Application of Manometric Method to Testing Chemical Agents *in vitro* for Interference with Poliomyelitis Virus Synthesis.*† (21152)

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The need for a reliable *in vitro* screening procedure to expedite the search for a chemical inhibitor(s) of mammalian virus infection has led to a variety of approaches to this problem. Advantage may be taken of the observation that a human epithelial cancer cell, strain HeLa, and any of the 3 types of poliomyelitis virus possess attributes which provide in combination a model cell-virus system for quantitative studies(1-3). Thus, poliomyelitis virus excels as a test agent since it is stable under widely different conditions, and it can be readily handled and accurately quantitated. Similarly, the HeLa cell strain in continuous culture has extraordinary properties: It is a) stable, b) yields cells on a 2-dimensional surface such as glass for uniform exposure to virus, c) provides a cell population that can be accurately measured, and d) upon infection by a cytopathogenic virus responds by rapidly progressive total destruction. Direct observation for cytopathology permits recognition of any interference of viral infection that may result from a test chemical inhibitor. These properties of host cell and of virus make it possible to study a test material for an alterative effect upon the host cell, or upon virus synthesis, or both. A relationship of virus synthesis to actively metabolizing cells is recognized. For example, other workers have related the dependence of virus synthesis on oxidative energy of cellular respiration for the influenza-embryonic chick system(4), for the influenza-chorioallantoic membrane system (5), and for the feline pneumonitis-yolk sac system(6). If a similar relationship were to hold for the synthesis of poliomyelitis virus by strain HeLa cells, it would render suspect the usefulness of a chemical that reduces concurrently synthesis *in vitro* and the respiratory rate of the host cell. Moreover, the respiration of the surviving cells reflects the progress of infection by a cytopathogenic virus. The present study reports findings of manometric studies which employed cultures of strain HeLa cells to test the effect of antimetabolites upon poliomyelitis infection. These studies demonstrate that the chemical agents employed can influence the replication of poliomyelitis virus only when used in a concentration that inhibits markedly cellular respiration.

Materials and methods. Microbial contamination was avoided by aseptic technic and the employment of penicillin and streptomycin in culture medium at levels of 300 units per ml and 100 μg per ml respectively.

Assay for virus. Virus titrations were conducted essentially as described(1,2) with the following exceptions: a) cell cultures in preparation for incubation were rinsed thrice instead of twice; b) 5 replicate tubes were set up at each successive geometric dilution so to permit by use of standard statistical tables computation of most probable numbers of tissue culture infectious doses (TCID). Final virus dilutions were made in a solution consisting of 1 part chicken serum to 9 parts of maintenance solution; 1.0 ml of each dilution was added per tube. The reproducibility of such titrations in this laboratory has been found to be of the order of $\pm$0.3 log. *Virus:* Poliomyelitis virus, Type 1, Mahoney strain was employed. The immediate source of virus was a pool of supernatant fluids from cultures representative of the 63rd passage of the virus in strain HeLa cells. Virus suspensions were stored in 2 ml glass ampules at −70°C. A small amount of virus (15-30 TCID) was employed in each experiment so that several cycles of virus synthesis could be followed. *Host cell:* Strain HeLa cells were employed,

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

† Presented at the Annual Meeting of the Society of American Bacteriologists, Pittsburgh, Penn., May 2-7, 1954.

‡ The material in this paper represents in part the thesis by George E. Gifford in partial fulfillment of the requirements for the Ph.D. degree.

cultivated, maintained, and enumerated as described previously(1-3). Cells were grown in planar bottles with a nutritive medium composed of 40% human adult serum, 2% chicken embryonic extract, and 58% Hanks' balanced salt solution (HAS-40, EE-2, BSS-58).

Metabolic analogues. The metabolic analogues, DL-ethionine,§ benzimidazole,§ β-2-thienylalanine, 2,6-diaminopurine, and the metabolite DL-methionine§ were used as isotonic solutions prepared by dilution with BSS to a final ionic concentration of 0.33 M. All solutions were sterilized by filtration through ultrafine sintered glass filters.

Phosphite buffer-salt solution (BPS). A sodium hydrogen phosphite buffer¶ replaced MS for experiments in which additional buffering capacity was desired. A 0.20 M stock solution of H_3PO_3 was prepared from Baker's purified 30% solution and checked acidimetrically. 100 ml of this stock, adjusted to pH 7.6 with NaOH, and diluted to 200 ml with distilled water, yielded an isotonic 0.1 M solution of NaH_2PO_3-Na_2HPO_3 of pH 7.6. This solution was sterilized by passage through an ultrafine sintered glass filter. Phosphite anion has been found(7) to be a relatively non-toxic buffer with an useful buffering range of pH 5.5 to 7.5. It is compatible with calcium and magnesium salts, does not interfere in known enzymic reactions of phosphate, and when present at 0.02 M concentration in the medium, does not reduce the growth rate of strain HeLa cells in stationary culture nor reduce the rate capacity of the cell to synthesize poliomyelitis virus.

Manometric methods. The Krebs' modification of Pardee's carbon dioxide buffer(8) was employed in respiration studies at an equilibrium concentration of 2% carbon dioxide in the gas phase. Carbon dioxide buffer, 0.5 ml, was placed in the center well fitted with a filter paper wick and another 0.5 ml placed in the side arm.

A convenient rate of respiration was found with from 4 x 10^5 to 2 x 10^6 cells per flask. To obtain the requisite number of cells for each experiment, cultures of several bottles were trypsinized, pooled, concentrated by centrifugation at 160 g for 10 minutes, washed by resuspension in BSS to original volume and recentrifuged as before. Cells were suspended in a volume of MS-100 so that the desired number of cells would be contained in the 1 ml aliquots transferred to the main compartment of each sterile Warburg flask. Flasks then received 0.2 ml of ChS-100 and 0.7 ml of either BSS or test chemical in BSS. Cultures were infected by inoculation with 0.1 ml of virus suspension containing 15 to 30 TCID; control flasks received 0.1 ml of BSS in lieu of above. To reduce the hazard of contamination, the ground glass connections of the manometers were sterilized by repeated immersion in 95% ethanol and ignition and then lubricated with sterile petrolatum. The sterile venting tubes fitted with absorbent cotton filters were connected to the gas supply and inserted in the flasks. The flasks were mounted on the manometers, immersed in the water bath maintained at 35°C and gas was passed through the system for 1 hour while the flasks were shaken at a rate of 60 strokes per minute and an amplitude of 2.5 cm. Stopcocks were then closed and the respiration followed in the conventional manner for a period of several days.

Experimental. Pilot studies. The first experiments were designed to test the thesis that the energy derived from respiration was essential for synthesis of poliomyelitis virus by strain HeLa. The capacity of HeLa cells to produce poliomyelitis virus, Type 1, under aerobic and anaerobic conditions was compared. The amount of acid produced by the HeLa cells under anaerobic conditions was greater than under aerobic conditions. To maintain a comparable pH in both systems, it was necessary to add additional buffer. The possibility of using phosphite buffer at 0.02 M concentration was tested by observing the effect on the rate of respiration of HeLa cells. Two series of Warburg flasks were prepared

§ Nutritional Biochemicals Corp.

‖ Bios Laboratories, Inc.

¶ 200 ml of 0.1 M phosphite solution at pH 7.6, 1.0 g glucose, 6.25 g NaCl, 0.4 g KCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.14 g $CaCl_2$, 0.35 g $NaHCO_3$, 0.06 g KH_2PO_4, 0.06 g Na_2HPO_4, and distilled water to make 1 liter. The final concentration of phosphite is 0.02 M.

TABLE I. Effect of Sodium Acid Phosphite (.02M) upon Respiration of Strain HeLa.

Hr	O_2 uptake, μl MS* (control)	O_2 uptake, μl BPS† (test)	% of control
12	127	117	92
24	250	236	94
36	371	366	99
48	487	496	102
72	708	726	103

* Maintenance solution.
† Balanced phosphite solution.

with carbon dioxide buffer in the center well and side arm of each flask. Chicken serum, 0.2 ml, was added to the main compartment of each flask. HeLa cells, 1.1 million, suspended in either 1.8 ml of BPS or 1.8 ml of MS were added to each flask. The thermobarometer was prepared by adding a like amount of carbon dioxide buffer to the center well and side arm, and by placing medium without cells in the main compartment. Oxygen uptake was followed for 72 hours in the conventional manner. The results (Table I) indicated that 0.02 M phosphite did not affect the respiration of the HeLa cells.

Replicate Warburg flasks were prepared using BPS-90, ChS-10. An inoculum of from 20-30 TCID of virus in a volume of 0.2 ml of BPS was added to the side arm of each flask. The aerobic flasks were incubated in an atmosphere of 2% CO_2, 98% air with CO_2 buffer in the center well and side arm equilibrated in the usual manner. The anaerobic flasks were equilibrated in an atmosphere of 100% nitrogen (oxygen-free) with continuous shaking for 1 hour in the water bath. The contents of the side arm were dumped into the main compartment. The cells under anaerobic conditions remained viable, as evidenced by the production of carbon dioxide and progressive accumulation of acid. However, the virus yield from these cells was only 0.01% of the amount produced by an equal number of cells maintained aerobically. Thus, the anaerobic yield of virus in TCID/ml was $10^{3.1}$ as compared to an aerobic yield of $10^{7.2}$.

A comparative study of the respiratory rate and virus yield was made of cultures shaken continuously at 60 strokes per minute and an amplitude of 2.5 cm, and of cultures held stationary to learn whether the vigorous agitation imposed on HeLa cells in Warburg flasks might affect adversely their metabolic rate. If cellular respiration were affected markedly the results of such manometric studies might be difficult to relate to findings with stationary cultures. HeLa cells attach and grow readily on a glass surface; in agitated cultures they assume a position at the gas-liquid-glass interface. As shown by the data in Table II, the initial respiratory rate of agitated cultures was somewhat greater than that of static cultures, presumably from more rapid diffusion of oxygen through the medium. Nevertheless, the total O_2 uptake and virus synthesized were essentially unaltered by such agitation.

TABLE II. Effect of Agitation on Respiration and Virus Production by HeLa Cells.

	O_2 uptake, μl 12 hr	O_2 uptake, μl 24 hr	O_2 uptake, μl 44 hr	Virus yield, log TCID/ml (44 hr)
Shaken*	37	73	93	6.5
	38	74	92	6.7
Stationary	29	59	87	6.4
	32	62	92	6.5

* 60 strokes/min., amplitude of 2.5 cm.

Tests with metabolic analogues. The stationary tube method and the manometric method were compared for the determination of toxicity of benzimidazole for strain HeLa cells and for the resultant effect on virus yield. Parallel experiments were performed in duplicate Warburg flasks and in quintuplicate test tube cultures. Each Warburg flask was

TABLE III. Comparison of Virus Yield and Detection of Toxicity by Test Tube and Manometric Methods.

Benzimidazole, mg/ml	56 hr virus yield, log TCID/ml Manometric	56 hr virus yield, log TCID/ml Tube	Toxicity* to tube culture, 56 hr	Inhibition of respiration (%), 56 hr
.0	7.0	3.9		
	7.0			
.25	6.4	3.0	—†	36
	6.5			
.50	3.7	1.4	+	80
	3.5			

* Microscopic evidence for toxicity in test tubes was assessed by contraction of cytoplasm, by granularity, and/or cells leaving the glass.
† Control tubes incubated for an additional 24 hr showed questionable evidence of toxicity by slight rounding of cells.

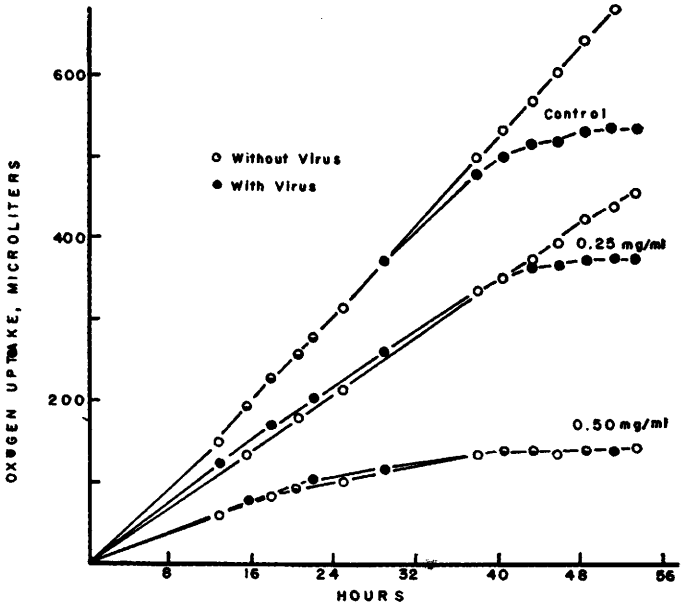

FIG. 1. Effect of benzimidazole upon respiration of strain HeLa.

charged with 5 x 10^5 cells; comparable tube cultures each contained approximately 5 x 10^4 cells. After 55 hours incubation at 35°C, the supernatant fluids were harvested separately and assayed for virus content. The results are given in Table III. The presence of 0.25 mg/ml of benzimidazole significantly depressed the virus yield in tube cultures over a period when control tubes showed no microscopic evidence of toxicity. However, this concentration of benzimidazole was found to markedly inhibit respiration. This interference with cellular respiration was apparent from the outset and progressive as shown in Fig. 1. Similar manometric studies were carried out with 3 other metabolic analogues, *viz.*, DL-ethionine, β-2-thienylalanine, and 2,6-diaminopurine. The results are summarized in Table IV. The effects of these antimetabolites on the respiration of HeLa cells are shown in Fig. 2 to 4. Supernatant fluids for virus titrations were removed shortly after respiration ceased. It was observed in each experiment that cellular death of infected cultures, as indicated by the cessation of respiration, was not influenced by the presence or absence of antimetabolite, or by the amount of virus produced. Thus again, significant inhibition of virus synthesis was limited to the concentrations of antimetabolite that provoked marked inhibition of the respiration of the host cell. Included in Table IV are the results of two attempts to reverse the toxic effects of ethionine. Methionine was able to partially reverse the inhibition of ethionine.

Discussion. DL-ethionine, 2,6-diaminopurine, benzimidazole, and β-2-thienylalanine did not inhibit the synthesis of poliomyelitis virus, Type 1, in strain HeLa cell cultures except at concentrations which were markedly inhibitory to the respiration of the host cell. Cessation of respiration of infected cultures occurred at the same time independent of the concentration of metabolic analogue employed, or of the final concentration of virus. The cytopathic changes reflected in reality the additive effects of chemical and virus. With the possible exception of benzimidazole, toxicity increased with duration of exposure to the antimetabolite. The respiratory inhibition provoked by ethionine was only partially reversed by the addition of methionine.

A concentration of benzimidazole, 0.25 mg/ml, which was non-toxic for HeLa cells as assessed by microscopic observations, was markedly inhibitory to the respiration of cells in a Warburg flask. This observation indicates that the detection of altered respiration

TABLE IV. Effect of Metabolic Analogues upon Respiration of Strain HeLa and upon Replication of Poliomyelitis Virus.

Exp. No.	Compound	mg/ml	% inhibition of respiration 24 hr	48 hr	72 hr	Virus yield, log TCID/ml	No. of cells per flask $\times 10^{-5}$
1.	Benzimidazole	0				7.0	5
		.25	32	34	38	6.4	
		.50	68	78	79	3.6	
2.	β-2-thienylalanine	0				6.5	5
		.10	0	0	0	6.6	
		.25	0	10	17	6.4	
		.50	9	24	33	5.8	
3.	2,6-diamino-purine	0				6.5	5
		.025	0	8	24	6.3	
		.050	4	13	30	6.8	
4.	2,6- " "	0				6.7	6
		.5	34	54	63	3.7	
5.	DL-ethionine	0				7.5	8
		.68	0	3	19	7.5	
6.	DL- "	0				7.3	12
		1.3	27	44	56	6.3	
		2.7	33	57	71	5.3	
	DL-ethionine + DL-methionine	1.3 + 1.2	23	37	42	6.6	
7.	Dl-ethionine	0				7.6	10
		2.7	33	56	73	6.3	
	DL-ethionine + DL-methionine	2.7 + 2.5	27	42	51	7.5	

might prove generally a more sensitive criterion of drug toxicity than the subjective observation of any alteration in morphology.

The importance of the oxidative energy of respiration for the synthesis of poliomyelitis virus, Type 1, by HeLa cells was demonstrated. Anaerobically, such cells were able to produce less than 0.01% of the amount of virus produced by replicate systems under aerobic conditions. This small anaerobic production of virus may reflect either stored energy reserves of the host cell or incomplete removal of the oxygen from the medium during equilibration.

The importance of oxidative energy of respiration for the synthesis of viruses in other virus-host systems has been reported by several investigators(4-6). If an agent so interfered with essential metabolism that it reduced the respiration of the host cell and hence the supply of energy available for endergonic processes, it would not be remarkable if virus synthesis was also suppressed. Such non-specific interference with virus synthesis in homogeneous cell populations could be recognized readily by observations of altered respiratory rate. Thus, the homogeneity of HeLa cells in culture makes this cell strain distinctly superior to any mixed cell culture, for in the latter, the proportion of cells susceptible to virus or to chemical agent is variable and may be small.

HeLa cells in each of the cultures here employed have been susceptible to infection and subsequent destruction by poliomyelitis virus. This evidence for cellular uniformity in susceptibility to the test virus permits one to assign the observed effects of added agents on respiration of such cultures to the effect of the agent on those cells capable of propagating the virus. Such a system of uniform cells can serve as an index of the progress of infection by cytopathogenic viruses. Presumably any agent capable of interference with virus synthesis without blocking essential metabolism of the host cell would behave by prolonging the respiration of infected cultures.

The desirability of interfering with virus synthesis *per se* rather than with essential metabolism of the host cell may not be realized

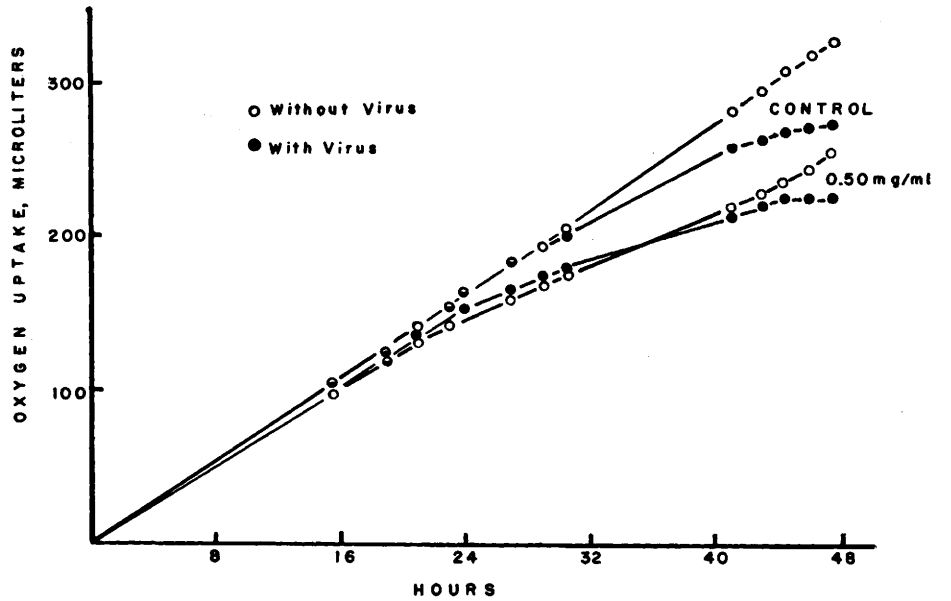

FIG. 2. Effect of β-2-thienylalanine upon respiration of strain HeLa.

without careful assessment of the toxicity of such chemicals. The demonstration of recovery of cells after removal of drug, or upon addition of metabolite, has been generally used in heterogeneous tissue culture to indicate the degree of permanent damage inflicted on the host cell by the test agent. More recently, cell cultures amenable to visual observations have permitted changes in morphology or growth rate to serve as a measure of toxicity of such added agents. Determination of toxicity by observation of the effect of added chemical agents on cell respiration has been employed in mixed cell cultures(5,9,10). Since the proportion of cells susceptible to virus or to chemical was not established by

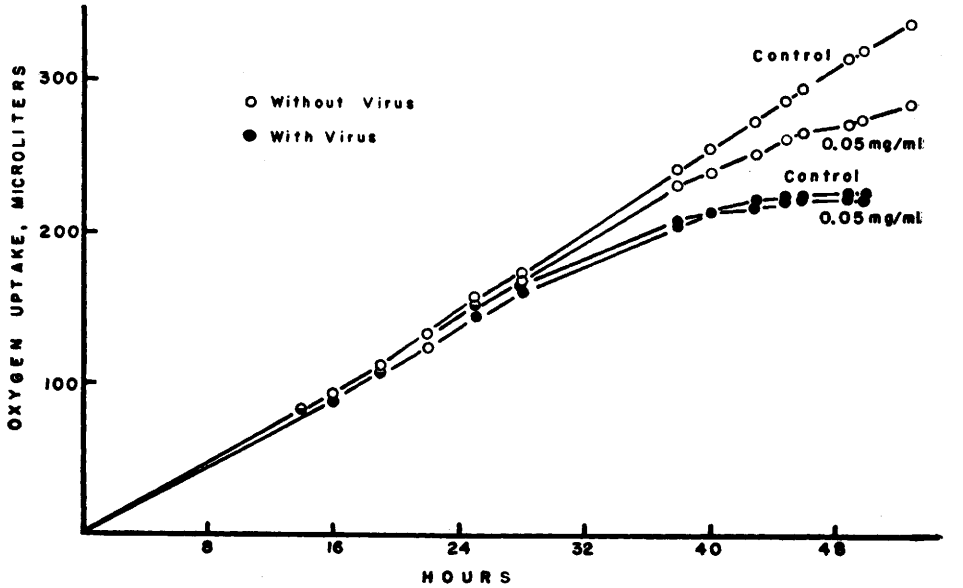

FIG. 3. Effect of 2,6-diaminopurine upon respiration of strain HeLa.

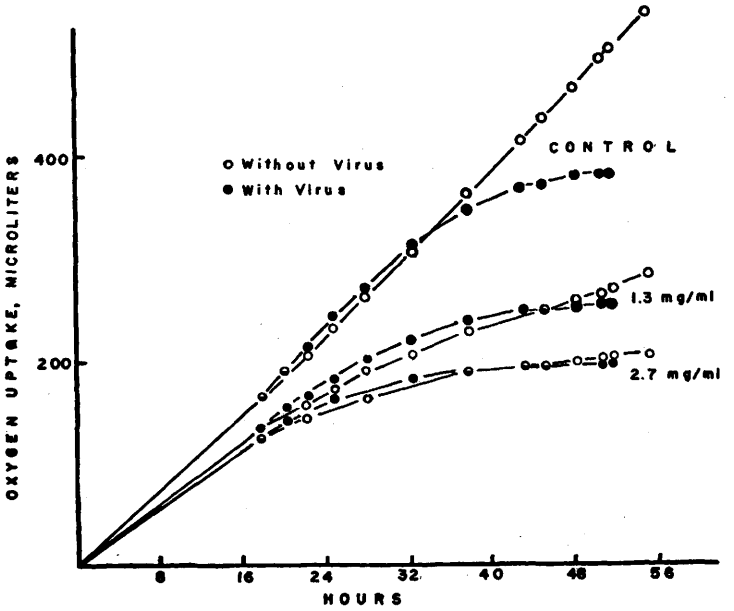

FIG. 4. Effect of ethionine upon respiration of strain HeLa.

these investigators, it is our opinion that the results do not permit definite conclusions.

Much effort is being expended in the search for effective chemotherapeutic and chemoprophylactic agents for virus diseases. Numerous chemical agents have been reported to inhibit virus synthesis in tissue culture. For example, Brown and Ackermann(11) and Brown(12) reported that many chemical agents inhibit poliomyelitis virus replication in tissue culture. It is our experience(13) that the replication of poliomyelitis virus in strain HeLa cellular cultures can not be inhibited by the incorporation of ethionine, benzimidazole, β-2-thienylalanine, 2,6-diaminopurine, pyridine-3-sulfonic acid, DL-desthiobiotin, pantoyl taurine, 3-acetyl pyridine, and desoxypyridoxine HCl in concentrations that do not alter the morphology of HeLa cells. It was shown that at levels of doubtful toxicity, inhibition of virus synthesis was occasionally manifested. These results led to the use of the measurement of oxygen uptake as a more sensitive and objective means for the determination of toxicity. Such non-specific interference with virus synthesis can be recognized readily by the observation of an altered respiratory rate. The existence of a variety of agents, such as 2,4-dinitrophenol, which waste the energy of oxidative metabolism without impairing respiration, would not reduce the value of recognizing those agents which interfere with essential metabolism in a less subtle way.

Summary. DL-ethionine, 2,6-diaminopurine, benzimidazole, and β-2-thienylalanine did not inhibit the synthesis of poliomyelitis virus, Type 1, in strain HeLa cell cultures except at concentrations which were markedly inhibitory to the respiration of the host cell. Cessation of respiration of infected cultures occurred at the same time independent of the concentration of the metabolic analogue employed or of the final concentration of virus. The ethionine-induced inhibition of respiration was only partially reversed by the addition of methionine. A concentration of benzimidazole, 0.25 mg/ml, which was not toxic for HeLa cells, as assessed by microscopic observations, was markedly inhibitory to the respiration of cells in a Warburg flask. The importance of the oxidative energy of respiration for the synthesis of poliomyelitis virus, Type 1, by HeLa cells was demonstrated. Anaerobically, such cells were able to produce less than 0.01% of the amount of virus produced by replicate systems under aerobic conditions. The advantages of a homogene-

ous cell culture over mixed cell cultures for studies of chemical inhibition of virus synthesis are discussed. Cellular uniformity permits precise allocation of the observed effects of a test agent to cells capable of propagating the virus.

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Received June 7, 1954. P.S.E.B.M., 1954, v86.

Intestinal Absorption of Vitamin B_{12} in Humans as Studied by Isotope Technic.* (21153)

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The intestinal absorption of vit. B_{12} appears to be variable from one individual to another(1-3) and its underlying principles seem to be contradictory and confusing. Only a small part of vit. B_{12} ingested is absorbed in the intestine(4-6) and the hematopoietic responses of patients with pernicious anemia to oral administration of vit. B_{12} even in association with potent sources of intrinsic factor from human or animal origin are, at their best, lower than those observed after parenteral administration of similar doses of vit. B_{12}(1-3,7-10). On the other hand, fecal excretory studies done with radioactive vit. B_{12} tend to indicate that as much as 90-95% might be absorbed when 0.5-1.0 μg is given orally to normal individuals(11-13).

Recently, we have used surface scintillation measurements of the uptake of radioactive vit. B_{12} by the liver following parenteral and oral administration of this vitamin for the study of B_{12} metabolism(14-16). These investigations have shown that in humans under normal conditions the liver is the target organ for the uptake of radioactive vit. B_{12}, especially after its oral administration and that the scanning technic can be successfully applied to evaluate the intestinal absorption of vit. B_{12}.

Method. The measurements were made with the method described previously(16) in 20 normal individuals or patients with irrevalent disorders, following ingestion of standard doses of Co^{60}-containing vit. B_{12}† to which variable doses of crystalline nonradioactive vit. B_{12} were added. Between the 6th and 10th day after ingestion of radioactive vit. B_{12}, when most of the radioactive material had left the gastrointestinal tract, counts were made with scintillation counter (NaI Thallium 1″ x 1″ crystal) in close approximation with the skin at 1000 volts over 3-4 anterior,

* Aided by the Grant-in-Aid No. A-68 (R & C) of the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service.

† Generously supplied through the courtesy of Dr. Charles Rosenblum from Merck Co., Inc., Rahway, N. J., on allocation of the Atomic Energy Commission.