supplemented with vitamin K_1 had a normal prothrombin time (Table 1). In both cases, the livers were about 2·9% of the body weight. The glycogen content averaged 13·3 ± 3·2 and 13·3 ± 4·9 mg/g wet weight for the controls and experimentals respectively.

A summary of the adenine nucleotide content of the livers is presented in Table 1. There was no difference between control and experimental livers in any of the nucleotides, individually or in total amount. Similarly, the inorganic phosphate contents were about the same. The creatine phosphate content was too low to be measured accurately and therefore was not included in this analysis. The amount of ATP, compared with ADP and AMP, was relatively low in contrast to the proportion found in the livers of mature rats, which contained about 2·0 μmoles ATP/g.[10] Since the methods and assay procedures used were the same in each case, it would appear that the variations in values obtained are reflections of the differences between the species or ages of the animals rather than differences in method of isolation and assay. The similarity in glycogen and inorganic phosphate content of control and experimental animals does not indicate a shift to glycolysis. The possibility that the ATP levels were maintained by accelerated rates of metabolism or greater utilization of other substrates can not be excluded. However, if such an increase or shift had taken place in order to maintain the ATP levels, it would not seem likely that the synthesis of the clotting factors would be affected. Since no difference was found between the ATP levels of control and experimental animals, there is no evidence from these studies to support the view that a vitamin K deficiency produces changes in oxidative phosphorylation which would account for a hemorrhagic condition.

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Evidence for pilocarpine transformation by serum*

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PILOCARPINE is being extensively used for inducing the copious flow of prostatic secretion,[1, 2] although the mechanism of stimulation has barely been examined.[3, 4] Since the pharmacologic stimulation of

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prostatic secretion is induced by the intravenous administration of pilocarpine, it was of interest to examine serum effects on the drug. Biochemical data are meager on this point, but the reports of Beutner[5] and Augustinsson[6] suggested pilocarpine decomposition in serum. Contrary results implicating cholinesterase have been obtained.[6-8] Only the work of Nakanishi and Sumiya[9] *in vitro* directly established hydrolysis of pilocarpine by rabbit lymph. The present investigation explored possible enzymic transformation of pilocarpine in rat serum and methods of inhibiting the transformation.

METHODS

Rat blood was obtained by cardiac puncture, the blood allowed to coagulate for 1 hr, spun at 2,000 rev/min for 15 min and the serum refrigerated until used. One tenth ml pilocarpine hydrochloride (10 mg/ml) was added to each tube containing 1 ml serum and incubated at 37°. A 10-ml pool of rat blood was employed for intermittent analyses in conjunction with spaced additions of pilocarpine: after a 1-hr incubation with 1 mg pilocarpine/ml, 4 ml of the incubate was removed and 6 mg of fresh pilocarpine added to the remaining system; after another 1-hr incubation, 3 ml of incubate were removed and 3 mg of fresh pilocarpine introduced into the remaining medium for a third 1-hr incubation. The samples taken at hourly intervals were analyzed separately.

The reaction of samples was stopped by adjusting the pH to 9·0 with 10 N NaOH and the pilocarpine extracted by adding 3 ml of ethylene dichloride and shaking for 10 min. The sample separated into two phases, the organic phase being filtered and brought to dryness under vacuum. The samples were taken up in either water or ethanol, dependent on whether colorimetry or chromatography was to be employed for detection.

Inhibition studies were carried out with 10^{-2} to 10^{-4} M ethylenediaminetetracetate (EDTA), *p*-chloromercuribenzoic acid (PMBA), sodium fluoride potassium cyanide, paroaxon (diethyl-*p*-nitrophenyl phosphate), and eserine salicylate by addition of 0·1 ml of the inhibitor solution to 1 ml rat serum in the presence and absence of pilocarpine.

Several aliquots of rat serum incubates were subjected to bioassay by classical pharmacologic techniques. Rat cardiac stomach strips, 2 to 3 mm × 22 to 25 mm were mounted in Tyrode's solution at pH 7·6. The strips were acclimated for 1 hr at 30° and a dose–response curve to pilocarpine recorded. Varying aliquots of solutions containing amounts of extracted pilocarpine were then tested. Other aliquots of rat serum incubates were chromatographed according to the method of Waldi *et al.*[10] on commercially prepared thin-layer plates (Custom Service Chemicals). Both silica gel G and alumina oxide G plates were used after being preheated for 15 min at 90° and desiccated during cooling. Pilocarpine was detected by the imidazole reaction of Cochin and Daly[11] or the lactone reaction of Abdel-Akher and Smith.[12] Quantitative colorimetric measurement of recovered pilocarpine was achieved by the procedure of Hestrin,[13] although the limitation of detection was 50 μg.

RESULTS

In order to establish whether pilocarpine could be recovered and identified by the procedure of extraction employed, thin-layer chromatography was performed on several extracts. Both the imidazole and lactone reactions gave similar results. It can be seen in Table 1 that only one zone was detected with an R_f in agreement with that reported in the literature.[10] Incubations of serum with pilocarpine yielded only one detectable zone at the proper R_f. Although a loss in pilocarpine content was obvious from the reduced intensity of the color reaction, no metabolites appeared after staining with the color reagents.

Since only one reactive zone could be discerned after thin-layer chromatography, this step was eliminated in favor of quantitation by photoelectric colorimetry of the recoverable pilocarpine. The disappearance of pilocarpine was determined in heated serums from rat and in Krebs-Ringer phosphate buffer as control measures. Quantitative recovery (95–99%) was obtained in all samples, although difficulty in extracting pilocarpine from the heterogeneous coagulated serum system required two or three extractions.

Incubations of pilocarpine *in vitro* with heat-untreated rat serum was followed by colorimetric and bioassay methods over various intervals of time. The results are reported in Table 2 and reveal the close check of the methods employed. Transformation of pilocarpine occurred within 10 min

and was approximately completed in 1 hr. The control incubations at 0 time and at 5° for 60 min demonstrated little transformation of added pilocarpine.

The ability of rat serum to continue the transformation of sequential additions of exogenous pilocarpine is demonstrated by the data in Table 3. It can be seen that approximately 70% of each pilocarpine addition was transformed in 60 min. It seemed obvious that the converting system was not exhausted after three additions of pilocarpine.

TABLE 1. THIN-LAYER CHROMATOGRAPHY OF PILOCARPINE

Sample	Time (min)	Amount of pilocarpine applied *RJ*	(μg)	Amount estimated from color intensity (μg)
Water solution		10	0·69	10
		20	0·68	20
		50	0·68	50
		100	0·68	100
Literature			0·68	
Serum, *in vitro*,	10	50	0·69	25
Serum, *in vitro*,	20	50	0·68	15
Serum, *in vitro*	60	50	0·68	0
Serum, *in vitro*,	60	0		0
Serum, *in vivo*,	0	*		0
Serum, *in vivo*,	1	*	0·69	25
Serum, *in vivo*,	3	*	0·69	12
Serum, *in vivo*,	10	*		< 5

*The total extract of 1 ml serum was applied. Three rats were injected with 10 mg pilocarpine hydrochloride/kg.

TABLE 2. HYDROLYSIS OF PILOCARPINE BY SERUM *in vitro**

Incubation time (min)	Colorimetric method			Bioassay method		
	Recovered (μg)	Lost (μg)	Transformed (%)†	Recovered (μg)	Lost (μg)	Transformed (%)
0	950	50		940	60	
10	610	390	39	650	350	31
20	410	590	59	460	540	51
30	310	690	69	390	610	58
60	82	918	92	107	893	89
120	0	1,000	100	0	1,000	100
60‡	950	50	0			

* All experiments were conducted with 1,000 μg pilocarpine hydrochloride.

† Transformation values were calculated on the basis of amount of pilocarpine recovered at 0 time.

‡ Incubation performed at 5°.

Various known inhibitors were tested for their ability to prevent the transformation of pilocarpine by rat serum. The results have been summarized in Table 4, and reveal good inhibitory activity by EDTA and PMBA. Eserine at 10^{-3} M and paroaxon at 10^{-2} M suppressed pilocarpine conversion by 62% and 48% respectively. Sodium fluoride exhibited a low order of inhibition as did potassium cyanide at 10^{-2} M concentration.

Because EDTA is a known chelating agent of cations, the addition of various mineral cofactors to dialyzed rat serum was explored. It was observed that the ability of rat serum to transform pilocarpine was completely lost after a 24-hr dialysis against running tap water and could not be restored by 10^{-3} M Ca^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+} and $Zn^{2}{}_{2}$ as the sulfates.

Transformation of pilocarpine *in vitro* was also confirmed in other species; dog serum converted 31%, monkey 60%, human 68%, and rabbit 100% of the added pilocarpine during a 60-min incubation.

TABLE 3. TRANSFORMATION OF PILOCARPINE ADDED AT SPACED INTERVALS*

Incubation time (min)	Amount added (μg)	Amount recovered (μg)	Transformed† (%)
0	1,000	950	0
60	10,000	2,240	78
120	8,240	2,400	71
980	5,400	1,540	70

* Ten ml rat serum containing 1 mg pilocarpine/ml served as the initial incubation system. After 60 min, 4 ml was withdrawn for analysis and an additional 1 mg pilocarpine/ml was added to the remaining volume. At the end of 120 min, a 3-ml aliquot was taken and 1 mg pilocarpine/ml was introduced into the remaining 3 ml for a third incubation period.

† Corrected for 95% recovery based on control value.

TABLE 4. INHIBITION OF PILOCARPINE TRANSFORMATION IN RAT SERUM*

Inhibitor	Conc. (M)	Amount recovered (μg)	Transformed† (%)	Inhibition (%)
None		950	0	
EDTA	10^{-4}	845	11	89
p-Chloromercuribenzoic	10^{-4}	900	16	84
Eserine salicylate	10^{-3}	586	38	62
Paroaxon	5×10^{-3}	124	87	13
Paroaxon	2×10^{-2}	458	52	48
Sodium fluoride	10^{-2}	139	86	14
Potassium cyanide	10^{-2}	87	92	8

* All experiments were conducted with 1,000 μg pilocarpine hydrochloride; incubation time was 60 min.

† Transformation values were calculated on the basis of the amount of pilocarpine recovered in the absence of inhibitor.

Since pilocarpine stimulates prostatic secretion which contains several hydrolytic enzymes, its measurement in prostatic fluid and tissue seemed worthwhile. Dog whole prostatic fluid or purified enzyme fractions of prostatic fluid did not initiate transformation of pilocarpine during periods between 0 and 60 min. In one experiment a 20% rat homogenate in isotonic saline was utilized as a possible enzyme source, but no transformation of pilocarpine occurred between 0 and 60 min.

A few experiments were performed *in vivo* in which serum was examined for pilocarpine after an i.v. injection of 10 mg/kg in rats and 3 mg/kg in two dogs. Because of the lack of sensitivity of the method available at the moment, only preliminary results can be mentioned. Intact pilocarpine could not be detected in the blood after 5 min and, in the dogs, approximately 2% of the injected pilocarpine appeared in the prostatic fluid and 3·5% in the urine within 2 hr.

The observations made in this study suggest the presence of a serum system capable of transforming pilocarpine to a form(s) not detectable by the imidazole or lactone reactions. The transformation system is dependent on dialyzable components.

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The rôle of octopamine in tachyphylaxis to tyramine

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BURN AND RAND[1] suggested that the sympathomimetic action of tyramine is a consequence of release of norepinephrine from tissue stores. Direct evidence to support this hypothesis has been forthcoming from a number of laboratories. When repeated doses of tyramine are administered tachyphylaxis develops. With each successive dose of tyramine there is diminished release of norepinephrine.[2] Tachyphylaxis, however, develops before the norepinephrine stores are completely depleted. It has been demonstrated recently that tyramine is taken into sympathetic nerves and converted by β-hydroxylation to octopamine.[3, 4] Octopamine is retained in part by the "microsomal" fraction, which contains the norepinephrine storage granules.[5] It can be released by nerve stimulation[6, 7] and by drugs that deplete norepinephrine.[7] We have examined the octopamine content of the heart during the development of tachyphylaxis to tyramine.

Tyramine-^{3}H (G.L.), obtained from New England Nuclear Corp., Boston, Mass., was diluted with unlabeled tyramine to a specific activity of 5 μc/μmole. Enzymatic conversion of this tyramine-^{3}H to octopamine-^{3}H did not result in production of significant amounts of tritiated water (unpublished observations). Male Sprague-Dawley rats weighing 200 to 250 g received one, two, or three doses of the labeled amine (10 mg/kg, i.m.) at 15-min intervals. One group of rats which had received three injections of the labeled amine was given a fourth dose of unlabeled tyramine (10 mg/kg). Another group received three doses of unlabled tyramine (10 mg/kg) and a fourth dose of labeled tyramine (10 mg/kg). The animals were killed by a blow on the head 15 min after the last dose of tyramine. The hearts were removed, homogenized in ice-cold 0·4 N perchloric acid, and the supernatant analyzed for norepinephrine[8] and tritiated octopamine.[5]