Aspergillus (usually *A. fumigatus*)[4-7] and by *Penicillium*.[8,9] Unfortunately little is reported of the bacterial spectra or other characteristics of these agents. However, "aspergillin"[7] may resemble the antismegmatis factor in that preparations inhibiting *M. phlei* at dilutions of 1:50 to 1:100 and *M. tuberculosis* at 1:10 to 1:20 failed to inhibit 2 strains of *Staphylococci*. Both "aspergillin" and "mycoidin"[6] are thermostable, as is the antismegmatis factor. Such *Mycobacterium*-inhibiting agents as streptothricin and streptomycin are clearly differentiated from the antismegmatis factor because they inhibit *Escherchia coli* and *Staphylococcus aureus* to the same degree they inhibit *Mycobacteria*.[10]

Of the older antibacterial agents, the most specific against *Mycobacteria* are the chaulmoogrates.[11] Hesse and Meisser[12] report that activity of several heterocyclic organic compounds against *M. tuberculosis* is correlated with their basic nature. The marked increase in activity of the antismegmatis factor with increasing pH, a property suggestive of a basic compound (see studies on streptothricin and streptomycin[13]) is of interest in this connection.

Although the antismegmatis factor failed to inhibit one strain of pathogenic *M. tuberculosis*, variety *bovis*, the fact that *M. tuberculosis* variety *hominis* No. 607 was inhibited, suggests that pathogenic strains of *M. tuberculosis* may be sensitive to preparations of the antismegmatis factor more concentrated than the crude culture filtrates we used and that there may be variations in sensitivity of strains within the species.

Summary. An actinomycete (A-82) forms an antibiotic which when tested against more than 60 species, including more than 20 genera of bacteria inhibited only *M. smegmatis*, *M. phlei*, and a nonpathogenic, rapidly growing strain of *M. tuberculosis*, variety *hominis* No. 607. A pathogenic *M. tuberculosis*, variety *bovis*, was not inhibited. The specificity of this antibiotic, referred to as the antismegmatis factor (because of the sensitivity of *Mycobacterium smegmatis*), for the genus *Mycobacterium* is discussed. Methods for producing the crude antismegmatis factor are outlined. The antibiotic is thermostable, and is more active in alkaline media than in acid media. No chemical purification of the antismegmatis factor was done.

4 Asheshov, I. N., and Strelitz, F., *Science*, 1945, **101**, 119.

5 Kallos, P., *Nature*, 1945, **155**, 300.

6 Gerber, I. E., and Gross, M., *Science*, 1946, **103**, 167.

7 Soltys, M. A., *Nature*, 1944, **154**, 550.

8 Miller, D. K., and Rekate, A. C., *Science*, 1944, **100**, 172.

9 Smith, M. I., and Emmart, E. W., *Pub. Health Rep.*, 1944, **59**, 417.

10 Schatz, A., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 244.

11 Walker, E. L., and Sweeney, M. A., *J. Inf. Dis.*, 1920, **26**, 238.

12 Hesse, E., and Meissner, G., *Arch. Exp. Path. u. Pharmakol.*, 1931, **159**, 676.

13 Waksman, S. A., *Microbial Antagonisms and Antibiotic Substances*, 1945, The Commonwealth Fund, N.Y.

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The Biological Activity of Calciferol (Vitamin D_2).*

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The international unit of vitamin D is the activity of 1 mg of the international Standard of Reference which, in turn, is defined in terms of a solution of irradiated ergosterol in olive oil. Collaborative study[1] in 1940 showed

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1 Coward, K. H., *Bull. Health Org., League of Nations*, 1940-1, **9**, 425.

that the international unit represents the activity of 0.025 μg of pure crystalline calciferol, *i.e.*, 1 μg is equivalent to 40 international units. The U.S.P. unit of vitamin D, by definition,[2,3] is equivalent to an international unit. However, recently we have observed that calciferol† (vitamin D_2) exhibits greater activity than 40 U.S.P. units per μg when compared against the U.S.P. reference standard. This appears to be evidence that the present U.S.P. reference cod liver oil (No. 2) contains less vitamin D than would be expected from the original assay values.[4] If this observation is confirmed generally the activity of the U.S.P. standard should be reconsidered. There is considerable importance attached, therefore, to examining the data regarding the standard currently in use.

Experimental. Samples. The samples of calciferol tested were from current commercial production and were of a purity comparable to that described in an earlier publication from these laboratories.[5] The physical constants used as a check on the purity of the synthesized vitamin were melting point, specific rotation, and specific absorption.

For the tests, 20 to 30 mg of the samples were dissolved in corn oil and diluted so as to supply in 6 days approximately 5.0, 7.5, 8.0, or 8.65 U.S.P. units of vitamin D per rat.

Method of testing. The diluted calciferol samples were assayed by the U.S.P. XII procedure.[3] The test animals were from our own stock colony so that the healing response to the standard or test doses was moderately uniform. Groups of 8 to 10 animals were fed the vitamin D supplements for 6 successive days and were killed on the 8th day in accordance with the provisions of the U.S.P. method. Rats that did not gain weight properly were discarded.

The U.S.P. reference cod liver oil is fed routinely at 2 levels in our laboratory, while the test materials are usually fed at one level. Such tests lend themselves best to graphic analysis which method has been used for the potency estimations of single-level assays reported in this study. For tests which are designed to supply an estimate of potency along with an estimate of the standard error it is often preferred to test the unknown material at 2 levels. Under these conditions, any of several methods may be used to calculate the standard error of the determination, the one by Miller, Bliss and Braun[6] being used here. Potency estimates were calculated by the method of Bliss and Marks.[7]

Results. The results of the 1- and 2-level assays of the test calciferol samples are given in Table I.

It will be observed (Col. 8) that the calciferol samples were diluted on the assumption, based on past experience, that they would supply 49 U.S.P. units per μg; this assumption has no effect on the outcome of the test, of course, but must be mentioned to clarify the procedure used.

It will be seen that the single-level assays uniformly indicated slightly over 50 units per μg. The 2-level assays, Nos. 470 and 471, show potencies very close to 50 units per μg for all lots except Lot 60; the value for this sample by the 2-level assay (No. 471) was 46.5 ± 4.7 units per μg which is not significantly different from the value of 50.7 units per μg (Assay No. 458) obtained by the single-level assay on the sample.

Discussion. The data presented here leave no doubt as to the consistency with which these calciferol samples have shown more than 40 U.S.P. units per μg. The weighted mean of the 8 results of the 2-level assays is 49.7 with a weighted average standard error of $\pm$ 2.1 U.S.P. units per μg (calculated by the method of Miller, Bliss, and Braun.[6])

[2] Nelson, E. M., *The Vitamins*, A symposium, Chicago, 1939, 475-81.

[3] *U. S. Pharmacopoeia* XII, Easton, 1942, 635.

† Crystalline vitamin D_2 is most conveniently termed calciferol to distinguish it from Viosterol which represents irradiated ergosterol. The latter mixture contains vitamin D_2, unchanged ergosterol, and under—and over—irradiation products.

[4] U.S.P. Vitamin Advisory Board, *J. Am. Pharm. Assn.*, 1942, **31**, 124.

[5] Huber, W., and Barlow, O. W., *J. Biol. Chem.*, 1943, **149**, 125.

[6] Miller, L. C., Bliss, C. I., and Braun, H. A., *J. Am. Pharm. Assn.*, 1939, **28**, 644.

[7] Bliss, C. I., and Marks, H. P., *Quart. J. Pharm. and Pharmacol.*, 1939, **12**, 182.

TABLE I.

Biological Activity of 8 Lots of Calciferol Tested at One- and Two-levels of Assay at 6 Different Times.

U.S.P. reference oil					Calciferol				
			Healing score				Healing score		Potency* U.S.P. units per μg
Test No.	No. of rats	Dose per rat U.S.P. units	Range	Mean	Lot No.	No. of rats	Dose per rat U.S.P. units	Range	Mean	
457	7	5.0†	1.5-1.75	1.64	59	9	8.65‡	1.75-3.0	2.28	51.6
	8	8.65	1.75-3.0	2.22	62	9	8.65	1.75-3.0	2.31	53.7
458	9	5.0	1.5-1.75	1.67	60	9	8.65	1.75-3.0	2.22	50.7
	9	8.65	1.75-3.0	2.19						
459	8	5.0	1.0-2.0	1.66	61	8	8.65	1.50-3.0	2.43	50.7
	7	8.65	1.75-3.0	2.39	63	7	8.65	1.75-3.0	2.43	50.6
					64	8	8.65	1.75-3.0	2.43	50.6
470	9	5.0	1.5-1.75	1.58	94	8	5.0	1.0-1.75	1.61	50.9 ± 5.9
	10	7.5	1.5-2.0	1.80		9	7.5	1.5-2.0	1.80	
					59	7	5.0	1.5-1.75	1.71	50.7 ± 5.4
						9	7.5	1.75-2.75	2.05	
					60	5	5.0	1.50-1.75	1.65	46.5 ± 4.7
						8	7.5	1.75-2.5	1.97	
					61	8	5.0	1.50-2.0	1.72	49.3 ± 5.9
471	6	5.0	1.5-1.75	1.67		8	7.5	1.75-2.75	2.00	
	6	7.5	1.75-2.5	2.04	62	5	5.0	1.50-2.0	1.75	51.4 ± 6.8
						7	7.5	1.75-2.75	2.04	
					63	7	5.0	1.50-1.75	1.71	51.1 ± 6.5
						7	7.5	1.50-2.75	2.07	
					64	5	5.0	1.50-1.75	1.65	49.6 ± 5.9
						5	7.5	1.75-2.75	2.08	
476	8	5.0	(all 1.75)	1.75	103	8	5.0	(all 1.75)	1.75	49.4 ± 7.8
	8	8.0	1.75-2.5	1.97		8	8.0	1.75-2.5	1.98	
					Mean (2-level assays)					49.7 ± 2.1

* For the estimation of potency of the calciferol samples tested at a single level (assay Nos. 457, 458 and 459), a curve was constructed (healing vs. log dose) on the basis of the 2 levels of the reference oil standard. The average healing score for each sample was converted to units of biological activity on the basis of the straight line curve of the respective assay.

The potency estimates of the 2 level assays were calculated statistically in accordance with the method of Bliss and Marks[7] from the potency formula $M = \frac{ID}{B}$ where M = antilog of the estimated potency of the unknown as compared with the standard; I = difference between the logarithms of the doses; D = difference in the responses to the sample and to the standard; B = difference in response between levels for the sample and the standard.

In view of the fact that all groups did not have the same number of animals (some of the rats could not be used since they did not gain sufficient weight during the test period or showed "starvation healing"), the mean healing scores were substituted for the missing values in order to bring the groups up to equal size. This does not affect the calculated standard error significantly as long as fewer than one-quarter of the total observations for each calculation are substituted values.

† The U.S.P. reference cod liver oil was diluted for test on the basis of 115 U.S.P. units per g in accordance with the label statement. Several bottles of the reference oil were used in the course of these studies.

‡ The calciferol samples were diluted for test on the assumption that they would supply 49 U.S.P. units per μg.

This result obtained on different lots of calciferol indicates that, as currently produced, calciferol has a potency much greater than 40 of the present U.S.P. units per μg. The error of the assay is such that no one sample can be said to be significantly more potent than 40 U.S.P. units per μg, *i.e.*, the odds are only about 1 in 10 in support of such a conclusion. However, taken together and considering all samples as representative of current commercial production, the average indicates that the odds are only 1 in 1000 that the value of 49.7 U.S.P. units per μg is a chance occurrence. These results agree closely with those obtained in a recent collaborative study on vitamin D_3 with chicks;[8] the grand average value compiled by Waddell therein indicated the latter vitamin to have slightly over 49 A.O.A.C. units per μg. Since the A.O.A.C. unit and the U.S.P. unit are equivalent by rat assay,[9] additional and independent evidence is thus at hand to indicate that vitamin D_3, as well as calciferol, assays at more than 40 U.S.P. units per μg.

As indicated above, a collaborative study carried out some years ago (in which 9 laboratories participated) showed that calciferol‡ has a potency of 40 international units per μg. Since the physical constants of our calciferol do not appear to be different from those given for the material used in the study reported by Coward,[1] ours should not show 25% greater activity. We must turn to the alternative explanation that the current U.S.P. reference cod liver oil is less potent than the original international standard. The U.S.P. unit must be defined in terms of the U.S.P. reference standard to satisfy legal requirements. Thus it cannot be said that the U.S.P. unit is too small. It does appear clear, however, that at present one U.S.P. unit has only 0.8 the activity exhibited by 1 mg of that international standard which some years ago was found equivalent to 0.025 μg of calciferol.

Brief mention may be made of the findings of Gridgeman[11] that pure calciferol showed only 35.7 international units of vitamin D per μg while vitamin D_3 was equivalent to 46.6 international units per μg. This observation that vitamin D_3 is 1.31 times as potent as calciferol is so markedly at variance with earlier data obtained in the extensive collaborative studies reported by Coward[1] that independent confirmation is needed. The data presented in this communication and in the A.V.R.C. report[8] based on chick assays, agree in showing that both calciferol and vitamin D_3 assay 49 to 50 U.S.P. units per μg when referred to the U.S.P. or A.O.A.C. standard (these are equivalent[9]). These data are interpreted as showing a 1:1 ratio of the potencies.

Summary. Assays of 8 samples of calciferol (vitamin D_2) using the U.S.P. biological assay with rats indicated in every case a potency significantly greater than 40 U.S.P. units per μg. Our assays gave an average value of 49.7 ± 2.1 units per μg. This is interpreted to mean that U.S.P. reference cod liver oil No. 2 has less than its generally accepted potency. In view of this finding, a re-evaluation of the relationship between calciferol, the international standard and the U.S.P. standard for vitamin D would seem to be indicated. Serious consideration should be given to adopting a fixed weight of pure crystalline calciferol or vitamin D_3 as the legal unit instead of relying on designated weights of reference standards consisting of solutions of impure and possibly unstable mixtures.

8 News Item, *Chem. Eng. News*, 1944, **22**, 2053.

9 Assn. Official Agr. Chem., *Official and Tentative Methods of Analysis*, 5th ed., Washington, 1940, 372.

‡ The international standard is not calciferol in oil as stated by Reed, Struck, and Steck.[10]

10 Reed, C. I., Struck, H. C., and Steck, I. E., *Vitamin D: Chemistry, Physiology, Pharmacology, Pathology, Experimental and Clinical Investigations*, Chicago, 1939, 58.

11 Gridgeman, N. T., *Quart. J. Pharm. and Pharmacol.*, 1945, **18**, 24.