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THE REABSORPTIVE FUNCTION OF THE GALL-BLADDER

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Most vertebrates possess a gall-bladder in which hepatic bile, the immediate secretion product of the liver, is stored between meals before being discharged into the duodenum during digestion. Although the volume of the gall-bladder may be only one thirtieth of the volume of bile secreted daily by the liver, the gall-bladder selectively concentrates the bile and can thereby hold up to one half of the daily output of hepatic bile (Schmidt & Ivy, 1937). During its stay in the gall-bladder the bile may be reduced in volume by about 90 %, but its physiologically significant components—the bile pigments, steroid bile acids, and cholesterol—undergo practically no reabsorption and therefore increase up to tenfold in concentration. Since the bile pigments and cholesterol are sometimes already near the saturation point in hepatic bile, the concentrating activity of the gall-bladder is thus a primary cause of gall-stone formation. The mechanism of this selective absorption is unknown, and will be the subject of the present series of papers.

The most unequivocal evidence on concentration changes during absorption comes from the classical experiments of Ravdin, Johnston, Riegel & Wright (1932), who introduced hepatic bile into the gall-bladders of unanaesthetized dogs and periodically withdrew samples for analysis. They observed that the normal gall-bladder absorbed about 16 % of its volume of bile per hour, with concomitant increases in the concentrations of calcium and bile acid and decrease in those of chloride and bicarbonate. Sodium concentration consistently dropped slightly at first, then rose, often to double its initial value. Since the *amounts* of bile pigment, bile acid, and cholesterol normally absorbed were negligible, their increase in concentration was due simply to the decrease in volume. Absorption became slower with time, and finally came to a halt when the bile had been reduced to 8–10 % of its original volume. Table 1 summarizes the differences in chemical composition between canine bile recovered before and after storage in the gall-bladder. One further point emphasized in all freezing-point studies (e.g. Brand, 1902; Gilman & Cowgill, 1933) is that hepatic bile, and gall-bladder bile at all stages of its reabsorption, are isotonic with

plasma. This implies that some of the constituents of bile must be osmotically inactive, since the sum of their concentrations often doubles in the course of reabsorption.

Ravdin's group also found that inorganic salt solutions, including isotonic NaCl and NaCl-$NaHCO_3$ mixtures, could be absorbed (Ravdin, Johnston, Austin & Riegel, 1932). This simple observation vitiates attempts to explain absorption by invoking the Donnan equilibrium, with either the bile acids (Sobotka, 1937) or negatively charged mucins (Frey, 1934) serving as the fixed anion. However, when the gall-bladder was damaged or infected, absorption ceased. The human gall-bladder may also cease to absorb when damaged (e.g. by gall-stones), as evidenced by the concentration profile of gall-bladder bile approaching that of hepatic bile. In addition, two conditions were detected under which absorption is inhibited

TABLE 1. Composition of canine bile (mM)

	Hepatic bile	Gall-bladder bile
Na^+	174	220–340
K^+	6·6	6–10
Cl^-	55–107	1–10
HCO_3^-	34–65	0–17
Bile acids	28–42	290–340
Ca^{2+}	6	25–32
Mg^{2+}	3·6	—

From the analyses of Ravdin, Johnston, Riegel & Wright (1932), Reinhold & Wilson (1934), and Wheeler & Ramos (1960).

without anatomical signs of damage. First, Johnston, Ravdin, Austin & Morrison (1932) observed that absorption by the canine gall-bladder proceeded three times more slowly at night than during the day. Secondly, it was noticed by an obstetrician that the gall-bladder was highly distended in 75 % of pregnant women undergoing Caesarean section (Potter, 1936). The gall-bladder wall itself appeared normal, but the bile resembled hepatic bile, for instance in its high chloride and low bile-acid concentration. The highest bile-acid level recorded in the gall-bladders of thirty-four Caesarean patients was only one half of the normal level (Riegel, Ravdin, Morrison & Potter, 1935). Evidently the absorptive function of the gall-bladder practically ceases during parturition. The regulatory factors responsible for this inhibition and for the diurnal variations are obscure.

The experimental results to be presented here have been mostly obtained from an *in vitro* preparation of the gall-bladder of fresh-water fish. The first paper will be concerned with a rationale for the concentration changes in hepatic bile effected by the gall-bladder, and the regulation of the absorptive process; the second and third will be devoted to the mechanism of absorption of solute and water, respectively. These problems are of

course not confined to the physiology of the gall-bladder, but are also relevant to general questions of how substances pass through biological membranes and how fluids are transported by other secretory or absorptive epithelia, such as those of the intestine, kidney and other glands. It will be shown that the gall-bladder can selectively absorb isotonic Ringer's solution in a manner reminiscent of many other epithelia; and that this ability, coupled with purely physical properties of the steroid bile salts, is sufficient to explain the main features of bile reabsorption. In addition, a hitherto unsuspected role of the pituitary hormone oxytocin has been discovered. A preliminary account of some of this work has already appeared (Diamond, 1960).

METHODS

Dissection. Most of the fish used in this study were roach (*Rutilus rutilus*), caught on rod and line in nearby rivers and 6–13 in. (15–33 cm) in length. When other species were used (king carp, *Cyprinus carpio*; bream, *Abramis blicca*; and pike, *Esox lucius*), this is indicated in the text. The fish were kept in a large tank with constant running tap water, and fed weekly on maggots. In all these species several hepatic ducts unite with the cystic duct to form the common bile duct. Ducts leading directly from the liver to the gall-bladder, such as have been reported in salmon, have never been observed in over 200 dissections during the present study. After the fish had been pithed and the viscera removed to Ringer's solution, the hepatic ducts and organs surrounding the gall-bladder were carefully cut away. The common duct was cut just before it reached the duodenum. With a syringe and blunt-tipped needle inserted up the common duct into the gall-bladder, the deep yellow bile was withdrawn, and the organ washed out with Ringer's solution until the lumen appeared colourless through the transparent wall. The external Ringer's solution was changed several times during this stage of the dissection, to prevent bile from damaging the preparation. Finally, a 4·2 cm length of fine nylon tube (0·5 mm int. diam., 0·63 mm ext. diam.) was advanced down the common duct and cystic duct until its tip could be seen a few millimetres inside the gall-bladder. This cannula was secured by two tight ligatures where the cystic duct left the gall-bladder, well distal to all the hepatic ducts. The exterior end of the cannula could then be closed with a small glass plug to which a fine wire hook had been tied. The interval between pithing and the cannulation of the gall-bladder averaged 20 min.

Replacement of the luminal solution was accomplished by means of polythene tubing whose tip had been drawn out into a capillary of length 4·6 cm and internal diameter 50–100 μ. With the wide end of the tubing mounted on a hypodermic needle and syringe, the whole of the capillary part was advanced down the unplugged cannula into the lumen, where its tip would then be projecting 0·4 cm beyond the end of the cannula into the gall-bladder, as could also be confirmed visually. The luminal fluid was then aspirated, and the gall-bladder refilled with fresh solution until the fluid was forced back up the space between the capillary and cannula and overflowed out of the open end of the cannula. Repetition of this procedure three more times sufficed to change the luminal solution completely, as judged, for instance, by the disappearance of all dye when the gall-bladder initially contained an intensely coloured solution. After the final filling the cannula was replugged.

Experiments on absorption. The principle used to follow absorption was that as the gall-bladder transfers fluid from its lumen to the outer solution, the preparation loses weight (generally the luminal fluid represented 70–90 % of the total weight). Accordingly the preparation was suspended in a 30 ml. beaker of Ringer's solution and weighed every 5 min to $\pm 0{\cdot}1$ mg on a Baird & Tatlock double-pan balance. As the principle source of error is

the variable amount of surface water clinging to the outside of the gall-bladder, a consistent procedure was adopted whereby the gall-bladder was drained six times in rapid succession against the smooth lip of a cylindrical glass beaker, going progressively around the circumference of the beaker so that all sides of the gall-bladder would be drained. The entire weighing procedure involved the gall-bladder being out of the solution for 25–30 sec. In all manipulations the preparation was held by the wire hook with forceps and during weighing was suspended by the wire hook from a hook provided above the weighing-pan of the balance. The experimental solutions were stirred by a stream of oxygen bubbles saturated with water vapour by passage through Ringer's solution. All experiments were carried out at ambient room temperature (17–23° C), except for a few measurements of temperature coefficients, in which the experimental beaker rested in an electrically heated water-bath whose temperature was kept constant within $\pm 0{\cdot}2°$ C by a thermostat.

At the end of an experiment the drained gall-bladder was cut over a tared glass tube, into which the luminal fluid was collected. From the weight of the promptly stoppered tube and the known density of the experimental solutions (always in the range 1·004–1·033 g/ml.), the volume of the recovered fluid was computed, and it could then be diluted for analysis. The drained gall-bladder wall was weighed either directly or after surface fluid had been lightly blotted off with Whatman No. 542 low-ash filter paper (since the extracellular layer is on the outside, this procedure should not damage the cells). From the weight of a gall-bladder wall in air and under water the specific gravity was calculated to be 1·1. Hence the average thickness of the wall was estimated as the quotient of the blotted weight divided by the area and specific gravity. In many cases the ratio of dry weight to wet weight was determined by weighing again after drying overnight in an oven at 110° C and then over concentrated sulphuric acid in a desiccator. This ratio was 0·154 for unblotted and 0·183 for blotted gall-bladders. Hence unblotted gall-bladders have 1·03 g more water per gram dry weight than blotted gall-bladders, the difference presumably representing extracellular water. For analysis the gall-bladder was extracted with boiling distilled water. The efficiency of this procedure was confirmed by the observation that it removed 99·7 % of the ^{82}Br from a gall-bladder wall incubated for 1 hr in 'Ringer's solution' containing ^{82}Br.

The size of the extracellular space was determined by leaving gall-bladders for 1 hr in Ringer's solution containing 1 mM inulin (5 g/l.) both in the lumen and outside, then analysing the extract for inulin. The 'inulin blank' value for gall-bladders from Ringer's solution without inulin was found to be nil. The average extracellular space for blotted gall-bladders was $34{\cdot}2 \pm 4{\cdot}2$ % (this and all other errors to be reported are standard deviations). If all the water removed by blotting was extracellular, an unblotted gall-bladder should have an extracellular space of $46{\cdot}5 \pm 3{\cdot}6$ %, which is in satisfactory agreement with the one measured value of 49·8 %.

To compute the area of the gall-bladder from the volume by the formulae for a sphere the volume was calculated from the weight of the 'full' lumen, taken arbitrarily as the difference between the weight of the preparation at the beginning of the experiment and the final weight with the luminal fluid drained out. This procedure was adopted because the initial weight was replicable to ± 2 % on successive refillings, and the luminal surface of a filled and fixed gall-bladder showed no folds in the light microscope. Strictly speaking, the shape of the organ is not quite a sphere but more nearly an oblate spheroid whose axis of revolution is up to 30 % shorter than its major axis. However, this approximation is quite sufficient for the purpose of standardizing results from different preparations on the basis of surface area and for comparison with other tissues.

Preparation of solutions. Table 2 gives the composition of the principal solutions employed. It may be assumed that solution *A* was used in all cases where the solution is not specified. Anisotonic solutions were prepared by mixing *E* or *F* with *A*, and *J* or *K* with *G*; solutions in which varying amounts of NaCl were replaced with KCl or sucrose were prepared by mixing *C* or *D* with *A*, and similarly *H* or *I* with *G* for replacements of Na_2SO_4 with

TABLE 2. Composition of solutions (mM)

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*
NaCl	144	144	—	—	—	—	—	—	—	—	—	—
K phosphate	2·5	—	2·5	2·5	2·5	2·5	2·5	2·5	2·5	2·5	2·5	2·5
$CaCl_2$	1·8	1·8	1·8	1·8	1·8	1·8	—	—	—	—	—	—
$MgCl_2$	1·0	1·0	1·0	1·0	1·0	1·0	—	—	—	—	—	—
Glucose	5·0	5·0	5·0	5·0	5·0	5·0	5·0	5·0	5·0	5·0	5·0	5·0
Na phosphate	—	2·5	—	—	—	—	—	—	—	—	—	—
KCl	—	—	144	—	—	—	—	—	—	—	—	—
Sucrose	—	—	—	261	—	600	93·1	269·5	93·1	—	600	—
Na_2SO_4	—	—	—	—	—	—	72	—	—	—	72	—
K_2SO_4	—	—	—	—	—	—	—	—	72	—	—	—
$CaSO_4$	—	—	—	—	—	—	7·97	2·75	7·97	2·75	7·97	2·75
$MgSO_4$	—	—	—	—	—	—	1·0	1·0	1·0	1·0	1·0	1·0
$NaCH_3SO_4$	—	—	—	—	—	—	—	—	—	—	—	144

The stock solution of K phosphate was a mixture of K_2HPO_4 and KH_2PO_4 containing 4·5 m-mole of K for each 2·5 m-mole of phosphate; thus [K] is 4·5 mM in all solutions except *B*, *C*, and *I*. *B* is a K-free solution. In the text solutions are frequently named after their principal component: e.g. *A* is NaCl Ringer's solution, *D* 'sucrose solution', *G* 'Na_2SO_4 solution', and *L* is '$NaCH_3SO_4$ solution'.

K_2SO_4 and sucrose. 'Bromide Ringer' differed from *A* only in the replacement of chloride salts by the corresponding bromides. In the tetra-ethyl ammonium (TEA) chloride solution, the NaCl of *A* was replaced by 144 mM TEACl. All solutions were buffered with 2·5 mM phosphate at pH 7·3, checked occasionally with the glass electrode and Pye pH meter. Glucose, sucrose, $NaCH_3SO_4$, and TEACl were weighed out fresh for each experimental solution, and other salts were taken from concentrated stock solutions. Experimental solutions were kept in a refrigerator when not in use, and were discarded after a maximum of 5 days. Pyrex glass-ware was used throughout, and reagents were Analar, except those not manufactured in this grade. From cryoscopic data in the International Critical Tables, solutions *A–D*, *G–I*, and *L* were calculated to be isosmotic with 154 mM-NaCl, which has approximately the same freezing-point depression (0·509° C) as the blood of the fresh-water fish used (0·497–0·530° C). $NaCH_3SO_4$ and TEACl, for which cryoscopic data are not available, were assumed to have the same osmotic coefficient as NaCl. If the dissociation constant of $CaSO_4$ is taken as 5·3 mM, the concentration of ionized calcium is 0·5 mM in solutions *G*, *I*, and *K*, and 1·8 mM in *H*, *J*, and *L*.

Cyanide and iodoacetate were added as a neutral isotonic stock solution (103 mM NaCN, 103 mM iodoacetic acid), to give a final concentration of 3 mM for each. Diamox, a generous gift of Lederle Laboratories, was also added as an isotonic stock solution (154 mM sodium acetazolamide, titrated to pH 7·3 with HCl). All other pharmacological agents were used at such low concentrations as to have no effect on the over-all osmolarity, and were made up with the experimental solutions. Since ouabain was dissolved in 80 % (v/v) ethanol before being incorporated into Ringer's solution, to give a final alcohol concentration of 0·1 % (v/v), ethanol was added to the control solutions to the same concentration.

Analytical methods. Sodium and potassium were analysed in the EEL flame photometer. Controls showed that in the range of concentrations used for analysis (sodium 0–2400 μM, potassium 0–120 μM) varying concentrations of sodium had no effect on the potassium results, and vice versa. Chloride and bromide were titrated potentiometrically with 10 mM-$AgNO_3$ (Sanderson, 1952). The molarity of the $AgNO_3$ was checked against a standard NaCl solution, and the reagent blank with a 2 ml. distilled water sample was found to be 0·003 ml. of 10 mM-$AgNO_3$. The standard deviation of six replicate samples containing 5 μmoles Cl was $\pm 1 \cdot 1$ %. Sucrose and inulin were determined by a colorimetric resorcinol method (Schreiner, 1950), with the Hilger spectrophotometer. Haemoglobin was measured at 407 mμ and Evans Blue at 610 mμ with the same instrument.

Sodium activities in bile salt solutions were determined with a sodium-glass electrode (made by Dr J. A. M. Hinke) and vibrating-reed electrometer ('Vibron' model 33B, Electronic Instruments, Ltd). Provided that the pH is not too low, this glass behaves as a reversible electrode for the sodium ion (Hinke, 1961). Sodium activities (a_{Na}) were calculated from the equation $E = E^o + S \log a_{Na}$, where E is the potential between an Ag-AgCl wire in a saturated KCl bridge and the sodium electrode, both dipping into the unknown solution. E^o and S were determined by including samples of 10 and 100 mM-NaCl with each series of unknowns. The sodium activity coefficients γ_{Na} in 10 and 100 mM-NaCl were taken as 0·909 and 0·787, respectively. A typical value for E^o was -35 mV, for S 58·1 mV. The measurements were carried out at the Plymouth Marine Biological Laboratory at an ambient room temperature of 24–25° C. As the sodium glass is not completely selective for sodium, the full equation is $E = E^o + S \log (a_{Na} + ra_K)$, but since r is about 1/300 and the solutions contained only trace impurities of potassium as opposed to sodium at 5–300 mM, the ra_K term may be neglected.

RESULTS

Absorption of Ringer's solutions

Absorption measured by changes in weight. Figure 1 depicts the experimental values for the weight of a gall-bladder preparation with NaCl Ringer's solution as both the luminal and outer solutions. The weight declined steadily for 3·5 hr, but levelled off to a constant value half an hour after the addition of cyanide and iodoacetate. Of the initial weight of 837·8 mg, the gall-bladder wall accounted for 126·9 mg, the cannula, glass plug, and wire hook 31·7 mg, and the luminal fluid 679·2 mg. During the course of the experiment the preparation lost 151·6 mg. If this was

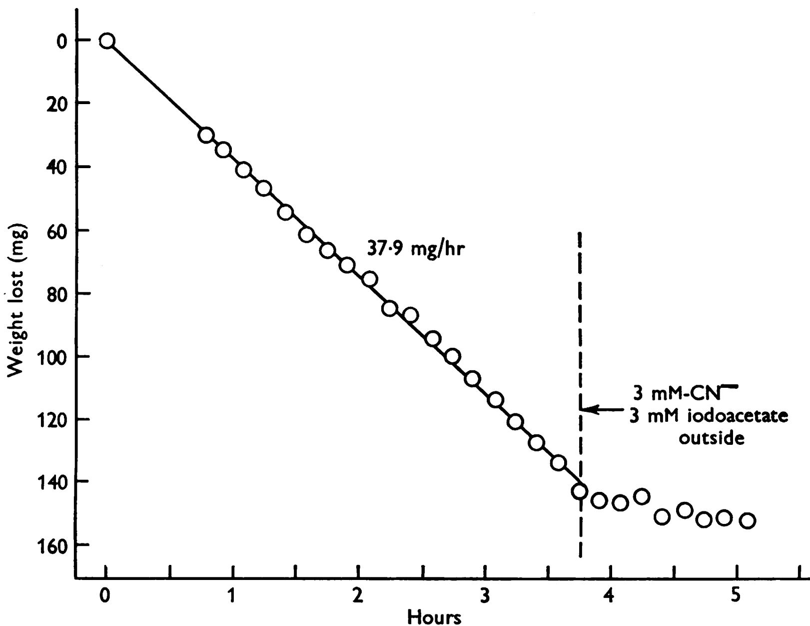

Fig. 1. Effect of cyanide-iodoacetate on absorption of (NaCl) Ringer's solution. Ordinate, decline in weight of a gall-bladder preparation since $t = 0$. Both sides of the gall-bladder were initially bathed in Ringer's solution (solution *A*, Table 2). At $t = 3$ hr 48 min, 0·9 ml. of a neutral isotonic solution of 103 mM-NaCN, 103 mM iodoacetic acid, was added to 30 ml. of the outer solution.

entirely at the expense of the luminal fluid, the rate of weight loss (henceforth referred to as dW/dt) represented 5·6 % of the initial luminal contents per hour, or 10·2 μl. fluid/hr.cm^2 of gall-bladder surface. From similar experiments on a total of 64 roach gall-bladders the average value and standard deviation of dW/dt was $9·8 \pm 4·7$ % hr, or 15·3 μl./hr.cm^2. Corresponding figures for two other species of fish were $2·5 \pm 0·6$ %/hr for

ten bream, and $2{\cdot}6 \pm 1{\cdot}0$ %/hr for three carp. Losses of weight sensitive to cyanide-iodoacetate were also observed in a few cursory experiments on frog gall-bladder. Canine gall-bladder *in vivo* absorbs 16 % of its volume of isotonic NaCl per hour (Ravdin, Johnston, Austin & Riegel, 1932) or 66 μl./hr.cm^2 (Grim & Smith, 1957). Roach and canine gall-bladder thus have comparable absorptive powers on a volume-per-cent basis, but canine gall-bladder must absorb more rapidly on a flux–area basis because of its larger size and higher volume: area ratio.

The weighings themselves caused no change of weight, since the rate of change of weight remained constant when the preparation was weighed alternately every 5 and 20 min. As an illustration of the replicability of the weighing operation, one may consider a gall-bladder which had been poisoned and was undergoing no net changes of weight. The average of ten successive weighings at 5 min intervals was $523{\cdot}2 \pm 0{\cdot}6$ mg. Of this, 110·6 mg was the wall of the gall-bladder itself, 45·1 mg the combined weight of the hook, plug, and cannula, and 367·5 mg the luminal fluid. The weighing error is thus 0·6/367·5, or 0·16 % of the luminal fluid. In general, the volume of the lumen fell in the range 0·2–1·8 ml.

In order to determine whether absorption from the lumen accounts quantitatively for this loss of weight, ox methaemoglobin (0·038 mM) and Evans Blue (0·005 mM) were added to the Ringer's solution in the lumen of five gall-bladders. At times varying from 4 to 18 hr the experiments were terminated, and the luminal fluid analysed for methaemoglobin and Evans Blue. No traces of either dye could be detected in the outer solution at the end of the experiments; the ratio of methaemoglobin to Evans Blue in the recovered samples was on the average 1·01 times the ratio in the solution introduced; and the absorption spectra of the introduced and recovered fluids were indistinguishable. Thus, the fraction of the luminal contents absorbed may be computed directly from the change in concentration of either dye by the formula

$$\text{fraction absorbed} = (r-1)/r,$$

where r is the ratio of the final to the initial concentration. In Table 3 this is compared with the percentage absorption as determined by the weighings, on the assumption that all weight lost represents luminal fluid absorbed. The agreement of the two sets of figures within experimental error, as shown in the last column, implies that loss of weight from the gall-bladder wall itself or by bulk leakage of luminal contents must be negligible, and confirms the validity of the weight method for following absorption.

In general, the rate of weight loss began to decline by the fifth hour after cannulation, and accordingly almost all experimental results on absorp-

tion were obtained within this period. By the end of an experiment up to 80·8 % of the original luminal contents had been absorbed. No significant variations of the absorption rate with season or the sex of the fish were noticed. Complete lack of an absorptive capacity was encountered in only two completed dissections, one of which was a hypertrophic gall-bladder (contents 7·2 ml., 14 times normal and almost as large as the same fish's stomach), and the other of which was found to contain gall-stones. *In vivo*, a similar failure of the human gall-bladder to concentrate during pathological conditions (e.g. when filled with gall-stones) is a familiar clinical

TABLE 3. Absorption measured simultaneously by changes in weight and in luminal dye concentrations

Initial wt. of luminal contents, (mg) a	Final wt. of luminal contents, (mg) b	A $\frac{a-b}{a}$	Evans Blue r	Evans Blue B $\frac{r-1}{r}$	Haemoglobin r	Haemoglobin C $\frac{r-1}{r}$	$\frac{B}{A}$ or $\frac{C}{A}$
500	203	0·59	2·30	0·57			0·97
					2·32	0·56	0·95
1216	768	0·37	1·64	0·39			1·05
					1·66	0·40	1·08
1040	441	0·58	2·36	0·58			1·00
					2·34	0·57	0·98
1215	468	0·61	2·38	0·58			0·95
					2·41	0·59	0·97
870	378	0·57	2·16	0·54			0·95
					1·98	0·50	0·88
						Average	0·98 ± 0·06

Both the luminal and outer solutions were NaCl Ringer's solution. The luminal solution contained Evans Blue and haemoglobin. The gall-bladders were allowed to lose weight for several hours, then the luminal fluid was analysed for Evans Blue and haemoglobin. r is the ratio of the final to the initial concentration of Evans Blue or haemoglobin. The numbers in the last column should be 1·00 if changes in weight are due solely to absorption of luminal fluid.

finding. In these circumstances of identical solutions on both sides of the gall-bladder there is no chemical driving force for fluid transport, and the cessation of absorption after poisoning suggests that the energy for transport is provided by the tissue itself, either from stored metabolic energy or from metabolism of the glucose in the medium.

Composition of the absorbate. For analysis of ion shifts fifteen gall-bladders were prepared with the same Ringer's solution initially in the lumen as in the outer solution. NaCl Ringer's solution (A) was used in twelve cases, sulphate solution once, a K-rich solution once (72·7 mM-K, 76·6 mM-Na), and a K-free solution once. After different lengths of time the experiments were terminated and the luminal contents analysed, so that the percentage of the initial volume absorbed varied from 4·6 to 80·8 %. Comparison of the amounts of Na and K absorbed showed that sodium

generally represented at least 98 % of the monovalent cation in the absorbate. In all experiments luminal [K] was higher at the end than at the beginning, and in eight of the fifteen cases the actual amount of K recovered was higher than that introduced, suggesting that small net quantities of K (on a molar basis, less than 1/50 of the Na absorbed) either were back-transported or leaked out of the gall-bladder wall. Even in the experiment where [Na] and [K] in the lumen were initially approximately

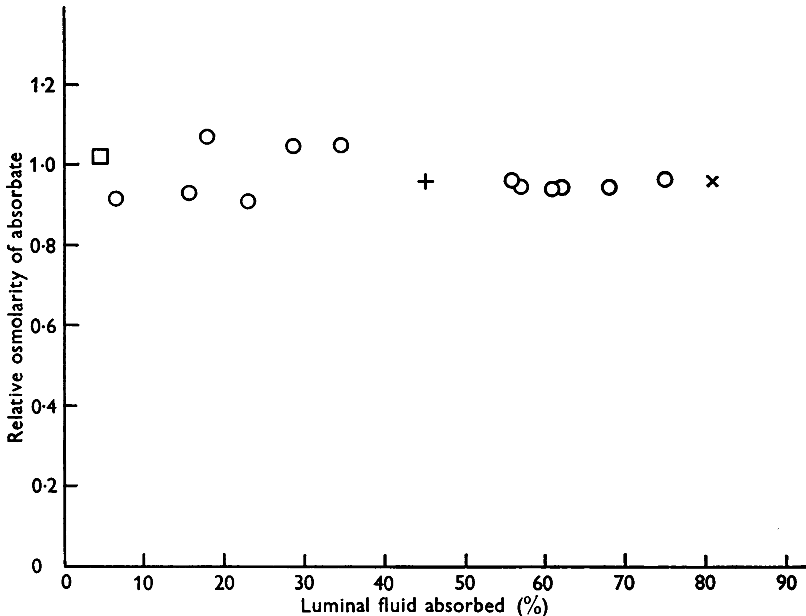

Fig. 2. Osmolarity of the absorbate, relative to Ringer's solution, at various stages in the absorption process. Abscissa, percentage of the initial luminal volume reabsorbed by the end of the experiment; ordinate, ratio of the osmolarity of the absorbate to the osmolarity of Ringer's solution. Gall-bladders were allowed to absorb varying fractions of the luminal fluid, then the absorbate osmolarity was computed from the amounts of water and ions absorbed.

Identical solutions bathed both sides of the gall-bladder except in the experiment indicated by ×, in which the outer solution was Ringer's solution (solution *A*) and the luminal solution K-free solution (*B*). ○ Ringer's solution; □ Na_2SO_4 solution; + a K-rich solution (72·7 mM-K, 76·6 mM-Na).

equal, almost $2\frac{1}{2}$ times as much Na as K was absorbed. However, this does not represent the Na:K ratio in the primary absorbate, since K must have diffused out, and Na in, down the concentration gradients established by absorption (at the end of the experiment [Na] and [K] were 55·2 and 94·9 mM, respectively, in the lumen, and 76·6 and 72·7 mM in the outer solution throughout). In fact, it turns out that these final luminal con-

centrations are what one would expect from the passive permeabilities if there were no primary transport of K.

The concentrations of Na and K in the absorbate were calculated from the amounts of these ions absorbed, divided by the volume absorbed. The osmolarity of the absorbate was then computed on the assumption that net movements of Na and K are accompanied by chloride (or, in sulphate solution, by sulphate); and that net fluxes of the minor components of Ringer's solution (calcium, magnesium, and glucose) are negligible. Figure 2 depicts the ratio, osmolarity of absorbate: osmolarity of solution, for these fifteen experiments. The fact that this ratio is nearly 1 (average value $0{\cdot}98 \pm 0{\cdot}05$), independent of what fraction of the luminal volume has been absorbed, argues that the absorbate is essentially isotonic NaCl (Na_2SO_4 in sulphate solution) at all stages of the absorption process. This conclusion also holds independently of variations in luminal [K] from 0 to 94·9 mM and has been confirmed in two other species of fish (bream and carp) besides roach. In a few instances the residual fluid in the lumen was also analysed for chloride, which was found to be at virtually the same concentration as in the original solution. This also implies an isotonic absorbate, since chloride accounts for 98 % of the total anion in Ringer's solution.

Specificity of the absorption process. To determine whether the gall-bladder can absorb solutions in which the principal salt is no longer NaCl, weighing experiments were carried out with four other solutions (see Methods for formulae). In one experiment both NaBr (bromide solution *A*) and NaCl were tested on the same gall-bladder. The rate of weight loss (dW/dt) with bromide solution *A* in both the lumen and outer solution, 22·1 mg/hr, hardly differed from the average of the rates with NaCl Ringer's solution (23·3 mg/hr), measured immediately before and afterwards. For four gall-bladders in bromide solution *A* the average rate of absorption of the luminal contents was $6{\cdot}6 \pm 3{\cdot}2$ %/hr, slightly lower than the over-all average for the solution based on NaCl ($9{\cdot}8 \pm 4{\cdot}7$ %/hr). In Na_2SO_4 solution, however, dW/dt declined to $2{\cdot}2 \pm 0{\cdot}2$ %/hr, and the ratio of the absorption rate with Na_2SO_4 to the rate with NaCl was $0{\cdot}17 \pm 0{\cdot}08$ in three cases where both rates were determined on the same gall-bladder. Replacement of all chloride by sulphate in the outer solution alone did not impair a gall-bladder's ability to absorb NaCl Ringer's solution from its lumen; and a gall-bladder filled with Na_2SO_4 solution did not lose weight more rapidly when the outer solution was changed to chloride instead of sulphate. The sulphate effect is therefore due solely to sulphate in the lumen. Similarly, $NaCH_3SO_4$ and TEACl yielded absorption rates 24 and 18 % of these measured with NaCl.

Thus, the absorption process will transport NaCl and NaBr much more

efficiently than Na_2SO_4, $NaCH_3SO_4$, TEACl, or KCl. Such selectivity is characteristic of other epithelial tissues which transport NaCl and water. Generally, no other cation can replace sodium: for example, isolated rat small intestine absorbs sodium, but not potassium, lithium, caesium, or the TEA cation (Clarkson & Rothstein, 1960). Salivary gland (Lundberg, 1957) and frog skin (Krogh, 1937) can transport bromide as well as chloride, while the intestine absorbs chloride but not sulphate (Ingraham & Visscher, 1936).

Absorption against a concentration gradient. The experiments described so far have been dealing with the transport of salt and water between two identical solutions. In six further experiments the ability of the gall-bladder to absorb against a salt concentration gradient was tested by replacing varying fractions of the NaCl in the lumen by sucrose. From analyses of the outer solution for sucrose in three instances, only $3{\cdot}4 \pm 2{\cdot}5$ % of the sucrose initially present in the lumen was found to have come out after 1 hr, so that sucrose may be considered as an effectively impermeant molecule in this preparation. The procedure was to compare dW/dt in the same gall-bladder when the lumen was filled with NaCl Ringer's solution and with a solution in which NaCl had been partially replaced. The latter rate is expressed as a percentage of the former in Fig. 3, where the crosses represent the experimental findings.

The first point to note is that with sucrose substituted for all NaCl in the lumen the gall-bladder *gained* weight. This is a passive property of the membrane and is not caused by the absorptive process working in reverse, since the gain of weight persisted in the presence of cyanide and iodo-acetate, which prevent normal absorption. Analysis of the luminal contents showed that Na and Cl had entered as well as water. Presumably NaCl is diffusing down its concentration gradient into the lumen, accompanied by water, since sucrose cannot diffuse out. Secondly, no net change of weight occurs when luminal [Na] = 10·1, [Cl] = 15·7 mM. At this point the rate of outward transport of NaCl must equal the rate of diffusion inwards, and net absorption of Na can therefore take place against all concentration gradients less than 144/10·1 = 14·3 (149·6/15·7 = 9·5 for Cl). Without knowledge of the electrical potential between the lumen and outside it is not possible to say whether it is Na or Cl that moves against an electrochemical gradient. The gall-bladder is thus relatively efficient by biological standards, since net absorption of NaCl from the proximal tubule (Windhager, Whittembury, Ojen, Schatzmann & Solomon, 1959) and small intestine (Curran & Solomon, 1957) can take place against concentration gradients of only about 1·5 and 2·5 respectively. However, salt-depleted frogs can absorb NaCl through their skins against a concentration gradient of 100 (Krogh, 1937).

Since dW/dt in these experiments is the resultant of two processes, diffusion inwards and transport outwards, further assumptions must be made in order to derive the concentration dependence of transport from the experimental data. As a first approximation the rate of diffusion inwards may reasonably be taken as proportional to the NaCl concentration gradient (Fick's law) and this rate is accordingly portrayed by the straight line in Fig. 3. The difference between the experimental weight change and this assumed diffusion rate is then the rate of transport as a

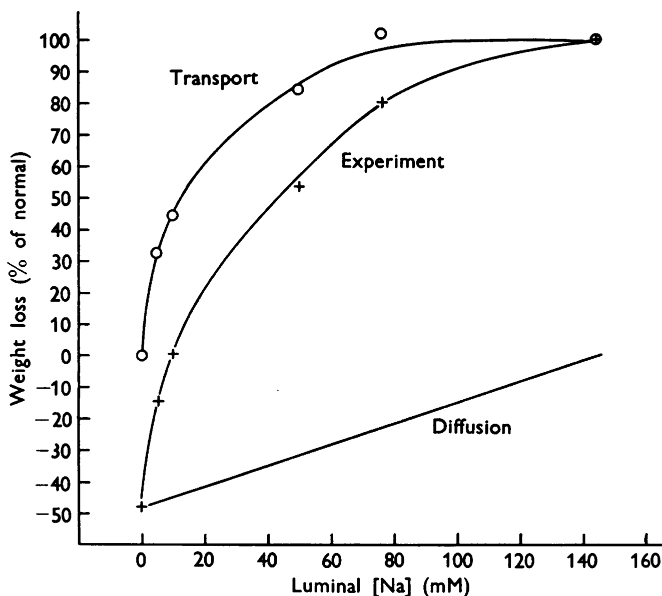

Fig. 3. Saturation of the NaCl transport mechanism at higher luminal NaCl concentrations. Abscissa, luminal [Na]; ordinate, rate of weight loss, as percentage of rate when lumen was filled with NaCl Ringer's solution ([Na] = 144 mM). Luminal [Na] was reduced below 144 mM by substituting sucrose for NaCl; i.e. by mixing solutions *A* and *D*. The outer solutions remained Ringer's solution in all cases. [Cl] was 5·6 mM higher than [Na] for all solutions, and [K] remained at 4·5 mM. + experimental values; straight line, estimated water movement associated with inward diffusion of NaCl (net diffusion is zero at luminal [Na] = 144 mM, when the luminal and outer solutions are identical); ○ water movement associated with outward transport of NaCl, estimated as the difference between the experimental results and the diffusion curve.

function of luminal [NaCl], given by the circles in Fig. 3. The transport mechanism apparently becomes saturated at the higher values of luminal [NaCl], and the rate of absorption decreases to half its original value only when 90 % of the NaCl has been removed from the lumen. Similar results have been observed for the concentration dependence of NaCl absorption by small intestine (Curran & Solomon, 1957) and of K uptake and Na

extrusion by human red cells (Glynn, 1956). These saturable kinetics are often assumed to support the hypothesis that the transported ions cross the cell membrane in combination with a specific carrier, most of whose available binding sites are occupied at higher ion concentrations.

Absorption transients. In the series of experiments illustrated in Fig. 3, about 15 min elapsed between removal of one solution from the lumen and the final filling with a fresh luminal solution. No information could be gathered about the time course of $\mathrm{d}W/\mathrm{d}t$ while the direction of net water flow was reversing. However, in four additional experiments the luminal solution was changed as rapidly as possible, so that the first weighing was taken 105–360 sec after the introduction of a new solution into the gall-bladder. The sequence was to measure $\mathrm{d}W/\mathrm{d}t$ in NaCl Ringer's solution, then to replace luminal NaCl completely by sucrose or glucose. It was found that the preparation continued to lose weight for about 10 min, after which the water flow reversed to give steady-state weight gains, as discussed in the preceding section. Extrapolation of the initial loss to zero time (the moment of introduction of a new solution) showed that a total of 7–49 mg was lost during the transient. Since the gall-bladder walls themselves weighed 25–230 mg and there was no NaCl in the lumen, the most likely interpretation is that this weight came from the cells of the gall-bladder wall—i.e. that some NaCl (and water) remained in a part of the wall from which it could not be washed out by renewing the luminal fluid but could still be transported into the outer solution.

Structurally, the gall-bladder consists of two layers, mucosa and serosa. On the inside, facing the lumen, is a single layer of tall epithelial cells (the mucosa), supported on the outside by a connective-tissue layer (the serosa) of about equal thickness, possibly containing some bundles of smooth-muscle fibres. From the known area of each wall, the water lost in the transients must have represented a layer $51 \pm 18\,\mu$ (4) thick. For comparison, the average thickness of a wall is $217\,\mu$, of which the epithelial-cell layer accounts for about half. The fluid lost in the transient would thus be about half the volume of the epithelial cells.

This hypothesis was tested directly in one experiment. The principle used was that if the wall loses an appreciable fraction of its intracellular water, the inulin extracellular space on a percentage basis should increase. Since sucrose and inulin are equivalent in the colorimetric resorcinol method for analysing the latter, luminal NaCl was in this case replaced by glucose rather than sucrose, and inulin was in both the lumen and the outer solution. The first weighing was taken 105 sec after the luminal solution had been changed: 12 mg was lost during the transient, and the total remaining water in the blotted gall-bladder wall was 40·5 mg, so that the total original water must have been 52·5 mg. The average extracellular space of

four blotted normal gall-bladders was 34·2 ± 4·2 %. Hence the gall-bladder of this experiment originally had (0·342) × (52·5) = 18·0 mg extracellular and 34·5 mg intracellular water. If the 12 mg lost in the transient was entirely extracellular, the measured extracellular space should have been (100) × (18 – 12)/40·5 = 14·8 %; if intracellular, (100) × (18)/40·5 = 44·4 %. The actual value was 48·8 %, which supports the assumption that the weight loss in the transient was at the expense of intracellular water. This conclusion is corroborated by preliminary evidence from electron microscopy. The epithelial cells of gall-bladders fixed at the end of an absorption transient have much denser cytoplasm and closer packing of the mitochondria than normal, implying that the cells have lost water.

Evidently, in the normal gall-bladder fluid enters the cells from the lumen as fast as it is pumped out the other end of the cells. When all NaCl is removed from the lumen, the pump continues to remove a substantial fraction of the NaCl left in the cells, reducing their volume. Similar volume shrinkage of cells responsible for fluid transfer has been noted in salivary gland. The observation that the cells of the gall-bladder can partially pump out their own contents eliminates theories of absorption which postulate that the transport process occurs entirely at the luminal border of the epithelial cells, or that the absorbate passes between the cells, as Linderholm (1954) postulated for frog skin.

Intracellular concentrations of ions. An obvious corollary of the preceding section is that the gall-bladder wall should normally contain substantial amounts of intracellular Na and Cl. The actual concentrations of intracellular ions were calculated from the total amounts of water and ions in the wall and the extracellular-space value of 34·2 % for blotted and 46·5 % for drained gall-bladders, on the assumption that ions are at the same concentration in the extracellular space as in the surrounding solution. For twelve roach gall-bladders in normal Ringer's solution the cellular fluid thus computed is:

$$[K]_i = 81 \pm 15 \text{ mM}, \quad [Na]_i = 54 \pm 15 \text{ mM}, \quad [Cl]_i = 91 \pm 22 \text{ mM}$$

(subscript i refers to intracellular). Corresponding figures for eight gall-bladders left in Ringer's solution containing cyanide and iodoacetate for 1–4 hr are:

$$[K]_i\, 46 \pm 13 \text{ mM}, \quad [Na]_i\, 102 \pm 27 \text{ mM}, \quad [Cl]_i = 113 \pm 29 \text{ mM}.$$

Thus, the cells of the gall-bladder resemble most other animal cells in normally maintaining a K:Na ratio much higher (1·5) than that in the external fluid (0·03), and $[Cl]_i$ is lower than external [Cl]. By analogy with other tissues these concentration gradients probably depend for their maintenance upon metabolic energy, directly, or else indirectly through

the resting potential. This metabolic dependence is also indicated by the fact that $[K]_i$ decreased and $[Na]_i$ and $[Cl]_i$ increased after poisoning. $[Na]_i$ and $[Cl]_i$ of unpoisoned gall-bladders, even those taken for analysis within 15 min of removal from the fish, are nevertheless higher than the figures reported for many vertebrate tissues: for example, $[K]_i$, $[Na]_i$, and $[Cl]_i$ are respectively, 142, 21·6, and 48·3 mM in rat parotid gland, 156, 18·5, and 38·3 mM in rat pancreas (Schneyer & Schneyer, 1960), and 139, 22, and 3·8 mM in frog sartorius muscle (Adrian, 1956, 1960). Equally high $[Na]_i$ and $[Cl]_i$ have been observed in gall-bladders of two other species of fish, bream and pike. For example, two bream gall-bladders on which the extracellular space was determined to be $24·5 \pm 1·0$ % and which were taken for analysis 1 hr after removal from the fish gave: $[K]_i$ 65 and 67, $[Na]_i$ 84 and 76, and $[Cl]_i$ 105 and 95 mM. Hence these values for the intracellular ions should not be regarded as a species peculiarity or an experimental artifact. They are what one would expect for cells which devote half their volume to the immediate precursor of an isotonic NaCl absorbate.

Pharmacology of absorption

Cyanide and iodoacetate, both at 3 mM, reduced dW/dt to zero in fourteen gall-bladders, of which nine were in NaCl Ringer's solution, four in bromide solution, and one in sulphate solution. The effect usually was irreversible, complete after 30–40 min (see Fig. 1), and occurred whether the poisons were present on both sides or only in the outer solution. Reduction was partial in three cases, one in bromide solution and two in NaCl Ringer's solution. At 2–4 mM, cyanide alone, whether in both solutions or only in the outer solution, decreased dW/dt by 59 ± 13 % (5) in NaCl Ringer's solution and by 67 % (1) in sulphate solution, but never reduced it to zero. Evidently the energy for absorption is provided both by oxidative metabolism and by anaerobic glycolysis, but the complete inhibition produced by cyanide and iodoacetate together in fourteen out of seventeen cases implies that reserves of high-energy phosphates normally either are not utilized for absorption or else are quantitatively negligible. There may, however, be some stored carbohydrate, since five gall-bladders incubated without glucose sustained absorption rates of $3·5 \pm 2·2$ %/hr (5) for 1 hr.

Removal of potassium from the outer solution inhibited absorption reversibly within 25–40 min and the effect could be repeated several times on the same gall-bladder (Fig. 4). Removal of K from the luminal solution was without effect, and when K was initially absent on both sides of the gall-bladder, only restoration of K to the outer solution could restore the rate of absorption. The average degree of inhibition produced by K-free solutions was 52 ± 18 % (5) if the value of dW/dt was compared with the

succeeding period, when 4·5 mM-K was restored to the outer solution. After 3 hr in K-free solution the inhibition became irreversible. Inhibition produced by removal of K from the outer solution a second time was never as great as the first time on the same gall-bladder. The K-free effect was also obtained on the gall-bladder of one bream (inhibition 53 %).

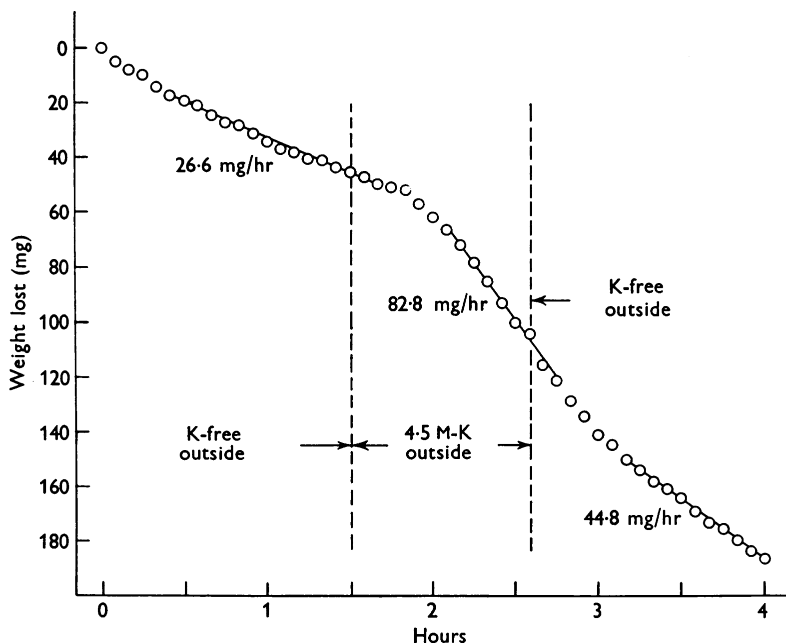

Fig. 4. Effect of K in the outer solution on absorption of Ringer's solution. Ordinate, weight lost by a gall-bladder preparation since $t = 0$. The luminal solution was solution *B* (K-free) throughout. The outer solution was initially Ringer's solution (*A*), then solution *B*, and finally solution *A* again.

Other cells and tissues in which a similar and often greater potassium dependence of sodium movements has been observed include skeletal muscle (Keynes, 1954), uterine smooth muscle (Daniel & Robinson, 1960*a*), nerve (Hodgkin & Keynes, 1955), red blood cells (Harris & Maizels, 1951), frog skin (Ussing, Kruhøffer, Hess-Thaysen & Thorn, 1960), the alga *Nitella* (E. A. C. MacRobbie, unpublished), urinary bladder (Bentley, 1959), placenta (Crawford & McCance, 1960); iodine movements are affected in thyroid, submaxillary gland, mammary gland, and a thyroid tumour (Wolff & Maurey, 1961); and chloride transport in cat sublingual gland (Lundberg, 1957). In the case of the four epithelial membranes (frog skin, placenta, urinary bladder, and gall-bladder) inhibition is produced only when K is removed from the surface of the membrane towards which the net movement of Na is occurring—i.e. the inside of frog skin,

maternal side of placenta, serosa of urinary bladder, and outside of gall-bladder. This polarity is also observed with the above-mentioned cells and cellular tissues which are extruding Na into the external solution; although it has not yet been possible to substantiate the absence of a K-free effect on the inside of the cells.

The cardiac glycoside ouabain inhibited absorption completely and irreversibly within 30 min when added to the outer solution at 10^{-4}M (see Fig. 5). 10^{-5}M inhibited partially and 10^{-6}M not at all, and even 10^{-4}M was without effect when in the lumen.

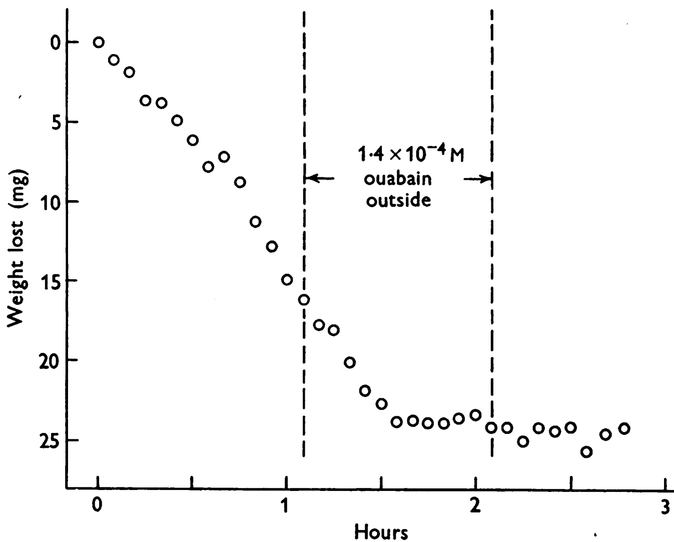

Fig. 5. Effect of ouabain on absorption of NaCl Ringer's solution (*A*). Ordinate, weight lost by a gall-bladder preparation since $t = 0$. Both the outer and luminal solutions were Ringer's solution. The outer solution contained ethanol 0·1 % (v/v) throughout.

The list of tissues in which ouabain, usually at even lower concentrations, inhibits ionic movements includes all victims of the K-free effect on which it has been tested, and other preparations as well: Na movements in skeletal muscle (Johnson, 1956), uterine smooth muscle (Daniel & Robinson, 1960*b*), nerve (Caldwell & Keynes, 1959), red blood cells (Glynn 1957), frog skin (Koefoed-Johnsen, 1958), the alga *Nitella* (MacRobbie, unpublished), heart muscle (Vick & Kahn, 1957), kidney proximal tubule (Schatzmann, Windhager & Solomon, 1958), mouse ascites tumour cells (Maizels, Remington & Truscoe, 1958), lens (Kinoshita, Kern & Merola, 1961), bicarbonate transport in the intestine (Cooperstein & Brockman, 1959), H^+ and chloride transport in the stomach (Cooperstein, 1959), and iodine transport in thyroid, submaxillary gland, mammary gland, and a

thyroid tumour (Wolff & Mauery, 1961). Whenever checked (frog skin, nerve, and gall-bladder), only the side of the preparation towards which the net movement of Na is directed appears sensitive to ouabain, as is also true of the K-free effect.

Temperature coefficient. In one experiment dW/dt was measured successively at 16·9, 31·2 and 16·9° C, and values of 19·3, 51·2 and 22·4 mg/hr, respectively, were obtained: dW/dt appeared to have changed already by the first weighing 5 min after the change in temperature. Hence the Q_{10} of absorption between 16·9 and 31·2° C is 1·7. For two other experiments at 32·0° C the average dW/dt was 17·1 %/hr, 1·7 times the average value of 9·8 %/hr for the normal 17–23° C range. The results of Hodgkin & Keynes (1955) suggest a Q_{10} of 2·2 between 18 and 28° C for Na efflux in cuttlefish nerve, and indicate that the Q_{10} falls off with increasing temperature. Hence a higher Q_{10} would probably have been observed at lower (and more physiological) temperatures in the present experiments; but in any case a Q_{10} of 1·7 might suggest that absorption is dependent upon chemical processes.

Neurohypophysial hormones. Of the three hormones of the vertebrate posterior pituitary gland, the antidiuretic hormone (otherwise known as vasopressin or ADH; Parke, Davis Pitressin) had no effect on dW/dt at any concentration tested from 8 to 400 m-u./ml. Arginine vasotocin could not be obtained. Oxytocin (Parke, Davis Pitocin) had no effect at 4 or 40 m-u./ml., and produced a slowly developing reduction at 200–286 m-u./ml. At 400 m-u./ml. the inhibitory effect of oxytocin was complete after 10 min and not readily reversible (Fig. 6). This action has been noted on gall-bladders from both male and female fish. The effect has also been obtained with pure synthetic oxytocin (Syntocinon, a generous gift of Sandoz Products, Ltd). The same polarity was observed as with the K-free effect and ouabain—i.e. full inhibition was obtained with oxytocin in the outer solution only, and oxytocin in the lumen alone was ineffective. The effect must be a direct one on the absorptive process, since the muscular layer and intraluminal pressure will be shown later to be irrelevant to absorption, and in any case it is uncertain whether oxytocin contracts or relaxes gall-bladder musculature.

It is remarkable that oxytocin arrests absorption by the gall-bladder three times as fast as any other inhibitor. The effect is even more surprising because all previously reported actions of the neurohypophysial hormones on salt and water transport have been stimulatory: (1) vasopressin and arginine vasotocin, but probably not oxytocin, increase reabsorption of water by the kidney (van Dyke, Adamson & Engel, 1955); (2) vasopressin, arginine vasotocin, and oxytocin stimulate Na transport and fluid absorption in toad urinary bladder (Leaf, Anderson & Page, 1958); (3) vaso-

pressin, arginine vasotocin, and oxytocin stimulate Na transport and water uptake by frog and toad skin (Ussing *et al.* 1960) (all arginine vasotocin references, Sawyer, Munsick & van Dyke, 1961); (4) oxytocin but not vasopressin stimulates Na uptake by the gills of fresh-water fish (Maetz & Juien, 1961); (5) neurohypophysial hormones (identity not stated) stimulate transport by large intestine (Ussing *et al.* 1960). However, the stimulatory effect in urinary bladder, large intestine, frog skin, and possibly kidney and fish gill has the same polarity as the inhibitory effect in gall-bladder—i.e. the hormones act only from the blood side, as is to be expected, since they would reach organs *in vivo* only from this side.

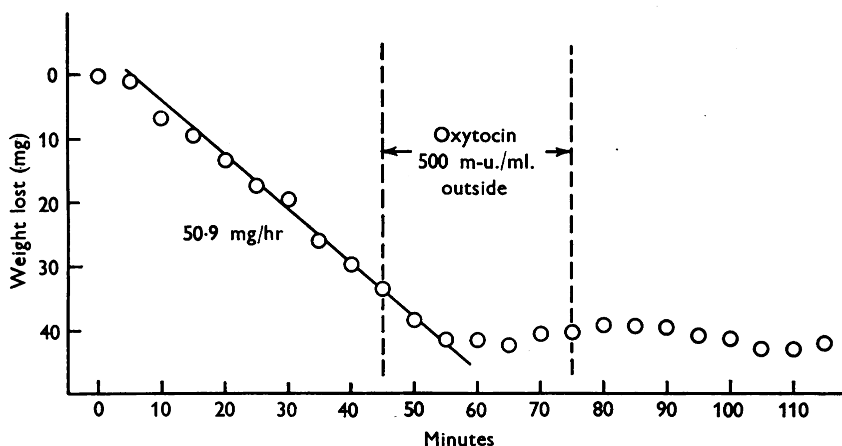

Fig. 6. Effect of oxytocin on absorption of NaCl Ringer's solution (*A*). Ordinate, weight lost by a gall-bladder preparation since $t = 0$. Both the outer and luminal solutions were NaCl Ringer's solution. The oxytocin preparation added to the outer solution between the arrows was Parke, Davis Pitocin.

Fish pituitary contains oxytocin and arginine vasotocin, but probably not vasopressin (Pickering & Heller, 1959). The absence of a vasopressin effect on the gall-bladder might conceivably be related merely to the choice of experimental animal, since vasopressin is absent in fish. However, this explanation seems doubtful because vasopressin, though also absent from the frog, can nevertheless stimulate frog skin, kidney, intestine, and urinary bladder.

Other agents. Acetylcholine was added at 4×10^{-4} M to both the outer and luminal solutions in one experiment and stimulated absorption reversibly by 18%. When it was present on the outside alone at 12×10^{-4} M, dW/dt increased by 25%. As acetylcholine was not tested in the lumen alone, the question remains open whether the effect is strictly unilateral. Adrenaline present simultaneously on both sides decreased dW/dt by 28% at $1 \cdot 3 \times 10^{-4}$ M in one experiment, and by 13% at 2×10^{-4} M in another. As

the effects were small, they were not further investigated. It should be noted, however, that Westphal, Gleichmann & Soika (1931), studying the gall-bladder of the anaesthetized dog *in vivo*, found that stimulation of the peripheral end of the divided vagus nerve, whose fibres are presumably cholinergic, increased the rate of absorption of chloride and water, while injection of adrenaline produced an opposite action.

The pH of the outer solution was varied in one experiment between 6·5 and 7·9 by phosphate buffers, without any effect on dW/dt.

In a few early experiments the outer solution was stirred by air or by 95 % O_2–5 % CO_2 in conjunction with a bicarbonate buffer, and dW/dt fell within the range of values obtained with O_2 stirring in the main series of experiments. Since turning off the O_2 stirrer in the middle of one experiment left dW/dt unchanged, oxygenation of the preparation cannot be a critical factor.

The carbonic-anhydrase inhibitor diamox (acetazoleamide), even when present on both sides of the gall-bladder at the highest concentration that could be conveniently got into solution (10 mM), had no effect on dW/dt. Hence carbonic anhydrase is probably unnecessary for NaCl transport by the gall-bladder.

Experiments on bile

The state of sodium in bile. Before these results concerning the absorption of Ringer's solutions can be extrapolated to bile, the peculiar osmotic properties of bile require consideration. It has frequently been observed that bile and plasma have the same freezing-point depression but that the former nominally contains up to twice as many solute molecules per unit volume as the latter. One method of overcoming this apparent contradiction was adopted by Ravdin, Johnston, Riegel & Wright (1932) who pointed out that if one ignored the bile-acid anions, the remaining solutes (largely chloride, bicarbonate, and inorganic cations) accounted well for the observed freezing-point depression. Similarly, Wheeler & Ramos (1960) found that the freezing point of hepatic biles of widely differing composition could be rationalized by assuming that all the bile-acid anions were osmotically inactive and all other molecules completely free. This empirical procedure of disregarding the bile acids derived some theoretical support from the low freezing-point depressions and osmotic coefficients of pure bile-salt solutions (i.e. sodium salts of the bile acids). These physical measurements suggested that at higher concentrations bile salts associate into micelles to an increasing extent. The tendency has thus been to make the tacit assumption that these micelles involve principally the bile-acid anions (which are steroids) rather than the inorganic cations.

The recent development of a glass which behaves as an almost perfect sodium electrode above pH 5·6 offered a direct method for determining

whether the sodium in bile is free and the bile-acid anions bound. At the Plymouth Marine Biological Laboratory, where the experiments with this electrode were carried out, the most convenient sources of gall-bladder bile were rays, marine elasmobranchs. Accordingly the sodium concentration and activity were determined on bile specimens from four rays by flame photometry and the sodium-glass electrode, respectively. The results are given in Table 4, where the sodium activity coefficient (γ_{Na}) comes out on the average $0{\cdot}56 \pm 0{\cdot}07$ (4). γ_{Na} in NaCl over the same concentration range is 0·69–0·71. Hence γ_{Na} is depressed in bile with respect to NaCl at the same sodium concentration.

TABLE 4. Sodium activity coefficient γ_{Na} in ray bile

Species	Na concentration (mM)	Na activity (mM)	γ_{Na}
Raia clavata			
1	320	197	0·62
2	300	177	0·59
3	299	167	0·56
Raia montagua	434	198	0·46
		Average	$0{\cdot}56 \pm 0{\cdot}07$

γ_{Na} is the quotient of the sodium activity measured with the sodium-selective glass electrode, divided by the sodium concentration measured by flame photometry.

The predominant bile acid in elasmobranchs is scymnol sulphate, about whose osmotic properties nothing is known. In order to be able to correlate γ_{Na} with the degree of micelle formation, sodium activity was determined in solutions of a better characterized bile salt, sodium deoxycholate. The results appear in Fig. 7 along with the osmotic coefficient of sodium deoxycholate from the work of Johnston & McBain (1942). It is at once clear that changes in γ_{Na} parallel changes in the osmotic coefficient of the whole salt remarkably closely. Hence the micelles must involve sodium as well as the bile-acid anions, and it is improper to regard the freezing-point depression of bile as resulting from the osmotic inactivity of the bile-acid anions alone. That this incorrect assumption, nevertheless, gave fair agreement with the observed freezing-point depression must be regarded as fortuitous, and its apparent success is probably due to the fact that many natural bile salts have osmotic coefficients around 0·35–0·64, in the physiological concentration range. Thus, dismissing the bile-acid anions as entirely bound would lead to a prediction of the same freezing-point depression as the correct assumption, that about half of the bile-acid anions and a corresponding quantity of Na are bound. However, the common practice of regarding the bile-acid anions as bound and equating bile Na concentration with activity is certain to lead to serious error in any attempt to interpret the electrical potential difference or Na activity

gradient between gall-bladder bile and plasma. For example, E. Grim (unpublished) found a potential difference of 15 mV, lumen negative, across the *in vivo* canine gall-bladder filled with gall-bladder bile (290 mM-Na, versus 140 mM-Na in plasma). Other evidence suggests that canine gall-bladder is equally permeable to Na and Cl, and the assumption that Na in bile is free would lead to the erroneous prediction of 28·3 mV, from

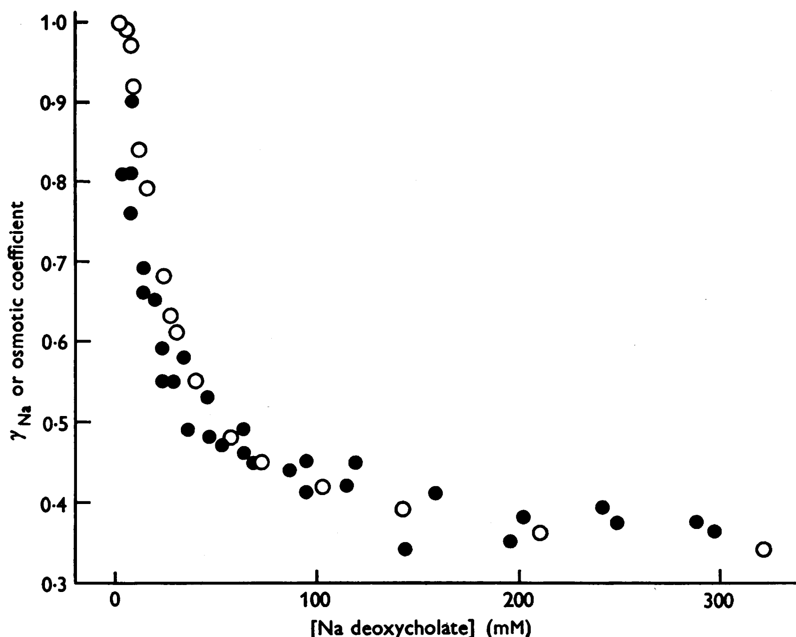

Fig. 7. Sodium activity coefficient γ_{Na} in solutions of sodium deoxycholate, as measured by the sodium-selective glass electrode. ●, γ_{Na}; ○, osmotic coefficient of sodium deoxycholate, as measured by Johnston & McBain (1942).

the constant-field equation. However, if one takes into account sodium-binding in bile, and uses the relevant sodium activity coefficient from Fig. 7, the expected potential difference is 17·8 mV, in much better agreement with experiment.

Comparative chemistry of bile. Table 5 gives the results of analyses, performed during the course of this study, on bile of thirty-five animals belonging to nine species. These results are compared with ion concentrations in the plasma.

The most significant conclusions to emerge from this table are that bile [Na] is higher than plasma [Na] for all nine species, in several cases more than twice as high; and that bile [Cl] is well below plasma [Cl] in the four species where [Cl] was measured. Bile [K] is also above plasma [K] in all

TABLE 5. Comparative bile chemistry

Species	Number of specimens	Bile: Pigment (O.D. units)	Bile: [K] (mM)	Bile: [Na] (mM)	Bile: [Cl] (mM)	Plasma: [K] (mM)	Plasma: [Na] (mM)	Plasma: [Cl] (mM)	Source of plasma analysis
Raia clavata	3	—	6·7	306	171 (2)	4·5	236	230	Smith (1929)
Raia montagua	1	—	38·9	434	—	4·5	236	230	Smith (1929)
Rutilus rutilus, roach	18	56 (10–73)	4·5 (3·4–5·7)	212 (101–267)	28·5 (8) (7·5–81)	2·3	117	88	Present study
Cyprinus carpio, carp	1	124	7·5	250·5	—	2·3	117	88	Present study (roach)
Abramis blicca, bream	4	109	6·0	218	7·4	1·8	134	106	Present study
Esox lucidus, pike	2	118	4·9	230·0	43·7 (1)	2·3	117	88	Present study (roach)
Electrophorus electricus, electric eel	4	15·4	12·3	191	—	5·5	170	—	Hargreaves & Moreira (1949)
Frog	3*	168	15·8	185·3	—	2·5	103·8	74·3	Fenn (1936)
Grass snake	1	32	10·4	164·3	—	—	132	75	Hutton (1958) (king snake)

All the bile analyses were obtained during the present study. Since plasma analyses were not available for the carp, pike, or grass snake, figures for related species have had to be used for comparison. The third column gives the optical density of bile at the absorption maximum between 390 and 420 mμ due to bile pigments.

* Pooled.

cases. Freezing-point depressions were determined for the plasma, bile, and aqueous humour of one bream as 0·488, 0·502, and 0·492° C, respectively. The principal chemical characteristics of mammalian gall-bladder bile—an isotonic, Na-rich, Cl-poor fluid—thus apply as well to representatives of the three classes of cold-blooded vertebrates.

The results of [Na] and pigment analyses on the nineteen fish where these were determined concurrently are depicted in Fig. 8. There will of course be variations in the pigment content of hepatic bile itself, but during reabsorption of bile by the gall-bladder, pigment remains entirely unabsorbed, and its concentration consequently rises by a factor of up to ten.

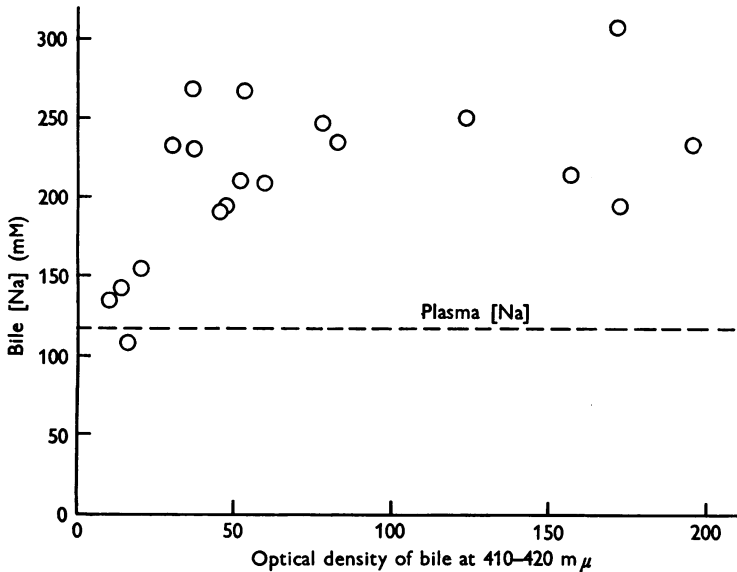

Fig. 8. Relation between Na and bile-pigment concentration in roach bile. Abscissa, optical density of bile at the absorption maximum between 410 and 420 mμ due to bilirubin; ordinate, [Na] in bile. The horizontal interrupted line is [Na] in plasma.

For example, the bile-filled gall-bladders of four fish and nine frogs have been incubated in Ringer's solution for up to 2 days without a trace of pigment permeating into the external solution. Hence the optical density of roach gall-bladder bile at the 410–420 mμ absorption maximum for bilirubin approximately reflects the degree to which it has been concentrated. It appears from Fig. 8 that bile [Na] is initially near plasma levels but attains and remains at a level twice that of plasma after the bile has been concentrated by a factor of two or three. As was mentioned above (p. 442), Ravdin, Johnston, Riegel & Wright (1932) also found a rise in

canine bile [Na] during the concentration process, though the present results give no hint whether their observation of an initial fall preceding the rise also applies to fish.

DISCUSSION

Mechanism of reabsorption of bile

The experiments described here concerning the absorption of Ringer's solutions make it clear that fish gall-bladder *in vitro* can utilize metabolic energy to transport a solution approximating to isotonic NaCl from its lumen to the outside. This ability has also been demonstrated for canine gall-bladder. In fact, a rather similar property was postulated for the gall-bladder nearly 60 years ago by Brand (1902), who suggested that concentration of the bile might occur by reabsorption of isotonic saline ('mit Blut isotonische Salzlösung'). As in many other epithelia, the absorptive process is specific for NaCl, shows saturable kinetics, and is unilaterally sensitive to cardiac glycosides or absence of potassium. It remains to be seen whether this demonstrated ability to absorb isotonic NaCl is sufficient to account for the observed differences between hepatic and gall-bladder bile. Such an absorbate would obviously lead directly to a reduction in bile volume, consequent increase in concentration of such non-diffusible components as the bile acids and bile pigments, and reduction of bile chloride. However, this simple hypothesis as it stands faces two apparent obstacles: (1) at first sight one might expect the concentration of sodium in gall-bladder bile to be lower than in hepatic bile, whereas actually it is up to twice as high; (2) the inorganic anions of hepatic bile seem quantitatively insufficient to account for the observed extent of reabsorption. Since bile acids are not reabsorbed and the inorganic anions account for up to 79 % of the total anion in hepatic bile, absorption should apparently cease with at least 21 % of the bile remaining, even if the inorganic anions were completely removed. In fact, the experiments of Ravdin, Johnston, Riegel & Wright (1932) often show reabsorption of all but 7–10 % of the volume of bile.

Both these apparent difficulties in the hypothesis of an isotonic NaCl absorbate can be explained by progressive formation of bile-salt micelles, resulting in a decrease of bile-salt osmotic coefficients with concentration. Hepatic bile (Table 1) consists largely of isotonic NaCl-$NaHCO_3$ solution, whereas in the gall-bladder the NaCl-$NaHCO_3$ is reabsorbed to leave an isotonic solution of bile salt (or NaB where B is an abbreviation for bile-acid anions). Since the osmotic coefficient of NaB is usually about half that of NaCl in the physiological concentration range, [Na] in isotonic NaB must be about double [Na] in isotonic NaCl. Furthermore, as is seen from Fig. 7, the osmotic coefficient of NaB (when B = deoxycholate) declines

from 0·62 to 0·33 between 28–42 and 220–340 mM, the concentrations in hepatic and gall-bladder biles respectively—i.e. the extent of micelle formation increases over this concentration range. Hence additional water must pass out of the gall-bladder so that increased micelle-formation may not leave the bile hypotonic.

The role of osmotic coefficients may best be illustrated by semi-quantitative consideration of the case in which the hepatic bile of Table 1 becomes concentrated down to gall-bladder bile of Table 1. For osmotic calculations the hepatic bile may be idealized as 142 mM-NaCl (combining 47 mM-HCO_3 with 95 mM-Cl), 36 mM-Na*B*, and 17 mM-*X* (largely K, Ca, Mg and bilirubin). Let the absorption process be idealized as the removal of all NaCl but no Na*B* or *X*. The relevant osmotic coefficients are: 142 mM-NaCl, 0·93; 36 mM-Na*B*, 0·57 (from the values for sodium deoxycholate in Fig. 7); 200–350 mM-Na*B*, 0·35; *X*, 0·9 (assigned arbitrarily, as the exact value will be uncertain and variable and has little significance for the calculations). The osmolarity of hepatic bile is thus $(2) \times (142) \times (0{\cdot}93) + (2) \times (36) \times (0{\cdot}57) + (0{\cdot}9) \times (17) = 320$ m-osmolar. If all the NaCl is absorbed isotonically as 171 mM ($0{\cdot}93 \times 2 \times 171 = 320$ m-osmolar), the absorbate would represent $(142) \times (100)/171 = 83\,\%$ of the total original volume. The residue would then be $(100) \times (17)/(100-83) = 100$ mM-*X* and $(100) \times (36)/(100-83) = 213$ mM-Na*B*. However, because of the drop in the osmotic coefficient of Na*B* this residue would be hypotonic ($212 \times 0{\cdot}35 \times 2 + 100 \times 0{\cdot}9 = 238{\cdot}4$ m-osmolar), and more water would therefore leave until the residue became isotonic at $X = 134$ mM, $NaB = 284$ mM ($0{\cdot}9 \times 134 + 2 \times 0{\cdot}35 \times 284 = 320$ m-osmolar), with 87 % of the initial volume reabsorbed. This division of the absorbate into 83 % isotonic NaCl followed by 4 % distilled water is of course artificial, and in practice the absorbate would be slightly hypotonic throughout. Thus, the effect of micelle formation would be to raise [Na] in gall-bladder bile to 284 mM and the reabsorbate volume to 87 %, in accord with Ravdin's experimental values of 224–305 mM and 90 %.

It has so far been assumed that bile components other than NaCl-$NaHCO_3$ remain strictly unabsorbed. This is certainly true for bilirubin *in vivo* (Riegel, Johnston & Ravdin, 1932) and, as judged from its impermeability, in the present *in vitro* preparation. The same conclusion holds approximately for the bile-acid anions (Ivy, 1934). However, potassium is present in canine gall-bladder at a concentration scarcely higher than that in hepatic bile (Table 1); hence it presumably diffuses readily out of the gall-bladder down the concentration gradient created during reabsorption. To a less extent there must also be some outward diffusion of calcium, since the (gall-bladder bile) : (hepatic bile) concentration ratio for calcium is less than that for the bile-acid anions (4·8 against 8·7). This diffusion of

potassium and calcium is presumably accompanied by water in approximately isotonic proportions, and if, for example, half the X fraction (molecules other than NaCl, $NaHCO_3$, and NaB) diffused out, the proportion of the initial volume of bile reabsorbed would be increased from 87 to 89·5 % in the model calculation.

Because of the uncertainty inherent in quantitative extrapolations to bile from simple solutions of particular bile salts, the numbers in these calculations should be regarded merely as illustrative. However, the direction of the calculated effects does suggest that the anomalous osmotic properties of the bile salts provide a purely physical explanation for the two apparent difficulties in supposing concentration of the bile to occur by primary reabsorption of isotonic NaCl. In conjunction with the impermeability of the gall-bladder to bilirubin and the bile-acid anions, low permeability to Ca, and micelle formation by the bile salts, the saline pump will account for the following changes in hepatic bile effected by the gall-bladder: (i) the virtual disappearance of chloride; (ii) up to a twofold increase in [Na]; (iii) eight- to tenfold increase in bilirubin and bile-acid concentrations; (iv) rise in calcium concentration; and (v) decrease in volume by up to 90 %. Two outstanding chemical changes about which the present study gives no information are the drop in pH and disappearance of HCO_3. Since [Na] is higher and [Cl] lower in the bile than in the plasma of other cold-blooded species (Table 5), as well as in virtually all published reports on mammals, it will not be surprising if the same factors prove to be responsible for absorption by the gall-bladder in other vertebrates.

The absorption of isotonic NaCl may thus be regarded as the biological driving force for the concentrating activity of the gall-bladder, and will accordingly be the subject of the experiments to be described in following papers.

Regulation of absorption

Physiological regulators of the absorptive process may be divided into factors determining its final extent, and factors setting the rate at which it approaches this limit. It is essential to the function of the valve of Heister (Löhner, 1926) that the bile should never be completely absorbed. The limit to absorption is set approximately by the residue of non-absorbable bile salts and bile pigments, but more exactly by the fact that luminal [Cl] itself is not reduced quite to zero—for example, only to about 5 mM in the canine gall-bladder. Of the eight chloride analyses on roach gall-bladder bile in the present study, two on freshly fed fish (whose gall-bladders must have been recently emptied and refilled with fresh hepatic bile) gave values over 40 mM, while the other six fell into the range 7·5–24·7 mM. This range is in satisfactory agreement with the value of luminal [Cl] = 15·6 mM, at which the net flux in Ringer's solution ceases because

the rate of diffusion inwards equals the rate of pumping outwards. Hence no factor other than the observed dependence of the pump on the luminal chloride concentration need be postulated to account for the final limit of absorption.

At higher luminal salt concentrations the rate of pumping may be varied to a limited extent by acetylcholine and adrenaline, which have slight excitatory and inhibitory effects, respectively, on the *in vitro* preparation. Similar effects have been noted *in vivo* (Westphal *et al.* 1931) and might operate through the branches of the vagus and splanchnic nerves to the gall-bladder. However, these effects appear too small both *in vivo* and *in vitro* to mediate the threefold diurnal variations and the complete inhibition at child-birth observed by Ravdin.

A more promising alternative is that regulation of the physiological rate of absorption depends upon oxytocin, which fully inhibits absorption *in vitro*. The dose required for this effect *in vitro* is much higher than the levels of oxytocin probably circulating *in vivo*, but posterior-pituitary hormones generally act at much lower doses *in vivo* than *in vitro*. Both Ravdin's observations concerning natural variations in gall-bladder activity point to an effect of oxytocin *in vivo*. First, the absorptive function of the gall-bladder disappears during child-birth, when oxytocin is released to contract the uterus. Secondly, absorption is inhibited at night, when increased amounts of posterior-pituitary hormones are circulating (the character of nocturnal antidiuresis points to increased ADH release at night, and ADH and oxytocin are believed generally to be released together). Thus, the oxytocin effect on the gall-bladder is also elicited *in vivo* under at least some conditions of endogenous release. The only other established physiological role of this hormone, in addition to stimulating contractions of the uterus at birth, is to cause secretion and ejection of milk in lactating female mammals. However, oxytocin is also found in non-pregnant and non-lactating female mammals, male mammals, and the four non-mammalian classes of vertebrates, where its function has been obscure and can obviously have no relation to mammary glands and placental birth. The potential clinical value of a specific inhibitor of reabsorption by the gall-bladder in preventing growth of gall-stones makes it particularly important that the oxytocin effect be tested on *in vivo* preparations.

SUMMARY

1. An *in vitro* preparation of fish gall-bladder has been used to study the mechanism by which the gall-bladder concentrates bile, and the factors regulating this process. Absorption of fluid from the lumen was measured by weighing the organ at 5 min intervals.

2. Like many other epithelia, the gall-bladder absorbs isotonic NaCl and NaBr, but not Na_2SO_4, $NaCH_3SO_4$, KCl, or tetraethyl ammonium chloride.

3. The relation between the absorption rate and luminal [NaCl] indicate saturable kinetics. Absorption may proceed at the expense of the contents of the epithelial cells themselves.

4. Absorption is inhibited by cyanide-iodoacetate, ouabain, oxytocin, or lack of potassium. The last three agents are effective only from the serosal surface.

5. In bile, both sodium and the bile acids exist partly in a bound form (e.g. micelles).

6. The transport of isotonic NaCl, in conjunction with the concentration-dependent osmotic coefficients of bile salts, suffices to explain most concentration changes during reabsorption of bile by the gall-bladder. The limit to absorption probably is regulated by the dependence of the transport mechanism on luminal chloride concentration.

7. The physiological rate of absorption may depend upon the hormone oxytocin.

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