[CONTRIBUTION FROM THE UNIVERSITY OF TEXAS, BIOCHEMICAL INSTITUTE, AND THE CLAYTON FOUNDATION FOR RESEARCH]

The Assay of Purified Proteins, Enzymes, Etc., for "B Vitamins"

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Microbiological assay methods for "B vitamins" have been used extensively in this Laboratory to assay small samples of tissues and foods.[3,4] In the present study the same methods were applied to various purified proteins, etc., as indicated in Table I.

The purpose of the investigation was to gain insight into the possible importance of "B vitamins" as prosthetic groups in proteins known to possess unusual physiological functions. It is common to assume that those B vitamins whose functions are unknown will ultimately be found to have a catalytic role and to be essential parts of specific enzyme systems. The possibility that some of these B vitamins should be constituent parts of already recognized enzymes and other physiologically active substances seemed worth exploring. Obviously in the case of comparatively low molecular weight proteins, a vitamin in order to be a constituent part of the molecule must be present in relatively large amounts, and relatively crude tests may serve to settle the question. It was of additional interest, however, to know in some cases whether specific proteins contained substantial amounts of B vitamins even though the vitamins were not present in amounts sufficient to be integral parts of the protein molecules, and were presumably held only by adsorption.

The results of the assays are given in Table I.

Of the 33 samples tested, the urease, ferritin and two ribonuclease samples were prepared by one of us (F. S.) using published methods[5,6,7,8,9]; the casein samples were obtained commercially. The others are described and their origin indicated in Table I.

The various preparations were not in general assayed for riboflavin because it is a highly colored substance and could hardly be an integral part of a purified protein (except highly colored ones) without revealing its presence visually.

Quantitative determinations on poor sources of B vitamins such as those investigated in this study are, in the nature of the case, subject to large percentage errors. The method for inositol is not on a par with the other methods because the organism (yeast) is far less sensitive to it, and the enzyme preparation used for digestion is comparatively rich and introduces a high correction. For this reason the low values for inositol content are relatively unreliable.

For determination of biotin and *p*-aminobenzoic acid, acid digestion replaced enzyme digestion; hence there was no "enzyme blank" to be used as a correction. The determinations of *p*-aminobenzoic acid were made with slight modifications according to the method of Lewis.[10]

Discussion

Most of the preparations of enzymes, hormones, proteins and other principles assayed contained measurable amounts of the various B vitamins. In most cases, however, the amounts are so small that the vitamins could not constitute an integral part of the molecules concerned.

Two samples yielded results of unusual interest, namely, pancreatic amylase and the yeast carboxylase. The pancreatic amylase preparation was capable of yielding 11,000 times its weight of maltose under standard test conditions. This is as high a potency as has been reported for any amylase preparation. Its inositol content is sufficiently high (4.1 mg. per gram) so that in order to be an integral part of the enzyme, the molecular weight of the latter would need to be no more than 44,000. That this inositol content is real and not due to error is indicated by the fact that a duplicate set of assays using acid extraction instead of enzymatic extraction gave results which were in substantial agreement (4.4 mg. per gram). This interesting observation regarding the amylase will be investigated further in collaboration with Dr. Caldwell.

Both yeast carboxylase preparations yielded an amount of nicotinic acid which was far above that obtained from other proteins. Preparation 1 was approximately 25% pure. The second preparation was "put through the same purification procedure that leads to the purest material recorded in our paper. However, the purity of this preparation is certainly far below what it ought to be. I have no explanation for this discrepancy except to point out that the activity of our starting material was 10% of that we used in England."[11] The possible existence of nicotinic acid in carboxylase will require further investigation, but the results obtained are suggestive.

The fact that for the most part B vitamins are found not to be constituents of the enzymes, etc., tested indicates, assuming that the less known B vitamins are enzyme constituents, that there exists a multiplicity of enzymes which are not at present available in concentrated form.

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(3) R. J. Williams, *et al.*, *Univ. Texas Pub.*, **4137** (1941).

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(5) J. B. Sumner, *J. Biol. Chem.*, **69**, 435 (1926).

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(7) M. Kunitz, *J. Gen. Physiol.*, **24**, 15 (1941).

(8) S. Granick and L. Michaelis, *J. Biol. Chem.*, **146**, 451 (1942).

(9) S. Granick and L. Michaelis, *ibid.*, **147**, 91 (1943).

(10) J. C. Lewis, *ibid.*, **146**, 441 (1942)

(11) Personal communication from Dr. David E. Green.

TABLE I

B VITAMIN CONTENT OF ENZYMES, ETC.

Preparation tested	Description	Source[a]	Thiamin, γ/g.	Niacin, γ/g.	Pantothen, γ/g.	Pyridoxin, γ/g.	Biotin, γ/g.	Inositol, γ/g.	PAB, γ/g.	Folic acid, γ u./g.
Urease	Crystalline	..	1.8	2.4	0[b]	0.47	0.096	0[c]	...	0.3
Trypsin	Cryst. + $MgSO_4$	MB	1.0	3.1	1.2	0.16	.036	0[b]	0.36	0[b]
Chymotrypsin	Cryst. + $MgSO_4$	MB	3.1	3.5	1	0.1	.041	270	...	0[b]
Ribonuclease	5 × crystallized	MK	1	4.6	0[b]	0[b]	0[b]	0[c]	0.61	0[b]
Ribonuclease	Preparation 1	..	0.7	7.3	5.2	.4	0.037	0[b]	1.3	0[b]
Ribonuclease	Preparation 2	..	0.1	6.9	5.7	.25	0.012	0[b]	*ca.* 1.4	0.4
Carboxylase	Preparation 1[d]	DG	940	145	6.7	.27	[c]	0[c]	...	0[b]
Carboxylase	Preparation 2. See text	DG	530	131	1.3	.45	[c]	0[b]	...	0[b]
Muscle phosphorylase	[e]	GC	0.24	1.2	2.1	..	.019	0[b]	...	0[b]
Pancreatic amylase	[f]	MC	...	19.7	1.3	..	.013	4100	...	13
Myokinase	Impure[g]	HK	2.7	8.3	1.8	..	.026	0[b]	0.31	0[b]
Tyrosinase	Impure[h]	JN	3.7	24	..	0	.2	120	...	low
Cytochrome reductase	6.2% pure	EH	19.3	84	6.6	2.8	1.4	0[c]	...	0.3
Alkaline phosphatase	Preparation 1[i]	GS	0[c]	11	..	..	0.017	0[c]	...	low
Alkaline phosphatase	Preparation 2[j]	GS	1	...	..	0.1	.15	278	...	low
Insulin	Crystalline	VV	0.33	1.6	2.5	.13	.008	60	...	0[b]
Lactogenic hormone	About 90% pure	HE	0.87	0[b]	3	.47	.023	0[c]	*ca.* 1.5	low
Renin	[k]	IP	2.3	20	18	.30	.048	158	1	11
Heparin	Pure	TJ	0.3	1.8	0.6	.06	.005	60[c]	0.26	0[b]
Silk fibroin	[l]	MB	1.4	3.4	2.1	.27	0[b]	0[c]	...	0[b]
Nucleoprotein	[m]	AM	22	2.6	0.8	.41	0.023	330	...	0[b]
Lactoglobulin	3 × recryst.	MB	0.5	1.2	2.1	0[b]	0[b]	0[b]	0.08	0[b]
Horse hemoglobin	1 × recryst.	MB	0[b]	3.2	0.8	0[b]	0[b]	0[b]	0.19	0[b]
Horse hemoglobin	3 × recryst.	MB	.35	1.7	0.9	0[b]	.033	0[b]	0.25	0[b]
Egg albumin	[n]	MB	.7	0[b]	0[b]	0[b]	.008	0[c]	...	0[b]
Ferritin	Preparation 1	..	.33	0[c]	0[b]	0[c]	.012	0[c]	...	0[b]
Ferritin	Preparation 2	..	.06	2.5	0[b]	0[c]	...	0[c]	...	0[b]
Casein	Vit. free 9763	..	.13	0[b]	.7	.4	.006	0[c]	...	0[b]
Casein	Vit. free 9884	..	.18	0[b]	.8	.3	.008	0[c]	...	0[b]
Casein	Vit. free 9885	..	.13	0[b]	.8	.4	.010	0[c]	...	0[b]
Casein	Vit. free 10088	..	.21	0[b]	.7	.4	.007	110	...	..
Casein	Vit. free 10089	..	.28	0[b]	.9	.4	.005	110	...	..
Tobacco mosaic virus[o]	[p]	WS	2.3	1.1	.3	.18	.017	0[b]	...	0[b]
Enzyme mixture "papain" and "taka-diastase" used for digestion (usually at 10% level)		..	1.7	50.5	22.6	1.5	...	8000	...	3.8

[a] Symbols stand for the following sources: MB = Max Bergmann, MK = Moses Kunitz, DG = David E. Green, GC = Gerty T. Cori, MC = Mary L. Caldwell, HK = Herman M. Kalckar, JN = J. M. Nelson, EH = Erwin Haas, GS = Gerhardt Schmidt, VV = Vincent du Vigneaud (Eli Lilly product), HE = Herbert M. Evans, IP = Irvine H. Page, TJ = T. H. Jukes (Lederle Labs.), AM = Alfred E. Mirsky, WS = W. M. Stanley. [b] Values obtained were not necessarily zero, but were so low as to be probably negligible. [c] These tests were not entirely satisfactory. In most cases a slight growth inhibition was observed and this makes the result open to some question. [d] Approximately 25% pure, contained about 15 mg. dry wt. protein and approximately 20 units carboxylase activity per cc. [e] Many times recrystallized, washed with potassium chloride, dried *in vacuo*. [f] Highly purified, will produce approximately 11,000 times its weight of maltose under standard conditions. [g] Three to four times less active than material which catalyzes transfer of 5γ P per 1γ protein per minute. [h] From common mushroom; one cc. contains 10,200 Miller–Dawson catecholase units. [i] One mg. of dry enzyme forms 20 mg. of inorganic P within fifteen minutes from glycerophosphate. [j] About ten times more concentrated than preparation 1. [k] Relatively impure, but very potent. [l] N = 18.9% dry wt., ash = 0.08% (sulfate). [m] Purified from calf thymus. [n] Three times recrystallized, then denatured. [o] Partial assays were made of three other viruses, samples of which were kindly furnished by Dr. W. M. Stanley, *e. g.*, tobacco necrosis virus, bushy stunt virus and a centrifugally purified preparation of PR8 influenza virus. In no case did any vitamin appear to be present in substantial amounts; their contents of B vitamins appeared to be of a similar order to those of the tobacco mosaic virus. The small amounts of the other viruses which were available made the assays less satisfactory and less complete. [p] Crystalline, prepared by three differential centrifugative cycles.

The existence of appreciable amounts of the various B vitamins in all forms of living matter which have been investigated suggests the possibility of utilizing the presence or absence of these vitamins as a criterion with regard to the animate and inanimate nature or origin of a material in question. If we accept this criterion and the probability that viruses maintain their identity in the absence of all B vitamins, we must conclude that they are what we call inanimate.

Acknowledgment.—We wish to acknowledge most gratefully the kindness of the numerous

individuals and laboratories referred to in Table I for furnishing us with the samples which made this investigation possible.

Summary

A series of protein, enzyme, hormone, and miscellaneous preparations have been assayed for B vitamins. Only traces have been found in most; however, inositol appears to be a constituent of purified amylase and the impure carboxylase preparations available contained a suggestively high content of nicotinic acid in addition to the thiamin already known to be present. The viruses investigated were found to be nearly devoid of B vitamins, and in this respect they appear to resemble "inanimate" rather than "animate" matter.

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[FROM THE CHEMICAL LABORATORIES OF HARVARD UNIVERSITY AND RADCLIFFE COLLEGE]

Some Experiments on the *in vitro* Formation of Thyroxine from Diiodotyrosine

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Following the report of von Mutzenbecher[1c] that thyroxine is readily formed *in vitro* from diiodotyrosine as well as from iodinated proteins,[2] considerable interest has been expressed in the nature of the reaction. As normally carried out, slightly alkaline digestion of diiodotyrosine over a period of two weeks gives a very small quantity of thyroxine. Block[3] repeated von Mutzenbecher's work using completely synthetic diiodotyrosine in order to eliminate the possibility that a trace of impurity, responsible for the thyroxine formation, had accompanied the tyrosine from its natural source. Johnson and Tewkesbury[4] made a further study of the reaction, obtaining a slight increase in the yield of thyroxine on the addition of hypoiodite to the solution and establishing pyruvic acid and ammonia as further products. As a result of their work, they postulated a mechanism evolved from the work of Pummerer[5] on the oxidation of *o*- and *p*-substituted phenols in alkaline solution. Their paper may be consulted for the proposed mechanism. We have made additional observations on the nature of the reaction, which throw further light on several points.

It appeared plausible that thyroxine may have been formed by the oxidizing action of the iodine solution originally used for the preparation of the diiodotyrosine rather than during the incubation of the diiodotyrosine. This possibility was eliminated when a sample of crude diiodotyrosine, as it was obtained from the iodination reaction, was subjected to butyl alcohol extraction for thyroxine. No thyroxine was found.

It was equally possible that a small amount of precursor was formed during the iodination and that this and not diiodotyrosine was converted into thyroxine during the long incubation period. A quantity of diiodotyrosine was therefore very carefully purified, but the yield of thyroxine after digestion was not altered.

To further eliminate the role of a small amount of accompanying impurity, diiodotyrosine which had already yielded thyroxine by incubation, was recovered, purified and re-incubated. Again thyroxine was formed.

In order to prove or disprove that oxidation, presumably by air, is involved in the formation of thyroxine, a solution of diiodotyrosine was incubated in an oxygen-free system under the usual conditions; it yielded no thyroxine. Whereas diiodotyrosine recovered from experiments carried out in the presence of air contain much brown, amorphous material, the diiodotyrosine recovered from this experiment was crystalline and only slightly darker than the starting material.

Conversely, the yield of thyroxine from a given amount of diiodotyrosine was increased by passing a slow stream of carbon dioxide-free air through the solution during the incubation.

In view of the work of Pummerer, *et al.*,[6] it was thought that incubation in the presence of a small amount of potassium ferricyanide might increase the yield of thyroxine. This appeared to be too drastic an oxidizing agent, however, as no thyroxine whatever was formed and the diiodotyrosine was converted to a dark brown amorphous substance.

A possible intermediate in the formation of thyroxine would be 3,5-diiodo-4-hydroxybenzoic acid, formed through the oxidative degradation of diiodotyrosine. An incubation carried out in the presence of this compound, instead of increasing the yield of thyroxine, completely prevented the conversion.

It was also found that 2,4,6-triiodophenol would not act as an intermediate to increase the

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(1) (c) von Mutzenbecher, *Z. physiol. Chem.*, **261**, 253 (1939).

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