STUDIES ON AN ANTIDIURETIC SUBSTANCE IN THE BILE

REPORT II. PHYSIOLOGICAL FATE OF THE BILIARY ANTIDIURETIC SUBSTANCE

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In the previous communication[1], we reported an antidiuretic substance extracted from human bile (biliary ADS). In the present study experiments were undertaken to ascertain the physiological fate of the biliary ADS in the gall bladder, gastrointestinal tract and liver in comparison with that of Pitressin.

(I) Fate of the biliary ADS in the gall bladder

1) Clinical experiments

Method:

Antidiuretic activities were assayed on 20 ml. of human C-bile and B-bile obtained by duodenal drainage, according to thc technique described previously.[1] The B-bile was diluted with water (diluted B-bile) to the extent that its bilirubin concentration became the same as C-bile, and its antidiuretic activity was compared with the latter.

Results:

It was difficult to compare the antidiuretic activities of B-and C-bile, when its ADS-index was close to zero (high potency in antidiuretic activity), but all the B-bile had distinctly greater antidiuretic activity than the C-bile in cases in whom the ADS-index was high (low potency in antidiuretic activity) (Fig. 1).

Antidiuretic activity of the diluted B-bile was less potent than the corresponding C-bile (Fig. 1). Accordingly it was suspected that a part of antidiuretic substance might be lost in the gall bladder.

2) Animal experiments

Method:

An isolated gall bladder was made in a rabbit in the following manner. Ligation of the cystic duct was performed after cautious separation of the blood vessels from

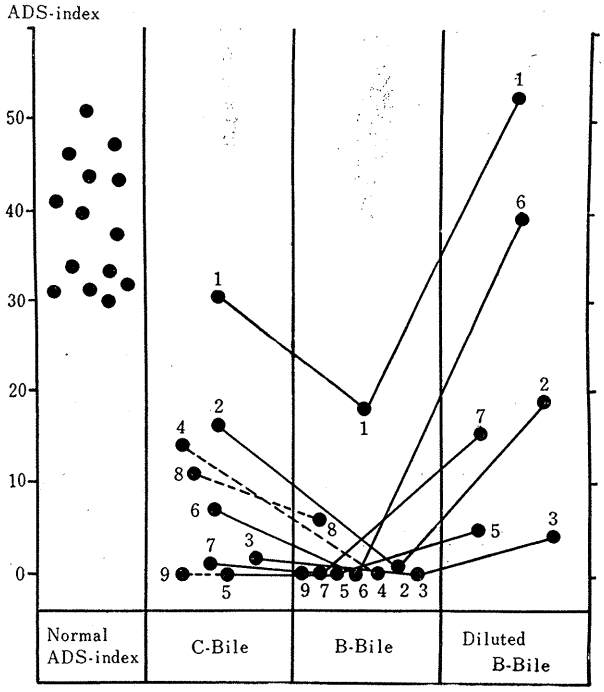

Fig. 1 Comparison of Human C- and B-Bile (ADS-index)

The figures of the same individual bear the same number and are connected with a line. "Diluted B-bile" means the B-bile diluted with water to the extent where its bilirubin concentration is the same as that of C-bile.

it. A fine vinyl tube was then inserted into the head of the cyst and the inserted part was ligated to prevent leakage of the cystic content. In such animals, an intestinal fistula for water infusion was also made with an insertion of a vinyl tube into the intestine about 10 cm below the Oddi's sphincter, through which 7 doses of 10 ml. of warm water were administered at hourly intervals. The urine output was measured at 30 minutes' intervals through an urinary catheter. The other ends of these two vinyl tubes were kept outside the body and the abdominal wall was closed. The animals were fixed in a supine position throughout the experiment.

A conspicuous diuresis and hypochloruresis occurred 3 to 3.5 hours after the first water loading onward. At 4 hours of the experiment, 0.5 ml. of normal saline solution with or without 2,000 mU of Pitressin, or the biliary ADS which was obtained from 20 ml. of human C-bile, was slowly introduced into the isolated gall bladder through the biliary canula. The chloride concentration was also estimated in each urinary specimen.[2)]

Results:

Introduction of the biliary ADS or Pitressin into an isolated gall bladder at the

diuretic stage immediately lead to a cessation of urine outflow, whereas introduction of normal saline solution resulted continuous diuresis (Fig. 2). Introduction of

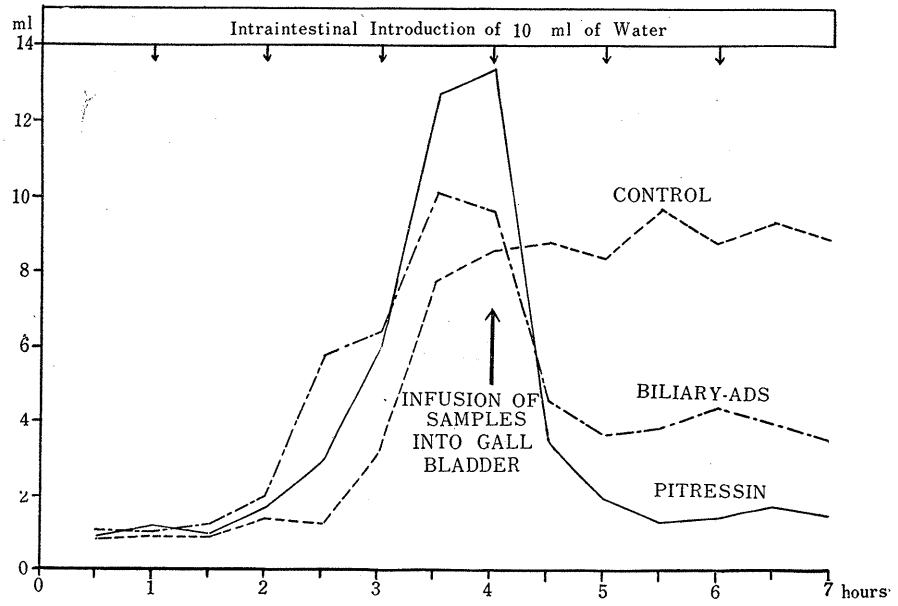

Fig. 2 Absorption of ADS from Isolated Gall Bladder (Urinary Outflow)

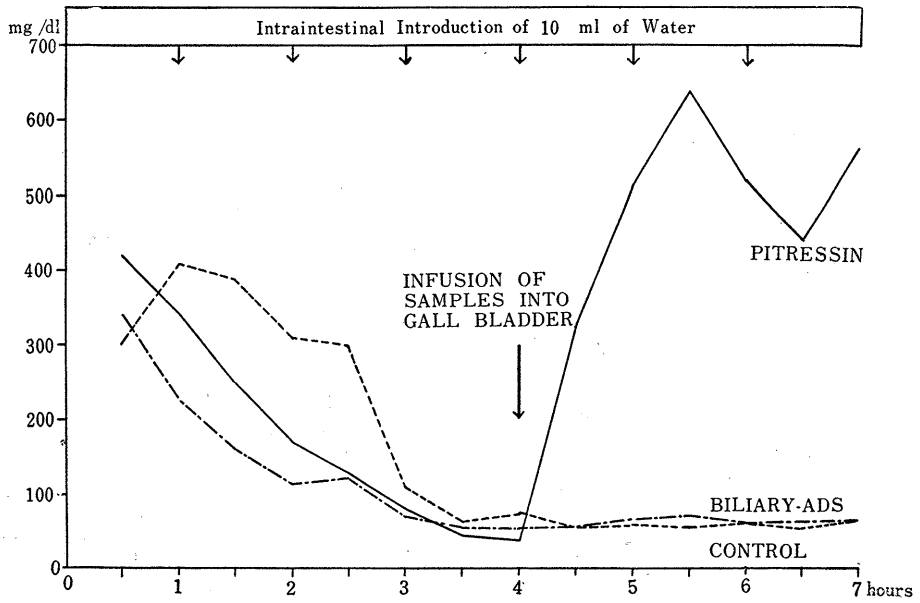

Fig. 3 Absorption of ADS from Isolated Gall Bladder (Urinary NaCl Concentration)

Pitressin into the cyst immediately increased the urinary chloride concentration, whereas, in the control group, it began to decrease prior to the onset of the diuresis and this condition continued till the end of the experiment (Fig. 3). Introduction of the biliary ADS into the cyst caused no change on hypochloruresis.

That Pitressin introduced into an isolated gall bladder immediately decreased urine outflow and increased chloride concentration suggests a direct action of Pitressin on the kidney, Pitressin being absorbed into blood stream from the gall bladder, because Pitressin has been found to have such effects on urinary condition.[1] A similar effect was obtained with the biliary ADS which, upon introduction into an isolated gall bladder, caused immediate cessation of urine outflow. As expected, introduction of the biliary ADS into the cyst did not increase the urinary chloride concentration (Fig. 3), because the extracted biliary ADS has no such effect on rats.[1]

(II) Inactivation of antidiuretic substance by digestive juices and enzymes

Methods:

(i) Preparation of ADS: The biliary ADS was prepared in such an amount that the extract from 20 ml. of the human bile could be injected intraperitoneally into a group of three rats, and Pitressin was prepared in such an amount that 200 or 500 mU could be injected per three rats.

(ii) Inactivation of ADS by human gastric juice: Pitressin or the biliary ADS was added to 20 ml. of a human gastric juice which was obtained through a gastric tube after introduction of Katsch-Kalk's solution. Ten milliliters of this sample was kept in an incubator at 37°C for three hours and the remaining sample was kept in an icebox for three hours. After these procedures both samples were assayed for antidiuretic activity according to the same procedure as employed for extraction of the biliary ADS.

(iii) Inactivation of ADS by the human bile: One thousand milliunits of Pitressin was added to 20 ml. of human C-bile; 10 ml. of this sample was kept in an incubator at 37°C for three hours and the remaining sample was kept in an icebox for three hours. For the purpose of examining the stability of the biliary ADS in the bile, antidiuretic activities of the following samples were also assayed; a) 20 ml. of the bile incubated at 37°C for three hours and b) the same samples which was kept in an icebox for the same length of time.

(iv) Inactivation of ADS by pepsin: Four hundred milliunits of Pitressin (0.2 ml.) was brought to 10 ml. with citric acid buffer solution (pH 3). The biliary ADS was dissolved in 10 ml. of this buffer solution. To each 2.0 ml. of these solutions 0.2 ml. of active pepsin solution (50 mg/dl) was added. These samples were incubated for five hours at 37°C, and were heated at 100°C for 10 minutes. The heated sample was immediately cooled, neutralized with 1N NaOH and filtered. Each 0.5 ml. of this filtrate was injected intraperitoneally into three rats for the assay of

antidiuretic activity.

The control was kept in an icebox for five hours after addition of pepsin. Both Pitressin and the biliary ADS were still active despite 10 minutes' boiling at 100°C, whereas pepsin was inactivated by this heat treatment.

(v) Inactivation of ADS by trypsin: The effect of trypsin (50 mg/dl) was similarly tested. The buffer solution used was a phosphate buffer of pH 8. For the purpose of inacivating trypsin, the samples were boiled at 100°C for 10 minutes after adjustment of pH to 3 with 1N HCl, and were then neutralized with 1N NaOH for intraperitoneal injection.

Results:

(1) Inactivation of ADS by human gastric juice:

Antidiuretic activity was negative in the human gastric juice which was extracted for ADS with the procedure used for biliary ADS (Table 1).

Antidiuretic activities of the biliary ADS and Pitressin were unchanged after an incubation in gastric juice for 3 hours at 37°C (Table 2).

Table 1 Antidiuretic Activity of the Gastric Juice

No.	ADS-index	Cl-index
1	35.6	1.0
2	26.8	1.1
3	28.1	1.1

Normal ADS-index: 38.0±6.8

Table 2 Stability of the Biliary ADS and Pitressin in Gastric Juice (Incubated for 3 hours at 37° C)

	No.	Pre-Incubation ADS-index	Post-Incubation ADS-index
Biliary ADS	1	14.1	12.2
	2	3.7	4.3
	3	4.8	3.4
	4	4.2	2.7
	5	4.2	2.7
Pitressin	1	13.4	13.8
	2	2.2	0.3
	3	14.1	12.2

(2) Inactivation of ADS by human bile:

Antidiuretic activity of Pitressin was unchanged after an incubation in the human bile for 3 hours at 37°C (Table 3). Since the amount of bile from which Pitressin was extracted was as small as 10 ml. and it did not have any antidiuretic potency, it was presumed that the data of these experiments were interfered by the bile. Accordingly, it was concluded that Pitressin is stable in the bile.

Antidiuretic activity of the bile after 3 hours' incubation at 37°C was the same as

that before incubation (Table 4). The results suggest that the biliary ADS is stable in the bile.

Table 3 Stability of Pitressin in the Bile
(Incubated for 3 hours at 37°C)

No.	Pre-Incubation ADS-index	Post-Incubation ADS-index
1	0.6	2.6
2	5.6	10.5
3	5.1	7.3

Table 4 Stability of the Biliary ADS in the Bile
(Incubated for 3 hours at 37°C)

No.	Pre-Incubation ADS-index	Post-Incubation ADS-index
1	0.9	2.6
2	8.5	8.4
3	5.5	7.3

(3) Inactivation of ADS by pepsin:

The biliary ADS, as well as Pitressin resisted pepsin treatment (Table 5).

Table 5 Inactivation of the Biliary ADS and Pitressin with Pepsin
(pH 3; 37°C for 5 hours)

	No.	Pre-Incubation ADS-index	Post-Incubation ADS-index
Pitressin	1	2.1	8.4
	2	2.2	3.5
	3	0.8	0.0
	4	11.8	17.7
	5	9.3	2.1
	6	6.0	5.6
	Average	5.4	7.5
Biliary ADS	1	6.2	8.5
	2	7.8	11.4
	3	10.4	14.2
	4	6.2	9.8
	5	8.4	12.6
	Average	7.8	11.3

(4) Inactivation of ADS by trypsin:

The biliary ADS was inactivated by trypsin treatment, because the ADS-index was elevated after this treatment. In contrast, Pitressin was found to be stable against trypsin treatment (Table 6).

(III) Inactivation of ADS by the extract of intestinal mucosa

Method:

About one gram of the small intestinal mucosa of rats was homogenized in 10 ml.

Table 6 Inactivation of the Biliary ADS and Pitressin with Trypsin (pH 8; 37°C for 5 hours)

	No.	Pre-Incubation ADS-index	Post-Incubation ADS-index
Pitressin	1	18.2	9.7
	2	18.2	12.2
	3	10.9	9.4
	4	10.9	14.3
	5	10.9	11.3
	6	3.1	5.6
	7	3.1	4.5
	8	14.4	12.2
	9	23.0	23.6
	10	3.6	8.0
	11	12.3	14.4
	12	4.0	6.2
	Average	11.1	11.0
Biliary ADS	1	17.8	29.1
	2	22.2	25.8
	3	7.1	18.2
	4	23.8	42.6
	5	22.2	45.4
	Average	18.6	34.2

of pH 8 phosphate buffer solution. The homogenate was filtered. One milliliter of water solution of the biliary ADS extracted from 20 ml. of the bile, or 1.0 ml. of water solution containing 5,000 mU of Pitressin, was added to 1.0 ml. of the filtrate. These solutions were made up to 5 ml. with the buffer solution and were incubated at 37°C. Samples of the Pitressin group were assayed for their antidiuretic activities at 0, 60 and 120 minutes of incubation. Samples of the biliary ADS group were assayed at 0 and 90 minutes.

Results:

Value of the ADS-index of Pitressin group was already elevated at 60 minutes of incubation with an extract of the intestinal mucosa and the elevation became more marked at 120 minutes (Table 7). The results suggest that it is inactivated by an extract of the intestinal mucosa with the lapse of incubation time.

The biliary ADS was also inactivated at 90 minutes of incubation with the mucous extract (Table 8).

Table 7 Inactivation of Pitressin with the Extract of Intestinal Mucosa (pH 8, 37°C)

No.	Pre-Incubation ADS-index	Post-Incubation (60 minutes) ADS-index	Post-Incubation (120 minutes) ADS-index
1	1.2	4.7	13.5
2	0.6	9.6	20.1
3	15.3	33.7	35.5
4	23.3	31.1	35.3
5	21.0	20.0	33.9
Average	12.3	19.8	27.7

Table 8 Inactivation of Biliary ADS with the Extract of Intestinal Mucosa (pH 8, 37°C)

No.	Pre-Incubation ADS-index	Post-Incubation ADS-index (90 minutes)
1	8.3	15.9
2	0.9	2.2
3	12.7	26.1
4	3.5	16.4
5	10.3	18.5
Average	7.1	15.8

(IV) Absorption of ADS from the intestinal wall

Methods:

(i) Experiment in rats: Instead of intraperitoneal injection, the test materials were infused orally through a rubber tube into the stomach of the rats which had been prepared for the assay of antidiuretic activity. The test material given to a rat was 1,000 mU of Pitressin or the biliary ADS extracted from 10 ml. of the human bile. Values of the both indices were presented as an average of three rats.

(ii) Experiment in rabbits: Urine output and urinary chloride concentration were estimated in the rabbits in diuretic state, without ligation of the cystic duct as described. The test material was introduced through an intestinal tube into the small bowel 4 hours after the first water loading. The test material given to a rabbit was 2,000 mU of Pitressin or the biliary ADS extracted from 20 ml. of the human bile.

Results:

(1) Experiment in rats

The ADS-index, as well as the Cl-index of the Pitressin group was nearly the same as that of the control group, and no significant difference was noted between the index of the biliary ADS group and that of the control group (Table 9). The results suggest that Pitressin, like the biliary ADS, is not absorbed from the gastrointestinal cannal.

Table 9 Oral Introduction of Pitressin or Biliary ADS

	No.	ADS-index	Cl-index
Pitressin	1	28.5	0.95
	2	40.6	0.95
	3	38.3	0.93
	Average	35.8	0.94
Biliary ADS	1	23.4	1.07
	2	29.6	0.89
	3	27.1	0.94
	4	41.1	0.97
	Average	30.3	0.97

(2) Experiment in rabbits

In the control group a remarkable diuresis occurred from 3 to 3.5 hours onward

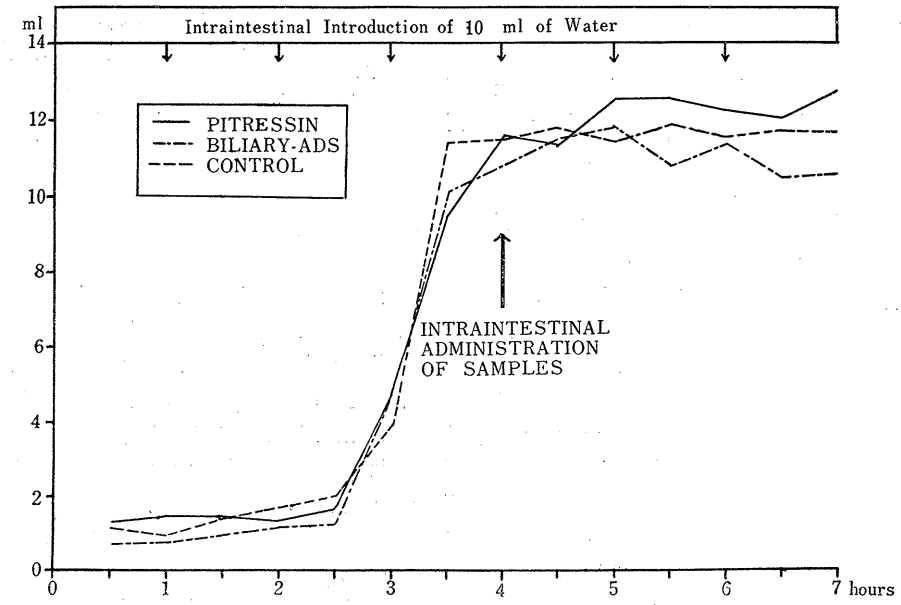

Fig. 4 Absorption of ADS from the Intestine (Urinary Outflow)

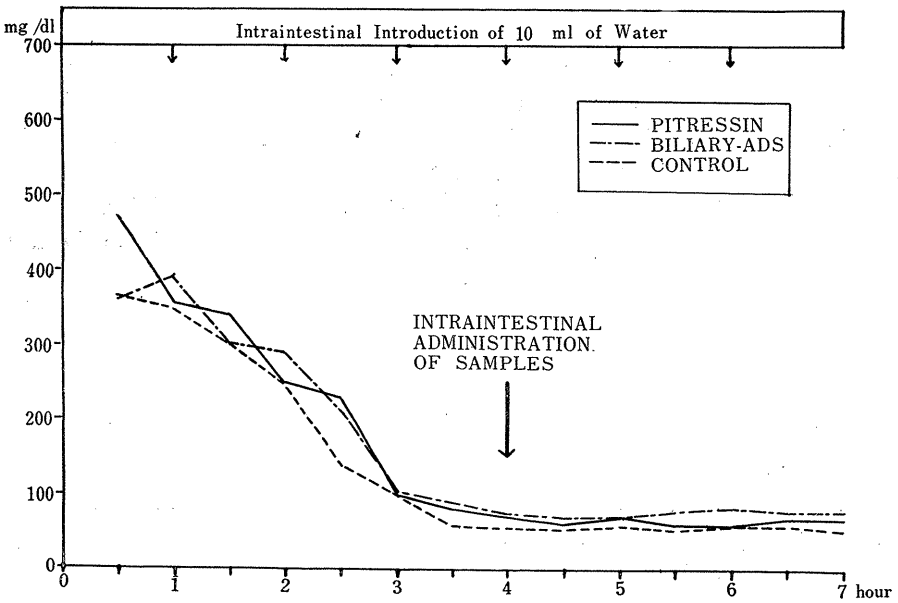

Fig. 5 Absorption of ADS from the Intestine (Urinary NaCl Concentration)

after the first water loading. The intestinal administration of Pitressin or the biliary ADS at this diuretic stage did not decrease the urine output (Fig. 4). Immediately prior to the onset of diuresis the urinary chloride concentration was markedly decreased and remained low till the end of the control experiment (Fig. 5).

The intestinal administration of these two antidiuretic substances had no elevating effect on the urinary chloride concentration (Fig. 4, 5). The results also suggest that Pitressin, as well as the biliary ADS, is not absorbed from the intestinal canal.

(V) Inactivation of ADS by the liver

Methods:

(i) Inactivation of ADS by the liver extract: The liver tissue was obtained from a rat of about 120 gm under ether anesthesis. Blood and fibrous tissue were removed as much as possible. One gram of the liver tissue was homogenized in 10 ml. of a phosphate buffer solution of pH 6.5. The homogenate was centrifuged and filtered. The filtrate was used as the liver extract in the following experiments.

Five hundred milliunits of Pitressin or the biliary ADS, which was extracted from 10 ml. of a mixture of human C- and B-bile, was made up to 1.0 ml. with the phosphate buffer of pH 6.5. One milliliter of the liver extract and 3 ml. of the phosphate buffer was added to this ADS solution. One portion of this sample was incubated at 37°C for 60 minutes and another portion was kept in an icebox for 60 minutes.

A mixture of 1.0 ml. of liver extract and 4.0 ml. of the buffer solution served as the control sample.

(ii) Inactivation of ADS by perfusion through an isolated rabbit liver: Rabbit liver was removed for the perfusion experiment. Perfusion fluid was a mixture of defibrinized blood and Ringer's solution. Perfusion apparatus and procedure were the same as those described by Ohishi.[4] At 0, 60 and 120 minutes of the perfusion, 5 ml. of each perfusate was taken for assay of the antidiuretic activity. The perfusate was centrifuged and the supernant fluid was injected intraperitoneally in such an amount that each rat recieved 0.5 ml. of the sample.

In the inactivation experiments of ADS, 500-1,000 mU of Pitressin or the biliary ADS extracted from 100 ml. of the normal human bile was added to the perfusion fluid.

Results:

(1) Inactivation of ADS by the rat liver extract

The ADS index of the Pitressin group, as well as that of the biliary ADS group was increased after the incubation with the rat liver extract, while that of the control group which consisted of the liver extract alone without ADS solution was unchanged (Fig. 6, Table 10, 11, 12). The results indicate that the biliary ADS, like

Pitressin, is inactivated by the liver extract.

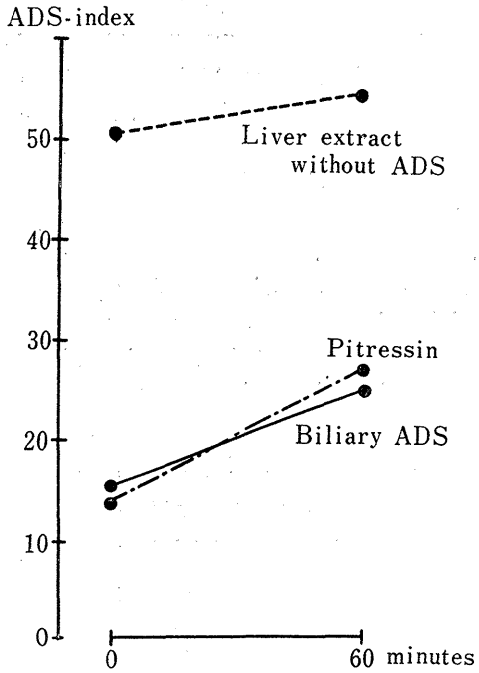

Fig. 6 Inactivation of ADS by Liver Extract

Table 10 ADS-index of the Liver Extract (Control Experiment)

Incubation time	No. 1	No. 2	No. 3	No. 4	No. 5	Average
0 hr	48.9	55.1	42.9	47.8	56.2	50.2
1 hr	57.3	39.5	39.4	67.3	67.9	54.5

Table 11 Inactivation of Pitressin with liver Extract

Incubation time	No. 1	No. 2	No. 3	No. 4	No. 5	Average
0 hr	23.7	15.6	3.8	20.2	5.1	13.7
1 hr	52.3	22.2	11.2	26.3	22.0	26.8

Table 12 Inactivation of the Biliary ADS with Liver Extract

Incubation time	No. 1	No. 2	No. 3	No. 4	No. 5	Average
0 hr	21.3	22.1	30.2	0.3	1.9	15.2
1 hr	46.6	28.6	28.3	5.4	6.8	25.1

(2) Inactivation of ADS by perfusion through an isolated rabbit liver

The ADS-index of the normal perfusate was 36.1 on an average (30.8−41.0), nearly the same as that of a control (38.0±6.8) which received an intraperitoneal injection of normal saline solution. The ADS-index of the normal perfusate was decreased distinctly after two hours' perfusion (Fig. 7, Table 13). The results suggest that a newly produced antidiuretic substance may be liberated from the liver.

The ADS-index of the perfusate in which Pitressin had been added was increased in the first one hour of the perfusion and returned to the previous level during the second one hour (Fig. 7, Table 14). A phenomenon that antidiuretic activity once decreased was brought back to the previous level by prolonged perfusion probably suggests that a new antidiuretic substance was produced from the anoxic or impaired liver which underwent a prolonged perfusion. This antidiuretic substance, if present, may be called as "hepatic antidiuretic substance."

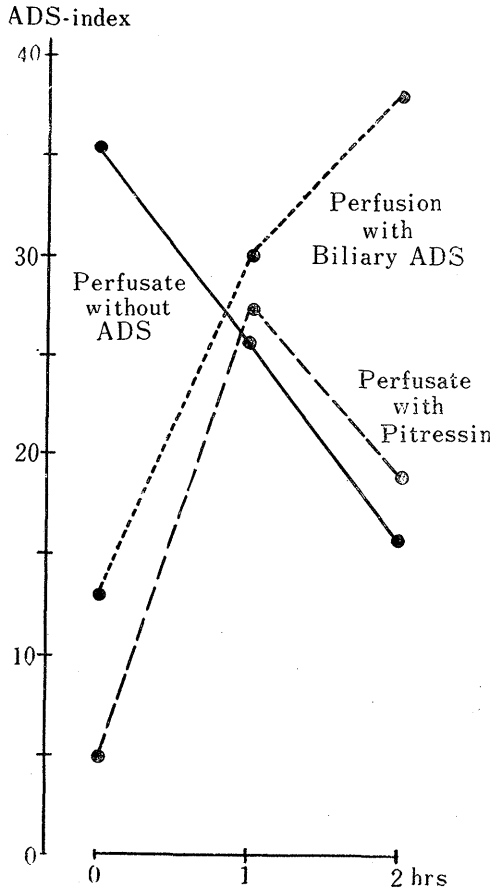

Fig. 7 Perfusion Experiment on Normal Isolated Liver

Table 13 ADS-index of the Normal Perfusate in Liver Perfusion Experiment

Perfusion's time	No. 1	No. 2	No. 3	No. 4	No. 5	Average
0 hr	39.4	30.8	37.0	32.4	41.0	36.1
1 hr	31.5	26.3	26.3	24.0	23.2	26.3
2 hrs	18.5	18.3	15.4	15.9	14.2	16.5

Table 14 ADS-index of the Perfusate with Pitressin in Liver Perfusion Experiment

Perfusion's time	No. 1	No. 2	No. 3	No. 4	No. 5	Average
0 hr	9.8	0.5	2.6	5.9	3.9	4.5
1 hr	15.4	27.7	33.6	27.6	32.3	27.3
2 hr	10.3	18.8	13.3	26.2	19.6	17.6

Table 15 ADS-index of the Perfusate with the Biliary ADS in Liver Perfusion Experiment

Perfusion's time	No. 1	No. 2	No. 3	No. 4	No. 5	Average
0 hr	18.6	17.0	10.7	11.4	7.3	13.0
1 hr	32.3	22.0	19.7	53.8	22.2	30.0
2 hrs	39.3	40.6	30.8	52.8	27.8	38.3

The antidiuretic activity of the perfusate in which the biliary ADS had been added became less potent after two hours' perfusion (Fig. 7, Table 15). Namely, there was no production of a new antidiuretic substance which was produced from the liver after two hours' perfusion under the preceding conditions. It may be that this phenomenon resulted from a hepatotonic substance, which was possibly contained in the biliary ADS as a contaminant and capable of enhancing hepatic functions to relieve tissue exhaustion and prevent the production of hepatic antidiuretic substance. Further investigation on this problem will be reported elsewhere.

DISCUSSION

It has been demonstrated that the biliary ADS is reabsorbed from the gall bladder, stable in gastric juice and bile, inactivated by trypsin or intestinal mucosa extract, while it is resistant to pepsin treatment. It is not reabsorbed from the intestinal wall, and is inactivated in blood stream while passing the liver. In other words, the biliary ADS may have a physiological significance for the fluid balance of the body only in that it is reabsorbed from the gall bladder.

So far as these experiments are concerned the biliary ADS has the same properties as those of Pitressin except that Pitressin is resistant to trypsin treatment. Our data contradicted the reports that vasopressin, a posterior pituitary hormone, resists pepsin treatment, but not trypsin treatment[4]. Presumably such discrepancies are related to the fact that Pitressin is a commercial preparation which has been treated for prolonged preservation. Further studies into this problem are necessary.

The results that Pitressin is inactivated by a liver extract or by perfusion through an isolated liver are in agreement with many other reports.[5)6)]

On the basis that an antidiuretic activity was negative in the gastric juice, it seems likely that the antidiuretic substance of the duodenal juice was derived from the bile or pancreatic juice. Furthermore, it may be concluded that the ADS of the duodenal juice was originated from the bile itself, because this substance was demonstrated in the cystic bile obtained under a surgical operation and the pancreatic juice contains trypsin which has potent activities to inactivate the ADS.

SUMMARY AND CONCLUSION

1) Human B-bile was distinctly greater in antidiuretic activity than human C-bile. The B-bile was diluted with water to the extent that its bilirubin concentration equaled that of C-bile, and its antidiuretic activity was assayed in comparison with the C-bile. The diluted B-bile was found to be less potent in activity than the C-bile. It was therfore thought that a part of antidiuretic substance might be reabsorbed from the wall of the gall bladder.

2) Direct infusion of the biliary ADS into an isolated gall bladder of a rabbit, in which continuous diuresis had been induced, immediately lead to a cessation of urine outflow. This also indicates that the biliary ADS is reabsorbed from the gall bladder.

3) No antidiuretic activity was demonstrated in human gastric juice.

4) The biliary ADS was stable in human gastric juice or C-bile.

5) The biliary ADS was resistant to pepsin treatment but was inactivated by trypsin or intestinal mucosa extract.

6) Introduction of the biliary ADS into the rat stomach or into the rabbit intestine resulted in no depression of diuresis.

7) The biliary ADS was inactivated by perfusion through an isolated rabbit liver and by rat liver extract.

8) The biliary ADS probably has the same properties as Pitressin except that the latter is stable during trypsin treatment.

9) It has been concluded that the biliary ADS is reabsorbed from the gall bladder and inactivated in the intestine. Even though it is not completely inactivated in the intestine, there is no absorption from the intestinal wall. While circulating in the blood stream, it is inactivated by the liver.

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