catalase iron is reduced to ferrous iron during its action on H_2O_2 . Only ferrous haem compounds are oxidized by H_2O_2 to bile pigment haem compounds. Ascorbic acid does not reduce catalase, but may protect the ferrous H_2O_2 -catalase compound against autoxidation.

SUMMARY

1. A simplified procedure for the isolation of crystalline horse liver catalase is described.

2. By splitting with acetic acid and extracting with ether, biliverdin and small amounts of biliverdin are obtained from crystalline ox and horse liver catalases.

3. The molar ratio of (bile pigment)/(bile pigment + haem) from impure and pure horse and ox liver catalases was found to vary from 0.3 to 0.5.

4. The CO-haemochromogens of these catalase preparations had, in addition to the absorption bands in CO-protohaemochromogen, an absorption band in the same position as that of CO-cholehaemochromogen. Spectrophotometric analysis showed that the height of this band was proportional to the bile pigment content as found by the splitting with acids, and that the same values for the ratio of

(bile pigment Fe)/(bile pigment Fe + haem Fe) were found by both methods. This shows that the bile pigments are derived from a bile pigment haemoprotein.

5. If the presence of inactive haem-free protein in the impure preparations is taken into consideration, it can be shown that the specific activity (Kat.-f.) increases with decreasing bile pigment haem content, being about 50,000 for catalase containing protohaem only, and nil for the bile pigment haemoprotein.

6. A theoretically sound method for measuring catalase activity is described, which gives also the rate of destruction of the enzyme by H_2O_2 .

7. Ascorbic acid inhibits catalase by accelerating the destruction of the enzyme by H_2O_2 .

8. By the action of H_2O_2 on catalase in the presence of ascorbic acid, bile pigment haemoprotein is formed from protohaem catalase.

9. The significance of these findings for the explanation of the bile pigment haem content in liver catalase is discussed.

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The Technique of the Biological Vitamin A Assay

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The publications of Burn [1937], Irwin [1937], Coward [1938] and Bliss & Marks [1939], to cite a few of the more important papers, testify to the existence of a good measure of agreement on the

general principles of the graded-response biological assay. At the same time assays of any particular substance usually necessitate the use of some *ad hoc* technique. The rat-growth (curative) vitamin A

assay is a notable example, and we here outline recent developments in the conduct of this assay in our laboratory.

Some special considerations regarding the vitamin A assay are listed below:

(i) The criterion of vitamin A depletion in the experimental animals is cessation, or marked retardation, of growth, a condition liable to cause secondary reactions that may jeopardize the response to vitamin A.

(ii) The useful range of the growth-response curve is limited at its lower end by an abrupt fall at the point where the dose is just insufficient to prevent the onset of a pathological state, with consequent invalidity of response, i.e. the curve does not rise smoothly from zero.

(iii) If the behaviour of the rats during the depletion period is 'good' there will be occasional cases of death or loss of weight. There is no biometrically acceptable method of incorporating the records of such animals into the assay: if the rats die during the test period there is clearly no alternative to the somewhat unsatisfactory procedure of, in effect, ignoring the existence of the behaviour records; and because of this, the records of rats that lose weight have also, *a fortiori*, to be set aside. Otherwise we should have the paradox that while a negative growth pulled down the group average, loss of weight culminating in early death left the average undisturbed. It is therefore desirable that the assay conditions be such that there is a maximum number of positive growth responses at all dosage levels.

(iv) The International Standard is not vitamin A but the provitamin β -carotene. Some assurance that the two substances give a common kind of response under all conditions of the assay is therefore desirable, i.e. evidence of the constancy of the conversion of β -carotene to vitamin A is required. There is therefore need to demonstrate a substantial identity between the normal variations of response, and between the constants of slope of the curves of response, to Standard and test materials.

The inaugural work on our assay technique has been described [Morgan, 1934]. Minor changes have since been introduced, e.g. the vitamin A-deficient diet now in use is:

	%
Casein A/E	10
Coconut-cake meal	30
Flaked rice	48
Dried yeast	8
Steenbock's no. 40 salt mixture	4
	100

The addition of vitamin D has been shown to be unnecessary with this diet. The animals (bucks only) become depleted of vitamin A at the age of about 50 days, litters always *en bloc*, so that the members of each litter are started on the 3-week test

on the same day. Typical average behaviour on the diet is portrayed in simplified form in Fig. 1.

Morgan [1934] found that for the then existing regime the logarithm of the dosage was linearly related to the square of the growth response. In practice the differences between the squares of

Fig. 1. Composite growth curves of 834 recent test rats.

Curve A. From weaning to vitamin-A-depletion point.

Curve B. Test period; positive controls; on ± 20 i.u./day.

Curve C. Test period; on 2 i.u./day.

Curve D. Test period; on 1.2 i.u./day.

Curve E. Test period; negative controls; no vitamin A.

response in weight-matched, isogenic, Standard (T):test substance (S) pairs were taken as metameters* with which to calculate directly $\sigma_{(S-T)}$ and to evaluate the 'dose ratio' (S/T), and hence the test-material potency, by substitution in

$$G_S^2 - G_T^2 = b \log S/T.$$

Until recently we have continued to use this equation. At approximately 6-monthly intervals the accumulated test data, covering a dosage range of about 0.5–3.0 i.u./day, were summarized to check the validity of the equation and to recalculate b . There is, of course, no *a priori* reason why the growth:log-dose curve should be linear in the vitamin A, or in any other, biological assay; the important consideration is whether accuracy is enhanced by the employment of a precise equation rather than the convenient linear approximation.

One of the consequences of the above technique was that the necessity of pairing left an unusable residuum of 10–15% of the animals. This wastefulness caused us to investigate the rationale of rat selection; at the same time the biometry of the assay was reviewed, special attention being given to the stability of equation constants.

* Metameter—the measurement used in a biological assay (see *Biochem. J.* 1942, 36, 411, footnote).

arbitrary. The choice of a dosage interval is influenced by the fact that the smaller it is the less frequently will responses fall outside the critical range (this is especially important in assays of materials that are unexpectedly high or low in potency), whereas the larger the interval is the more sensitive and, other things being equal, the more accurate the assays will be. Our values are a working compromise arrived at by accumulated experience coupled with certain dosimetric considerations.

The object of the scheme is to have available at the end of the test the following four, equally weighted, isogenic pair-difference means of growth response:

$$\left. \begin{array}{l} (S_{1.2} - T_{1.2}) \text{ and its s.e.} \\ (S_{2.0} - T_{2.0}) \text{ and its s.e.} \\ (S_{2.0} - S_{1.2}) \text{ and its s.e.} \\ (T_{2.0} - T_{1.2}) \text{ and its s.e.} \end{array} \right\} \begin{array}{l} \text{hence overall } (S - T) \pm \epsilon_{S-T} \\ \\ \\ \text{hence overall } b \pm \epsilon_b \end{array}$$

Concentration upon pair differences of response as the metameters *ipso facto* eliminates interlitter variations, and the given arrangement enables this to be done thoroughly despite the limitations and variations of litter size. From the data the assay and the approximate error (incorporating the error of the slope *b*) can readily be calculated. Furthermore, in a series of assays, fluctuations of *b* can be followed and any significant differences between the *b*'s of Standard and test material detected.

More than 30 assays have been carried out to date on this plan. In several of the larger ones enough tetrad (four buck) litters were incorporated to permit analysis of variance of growth response and of covariance of initial weight and growth response. Nine such calculations have been made (Table 2).

Note, first, the renewed emphasis on interlitter variations; secondly, that there is further evidence of the negligible influence of initial weight on subsequent growth response; thirdly, that in only one case, that of assay 454, does departure from parallelism approach statistical significance, i.e. the (*P* = 0.95) level of variance ratio for the particular circumstances is 4.2, whereas in this assay it is, at most, 4.8; fourthly, that in all cases but one, variations due to slope are highly significant, assay 465 being a borderline exception.

For the purposes of statistical analysis these part assays have each been evaluated from 'internal' estimates of *b*. In practice, however, this is unsatisfactory. The sampling error of a single-assay estimate of the slope is large. And though the error of an estimate from a number of assays is, of course, correspondingly small, the use of such an estimate would be justified only if it were proven that absolute changes in *b* did not take place within the covered period of time. In the case of these rats it was found that absolute changes did take place

Table 2. Analysis of variance and covariance of growth response (GR) and initial weight in nine vitamin-A part-assays composed of isogenic tetrads of buck rats on four different doses, two of standard (S) and two of test material (T)

Assay no.	No. of isogenic tetrads	Calculations based on GR taken (a) alone and (b) adjusted for init. wt.		Residual σ of GR	Variances of GR due to three specified factors expressed as multiples of the corresponding variances due to residual error			Coefficient of regression of GR on init. wt.	'Unadjusted' assay as % of 'adjusted' assay	Upper limit of error (P=0.95) expressed $\equiv$ 10 pairs %
					Diff. between litters	Slope b	Departure from parallelism			
454	11	(a)	4.66	2.44	26.76	4.28	- 0.563	96.3	{140.1 143.6	
		(b)	4.36	2.80	24.73	4.82				
455	17	(a)	4.79	3.44	20.77	0.04	- 0.125	101.7	{150.0 149.6	
		(b)	4.78	3.53	20.95	0.06				
459	7	(a)	3.93	13.77	9.19	2.23	- 0.123	99.8	{160.6 158.5	
		(b)	3.95	13.89	9.74	2.13				
460	9	(a)	5.73	2.29	11.78	0.12	- 0.201	105.4	{151.2 141.6	
		(b)	5.30	1.43	15.84	0.08				
465	9	(a)	7.35	2.06	4.35	0.02	- 0.115	100.9	{206.7 211.4	
		(b)	7.50	2.26	4.04	0.02				
469	11	(a)	6.62	3.53	53.17	0.03	+ 0.297	102.3	{133.5 131.1	
		(b)	6.55	3.99	57.54	0				
472	9	(a)	7.53	2.25	23.91	0.11	- 0.253	105.8	{140.9 134.9	
		(b)	7.58	1.55	28.29	0.11				
474	10	(a)	6.96	1.78	40.46	1.40	- 0.126	100.2	{125.8 125.7	
		(b)	7.02	1.63	40.62	1.00				
481	9	(a)	4.73	2.11	25.39	1.89	- 0.900	97.7	{133.0 135.5	
		(b)	4.77	2.13	23.22	1.78				
Weighted means (92 total)		(a)	5.89	3.44	24.89	1.05	- 0.142	101.1	{150.0 149.4	
		(b)	5.85	3.44	25.85	1.05				

and it was therefore thought advisable to use the weighted mean of the estimate of b for any given assay plus those of the few (latterly two) immediately preceding assays: a balance is thereby struck between avoidance of short-term apparent variations of b on the one hand and recognition of real variations over longer periods on the other. Since the introduction of the new regime, the moving averages employed have revealed definite variation trends. All these averages are charted in Fig. 2.

Fig. 2. Variation in b , the constant of slope in the rectilinear growth/log-dosage equation during the current year. Mean and limits of S.E. are given. The whole constitutes an assay-to-assay moving average, each estimate being based on the data of the particular assay together with those of (generally) the two preceding assays—an average of 62 values. (By one ‘value’ of b is meant the estimate from one isogenic pair difference of response.) The mean interval between assays—and therefore between estimates of b —is 8 days.

It is clear from Fig. 2 that real variations of slope have occurred. If the component series of separate assay estimates is roughly trisected the following averages are obtained (Table 3):

Table 3. Variations in b , the constant of slope		
	Mean b	Standard error
Earliest group (158 values)	24.5	3.23
Middle group (173 values)	57.5	3.43
Latest group (157 values)	42.0	2.95
488 values	41.8	

It is easily demonstrable that any one of these three means is significantly different from the other two. Thus there is danger in using a fixed b over even a few months.

Long-period fluctuations in b are of interest. The mean annual estimates over the past few years (re-

calculated from the records, because until recently the ‘square-growth’ b was in use) are given below, together with the responses to 2 units/day and the mean ($P=0.95$) error of a 10-pair assay in which the growth responses to nominally equivalent dosage levels of Standard and test material are equal and the calculated dose-ratio is therefore unity. Under these conditions the error is at a minimum.

Table 4. Annual fluctuations in certain mean values in vitamin A assays				
Year	Mean growth on 2 units Standard g.	Mean b from isogenic pair-differences only (No. of values)	S.D. of pair-differences of growth $\sigma_{(S-T)}$	Upper limit of error ($P=0.95$) for a 10-pair assay with equal S and T growth responses %
1936	45	48 (141)	7.5	125
1937	51	43 (135)	7.2	127
1938	48	52 (233)	9.6	130
1939	41	36 (107)	10.5	152
1940	38	30 (142)	6.8	138
1941	50	54 (104)	7.0	120
1942	41	42 (488)	9.0	136

(7½ months)

In the first 7½ months of 1942 the estimation of b has been made from more than twice as many values as in any previous year. Future variations of all important factors will be followed with correspondingly greater accuracy. The highest average error for any one year for ($P=0.95$), 3-weeks, 10-pairs, is 66 to 152 %, which is a little lower than the British Pharmacopoeia error for a 40-pair assay (63–159 %).

Finally, the records of assays carried out on the new plan have yielded information on the comparability of estimates of b from Standard and from test materials. Altogether there were 24 assays, involving 416 values, in which β -carotene Standard was compared with test-materials in which vitamin A itself supplied the whole of the vitamin A activity (Table 5).

Table 5. Comparison of estimates of slope from response to β -carotene and to vitamin A					
	No. of values	Mean b	S.E.	Variance	Degrees of freedom n
β -Carotene (S)	221	43.6	2.69	1601	197
Vitamin A (T)	195	39.6	3.02	1781	171
Difference		4.0	4.05		
Variance ratio = 1.112. $z = \frac{1}{2} \log_e 1.112 = 0.0531$.					
S.E. of $z = \sqrt{\frac{1}{2} (1/n_S + 1/n_T)} = 0.0739$.					

Neither the two b 's nor the variances are significantly different; thus an important condition of satisfactory assay work is fulfilled. This conclusion depends to some extent on the mean Standard: test-

material dose-ratio of the component assays, because in a series in which the mean dose-ratio is much higher or lower than unity a systematic difference due to departure from parallelism may invalidate the result. The mean dose-ratio of the above 24 assays is sufficiently close to unity, being approximately 1.1.

SUMMARY

(i) For the vitamin A-test rats in this laboratory interlitter variations in response are large enough to justify strict adherence to isogenic comparisons.

The ratio of the numbers of isogenic or anisogenic pairs required for equal accuracy is about 1 : 2.

(ii) A new assay plan for routine work is described. A moving average estimate of b , the constant of slope, is employed.

(iii) Real variations in b are found to occur over comparatively short periods.

(iv) The b 's for vitamin A and β -carotene are shown to be substantially similar.

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Further Studies of Cryptogam Tissue Proteins: Protein Composition and Tissue Function in Plants

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In an earlier article [Lugg, 1940] the amide and some amino-acid contents of the photosynthesizing tissues of *Lunularia cruciata*, *Pteridium aquilinum* and *Selaginella uncinata*,* and similar data for leaf protein of *Trifolium subterraneum*, were reported. It was pointed out that the amide, tyrosine and tryptophan contents revealed no marked changes in the compositions of the proteins to correspond with the different degrees of evolutionary development of the three cryptogams and of the phanerogams (i.e. *Trifolium subterraneum*) which have been examined.

In this article, the results of further analytical work with the same protein preparations will be reported. Some data are given for one of the extracted protein preparations from pinnae of *Pteridium aquilinum*, and the protein in the thalli of *Ulva lactuca*.

METHODS

The protein preparations. With the exception of the *Ulva lactuca* preparation, the protein prepara-

tions and the tissues from which they were obtained, have been described [Lugg, 1940].

The *U. lactuca* preparation was made from thalli collected on 11 April 1941 from a rocky ledge in the Southern Ocean near Port Elliot. The ledge was completely submerged at high water but merely washed over by the waves at low water. The thalli, thoroughly wet with sea water, were examined about 36 hr. after collection. They did not differ in general appearance from freshly collected material, nor appear more flaccid. They were rinsed with distilled water and pressed between sheets of blotting paper. After being weighed, they were dried at 65° in a current of air and then powdered, prior to removal of non-coagulable and other non-protein nitrogenous substances [Lugg, 1939]. Complete drying was effected by heating at 105° for about 8 hr. and exposing at low pressure over H_2SO_4 . Nitrogen estimations were made by the Kjeldahl method.

The fresh thalli contained 15.8% dry material consisting of 1.18, 0.90 and 0.83% of 'total', 'coagulable' and 'protein' N respectively. The dried 'whole protein preparation' contained 2.04% N, and 11.5% ash (by ignition in air at about 600°). The ash contained large amounts of $CaSO_4$.

Partial analyses of the protein preparations. The

* Previously reported as '*Selaginella* sp. (probably *Jungermannioidea* group)'. I am indebted to Prof. J. G. Wood for completing the identification of this plant. *S. uncinata* is not in the *Jungermannioidea* group.