LXX. THE ACTION OF NITROUS ACID ON *p*-CRESOL AND TYROSINE

By JOHN ST LEGER PHILPOT AND PERCY ARTHUR SMALL

From the Department of Biochemistry, Oxford

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IN connexion with an investigation of the inactivation of pepsin by nitrous acid, the results of which appear in the next paper, we have been led to examine the action of nitrous acid on tyrosine. It seems to be generally supposed [Gibbs, 1926-27] that the yellow product obtained when nitrous acid acts on dilute aqueous tyrosine or *p*-cresol is an *o*-nitroso compound. We have now obtained evidence that although this compound is first formed, it does not accumulate, but at once undergoes further reaction. The usual product is chiefly a diazo compound accompanied by a nitro compound and photolysis products from the diazo compound (possibly also condensation products), the proportions varying with the conditions. We have also proved that substances belonging to the class of *o*-nitrosophenols are formed in the Millon reaction, but that they differ from the simple derivatives.

Reaction scheme for p-cresol and tyrosine

The scheme in Fig. 1 summarizes the reactions which we have studied. It applies to both tyrosine and *p*-cresol, which differ only kinetically, apart from the deamination of the tyrosine; and it permits a wide choice of reactions according to conditions. The evidence for the scheme is based partly on conditions of formation and partly on the properties of the products, and is therefore diffused throughout the paper without being given separate consideration. Tyrosine and *p*-cresol will be referred to collectively as "phenols", since other monohydric phenols probably conform.

The chief possibilities are as follows:

(1) With dilute nitrite and phenols in acetate buffer plus CuSO_4 at temperatures from 0 to 40° the nitroso compounds are trapped as the very stable Cu derivatives.

(2) Under similar conditions in absence of CuSO_4 little or no nitroso compound can be detected at any stage of the reaction by adding CuSO_4 since it is rapidly transformed further. If, however, very dilute nitrite is added to a large excess of *p*-cresol, considerable amounts of nitroso compound are formed.

(3) The products of further reaction depend on the concentrations of HNO_2 and phenols, and on the temperature. If the ratio $[\text{HNO}_2]/[\text{phenol}]$ is low, red substances are obtained. Their structure and origin are at present unknown, but the product from *p*-cresol sometimes crystallizes directly from the reaction mixture. If the concentration of HNO_2 is increased and the concentration of phenol is not too high the chief product in the dark is diazo compound, unless air or nitrogen is bubbled through the solution. (Such treatment removes nitric oxide as explained below.) At still higher concentrations of nitrous acid, the yield of

diazo compound falls off, probably in favour of mono- or di-nitro compound. In the case of *p*-cresol these three regions are telescoped, while with tyrosine the middle one is much the most extensive.

Fig. 1. $R = \text{CH}_3$, $\text{CH}_2 \cdot \text{CH}(\text{NH}_2)\text{CO}_2\text{H}$ or $\text{CH}_2 \cdot \text{CHOH} \cdot \text{CO}_2\text{H}$.
Substances 3, 4, 6, 7, and 9 have been obtained crystalline.

The decomposition equilibrium of nitrous acid

The above conditions can be partly explained by consideration of the decomposition equilibrium of nitrous acid.

The reactions

have been described by Abel *et al.* [1930].

The equilibrium point of reaction 3 normally lies well towards the right, but it is established much more slowly than the other two equilibria, which maintain the curious relationship:

$$\frac{[\text{NO}]^2 [\text{N}_2\text{O}_4]}{[\text{HNO}_2]^4} = \text{const.}$$

Explanation of above-mentioned conditions. (1) Dilute HNO_2 is used to avoid the action of N_2O_4 on the phenols. Acetate buffer is better than mineral acids because it allows the HNO_2 to be continuously replenished from an internal reserve of NO_2 . The $p\text{H}$ of HNO_2 is about 3.3; with the concentrations of nitrite commonly used by us, e.g. $N/16$, the concentration of HNO_2 ranges from about 0.02 N at $p\text{H}$ 3.7 to about 0.002 N at $p\text{H}$ 4.7. A further reason for using this region of $p\text{H}$ is that the Cu derivative is more stable than at lower $p\text{H}$ values.

(2) The rate of formation of nitroso compound has been shown to be proportional to $[\text{HNO}_2] \times [\text{phenol}]$ by kinetic experiments which will be published later, while its further transformation into diazo compound will probably depend on $[\text{nitroso compound}] \times [\text{NO}]^2$. Hence low values of $[\text{HNO}_2]$ and high concentrations of phenols favour accumulation of nitroso compound.

(3) The unknown red substances are obtained whenever there is not enough NO or NO_2 or Cu^{++} present to react with the nitroso compound as fast as it is formed. We think we have excluded the possibility of their formation from the nitroso compound, either by further reaction with the HNO_2 system, or by condensation with itself or anything else. (Autocondensation does occur, but quite slowly and to a different product.) We are almost driven to postulate a precursor of the nitroso compound, e.g. an addition product of the phenol and HNO_2 , as postulated by Veibel [1930, 1, 2]. This precursor could undergo side reactions to give the red substance unless it were rapidly removed in other ways.

Preparations and properties of products

Nitroso compounds. The purple Cu chelated derivatives of the *o*-nitroso-phenols are very stable between $p\text{H}$ 3 and 8, particularly in presence of an excess of cupric salts. They are therefore formed under these conditions preferentially to any other derivatives. The similar derivative of phenol itself has been described by Baudisch [1918] though the preparation here described seems to be new. There are two derivatives from *p*-cresol. One, formed in presence of one equivalent of Cu is insoluble in water, but soluble in many organic solvents, while the other, formed in presence of excess Cu, is soluble in water and not extracted from aqueous solution by organic solvents. These are presumably the di- and mono-derivatives respectively; a supposition which agrees with the analysis of the supposed Cu di-derivative, given below. The corresponding derivatives from tyrosine are both water-soluble and not easily separated.

Preparation of Cu di-o-nitroso-p-cresol, and o-nitroso-p-cresol

10.8 g. *p*-cresol (0.1 mol.) were dissolved in 400 ml. of acetate buffer of $p\text{H}$ 4.6, and 50 ml. of M CuSO_4 were added. 59.3 ml. of 4.22 N NaNO_2 were added and the solution made up to 1 l. and left 2 days at room temperature. Black crystals separated and were sucked off; the yield was 22 g. of damp cake. It was recrystallized by dissolving in hot alcohol and precipitating with water, or better, by evaporating a solution in a 2:3-alcohol:chloroform mixture. (Found: Cu 19.15%. $\text{C}_{14}\text{H}_{12}\text{N}_2\text{O}_4\text{Cu}$ requires Cu 18.95%.) The substance can conveniently be kept as an $M/10$ solution in the alcohol-chloroform mixture. It is also readily soluble in dioxane.

To obtain free *o*-nitroso-*p*-cresol for any experiment, the alcohol-chloroform solution may be shaken twice with N H_2SO_4 , to decompose the Cu complex, and washed twice with water, a chloroform solution of the nitroso compound resulting. The nitroso compound may be obtained solid by extraction of such a solution with a small amount of N NaOH , and acidifying at 0° , when orange

crystals separate. They may be sublimed at $38^{\circ}/20$ mm. pressure, m.p. 58.5° (uncorr.). Evaporation of the chloroform solution gives nearly colourless crystals, but much is lost through volatilization.

Properties. The Cu di-*o*-nitroso-*p*-cresol is very stable, but decomposes explosively on heating. It is readily interconvertible with the water-soluble mono-derivative, according as nitroso-*p*-cresol or Cu is in excess.

It may be mentioned here that ordinary phenol also gives Cu *o*-nitroso-phenols under conditions such as described above, whereas in ordinary nitrosation it gives very little *o*-derivative.

The free *o*-nitroso-*p*-cresol is fairly stable in dilute aqueous solution, but solutions stronger than *N*/10 apparently undergo condensation fairly rapidly in either water or organic solvents. It is acted on very rapidly by 50% conc. HCl. A solution of the orange-red Na salt, made by shaking *N*/10 NaOH with an excess of the chloroform solution, is rather more stable, but seems to oxidize in the air in the course of some weeks. The compound gives the Liebermann nitroso reaction, and forms bright red nickel and mercuric derivatives. These properties, and the smell and volatility, agree with those of *o*-nitrosophenol as described by Baudisch [1918].

The purple colour of the Cu mononitroso-derivative may be used for its colorimetric estimation. Since the Cu solutions used are coloured green before any purple nitroso compound is present, monochromatic (sodium) light was used to avoid difficulties of colour matching. Colour intensities were measured in terms of glass filters as permanent standards. A solution was assigned a value of 100 when it matched a certain filter at a setting of 10 mm. The increase in colour units was taken as proportional to the amount of nitroso compound formed. By this means the yield of Cu *o*-nitroso-*p*-cresol was measured under various conditions, for which purpose a solution prepared by weighing out the solid di-derivative, and dissolving in an excess of buffered CuSO_4 , thus forming the mono-derivative, was used as standard. The yield was found to be $95\% \pm$ about 2% in experiments at 38° , room temperature and 0° .

Experiments were also made to determine the effect of bubbling N_2 through the solution on the yield of the derivative from tyrosine. A solution was made up, at pH 3.7, containing 2 mg. tyrosine per ml., and NaNO_2 at a concentration of *N*/8. This was split into two parts, and each was heated at 38° for 6 hr., till the reaction was nearly complete, one being gassed with N_2 and the other not. Portions were removed at intervals, diluted 1:6 with buffer and their colour intensities determined. The final intensities were for the gassed solution 130 units, for the untreated 122 units; the slight excess in the former is attributable to evaporation. There is no significant difference; that is, neither NO nor NO_2 attacks the Cu compound with appreciable velocity, a conclusion which is supported by various qualitative experiments.

Tryptophan gives no coloured complex with HNO_2 in presence of Cu.

Nitrosophenols and the Millon reaction

We have examined briefly the relation between the formation of *o*-nitroso-phenols and the Millon reaction. *o*-Nitroso-*p*-cresol forms an intensely red mercuric derivative, at pH about 3-4, and may be prepared from *p*-cresol using Hg instead of Cu. This complex is not identical with the red substance obtained in the Millon reaction, since it is chloroform-soluble and instantly dissociated in $2N$ H_2SO_4 . The red precipitate, obtained by performing the modified Millon reaction [Cole, 1933] on *p*-cresol, is not soluble in chloroform, and is fairly stable in $2N$ H_2SO_4 acid, but is converted by shaking with acetate buffer of pH 4.6 into

a chloroform-soluble substance, apparently identical with the mercuric complex of *o*-nitroso-*p*-cresol. On shaking the red chloroform solution with 2*N* H₂SO₄ even in presence of much HgSO₄, a yellow chloroform solution is obtained, which, when shaken with buffered CuSO₄ at pH 4.6, gives a purple aqueous layer; this behaviour is identical with that of the mercuric complex of *o*-nitroso-*p*-cresol.

If an excess of NaCl is added to the mercurial phenol obtained in the modified Millon test by heating *p*-cresol with a solution of HgSO₄ in 2*N* H₂SO₄, the solution so obtained fails to give the Millon test on addition of NaNO₂ as is well known. If not too much nitrite is added, a yellow substance is obtained soluble in water but not in chloroform, which gives a red colour with excess of HgSO₄, or a purple solution with CuSO₄, even in 2*N* H₂SO₄. It is therefore an *o*-nitrosophenol, though not *o*-nitroso-*p*-cresol itself, since the latter is soluble in chloroform and does not form complexes at so low a pH. If an excess of nitrite is used in the above experiment, diazo compound can be detected in the resulting yellow solution.

This behaviour can be explained on the assumption that the products insoluble in chloroform are mercurated in the nucleus, so that the red substance responsible for the Millon reaction would be a mercurated mercury complex of an *o*-nitrosophenol. The red substance obtained under similar conditions from tryptophan must be different in nature, and when it is decolored with NaCl, the product does not develop a colour with cupric salts.

Lugg [1937] has been led to postulate two classes of *o*-nitrosophenols, one of which gives red mercuric complexes, while the other, obtained from tyrosine and other phenols in absence of mercuric salts, does not. It is clear that the latter derivatives are what we have shown to be diazo compounds. There are, as we have shown above, two classes of nitroso compound, but both give red mercuric complexes under appropriate conditions.

The distinction drawn by Lugg between *o*-quinone monoximes and *o*-nitrosophenols seems now to be unnecessary; we have found no trace of such tautomerism when dealing with free *o*-nitroso-*p*-cresol, and the chelated metallic derivatives have a structure which is a resonance hybrid of the two types.

Diazo compounds

Diazo derivatives are formed with great ease by the action of HNO₂ on tyrosine and *p*-cresol. The one formed from tyrosine is much the more stable against further action of nitrous acid, and is formed under almost all conditions when tyrosine and HNO₂ react between 0 and 40°. Even if only one equivalent of HNO₂ is present, some diazo compound is formed from tyrosine, so that the reaction can compete with the deamination.

We have not isolated the diazo compounds, but they show characteristic colour reactions, by which they may be detected and estimated. They give red or purple coupling products in alkaline solution with such phenols as β -naphthol, resorcinol or phloroglucinol, and, if exposed to light in alkaline solution, form a red substance, which is photosensitive itself, so that the colour shows a maximum with time. This colour has been shown to be due to the coupling of unchanged diazo compound with a catechol derivative formed on irradiation by loss of nitrogen. Such a reaction of *o*-diazophenols has already been observed by Schmidt & Meier [1931]. If irradiated in sufficiently acid solution, these compounds give no colour, but if the irradiated solution is made alkaline, it will couple with fresh diazo compound. Though catechol does not readily couple, homocatechol, and more readily, "dopa" (3:4-dihydroxyphenylalanine) will couple, in alkaline solution, with diazodeaminotyrosine.

The diazo compounds do not couple with monohydric phenols, or with aniline, benzidine or dimethylaniline. They do, however, couple with α -naphthylamine, slowly in the cold, more rapidly on warming, at pH about 3.

The yield of diazo compound from tyrosine, in presence of various concentrations of HNO_2 has been investigated at 0 and 38°; a maximum is found at intermediate concentrations. The following figures give the amount of diazo compound formed in arbitrary units, measured colorimetrically by coupling with resorcinol, in presence of the respective concentrations of NaNO_2 , at pH 3.7. One set of experiments at 0° was carried out with a layer of paraffin floated on the solution (as explained above this increases the NO concentration). The times before sampling were inversely proportional to the nitrite concentrations, so that an equal fraction of the reaction had occurred in each case (about 90 %, as deduced from kinetic measurements).

Final conc. NaNO_2	0°	0° paraffin	38°
2 <i>N</i>	90	—	73
<i>N</i>	95	99	81
<i>N</i> /2	97	99	83
<i>N</i> /4	102	107	81
<i>N</i> /8	100	104	65
<i>N</i> /16	94	95	53
<i>N</i> /32	85	—	—

The results indicate a definite optimal concentration of HNO_2 .

The reaction mechanism by which diazo compounds are formed in this reaction has been determined. Kinetic data, to be given in detail in a later paper, indicate that the nitroso compound is a precursor of the diazo compound. It is known that "nitrous fumes" can convert other nitroso compounds into diazo compounds in ethereal solution, the reaction being

We have shown that *o*-diazo compounds are formed from ethereal or chloroform solutions of the *o*-nitrosophenols in the same way, and the above-mentioned effect of bubbling N_2 supports the view that the reaction is the same in aqueous solution. Ethereal N_2O_3 or NO_2 give no *o*-diazo compounds.

These diazo compounds are extremely stable in absence of excess HNO_2 ; their solutions may be boiled without decomposition, and they do not undergo the Sandmeyer reaction even with HI except at 100° and then slowly. They react rather slowly with excess HNO_2 , forming products whose nature has not been investigated. They may be reduced, presumably to amino or hydrazino derivatives, by the action of TiCl_3 or $\text{Na}_2\text{S}_2\text{O}_4$. A solution of diazopepsin titrated with TiCl_3 was found to require 3.8 atoms of H per diazo group corresponding with a reduction $R.\text{N}_2.\text{OH} \rightarrow R.\text{NH}.\text{NH}_3 + \text{H}_2\text{O}$.

The coupling product of diazocresol and β -naphthol has been isolated. The preparation is as follows.

40 ml. of 0.2 *M* *p*-cresol in water (0.86 g.) and 10 ml. conc. HCl were made up to 1 litre with water, and 10 ml. 4 *N* NaNO_2 added. After 1 hr. the bright yellow solution was made strongly alkaline with 10 *N* NaOH, and 2 g. solid β -naphthol added. After 1 hr. more the solution was extracted with ethyl acetate, the extract washed with dil. NaOH to remove β -naphthol, and then twice with water, and the ethyl acetate dried with CaCl_2 , filtered and evaporated on a water bath; the residual liquid was allowed to evaporate at room temperature; 1.3 g. of crystals

were obtained, this being a yield of 60 %. The product can be purified by sublimation at low pressure, or better by recrystallization from ethyl acetate; m.p. 109° (uncorr.). The substance is generally similar to the phenolazo- β -naphthol described by Niementowski [1902], though of a lower m.p. (phenolazo- β -naphthol melts at 193°). The yield of 60 % shows that, at least at one stage of the reaction between *p*-cresol and HNO_2 , the amount of diazo compound present must be large.

Nitro compounds and condensation products

The study of the other products has been greatly hindered by lack of colour reactions for their detection and estimation. It was thought at first that the product obtained by the action of HNO_2 gassed with N_2 on *p*-cresol might be a nitro compound; but though the product of the reaction was extractable by ether, no definite compound could be isolated. By shaking a dilute ethereal solution of *p*-cresol with an aqueous mixture of acetate buffer and moderately concentrated NaNO_2 and evaporating the separated ether layer a good crop of crystals was obtained. These were recrystallized from ether plus light petroleum and proved to be dinitro-*p*-cresol, m.p. 81°. The same compound was got by the action of mixtures of NaNO_2 and 60–90 % conc. HNO_3 , both from cresol and nitrosocresol. The mononitro compound, m.p. 33°, was obtained by the action of moderately concentrated nitrite and pH 3.7 buffer on very dilute *p*-cresol, 0.5 mg./ml., along with some diazo compound. We think, but cannot prove, that these are all cases of direct nitration by N_2O_4 .

Various attempts were made to produce the nitro compound from the nitroso compound under gentle conditions in acid solution by oxidation, but without success. Titration of a solution of nitroso-*p*-cresol in pH 3.7 buffer with KMnO_4 showed that each mol. took up 6O, which presumably indicated the opening of the benzene ring.

Application to pepsin. Some, but not all, of the tyrosine groups in pepsin can be converted into diazo derivatives or Cu nitroso derivatives, without denaturing the protein or completely inactivating it. This will be described in the succeeding paper.

SUMMARY

1. The first recognizable product of the action of nitrous acid on monophenols is a nitrosophenol.
2. The *o*-nitrosophenol, however, rapidly undergoes further reaction, chiefly with nitric oxide to form the *o*-diazophenol.
3. The *o*-nitrosophenol can be trapped and isolated, via the very stable copper complex.
4. The red product of the Millon reaction is not the simple mercury-*o*-nitrosophenol, but is readily converted into it. The reverse change has not proved possible.

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REFERENCES

- Abel (1930). *Z. phys. Chem. A*, **148**, 337.
Baudisch (1918). *Ber. deutsch. chem. Ges.* **51**, 1058.
Cole (1933). *Practical Physiological Chemistry*, p. 80. (Heffer, Cambridge.)
Gibbs (1926-27). *J. biol. Chem.* **71**, 445.
Lugg (1937). *Biochem. J.* **31**, 1422.
Niementowski (1902). *Chem. Zbl.* 938.
Schmidt & Meier (1931). *Ber. deutsch. chem. Ges.* **64**, 778.
Veibel (1930, 1). *Ber. deutsch. chem. Ges.* **63**, 1577.
—— (1930, 2). *Z. phys. Chem. B*, **10**, 22.

Note added March 1938. The irradiation products of the diazophenols differ from "Dopa" and other catechol derivatives in that they couple much more rapidly with unchanged diazophenols at $pH\ 10.4$, and give less blue colour with Folin's "phenol" reagent at $pH\ 5$ and 100° , and practically no blue colour with arsenomolybdate at $pH\ 3.7$ and 100° . In these respects they resemble resorcinol very closely indeed. Attempts to obtain a fluorescein reaction with them were unsuccessful, possibly owing to insufficient purification; nevertheless it seems possible that they are 1:3- instead of 1:2-hydroxy derivatives.