The stability of noradrenaline in physiological saline solutions

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Although noradrenaline is known to be stable for at least 4 h at room temperature in sterile solutions for intravenous infusion (Haggendal & Johnsson, 1967), it is well appreciated that noradrenaline is highly susceptible to chemical degradation by oxidation especially under the conditions found in isolated tissue baths (physiological saline, pH 7·4, 37°, oxygenation). For this reason many workers include stabilizing agents (typically ethylenediaminetetraacetic acid (EDTA) or ascorbic acid) in the physiological saline during incubation of tissues with radiolabelled noradrenaline or when perfusing tissues with solution containing noradrenaline. A variety of concentrations of EDTA or ascorbic acid have been used by different workers but there appears to be little information in the literature concerning the effectiveness of these concentrations or about the actions of these materials on relevant biological processes, noradrenaline uptake for example.

To clarify these points (−)-noradrenaline bitartrate (Sigma) (312 nM) was incubated in a stirred isolated tissue bath (100 ml) with physiological saline (NaCl 118, KCl 4·7, $CaCl_2$ 2·5, $MgSO_4$ 1·2, $NaHCO_3$ 25, KH_2PO_4 1·2, glucose 11·1mM; aerated with 5% carbon dioxide in oxygen (30 ml min^{-1}); pH 7·4) at various temperatures. At appropriate times a portion of the bath fluid was removed and sufficient 2·0M acetic acid was added to give 0·1 ml 2·0M acetic acid per 0·9 ml bath fluid. The solution was then assayed for noradrenaline by the following fluorimetric method using internal standards. To a 1·0 ml sample was added 0·2 ml 1·0M K_2HPO_4 and 0·1 ml 6·07 mM $K_3Fe(CN)_6$ and the solution was mixed well. After 5 min 0·5 ml of a freshly prepared mixture consisting of 6·25 ml 10N NaOH + 3·25 ml 1·59M Na_2SO_3 + 0·5 ml 90·9mM ascorbic acid was added. After a further 5 min 0·5 ml of glacial acetic acid was added and the solution left to stand for 5 min. Fluorescence of the sample was measured in a Perkin Elmer MPF 3 fluorescence sepectrometer using an excitation wavelength of 395 nm, an emission wavelength of 480 nm and a band pass of 10 nm. When noradrenaline assays were carried out with bath solutions containing EDTA or 284·1 μM ascorbic acid it was found necessary to increase the $K_3Fe(CN)_6$ to 0·2 ml and 0·3 ml respectively.

This procedure provided a measure of the amount of noradrenaline in the bath fluid together with any preformed oxidation products expressed in terms of noradrenaline. Two types of blanks were used in conjunction. Firstly, 'faded blanks' (which assess the contribution of the reagents to the total fluorescence) were prepared by adding the NaOH component of the mixture at the normal time but delaying addition of the Na_2SO_3 and ascorbic acid until immediately before the addition of the acetic acid. Secondly, a 'reversed oxidant blank' was prepared by adding the $K_3Fe(CN)_6$ after the alkaline mixture and immediately before the acetic acid. This modification fails to oxidize the noradrenaline present which therefore does not form the fluorophore while any preoxidized noradrenaline present will go through the reaction and form the fluorophore normally. Using these three measurements the amount of noradrenaline and the amount of preformed oxidation products existing in a sample of bath fluid can be assayed separately.

The fluorescence derived from freshly prepared solutions of noradrenaline (in 0·01N HCl) was linearly related to the concentration of noradrenaline up to 800nM (the highest concentration tested) and the reversed oxidant blank and faded blank showed equal fluorescence indicating that, as expected, no preoxidized noradrenaline existed in these solutions. During incubation in physiological saline however a steady rise was observed in the fluorescence of the reversed oxidant blank (derived mainly from pre-oxidized noradrenaline) indicating that progressive oxidative degradation was taking place. Fig. 1 shows the amount of remaining noradrenaline expressed as a percentage of the initial concentration plotted against duration of incubation at temperatures of 37°, 32° and 27°. Degradation is clearly temperature dependent and the mean $t_{\frac{1}{2}}$ for decay of

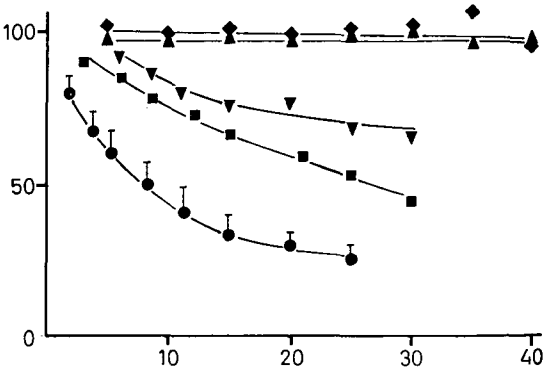

FIG. 1. Showing the percentage of noradrenaline remaining undegraded after various times of incubation in physiological saline. The values shown are means and the error bars represent standard errors. These have been omitted from most points for clarity but were less than 10% in every case. The following solutions were used: physiological saline alone at 37° (●, n = 4), at 32° (■, n = 3), at 27° (▼, n = 3) and at 37° in the presence of ascorbic acid 113·6 μM (▲), n = 3) or ethylenediaminetetraacetic acid 54·1 μM (EDTA; ◆, n = 3). The initial concentration of (—)-noradrenaline in the bath was 312 nM. Ordinate: Noradrenaline (% of initial concentration). Abscissa: Incubation time (min).

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noradrenaline averaged over 4 separate experiments at 37° was 8·5 ± 1·7 min (m ± s.e.). Thus under these conditions destruction of noradrenaline is much more rapid than that encountered during incubation at 37° in 0·9% NaCl in the absence of oxygenation when approximately 95% of the noradrenaline remained after 30 min (West, 1952).

The inclusion of ascorbic acid (56·8, 113·6 or 284·1 μM) or EDTA (27·0, 54·1 or 135·1 μM) in the physiological saline preserved noradrenaline from destruction and increased the $t_{\frac{1}{2}}$ to >180 in each case (Fig. 1). Lower concentrations also provided some protection though this was not always complete. It is apparent therefore that noradrenaline does oxidize rapidly in physiological saline solution and that these concentrations of ascorbic acid and EDTA provide effectively complete protection under the conditions of these experiments.

To investigate the effects of these concentrations of ascorbic acid and EDTA on the uptake or noradrenaline, groups of isolated vasa deferentia from Tuck No. 1 mice were incubated for 15 min with (−)-[^{3}H]noradrenaline (0·98 μM; 0·98 μCi ml^{-1}; Radiochemical Centre, Amersham) at 37° (i) in physiological saline alone, (ii) in the presence of 113·6 μM ascorbic acid and various concentrations of EDTA or (iii) in the presence of 34·1 μM EDTA and various concentrations of ascorbic acid. At the end of the incubation the tissues were washed for 10 min at 4°, blotted dry, weighed and combusted in a Packard Tri-Carb Oxidiser (305). The resulting samples were counted for tritium in a Packard Liquid scintillation spectrometer (3350) and the tritium content expressed as ng noradrenaline per mg tissue on the assumption that all the tritium in the tissue was present as noradrenaline. The results which are shown in Table 1 were not corrected for recovery from the combustion process which was 97·0 ± 1·8% (m ± s.e., n = 10).

Several points of interest are apparent. Firstly, increasing the incubation time to 30 min increased the amount of tritium present in the tissues indicating that uptake was not maximal at the shorter incubation time. Secondly, increasing the concentration of ascorbic acid in the incubation mixture in the presence of a fixed concentration of EDTA reduced the uptake of tritium and this reduction was concentration dependent. Thirdly, increasing the concentration of EDTA in the presence of a fixed concentration of ascorbic acid produced no concentration dependent reduction in uptake but fourthly, and most surprisingly, inclusion of ascorbic acid or EDTA reduced uptake of noradrenaline very considerably compared with the tissues incubated in physiological saline alone in the absence of any stabilizer.

We had expected that uncontrolled oxidation of noradrenaline in the physiological saline would reduce the concentration of noradrenaline and therefore reduce uptake but this did not occur. One possibility which would explain this unexpected result is that in the presence of animal tissue noradrenaline is considerably less susceptible to oxidation. We have therefore investigated the chemical stability of noradrenaline using the fluorimetric method above but carrying out the incubations in the presence of animal tissue (isolated vasa deferentia; 1 mg wet weight per ml of incubation fluid). Under these conditions the $t_{\frac{1}{2}}$ for noradrenaline was in excess of 160 min in each of two experiments as compared with a value of 8·5 ± 1·7 min obtained previously in the absence of animal tissue. Clearly the presence of the animal tissue stabilizes noradrenaline against chemical degradation.

Table 1. *Uptake of* (−)-[^{3}*H*]*noradrenaline (ng mg*$^{-1}$ *wet weight; m ± s.e.) by isolated mouse vasa deferentia.* Six tissues contributed to each group and each incubation was for 15 min except where indicated. All groups show a statistically significant difference in uptake from the control group incubated in the absence of stabilizers ($P<0·01$ in each case: Student's *t*-test).

Concentration (μM) of Ascorbic acid	EDTA	^{3}H-NA content
—	—	1·12 ± 0·06
—	—	1·38 and 1·43*
—	54·1	0·86 ± 0·02
113·6	54·1	0·83 ± 0·04
284·1	54·1	0·70 ± 0·03
1136	54·1	0·60 ± 0·04
113·6	—	0·64 ± 0·05
113·6	54·1	0·71 ± 0·04
113·6	135·1	0·56 ± 0·05
113·6	541·0	0·62 ± 0·03

* Incubation for 30 min; individual results.

A similar observation has been reported by Haggendal & Svedmyr (1967) who found that the presence of rat diaphragm tissue stabilized adrenaline in physiological saline. In a single experiment we have confirmed their result and the observations reported above extend this phenomenon to noradrenaline.

These results have two consequences. Firstly, in solutions used for perfusion, where noradrenaline is contained in physiological saline, there may be a considerable loss of noradrenaline unless stabilizers are added. Secondly, in solutions used to incubate tissues in the presence of noradrenaline it is not necessary to include a stabilizer under the conditions we have used and indeed, the inclusion of either EDTA or ascorbic acid will apparently reduce the uptake of noradrenaline. This result was surprising as we know of no reports in the literature suggesting that EDTA or ascorbic acid will potentiate the response to noradrenaline in isolated tissues which would be expected if noradrenaline uptake was inhibited. It should be noted however that we have measured the tritium remaining in the tissues at the end of the experimental procedure which may be influenced not only by the amount taken up but also by the amount released during the wash procedure either as noradrenaline or as metabolic products.

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N-(2-Carboxyphenyl)-4-chloroanthranilic acid disodium salt: prevention of autoimmune kidney disease in NZB/NZW F_1 hybrid mice

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NZB × NZW F_1 hybrid (B/W) mice spontaneously develop antinucleic acid antibodies and immune complex glomerulonephritis which resembles human systemic lupus erythematosus (Helyer & Howie, 1963a; Lambert & Dixon, 1968). Cell-mediated immunity is deficient with age in B/W mice, e.g. the spleen cells of old mice lack the ability to induce a graft-*vs*-host reaction (Gerber, Hardin & others, 1974), and to respond to mitogens (Leventhal & Talal, 1970), and the rejection process of these mice against skin allografts (Gelfand & Steinberg, 1973) and some tumors (Gazdar, Beitzel & Talal, 1971), is impaired. Evidence has accumulated suggesting that the deficiency of suppressor T-cell activity allows the formation of autoantibody and the development of autoimmune diseases in NZB and B/W mice (Allison, Denman & Barnes, 1971; Chused, Steinberg & Parker, 1973; Barthold, Kysela & Steinberg, 1974; Steinberg & Talal, 1975; Krakauer, Waldmann & Strober, 1976; Talal & Steinberg, 1976). In addition, neonatal thymectomy results in a high incidence of disorders in B/W mice (Helyer & Howie, 1963b).

Gershwin & Steinberg (1975a) reported that biweekly injections of thymocytes from young NZB mice, which include suppressor T lymphocytes, inhibited the pathogenesis of autoimmune haemolytic anaemia in syngeneic mice. Very recently a similar prophylactic effect was observed in B/W mice treated with the soluble factor from suppressor T lymphocytes (Krakauer, Strober & others, 1977). Further, it has been reported that injections of concanavalin A (Gershwin & Steinberg, 1975b) or thymosin (Gershwin, Ahmed & others, 1974; Talal & Steinberg, 1976) depressed immunologic abnormality and delayed the onset of, or prevented development of, autoimmune diseases in NZB or B/W mice.

From the above evidence, we considered that agents activating the T-cell function might prevent the appearance of autoantibody and the development of autoimmune desease in B/W mice.

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N-(2-Carboxyphenyl)-4-chloroanthranilic acid disodium salt (CCA), a newly synthesized drug, has immunostimulating activities, i.e. it enhances antibody formation against sheep erythrocytes (Ohsugi, Nakano & others, 1977a) and transformation of spleen cells by concanavalin A both in mice and rats (in preparation). We also reported that CCA inhibited adjuvant arthritis in rats and we thought that its activity might be based upon its stimulation of thymus regulatory function (Ohsugi, Hata & others, 1977b). We therefore examined the effect of CCA on the development of autoimmune desease in female B/W mice. These were bred in our laboratories, and at 9 weeks old, were divided into 3 groups. The first group (controls) consisted of 13 mice given water. The second and third groups had 8 mice each and these were given CCA by mouth daily doses of 5 or 50 mg kg^{-1}, respectively. The drug was administered every day except Sundays until the end of the experiment.

As shown in Figs 1 and 2, in the control group, proteinuria began to appear at age 28 weeks. it then rapidly increased, 9 of 13 mice dying of renal failure at 45 weeks while intense proteinuria was observed in the surviving mice. In contrast, 3 of 16 mice treated with CCA died at the same age as the control group, and proteinuria was markedly depressed. The mean survival times of control and CCA-treated groups at a dose of 5 or 50 mg kg^{-1} was 38, 60 and 62 weeks, respectively.

In the next experiment, B/W mice aged 13 weeks were treated with CCA. At 38 weeks, 6–7 mice in each group, were autopsied, and the kidneys were examined histopathologically. Judgement was made in a blind fashion. In the controls, severe lesions such as hypercellularity, hyaline thickening of capillary walls resembling 'wire loop lesions', and fibrinoid necrosis were observed in glomeruli of most of animals. Mice, treated with CCA, 5 or 50 mg kg^{-1}, showed less glomerular lesions than controls and had almost normal glomeruli.

In the third experiment, administration of CCA was begun at 9-weeks old. Mice were killed at 28 weeks.