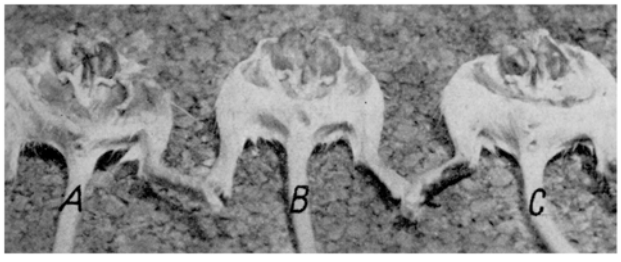

Abb. 2: A. Maus mit Kohlextrakt behandelt; B. Kontrollmaus ohne Behandlung; C. Maus mit Östradiolbenzoat behandelt; 1,5fache lineare Vergrösserung

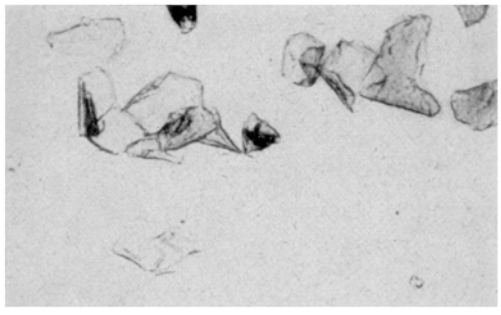

Abb. 3: Vaginalanstrich einer mit Kohlextrakt behandelten kastrierten Ratte

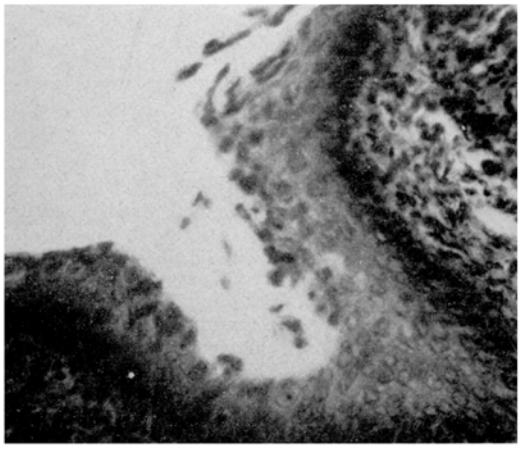

Abb. 4: Vaginalschnitt einer infantilen mit Rotklee-Extrakt behandelten Maus

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Summary

The quantity of phyto-estrogens in red clover, hop, peas, and cabbage was estimated by titration on castrated female rats and infantile mice. The level of phyto-estrogens was as follows: in hop 1050–2000 γ/kg, in peas 4–6 γ/kg, in cabbage 24 γ/kg, and in red clover 6–9 γ/kg. The author supposes that the sexual disorders described by Rosenberger[14] in cows fed with cabbage, can be explained by the presence of phyto-estrogens in this plant.

Notes on the Hydroxamate Assay for Amino Acid Activating Enzymes[1]

The amino acid activating enzymes, first described in mammalian tissues by Hoagland[2] are now quite generally accepted to catalyse the first step in protein synthesis. A worrisome aspect of this concept, however, has been the fact that, while all AA[3] are incorporated into protein, most tissues seem to possess activating enzymes for only a few [4–7]. In some cases, this anomaly seems to be due to a rapid inactivation of some of the enzymes during the extraction and/or purification procedures[8,9], but it now appears that the assays may also be at fault. Webster[10] has discussed reasons for failing to obtain an ATP-PP^{32} exchange with some amino acids; and it is the purpose of the present note to point out some sources of error when the hydroxamate assay is used. In order to obtain meaningful results with the latter, it is essential not only to have a reliable method for the determination of the hydroxamate formed, but also to ascertain that the NH_2OH is not interfering with any of the enzymes, and that the extract used does not destroy the hydroxamate formed or otherwise interfere with its determination.

The Determination of Hydroxamate: For hydroxamates of AA, the method of Schweet[11] is preferable to that of Lipmann and Tuttle[12], which was developed for the determination of acetyl hydroxamate. Instead of strong mineral acid, the former employs concentrated TCA, adjusted to pH 0·9, which is optimum for color development with AA hydroxamates. Under assay conditions, however, when crude extracts are used and in the presence of $NH_2OH \cdot KCl$, the pH (i. e., the empirical reading obtained with a Beckman Model G glass electrode) is often as low as 0·3. Since the color yield in this reaction is inversely proportional to pH between pH 0·3 and 0·9[13] and decreases 20% (0·027 O. D. units at 540 mμ for 1 μM LeuH) for every 0·1 pH unit, very considerable errors in the determination of the AAA-activity are introduced unless the final pH of the reaction mixture (after addition of $FeCl_3$) is taken into consideration.

The Effect of Hydroxylamine on the Assay: It was originally reported by Hoagland[2] that high concentrations of salt-free NH_2OH did not seem to inhibit the AAA-enzymes in liver extract, although $NH_2OH \cdot HCl$ neutralized with NaOH was highly inhibitory. Schweet *et al.*[14], however, found that this inhibition was most likely

[1] This investigation was aided by U. S. Public Health Service Training Grant CRTY-5028.

[2] M. B. Hoagland, Biochim. biophys. Acta *16*, 288 (1955).

[3] The following abbreviations are used throughout: ATP, adenosine triphosphate; AA, amino acid; AAA-enzyme, amino acid activating enzyme; LeuH, leucine hydroxamate.

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[13] I. D. Raacke, Biochim. biophys. Acta *27*, 416 (1958).

[14] R. S. Schweet, R. W. Holley, and E. H. Allen, Arch. Biochem. Biophys. *71*, 311 (1957).

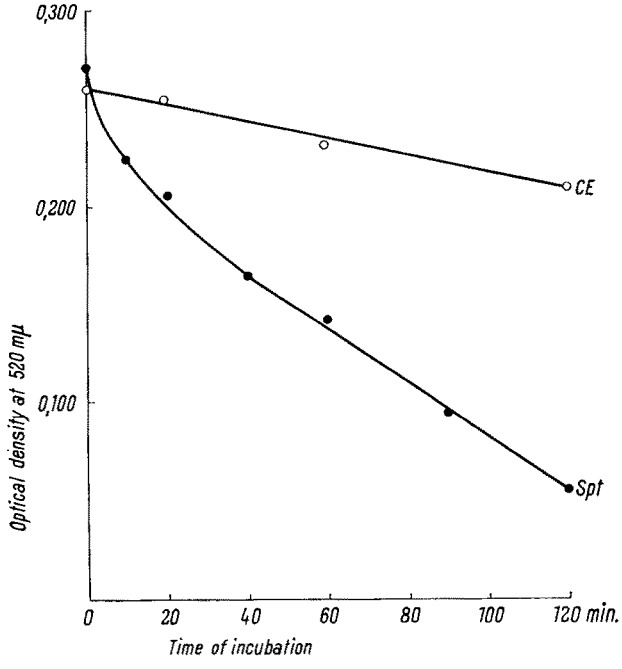
due to the Na^+ (which inhibited the activity of both crude and purified AAA-enzymes) and that if KOH was used for neutralization, the high concentration of K^+ was found to overcome some of the inhibitory effects of Na naturally present in the extracts, and the results were identical or better than those obtained with saltfree NH_2OH. The method had reportedly the further advantage that, contrary to the free base, the hydrochloride was stable and could be stored in the refrigerator, being neutralized just before use. In a recent investigation on the AAA-enzymes in sub-cellular fractions of spinach leaves, BOVÉ and RAACKE[15], however, found that over a period of several weeks there was a continuous and drastic decline in the apparent activity of freshly prepared extracts, and more disturbingly yet, there was a gradual increase in the effect of ATP on the amount of hydroxamate formed, both in the presence and in the absence of AA, and a gradual decrease in the amount of AA-dependent hydroxamate. These drifts were eventually found to be correlated with the age of the $NH_2OH \cdot HCl$ (Eastman, sulfate-free) solution. The results obtained with cytoplasmic supernatant (Spt) and chloroplast extract (CE) are shown in the Table. It is seen that whatever the causative agent may be, its concentration in a 21-day old solution of $NH_2OH \cdot HCl$ is sufficient to decrease the apparent activity of CE to one third, but does not yet affect that of Spt. In the light of the different relative behaviour of the two extracts with the same NH_2OH solution, one may wonder about the quantitative significance of the effects obtained even with freshly prepared reagent.

Effect of Age of $NH_2OH \cdot HCl$ Solution on AAA-Assay by the Hydroxamate Method

Fraction	Age of $NH_2OH \cdot HCl$ Days	Specific Activity in μM/mg protein				Percentage increase due to			
		−AA		+AA		ATP		AA	
		−ATP	+ATP	−ATP	+ATP	−AA	+AA	−ATP	+ATP
CE	1	0·15	0·27	1·05	1·05	80	0	600	290
CE	7	0·16	0·26	0·81	1·03	62	27	405	300
CE	21	0·06	0·14	0·19	0·30	140	58	220	107
CE	28	0·05	0·16	0·20	0·39	220	95	300	145
CE	48	0·04	0·12	0·09	0·17	200	90	125	145
CE	52	0·04	0·13	0·08	0·20	225	150	100	54
Spt	1	0·26	0·33	0·91	0·92	21	1	250	180
Spt	21	0·25	0·40	0·91	0·87	60	−5	265	120
Spt	28	0·12	0·23	0·14	0·28	92	100	17	22
Spt	48	0·07	0·13	0·12	0·16	86	72	23	33

For preparation of the extracts see [15]. The incubation mixtures for the assay contained, in a total volume of 3 ml: 100 μM 'Tris', pH 7·5, 15 μM $MgCl_2$, 3000 μM $NH_2OH \cdot KCl$, 5 μM each of Ala, Arg, Asp, Cys, Gly, Glu, His, Leu, Lys, Met, Phe, Pro, Ser, Thr, Try, and Val, 2 μM of Tyr, 20 μM ATP and 1 ml of extract

The Interaction of the Extract with the Hydroxamate Formed: Extracts of most tissues will form some hydroxamate without the addition of either ATP or AA. This intrinsic blank is particularly high in plant tissues, and it then becomes important to run internal standards to make sure small amounts of added hydroxamates can be recovered quantitatively. With the system from spinach leaves[15], added LeuH could not be recovered from the Spt under assay conditions. It is now found that even in the absence of added NH_2OH, LeuH is rapidly destroyed by Spt but not by CE, as is seen from the Figure. As a result of this activity (which is heat-labile and presumably enzymic in nature) the apparent concentration of AAA-enzymes in Spt is much reduced. If it turns out that a given tissue extract can destroy different AA hydroxamates at different rates, this might be an important contributing factor towards the inability of demonstrating certain enzymes.



Hydrolysis of Leu Hydroxamate by Spt and CE. The incubation mixtures contained, in a total of 3 ml: 1 ml extract (4·9 mg protein), 100 μM 'Tris' pH 7·5, 10 μM $MgCl_2$, 10 μM ATP, and 2·0 μM LeuH. The incubations were carried out in 20 ml beakers in a Dubnoff metabolic incubator at 25°C. The reaction was stopped by the addition of 1·4 ml of 100% (w/v) TCA. The pH after addition of $FeCl_3$ was 0·3. The O. D. was corrected for a blank of 0·170 given by Spt and $FeCl_3$ alone

I. D. RAACKE and J. BOVÉ*

Virus Laboratory, University of California, Berkeley, November 19, 1959.

Zusammenfassung

Die durch die Hydroxamatmethode bestimmte Aktivität von Aminosäureaktivierungsenzymen wird durch das Alter der angewandten $NH_2OH \cdot HCl$-Lösung, durch manchmal vorhandene Hydroxamat spaltende Enzyme sowie durch Unzulänglichkeiten in der chemischen Hydroxamatbestimmungsmethode beeinflusst.

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[15] J. BOVÉ and I. D. RAACKE, Arch. Biochem. Biophys. *85*, 521 (1959).