REFERENCES

- Baker, J. W. (1946). *J. chem. Soc.* (in the press).
 Baker, J. W. & Happold, F. C. (1940). *Biochem. J.* **34**, 657.
 Baker, J. W. & Happold, F. C. (1942). *Chem. & Ind.* **61**, 723.
 Dawson, J. & Happold, F. C. (1943). *Biochem. J.* **37**, 389.
 Happold, F. C. & Hoyle, L. (1934). *Biochem. J.* **28**, 1171.
 Krebs, H. A., Hafez, M. M. & Eggleston, L. V. (1942). *Biochem. J.* **36**, 306.
 Redemann, C. E. & Dunn, M. S. (1940). *J. biol. Chem.* **130**, 341.
 Tatum, E. L. & Bonner, D. (1944). *Proc. nat. Acad. Sci., Wash.*, **30**, 30.
 Wieland, H., Konz, W. & Mittasch, H. (1934). *Liebigs Ann.* **513**, 19.

Comparative Microbiological Assays of Members of the Vitamin B Complex in Yeast and Liver Extracts

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Although a large number of different micro-organisms have been used for the microbiological assay of members of the vitamin B complex, comparative assays on the same substances do not appear to have been carried out except with micro-organisms belonging to the same genera, e.g. *Lactobacillus arabinosus* and *L. casei* (Skeggs & Wright, 1944). Since it has frequently been reported that yeast and liver extracts contain unidentified growth factors, it seemed particularly desirable in assaying such preparations to confirm the specificity of the response of any organism to any one member of the vitamin B complex by carrying out a comparative assay using another organism, preferably one belonging to a different botanical class. It was also considered of interest to see whether any organisms could be found that responded to these hypothetical growth factors in yeast or liver extracts.

Pantothenic acid can be assayed by means of *Proteus morganii* (Pelczar & Porter, 1941), *Lactobacillus helveticus* (Pennington, Snell & Williams, 1940; Strong, Feeney & Earle, 1941) and *L. arabinosus* (Skeggs & Wright, 1944; Hoag, Sarett & Cheldelin, 1945); pyridoxin by means of an X-ray-induced mutant of *Neurospora sitophila* (Stokes, Larsen, Woodward & Foster, 1943), *Saccharomyces carlsbergensis* (Johnson, 1944; Carpenter & Strong, 1944), and an unspecified yeast (Atkin, Schultz, Williams & Frey, 1943); biotin by means of *L. arabinosus* (Wright & Skeggs, 1944), and a 'cholineless' strain (i.e. one unable to synthesize choline) of *Neurospora crassa* (Beadle, 1944) and by *Saccharomyces cerevisiae* (Woolley, 1941; Jurist & Foy, 1944); and aneurin by *Phycomyces blakesleeana*

(Meiklejohn, 1937; Sinclair, 1938, 1939), an unspecified yeast (Schultz, Atkin & Frey, 1942; Scrimshaw & Stewart, 1944), *Streptococcus salivarius* (Niven & Smiley, 1943) and *Lactobacillus fermenti* (Sarett & Cheldelin, 1944).

Burkholder (1943), Burkholder & Moyer (1943), and Burkholder, McVeigh & Moyer (1944) described the vitamin B requirements of a large number of different species and varieties of yeasts, and used *Kloeckera brevis* for the assay of inositol. Through the kindness of Prof. Burkholder, we have been able to examine a number of these, together with other yeasts obtained elsewhere, with the results recorded in Table 1.

It will be observed that no yeast was found for which either riboflavin or nicotinic acid was essential, whereas according to Burkholder *et al.*, several required nicotinic acid. Many of the yeasts required other members of the vitamin B complex, however, and appeared to be of potential value for use in microbiological assays. In no instance was the response produced by liver extracts plus all seven vitamins markedly greater than with the vitamins alone, so that none of the organisms indicated the presence of any new growth factor in the preparations.

For purposes of microbiological assay, the most interesting species, as already noted by Burkholder, is *Kloeckera brevis*, which requires the addition of six members of the vitamin B complex for optimal growth, namely, aneurin, nicotinic acid, pantothenic acid, pyridoxin, biotin and inositol. It should therefore be possible to assay the other five factors in addition to inositol by means of this organism.

Table 1. Growth of yeasts in Burkholder (1943) medium with supplements of vitamins or liver extract

The values represent relative turbidities after incubation for 72 hr. at 24°, measured on the Spekker absorptiometer, using a water blank and neutral filters. (Setting for maximum growth = 1.2 except where stated.)

Glaxo Laboratories Ref. no.	Organism	- 7 vitamins										+ 7 vitamins + liver	
		- Aneurin	- Ribo- flavin	- Panto- thenate	- Nicotinic acid	- Biotin	- Inositol	- Pyri- doxin					
B782	<i>Candida breve</i>	0.63	1.02	1.2	1.2	0.72	1.2	1.2					1.2
758	<i>C. chevalieri</i>	1.2	1.2	1.2	1.2	1.2	1.2	1.2					1.2
759	<i>C. guilliermondia</i>	0.0	0.65	0.49	0.54	0.1	0.48	0.52					0.44
760	<i>C. pelliculosa</i>	1.04	1.2	1.2	1.2	1.2	1.2	1.2					1.2
783	<i>Chalara mycoderma</i>	0.02	0.15	0.08	0.1	0.02	0.15	0.1					0.13
784	<i>Debaryomyces tyrocola</i>	0.15	0.56	0.77	0.74	0.43	0.71	0.66					0.71
769	<i>Hansenula anomala</i>	0.5	1.2	1.2	1.2	1.2	1.2	1.2					1.2
786	<i>H. margaritae</i>	1.2	1.2	1.2	1.2	1.2	1.2	1.2					1.2
768	<i>Kloeckera brevis</i>	0.0	0.23	0.08	0.88	0.3	0.0	0.1					1.1
780	<i>Mycoderma chevalieri</i>	1.2	1.2	1.2	1.2	1.2	1.2	1.2					1.2
761	<i>M. decolorans</i>	0.40	1.2	1.2	1.2	1.2	1.2	1.2					1.2
778	<i>Mycotorula psilosis</i>	0.45	0.48	1.2	1.2	1.2	1.2	1.2					1.2
779	<i>M. sinensis</i>	0.47	0.53	1.2	1.2	0.65	1.2	1.2					1.2
775	<i>Pichia hyalosporea</i>	0.15	0.23	0.21	0.22	0.21	0.22	0.22					0.28
* 767	<i>P. Kluyveri</i>	0.15	0.53	0.83	0.86	0.38	0.86	0.07					0.99
770	<i>Rhodotorula minuta</i>	0.12	0.15	0.15	0.15	0.11	0.11	0.11					0.24
* 762	<i>Saccharomyces cerevisiae</i> var. <i>ellip- soides</i>	0.0	0.66	0.0	1.0	0.93	0.36	1.01					1.32
* 763	<i>S. macedoniensis</i>	0.0	0.25	0.89	1.18	1.24	1.28	1.24					1.33
781	<i>S. validus</i>	0.43	1.2	1.2	1.2	1.2	0.7	1.2					1.2
* 764	<i>Saccharomycodes ludwigii</i>	0.0	1.2	0.0	1.20	1.05	1.4	0.06					1.42
785	<i>Schizosaccharomyces pombe</i>	0.02	0.7	0.1	0.79	0.15	0.02	0.85					0.98
771	<i>Schizoblastosporion Starkeyi-Henrici</i>	1.06	1.2	1.2	1.2	1.2	1.2	1.2					1.2
776	<i>Torulopsis pulcherrima</i>	0.3	0.97	1.2	1.2	1.2	1.2	1.2					1.2
777	<i>T. sphacra</i>	0.075	0.44	0.38	0.6	0.34	0.61	0.62					0.67
* 765	<i>T. sphacra</i>	0.0	0.96	0.6	0.79	0.68	0.78	0.99					0.84
787	<i>Zygosaccharomyces nadsosii</i>	0.1	0.86	0.1	0.92	0.27	0.89	0.9					1.05
719	Unidentified yeast isolated from orange juice	0.14	1.2	0.15	1.2	1.08	1.2	1.2					1.2
739	Do.	0.4	0.52	1.06	0.91	0.43	0.95	0.75					1.2

* Setting for maximum growth = 1.5.

It can also be seen from the table that *Candida guilliermondia* is sensitive to biotin, *Pichia Kluyveri* to pyridoxin, *Saccharomycodes ludwigii* to pantothenic acid and pyridoxin, *Saccharomyces macedoniensis* to aneurin, *Schizosaccharomyces pombe* to pantothenic acid, biotin and inositol, and the unidentified yeast B719, *Saccharomyces cerevisiae* var. *ellipsoideus* and *Zygosaccharomyces nadsonii* to pantothenic acid.

Methods of assaying five of the vitamins with *Kloeckera brevis*, *Saccharomyces macedoniensis*, *S. cerevisiae* var. *ellipsoideus*, *S. pombe* and *Candida guilliermondia*, were devised and used to check the results obtained by published methods.

EXPERIMENTAL

Vitamin requirements of yeasts

Stock cultures of the yeasts were maintained on a potato-starch agar medium and subcultured monthly. The following double-strength basal medium (Burkholder, 1943) was prepared: glucose, 20 g.; *dl*-asparagine, 2.0 g.; KH_2PO_4 , 1.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.33 g.; $(\text{NH}_4)_2\text{SO}_4$, 2.0 g.; KI, 0.1 mg.; solution containing inorganic constituents, 1 ml.; distilled water to 500 ml. The pH was adjusted to 5.0 with NaOH.

The solution of other inorganic constituents consisted of boric acid, 0.1 g.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.04 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.045 g.; ammonium molybdate, 0.02 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g. in 1 l. of water.

Stock solutions of the seven vitamins were prepared as follows: aneurin, 40; riboflavin, 100; pyridoxin, 100; nicotinic acid, 100; biotin, 1; pantothenic acid, 100; inositol, 500 $\mu\text{g.}/\text{ml.}$

Into each of a series of test-tubes were put 2.5 ml. of the double-strength basal medium, and sufficient of the vitamin solutions added singly or in combination to give the following concentrations ($\mu\text{g.}/\text{l.}$) in the final medium: aneurin, 200; riboflavin, 100; pyridoxin, 200; nicotinic acid, 200; biotin, 0.2; pantothenic acid, 200; inositol, 1000. The volume in each tube was made up to 5.0 ml. with distilled water, and the tubes were covered with inverted glass specimen tubes, autoclaved at 15 lb. pressure for 15 min. and then inoculated with one drop of a yeast suspension, obtained by transferring a loopful of a 24 hr. culture to 5 ml. of the vitamin-free basal medium. The tubes were incubated at 24° for 72 hr., and the turbidity was measured in a Hilger Spekker Absorptiometer against a water blank with the neutral grey-green filters. The figures given in Table 1 represent the relative turbidities, read directly from the scale of this instrument.

Vitamin assays

A procedure similar to the above was followed in assaying yeast and liver extracts with the selected yeasts. Into each of a series of test-tubes were put 2.5 ml. of the basal medium containing the above quantities of all seven vitamins with the exception of the one under investigation.

To one series of tubes (in duplicate or triplicate) were added 2.5 ml. quantities of solutions of the particular vitamin over a suitable range of concentrations, or 2.5 ml.

quantities of solutions of the extract containing approximately the same concentrations of the vitamin.

The tubes were covered with inverted specimen tubes, sterilized, inoculated and incubated as described above. The turbidities of the standard solutions and of the unknowns were measured in the Spekker Absorptiometer, and plotted against the concentrations of the vitamin in the standard solutions and dilutions of the material under assay. The vitamin content of the latter was calculated by averaging the results read off from the standard curve obtained from the former; the 'slope ratio' method (Wood, 1945, 1946) was not used in these exploratory tests.

Pantothenic acid assays

The pantothenic acid contents of several yeast and liver extracts were estimated by means of *Lactobacillus helveticus* (Pennington *et al.* 1940), and *L. arabinosus* (Skeggs & Wright, 1944), and the same samples tested against *Saccharomyces cerevisiae* var. *ellipsoideus*. Standard solutions containing 0.0.5 and 0.0.2 $\mu\text{g.}$ respectively of calcium *d*-pantothenate per tube were used for constructing the two standard curves shown in Figs. 1 and 2. The results are recorded in Table 2. The agreement is on the whole satisfactory, though with some samples greater discrepancies were obtained than could be accounted for as experimental error. Satisfactory agreement was obtained with two other liver extracts, using *Lactobacillus helveticus* and *Kloeckera brevis*, but a third extract gave a response with the latter organism much smaller than expected. Increasing the amounts of the B vitamins in the medium as suggested by Rogosa (1944) did not affect this last result.

Pyridoxin assays

Six liver extracts and five yeast extracts were assayed by the method of Stokes *et al.* (1943), using the X-ray induced mutant of *Neurospora sitophila*, and the results compared with assays on the same samples using *Kloeckera brevis*. Solutions containing 0.0.005 $\mu\text{g.}$ of pyridoxin per tube were used for constructing the standard curve for *K. brevis*, whereas for *Neurospora sitophila*, the range was 0.1.0 $\mu\text{g.}$ per tube. The standard curves are shown in Figs. 3 and 4 and the results are recorded in Table 2.

With *Neurospora*, as pointed out by Stokes *et al.* (1943), aneurin must be destroyed, since it stimulates the growth of this organism. Agreement between the two sets of assays was less satisfactory with pyridoxin than with any of the other vitamins, possibly because the two organisms respond to the different forms of vitamin B₆ (e.g. pyridoxal, pyridoxamine, etc.) to different extents. Several of the extracts gave discrepancies well beyond the limits of experimental error, *Neurospora* giving the higher result with most of the yeast extracts, and *Kloeckera brevis* the higher result with one of the liver extracts.

Fig. 1. Response of *S. cerevisiae* to calcium *D*-pantothenate.

Fig. 2. Response of *L. helveticus* to calcium *D*-pantothenate.

Table 2. Vitamin contents ($\mu\text{g./g.}$) of yeast and liver extracts

Assay organism Extract	...	Aneurin		Pyridoxin		Pantothenic acid		Biotin		Inositol	
		<i>Lactobac. fermenti</i>	<i>Sacch. macedon.</i>	<i>Kloeckera brevis</i>	<i>Neurospora sitophila</i>	<i>Lactobac. helveticus (L. arabinosus)</i>	<i>Sacch. cerevis.</i>	<i>Lactobac. arabinosus</i>	<i>Candida guillerm.</i>	<i>Kloeckera brevis</i>	<i>Sacch. pombe</i>
Proteolyzed liver extract	A	—	—	20	20*	561	562	5.2	5.2	—	—
"	E	—	—	30	22*	506	570	4.3	4.1	—	—
"	G	—	—	27	23*	510	603	5.0	5.0	2230	2470
Crude liver extract	E	10.7	11.7	—	—	125 (132)	105	0.74	0.63	—	—
"	P	10.0	17.5	22	7.8	390 (420)	425	0.15	0.16	2100	2267
"	PE	—	—	11	11.5	32.4 (45.8)	35.7	—	—	—	—
Refined liver extract	E	{40.7 41.0	{45.6 43.0	6.8	7.7	5.6 (6.6)	—	—	—	24.7	25.8
"	US	—	—	—	—	—	—	0.10	0.11	—	—
"	PE	30.0	32.2	—	—	1.9 (2.2)	—	—	—	—	—
Yeast extract	G	100	140	54	67	119	112	—	—	3060	2930
"	GL	100	110	69	57	386	421	—	—	—	—
"	M	—	—	20	58	100	80	—	—	1680	1740
"	N	—	—	81	185	640	658	1.4	1.5	1940	1997
"	Y	26	125	14	32	93	58	—	—	1260	1302

* Aneurin not destroyed.

Aneurin assays

The aneurin contents of five liver extracts and three yeast extracts were estimated by the method of Sarett & Cheldelin (1944), using *Lactobacillus fermenti* 36, and with *Saccharomyces macedoniensis*. The results (Table 2) for the majority of liver extracts were in good agreement, but two of the yeast extracts gave considerably higher results with *S. macedoniensis* than with *Lactobacillus fermenti*.

Standard curves are shown in Figs. 5 and 6. The concentrations ranged from 0 to 0.06 and 0 to 0.05 $\mu\text{g.}$ per tube respectively.

Biotin assays

Biotin was estimated by the method of Wright & Skeggs (1944) using *Lactobacillus arabinosus*, and the results compared with those obtained using *Candida guillermundia*. Excellent agreement was obtained in all instances (Table 2). The standard

curves are shown in Figs. 7 and 8. The range of concentrations for *Lactobacillus arabinosus* was 0–0.0005 $\mu\text{g.}$ per tube and for *Candida guilliermondia* 0–0.001 $\mu\text{g.}$ per tube.

Burkholder *et al.* (1944). We compared the results obtained with these two organisms and with *Schizosaccharomyces pombe*. Whereas the results obtained with yeast and liver extracts were in close

Fig. 3. Response of *K. brevis* to pyridoxin.

Fig. 4. Response of *N. sitophila* to pyridoxin.

Fig. 5. Response of *K. brevis* to aneurin.

Fig. 6. Response of *L. fermenti* to aneurin.

Inositol assays

The estimation of inositol with *Kloeckera brevis* and *Saccharomyces cerevisiae* was described by

agreement when *Kloeckera brevis* and *Schizosaccharomyces pombe* were used, *Saccharomyces cerevisiae* var. *ellipsoideus* gave less satisfactory values. The results with the first two organisms are recorded in Table 2

and the standard curves in Figs. 9 and 10. With *Kloeckera brevis* the concentrations ranged from 0 to 5 $\mu\text{g.}$ per tube and with *Schizosaccharomyces pombe* from 0 to 3 $\mu\text{g.}$ per tube.

investigated. Several species failed to grow appreciably in absence of one or more of these factors, whilst *Kloeckera brevis* required six of them for growth.

Fig. 7. Response of *C. guilliermondia* to biotin.

Fig. 8. Response of *L. arabinosus* to biotin.

Fig. 9. Response of *K. brevis* to inositol.

Fig. 10. Response of *S. pombe* to inositol.

SUMMARY

1. The requirements of several species of yeasts for seven members of the vitamin B complex were

2. The vitamin B contents of a number of yeast and liver extracts were estimated by measuring the growth responses of several of these yeasts, and the results were compared with those obtained by

published microbiological methods of assay. *K. brevis* was found to be satisfactory for the assay of pantothenic acid and inositol, *Saccharomyces cerevisiae* var. *ellipsoideus* for pantothenic acid, *S. macedoniensis* for aneurin, *Candida guiller-*

mondia for biotin and *Schizosaccharomyces pombe* for inositol.

3. *Kloeckera brevis* was used for the assay of pyridoxin, but the results were frequently at variance with those given by *Neurospora sitophila*.

REFERENCES

- Atkin, L., Schultz, A. S., Williams, W. L. & Frey, C. N. (1943). *Industr. Engng Chem. (Anal. ed.)*, **15**, 141.
- Beadle, G. W. (1944). *J. biol. Chem.* **156**, 683.
- Burkholder, P. R. (1943). *Amer. J. Bot.* **30**, 206.
- Burkholder, P. R., McVeigh, I. & Moyer, D. (1944). *J. Bact.* **48**, 385.
- Burkholder, P. R. & Moyer, D. (1943). *Bull. Torrey Bot. Cl.* **70**, 372.
- Carpenter, L. E. & Strong, F. M. (1944). *Arch. Biochem.* **3**, 375.
- Hoag, E. H., Sarett, H. P. & Cheldelin, V. H. (1945). *Industr. Engng Chem. (Anal. ed.)*, **17**, 60.
- Johnson, B. C. (1944). *Proc. Soc. exp. Biol., N.Y.*, **55**, 199.
- Jurist, V. & Foy, J. R. (1944). *J. Bact.* **47**, 434.
- Meiklejohn, A. P. (1937). *Biochem. J.* **31**, 1441.
- Niven, C. F. & Smiley, K. L. (1943). *J. biol. Chem.* **150**, 1.
- Pelczar, M. J. & Porter, J. R. (1941). *J. biol. Chem.* **139**, 111.
- Pennington, D., Snell, E. E. & Williams, R. J. (1940). *J. biol. Chem.* **135**, 213.
- Rogosa, M. (1944). *J. Bact.* **47**, 159.
- Sarett, H. P. & Cheldelin, V. H. (1944). *J. biol. Chem.* **155**, 153.
- Schultz, A. S., Atkins, L. & Frey, C. N. (1942). *Industr. Engng Chem. (Anal. ed.)*, **13**, 45.
- Scrimshaw, N. S. & Stewart, W. B. (1944). *J. biol. Chem.* **155**, 79.
- Sinclair, H. M. (1938). *Biochem. J.* **32**, 2185.
- Sinclair, H. M. (1939). *Biochem. J.* **33**, 1816, 2027.
- Skeggs, H. R. & Wright, L. D. (1944). *J. biol. Chem.* **156**, 21.
- Stokes, J. L., Larsen, A., Woodward, C. R. & Foster, J. W. (1943). *J. biol. Chem.* **150**, 17.
- Strong, F. M., Feeney, R. E. & Earle, A. (1941). *Industr. Engng Chem. (Anal. ed.)*, **13**, 566.
- Wood, E. C. (1945). *Nature, Lond.*, **155**, 632.
- Wood, E. C. (1946). *Analyst*, **71**, 1.
- Woolley, D. W. (1941). *J. biol. Chem.* **140**, 453.
- Wright, L. D. & Skeggs, H. R. (1944). *Proc. Soc. exp. Biol., N.Y.*, **55**, 95.

The Sedimentation Constant of Insulin

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In view of other evidence, to be published elsewhere by one of us (H.G.) on the molecular size and stability of insulin, we undertook a re-examination of the sedimentation constant over a range of pH and at different concentrations. We are unable to agree with Sjögren & Svedberg (1931) and Miller & Andersson (1942) about the value of the sedimentation constant or the range of stability. Sjögren & Svedberg found that a fall in the sedimentation constant occurs above pH 7 and below pH 4.5. Miller & Andersson confirmed a fall between pH 7.5 and 8.5 and also concluded that at pH 8.5 the insulin is becoming heterogeneous. Miller & Andersson obtained a sedimentation constant of 3.55×10^{-13} at pH 7 which agreed well with Sjögren & Svedberg's uncorrected value of 3.47×10^{-13} . Both these workers used recrystallized insulin. Miller & Andersson worked at a concentration of 1 % insulin.

EXPERIMENTAL

Sedimentation was observed in a Svedberg oil-turbine ultracentrifuge by the method of Philpot (1938); the speed was 1000–1020 rev./sec. In view of the difference of our results from those of previous workers we should mention the precautions that have been taken to make our estimates of the sedimentation constant accurate: the constants of the optical system were carefully and directly measured (by A. G. Ogston and J. P. Johnston); the vibrating reed frequency-meter had been calibrated against a crystal oscillator (with the kind collaboration of Dr R. P. Martin of the Clarendon Laboratory) and had been shown to give stable readings over a period of 2 years; the reading of the rotor temperature had been checked at 300 rev./sec. (Ogston, 1943) and found correct; in addition we found that the thermocouple reads correctly the temperatures encountered at 1000 rev./sec., but we have not tested whether these temperatures actually correspond with those of the rotor.