acid-like compounds were tested for activity. Vitamin B_{10} and B_{11} concentrates which are prepared from the crude norite eluate apparently do not possess activity for the monkey but do contain compounds which are available for the chick. Crystalline vitamin B_c or the synthetic *Lactobacillus casei* factor can replace the norite eluate concentrate in the ration of the monkey each possessing about equal activity. Evidently the B_c conjugate is less active than either of these two compounds.

A deficiency in the monkey anti-anemia factor complicated the folic acid deficiency; similar conditions have been observed in riboflavin,[11] B_6 and pantothenic acid[42] deficiency in the monkey. This deficiency is characterized by a suboptimal hemoglobin level, lack of growth and a reversal in the neutrophile-lymphocyte ratio. In the normal monkey the lymphocytes constitute 60-70% of the total leucocytes and the neutrophiles 25-35%. However, in monkeys deficient in the monkey anti-anemia factor the ratio is reversed and the neutrophiles rise to 60-70% of the total leucocytes and the lymphocytes fall to 25-35% while the total leucocyte count is not appreciably reduced. There is, therefore, an actual increase in the number of neutrophiles and a decrease in the number of lymphocytes. Therapy with whole liver corrects this reversal, and produces a normal hemoglobin level and a resumption of growth.

The folic acid deficiency evidently precipitated a deficiency of this factor in the monkeys which had never received a ration containing whole liver. Thus in monkey 219 the low leucocyte count due to folic acid deficiency was corrected by vitamin B_c but whole liver was necessary to correct the neutrophile-lymphocyte ratio reversal, the low hemoglobin levels and the loss in weight. On the other hand monkey 218 was fed 3% whole liver powder in the ration for 3 months and consequently had an adequate store of this factor when the folic acid deficiency developed. This animal showed the best response to the various crystalline supplements since the folic acid deficiency was uncomplicated. Monkey 217, like monkey 219, showed a good leucocyte response to therapeutic doses of vitamin B_c. The weight response on the other hand was poor and blood studies showed that a dyscrasia existed typical of a deficiency of the monkey anti-anemia factor.

Summary. Vitamin B_{10} and B_{11} concentrates were inactive as a source of folic acid for the monkey.

Crystalline vitamin B_c and the synthetic *Lactobacillus casei* factor were active as a source of folic acid for the monkey when fed at a level of 100 γ per day. The B_c conjugate had less activity when fed at levels of 200 and 300 γ per day.

Folic acid deficiency precipitates a deficiency for the monkey anti-anemia factor which is characterized by lack of growth, suboptimal hemoglobin levels and a reversal in the lymphocyte-neutrophile ratio.

15237

A Differential, Microbiological Assay for O-Heterobiotin.*

T. D. LUCKEY, P. R. MOORE, AND C. A. ELVEHJEM.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison, Wis.

O-Heterobiotin (an analog of biotin in which the sulfur atom has been replaced by an oxygen atom) has biotin-like activity for *L. casei*,[1,2,3] *S. cerevisiæ*,[1,2,3] *R. trifolii*,[4] *L. arabinosus*,[3,5] chicks,[3] and for counteracting egg white toxicity in rats.[3,5] Hofmann *et al.* have developed an assay for O-heterobiotin with *S. cerevisiæ* after the biotin in the sample

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Wisconsin Alumni Research Foundation and Swift and Company.

We thank Merck and Co., Inc., Rahway, N.J.,

has been destroyed by permanganate oxidation.[4] In an attempt to develop a differential microbiological assay for O-heterobiotin we have tested its activity for representative microörganisms from several different genera. The results with *L. arabinosus* 17-5 and *S. faecalis* R are presented here.

Experimental. The general procedures used and the preparation of the basal medium constituents are similar to those used in the assay of folic acid.[6] A 24-hour-old inoculum of each microörganism is centrifuged and resuspended separately in sterile saline solution. One drop of each suspension is inoculated separately into duplicate sterilized tubes which contain 5 ml of basal medium (Table I), the sample, and distilled water to give a total volume of 10 ml. Standard curves with biotin and O-heterobiotin are run simultaneously with graded levels of the sample. The tubes are incubated at 30° for 16-20 hours and the growth is measured with the Evelyn photoelectric colorimeter.

Since the activity of O-heterobiotin for *L. arabinosus* may vary from one assay to another, a factor relating the activity of O-heterobiotin to that of biotin must be obtained for each assay. An average of 3-5 ratios obtained from different sections of the standard curves gives a reliable activity factor since the ratios are constant within each assay.

The biotin content of a sample may be determined directly from the standard biotin curve obtained with *S. faecalis*. The O-heterobiotin content may be calculated by multiplying the activity factor for O-heterobiotin by the difference between the apparent biotin content as measured with *L. arabinosus* and the biotin content obtained with *S. faecalis*. These calculations may be summarized:

O = k (Ba-Bt), where
O = O-heterobiotin content of the sample,
k = activity factor for O-heterobiotin compared to biotin with *L. arabinosus*, or

$$k = \frac{\text{amount of O-heterobiotin needed to produce x growth}}{\text{amount of biotin needed to produce x growth}}$$

Ba = apparent biotin content as determined with *L. arabinosus*, and
Bt = true biotin content obtained with *S. faecalis*.

The following procedure is used to liberate bound biotin: Add one gram of sample to 25 ml of $2N/H_2SO_4$, autoclave at 122° for 2 hours, neutralize with $4N/NaK(OH)_2$ (a mixture of sodium and potassium hydroxide is used to keep a balance of these ions in the medium), filter and dilute as needed. When a

TABLE I.
Medium for O-Heterobiotin Determination.

Constituent	Amt for 10 tubes (100 cc of complete medium or 50 cc basal)
Casein	0.5 g
Tryptophane	0.02
Cystine	0.01
Glucose	1.0
Na acetate	0.4
K_2HPO_4	0.5
Speakman's salts B	0.5 cc
Adenine · SO_4	1.0 mg
Guanine · HCl	1.0
Xanthine	1.0
Uracil	1.0
Thiamin · HCl	50 γ
Riboflavin	50
Nicotinic acid	80
Pyridoxine · HCl	50
Pyridoxal · HCl	0.2
α-Pyracin*	0.2
Biotin	00.0
Synthetic folic acid†	0.2
Ca pantothenate	50
p-Aminobenzoic acid	10
Water	50 cc
pH	6.8

* 5-Pyridoxic acid, or the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine are other names for this compound obtained from Merck and Co. through the courtesy of Dr. Karl Folkers.

† Obtained through the courtesy of Dr. B. L. Hutchings at Lederle Laboratories, Inc.

for the crystalline vitamins and Dr. S. H. Rubin and Dr. J. A. Aeschlimann of Hoffmann-LaRoche, Inc., Nutley, N.J., for the O-heterobiotin.

1 Duschinsky, R., Dolan, L. A., Flower, D., and Rubin, S. H., *Arch. Biochem.*, 1945, **6**, 480.

2 Pilgrim, F. J., Axelrod, A. E., Winnick, T., and Hofmann, K., *Science*, 1945, **102**, 35.

3 Hofmann, K., McCoy, R. H., Felton, J. R., Axelrod, A. E., and Pilgrim, F. J., *Arch. Biochem.*, 1945, **7**, 393.

4 Hofmann, K., and Winnick, T., *J. Biol. Chem.*, 1945, **160**, 449.

5 Rubin, S. H., Flower, D., Rosen, F., and Drekter, L., *Arch. Biochem.*, 1945, **8**, 79.

6 Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

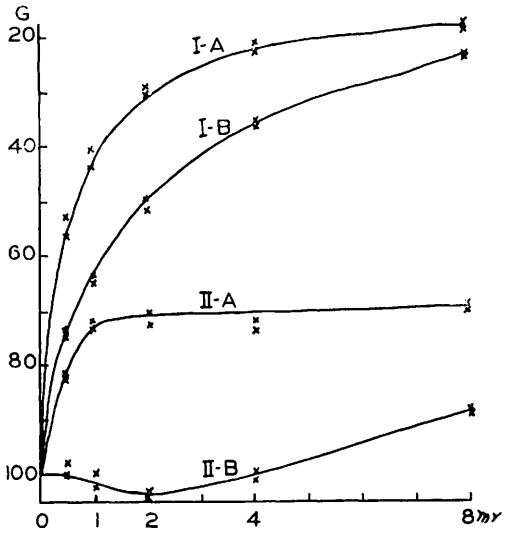

FIG. 1.

Standard curves with biotin (A) and O-heterobiotin (B) obtained with *L. arabinosus* (I) and *S. faecalis* (II).

* G = Galvanometer reading with inoculated blank set at 100.

TABLE II.

Effect of pH on the Activity of *d,l*-O-Heterobiotin.

pH	% activity compared to *d*-biotin		Tubes used for each value
	*S.f.**	*L.a.*†	
6.6	0.0	33	20
6.8	1.7	44	40
7.0	2.5	39	40
7.3	7.5	29	20

* *S.f.* = *Streptococcus faecalis.*
† *L.a.* = *Lactobacillus arabinosus.*

small amount of O-heterobiotin was hydrolyzed its activity for *L. arabinosus* decreased slightly.

Throughout this work *d*-biotin has been compared to *d,l*-O-heterobiotin. If only the *d*-form of O-heterobiotin is active, the values given here should be increased correspondingly.

Results. A typical set of curves for the assay of O-heterobiotin (Fig. 1) indicates that its activity relative to biotin is constant throughout the curves obtained with *L. arabinosus*. Low levels of O-heterobiotin are somewhat inhibitory for *S. faecalis* while higher levels are about 2% active. The activity of O-heterobiotin for *S. faecalis* may be disregarded since any amount of O-heterobiotin which will produce appreciable growth for this organism (more than 8mγ) gives maximum growth for *L. arabinosus*.

In an attempt to find why the activity factor for O-heterobiotin varied with different assays, standard curves were run at various pH levels. The results (Table II) indicate that the activity of O-heterobiotin relative to biotin for *S. faecalis* increases as the pH is increased from 6.6 to 7.3, while the activity of O-heterobiotin relative to biotin for *L. arabinosus* is greatest at pH 6.8. Thus, pH 6.8, which was originally used, gives the greatest differentiation.

In order to test the effect of biotin upon the activity of O-heterobiotin graded levels of O-heterobiotin were added to tubes containing a known amount of biotin. The amount of O-heterobiotin found corresponded well with the amount added (Table III). The amount of biotin found in tubes containing large amounts of O-heterobiotin was greater than the amount of biotin added. This may be due to an increased sensitivity in both bacteria for O-heterobiotin in the presence of biotin.

When certain natural materials were analyzed for O-heterobiotin the results (Table IV) indicated that if this compound occurs naturally its extent is limited. When O-heterobiotin was added to fresh kidney, after acid hydrolysis, the biotin content as measured with *S. faecalis* was identical to the value obtained for kidney without the added O-heterobiotin. The apparent biotin content, as measured with *L. arabinosus* was higher. When the values obtained were applied in the above formula, the recovery of O-heterobiotin was very satisfactory (Table IV, No. 8).

Discussion. The name O-heterobiotin is used in this paper in conformity with the first common name to appear in the literature[1] rather than the later name oxybiotin[2] since the latter does not definitely distinguish between this compound and such compounds as biotin sulfoxide and biotin sulfone.

Since O-heterobiotin does not seem to occur naturally, biotin values previously obtained are probably not invalidated by this new compound. However, there does seem to exist naturally a compound in chicken muscle and fresh lamb kidney which has more biotin

TABLE III.
Recovery of O-Heterobiotin.

Biotin per tube, mγ	O-heterobiotin per tube, mγ	Biotin found,* mγ	O-heterobiotin found,† mγ	% recovery of O-heterobiotin
0.5	0.5	0.5	0.48	96
0.5	1	0.6	1.14	114
0.5	1	0.6	0.96	96
0.5	2	0.75	2.10	105
0.5	4	0.75	4.35	109

* *S. faecalis* value.
† (*L.a.* value—*S.f.* value) × factor = O-heterobiotin.

TABLE IV.
O-Heterobiotin Content of Naturally Occurring Materials.

Material	γ "Biotin"/g		γ O-heterobiotin/g
	S. faecalis	*L. arabinosus*	
1. Fresh beef liver	1.2	1.1	0.0
2. Fresh chick liver	0.90	0.88	0.0
3. Fresh chick leg muscle	0.15	0.02	0.0
4. Difco yeast extract	3.3	3.3	0.0
5. Grass juice powder	0.40	0.39	0.0
6. Vitab	1.0	0.93	0.0
7. Fresh lamb kidney	0.90	0.66	0.0
8. Fresh lamb kidney + 1 γ O-heterobiotin	0.90	1.61	1.0

activity for *S. faecalis* than for *L. arabinosus*. When O-heterobiotin is fed or injected into animals, it might be expected to occur to some extent in the tissues.

Summary. A differential microbiological assay for O-heterobiotin with *S. faecalis* R and *L. arabinosus* 17-5 has been developed. Assays of several natural materials indicated that O-heterobiotin does not occur generally, but that a metabolite does exist in animal tissues which has biotin activity for *S. faecalis* and not for *L. arabinosus*.