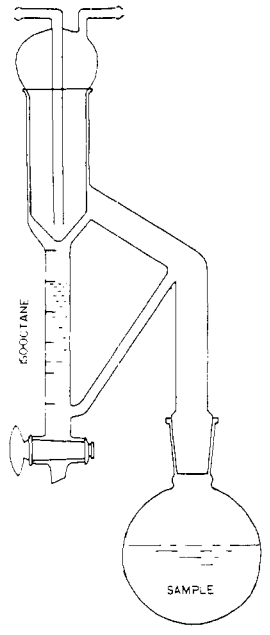

Figure 1. Apparatus for recovery of EPTC-S^{35} residue in crops

procedure to be satisfactory in amounts ranging from 10 to 400 γ. The precision of the method on the recovery of EPTC is approximately 99 $\pm$ 3%. The lower limit of detection is 0.5 γ when a thin mica end window Geiger-Müller counter is employed. When a gas flow windowless counter is used for the detection of radioactivity, the sensitivity is increased to 0.2 γ in the sample tested. The recommended 1-hour boiling time is generally sufficient to remove all EPTC-S^{35} residue from crops, as indicated by a study of continuous extraction of the distillate with an hourly change of iso-octane. No radioactivity is found in the iso-octane solution after the first hour.

Twenty-three different samples of various crops grown in EPTC-S^{35} treated soil were analyzed for free EPTC (Table II). Examination of the radioactivities of the water extract and of the plant tissue after complete removal of the volatile EPTC-S^{35} by boiling, revealed that approximately 80 to 90% of the absorbed radioactivity remains in the water solution. The largest amount of free EPTC-S^{35} in the tissues was less than 3% of the total amount absorbed. This suggests that EPTC is rapidly metabolized by plants and therefore should present little residue problem for the crops tested.

Table II. EPTC-S^{35} Residue in Plant Tissues from Crops Which Received Various Rates of Pre-emergence Treatment

Crops	Plant Part	Fresh Weight, Grams	EPTC-S^{35} Application, Lb./Acre	Treatment to Harvest, Days	Free EPTC-S^{35} Residue, γ	Concentration of EPTC-S^{35} in Plants, P.P.M.
Kidney bean	Whole plant	7.5	4	14	2.40	0.32
	Whole plant	30	4	38	1.97	0.066
	Flowers, buds	5	4	38	0	0
	Root	5	4	38	1.98	0.396
	Leaves, stem	23	4	38	0	0
	Pods	5[a]	1	66	0	0
Corn	Whole plant	7.5	4	14	1.20	0.16
	Leaves	30	4	38	0	0
	Ear	35	1	114	0	0
	Roots	1.2[a]	4	38	0.34	...
Pea	Whole plant	5.0	4	14	0.92	0.184
	Leaves, stem	0.68[a]	4	38	0.57	...
	Whole plant	10	4	38	2.47	0.247
Radish	Whole plant	5.9	4	54	0.45	0.076
	Leaves	21.6	4	55	0.51	0.024
	Roots	5.5	4	55	0	0
Carrot	Whole plant	1.5[a]	4	67	0.94	...
	Root	3.5	1	83	0	0
Beet, table	Leaves	3.3	4	38	0.26	0.079
	Whole plant	10	4	64	0.42	0.042
Sugar beets	Whole plant	5	4	64	0.66	0.132
Cabbage	Leaves	10	1	83	0	0
Mustard	Leaves	10	1	83	0	0

[a] Dry weight.

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VITAMIN B_{12} IN FOODSTUFFS

Comparative Vitamin B_{12} Assay of Foods of Animal Origin by *Lactobacillus leichmannii* and *Ochromonas malhamensis*

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OF THE MICROBIOLOGICAL METHODS available for the determination of vitamin B_{12}, two appear to have special merit, the *Lactobacillus leichmannii* (*9*) and the *Ochromonas malhamensis* (*1, 5*) methods. The *L. leichmannii* assay has, in several modifications, been more widely accepted because of its rapidity, uniformity of response, and precision of results. Comparative data on the vitamin B_{12} content of foods of animal origin as assayed by the *L. leichmannii* and *O. malhamensis* methods are presented.

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Experimental Procedure

To provide comparative data, a number of food products were selected so as to comprise a cross-sectional survey of the various classes of foods of animal origin in customary use. These were assayed by both the *L. leichmannii* and *O. malhamensis* methods. Food products with a fermentation history and organ

Few directly comparative data on the vitamin B_{12} content of foods, as assayed by the *Lactobacillus leichmannii* and *Ochromonas malhamensis* methods, are available as a basis for judging the relative validity of the two methods. This report presents the results of parallel assays using the two methods on a series of foods of animal origin. While results of the assays were generally of the same order of magnitude in 15 of the 27 samples tested, the vitamin B_{12} activity as measured by the O. *malhamensis* method was significantly higher at the 5% level than when measured by the *L. leichmannii* method. These results, unexpected in view of the reported greater specificity for vitamin B_{12} of O. *malhamensis* as compared to *L. leichmannii*, may be due to the presence in certain foods of animal origin of substances other than vitamin B_{12} which stimulate the growth of O. *malhamensis*.

meats were included, particularly because of the possibility that their extracts might contain nonspecific material analogous to vitamin B_{12} (*3*, *7*, *12*) or of deoxyriboside nature (*6*, *14*, *17*, *18*). Both of the latter groups of substances could contribute to erroneously high potency values on the foods containing them when assayed with the less specific *L. leichmannii* method; and should thus be sensitive indicators of the reliability of that method.

Attention was focused on animal materials to the exclusion of food products of higher plant origin because the evidence available from the literature (*13*, *15*, *16*), as well as from unpublished work in this laboratory support the view that higher plants, except for occasional chance absorption, are devoid of vitamin B_{12}.

The samples assayed were purchased in the local Washington, D. C., market, with the exception of lamb, pork loin, chicken liver, and chicken gizzard, which were obtained from the Agricultural Research Center, Beltsville, Md., and the dried egg, which was purchased directly from the processor. The pork loin sample and the lamb samples were each taken from one cut from a single animal. Except for the yogurt, and the dried skim milk samples, which were obtained in two lots, the market samples were purchased in three or four lots of different brands or from different suppliers, and were composited on an equal weight basis. Chicken liver and gizzard samples were obtained in 10 or more lots, and were composited without regard to weight. The dried egg sample was obtained in a single lot. Meat samples were trimmed of visible fat, cubed, stored at −40° C. until needed, then ground and blended. Liquid samples were stored at −40° C. until needed, then they were blended. Dried samples were mixed by tumbling; dried egg was refrigerated at −5° C. until needed for assay, whereas dried milk was kept at room temperature. At least two subsamples were taken from each sample, and each subsample was assayed for vitamin B_{12} on a different date. Moisture determinations by AOAC me hod were run parallel to the vitamin B_{12} assays.

Extracts for use with both assay methods were, except for dried egg, prepared by the AOAC extraction procedure (*9*) based on autoclaving for 10 minutes in the presence of a citrate-phosphate buffer at pH 4.5 and which contained 1% of sodium metabisulfite. The concentration of the latter was reduced, when necessary, not to exceed 0.025 mg. per assay tube. This limit is lower than that specified in the 1954 modification of the method (*8*) as bisulfite concentrations in excess of this were inhibitory to *O. malhamensis*. Dried egg was autoclaved for 30 minutes. Tests in the authors' laboratory had shown that shorter treatments resulted in the release of lesser quantities of vitamin B_{12}. In the case of more than half of the samples, both the *L. leichmannii* and *O. malhamensis* assays were run on identical extracts. Examination of the analytical data showed that assay variance was not significantly different for determinations made on separate and on identical extracts.

The *L. leichmannii* procedure used was basically the 1955 modification of the AOAC method (*9*). The vitamin B_{12} standard was given the same extraction treatment as the samples. The concentration of inoculum was adjusted in accordance with data of a previous publication from this laboratory (*10*).

The *O. malhamensis* procedure used was Ford's (*5*) but with some modifications. Maintenance cultures were incubated at 28–29° C., one foot from a 15-watt fluorescent lamp, and were transferred at 5-day intervals. The 5× basal medium for assay was adjusted to pH 4.5. For inoculum, a maintenance culture, diluted to a transmittance of approximately 70% on the Beckman Model B spectrophotometer, was administered to the assay tubes dropwise (approximately 35,000 cells per drop). The tubes were not shaken before or during incubation. In order to provide adequate aerobic conditions with stationary incubation, 25 × 50 mm. aluminum capped vials were used as assay tubes. Incubation of the assay tubes was carried out at 28 to 29° C. for 5 to 6 days. Under these conditions, the assay range was 0.02 to 0.10 milligamma of vitamin B_{12}, identical with that of the *L. leichmannii* method.

The following criterion of acceptability was applied to the data of both methods. In the evaluation of individual tube potencies for the calculation of mean potency values of single assays, all values including extrapolated ones were accepted when they fell within 10% of the provisional means. If fewer than two thirds of the individual tube values fell within the 10% limits, the data were considered insufficient for calculation of the sample potency.

Bacto liver was included as a standard sample in each assay run.

The degree of recovery of vitamin B_{12} from an admixture with a crude material, similar in nature to the foods tested, was determined by assaying identical extracts of mixtures in the proportions of 1 to 1 and 3 to 1 of vitamin B_{12} and Bacto liver by both the *L. leichmannii* and *O. malhamensis* methods.

Results and Discussion

The tabulated data (Table I) indicate general agreement between the vitamin B_{12} potency values obtained by the *L. leichmannii* and *O. malhamensis* methods. However, analysis of the data showed that some of the differences were statistically significant. Analyses of variance were done on the logarithms of the vitamin B_{12} potency data for each method. Logarithmic transformation was necessary in order to obtain homogeneity of variance. A *t*-test revealed that the over-all means of the two methods were significantly different at the 5% level. The sums of within groups squares for each method were pooled and used for *t* tests on individual samples. Where the *t*-tests indicated significantly divergent mean values, the mean *O. malhamensis* values were generally higher than the *L. leichmannii* values. The *O. malhamensis* values were significantly higher than

those of the *L. leichmannii* method with 15 of the 27 samples tested, the mean differences being 13% or more. Only one sample, yogurt, gave a significantly higher value in the *L. leichmannii* than in the *O. malhamensis* assay, the difference being 26%.

In six of 11 samples containing bacterial growth products, differences between the mean *O. malhamensis* and *L. leichmannii* values were significant. In five of the six, the *O. malhamensis* values exceeded the *L. leichmannii* values by 13% or more, and in one, yogurt, the reverse was the case. Such data indicate that even those foods which were exposed to bacterial activity prior to assay contained, in most cases, insufficient quantities of nonspecific activity to seriously interfere with the *L. leichmannii* method. Similarly, data for the organ meats indicate that they contained no significant quantities of nonspecific activity for *L. leichmannii*.

Upper and lower 95% confidence limits were computed for each mean. For ease of comparison the per cent deviation of each limit from its mean was calculated. The means of these percentage deviations were +7.3 and −7.0 for determinations by the *L. leichmannii* method and +8.8 and −8.1 for those by the *O. malhamensis* method.

On the Bacto liver standard samples, assays by the *L. leichmannii* (27 samples) and *O. malhamensis* (24 samples) methods yielded mean values of 77.3 and 80.8 and standard errors of 0.976 and 1.64 γ per 100 grams, respectively.

In duplicate tests on mixtures of vitamin B_{12} with Bacto liver, added vitamin B_{12} was recovered from 1 to 1 mixtures to the extent of 95.2 and 93.5% by the *L. leichmannii* and *O. malhamensis* methods, respectively. Similar tests on 3 to 1 mixtures resulted in recoveries of 96.9 and 94.7%, respectively.

The experience in this laboratory of obtaining higher potency values on many of the food samples by the *O. malhamensis* method than by the *L. leichmannii* is similar to that reported by Williams *et al.* (*19*). The *O. malhamensis* method, in their hands, yielded higher values than did the *L. leichmannii* method on approximately half of the 34 feed supplement samples assayed by both methods. The divergence in values between the two methods could be the result of stimulation of the growth of *O. malhamensis* by nonspecific substances of unknown nature in the food samples. Such stimulation has been reported to occur in serum, spinal fluid, and spleen (*2, 5, 11*).

With other vitamins, biological methods have been available as standards of reference, in comparison with which it is possible to evaluate the accuracy of microbiological methods. This does not hold true for vitamin B_{12}. The chick and rat assays, which have been tested in other laboratories, tend to overestimate vitamin B_{12} in crude materials containing unidentified growth factors, and are thus unsuitable for that purpose (*4*). In the absence of a standard of reference, it is not feasible to determine which of the two methods, *L. leichmannii* or *O. malhamensis*, is capable of yielding more biologically accurate vitamin B_{12} potency values. The data indicate that while, in general, both the *L. leichmannii* and *O. malhamensis* assay

Table I. Vitamin B_{12} Potencies of Selected Foods as Determined by *L. leichmannii* and *O. malhamensis*

		Vitamin B_{12}, γ/100 Grams						
		L. leichmannii			*O. malhamensis*			
		Moist Basis			Moist Basis			
Food	Moisture, %	Mean	Standard error of mean	Dry basis mean[a]	Mean	Standard error of mean	Dry basis mean[a]	Ratio O. m. / L. l.
Dairy Products								
Buttermilk, cultured[b]	89.4	0.198[c]	0.00458	1.87	0.240	0.0135	2.26	1.21
Cheese:								
Cheddar[b]	33.8	0.990	0.00600	1.50	1.17	0.0245	1.77	1.18
Limburger	49.1	1.04	0.0245	2.04	1.01	0.0640	1.98	0.97
Roquefort[b]	39.1	0.587	0.0285	0.964	0.664	0.00748	1.09	1.13
Swiss	36.5	1.65	0.0300	2.60	1.71	0.0548	2.69	1.04
Milk, skim, dried[b]	1.2	3.76	0.0245	3.81	4.92	0.135	4.98	1.31
Yogurt[b]	86.2	0.0809	0.00595	0.586	0.0601	0.000600	0.436	0.74
Meats								
Beef:								
Heart[b]	76.6	11.3[c]	0.361	48.3	14.2	0.600	60.7	1.26
Liver	68.1	116.[c]	3.85	364.	122.	5.00	382.	1.05
Round steak	72.6	1.93	0.0447	7.04	2.06[c]	0.0321	7.52	1.07
Thymus	80.5	2.13	0.0346	10.9	2.30	0.115	11.8	1.08
Lamb:								
Leg (6–8 mos.)[b]	72.3	1.40	0.0245	5.05	1.63	0.000	5.88	1.16
Leg (14–24 mos.)[b]	70.4	1.78	0.0900	6.01	2.04	0.165	6.89	1.15
Leg (6–7 yrs.)[b]	71.6	2.34	0.0300	8.24	3.10	0.0949	10.9	1.32
Lebanon bologna	50.3	1.97	0.120	3.96	1.90	0.185	3.82	0.96
Pork:								
Ham, cured[b]	63.1	0.586[c]	0.0195	1.59	0.797	0.00548	2.16	1.36
Loin[b]	70.6	0.555	0.0160	1.89	0.699	0.0165	2.38	1.26
Poultry Products								
Chicken:								
Breast[b]	74.3	0.415	0.00300	1.61	0.498	0.0415	1.94	1.20
Egg, whole, dried[b]	1.8	10.0	0.300	10.2	12.1	0.346	12.3	1.21
Gizzard[b]	78.6	2.31	0.0141	10.8	3.19[c]	0.0493	14.9	1.38
Liver	71.3	22.3	1.20	77.7	24.1	0.245	84.0	1.08
Thigh[b]	74.6	0.359	0.0240	1.41	0.473	0.00500	1.86	1.32
Seafood								
Crab, cooked[b]	74.7	7.30	0.0849	28.9	8.52	0.470	33.7	1.17
Oyster:								
Solids	89.4	21.4	0.100	202.	21.2	0.400	200.	0.99
Liquid	96.6	2.46	0.155	72.4	2.44	0.140	71.8	0.99
Salmon:								
Canned, solids	69.2	6.89	0.230	22.4	7.46	0.0141	24.2	1.08
Canned, liquid	86.8	4.08	0.0748	30.9	4.28	0.0949	32.4	1.05

[a] Ratio of standard error of mean to the mean is the same for both moist and dry basis.
[b] Mean values obtained by *L. leichmannii* and *O. malhamensis* method are significantly different at 5% level.
[c] Three replicates, all others two replicates.

procedures yielded values of the same order of magnitude, by statistical criteria, more than half of these were significantly higher by the *O. malhamensis* method than by the *L. leichmannii* method. The *L. leichmannii* method is better suited to routine practice. The data do not preclude the possibility that some hitherto untested type of animal food product might contain sufficient nonspecific active material to invalidate its *L. leichmannii* potency value. In view of this possibility it would be wise to assay by both methods products not previously so tested.

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FREEZE-PROCESSING EFFECTS ON RICE

Effect of Freeze-Processing on Amyloclastic Susceptibility, Crystallinity, and Hydration Characteristics of Rice

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This study was initiated to determine if starch retrogradation accompanied freeze-processing of rice and to obtain additional information on changes occurring in rice during processing. Freeze-processing resulted in development of a *B*-type x-ray diffraction pattern, and a lowered β-amylase susceptibility. This resistance was almost completely neutralized by heating an aqueous suspension 45 minutes at 45° C. or higher. The changes in x-ray pattern and amyloclastic susceptibility were similar to those associated with retrogradation. The water-holding capacity of whole-grain freeze-processed rice was much greater than that of either whole-grain raw or cooked unfrozen controls. Where the effects of gross physical structure were minimized by grinding, raw rice (at 80° C. and above) had the greatest water absorption. These studies demonstrated a physical as well as a chemical effect due to freezing.

When cooked rice was frozen under defined conditions and then air-dried, it exhibited an exceptionally rapid rate of water uptake, even at room temperature (*11*). It became chalky, took on a characteristic porous or spongelike texture, and had a greater specific volume than either a raw or an unfrozen control—i.e., one that had been cooked and then dried. It was postulated that this starch, spongelike formation was due to either retrogradation or a closely related mechanism.

Previous investigators (*10*, *13*) reported that measurement of the suceptibility of starch to attack by amylases was valuable in following the effects of physical treatment and "aging" on starch. They concluded that the amyloclastic susceptibility diminished as a result of retrogradation. Katz and van Itallie (*8*) reported that retrograded starch was in a semicrystalline state that could be characterized by a *B*-type x-ray pattern.

The present study was initiated not only to clarify whether retrogradation took place during the freeze-processing of rice, but also to obtain additional information on the changes in rice starch during processing. The measurement of amyloclastic susceptibility and the determination of the degree of crystallinity by x-ray appeared to be the most generally accepted criteria for the detection of retrogradation. However, because hydration characteristics have been used (*6*) to differentiate varieties of rice, it was anticipated that they also would be of value in differentiating rice processed by various methods. Therefore, to demonstrate possible retrogradation and differences in the properties of rice due to processing, the amyloclastic susceptibility, x-ray diffraction pattern, and hydration characteristics of freeze-processed rice were compared to those of an unfrozen control and to raw rice.