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ISOLATION AND STRUCTURE OF THE FLUORESCENT SUBSTANCES FORMED IN THE OXIDATIVE REACTION OF ADRENALINE AND NORADRENALINE WITH ETHYLENEDIAMINE

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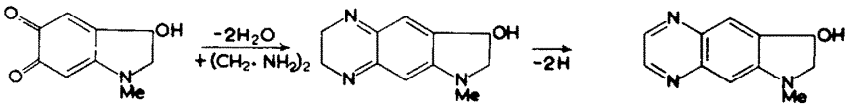
Abstract—One of the fluorescent products formed in the oxidative reaction of adrenaline with ethylenediamine has been characterized as 2,3-dihydro-3-hydroxy-1-methylpyrrolo-4,5-g quinoxaline (III). In the case of noradrenaline, the only fluorescent product found has been characterized as 1,2,3,4-tetrahydro-1,4,5,8-tetra-aza-anthracene (VII). The same tetrahydrotetra-aza-anthracene is also obtained from catechol, 2-methylnoradrenaline, adrenalone and 3,4-dihydroxymandelic acid. The chemistry and mechanism of formation of III and VII are discussed.

THE formation of a stable fluorescent compound by the reaction of adrenaline with ethylenediamine in the presence of air was demonstrated by Natelson *et al.*[1] Based on this reaction, a method for the determination of adrenaline and noradrenaline has been developed by Weil-Malherbe and Bone.[2] Further investigation by Burn and Field[3] has indicated that the reactions give rise to more than one product in each case, and an attempt to isolate these products and elucidate the chemistry involved seemed desirable.

The case of adrenaline was first examined. Weil-Malherbe and Bone had shown that the reaction proceeds via the formation of adrenochrome (I), and accordingly we oxidized adrenaline with silver oxide in methanol to adrenochrome and treated this solution with an excess of ethylenediamine. After dilution with water, extraction with ethyl acetate and concentration, chromatography on alumina gave a small (7 per cent), but easily reproducible, yield of a highly crystalline yellow material exhibiting an intense green fluorescence in solution. Elementary analysis indicated the formula $C_{11}H_{11}ON_3$, thus showing that the compound was formed from one mole each of adrenochrome and ethylenediamine with the loss of two moles of water and two hydrogen atoms. Moreover, the compound was optically active, showing that the secondary hydroxyl group was unaffected, and its u.v. spectrum (Fig. 1) resembled that of 7-aminoquinoxaline and showed a similar bathochromic shift on acidification.[4] It was therefore assigned the structure (—)-2,3-dihydro-3-hydroxy-1-methylpyrrolo-4,5-g-quinoxaline (III), and we suggest that it is formed via II as an intermediate. The same material was also obtained, though in lower yield, by the autoxidation of adrenaline in aqueous solution in the presence of ethylenediamine. In cold dilute mineral acids, the compound gives deep red non-fluorescent solutions, and is recovered unchanged on basification. However, if the acid solution is boiled

[1] S. Natelson, J. K. Lugovoy and J. B. Pincus, *Arch. Biochem. Biophys.* **23**, 157 (1949).
[2] H. Weil-Malherbe and A. D. Bone, *Biochem. J.* **51**, 311 (1952); **58**, 132 (1954); **67**, 65 (1957).
[3] G. P. Burn and E. O. Field, *Nature, Lond.* **178**, 542 (1956).
[4] A. R. Osborn, K. Schofield and L. N. Short, *J. Chem. Soc.* 4191 (1956).

for a few minutes and then basified, a less soluble dehydration product is obtained. This is the fully aromatic pyrrolo-quinoxaline (IX), and this dehydration can also be effected with acetic anhydride.

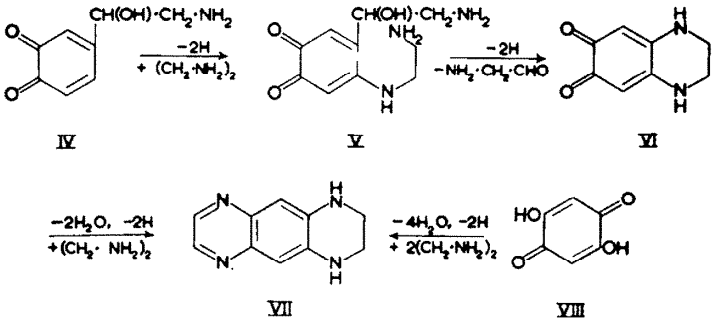


I II III

Run on paper (butanol–water), III has R_F of 0·69, identical with that of the fast-running green fluorescent spot described by Burn and Field. These authors also showed that a butanol extract of the oxidation mixture gave another slower-running spot ($R_F = 0{\cdot}37$) with a yellow fluorescence. This material was absent from our ethyl acetate extracts, but on repetition of our experiments using butanol as extractant we also observed this second product in addition to III. This material proved very intractable to chemical treatment and we have not as yet been able to obtain it in pure form. However, we have been able to show that it is not identical with adrenolutine (3,5,6-trihydroxy-1-methylindole) and that it is not a reaction product of adrenolutine and ethylenediamine.

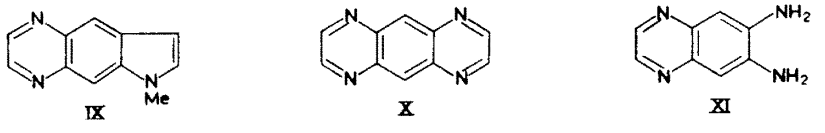
Attention was next turned to noradrenaline. The conditions reported by Burn and Field as optimal (autoxidation at 50°–70° and pH 11) were adopted, and an ethyl acetate extract was chromatographed on alumina as before, yielding a yellow crystalline substance exhibiting an intense green fluorescence in water and ethanol and a blue fluorescence in ethyl acetate. Elementary analysis indicated the empirical formula $C_5H_5N_2$ and this proved at first difficult to interpret, since a compound of such composition cannot be simply derived from the reactants. Weil-Malherbe and Bone observed that under their estimation conditions catechol gave a strong fluorescence, and on repetition of this work on a preparative scale we found that *the same product* (U.V. and I.R. spectrum and behaviour on heating) *is obtained from both catechol and noradrenaline.* We then found that this material can readily be obtained in quantity by the reaction of 2,5-dihydroxybenzoquinone (VIII) with ethylenediamine in hot aqueous solution in the presence of air. This clearly indicated that the empirical formula should be doubled to $C_{10}H_{10}N_4$, and that a tetrahydrotetra-aza-anthracene structure was probable, though the distribution of the hydrogen atoms in this system was not immediately clear, since at least ten alternative possibilities can be written. This matter was settled as follows: 6,7-diaminoquinoxaline (XI) was synthesized by treating 1,2,4,5-tetra-aminobenzene with glyoxal bisulphite. The U.V. spectra of this yellow fluorescent compound and of the tetrahydrotetra-aza-anthracene are given in Fig. 2, and the U.V. spectra of their colourless diacetyl derivatives in Fig. 3. The very close correspondence displayed makes it quite clear that our product is 1,2,3,4-tetrahydro-1,4,5,8-tetra-aza-anthracene (VII). The formation of this compound from catechol via *o*-benzoquinone and an intermediate such as VI does not present any particular chemical difficulties, following a not unusual pattern of quinone–amine condensation, and the same is true of its formation from 2,5-dihydroxybenzoquinone.

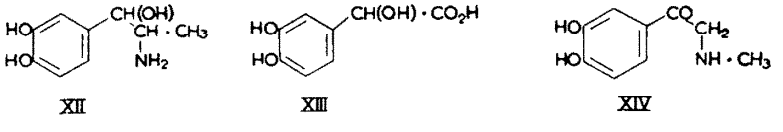
However, the formation of VII from noradrenaline is highly unexpected and requires comment. We interpret the reaction as follows: the first stage is the formation



of the *o*-quinone (IV). In the case of adrenaline, cyclization by intramolecular amine-quinone condensation occurs extremely rapidly leading to adrenochrome, but in the noradrenaline case this intramolecular process is slow, possibly due to the lower basicity of the primary amine function. Thus in the noradrenaline case a competing intermolecular process involving reaction with the ethylenediamine (present in large excess) occurs, leading to V. This intermediate then undergoes a further cyclization in which the side-chain is split off by a retro–aldol reaction giving VI, which then reacts further as in the catechol case above.

The above suggested mechanism is easily susceptible of test, since (a) other N-substituted noradrenalines should give products of the type of III and should not give VII; (b) any 4-substituted catechol with a side-chain hydroxyl *alpha* to the benzene ring (other than adrenaline types) should give VII irrespective of the nature of the side-chain; and (c) a 4-substituted catechol without a side-chain hydroxyl in the *alpha* position should not give VII, since elimination of the side-chain by a retro–aldol process would not then be possible. Each of these points has been verified experimentally as follows: (a) N-isopropylnoradrenaline (Isoprenaline) gave a crystalline product whose U.V. spectrum was almost superposable on that of III, and was thus clearly its isopropyl analogue; (b) 2-methylnoradrenaline (XII, Corbasil) and 3,4-dihydroxymandelic acid (XIII) both gave VII in yields comparable to those obtained from noradrenaline; (c) 4-methylcatechol and 3,4-dihydroxyphenylethylamine both gave a mixture of fluorescent products, none of which was identical (paper chromatography) with VII.





Run on paper (butanol–water), the tetrahydrotetra-aza-anthracene had $R_F = 0{\cdot}65$, corresponding very closely to the fast-running product ($R_F = 0{\cdot}64$) reported by

Burn and Field. These authors also reported a slower-running ($R_F = 0{\cdot}24$) weak spot derived from butanol extracts of noradrenaline–ethylenediamine reactions mixtures, but this we were unable to find. Although stable in the solid state, the tetrahydrotetra-aza-anthracene is photo-sensitive in solution, and on illumination the fluorescence disappears and the initial bright yellow colour becomes pale brown. This effect—presumably a result of photo-oxidation—is extremely marked in very dilute aqueous solution, the fluorescence disappearing in a few minutes on exposure to bright sunlight. This is undoubtedly the explanation of the findings of Goldfien and Karler[5] concerning the sensitivity to light of the noradrenaline estimation. These authors also noted that the adrenaline estimation was not light-sensitive and in agreement with this we find that solutions of the pyrrolo-quinoxaline (III) are quite stable to illumination and the fluorescence does not decay. A further consequence is that paper-chromatography of VII should be conducted in the dark.

We also investigated the behaviour of adrenalone (XIV) in the ethylenediamine reaction. In this case reaction proceeded very slowly and 5 hr heating at 90° was required to produce any substantial amount of fluorescent material. We isolated the product and found again that it was the tetrahydrotetra-aza-anthracene (VII). This was of interest, since the formation mechanism discussed above is not applicable in this case, and we suggest that the reaction here involves slow oxidative degradation of the side-chain ending with protocatechuic acid, which then gives *o*-benzoquinone with loss of carbon dioxide. We indeed confirmed that protocatechuic acid does readily and rapidly give VII with ethylenediamine and this finding is not surprising in view of the fact that the acid is known to give *o*-benzoquinone with decarboxylation on chemical oxidation. It may be noted in passing that on mechanistic grounds adrenalone might be expected to cyclize and give adrenolutine on oxidation, but numerous attempts to achieve this[6] proved unsuccessful, though the reasons for failure are not obvious.

Under normal conditions of oxidation, no trace of VII could be found in experiments with adrenaline; however, in one autoxidation experiment in which the mixture had been heated for 3 hr and then left standing for several days, a small amount of VII was isolated in addition to a larger amount of the normal product (III). We suggest that under these conditions, a small amount of adrenaline is autoxidized to adrenalone rather than to adrenochrome, and it is this adrenalone which gives rise to VII as above. It may also be noted that the noradrenaline analogue of III was never encountered in any of our experiments, and does not appear to be formed at all.

The chemistry of the tetrahydrotetra-aza-anthracene (VII) was further examined. On heating, the material does not melt, but decomposes at about 300° giving a small amount of almost colourless crystalline sublimate. This material gave analytical figures corresponding to a molecular formula of $C_{10}H_6N_4$ and its U.V. spectrum resembled that of phenazine. The same product was obtained in better yield when the tetrahydro compound was dehydrogenated with palladium–charcoal in boiling nitrobenzene. Evidently this product was fully aromatic 1,4,5,8-tetra-aza-anthracene (X), and formed almost colourless long needles giving pale yellow non-fluorescent solutions in water and ethanol and orange-yellow solutions in mineral acids. The

[5] A. Goldfien and R. Karler, *Science* **127**, 1292 (1958).
[6] J. D. Bu'Lock and J. Harley-Mason, unpublished.

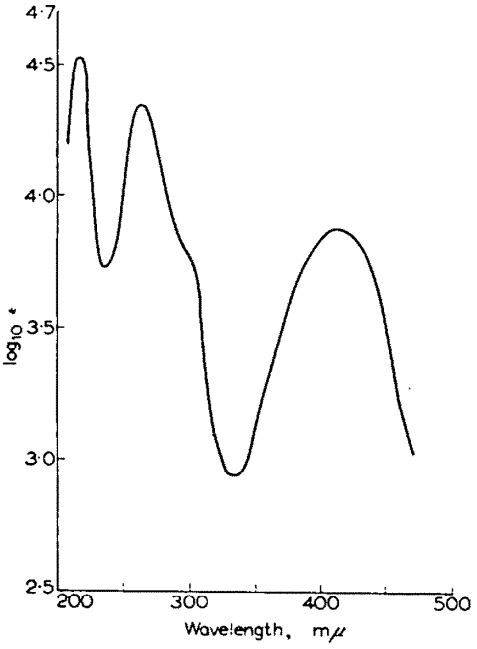


FIG. 1. Ultra-violet absorption spectrum of 2,3-dihydro-3-hydroxy-1-methylpyrrolo-4,5-g-quinoxaline (III).

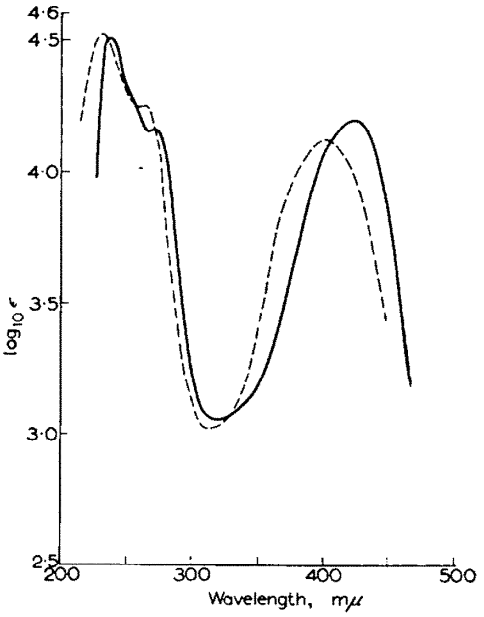


FIG. 2. Ultra-violet absorption spectra of:
——— 1,2,3,4-tetrahydro-1,4,5,8-tetra-aza-anthracene (VII).
- - - - 6,7-diaminoquinoxaline (XI).

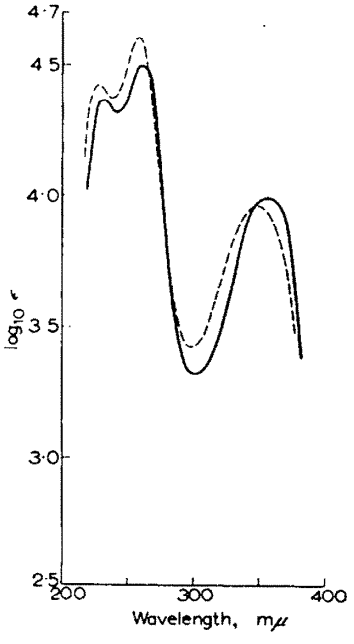


FIG. 3. Ultra-violet absorption spectra of:
——— 1,4-diacetyl-1,2,3,4-tetrahydro-1,4,5,8-tetra-aza-anthracene.
- - - - N N′-diacetyl-6,7-diaminoquinoxaline.

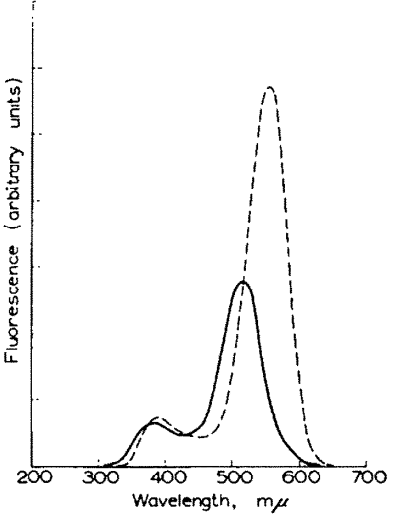


FIG. 4. Fluorescence spectra of:
——— 1,2,3,4-tetrahydro-1,4,5,8-tetra-aza-anthracene (VII). (Activation 295 mμ.)
- - - - 2,3-dihydro-3-hydroxy-1-methylpyrrolo-4,5-g-quinoxaline (III). (Activation 285 mμ.)

ethanolic solution was photo-sensitive, like that of the tetrahydro compound, but in this case, on illumination, a yellow colour and an intense green fluorescence developed. The tetrahydro compound dissolves in mineral acids giving deep red non-fluorescent solutions, and a dark red dihydrochloride was characterized. Fluorescence spectra of III and VII are given in Fig. 4.

A preliminary account of some of this work has been given earlier.[7]

EXPERIMENTAL

Ultra-violet spectra were determined in 95 per cent ethanol using a Cary recording spectrophotometer. Melting points are uncorrected.

Oxidative reaction of adrenaline with ethylenediamine

(a) L-Adrenaline (1 g) was dissolved in methanol (150 ml) containing formic acid (1 ml). Silver oxide (3 g) was added, and the mixture shaken for 7 min. The red solution of adrenochrome thus obtained was then filtered, and ethylenediamine (7 ml) added. After standing for 10 min, the solution was diluted with water (500 ml) and extracted with ethyl acetate (15 × 100 ml). The combined extracts were dried (Na_2SO_4), concentrated to small bulk, and chromatographed on an alumina column (15 cm × 1 cm^3). Elution of the bright yellow band with ethyl acetate–methanol (9 : 1), and evaporation of the eluate gave a yellow solid. Recrystallization from a small volume of ethyl acetate gave (−)-2,3-*dihydro*-3-*hydroxy*-1-*methylpyrrolo*-4,5 g-*quinoxaline* (III) (80 mg) as fine yellow needles, m.p. 171–172°, $[\alpha]_D^{20°} = -80°$ (ethanol, *c*, 0·7) (Found: C, 65·2; H, 5·6; N, 20·7. $C_{11}H_{11}ON_3$ requires: C, 65·6; H, 5·6; N, 20·9%). The product is readily soluble in water and the common organic solvents giving bright yellow solutions with an intense green fluorescence. The R_F in n-butanol–water was 0·69.

(b) L-Adrenaline (200 mg) was dissolved in dilute H_2SO_4 (1 ml) and an aqueous solution of ethylenediamine (5%; 100 ml) added. The solution was heated at 60° for 1·5 hr while a stream of air was drawn through it. After cooling, the mixture was extracted with ethyl acetate and worked up as before to give 15 mg of the same product.

(c) L-Adrenaline (200 mg) was treated as above, but in this case the reaction mixture was extracted with n-butanol (5 × 30 ml) instead of ethyl acetate. The butanol was removed *in vacuo* and the gummy residue was boiled with ethyl acetate (5 ml) and filtered. The filtrate was chromatographed on alumina as before, but in this case two yellow zones appeared. The faster-running material was eluted with ethyl acetate–ethanol (95 : 5) and proved to be the above pyrroloquinoxaline. The slower-running material was eluted with ethanol, and removal of the solvent gave a yellow amorphous product giving solutions displaying a strong yellow fluorescence. Further attempts at purification were unsuccessful. Run on paper (butanol–water) the material gave a rather diffuse spot of R_F approx. 0·36. A red colour was given with Ehrlich's reagent and a transient red colour with HCl. Light-absorption max, 262, 360 and 420 mμ.

1-*Methylpyrrolo*-4,5-g-*quinoxaline* (IX)

The foregoing hydroxy compound (25 mg) was dissolved in 3N HCl (2 ml), and the deep red solution was boiled for 3 min, cooled, and made alkaline with NaOH. The precipitated solid was collected, recrystallized from water, and then sublimed at 90°/10^{-4}mm, giving 1-*methylpyrrolo*-4,5-g-*quinoxaline* (20 mg) as yellow needles, m.p. 141–142°. (Found: C, 72·4; H, 4·9. $C_{11}H_9N_3$ requires: C, 72·1; H, 4·9%). The same product was obtained on boiling the hydroxy compound with acetic anhydride for 15 min. Light-absorption max, 226, 266, 352 and 410 mμ (ε, 24,000, 30,000, 8000 and 2000 respectively).

Oxidative reaction of noradrenaline with ethylenediamine

L-Noradrenaline bitartrate (250 mg) was dissolved in a 5% aqueous solution of ethylenediamine (125 ml) adjusted to pH 11 with H_2SO_4. The mixture was heated to 60° for 2 hr while a stream of air was drawn through it. The cooled solution was extracted with ethyl acetate (6 × 50 ml), the extracts dried (Na_2SO_4), and concentrated to small bulk. The concentrate was chromatographed on an alumina column (10 cm × 1 cm), the yellow band eluted with ethyl acetate, and the eluate

[7] J. Harley-Mason and A. H. Laird, *Biochem. J.* **69**, 59P (1958).

evaporated to give a yellow solid. This was recrystallized from ethanol giving 1,2,3,4-*tetrahydro*-1,4,5,8-*tetra-aza-anthracene* (VII, 40 mg) as small yellow plates (Found: C, 64·2; H, 5·6; N, 29·6. $C_{10}H_{10}N_4$ requires: C, 64·5; H, 5·4: N, 30·1%). The compound decomposes without melting at about 300° giving a sublimate of almost colourless needles (see below). The compound is soluble in water, ethanol and methanol giving yellow solutions with a powerful green fluorescence. In less polar solvents such as ethyl acetate, the fluorescence is blue. Treatment with ethanolic HCl gave a *dihydrochloride*, deep red prisms from aqueous ethanol (Found: C, 45·9; H, 4·3; N, 20·8. $C_{10}H_{10}N_4$. 2HCl requires: C, 46·4; H, 4·6; N, 21·6%). Light-absorption max, 262 and 478 mμ (ε, 39,000 and 25,000 respectively).

Boiling with acetic anhydride gave a *diacetyl derivative* which formed white needles from ethanol, m.p. 244–245° (Found: C, 62·3; H, 5·3; N, 20·7. $C_{14}H_{14}O_2N_4$ requires: C, 62·2; H, 5·2; N, 20·8%).

Other reactions leading to 1,2,3,4-*tetrahydrotetra-aza-anthracene*

(a) When the above experiment was repeated using 2-methyl noradrenaline and 3,4-dihydroxymandelic acid the same product was obtained in comparable yield in each case.

(b) Using adrenalone the above experiment was repeated except that the temperature was raised to 90° and the heating continued for 6 hr. The tetrahydrotetra-aza-anthracene was obtained as before.

(c) 2,5-Dihydroxy-*p*-benzoquinone (4·7 g) was dissolved in boiling water (300 ml), and ethylenediamine (10 ml) was added. The mixture was heated at 95° for 1 hr while a stream of air was drawn through it. After cooling, the brownish-yellow solid was filtered off and twice recrystallized from aqueous ethanol, giving the tetrahydrotetra-aza-anthracene (3 g; 50%). This is the most convenient method of preparation.

(d) A solution of catechol (12·5 g) and ethylenediamine (51 g) in water (1·0 l.) was heated at 50° for 2·5 hr while a stream of air was drawn through it. After cooling, the brown solution was extracted with ethyl acetate (5 × 100 ml), the combined extracts dried (Na_2SO_4), and the solvent removed, leaving a yellow solid. This was dissolved in dilute HCl (75 ml) and extracted five times with ether to remove unreacted catechol. The red aqueous phase was basified and then extracted with ethyl acetate (5 × 50 ml). Evaporation of this extract gave the tetrahydrotetra-aza-anthracene (50 mg).

1,4,5,8-*Tetra-aza-anthracene* (X)

(a) The tetrahydro compound (1·5 g) was refluxed in nitrobenzene (250 ml) with 10% Pd-charcoal (1 g) for 4 hr. After cooling and filtering, water was added and the mixture steam-distilled for 6 hr to remove nitrobenzene. The aqueous solution was concentrated, yielding a crude product which on recrystallization from water (charcoal) gave 1,4,5,8-*tetra-aza-anthracene* (0·5 g) as long, very pale yellow needles, m.p. 215–216°. For analysis, a sample was sublimed at 160°/10^{-3}mm (Found: C, 65·6; H, 3·0; N, 30·7. $C_{10}H_8N_4$ requires: C, 65·8; H, 3·3; N, 30·8%). Light-absorption max, 243 and 353 mμ (ε, 70,000 and 10,000 respectively). The compound is soluble in water and ethanol giving almost colourless non-fluorescent solutions; however, on exposure to light the solutions slowly turn yellow and acquire a green fluorescence.

(b) The tetrahydro compound (75 mg) was placed in a long glass tube heated in a sublimation block. When the temp was raised to 300°, the tetra-aza-anthracene (10 mg) sublimed out of the decomposing material as long needles, m.p. 215–216°.

6,7-*Diaminoquinoxaline* (XI)

To a solution of 1,2,4,5-tetra-aminobenzene hydrochloride[8] (0·5 g) and glyoxal bisulphite (0·5 g) in water (7 ml), solid $NaHCO_3$ was added until no more CO_2 was evolved. After warming briefly and then cooling, a yellowish-red crystalline solid separated, and was at once collected and recrystallized from water (charcoal), giving 6,7-*diaminoquinoxaline* (30 mg) as fine yellow needles, m.p. >360° (Found: C, 59·3; H, 5·4; N, 35·0. $C_8H_8N_4$ requires: C, 60·0; H, 5·0; N, 35·0%). Boiling with acetic anhydride gave the diacetyl derivative which formed colourless needles, m.p. 279–280° from ethanol.

Acknowledgements—The authors thank Professor R. T. Williams for permission to use the spectrophotofluorimeter at St. Mary's Hospital, London. One of us (A. H. L.) thanks the Department of Scientific and Industrial Research for a maintenance grant.

[8] R. Nietzki and E. Hagenbach, *Ber. Dtsch. Chem. Ges.* **20**, 328 (1887).