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The Formation of Hydrogen Peroxide During the Autoxidation of Ascorbic Acid

Ascorbic acid (Vitamin C) is usually described as a reducing agent, but several instances are known where it appears to act as an oxidizing agent. It has been shown that naphthalene is oxidized in aqueous acetone solutions of the vitamin[1], whilst under similar conditions, anthracene, phenanthrene and 3:4-benzpyrene are converted to the corresponding quinones[2]. The mechanism of these oxidations appears to be unknown but it has been suggested that hydrogen peroxide formed during autoxidation of the ascorbic acid is involved in the process. Since it has now been shown that 3:4-benzpyrene can be directly oxidized with hydrogen peroxide to products which include 8·0 H benzpyrene and the 5:8-quinone[3] the above explanation would appear more feasible. As there appears to be no record in the literature of the proof of peroxide formation in the course of reactions involving ascorbic acid further evidence on this point has been sought.

Two tests for hydrogen peroxide have been used:—the formation of yellow pertitanic acid from titanyl sulphate, and the formation of blue colour in the presence of ether, potassium dichromate and sulphuric acid. Both tests gave strongly positive results a few minutes after shaking ascorbic acid crystals with glass distilled water. Control tests of the water were negative. The possibility that the hydrogen peroxide found in the above experiments was, in any way, related to the addition of the test agents was eliminated by a further experiment. Ascorbic acid crystals were stirred into distilled water and after ten minutes the solution was shaken with ether; this being a reagent in which hydrogen peroxide, but not ascorbic acid, is soluble. After separation of the two layers some of the ether was withdrawn with a pipette and shaken with titanyl sulphate solution. The development of a yellow colour showed the presence of hydrogen peroxide. The ether used in this experiment had previously been tested and found free of contaminant peroxide.

It is evident from the above that ascorbic acid in solution will readily give rise to hydrogen peroxide. Whether a catalyst is essential for the reaction is uncertain. The experiments described were carried out in scrupulously clean glassware with reagents of the highest possible purity, but the possibility of contamination with traces of metal or other agent cannot be completely excluded.

These findings offer an explanation of the ability of ascorbic acid to act as an oxidizing agent under certain conditions and additionally may explain the anomalous results which have been obtained in electrode potential determinations[4].

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[1] W. P. JORRISSEN, Naturw. Tijsche. *19*, 15 (1937).
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Mount Vernon Hospital and The Radium Institute Northwood, Middx. England, September 21, 1950.

Zusammenfassung

Es konnte gezeigt werden, daß in Lösungen von Ascorbinsäure freies Wasserstoffsuperoxyd entsteht. Hiermit läßt sich wohl erklären, daß derartige Lösungen unter bestimmten Bedingungen oxydierend wirken können.

Photoreactivation in the near Ultra-violet of D-Glyceraldehyde-3-Phosphate Dehydrogenase

Partial reactivation of a crystalline preparation of triosephosphate dehydrogenase by irradiation in the near ultra-violet was observed during the course of an investigation of the inactivation of this enzyme by ultra-violet light and a measurement of the quantum yield for inactivation at 2537 Å.U.

The enzyme was prepared according to the method of CORI *et al.*[1] and recrystallized three times. In this form the enzyme has been shown to be firmly combined with a definite quantity of coenzyme (DPN) in the ratio of 1 mole DPN to 50,000 g triosephosphate dehydrogenase (TPD); neither recrystallization nor dialysis against distilled water remove the DPN[2]. CORI *et al.*[3] have recently shown that the DPN remains bound to the enzyme even after it has been reduced enzymatically in the presence of arsenate and glyceraldehyde phosphate; the method employed consisted of precipitation of the enzyme with ammonium sulphate following reduction of the DPN. During an independent study conducted at the same time it was found that reduced DPN (DPNH) could be readily dialyzed away against distilled water. The following test, involving no chemical alteration of the medium was therefore carried out: After reduction of the DPN in a 3 mg/cm³ sample of the enzyme in pyrophosphate buffer at p_H 8·5, 0·7 cm³ was centrifuged for 2 hours at 60,000 rpm in a Spinco ultracentrifuge using the analytical cell divided into two equal compartments by means of a diaphragm perpendicular to the radial axis of the cell, the purpose of which is to prevent mixing at the end of the run. The two fractions were then removed and their DPNH contents measured spectrophotometrically by means of the system pyruvate-lactic dehydrogenase. The optical densities at 3400 Å.U. of 5-times diluted samples of the inner and outer fractions and of a non-centrifuged sample were, respectively, 0·022, 0·050, 0·060. The fact that not all the DPNH is accounted for in the two fractions was due to deposition of some of the enzyme at the bottom of the cell. How-

[1] G. T. CORI, M. W. SLEIN, and C. F. CORI, J. Biol. Chem. *173*, 605 (1948).
[2] J. F. TAYLOR *et al.*, J. Biol. Chem. *173*, 619 (1948).
[3] C. F. CORI, S. F. VELICK, and G. T. CORI, Biochim. Bioph. Acta *4*, 160 (1950).

ever, in a couple of trials it was not found possible to relate the DPNH content of each fraction to its protein content due to masking of the absorption maximum of the enzyme at 2780 Å.U. by a rather pronounced non-selective band in the same region and due, in some way, to the glyceraldehyde phosphate. It is, however, quite clear that the DPNH remains bound to some heavy particle.

Enzyme activity is completely destroyed by the centrifugation. The enzyme also shows one main component which crosses the middle of the cell in about 1½ hours; and a very broad component the peak of which, during the same period, has not yet detached itself from the meniscus. This same behaviour has been noted with the DPN oxidized or reduced, with the enzyme completely inactive prior to the run, or for a mixture of these. The natural inference is that the enzyme does not consist of one component.

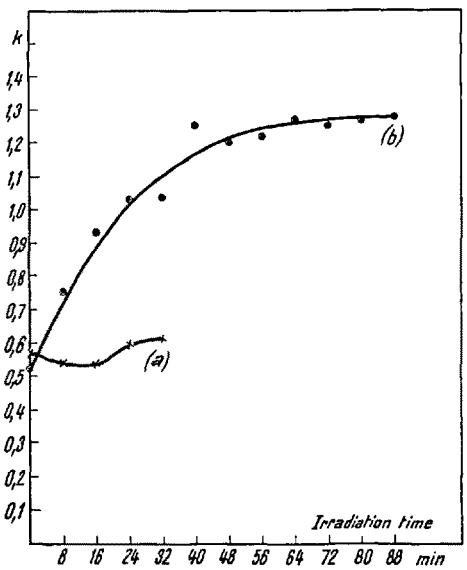

Fig. 1. — Activity (k) of triosephosphate dehydrogenase as a function of time of irradiation. (a) TPD-DPN; (b) TPD-DPNH.

A measurement of the quantum yield for inactivation of this enzyme must take account of the role played by the DPN, which accounts for about 35 % of the absorption of the TPD-DPN complex at 2537 Å.U. It was first found that reduction of the bound DPN resulted in no significant difference in the quantum yield.

However, the behaviour of the system is entirely different when it is irradiated with light in the region 3100–3800 Å.U., which comprises the absorption band of DPNH, and where the absorption of the enzyme itself is negligible. Fig. 1 shows the enzyme activity as a function of the time of irradiation in the presence of (a) DPN, (b) DPNH. The light source was an ordinary mercury arc with a glass filter. It can be seen that while the TPD-DPN complex is practically unaffected, the activity of the TPD-DPNH increases rather abruptly and then reaches a limiting value.

The following points of interest were noted:

(a) The rate of reactivation at 5°C and 25°C did not appear to differ appreciably. The total amount of reactivation was apparently lower at 5°C, but it is not known whether this is significant.

(b) The phenomenon is one of reactivation and not of activation. Muscle TPD is an SH enzyme which is generally partially oxidized and requires a reducing agent for full activity. The degree of photoreactivation is considerably less than the increase in activity obtained in the presence of cysteine. Furthermore, in a trial where the enzyme was first completely reduced with cysteine, no photoreactivation was found.

(c) Partial inactivation of the enzyme by irradiation below 3000 Å.U. results in the appearance of non-selective absorption above 3100 Å.U. Subsequent irradiation in this latter region produced, as was expected, no photoreactivation.

(d) Following photoreactivation of the enzyme, the DPNH absorption band at 3400 Å.U. remains unaltered and all the DPNH can be reoxidized with pyruvate and lactic dehydrogenase. However, a small, but very definite increase in absorption takes place between 2400 and 2800 Å.U.

(e) Limiting the irradiation to a band in the neighbourhood of 3650 Å.U. (at which the DPNH absorption is still about 50 % of that at 3400 Å.U.) by means of a Wood's filter only decreases the rate, but not the extent of photoreactivation. This is quite natural since, even at 3650 Å.U., the transmission of a Wood's filter is only about 40 %.

(f) The enzyme was not sufficiently inactivated to show the phenomenon of heat activation[1]. In any event in all of the above experiments the samples were at a sufficient distance from the light source to prevent any rise in temperature.

It is most likely that the phenomenon is due to reduction of some of the enzyme SS groups provoked by the absorption of energy by the bound DPNH in the vicinity of these groups[2]. The fact that only partial reactivation is obtained may be linked to the two-site hypothesis of CORI *et al.*[3] if one assumes that the DPNH provokes the reduction of only those groups to which it is more tightly bound. The existence of two types of active sites in TPD has been postulated as well from studies of the phenomenon of heat activation[1].

On the basis of some additional results, as yet incomplete, it is not improbable that photoreactivation may be provoked at wave-lengths below 3000 Å.U. There are a number of reports in the literature of purported activation of crude enzyme extracts by ultra-violet irradiation[4]. In general such behaviour is contrary to accepted concepts of the mechanism of ultra-violet inactivation and denaturation of enzymes and proteins, and such findings have therefore heretofore been regarded with scepticism. If, however, one bears in mind the crude nature of the extracts used and the fact that the sources were in general not monochromatic, it is not at all unreasonable to explain these findings on the basis of indirect effects similar to that found for the photoreactivation of TPD. Such effects would also have to be taken into consideration in the irradiation of cells and tissues. It is of interest in this connection to note that the photoreactivation of ultra-violet inactivated bacteriophage has been logically explained on the basis of an indirect mechanism[5].

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[3] C. F. CORI, S. F. VELICK, and G. T. CORI, Biochim. Bioph. Acta *4*, 160 (1950).

[4] F. F. HEYROTH, *Chemical action of ultra-violet rays* (Reinhold Publ. Co., New York, 1940).

[5] R. DULBECCO, J. Bacteriol. *59*, 329 (1950).

A few trials were carried out, in collaboration with Mlle ANNETTE PREVOT of the Institut de Chimie Physique, to see if it were possible to reactivate an enzyme preparation under the influence of X-rays which could conceivably cause reduction of SS groups by free radicals. The overall effect of X-irradiation on SH enzymes has been shown by BARRON *et al.*[1] to result in inactivation due to oxidation of the SH groups by free radicals; following irradiation, activity can be partially restored by means of a reducing agent. To eliminate the effect of oxidation, tests were conducted on an old spontaneously oxidized sample of enzyme which still retained an appreciable number of reducible SS groups (as shown by the appearance of enzymatic activity in the presence of cysteine). No measurable activity could be detected, however, following irradiation with increasing doses up to several thousand rœntgens. The inference is, therefore, that reduction of SS groups by free radicals does not take place or, if it does, that it is immediately reversed by oxidation. This result, incidentally, is in accord with the recent findings of BARRON and FLOOD[2] in regard to the X-irradiation of thiols; and would appear to indicate that oxidation, rather than reduction, by free radicals is the more important physiological result of irradiation by X-rays.

Full experimental details of the photoreactivation process will be presented in a forthcoming publication on the ultra-violet irradiation of triosephosphate dehydrogenase.

I should like to express my thanks to Mr. B. CHALOPIN for assistance with the ultracentrifugations, to Miss. B. TCHOUBAR for advice and assistance in the preparation of the glyceraldehyde phosphate and to Dr. DOROTHY N. NEEDHAM, F.R.S., for a sample of purified lactic dehydrogenase.

Note added December 12, 1950: Of interest in relation to the above results is an experiment reported by Calcutt (G. CALCUTT, Nature, *166*, 443 [1950], following the writing of this report. Cultures of Paramecium bursaria, submitted to the action of SH inhibitors, were found to be killed more rapidly when exposed to light from a mercury arc emitting radiations above 3300 A. The results lead the author to the conclusion that "radiation with wave-lengths greater than 3300 A. causes an increased exposure of intra-cellular sulphydryl groups".

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Résumé

On a constaté que le coenzyme (DPN) lié à la triosephosphate déshydrogénase reste lié après réduction. L'irradiation du complexe (enzyme)-(coenzyme réduit) dans l'ultra-violet proche réactive partiellement l'enzyme. Le mécanisme du phénomène est discuté et les résultats de quelques expériences d'irradiation par les rayons X sont donnés.

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[3] Present address: Laboratoire de Morphologie animale, Université libre de Bruxelles.

Über die tuberkulostatische Wirksamkeit einiger Derivate der para-Aminosalizylsäure

Seit der Einführung der para-Aminosalizylsäure (PAS) in die Therapie sind zahlreiche Isomere, Homologe und Derivate dieses Chemotherapeutikums synthetisiert und auf ihre tuberkulostatische Wirkung geprüft worden. Trotz der großen Zahl dieser Versuche wurden bisher die mit PAS erhaltenen Resultate nicht übertroffen, wiewohl PAS den Nachteil aufweist, aus dem Organismus sehr rasch ausgeschieden zu werden. Es kann daher ein Präparat klinisch interessant sein, wenn es zwar PAS in der tuberkulostatischen Wirkung nicht oder nur wenig übertrifft, jedoch infolge günstigerer Ausscheidungsverhältnisse die im Körper notwendige Blutkonzentration mit kleinern Mengen erhalten wird. Besonders aussichtsreich schienen PAS-Derivate zu sein, bei denen PAS mit Substanzen kombiniert ist, die selbst eine tuberkulostatische Wirkung aufweisen. Bekanntlich haben zahlreiche Autoren auf die günstige Wirkung einer Kombinationstherapie hingewiesen. Die Arbeiten von RIST[1], MÖSCHLIN[2] und HEILMEYER[3] haben gezeigt, daß bei gleichzeitiger Verabreichung von PAS und Streptomycin beziehungsweise Sulfonen oder Thiosemicarbazonen ein verstärkter therapeutischer Effekt erzielt wird. Es wurde untersucht, ob auch die Kombination von PAS mit dem zur Therapie der Tuberkulose früher vorgeschlagenen Chaulmoograöl bzw. Chaulmoograsäurederivaten günstigere Resultate ergibt, wie dies schon von WILDBOLZ[4] für die Kombination von Streptomycin und Moogrol gezeigt wurde. Es wurde festgestellt, daß schon die halbe Menge PAS, unter Zusatz von Chaulmoograöl bzw. Chaulmoograsäure oder gewissen Substanzen mit einem Zyklopentenylrest, die gleiche tuberkulostatische Grenzkonzentration ergibt, wie sie für das PAS bestimmt wurde, obwohl die Wirksamkeit dieser Substanzen bedeutend geringer ist als diejenige von PAS.

Nach WAGNER-JAUREGG[5] kommt für die Wirkung der Chaulmoograsäure dem im Molekül enthaltenen Zyklopentenylkern wesentliche Bedeutung zu. Es wurden deshalb einige PAS-Derivate, welche den Zyklopentenylrest enthalten, synthetisiert und in die Versuche einbezogen. Entgegen den Beobachtungen von LEHMANN[6], wonach die tuberkulostatische Wirkung durch Azylierung der Aminogruppe abgeschwächt wird, wurde gefunden, daß gewisse PAS-Derivate, die einen Zyklopentenylrest enthalten, bei *in-vitro*-Versuchen gar keine Abschwächung der tuberkulostatischen Wirkung zeigen. Auch die Ausscheidungsverhältnisse scheinen für einige dieser Substanzen günstiger zu liegen, indem z. B. die Präparate Nr. 612 und Nr. 622 nach oraler Applikation beim Kaninchen eine höhere und länger dauernde Blutkonzentration ergeben.

Die Wachstumsversuche von Tuberkelbazillen wurden sowohl nach der Oberflächenwachstums-Methode, wie sie von LEHMANN[6] und BLOCH[7] verwendet wurde, als auch nach der Tiefenwachstums-Methode nach SOLOMIDÈS[8] vorgenommen. Dabei zeigte sich, daß man im SAUTON-Milieu nach SOLOMIDÈS im *in-vitro*-Versuch verläßlichere Resultate erhält, als mit den Oberflächenkulturen. In den Tabellen haben wir denn auch nur die

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