rigidity as observed by touch was recorded. In most of the animals, death was preceded by a spasmodic stretching of the limbs, and it was thought that a nervous mechanism might play some part in the rigidity, but the experiments on the rats with one denervated limb and on other rats without Myanesin showed that rigor was delayed for only a minute or two. It follows that a fall in ATP concentration is not necessarily a concomitant of the stiffening which immediately follows death by DNOC poisoning. This being so, the physical change observed in skeletal muscle may be paralleled by a similar change in the muscle of the heart and diaphragm. In this connexion it is interesting to note that kymograph recordings of movements of the diaphragm and of the blood pressure of DNOC-poisoned rabbits indicate that respiration and the heart stop almost simultaneously (J. M. Barnes, personal communication).

SUMMARY

1. After lethal doses of 3:5-dinitro-*ortho*-cresol (DNOC), depletion of energy-rich phosphate compounds occurs in skeletal muscle, brain, heart and diaphragm.

2. The extent to which this change takes place in the muscle depends upon the survival time of the poisoned animal, but at all times nearly maximal loss of energy-rich phosphate compounds is found in the brain.

3. The rapid onset of rigor seen after the death of rats poisoned with DNOC is not necessarily associ-

ated with subnormal concentrations of adenosine triphosphate in muscle.

I am grateful to Dr J. M. Barnes for performing the denervation experiment and to Miss G. R. Reynolds for technical assistance.

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Formation of Formylkynurenine by the Action of X-Rays on Tryptophan in Aqueous Solution

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It is well known that the action of X-rays on water leads to the production of H atoms and OH radicals according to the net process (Weiss, 1944, 1947):

and, to a much smaller extent, also to a direct decomposition into molecular hydrogen and hydrogen peroxide (Allen, 1952; Weiss, 1953).

Although OH radicals themselves are known to be strong oxidizing agents, recent work has shown that certain specific oxidation processes appear to take place only if molecular oxygen is present in the

solution, e.g. in the formation, by irradiation with X-rays, of *o*-quinone from phenol (Stein & Weiss, 1951), nitrite from ammonia (Rigg, Scholes & Weiss, 1952), and hydroxylamine from primary aliphatic amines (Jayson, Scholes & Weiss, unpublished work).

Stein & Weiss (1949*a*) have previously also reported the formation of a dialdehydic substance when aqueous benzene solutions are irradiated with a mixed γ -ray neutron source; the opening of the benzene ring which has been observed under these conditions also takes place with X-radiation when

oxygen is present (Daniels, Scholes & Weiss, unpublished work). The biological conversion of tryptophan into kynurenine, under aerobic conditions (cf. Neuberger, 1944; Dalglish, 1951) likewise involves a ring opening, in this case of the indole ring, and this has recently been shown to proceed via the intermediate formation of formylkynurenine (Mehler & Knox, 1950). In the case of *Ephestia* and *Drosophila*, formation of kynurenine from tryptophan was shown to be responsible for the production of eye pigment (cf. Butenandt, 1949).

In view of these observations, it appeared to be of some interest to investigate the effects of X-rays on aqueous solutions of tryptophan, with particular reference to the influence of molecular oxygen. Stenström & Lohmann (1931) have reported that tryptophan is destroyed on irradiation with X-rays. More recently, Proctor & Bhatia (1952) have irradiated tryptophan solutions with high-voltage cathode rays and have measured microbiologically the disappearance of the amino acid. However, no definite chemical changes have been reported by any of these authors.

EXPERIMENTAL

Irradiations. The solutions (100 ml.) contained in Pyrex glass vessels were irradiated with X-rays generated from a Victor Maximar set operating at 200 kv and 15 ma (cf. Farmer, Stein & Weiss, 1949). The dose rate was 2700 r/min. (1.4×10^{17} ev/ml. min.) determined by the oxidation of 0.001 M-FeSO₄ in 0.8 N-H₂SO₄; for the dosimetry the value of 18 Fe³⁺/100 ev was used (Weiss, 1953). All solutions were prepared in triply distilled water, ordinary distilled water being redistilled in an all-glass still from alkaline permanganate and then from phosphoric acid.

Irradiations were carried out in the presence of O₂ and also *in vacuo*. Saturation with O₂ was effected by bubbling the gas through the solution for about 20 min. before irradiation. For complete evacuation, the solutions were pumped out by means of a Hyvac oil pump, shaken for a few min. and finally evacuated with a mercury diffusion pump. After this procedure less than 0.01 ml. non-condensable gas remained. Immediately after irradiation the solutions were concentrated by vacuum evaporation at relatively low temperatures (about 25°) or by freeze-drying. The freeze-drying apparatus used was essentially similar to that described by Holzman (1950). In all cases blank solutions were treated in an identical manner.

Paper chromatography. We were essentially concerned with the identification of small quantities of kynurenine and formylkynurenine in the presence of a large excess of tryptophan.

Of the several solvent systems examined, ethanol:sulphuric acid:water (160:1:39 v/v; Hellmann, 1951a) proved to be the most effective for the separation of kynurenine under these conditions. The chromatograms were run at 36° using Whatman no. 1 paper and the ascending technique described by Hellmann (1951b), at least 30 cm. solvent travel being allowed in all cases; the *R_F* values of tryptophan and kynurenine were 0.85 and 0.72, respectively. Since in the solvent containing sulphuric acid formyl-

kynurenine was hydrolysed to kynurenine, ethanol:acetic acid:water (155:20:25, v/v) was used for the detection of the formyl derivative, the chromatograms being run at room temperature. Although the separation was not particularly good in this solvent (*R_F* values for formylkynurenine and tryptophan were 0.20 and 0.23, respectively, formylkynurenine could be readily distinguished by the methods described below).

Paper chromatographic separation of mixtures of both kynurenine and oxindolylalanine (DL- α -amino- β -oxindol-3-ylpropionic acid) from relatively large quantities of tryptophan has been attempted but, as yet, no satisfactory result has been achieved. In the sulphuric acid solvent oxindolylalanine runs to almost the same position as kynurenine and is therefore obscured by it. In the absence of kynurenine, however, oxindolylalanine can be readily detected on the chromatograms.

Two reagents were used for the detection of the substance on the paper, (a) the Ehrlich reagent (a 2% (w/v) solution of *p*-dimethylaminobenzaldehyde in 20% (w/v) HCl (Hellmann, 1951a)) which gives an orange colour with kynurenine and formylkynurenine, a pale yellow with oxindolylalanine and a violet colour, changing to green, with tryptophan, and (b) the Ekman reagent (diazotization followed by coupling with ethyl- α -naphthylamine (Ekman, 1948; Hellmann, 1951a)) which gives a carmine red with kynurenine and with formylkynurenine. Known quantities of the substances were chromatographed and the limits of detection determined. As little as 1 μ g. of kynurenine or formylkynurenine could be detected by means of the Ekman reagent; the Ehrlich reaction was somewhat less sensitive, about 5 μ g. being the lower limit. (More recently it has been pointed out to us by Dr C. E. Dalglish that if the Ehrlich reagent is made with less acid (e.g. 5% HCl), kynurenine can be detected in much smaller amounts than 5 μ g.) Oxindolylalanine, on the other hand, owing to its weak Ehrlich reaction, could not be readily detected in quantities of less than about 15 μ g. In the actual irradiation experiments these limits corresponded to about 0.2 μ mole formylkynurenine (or kynurenine) and about 15 μ moles oxindolylalanine/100 ml.

The identity of the substances on the chromatograms were, in some cases, confirmed by their absorption spectra. For this purpose the spots were eluted with 5 ml. phosphate buffer (0.02 M Sørensen buffer, pH 7.2) and the spectra recorded between 230 and 400 μ . by means of a Unicam Spectrophotometer. The spots were located by markers and also by their fluorescence in ultraviolet light in which formylkynurenine and kynurenine exhibit a blue fluorescence.

Hydrolysis of the eluates was carried out in N-HCl at 100° for 10 min. (Mehler & Knox, 1950). The cooled solutions were neutralized with NaOH and again buffered before spectroscopic analysis.

RESULTS

Irradiation of tryptophan

(1) *In the presence of oxygen.* Unbuffered 0.1% (w/v) DL-tryptophan (Roche Products Ltd.) solutions (100 ml.) were irradiated with a total dose of 1.6×10^6 r. After irradiation the solutions exhibited a yellow colour which intensified on standing (cf.

Proctor & Bhatia, 1952). In the preliminary experiments the solutions were concentrated to a small volume by evaporation under reduced pressure. The resulting dark brown concentrate was then subjected to chromatographic analysis, using the ethanol:water:sulphuric acid solvent. Examination of the dried chromatogram under ultraviolet light showed that a considerable number of fluorescent substances had been formed on irradiation. The R_f value of one of the fluorescent spots corresponded to that of kynurenine and gave the colour reactions of this substance with both the Ekman and Ehrlich reagents. The absorption spectrum of the eluted spot was identical with that of kynurenine, except that the second maximum was at 355 m μ . instead of at 360 m μ . However, it was found, confirming an observation of Dalglish (1952), that an authentic specimen of kynurenine run on the chromatogram also showed the same depression of this maximum.

The presence of hydrogen peroxide in the irradiated tryptophan solutions was shown by means of the titanium sulphate reagent (Eisenberg, 1943). In view of the possibility that the observed kynurenine may have arisen from some oxidation by the hydrogen peroxide during the concentration of the solution, the irradiated solutions were concentrated by freeze-drying, a process in which the hydrogen peroxide could be completely removed from the system at low temperature. However, chromatographic analysis of the freeze-dried material still showed the presence of kynurenine, thus confirming that this substance was not due to any such secondary attack. In addition, blank experiments were carried out in which tryptophan and hydrogen peroxide (in concentrations equivalent to those present during irradiation) were evaporated together: no formylkynurenine or oxindolylalanine could be detected in the residues. Concentration by freeze-drying was used in all but the preliminary experiments.

Since it was found that formylkynurenine was hydrolysed to kynurenine on chromatograms run in the solvent which contained sulphuric acid it had to be ascertained which of these compounds was, in fact, the initial product of the irradiation. The freeze-dried irradiated tryptophan mixture was therefore chromatographed in an acetic acid solvent, in which it had been shown that no decomposition of formylkynurenine took place. The fluorescent area which gave the Ekman reaction was eluted and absorption spectra taken before and after acid hydrolysis. From the fact that the absorption band at 355 m μ . appeared only after acid hydrolysis, we have concluded that formylkynurenine is a product of the irradiation of tryptophan solutions in the presence of oxygen.

The yield of formylkynurenine was determined

by measuring the amount of kynurenine formed by acid hydrolysis, i.e. the increase in absorption at 355 m μ . A known volume of the concentrated irradiated mixture was run in the acetic acid solvent, and optical densities of the eluates taken before and after hydrolysis in N-HCl for 10 min. The amount of formylkynurenine formed was 2.5 μ moles/100 ml., corresponding to a yield G (molecules of formylkynurenine formed per 100 ev), of the order, $G \approx 0.2$.

Owing to the experimental difficulties described above, it was impossible to determine with certainty whether or not oxindolylalanine was formed in the irradiation. It was, however, established by separate experiments that, had any oxindolylalanine been formed, it would not have decomposed during the working-up procedure.

(2) *In vacuo*. Evacuated 0.1% (w/v) unbuffered tryptophan solutions were irradiated with a total dose of 1.3×10^6 r; only an extremely faint yellow coloration was produced. The solutions were freeze-dried and chromatographed in both the sulphuric and acetic acid solvents. No kynurenine or formylkynurenine could be detected by colour reactions or by ultraviolet spectroscopy; the Ehrlich reaction for oxindolylalanine was also negative.

Irradiation of oxindolylalanine

Unbuffered oxindolylalanine solutions (0.03%, w/v) were saturated with oxygen and irradiated with a dose of 1.3×10^6 r. As in the tryptophan experiments a yellow coloration was produced. The solutions were freeze-dried and chromatographed in both solvent mixtures. The dried chromatograms when examined under ultraviolet light, showed a somewhat different fluorescence pattern from those of the irradiated tryptophan chromatograms; a very faint Ekman reaction was observed, but the amounts giving this reaction were too small for any further identification procedure.

DISCUSSION

The chemical action of ionizing radiations in dilute aqueous solutions can be attributed to the free OH radicals and H atoms formed by the splitting of the water molecules according to equation (1). The G value for the production of radicals is about 4 (Weiss, 1953). It has been found that G for the formation of formylkynurenine, by irradiation of tryptophan in the presence of oxygen, is only of the order of about 0.2. The radicals, therefore, evidently attack more than one site in the tryptophan molecule: in fact, the relatively complicated nature of the paper chromatograms indicates that a number of different reactions take place during the irradiation. One such process results in the formation of

ammonia (under the above experimental conditions with a yield of $G_{\text{NH}_3} \approx 0.45$) and this must involve attack on the alanine side chain, it being well known that amino acids, in general, liberate ammonia on irradiation of their aqueous solutions (cf. Stein & Weiss, 1949b; Dale, Davies & Gilbert, 1949). Further, it is very likely that the free radicals will also attack the aromatic ring, leading to the formation of phenolic compounds (cf. Stein & Weiss, 1949c); in fact, there were definite indications that phenolic compounds were formed.

Regarding the mechanism of the formation of formylkynurenine it seemed of interest, in the first place, to ascertain whether this involved intermediate formation of oxindolylalanine. This possibility could not be ruled out, since it could not be established with certainty whether or not this substance was absent from the irradiated solutions. However, we were able to show that no significant amount of formylkynurenine was formed when oxindolylalanine itself was irradiated; it may, therefore, be concluded that this substance cannot act as an intermediate in this process.

Under the conditions described above, it has been shown that the presence of molecular oxygen is necessary for the formation of formylkynurenine. In general, the presence of oxygen during irradiation can have a twofold effect: (a) the formation of hydroperoxy radicals, following the initial process (1) viz.

(b) the reaction of oxygen with an organic radical (R) to produce an organic peroxy radical (cf. Johnson, Scholes & Weiss, 1953), viz.

It is not unlikely, therefore, that an organic hydroperoxide (RO_2H) may eventually be formed. In the case of tryptophan (I) the hydroperoxide resulting from initial attack by OH radicals at the 2-3 bond of the indole ring could decompose to give formylkynurenine (II) according to the above scheme.

Reaction c, i.e. conversion of the organic peroxy radical into the corresponding hydroperoxide, could result, for example, from dismutation between two peroxy radicals (cf. Johnson *et al.* 1953).

It is of interest to compare our findings with those of the biological oxidation of tryptophan. Recent experiments have shown that oxindolylalanine is not an intermediate in the *in vivo* conversion of tryptophan into formylkynurenine (Sakan & Hayaishi, 1950; Dalglish, Knox & Neuberger, 1951). It may be of some significance, therefore, that the irradiation of oxindolylalanine itself does not give kynurenine.

SUMMARY

1. It has been shown that formylkynurenine is one of the products formed when DL-tryptophan is irradiated with X-rays in aqueous solution in the presence of molecular oxygen.

2. It has been concluded that oxindolylalanine (α -amino- β -oxindol-3-ylpropionic acid) is not an intermediate in the reaction mechanism since irradiation of this substance itself does not lead to any significant amounts of formylkynurenine.

3. The mechanism of these processes has been discussed, with particular reference to the role played by molecular oxygen, and attention has been drawn to the similarity to the biological oxidation of tryptophan.

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Transglucosidation Reactions with Flavins

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The enzymic formation of riboflavinyl glucoside by a transfer of glucose from maltose to riboflavin was described by Whitby (1952), and the reaction may be written thus:

It was concluded that a transglucosidase catalysed this reaction, and the characteristics of this enzyme, particularly with regard to its specificity, have now been investigated in an attempt to assess the physiological significance of riboflavinyl glucoside.

EXPERIMENTAL

Materials

D-Riboflavin (Roche Products, Ltd.) was used, and riboflavinyl glucoside was prepared enzymically (Whitby, 1952). D- and L-Arabinoflavin and the 9-substituted-isoalloxazines used in this work were the generous gift of Prof. P. Karrer; galactoflavin, L-lyxoflavin and isoriboflavin were kindly supplied by Dr M. Tishler. Flavin solutions were at all times protected from light as much as possible.

6:7-Dimethyl-9-(5'-[β -D-glucopyranosyl]-D-ribityl)-isoalloxazine and 6:7-dimethyl-9-(4'-[α -D-glucopyranosyl]-D-glucityl)-isoalloxazine have been synthesized (Plant, 1952); small amounts of these two compounds, riboflavinyl β -D-glucoside and maltoflavin, respectively, were kindly provided by Dr Plant.

α -D-Glucose 1-phosphate (dipotassium salt) was prepared by the method of Hanes (1940).

Methyl maltoside was prepared by refluxing maltose with methanolic HCl (Fischer, 1893, 1895). Other carbohydrates and derivatives were commercial samples, except for melezitose and turanose, kindly supplied by Dr D. J. Bell, and isomaltose octaacetate, maltulose and panose which were kindly provided by Dr W. J. Whelan.

isoMaltose octaacetate (15 mg.) was saponified with sodium methoxide under conditions similar to those used by Wolfrom, Georges & Miller (1949); the methanolic solution was diluted with water at 0°, immediately neutralized with HCl, and the methanol removed *in vacuo* at 35°. The solid

residue was dissolved in water and the solution used for enzymic tests; it showed only one spot on chromatograms which had been developed with butanol:acetic acid:water (4:1:5, v/v/v) and sprayed with aniline hydrogen phthalate (Partridge, 1949), the spot having $R_F = 0.10$, compared with 0.12 for maltose.

Phosphate buffers were prepared with K_2HPO_4 and $NaH_2PO_4 \cdot 2H_2O$.

Enzyme preparation. An acetone powder of rat liver was made by the method of Whitby (1952), and the following manipulations were all carried out in a cold room at 2°. The powder (40 g.) was extracted for 20 min. in a mortar with 400 ml. of 0.1 M phosphate buffer, pH 7.4, and the suspension centrifuged for 15 min. at 1800 g; the supernatant was collected, the centrifuge extracted a second time with 400 ml. of buffer for 20 min., centrifuged, the supernatants combined (728 ml.) and the solid residue discarded. M-Phosphate buffer (182 ml.), pH 7.4, and 131 g. of $(NH_4)_2SO_4$ were added. After 30 min. the precipitate was collected by centrifuging at 1800 g for 10 min. and was discarded. $(NH_4)_2SO_4$ was added to increase the saturation from 0.25 to 0.55 and the precipitate was collected 1 hr. later by centrifuging for 5 min. at 15000 g (Servall Centrifuge); the supernatant was discarded. The precipitate was dissolved in 50 ml. of 0.05 M phosphate buffer, pH 7.4, and dialysed overnight with stirring against 4 l. of 0.05 M phosphate buffer, pH 7.4. The dialysed solution was frozen at -20° and dried *in vacuo* over H_2SO_4 and NaOH. The powder weighed 7.3 g. and is henceforth referred to as 'the enzyme'; its activity did not deteriorate over 6 months when stored *in vacuo* over $CaCl_2$. Attempts at further purification of the enzyme by preferential thermal inactivation of contaminating protein, with and without the addition of maltose to protect the enzyme, by adsorption and elution from calcium phosphate gel, by precipitation below pH 5.5, etc., have not been successful; however, this impure enzyme has proved to be satisfactory for the following experiments.

Methods

The pH was measured with a Cambridge pH meter (Cambridge Instrument Co.). The instrument was equilibrated at 35° for 2 days before determining the pH/activity relationship for synthesis of riboflavinyl glucoside.