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Simple methods for the detection of tropinesterase activity in rabbits*

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In experiments in which rabbits are to be given atropine or certain atropine-like agents, it is important to select animals whose plasma is deficient in the enzyme tropinesterase (atropinesterase). Three simple procedures for assessing the presence of this enzyme in rabbits *in vivo* have been examined. It is concluded that a different procedure is needed for albino rabbits than for animals with dark irides. In this way, 95–98% correct assessments of the presence or absence of tropinesterase have been made by simple procedures within 1 hr. Fully reliable assessments could only be made by *in vitro* methods, two of which (one quantitative and the other qualitative) are described. The latter enabled a reliable all-or-none assessment of tropinesterase activity to be made with only 0·02 ml of plasma within 1–2 hr.

FLEISCHMANN (1910) and Metzner (1912) were the first to attribute the known variability in the sensitivity to atropine of rabbits in different laboratories, to the ability of the blood of certain individuals to hydrolyse atropine rapidly. At that time, rabbits whose blood possessed this ability appear to have had a geographical distribution. Thus Cyon (1898) and Fleischmann (1910) in Berne, Simon (1913) in Parma, Metzner (1912) in Basle, and Tardieu & Roussin in Paris (1867) reported a high sensitivity to the pharmacological actions of atropine in the rabbits they used, whereas most workers in Germany and Austria found rabbits noticeably insensitive to the alkaloid (e.g. Fröhlich (1874) and Rossbach (1875) at Wurzburg, Harnack (1874, 1898) at Strassburg and Halle, Furth & Schwarz (1908) in Vienna, and Gnauck (1881) in Berlin). Fleischmann and Metzner showed that the blood of the atropine-insensitive rabbits at Berlin, St. Ludwig and Leipzig possessed the power of inactivating atropine *in vitro*, whereas the more sensitive animals in Berne and Basle did not.

Since then mixing of regional rabbit strains has presumably taken place. By 1924 Rothlin reported that rabbits in Basle (where Fleischmann had found earlier only atropine-sensitive animals) showed a hundredfold variation in sensitivity to vagal block by atropine. More recently, Levy (1945) in France, Ambache & Lippold (1949), Hobbiger & Lessin (1955) and Brown & Quinton (1957) in England, Werner & Würker (1959) and Lendle & Paul (1964) in Germany, have all found a wide variation in sensitivity to atropine amongst the rabbits they used, and have shown this to run in parallel with the presence or absence of the enzyme tropinesterase (atropinesterase) in each animal's blood.

Since the presence of the enzyme in a rabbit's blood is an inheritable characteristic, probably of a sub-dominant nature (Sawin & Glick, 1943), the incidence of rabbits possessing this enzyme varies widely between stock in different laboratories or obtained from different breeders. In

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the experience of the present author no British strain or breed has been found to be completely free of animals possessing tropinesterase in their blood.

The enzyme is not specific for atropine and (−)-hyoscyamine, but also hydrolyses hyoscine, homatropine, benactyzine and many other amino-alcohol esters of tropic, benzilic, diphenylacetic and related acids (Werner, 1961; Herz, 1963; Quinton, unpublished).

It is obviously advisable therefore that, where rabbits are to be used in any pharmacological experiment involving atropine or a structurally similar ester, only those whose blood lacks tropinesterase should be chosen. This paper reports results obtained with various published methods for the ready detection of tropinesterase activity in rabbits and describes new procedures.

Experimental

In vivo METHODS

Three different procedures were used to assess the degree of pupil dilatation produced by a low dose of atropine or hyoscyamine. In all instances, the pupils were illuminated by a 60 W bulb of an Anglepoise lamp, partly masked to give a light aperture of 4 cm and placed 30 cm from the eye. The diameters of both pupils were measured, with a transparent metric rule held close to the eye, immediately before and at a stated time after treatment. A change in pupil diameter of 1 mm and more was considered significant. Measurement of pupil size was performed by assistants who did not know what changes to expect.

(i) One drop containing 1 μg of hyoscyamine sulphate was placed in the conjunctival sac of one eye of a rabbit. The degree of pupil dilatation was measured 60 min later. Under these conditions rabbits lacking tropinesterase have been reported to give a positive mydriatic response, whereas those in which the enzyme is present do not (Werner & Würker, 1959).

(ii) Rabbits were injected intravenously with either 0·1 or 1·0 mg/kg atropine sulphate. The diameters of both pupils were measured again 15 or 60–75 min respectively after injection. Under both these conditions the pupils of rabbits possessing tropinesterase have been reported to regain the light reflex (i.e. to contract in bright light) whereas in those lacking the enzyme the reflex remains blocked (i.e. the pupils stay dilated) (Ambache, Kavanagh & Shapiro, 1964).

(iii) The conjunctival sac of one eye of a rabbit was flooded with 2–3 drops of a solution containing 2·5 mg/ml of physostigmine sulphate and 0·3 mg/ml of atropine sulphate in 0·9% saline; the estimated amounts instilled were about 300 and 36 μg respectively. Change in pupil diameter was measured 45 min later.

In vitro METHODS

Plasma was separated by centrifugation from blood withdrawn from an ear vein and heparinized. It was stored at −17° when not in use;

under these conditions no significant loss in tropinesterase activity was noted within 3 months.

Incubation of plasma with atropine, followed by bioassay of residual atropine. To 0·25 ml of plasma in a 10 ml measuring cylinder were added 8·75 ml of a phosphate buffer pH 7·4 (20 ml of 5% w/v NaH_2PO_4 plus 20 ml of 5% w/v $Na_2HPO_4.2H_2O$ in 1 litre 0·9% w/v NaCl, brought to pH 7·4 with 20% w/v NaOH). The cylinder was placed in a water-bath kept at 37·7 $\pm$ 0·2°. After 5 min, 1 ml of a 6 mg/ml solution of atropine sulphate was added and the cylinder inverted twice to mix the contents. The enzymatic action was stopped either 30 or 60 min later by adding three drops of N hydrochloric acid; two drops of Universal Indicator solution (B.D.H.) were added at the same time to check that the pH of the solution was below 3. Incubated solutions were always assayed within 30 hr, being stored at −17° overnight.

Residual atropine in the incubated solutions was assayed by its mydriatic action in mice (Pulewka, 1932). Details of the method have been described previously (Quinton, 1963). Male albino mice, 25–40 g, were injected subcutaneously with 0·1 ml solution/10 g and the right pupil diameter measured again 40 min after injection. The response was taken as the arithmetic difference between the initial and the final pupil diameter. The potency of a solution was determined by reference to a logarithmic dose-response line derived from two dilutions of a non-incubated solution of atropine sulphate prepared at the same time as the incubated solutions and in exactly the same manner, except that buffer solution replaced the 0·25 ml of plasma. Generally, dilutions of 1–60 and 1–150 (corresponding to doses of atropine sulphate of 1 and 0·4 μg/10 g) gave suitable mydriatic responses of about 75% and 25% respectively of the maximum possible; 10–15 mice were used for each solution. The significance of marginal differences in potency between any test and the standard solution was checked by Student's "*t*" test applied to the mydriatic responses obtained from identical dilutions of the two solutions. Tropinesterase activity in each plasma sample was expressed as μg atropine base/ml of incubated solution hydrolysed in 1 hr under the conditions specified here.

No significant non-enzymatic inactivation of atropine could be demonstrated after incubation for 3 hr.

Agar plate method. The method was derived from that of Sandi (1962) who devised it to detect organophosphate anticholinesterase insecticide residues in plant material. Atropine sulphate, 5 mg/ml, was dissolved in a suspension of 1·5% w/v agar in a weak phosphate buffer (4 ml 0·1 M KH_2PO_4 plus 40 ml 0·1 M Na_2HPO_4, to 1 litre with 0·9% saline, and brought to pH 8·0 with 0·1 N NaOH). The pH of this solution after adding the agar and atropine sulphate was about 7·6. Sufficient solution of bromothymol blue was added to the solution after boiling to give a final concentration of about 0·02% w/v, and the mixture poured into petri dishes.

In earlier work plasma was added to circular holes cut in the agar, but more rapid results requiring smaller amounts of plasma were obtained

if small filter paper discs (Whatman No. 42) were used instead. The discs were about 6 mm diameter, cut by an office stationery hole-punch and placed on the surface of the agar. For a petri dish of 8·5 cm diameter, 12–15 ml of agar solution gave a suitable depth of agar of 2–3 mm. Only 0·020 ml of plasma (applied as three drops from a syringe fitted with a number 18 hypodermic needle) were needed; the plasma was dropped on to three discs of filter paper placed one on top of the other.

For rapid results, the agar plates were incubated at 38 or 45°. It was found that plastic petri dishes could be floated safely in water-baths at these temperatures, with lids placed upside-down on top to protect the agar from any splashes. Under these conditions colour changes were visible within 30 min and quite distinct by 60–120 min. Around filter paper discs to which tropinesterase-containing plasma had been added, enzymatic hydrolysis of the atropine caused a fall in pH with a corresponding colour change of the indicator from blue to yellow. In the case of plasma not containing tropinesterase, the colour remained blue or green, but never turned to yellow.

Results

In vivo METHODS

The changes in pupil size caused by the three different treatments with atropine or hyoscyamine (see "Methods") were assessed in about 150 mature rabbits of seven breeds (and a number of crosses).

Results, expressed as the percentage of animals with or without tropinesterase which responded in the manner predicted by Werner & Würker or Ambache & others, are represented in Fig. 1. Totals are shown, plus separate histograms for albino and non-albino animals. The tropinesterase activity of the plasma of each rabbit was determined by incubation with atropine *in vitro*, and subsequent bioassay of the residual atropine.

After instillation of 1 μg hyoscyamine sulphate in the eye, 18 out of 24 rabbits whose blood lacked tropinesterase activity showed a significant mydriatic response. Of these, seven were albinos, all of which responded. Of the rabbits possessing tropinesterase, 23 out of 25 (nine out of 10 albinos) failed to show a significant pupil dilatation. In all, 84% of all rabbits (94% of albinos) gave responses in this test of the nature predicted (Werner & Würker, 1959).

In the second procedure (Ambache & others, 1964), the pupils of all 16 rabbits (including eight albinos) lacking tropinesterase showed significant dilatation when examined 15 min after an intravenous injection of 0·1 mg/kg of atropine sulphate. Of the animals in which the enzyme was present, 16 out of 25 (three out of eight albinos) failed to show dilatation. The overall proportion responding as expected was 78% (69% for albinos only).

After the alternative treatment (1·0 mg/kg atropine sulphate 60–75 min previously) only 13 out of 22 rabbits (three out of five albinos) gave mydriatic responses as expected.

Analysis of the failures of rabbits to respond as expected in these two tests suggested that the main source of error lay in the marked difference in sensitivity of albino and non-albino rabbits to the mydriatic action of atropine. Thus the pupils of five out of eight albinos with tropinesterase dilated significantly to 0·1 mg/kg of atropine, whereas six out of 24 non-albino animals lacking tropinesterase failed to show a significant mydriatic

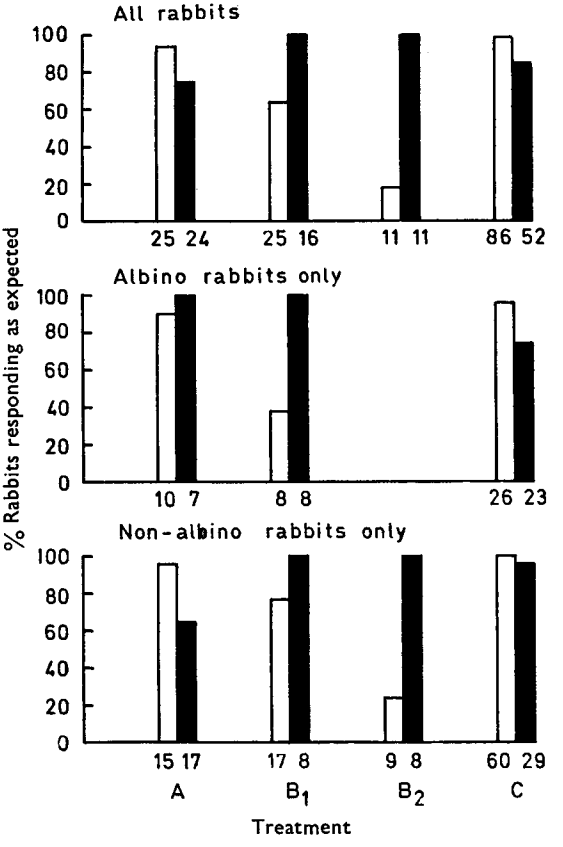

FIG. 1. Percentage of rabbits, with (open columns) or without (solid columns) significant plasma tropinesterase activity, responding by changes in pupil size as predicted (see text) to various treatments with atropine or hyoscyamine sulphate. Treatment: A, 1 μg hyoscyamine sulphate in eye; B1 or B2, 0·1 or 1·0 mg/kg atropine sulphate i.v.; C, 36 μg atropine sulphate + 300 μg physostigmine sulphate in eye. Figures indicate numbers of animals from which each percentage value is derived.

response to 1 μg hyoscyamine in the eye. These differences were shown also in the resting state since, under the lighting conditions used in this work, the pupils of albinos measured 4–5 mm before treatment, whereas those with dark irides were 5½–7 mm in diameter.

It was thought possible that any mydriatic action of atropine might be more readily discernible in the eyes of non-albino rabbits if the pupils

were first contracted by physostigmine. In the third type of test, therefore, physostigmine was instilled in the eye at the same time as atropine in a suitable concentration to cause mutual antagonism. Thus, in rabbits lacking tropinesterase, it was expected that the pupil sizes would not be altered significantly by this treatment, whereas in animals possessing the enzyme sufficient atropine would be destroyed for the physostigmine to produce miosis.

In the former group, the pupils of 44 out of 52 animals showed no change in size; of these 27 out of 29 were non-albino. In the case of rabbits with tropinesterase, 85 out of 86 displayed significant miosis. The animal which failed to respond in this way was an albino.

Thus, in the case of the *non-albino* rabbits, the method gave responses as expected in 87 animals out of 89, or 98%.

It was noted that even though the miotic action of physostigmine was presumably indirect, via inhibition of cholinesterase, its effects were noticeable within 10–20 min, before any atropine antagonism was seen. By 45 min after instillation, however, both drugs were exerting their effects and this was the time chosen for observation of any change in pupil size.

In vitro METHODS

Incubation of plasma with atropine, followed by bioassay of residual atropine. This method has been used routinely to determine tropinesterase activity in the plasma of over 300 rabbits during the past 8 years.

The limits of error of an estimate of the potency of an incubated solution were smallest when hydrolysis had been allowed to continue until its atropine content* was low, since it needed to be diluted less before assay. On the other hand, hydrolysis was not allowed to proceed beyond 75% of the atropine present, to ensure a nearly maximum rate of esterase activity. Except where an exact value for a particularly low hydrolytic activity was required, it was usually adequate to incubate solutions of atropine with plasma for either 30 or 60 min (depending on the tropinesterase activity). In these cases, significant tropinesterase activity could be detected down to values of about 90 μg atropine hydrolysed/ml/hr.

In rabbits possessing significant plasma tropinesterase activity, calculated values ranged from 78 to 720 μg atropine hydrolysed/ml solution/hr.

Agar plate method. This test has given consistently reliable results with rabbit plasma samples, in qualitative agreement with the assessment of tropinesterase activity made by incubation with atropine *in vitro* and subsequent bioassay of the residual atropine. Although the colour changes induced by tropinesterase-containing plasmas might be quantified by some colorimetric instrument, this has not been done since it was considered that the method could never attain the accuracy of the incubation/bioassay method described above.

* The presence of (+)-hyoscyamine in the incubated and assayed solutions was ignored, since it is hydrolysed only very slowly by tropinesterase (Werner & Brehmer, 1959) and has only 1/40–1/100 of the potency of the (−)-isomer in the mouse mydriatic test.

Degrees of colour change could however be observed by the naked eye and correlated with the tropinesterase activity of the plasma samples used. The time-courses of the colour changes produced by three plasma samples of differing tropinesterase activity and two samples lacking any activity are shown in Fig. 2, for three different temperatures of incubation.

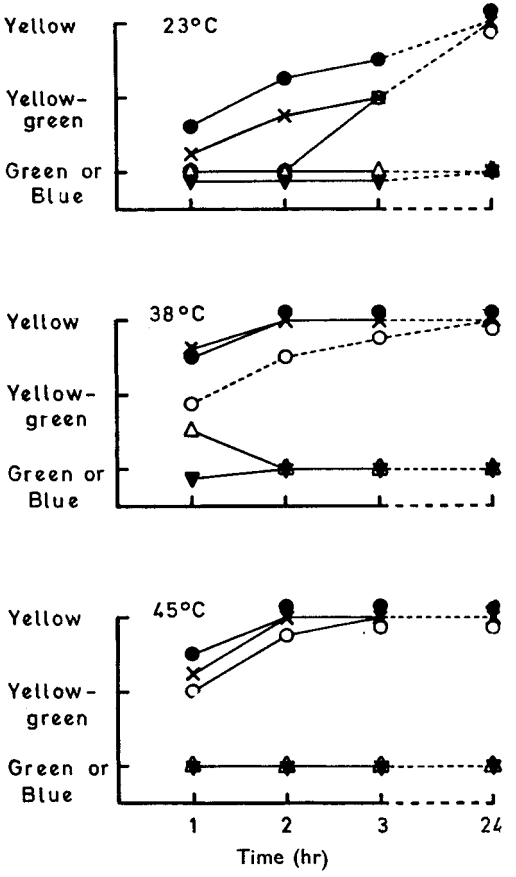

FIG. 2. Time-course of the colour changes caused in agar plates containing atropine sulphate (5 mg/ml) and bromothymol blue, by plasma (0·02 ml) of rabbits with various tropinesterase activities. ●, plasma tropinesterase activity of 678 μg atropine hydrolysed/ml solution/hr; ×, 272 μg/ml/hr; ○, 110 μg/ml/hr; △, no significant activity; ▼, no significant activity. Each point represents the mean of 2 or 3 observations.

With the plasma possessing the weakest activity, a colour change to yellow-green could be observed after 60 min at 38° or 45°, but was more convincing after 120 min. Plasma samples lacking tropinesterase occasionally produced a slight initial colour change towards yellow-green, but this was never seen after incubation for 120 min or more.

The parameters described for this method were chosen to give clear-cut

colorimetric responses within 60–120 min with the use of as little plasma and atropine as possible. Thus 0·02 ml of plasma and 5 mg/ml of atropine sulphate gave good responses whereas the colour changes by 0·007 ml or 2 mg/ml respectively were less convincing. The composition of the buffer solution did not appear critical providing it had only weak buffering capacity within the pH range 6·0–7·6. (Preliminary experiments in which a standard Sorensen phosphate buffer of pH 7·6 was used gave disappointing results before it was realised that the buffer was effectively resisting the pH change caused by hydrolysis of the atropine). Temperatures of 38 or 45° were found best for rapid colour changes, although perfectly adequate colour changes could be obtained even in plates kept at 4°, for 18–24 hr.

Enzymatic activity apparently fell off markedly below about pH 6, since, if bromocresol purple was used, it was changed to a pure yellow colour (pH 5·2) only after 12–24 hr at 38°. The small pH range suitable, about 6·0–7·4, therefore restricted the choice of indicators. Bromothymol blue, changing over exactly this range, gave clear-cut colour changes.

Attempts to use this method for whole blood were not fully successful, although distinctive colour-changes could be obtained from 0·015–0·03 ml blood. Since however the blood samples needed much longer incubation than did plasma, and colour changes were not so clear-cut, plasma was preferred.

Discussion

The object of this work was to examine various simple methods whereby tropinesterase activity could be assessed in rabbits, both in the whole animal and in the blood or plasma *in vitro*. Techniques requiring much equipment or expertise have not been considered (such as the Warburg manometric method, as used by many workers in this field—e.g. Glick, 1940). Particular attention has been paid to techniques which would permit a speedy all-or-none assessment of tropinesterase presence in the blood of a rabbit.

In the intact animal, none of the three methods described gave a completely reliable indication of the presence or absence of tropinesterase activity. This was probably due to marked differences in sensitivity to the mydriatic action of atropine or hyoscyamine in albino and non-albino rabbits. Thus, some albino animals possessing tropinesterase gave significant mydriatic responses to low doses of atropine or hyoscyamine administered topically or by intravenous injection. Conversely, the pupils of some rabbits with dark irides failed to show a significant dilatation to these agents even when no tropinesterase was present.

For these reasons it is recommended that albino and non-albino rabbits should be treated differently. Instillation of 1 μg of hyoscyamine sulphate in one eye of an albino rabbit has been shown, in 94% of animals, to cause significant mydriasis if the animal lacks tropinesterase, but no change in pupil size if its blood contains the enzyme. For rabbits with dark irides, instillation in one eye of a mixture of physostigmine sulphate and atropine

sulphate (about 300 μg and 36 μg respectively) has been shown, in 98% of animals, to produce significant pupil contraction in animals with tropinesterase but no significant change in pupil size in those lacking the enzyme. The two exceptions in the 89 non-albino rabbits tested by this procedure were tropinesterase-free but their pupils did contract significantly; in other words they reacted as if they possessed tropinesterase. All the rabbits whose pupils did *not* contract significantly were tropinesterase-free. The test therefore appears to offer a reliable means of selecting tropinesterase-*free* rabbits.

Under the standard lighting conditions used it was noted that, in their resting state, the pupils of albino rabbits were significantly more contracted than those of rabbits with pigmented irides. Possibly if the strength of lighting had been adjusted for each rabbit to give a constant initial pupil size, as was done by Herz (1963) when he used the procedure of Werner & Würker (1959), these differences in sensitivity to the mydriatic action of atropine or hyoscyamine might not have been observed. Lighting conditions were kept constant here, however, since it was felt that adjustment of lighting for each individual rabbit introduced an undesirable complication into what were intended to be simple procedures. Furthermore, experience had shown that the use of stronger illumination than that described here induced some rabbits to keep their eyes firmly closed.

Two methods, one quantitative and the other qualitative, have been described for the assessment of tropinesterase activity in rabbit plasma *in vitro*. The quantitative method, involving incubation of plasma with atropine followed by bioassay of the residual atropine, has been used routinely for the definitive assessment of tropinesterase activity in the rabbits used for the tests *in vivo*. The qualitative test makes use of the colour change induced in a pH indicator by the acid produced on hydrolysis of atropine by tropinesterase, in an agar plate. It needs very small quantities of plasma, 0·02 ml giving an adequate colour change even with plasma samples possessing feeble tropinesterase activity. It is simple and quick, a reliable qualitative assessment of enzyme presence being possible within 120 min.

If a rabbit of unknown tropinesterase activity should at any time be required to be used immediately for an experiment in which atropine or a closely-related ester is to be given, it is probably preferable to use a quaternary ammonium salt such as atropine methyl nitrate, which is not hydrolysed by tropinesterase (Dirner, 1937). Its ganglion-blocking activity (Bainbridge & Brown, 1960) and failure to pass the blood-brain barrier may, however, be disadvantageous. In such circumstances, (−)-tropine α-methyl tropate (Kramer, Maffii & Quinton, to be published) or oxyphencyclimine (Finkelstein, P'an, Niesler, Johnson & Schneider, 1959) could be used; these agents are not significantly hydrolysed by rabbit tropinesterase (Quinton, to be published).

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