pituitary pressor hormone exerts a pressor action on unanesthetized rabbits. This effect is so constant that it may be used in a new and convenient method for the estimation of the potency of pressor hormone preparations. Too few animals were available to us for a thorough investigation of the accuracy of this method. In previous experiments with unanesthetized animals the fall in blood pressure is often followed by an increase(9). As the pituitary glands used by the older investigators were often air dried, enzymatic activity and bacterial growth during the drying process may have caused contamination with depressor substances. Thus a proteolysis followed by bacterial decarboxylation might yield histidine and histamine.

The results here obtained show that doses of 0.5 unit per kg (and in other series also those of 1.0 and 2.0 units per kg) fall considerably below the curve. This is indicative of a secondary depressor mechanism interfering when larger doses are given. The reaction here involved is probably the constriction of the coronary arteries observed by other investigators.

In some experiments the hormone solutions were heated, either in 0.25% acetic acid as in the standard procedure for estimating the pituitary pressor hormone or in physiological saline. No change in activity was noticed. The pressure effect was not caused by a nonspecific reaction because repeated injections of proteins (lyophilized human plasma, egg albumin, edestin; 2.5 mg per kg) did not produce any significant rise (less than 10 mm, usually about 5 mm). Even after such sensitization an injection 4 weeks later did not produce any change, except a small transient decrease during the first 1-2 minutes.

Summary. The increase in blood pressure produced by injection of pituitary pressor hormone (pitressin, vasopressin) in unanesthetized rabbits may be used in the assay of this hormone.

1. Stehle, R. L., In *Vitamins and Hormones,* 1949, v7, 389. Academic Press, Inc., New York.

2. Waring, H., and Landgrebe, F. W., Hormones of the posterior pituitary. In Pincus and Thimann, *The Hormones,* 1950, v2, 427. Academic Press, New York.

3. Landis, E. M., Montgomery, H., and Sparkman, D., *J. Clin. Invest.,* 1938, v17, 189.

4. Page, E. W., and Ogden, E., PROC. SOC. EXP. BIOL. AND MED., 1939, v42, 770.

5. Grant, R. T., and Rothschild, P., *J. Physiol.,* 1934, v81, 265.

6. Pickering, G. W., and Prinzmetal, M., *Clin. Sci.,* 1938, v3, 211.

7. Schales, O., and Haynes, F. W., PROC. SOC. EXP. BIOL. AND MED., 1941, v47, 315.

8. Astrup, T., and Birch-Andersen, A., *Nature,* 1947, v160, 570.

9. Gruber, Ch. M., *J. Pharm. and Exp. Ther.,* 1929, v36, 155.

10. Sulkin, N. M., and Brizzee, K. R., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 125.

Received December 6, 1951. P.S.E.B.M., 1952, v79.

Uptake of Radioactive Vitamin B_{12} by Various Microorganisms. (19349)

ROBERT L. DAVIS, L. L. LAYTON, AND BACON F. CHOW.
From the Department of Biochemistry, School of Hygiene and Public Health of the Johns Hopkins University.

Microorganisms differ in their requirements for vit. B_{12}. Some can thrive successfully without any exogenous source of this vitamin, whereas others require it for propagation; still others have a need for vit. B_{12} only during the initial phase of growth. It is, therefore, of interest to study the possible incorporation of vit. B_{12} from the medium into bacterial cells of species representative of these three types. To this end, several strains of microorganisms were allowed to grow in a basal synthetic medium prepared according to Skeggs and Wright(1), to which radioactive vit. B_{12} (2) tagged with Co^{60} was introduced. The presence of the radioactive cobalt in the molecule facilitates the identification and tracing

TABLE I. Determination of Radioactivity in Cells Grown in Synthetic Medium Containing Vit. B_{12} Tagged with Co^{60} (8 mμg equivalent to 60 cpm).

Organism	Hr at 37°C	Spectrophotometer reading*	Total No. of microorganisms ($\times 10^9$)	Radioactivity in total microorganisms (cpm)	Molecules vit. B_{12} per microorganism
L. leichmannii	40	380	130	23	8
L. arabinosus	18	550	184	<3	<<1
Esch. coli	40	120	20	8	25
S. lactis R	23	180	32	25	40
B. subtilis	18	350	140	36	14
P. mirabilis	40	110	16	19	60
B. mycoides	22	195	36	20	30
Esch. freundii	40	125	20	24	60
S. dobson	40	200	96	23	12
L. lactis Dorner	24	250	44	23	25

* Spectrophotometer readings were obtained with Klett-Summerson photo-electric colorimeter using filter #42.

of that part of the vitamin molecule.

Experimental procedure. A. *Microorganisms chosen for study.* *L. leichmannii* is dependent on an exogenous source of vit. B_{12} for multiplication; while proliferation of *L. arabinosus* is independent of such a source. These strains were therefore studied as typical of the extreme cases. Exogenous vit. B_{12} can stimulate the growth of *L. lactis* Dorner during the initial phase of growth and it, therefore, is representative of the third category. Others were included in order to ascertain whether their requirements for vit. B_{12} if any, had any relationship to their uptake. B. *Measurement of the uptake of* Co^{60}. Varying amounts (4-8 mμg) of radioactive vit. B_{12}* containing $Co^{60(t.n.)}$ were added to 40-100 ml portions of media. After autoclaving, the enriched media were seeded with the selected organisms. At the end of the respective incubation periods listed in Table I the luxuriant growth which usually occurred was measured in terms of turbidity readings using a Klett spectrophotometer. Estimations of the number of viable and total microorganisms were performed by plate and direct counting methods respectively. The organisms were collected by centrifugation, washed twice with 0.85% saline solution and transferred into Kjeldahl flasks for digestion with nitric-sulfuric acid mixture. After complete destruction of the organic matter, the digests were transferred into beakers and the excess acid removed by heating. For measurement of radioactivity the inorganic residue was then transferred quantitatively to a planchet. As an alternative method, washed cells were placed directly in planchets. This latter method yielded results of essentially equal accuracy and since it is simpler, is the one of choice. In order to check the recovery of added radioactivity, it was necessary to estimate the vitamin remaining in the cell-free supernatant fluids. However, these fluids contained such a large amount of salt as to interfere with measurements of radioactivity. It was, therefore, essential to liberate the cobalt bound to the B_{12} molecule by autoclaving for 1-2 hr at 15 pounds pressure after addition of 3 to 4 ml of concentrated sulfuric acid in the presence of 0.5 ml of 0.1 N $CoCl_2$ carrier solution and 1 ml of 5% Na_2SO_3 solution. After cooling, the solutions were adjusted to pH 9-10 with 10 N NaOH solution and 1 ml of a 50% NaHS solution was added. The contents were heated over a steam bath for 15 minutes. The precipitates were collected and dried with acetone, and transferred quantitatively to planchets. To insure more complete recovery of Co^{60}, the supernatant fluids from the CoS precipitates were acidified to about pH 5-6 with 6 N HCl and heated on the steam bath until coagulation occurred. The resultant precipitates after centrifuging were washed with distilled water. Each was superimposed upon the planchet containing the previously separated CoS from the same

* The solution of radioactive vit. B_{12} kindly supplied by Merck and Company has a specific activity of 40 microcuries per mg of solid vitamin. Its microbiological activity was found to be identical with that of crystalline vit. B_{12} on an equal weight basis.

sample. Radioactivity was measured with a Cyclotron Specialties Counter equipped with a thin (3.1 mg/sq cm) mica end window counting tube.

Results and discussion. The results of determinations of uptake of radioactivity by different organisms are shown in Table I. They demonstrate that *L. leichmannii* which required vitamin B_{12} for growth took up radioactivity equivalent to about 25 cpm or about 4 mμg of the tagged vitamin in 45 ml vit. B_{12} media. On the other hand, *L. arabinosus,* for which an exogenous source of vit. B_{12} is not essential, took up negligible radioactivity. In the experiments with either organism, the radioactivity in the cell-free supernatant fluids and washings was also estimated. Our data showed that the total radioactivity in these fluids and in the intact cells accounted for virtually all of the radioactivity originally introduced into the system (90% for *L. leichmannii* and 85% for *L. arabinosus*).

In addition to these two cases, the uptakes of radioactive vit. B_{12} by 8 other strains are included in Table I. They demonstrate that 8 microorganisms could incorporate vit. B_{12} in the cells, even though they could grow equally well in the absence of this vitamin. It is worth noting that *L. arabinosus* was the only organism in our list which failed to show appreciable radioactivity in the cells.

The data on the last column of Table I give the approximate number of molecules of radioactive vit. B_{12} taken up by various growing organisms. These numbers varied from 10 to 60 molecules per organism, with the exception of *L. arabinosus,* whose uptake was essentially nil. The results of a typical experiment which illustrate the method of calculation showed that when 8 mμg of radioactive vit. B_{12} were added to 50 ml media, 3-4 mμg were found in the cells after 45 hours of incubation. The number of living cells in the media was estimated on a very small aliquot sample to be 1.7×10^{11} by plate count. Assuming the molecular weight of vit. B_{12} to be 1400(3), it was readily calculated that approximately 8 molecules of the vitamin were incorporated in each *L. leichmannii* organism. This is necessarily a maximal ratio since presumably radioactivity is a measure of vit. B_{12} in both dead and viable organisms while plate counts gave an estimate of living organisms only.

In order to ascertain whether the uptake of radioactivity by an organism such as *L. leichmannii* is due to vit. B_{12} *per se,* or to the formation of some cobalt complexes, the following experiments were performed. In one, comparable amounts of radioactive cobaltous chloride were used instead of radioactive vit. B_{12} in uptake experiments similar to those described above. Except where the strain under investigation absolutely required an exogenous source of vit. B_{12} (*e.g., L. leichmannii*) this series was carried out both with and without inclusion of non-radioactive vit. B_{12} in the media in amounts of 4-8 mμg. The data, except with *L. leichmannii,* demonstrate that the strains studied grew equally well regardless of the inclusion of B_{12} in the media and that in every case, including *L. leichmannii,* there was a negligible radioactivity in the washed cells even when the quantity of labeled $CoCl_2$ added was a several-fold excess over that offered as radioactive vit. B_{12} in the first series. These results, therefore, demonstrate that simple Co compounds are not retained in the cells and, inferentially, that the Co atom was not readily released from the vit. B_{12} molecule since there was no interchange between stable Co of the vit. B_{12} molecule and Co^{60} from the $Co^{60}Cl_2$.

In another experiment designed to explore further whether Co^{60} is likely to be present in the protoplasm as a part of the vit. B_{12} molecule, we disintegrated the bacterial cells (such as *L. leichmannii*) with an all glass homogenizer according to the procedure of Dochstader and Halvorsan(4). The homogenates, containing super-cell hyflo and cellular debris,

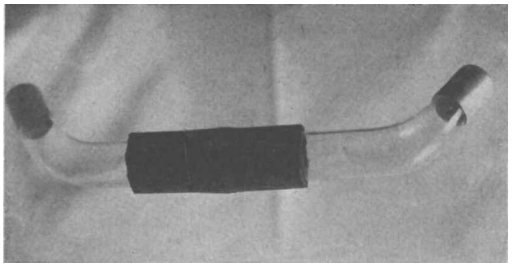

FIG. 1.

TABLE II. Determination of Radioactivity in *L. leichmannii* and a Second Microorganism Grown in a Medium Containing Radioactive Vit. B_{12}. (8 mμg equivalent to 100 c.p.m. added to each competitive system.)

	Total radioactivity in organisms (c.p.m.)	
	×*	*L. leichmannii*
L. arabinosus	3	34
Esch. coli	17	32
S. lactis R	28	26

* × = second organism.

were extracted with distilled water, and the aqueous extract analyzed for vit. B_{12} activity by the microbiological method of Skeggs and Wright(1). The results indicated that the vit. B_{12} measured microbiologically checked within 20% with that calculated from radioactivity due to Co^{60}.

Since the data obtained from the uptake experiments demonstrate that pure cultures of microorganisms incorporate radioactivity into their cells over a varied range when offered radioactive vit. B_{12}, it would be of interest to observe the fate of this tagged vitamin when introduced into a system of competitive organisms as is found in the intestinal tract. However, the physical separation of the (cultural) components of a mixed culture is, at present, a most difficult, if not impossible task. A partial solution of the problem of assaying individual species of bacteria for radioactivity while permitting all organisms access to media (containing Co^{60} labeled B_{12}) would be to grow such cultures in cellophane bags or in systems separated by cellophane membranes. In this way the organisms would be readily separable but the permeable barriers would allow change of low molecular components of the media such as vit. B_{12} as well as the metabolic products of the organisms under investigation. Fig. 1 is an illustration of the apparatus employed for this purpose. Table II summarizes the measurements of radioactive B_{12} uptake by organisms in the competitive systems, as estimated by the procedures outlined previously, and shows that *L. leichmannii* had taken up the same proportion of the added activity as when grown alone. Similar results of competitive systems of *L. leichmannii* against other bacterial organisms are also given in Table II.

Summary. A number of microorganisms were grown in a synthetic medium in which was incorporated radioactive vit. B_{12} tagged with Co^{60}. Among those studied, radioactivity was found in varying amounts in the cells of all species studied except *L. arabinosus*. The incorporation of the radioactivity was not related to the requirement of vit. B_{12} for growth of the bacterial cells. It was estimated that approximately 8 molecules of the tagged vitamin united with each *L. leichmannii* cell. Evidence is presented to show that the radioactivity in the cell is due to vit. B_{12} in that (1) cells grown in the presence of $Co^{60}Cl_2$ with or without addition of non-radioactive vit. B_{12} contained no radioactivity, and (2) the microbiological activity in homogenates of distintegrated cells paralleled that calculated from radioactivity due to Co^{60}. Measurements of uptakes of radioactive vit. B_{12} by two competing organisms (such as *L. leichmannii* and *E. coli*) separated by cellophane membranes readily permeable to this vitamin as well as other nutrients and metabolites demonstrate that radioactivity entered the cells of both organisms in essentially the same amounts as when each species was grown alone.

The authors wish to acknowledge grants-in-aid from Merck and Co., Upjohn Co., and Sharp and Dohme, Inc.

1. Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W., and Wright, L. D., *J. Biol. Chem.*, 1950, v184. 211.

2. Chaiet, L., Rosenblum, C., and Woodbury, D. T., *Science*, 1950, v111, 601.

3. Brink, N. G., Wolf, D. E., Kaczka, E., Rickes, E. L., Koniuszy, F. R., Wood, T. R., and Folkers, C., *J. Am. Chem. Soc.*, 1948, v71, 1854.

4. Dockstader, W. B., and Halvorson, H. O., *Science*, 1950, v112, 618.

Received December 27, 1951. P.S.E.B.M., 1952, v79.