activity to any extent, and in the moist state was a gummy looking mass which left a dry vitreous solid on evaporation of the alcohol. The amount of this solid extracted from the rubber was very small, so that a kilogramme of Para rubber had to be extracted in order to obtain sufficient material for chemical tests.

The peroxydase dissolved slowly in water, and the solution was found to be many times more active in presence of peroxide than the original aqueous extracts,¹ although it had not the slightest action in absence of hydrogen peroxide.

The specific activity of the aqueous extract of the peroxydase was destroyed by the addition of small quantities of potassium cyanide, sodium fluoride, or mercuric chloride to it, while in presence of mineral acids and alkalis, the activity also disappeared. The activity of the peroxydase was found to diminish gradually when the aqueous solution was left exposed to the air and light for some time.² Towards heat the peroxydase was remarkably stable, and it is interesting to note in this connection that when its solution in water was just raised to the boiling point, and then allowed to stand for some time, its activity gradually returned to a slight extent so that it again gave a marked reaction with tincture of guaiacum or with the indophenol reagent.³

By a series of rough measurements with tincture of guaiacum colorimetrically and with pyrogallol, the activity of the peroxydase was determined after exposure to different temperatures. It was found that heating the aqueous extract for 5 minutes to 80° C.

1. It is unnecessary to repeat here the various tests which were carried out in order to determine the peroxydase nature of the substance in solution. All the reactions, however, which have been described on page 169, were repeated many times during the course of the work, and always with more or less the same result.

2. Bach (*Ber. d. chem. Ges.*, Vol. XLI, p. 225, 1908) has determined a reduction in the activity of peroxydase due to the action of oxygen and light.

3. These observations agree with those of Woods (*United States Dep. of Agr. Bull.*, No. 18, p. 17) who found that the oxydase from tobacco juice when killed by boiling, gradually regained its activity on standing. Both Woods and Aso (*Bull. Coll. Agric., Tokyo*, Vol. II, p. 220) appear to be agreed on the existence of a zymogen, more stable towards heat than the oxydase, which changes slowly into the active enzyme.

destroyed the peroxydase activity completely, while $2\frac{1}{2}$ minutes reduced the activity by one half. Heated to 70° C. for 5 minutes the peroxydase was still active, whereas after 15 minutes the enzymic action was completely destroyed. The peroxydase when heated for 10, 20, 30 or 40 minutes to 60° C. was not destroyed.

The thermal optimum of the peroxydase was determined by similar colorimetric methods and it was found to be about 55° C.

In regard to the chemical nature of the peroxydase the following points were noted :—

Although the peroxydase contained a considerable amount of nitrogen, it gave no reaction either with Millon's reagent or by the biuret or other tests for protein. When fused with KOH, ammonia was evolved in quantity along with traces of pyrrol which gave the characteristic red coloration to a splint of pine-wood moistened with hydrochloric acid.¹ This point will be dealt with again in full when the properties of the oxygenase from the latex are discussed. The peroxydase, however, gave a slight reduction with Fehling's solution, and further gave the characteristic coloration for pentoses² with phloroglucin and hydrochloric acid, so that the pyrrol ring is probably formed from the pentose and ammonia set free in the process of fusion.

The peroxydase isolated from Para rubber was found to give a very marked reaction for iron. The coloration with potassium ferrocyanide or with ammonium sulphocyanide was only given, however, when the solution of the peroxydase was treated in such a way that the activity of the enzyme was destroyed. Thus for example, although no coloration was given by adding either of these reagents directly to the peroxydase solution when a drop of dilute hydrochloric acid was first added to the peroxydase or when the peroxydase

1. Tschirch was the first to observe that pyrrol is evolved when the oxydase from Japanese lacquer is distilled with KOH (*Archiv. der Phar.*, 243, 7, 504, 1905. Bach has recently confirmed Tschirch's observations in connection with the peroxydase from the radish, but has pointed out that ammonia as well as pyrrol vapours are evolved by this reaction (*Ber. d. deutsch. chem. Ges.*, Vol. XLI, p. 226, 1908).

2. From a review of the literature on this particular point it appears that a carbohydrate group of a reducing nature is to be found associated with most of the oxidizing enzymes, and that it is extremely difficult if indeed not impossible to separate this complex from the oxydase. Whether in reality a compound between carbohydrate and enzyme exists still remains to be shewn. See Bach and Chodat, *Ber. chem. Ges.*, XXXVII, p. 42, 1904; Cazeneuve, *Compt. rend.*, Vol. CXXIV, pp. 406 and 781, 1897; Tschirch u. Stevens, *Archiv. der Pharm.*, 243, 7, 535, 1905.

solution was boiled for a few minutes (although less marked in this case) and then treated with these reagents, a fine pink coloration was got with the ammonium sulphocyanide and a deep blue precipitate with the ferrocyanide reagent. Without going into a discussion of the present day position in regard to the function of the manganese,¹ which is usually associated with the oxydases or of the iron² which has already been found in an oxidizing-enzyme and is probably present in a colloidal form therein, from the above observations I am inclined to believe that considerable importance must be attached to the iron-content of the peroxydase from Para rubber.

RELATIONSHIP OF THE PEROXYDASE TO THE INSOLUBLE PROTEIN OF THE RUBBER

Having thus determined the presence of a peroxydase enzyme, it became of interest to study more closely its relation to the so-called insoluble constituent or protein in the rubber. The peculiar striped appearance of Para rubber, which, as I have already pointed out, falls closely in line with the distribution of the protein as revealed by the microscopical examination of stained sections, gave a valuable indication of the oxydase nature of the latter.

Indeed, the presence of a peroxydase alone in the insoluble body would not appear to be sufficient to account for the rapid darkening in colour observed when freshly cut rubber is left exposed to the air. According to Chodat and Bach the peroxydase is inactive unless in presence of the peroxide forming enzyme (oxygenase) or preformed peroxide, and this at once led me to examine the insoluble constituent of Para rubber for an oxygenase-complement which had not been extracted in previous experiments. Here, however, almost negative results were obtained, and it was only later when fresh latex from *Funtumia elastica* was obtainable, that I was able to prove the presence of an oxygenase as well as of a peroxydase in the fresh latex (see page 175).

1. Bertrand, *Ann. chim. phys.* (7) Vol. XII, p. 115; *Compt. rend.*, CXXIV, pp. 1032, 1355 (1897); *Bull. soc. chim.* (3) Vol. XVII, pp. 619, 753 (1897).

2. Sarthou, *Journ. pharm. chim.* (6) Vol. XI, p. 583, 1900; Vol. XIII, p. 464, 1902.

The method by which the Para rubber was tested for the presence of an oxygenase was as follows :—

A quantity of the insoluble protein was isolated from Para rubber by dissolving away the rubber in chloroform¹ in the cold. After three months' extraction the insoluble product left was washed with alcohol and dried. It was a hard, stringy, but non-elastic mass which gave a slight reaction for protein with Millon's reagent.

This body was allowed to act on peroxydase extracts in test tubes, but when the extracts were tested they shewed not the slightest activity in absence of hydrogen peroxide. As the oxygenase, as is conceivable, might have been destroyed by the long continued action of chloroform, thin sections of the raw rubber were cut and these were placed directly in a peroxydase extract with tincture of guaiacum. Whereas the tube containing the rubber and extract was only very faintly blue in colour and was hard to distinguish from the control with extract alone, the extract in presence of hydrogen peroxide gave a deep blue colour in a very short time.

Numerous experiments on these lines were tried in order to determine the presence of an oxygenase along with the peroxydase in the insoluble constituent of Para rubber, but the insuperable difficulties connected with the isolation of the 'insoluble constituent' from Para rubber made all attempts in this direction useless. One is led to the conclusion, therefore, that either the oxygenase is killed in extraction experiments with chloroform and toluol, and is not directly got at in the other tests on account of the large mass of the colloid material with which it is associated, or else that the oxygenase fails entirely in the rubber, being destroyed in the coagulation of the Hevea latex.² In view of the activity of the peroxydase in the rubber as evinced by the colour-changes produced, the first explanation seems to me to be the more probable of the two. The subsequent examination of a sample of *Funtumia elastica* latex and the isolation of an oxydase therefrom, determines with a fair degree of certainty the existence of an oxydase in the raw rubber.

In conclusion, further support was given to the theory of a stable zymogen in the raw rubber, by the fact that boiling the rubber with water alone or with water containing small quantities of cyanide, mercuric chloride, or formic acid for some time did not prevent the further darkening in colour of the rubber on prolonged keeping.

1. See 'Distribution of the Protein in Para Rubber,' *Quart. Journ. Inst. Comm. Res.*, Vol. III, No. 6, p. 51, 1908.

2. It would be interesting in this connection to know approximately the temperature at which coagulation of the Hevea latex is brought about.

OXYDASES IN THE LATEX

It may seem out of place that the examination of the latex emulsion was deferred until this point. Obviously, if any reaction is to be found it ought to be sought for in the fresh latex from which the rubber is formed. But various difficulties have to be overcome in working with latex, there being the question of supply of an emulsion which is not to be had in this country, and is withal exceedingly unstable and liable to coagulate. Hence it is that what should in the ordinary course of events have been studied first had to be left until late in the research.

A quantity of latex from *Funtumia elastica*, Stapf., preserved according to my directions, arrived here recently from Southern Nigeria.¹ The latex, which had been carefully collected and sealed up in well-filled pint bottles, arrived in this country for the most part in perfect condition. As only a couple of the samples of this latex come under consideration here, these only will be dealt with. The others will be considered elsewhere.

The two samples submitted to thorough examination for oxidising enzymes were :—

Sample I. The natural latex undiluted.

Sample II. One pint of latex treated with 2 tea-spoonfuls of ammonia (S.G. 0·880) in $\frac{1}{2}$ oz. of water.

Sample I was a clear white milky fluid which had not coagulated at all. On suitable dilution the globules of caoutchouc on examination under the microscope were found to be intact and in rapid brownian movement in the serum. This sample of latex although uncoagulated had developed a marked acidity to litmus² which is perhaps surprising, in view of the generally accepted theory in regard to the process of coagulation.³

1. This latex was collected for me through the kindness of Mr. T. F. Burrowes, the Acting Colonial Secretary for Southern Nigeria, and I should like to take this opportunity of expressing my thanks to him and to those who have assisted him in securing for me an ample supply of latex for further investigations.

2. Fresh *Funtumia elastica* latex is practically neutral! Experiments are already in progress which appear to show that the acidity developed in the natural latex is due to the oxydase therein.

3. This latex would indeed appear to be exceptionally stable in presence of acids; see Spence, *Quart. Journ. Inst. Comm. Research*, Vol. II, No. 4, p. 45, 1907; Schidrowitz and Kaye, *India Rubber Journal*, Vol. XXXIV, No. 7, p. 377, 1907.

Sample II. This sample had not coagulated at all and was of a pale yellow colour in consequence of the presence in it still of a considerable excess of ammonia.

Both samples were tested directly with tincture of guaiacum with and without hydrogen peroxide. The first sample was found to give a very marked peroxydase reaction, but no reaction in absence of hydrogen peroxide.

To free the latex, however, from products which would tend to interfere with the peroxydase reactions, both samples of latex were dialysed for twenty-four hours against running water and then examined.

The sample of untreated latex from which the free acid had been removed in this way was still found to give a very marked peroxydase reaction but no oxydase.

The sample of ammoniacal latex on the other hand now gave an intense peroxydase and a much less marked though positive oxydase reaction. This was confirmed by a positive reaction against boiled controls using the tincture of guaiacum, the indophenol, the *p*-phenylenediamine and the pyrogallol tests as indicators.

The oxygenase in Sample I had, therefore, been more or less destroyed by the acidity developed. The question as to whether new oxygenase is formed on removal of the acid from the sphere of action still remains to be proved. Experiments so far have not shewn whether the oxygenase like the peroxydase will recover its activity, although it would be remarkable, were this not so, that samples of rubber prepared from the two dialysed latices in the ordinary way should darken in colour at the same rate and to the same degree.

Experiments were now set on foot in order to isolate the oxydase from the latex. For this purpose several methods of separating the caoutchouc in the latex without at the same bringing down the protein with it were tried, and although the method in the case of the acid latex was simple, it being only necessary to dilute the dialysed latex with several volumes of distilled water in a separating funnel, and to allow the fine flakes of caoutchouc to rise to the surface and settle before drawing off the mother-liquor, the process in the case of the

ammoniacal product was not so easy, there being a great tendency for the protein to disappear in the rubber clot. The following method for the ammoniacal latex was found to be satisfactory :—

The dialysed latex was diluted by its own volume with water and it was then treated with 50 per cent. alcohol until the agglutination of the caoutchouc particles was complete. The fine flakes of caoutchouc were allowed to settle, after which the mother-liquor was filtered off and treated with a large excess of absolute alcohol. On adding excess of alcohol a gummy precipitate formed which was immediately filtered off and dried in vacuo. This precipitate gave a marked oxydase reaction.

PROPERTIES OF THE OXYDASES FROM THE LATEX

The product separated from the latex in this way was a dark brown vitreous-looking mass which dissolved only slowly in water, giving a somewhat opalescent solution. The aqueous solution was tested with the various reagents for oxydases both with and without hydrogen peroxide, and it was found to give a marked positive reaction *in absence of peroxide* and an intense coloration with the various reagents in presence of peroxide. It contained, therefore, the full oxydase complement (oxygenase-peroxydase) although the oxygenase appeared to be very feeble.

The enzyme was fairly stable towards heat and light in the dry state, but was readily destroyed in solution. It gave the xantho-proteic, Millon's, biuret, coagulation and precipitation tests for protein in a marked degree. It was also found to contain a considerable quantity of iron, and on fusion with alkali evolved ammonia and pyrrol, the latter being identified by the pinewood-HCl reaction already described. As in the case of the peroxydase from Para rubber the oxydase was also found to have associated with it a reducing substance which, from the violet red coloration (shewing an absorption band between D and E) given by it on warming in aqueous solution with an equal volume of HCl and a trace of phloroglucin, appears to be partly at least of the nature of a pentose. This being the case, it does not appear to me to be necessary or even correct to assume that a pyrrol ring is there *preformed* in the protein nucleus of the oxydase.¹ It seems far more probable that the pyrrol is formed

1. See Tschirch, 'Ueber die Gummi-Enzyme,' *Pharm. Zentralb.*, Nr. 31, 1905.

only in the process of fusion of the oxydase with the alkali, the ammonia liberated in this process reacting with the pentose or the partially oxidised pentose.¹ That such a reaction will actually result in the formation of a pyrrol derivative sufficient in amount to give a marked pinewood reaction, I have proved to myself to be the case by fusing together a mixture of pure arabinose, powdered potassium hydrate and ammonium sulphate. Although no coloration is given to the pinewood splint when the reaction is carried out in absence of ammonia, in the presence of this reagent I had no difficulty in getting, in repeated experiments, marked evidence of pyrrol formation.

The oxydase product isolated from the latex of *Funtumia elastica* did not appear to have proteolytic or amylolytic properties. It had a slight catalytic action.

In order to determine in how far the darkening in colour in rubber could be prevented practically by the removal of the nitrogenous products which form the 'insoluble constituent' of india-rubber, experiments were carried out with the *Funtumia* latex, the object being to separate the caoutchouc from the emulsion without at the same time coagulating the protein material in the latex. The following method was completely successful :—

The acid latex is diluted with many times its own volume of water in a separating funnel. The caoutchouc globules apparently coalesce in this way to form fine spongy flakes.² These flakes gradually separate and rise to the surface of the watery liquor, which is then drawn off. The flakes of caoutchouc are again shaken up with a fresh quantity of water, and when this process is repeated three times the caoutchouc is found to be practically nitrogen-free. The flakes are then worked up into a solid rubber clot by washing with alcohol or by pressure.

The rubber obtained in this way was snow-white and did not darken in the slightest on keeping. Samples prepared from the same latex by any of the usual methods of coagulation in which the protein is afterwards to be found in the rubber clot, darken rapidly in colour and become almost black in the course of a week. A sample of rubber prepared by the method described was found to be practically free from protein.³

1. Compare C. Paal, *Furfurane, thiophene and pyrrol syntheses*, Würzburg, 1890.

2. Coagulation does not take place.

3. The protein was found uncoagulated in the first washings. The value commercially of such a method of coagulation will be discussed elsewhere.

These observations prove conclusively that the darkening in colour of raw rubber is due to an oxydase which is associated with the protein or the so-called insoluble constituent of the rubber. In how far this oxydase is responsible for certain other changes occurring in raw rubber on keeping (decomposition, oxidation and the like), further experimental work, I hope, will shew.

In addition to its practical importance the presence of active oxydase enzymes in the latex of caoutchouc-producing plants appears to me to be of interest biologically when considered in the light of certain well-known facts regarding the chemistry of india-rubber. Up to the present no explanation of the function of the caoutchouc in the latex has been offered. The chemical inertness of india-rubber as we know it, and the fact that this product can be extracted in such quantities without any apparent injury to the tree, has led to the general belief that it is an excretory product of metabolism serving as a means of protection to the plant against injury¹ (*Wundverschluss*).

That the latex plays a useful part as a reserve store of water for the plant and as a means of protection against external injury, one need not doubt, but that these are the chief functions of the latex of caoutchouc-producing plants seems to me improbable. The economy and care with which the vital processes in nature are carried out and regulated, point on the very surface to a deeper and more important function for the caoutchouc in the plant, and it is scarcely to be accepted without further proof that a caoutchouc-producing tree continues to produce immense quantities of material in the nature of a hydrocarbon—the richest form of chemical energy that one can well imagine—solely as a means of protection against injury. From the very nature of the product, it seems to me we must far rather believe that it has a more important function in the plant, and there is considerable evidence on all hands to indicate this.

I would venture to suggest that the caoutchouc is probably a reserve food stuff for the plant, and to account for its formation and subsequent decomposition would bring the oxidizing enzymes under

1. See Czepek '*Biocemie der Pflanzen*,' Vol. II, pp. 698-701, 1905, and Tschirch, '*Die Harze und die Harzbehälter*,' 2nd edit., Vol. II, 1906.

consideration. Just as the glycogen in the liver is believed to be a reserve store of energy built up from the simpler sugars only to be broken down again into these and other products by the interaction of the glycolytic enzyme, so the caoutchouc may also be regarded as a reserve food stuff which is broken down by the oxydases in the plant as circumstances demand, into the simpler products from which it has been formed.

That the oxydases are capable of effecting such changes might appear at first sight hard to believe. But as evidence that such enzymes have already been shewn to bring about even more complicated changes, I should like to point out :—

The complete oxidation of sugar into CO_2 and H_2O by oxidizing enzymes has been shewn by the work of Sieber,¹ Palladin,² and others. The oxidation of complex fats into simpler fatty acids and CO_2 has been illustrated by the experiments of Tolomei³ on the oxidation of olive fat by olease—an oxidation enzyme in olives giving a marked tincture of guaiacum reaction. Finally, the oxidation of the protein decomposition product tyrosin by means of an oxidizing enzyme (the tyrosinase of Bertrand⁴) into homogentisinic acid, carbon dioxide and ammonia with the uptake of oxygen is sufficient evidence, I think, to shew that in complicated oxidation processes in both the animal and the vegetable kingdom oxidizing enzymes play an important rôle.

Nor does the decomposition of caoutchouc by oxidizing enzymes in the plant appear to me to offer insurmountable difficulties in the way of the theory suggested. Certain observations already made on the action of a peroxydase enzyme and hydrogen peroxide on the neutral latex emulsion of *Funtumia elastica* seem to indicate that the caoutchouc in the latex is not indifferent towards atomic oxygen liberated in this way. Further, the important discovery of Harries,⁵ that caoutchouc, even in the form in which it is known

1. Sieber, *Zeit. phys. Chem.*, Vol. XXXIX, p. 484, 1903.

2. Palladin, *Zeit. f. physiol. Chemie*, Vol XLVII, p. 407, 1906.

3. Tolomei, *Chem. Centr.*, Vol. I, p. 879, 1896.

4. Bourquelot and Bertrand, *Journ. pharm. chim* (6), Vol. 3, p. 177, 1896; Bourquelot, *Bull. Soc. Mycol.*, France, p. 65, 1897; v. Furth and Schneider, *Hofmeister's Beiträge*, Vol. I, p. 229, 1901; v. Furth and Jerusalem, *Hofmeister's Beiträge*, Vol. X, p. 131, 1907, and numerous others.

5. Harries, *Ber. chem. Ges.*, Vol. XXXVII, pp. 2708-2711, 1904, and Vol. XXXVIII, pp. 1195-1203, 1905.

commercially, is exceedingly sensitive towards small quantities of ozone, and indeed can be broken down by this means into products very nearly related to the pentoses¹ from which the caoutchouc is most probably formed by the plant, lends support to the view that the caoutchouc in the latex must be regarded as a reserve stuff which can be drawn upon as required and broken down by the oxidases always associated with it into simple carbohydrate products of value as food stuffs.²

If we go further, however, and assume that the reaction brought about by the oxydase is a reversible one, then by means of it we can account not only for the breakdown, but also for the building up of the caoutchouc from the sugars (from the pentoses probably as Harries suggested) by the plant. In this light, the presence of a pentose group associated with the oxydase from the latex of *Funtumia elastica* is highly interesting.³

The theory suggested to account for the formation and the function of the caoutchouc in the latex is supported further by experimental facts regarding the anatomy and physiology of caoutchouc-producing plants. It would explain, for example, the occurrence of a definite latex system in the embryo of certain caoutchouc-producing plants, and the complete disappearance of the latex from the tree under certain conditions, but these aspects of the question will be considered in full when further experimental evidence on the chemical side has been obtained.

In the meantime, I should like to reserve to myself the right to follow up the work on which I have already commenced, of investigating the action of oxidizing enzymes on latex emulsions.

In conclusion, I beg to express my thanks to Professor Benjamin Moore for the interest which he has taken in the progress of the work.

1. Laevulinic acid and hydrogen peroxide.

2. The peculiar state of division of the caoutchouc in the latex, and the fact that it is there most probably in a less complex form than in the coagulated product must not be overlooked. See Weber, *Ber. chem. Ges.*, Vol. XXXVI, pp. 3108-3115, 1903; Harries, *Ber. chem. Ges.*, Vol. XXXVII, p. 3842-3848, 1904.

3. Whether the so-called impurity always associated with the various enzymes may not represent minute quantities of the substrate in loose chemical combination or adsorption with the enzyme, in the process of change, is a point worthy of further consideration. If this were so then the nature of the so-called impurity so firmly attached to the enzyme ought to give a valuable indication of the function of the latter.