Evidence for the Presence of an Atropine-Degrading Enzyme in Goat Serum

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Pooled rabbit serum containing atropinesterase, pooled rabbit serum lacking atropinesterase, and pooled goat serum were tested for their effect on some pharmacological parameters of atropine and related compounds. Pooled goat serum was found to contain a previously undescribed enzymatic activity on certain of the effects of these compounds. These activities were compared to those of the rabbit sera. White male mice were used as the test animal.

A SURVEY OF the literature reveals no reference to any protective mechanism of the goat against atropine. Even in the folklore of ancient medicine, where there was widespread belief in the therapeutic powers of different parts of the goat's body, no indication of natural resistance of the goat to atropine, atropine-containing plants, or any atropine-related compounds is found (1).

Metabolic degradation of atropine has been studied in several species, including the mouse (2–4), rat, kitten, guinea pig (5), dog (6), and rabbit. The most extensive studies have been performed on rabbits, where a genetically inherited enzyme, atropinesterase, has been credited with the ability to impart resistance to the pharmacological effects of administered atropine in those rabbits containing the enzyme. Atropinesterase from rabbit serum has recently been purified and characterized by Margolis and Feigelson (7).

This paper presents evidence for the existence of a hitherto unreported atropine-degrading enzyme, present in pooled goat serum, which differs from atropinesterase in that it has no esterase activity.

MATERIALS AND METHODS

Chemicals.—Atropine sulfate (Merck and Co.), hyoscyamine sulfate (Nutritional Biochemicals), tropic acid (Nutritional Biochemicals), and tropine (K & K Laboratories) were all commercial products. Tropine was purified by vacuum distillation in this laboratory.

Sera.—Sterile pooled rabbit serum, no preservative, was obtained from Courtland Laboratories and Baltimore Biological Laboratories. Sterile, pooled, goat serum, no preservative, was obtained from Colorado Serum Co.

Eye Assay.—A modification of the method of Pulewka (8) was used. White, male, Swiss mice were used as the test animal. Initial pupil diameter, under the direct light of an 18-w. microscope maintained 10 cm. from the mouse eye, was obtained using an American Optical binocular microscope fitted with an ocular scale. Animals then received an intraperitoneal injection of 0.5 ml. of serum, followed by 0.2 ml. of atropine or hyoscyamine solution by the same route. Each animal received a total dose of 2 mcg. of the desired alkaloid, with stated exceptions. Pupil diameter was followed at 5-min. intervals until a maximum dilation was obtained. Maximum pupil diameter increase, in arbitrary units, was calculated, and the data subjected to a t test (9) for significance. Control animals received 0.5 ml. of water, followed by 0.2 ml. of alkaloidal solution.

Median Lethal Dose Determinations.—White, male, Swiss mice, 11–12 Gm. in weight, were randomized into five groups of 10 mice each. Each group was subjected to a different dose level, the per cent kills obtained for each group, and the results analyzed by probit analysis after the method of De Beer (10).

Toxicity Studies.—Mice in the test group received 0.8 ml. of serum, followed immediately by 0.2 ml. of the alkaloidal solution, containing a dose of alkaloid at the LD_{50} level. The control group received 0.8 ml. of water, followed immediately by 0.2 ml. of the alkaloidal solution. The total number of kills was obtained for each group, and the results tested for significance, using the χ^2 method of Batson (11). All injections were intraperitoneal.

Esterase Activity.—Warburg respirometer analyses were run on 2.5-ml. reaction mixtures, 0.04 M in phosphate, 0.04 M in bicarbonate, containing 1% of atropine free base and 20% serum, at an initial pH of 7.8. Carbon dioxide production, indicating the hydrolysis of atropine to tropic acid and tropine, was used as the criterion for esterase activity (12).

RESULTS AND DISCUSSION

In a preliminary attempt to compare the effect of esterase-containing and esterase-deficient rabbit serum, and goat serum, on the pharmacological action of atropine, the authors employed two parameters: mydriatic response and gross toxicity.

The presence or absence of esterase activity was determined by Warburg studies. Substantial carbon dioxide production was observed in the case of Courtland rabbit serum, but not with Baltimore rabbit serum or goat serum. On this basis, Courtland serum was judged to contain atropinesterase, and the latter two sera were classified as lacking the enzyme.

LD_{50} studies (Table I) show the relative toxicities of atropine and its components. Atropine, the racemate, and hyoscyamine, the levorotatory isomer, were found to be nearly equal in toxicity, atropine being somewhat more toxic. These results are in agreement with Buckett's observation (13) that, in white mice, the two isomers possess equal

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TABLE I.—MEDIAN LETHAL DOSES FOR THE COMPOUNDS STUDIED[a]

Compd.	LD_{50}, mg./Kg.	95% Confidence Limits of LD_{50}
Atropine sulfate	180	164–198 mg./Kg.
Hyoscyamine sulfate	210	181–244 mg./Kg.
Tropine sulfate	550	490–616 mg./Kg.
Tropic acid (sodium salt)	Nontoxic[b]	...

[a] Median lethal dose and confidence limits were determined by the method of De Beer (10). [b] No deaths were observed at maximum dosages of 1500 mg./Kg.

TABLE II.—PROTECTION OF SERA AGAINST THE LETHALITY OF ATROPINE SULFATE

Serum	Effect	$\chi^{2,a}$
Goat	Protection	16.16
Esterase positive rabbit	Protection	13.00
Esterase negative rabbit	Protection	5.12

[a] Critical values for χ^2: 5% = 3.84, 1% = 6.64, 0.1% = 10.83.

potencies at toxic levels. The authors observed that, upon standing in the laboratory, solutions of hyoscyamine appear to change in toxicity. A solution of hyoscyamine was followed for several hours using a polarimeter, without change in optical rotation. Therefore, racemization does not seem to be a possible explanation for the sometimes markedly different responses that were obtained from hyoscyamine.

Tropine was found to be about one-third as toxic as atropine, and tropic acid was found to be nontoxic. Therefore, the hydrolysis of atropine by those animals containing atropinesterase could serve as a protective mechanism against the lethality of atropine.

Hydrogen-ion concentration was not a factor in the toxicity of the injected drug. Atropine sulfate and hyoscyamine sulfate solutions have a pH of 5.5, and intraperitoneal injections of dilute solutions of sulfuric acid of the same pH caused no deaths or apparent discomforts in injected mice. Tropine solutions were too basic to be used as such, and were adjusted to pH 7.0 with dilute sulfuric acid. Tropic acid, in the acid form, is only very slightly soluble in water. This compound was administered as the sodium salt, pH 7.0, to solubilize enough of the tropic acid anion to permit toxicity studies.

When dose-response curves were run for mydriatic activity, the curves for hyoscyamine and atropine were indistinguishable from one another. Subjection of the data to a t test disclosed no significant difference in the mydriatic action of atropine and hyoscyamine in male, white mice. These findings are in disagreement with Buckett's (13) observation that the levorotatory isomer is 29 times as mydriatically potent as the dextrorotatory isomer. Although Buckett also used the mouse as the test animal, he employed subcutaneous or intravenous injections, while this laboratory employed the intraperitoneal route.

All three sera tested were found to protect against the lethality of atropine (Table II). Since esterase activity was present in only the Courtland serum, it is obvious that protection, while it may be a partial function of atropinesterase in Courtland serum, cannot be a function of esterase activity in the other sera.

Tropine, the alcohol portion of the ester, exhibited lesser toxicity than the parent compound. Goat serum and esterase-lacking rabbit serum were found to protect significantly against its toxicity (Table III). Since there are no ester links in this alkaloid, additional evidence is afforded that protection is due to something other than an esterase. There is an implied association, even in sera that contain atropinesterase, of protective action and ability to degrade or alter the tropine moiety.

The toxic effects of hyoscyamine were unmitigated by all three sera, hence, the protection is stereospecific and involves the dextrorotatory isomer (Table IV). This finding the authors regard as most significant. Atropinesterase is claimed to be specific for the levorotatory isomer (14), and if protection had been a function of hydrolysis, it would have been observed in the case of the pure substrate. This finding also eliminates the possibility that protection is due to serum binding of the administered drug. If this were the mechanism, the enzyme would have bound the natural substrate, the levorotatory isomer, just as much if not more so, as the racemic mixture of isomers.

Alteration of mydriasis showed both rabbit sera to significantly decrease the amount of mydriasis caused by an injected dose of atropine (Table V), again pointing to some activity other than enzymatic hydrolysis. Goat serum had no effect on mydriatic activity.

TABLE III.—PROTECTION OF SERA AGAINST THE LETHALITY OF TROPINE SULFATE

Serum	Effect	$\chi^{2,a}$
Goat	Protection	5.72
Esterase positive rabbit	Not run	...
Esterase negative rabbit	Protection	6.37

[a] Critical values for χ^2: 5% = 3.84, 1% = 6.64.

TABLE IV.—PROTECTION OF SERA AGAINST THE LETHALITY OF HYOSCYAMINE SULFATE

Serum	Effect	$\chi^{2,a}$
Goat	None	1.41
Esterase positive rabbit	None	0.06
Esterase negative rabbit	None	...

[a] Critical values of χ^2: 50% = 0.455, 5% = 3.84.

TABLE V.—PROTECTION OF SERA AGAINST THE MYDRIATIC ACTION OF ATROPINE SULFATE

Serum	Effect	t-Test[a]
Goat	None	1.1
Esterase positive rabbit	Protects	8.02
Esterase negative rabbit	Protects	5.67

[a] $t_{(20)}$ = 3.85 at 0.1%; $t_{(20)}$ = 1.73 at 10%.

CONCLUSION

These studies indicate that the protection rendered by these sera against the pharmacological effects of atropine cannot be ascribed to atropinesterase, or any other esterase. The protection against mydriasis, exhibited by the esterase-lacking rabbit serum, together with the observation that goat serum is ineffective under the same conditions, implies further that the mechanism of protection is of a different nature in the two sera. The evidence indicates that goat serum is specific for the dextrorotatory isomer of atropine, metabolizing it to a compound that is less toxic, but of equal mydriatic activity for the mouse. The rabbit sera data indicate an activity not involving the ester linkage, but metabolizing the compound to a substance of both lesser toxicity and lesser mydriatic activity for the mouse. The data further suggest that decreases in toxicity, with all sera tested, are very likely associated with alterations of the tropine moiety.

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Freezing Point Curve of Dimethyl Sulfoxide–Water Solutions

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The apparent freezing points have been determined for solutions of dimethyl sulfoxide and water. Several of the samples exhibited increasing viscosity as they were cooled and, at their solidification points, formed amorphous glasses rather than crystalline solids. It is proposed that these glasses form because of a modified lattice structure built of dimethyl sulfoxide and water molecules. The eutectic composition occurs in the region of 0.3 *M* dimethyl sulfoxide.

DIMETHYL SULFOXIDE (DMSO) has been used for many years as a solvent and a reaction medium. It will dissolve many inorganic salts and most classes of organic compounds. As a reaction medium it has been found to increase greatly the rates of many reactions beyond what would be expected (1). It also possesses a number of other unusual properties: a high dielectric constant for an aprotic solvent (2), miscibility with most organic solvents, a high heat of mixing with water (3), and a volume contraction when mixed with water (3). DMSO has also found use as a preservative and freezing medium for biological tissues (4). It has been found in this laboratory that DMSO will greatly lower the freezing point of water. This paper reports on the cryoscopic properties of DMSO–water solutions.

EXPERIMENTAL

Two sets of equipment were employed—one for those samples freezing above −40° and one for those freezing below. For the higher freezing solutions, the apparatus used for measuring the temperature was a copper-constantin thermocouple connected to a Westronics strip chart recorder. The recorder has a working range of −40° to +180° (or −1.46 to +8.24 mv.), with an accuracy of ±0.5°. The cooling chamber was a Dewar flask (12.3 cm. i.d.) containing the dry ice–glycol ether[1] cooling fluid (temperature about −75°).[2] About 2 ml. of a DMSO-water solution was placed in a 7.5 × 1 cm. glass test tube. The thermometric probe was threaded through a cork stopper in the test tube and positioned in the center of the liquid. This assembly was immersed in the cooling bath intermittently, to avoid supercooling, until the contents of the test tube had frozen. The assembly was then suspended from a clamp and allowed to warm at room temperature until the transition from solid to liquid was complete. The freezing point (melting point) of the mixture was taken as the point of inflection on the time-temperature curve.

For those samples with a freezing point below −40°, the equipment used was the Linde BF-3 biological freezer. This is a controlled rate freezer that uses liquid nitrogen as the cooling medium. A differential copper-constantin thermocouple probe is used to monitor the temperature difference between the sample and the cooling chamber. This temperature difference produces a millivolt signal, which is balanced against the controller voltage set to the desired temperature differential. As a result of the continuous comparison of these two voltages, liquid nitrogen is fed into the freezing chamber as needed, through a solenoid valve that is actuated by the controller. Thus, the freezing rate is established and maintained. After the conclusion of a particular freezing operation the sample vials are removed, the

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[1] Marketed as Dowonol 33B by Dow Chemical Co., Midland, Mich.
[2] Determined with a copper-constantin thermocouple and a Brown-Honeywell potentiometer.