Summary. Extracellular pH in the range 5.5 to 8.0 affects the yields of Herpes simplex virus obtained after one reproductive cycle in HEp-2 cells. For 3 strains of virus the extracellular pH for maximum yields are 6.5-6.7, 7.0, and 7.2-7.3, respectively. The data show that (a) extracellular pH for optimal virus multiplication is determined by the genetic constitution of the virus, and (b) HEp-2 cells support viral macromolecular synthesis when incubated in a medium buffered at pH as low as 6.0, *i.e.*, below the pH range for optimal cell multiplication and maintenance.

1. Lwoff, A., Cold Spring Harbor Symp. Quant. Biol., 1962, v26, 159.
2. Hoggan, M. D., Roizman, B., Am. J. Hyg., 1959, v70, 208.
3. Roizman, B., Aurelian, L., J. Mol. Biol., 1965, v11, 528.
4. Roizman, B., Cold Spring Harbor Symp. Quant. Biol., 1962, v26, 327.
5. Dulbecco, R., Vogt, M., Virology, 1958, v5, 220.

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Frog Gastric Mucosal ATPase.* (30366)

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In recent years interest in extramitochondrial ATPases has centered in large part on 'transport' ATPases. These enzymes have been prepared from a variety of tissue—such as crab nerve(1), red blood cells(2), kidney (3), rat brain(4), and other tissues(5). They share a certain number of features in common, namely a stimulation by Na^+ and K^+ and at least partial inhibition by ouabain. Furthermore, in the standard cellular fractionation methods, the ouabain-inhibitable Na^+ and K^+ sensitive component occurs in the microsomal fraction which is considered to contain a large proportion of fragmented membrane. Further evidence for the involvement of the microsomal fraction in transport involves (a) ATP-dependent Na^+ binding to the protein of brain microsomes(4); (b) P^{32} labelling of protein derived from ATP^{32} in r.b.c. membranes(6) and (c) the reduction of labelled phosphoserine from kidney ATPase preparations incubated with DFP^{32} in the presence of ATP and a possible identity of this ATPase and diglyceride kinase(7).

The gastric mucosa under normal conditions apparently contains at least 2 electrogenic pumps acting on Cl^- and H^+ respectively(8). The specificity of the pumps in the gastric mucosa may make it extremely difficult to characterize a 'pump' ATPase in the same manner as has been done for those described above. Thus the chloride mechanism has been shown to transport several univalent anions in the mucosa and to have some action on sulfate(9). Therefore the anion pump may be regarded as relatively nonspecific. The hydrogen ion pump is apparently specific for H^+. A demonstration of H^+ activation is naturally complicated by the pH activity curves obtained for enzymic reactions. The evidence therefore for a hydrogen ion pump must at present rest on the action of specific inhibitors.

In this connection Kasbekar and Durbin (10) have recently described a SCN^- sensitive ATPase in the microsomal fraction of the gastric mucosa, and in view of the possible importance of this observation in relation to H^+ ion secretion mechanism, which is inhibited by SCN^-, some of the properties of the mucosal ATPases have been investigated with particular reference to the action of SCN^-.

Methods-materials. All chemicals were reagent grade. Nucleotides were purchased from Sigma Chemical Co., labelled nucleotides

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from Schwarz Bioresearch, P^{32} from Abbott Laboratories, DEAE-cellulose from Gallard-Schlesinger.

Enzyme preparation. Starved *Rana pipiens* were decapitated and the stomach removed. The mucosal cells were scraped into ice cold 0.25 sucrose containing 0.1 M tris pH 7.4 and homogenized with a teflon homogenizer. The homogenate was fractionated into a nuclear, mitochondrial, microsomal and supernatant fraction by the Schneider-Hogeboom procedure(11). The microsomal fraction was used for these studies unless otherwise noted.

In some experiments further fractionation was carried out by layering an aliquot of the microsomal fraction or microsomal fraction containing 0.1% Triton X-100, on a linear gradient of sucrose from 0.7 to 2.5 M. After centrifuging for 2 hours at 39,000 r.p.m. in an SW39 rotor, fractions were collected *via* a tube-piercing device and assayed for protein and enzymatic activity.

ATPase studies. The standard incubation mixture contained 3 mM $MgCl_2$ 3 mM ATP 20 mM tris buffer pH 8.4, 0.5 mg protein in a final volume of 1 ml. Incubation was carried out in a water bath at 37° for 20 minutes. The reaction was stopped by addition of an equal volume of ice-cold 10% chloracetic acid, and the phosphate released was determined by the Fiske-Subbarow(12) procedure. For kinetic studies the Mg^{++}/ATP ratio was 1/1 throughout. Blanks were run with each determination and all assays were run in duplicate.

ATP-ADP exchange enzyme. The basic system contained 5 mM ATP, 5 mM $MgCl_2$, 1 mM ADP with c 20,000 cpm C^{14} ADP, 20 mM tris buffer pH 7.4 and c 0.5 mg enzyme in final volume of 1 ml. Incubation was carried out for 30 minutes at room temperature. The reaction was stopped by immersing tubes in boiling water for 4 minutes; control tubes showed there was no significant hydrolysis of the polyphosphates by this procedure. Control tubes containing boiled enzyme were run with each determination. After clarification by centrifugation, 10 μl were spotted on a DEAE-cellulose plate and developed for 30 minutes with 0.02 N HCl as solvent. The spots were located by U.V. light and eluted using 0.25 M NaCl pH 7.4. An aliquot was assayed for nucleotide content in a Beckman DU spectrophotometer at 260 mμ and another aliquot counted in a Nuclear Chicago scintillation spectrometer using a solvent system containing 70 g/l napthalene, 7 g/l PPO and 0.05 g/l POPOP in dioxane (spectroquality). Data were calculated from the average concentration of labelled precursor and average specific activity during incubation period(13).

ATP-Pi exchange. In this assay various concentrations of the reactants were used in an attempt to demonstrate activity. Thus Pi was added in a final concentration ranging from 1-10 mM, containing 5×10^5 cpm of P^{32} 20 mM tris pH 7.4-8.0, ADP 0-3 mM, ATP 5-10 mM, $MgCl_2$ 0-10 mM. SCN^- was also used to a final concentration of 3 mM. Assay was as in ATP-ADP exchange system.

Adenylate kinase. This activity was assayed by formation of ADP formed from labelled AMP (c 15,000 cpm) and ATP. The test system contained 5 mM ATP, 5 mM AMP, 5 mM $MgCl_2$, 20 mM tris pH 7.4, 0.5 mg enzyme and assay was performed as before with addition of carrier ADP prior to chromatography.

Nucleoside diphosphokinases. The reaction was estimated by the formation of C^{14} GTP from C^{14} GDP and ATP. The incubation mixture contained 3 mM ATP, 3 mM GDP containing 20,000 cpm, 3 mM $MgCl_2$, 20 mM tris pH 7.4 and 0.5 mg enzyme. Incubation was carried out for 30 minutes at room temperature and assay was performed as before. Protein was assayed by the Lowry method(14).

Results. ATPase activity. ATPases were found to be distributed in all the fractions of the gastric mucosa. 3 mM SCN^- inhibited all the fractions to approximately the same extent (Table I), and thus acted non-specifically with respect to the frog gastric mucosal ATPases. Further studies were carried out with the ATPase of the 'microsomal' fraction.

Metal ion specificity. A divalent metal cation was essential for activity and the greatest activity was obtained with Mg^{++}

TABLE I. SCN^- Sensitivity of ATPase in Subcellular Fractions μm Pi min^{-1} mg^{-1} Protein.

Nuclear	2.4
SCN^-	1.5
Mitochondrial	3.6
SCN^-	1.7
Microsomal	3.2
SCN^-	1.8
Supernatant	1.7
SCN^-	1.1

Assay performed with 3 mM ATP, 3 mM $MgCl_2$, 20 mM Tris pH 8.4 and 3 mM SCN^-, c 0.5 mg protein, 30 min, 37°.

(100%). However, both Mn^{++} (93%) and Ca^{++} (97%) could substitute for Mg^{++}. Various concentrations of Na^+ and K^+ (0-100 mM) were without effect on the ATPase activity. The optimal Mg/ATP ratio was found to be 1 in most preparations, while in 2 of 10 experiments the ratio was close to 2.

pH studies. The pH optimum curve of the enzyme showed a minor peak at pH 7.75 and a larger peak at 8.4. SCN^- eliminated the smaller peak and reduced the height of the peak at 8.4.

Specificity of ATPase. The highest activity was found with GTP (2.0 μM min^{-1} mg^{-1}) and ATP (1.8 μM min^{-1} mg^{-1}), and activity was about 50% lower toward CTP (1.3 μM) and UTP (1.2 μM). There was little hydrolysis of ADP (<0.25 μM). SCN^- was found to have an inhibitory action on the CTPase activity as well as the ATPase.

Inhibition of ATPase. Dinitrophenol, N-ethylmaleimide (NEM), cyanide, and ouabain were found to be without effect on the ATPase at 3×10^3 M. In contrast oligomycin, sodium nitrate, ammonium ion, cyanogen iodide and sodium thiocyanate were all found to have an inhibitory action (Table II). $HgCl_2$ was also found to be strongly inhibitory. Thus the -SH groups of the enzyme were reactive to mercurials but not NEM, but H^+ secretion is sensitive to both.

Na^+ and K^+ and ouabain insensitivity. Various concentrations of Na^+ and K^+, varying Na^+ from 0-100 mM and K^+ from 0-80 mM were without effect on enzyme activity. Ouabain at a concentration of 10^{-3} to 10^{-8} M was also without effect on the system. The exchange enzyme was similarly insensitive. Ouabain is without effect on the secretion of the *in vitro* frog mucosa.

Histamine effects. In 6 of 15 preparations, a variable degree of histamine sensitivity of the enzyme was found (about 30% stimulation), at concentrations of 10^{-3} to 10^{-4} M histamine. Various treatments such as Triton X-100, deoxycholate, diamine oxidase neither increased the sensitivity of the enzyme to such pharmacological doses, nor converted an insensitive preparation to a sensitive one.

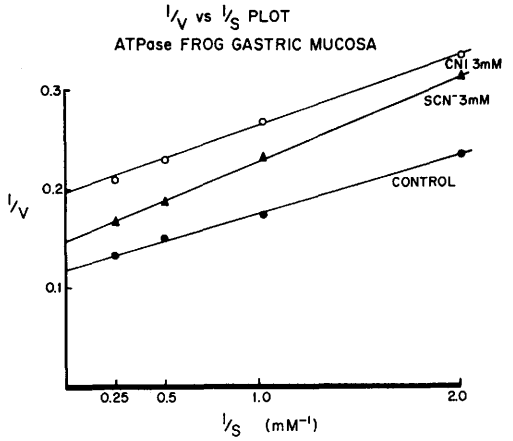

FIG. 1. 1/S *vs* 1/V plot for frog gastric mucosae microsomal ATPase, with SCN^-, CNl at 3 mM and ATP/mg ratio of 1, pH 8.4.

Kinetic studies. 1/S *versus* 1/V plots with 3×10^{-3} M thiocyanate show apparently a non-competitive mechanism of inhibition, with a calculated K_M for ATP of 5×10^{-4} M and a K_i of 12 mM for thiocyanate (Fig. 1). Similar studies for CNl showed that CNl also inhibited, apparently uncompetitively, but more potently with a K_i of 4 mM.

Exchange studies. A potent ATP-ADP exchange enzyme was found to be present in the microsomal fraction of the frog gastric mu-

TABLE II. Frog Gastric Mucosa Inhibition of Microsomal ATPase.

	% Inhibition
DNP, NEM, CN^- Ouabain	0
NO_2^-, NH_3	30
CNS^-	40
Oligomycin (10^{-5} M)	45
CNl	50
$HgCl_2$	100

Assayed as in Table I, with 3 mM inhibitor, except oligomycin as noted.

TABLE III. Frog Gastric Mucosa ATP-ADP, ATP-Pi Exchange mμmoles mg^{-1} min^{-1}.

(a)	ATP-ADP	350
	ATP-ADP + SCN^-	341
	Control boiled enzyme	<5
(b)	ATP-Pi	<15
(c, d)	Kinases (GDP, ATP, AMP)	<30

(a) ATP-ADP exchange assayed with 5 mM ATP, 5 mM $MgCl_2$, 1 mM C^{14} ADP, 20 mM Tris buffer pH 7.4, room temperature 30 min.

(b) ATP-Pi assayed with Pi^{32} 1-10 mM, ADP 0-3 mM, ATP 5-10 mM, $MgCl_2$ 0-10 mM, room temperature 30 min.

(c) GDP kinase assayed with C^{14} GDP 3 mM, ATP 3 mM, $MgCl_2$ 3 mM, 20 mM Tris pH 7.4, room temperature 30 min.

(d) Adenylate kinase with 5 mM ATP, 5 mM C^{14} AMP, 5 mM $MgCl_2$, 20 mM Tris pH 7.4, room temperature 30 min.

Nucleotides were separated by the thin layer chromatography.

cosa. The pH optimum was 7.4 and the average exchange rate was 350 mμM mg^{-1} min^{-1} (Table III). Thiocyanate, cyanogen iodide and oligomycin were found without effect on the exchange activity. No evidence could be found for an ATP-Pi exchange activity and the adenylic kinase and nucleoside diphosphokinases account for less than 10% of the exchange activity observed.

Gradient fractionation. Two peaks of ATPase activity were found, with the major peak of ATPase activity corresponding to the peak of ATP-ADP exchange activity. The density at which the enzyme activity fractionates corresponds to the region at which larger membrane fragments would fractionate. Triton X-100 apparently solubilized both enzyme activities (Fig. 2).

Discussion. ATPases were found in all fractions of the frog gastric mucosa. No very active inhibitor was found for the microsomal fraction apart from oligomycin and the mercurials. The SCN^- sensitivity of the microsomal ATPase was found to hold also for the other ATPases. The mechanism of inhibition of the microsomal ATPase with SCN^- appeared to be non-competitive. However, the high K_i found suggests that SCN^- is not a potent inhibitor of this system. A similar mechanism of inhibition was found for the brush border ATPase of the hamster intestine (15) and the rat liver mitochondrial ATPase. Thus the action of SCN^- on the microsomal ATPase apparently cannot be taken as evidence for the participation of the ATPase in hydrogen ion transport, and may rather be related to displacement of Cl^- from anion binding sites. The other inhibitors found, namely nitrite, ammonia(16), cynogen iodide (15), have also been found to inhibit gastric secretion *in vitro*. In the case of CNl, the mechanism of inhibition of the ATPase has been found to be uncompetitive. The lack of sensitivity to ouabain and to $Na^+ + K^+$ distinguished this ATPase from other 'transport' ATPases. It is not clear therefore from these experiments whether this ATPase is associated with transport and if so whether with hydrogen ion or anion transport, or with some other type of reaction.

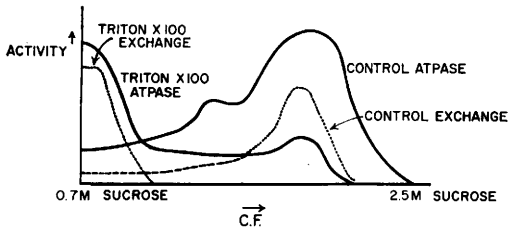

FIG. 2. Density gradient centrifugation of frog gastric mucosa microsomal ATPase, and ATP-ADP exchange enzyme between 0.7 M and 2.5 M sucrose, with and without Triton X-100.

The exchange enzyme demonstrated in the microsomal fraction shows similar properties to the other transport ATPases in that although ATP-ADP exchange activity is present, no ATP-Pi exchange activity was demonstrated(17). The exchange activity was not associated with adenylate kinase or NDP kinase. Since the ATPase inhibitors tested had no effect on the exchange enzyme their action must be confined to the second stage of the following scheme:

$$ATP + X \rightleftarrows P \sim X + ADP \quad (1)$$
$$P \sim X + H_2O \rightarrow Pi + X \quad (2)$$

Since no phosphate stimulation of the ATP-ADP exchange reaction was found, the above scheme is the simplest compatible with the data.

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1. Skou, J. C., Biochim. Biophys. Acta, 1957, v23, 394.

2. Post, R. L., Fed. Proc., 1959, v18, 121.
3. Skou, J. C., Biochim. Biophys. Acta, 1962, v58, 314.
4. Jarnefelt, J., von Stedingk, L. V., Acta Physiol. Scand., 1963, v57, 328.
5. Bonting, S. L., Simon, K. A., Hawkins, N. M., Arch. Biochem. Biophys., 1961, v95, 416.
6. Charnock, J. S., Post, R. L., Nature, 1963, v199, 910.
7. Hokin, L. E., Yoda, A., Proc. Nat. Acad. Sci., 1964, v52, 454.
8. Rehm, W. S., The Cellular Functions of Membrane Transport, Prentice-Hall, Englewood Cliffs, N. J., 1963, p231.
9. Hogben, C. A. M., Fed. Proc., 1957, v20, 139.
10. Kasbekar, D. K., Durbin, R. P., Biophysics Meeting, 1964.
11. Schneider, W. C., Hogeboom, J. Biol. Chem., 1950, v183, 123.
12. Fiske, C. H., Subbarow. Y., ibid., 1925, v66, 375.
13. Chiga, M., Plaut, W. E., ibid., 1959, v234, 3059.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., ibid., 1951, v193, 265.
15. Sachs, G., unpublished observations.
16. LeFevre, M. E., Gohmann, E. J., Jr., Rehm, W. S., Am. J. Physiol., 1964, v207, 613.
17. Skou, J. C., Biochim. Biophys. Acta, 1960, v42, 6.

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Urinary Excretion of Three Oral Cholecystographic Agents in Man. (30367)

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Data on the absorption and excretion of 2 orally administered cholecystographic agents have been reported from this laboratory(1,2). It was found that of a 3 g dose of iopanoic acid* to man, 36% was excreted in the urine within 132 hours, while 62% was excreted in the feces. Of the urinary excretory product about ⅞ was not extractable by ether at pH 6-7, therefore was presumed to be present as the glucuronide conjugate(1,3,4). Butyryl-iopanoic acid (tyropanoic acid†) was found to be 94% excreted within 108 hours following a 4.5 g dose of the sodium salt, but its partition between urine and feces was almost exactly 1:1. Although it was also shown that tyropanoate is excreted in the bile as a glucuronide, its partition between free and conjugated forms in the urine was not determined(2). It has been stated(5,6‡) that 70% of an oral dose of bunamiodyl§ is excreted in the urine. A more recent report, however, indicates that the urinary excretion in man is only 25% of the dose in the first 30 hours post-medication(7).

In view of several recent reports of renal damage in subjects receiving bunamiodyl(8-13), and the suggestion that cholecystographic agents might cause oliguria under certain circumstances by physical blockage(14), it has been of interest to determine the extent to which the comparatively insoluble contrast media are excreted in the urine in the non-conjugated form.

Materials and methods. Three inmates of the Oklahoma State Prison served as subjects for this experiment.‖ Each received orally, in experimental cross-over design, 3 g iopanoic acid, 4.5 g sodium tyropanoate, or 4.5 g of sodium bunamiodyl. There was a lapse of 2 weeks between the first and second medications, and a lapse of 4 weeks between the second and third medications. The radiopaques were given at 9 P.M. on the first day

* Available from Winthrop Laboratories, New York, under the trade name of Telepaque®.

† Winthrop Laboratories registered name for tyropanoic acid is Bilopaque.

‡ Although ref. 5 makes it clear that this statement applies specifically to the dog the idea has become fairly widespread that it also applies to man (see ref. 9).

‖ These samples were collected under the supervision of Dr. C. K. Wisdom of Stough-Wisdom Research, Inc.

§ Bunamiodyl is a product of E. Fougera Co., Hicksville, N. Y. (trade name, Orabilex).