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

By J. W. H. LUGG, *From the Nutrition Laboratory, Division of Animal Health and Nutrition, Commonwealth Council for Scientific and Industrial Research, at the University of Adelaide, South Australia*

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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 *Jungermannioides* group)'. I am indebted to Prof. J. G. Wood for completing the identification of this plant. *S. uncinata* is not in the *Jungermannioides* group.

amide, tyrosine, diiodotyrosine, tryptophan, cystine plus cysteine and methionine contents of the protein preparations were estimated by methods described in earlier articles [Lugg, 1937; 1938 *a, b, c*]. The arginine, histidine and lysine contents of one of

cluding hydrolysis) employed in the estimations [Lugg, 1938 *a, b*; Tristram, 1939].

In Table 1 are shown, in the order of the columns, the characteristic label of the preparation consisting of a number followed by W, E or R, signifying

Table 1. *Partial analysis of the plant-protein preparations by the method of Lugg (see p. 133)*

Preparation (see text)	Total N (%)	% total N							SO ₄ -S × 100/N	Ash
		Amide	Tyr.	Diiodo- tyr.	Try.	Cyst.	Meth.	Cyst. + meth.		
0W <i>Ulva lactuca</i> (thalli)	2.04	5.59	1.34	0.0	1.01	2.2	1.2	3.4	124.5	11.5
1W <i>Lunularia cruciata</i> (thalli)	3.92	5.52	2.43	0.0	1.70	1.31	1.36	2.67	0.51	3.5
2W <i>Pteridium aquilinum</i> (pinnae)	5.82	5.12	2.43	0.0	1.70	1.31	1.36	2.67	0.51	3.5
2E "	11.1	4.97	2.08	—	1.13	1.11	1.71	2.82	0.02	1.4
2R "	2.70	5.14	2.20	—	1.13	1.20	1.65	2.85	0.37	1.6
2E1 "	12.0	4.92	2.14	0.0	1.19	1.16	1.74	2.90	0.06	1.3
3W <i>Selaginella uncinata</i> (leaves and stems)	3.12	5.31	2.60	0.0	1.43	1.22	1.49	2.71	0.64	9.9
4W <i>Trifolium subterraneum</i> (leaves)	7.17	5.29	2.53	—	1.75	1.15	1.32	2.47	0.08	0.7
4E "	14.1	5.35	2.64	—	1.62	1.13	1.38	2.51	0.00	0.4
4R "	5.26	5.39	2.64	—	1.67	1.12	1.29	2.41	0.00	3.1

Estimations unaffected by presence of carbohydrate. Amino-acids are given as percentage of total N.

Percentage errors, from replicate estimations in present and earlier work; N in dry preparation 0.5, amide 1.5–2, tyrosine 2–3, tryptophane 3–4, cystine + cysteine 3–5, but 20 for 0W, methionine 4–6, but 30 for 0W; sum of last two 2–3, but 10 for 0W. The error for arginine, histidine and lysine estimations from the work of Tristram [1939].

the extracted protein preparations from pinnae of *Pteridium aquilinum*, were estimated by Tristram's [1939] methods. From tests which have been recorded with the descriptions of these methods of estimation, it seems probable that the considerable impurities in the protein preparations would not have vitiated the results obtained (Table 1). Estimations by the other methods (Table 2) are vitiated

respectively that the preparation was made from whole tissue, a tissue extract, or from tissue residues after extraction of some of the protein, followed by the name of the plant and tissue from which the preparation was derived, and the analytical data.

Most of the amide, tyrosine, diiodotyrosine and tryptophan contents have been already reported [Lugg, 1940], and the preparations are numbered as before. The *Ulva lactuca* preparation has the number 0. The numbers are in the order of what is customarily accepted as the increasing degree of evolutionary development of the plants.

Table 2. *Partial analysis of the protein preparations by other methods (see p. 133)*

	HI digestion and titration		Tristram [1939] (see p. 134)		
	Cyst.	Meth.	Arg.	Hist.	Lys.
2E1	1.03	1.80	13.5*	2.4	6.45
4E	0.93	1.16	—	—	—

Estimations affected by the presence of carbohydrate and not corrected for the effects.

Amino-acids are given as percentage of total N. Corrections were applied according to Tristram [1939]. Arginine + 2.6 mg. N in addition to solubility correction of Ag. arginine. Histidine + 0.3 mg. N. Lysine + 1.6 mg. N. Hydrolysis factors 1.06, 1.03 and 1.07 respectively.

* This value increased to 15.3 when corrected for effects of presence of carbohydrate impurity.

to some extent by impurities such as carbohydrates, and sometimes by small amounts of prosthetic carbohydrate. In both tables the amino-acid contents have been corrected for such losses as the pure free amino-acids undergo in the procedures (in-

DISCUSSION

From comparison of the partial compositions of 'whole', 'extracted' and 'residual' protein preparations (Table 1), the conclusion was drawn that 2E, 2E1 and 4E were reasonably representative of the 'whole' proteins in the tissues from which they were extracted, and the analyses shown in Table 2 are, therefore, assumed to be analyses of representative proteins, but they are undoubtedly subject to systematic errors, because such preparations contain impurities of a carbohydrate nature (e.g. pentosan or mucilage).

Lugg [1938*b*] found that these impurities affected the estimations of cyst(e)ine and methionine by the HI digestion procedure differently with different protein preparations and that the differences were

irregularly related to the amounts of impurity*, the standards of reference being estimations by the differential oxidation procedure. Thus corrections could not safely be attempted, and the results are only imperfect checks upon the values obtained by the less specific differential oxidation procedure. Note that the methionine content of 2E1 was actually slightly higher when estimated by the former procedure than by the latter. This may have been due to the presence of an impurity in the HI digest capable of reacting as methionine does in the titration procedure, but random errors could easily account for the discrepancy. Nearly all the cyst(e)ine plus methionine estimated by the differential oxidation procedure could be accounted for by the HI digestion procedure in this instance, which is unusual with preparations as impure as 2E1 appears to be (see below).

Tristram [1939] found that the influence of these impurities upon the estimations of histidine and lysine was irregularly related to the amount of impurity, but was small when the latter was not overwhelmingly large. For amounts of impurity such as those in 2E1, he considered the effects too small and uncertain to justify corrections. He found, however, in the estimation of arginine that the effects of small amounts of impurities were sufficiently regular to warrant the use of a correction factor calculable from the estimated percentage impurity.

Two methods of estimating the amount of impurity in preparations like 2E1, or of estimating the percentage N in the hypothetically 'pure' proteins, have been discussed elsewhere [Chibnall, 1939]. They are (1) computation from extensive amino-acid analyses of an impure preparation, and (2) calculation from the amount of humin precipitated at pH 2.5 after acid hydrolysis on the assumption that the impurity has behaved like a pentosan. These methods lead to substantially the same results when applied to extracted preparations from *Dactylis glomerata*. Since the leaf proteins of all phanerogams so far examined have very similar compositions, there is some justification for the assumption that the pure proteins all contain about 17% N. The pinna protein of *Pteridium aquilinum* has not been sufficiently examined to permit the use of method (1) except in a very loose way, i.e. since from Table 1 it corresponds fairly closely in composition with the leaf proteins of the phanerogams examined, it too probably contains about 17% N, if pure. Data for method (2) were obtained from both 2E and 2E1, which yielded respectively, 28.2 and 24.8% of 'humin', containing 7.4 and 6.9% of the protein N.

* Smith & Wang [1941], ignoring these complications, have contended from their analyses of pasture protein preparations by this procedure, that the protein composition varies somewhat with season.

Calculations from these data (ignoring ash) suggest that the 'pure' preparations would have contained 17.8 and 17.4% N respectively and it seems reasonable to accept the rounded value, 17%. The actual preparation must have contained 30% impurity, and from Tristram's [1939] correction table, the correction factor to be applied to the arginine content reported in Table 2 is 1.20/1.06. The corrected value is shown in a footnote to the table.

In Table 3 the partial composition of the proteins in the somatic photosynthesizing tissues of the cryptogams and those of various phanerogams are compared. The arginine estimations are corrected for impurities. The plants are grouped into families, orders, classes and divisions in the natural classification. The divisions have been arranged from left to right in the sequence of increasing evolutionary development, and the sequence has been maintained, as far as current evolutionary theory permits, down to the genera. Some of the extracted protein preparations are known not to have been fully representative of the tissue proteins, but they probably did not differ from them very much. These data have been drawn from material in the present article and from analyses by Lugg [1938c; 1939] and Tristram [1939]. Mean values are quoted where analyses have been made of more than one preparation from any particular plant species.

The striking feature of Table 3 is that among representatives of the bryophyte, pteridophyte and spermatophyte divisions, differences in tissue protein composition are small, the greatest variability being in the tryptophan content. The tissue protein of *Pteridium aquilinum* has a lower tyrosine and tryptophan content than those of the other plants belonging to these three divisions, and as compared with these its methionine content is rather high relative to its cyst(e)ine content; but in its amide, arginine, histidine and lysine content it is very similar to the others.

Unfortunately the cystine and methionine contents of the tissue protein of the only representative of the thallophyte division (*Ulva lactuca*) are subject to considerable errors, owing to the 2.5% of sulphate-S in the preparation. This was present possibly as sulphuric acid esters of substances of carbohydrate nature. The possibility of the presence of nitrogenous substances other than protein, in preparations like 0W, cannot be ignored. However, the rate of production of NH_3 by hydrolysis (in the amide-N estimation) was very similar to those found for phanerogam leaf-proteins [Lugg, 1938c], and the presumption is that non-protein nitrogenous substances had been removed completely. The cystine content of the tissue protein is rather high and the tryptophan rather low, in comparison with those of the other proteins, and though there is nothing unusual in the amide content, the tyrosine

content is decidedly low. Many marine algae (notably members of the class Phaeophyceae) contain fairly large amounts of iodine compounds, but this *U. lactuca* preparation (like the other preparations examined) contains no diiodotyrosine.

bility than are revealed in Table 3. It is not known whether the composition of whole proteins of cryptogam tissues are as independent of climate, locality of growth, manurial treatment and tissue age, as are those of the phanerogams. Small varia-

Table 3. *Partial compositions of the proteins of the somatic, photosynthesizing tissues of plants*

Division	Thallophyte	Bryophyte	Pteridophyte		Spermatophyte				
Subdivision	Algae	Hepaticae			Angiospermae				
Class	Chlorophyceae				Monotocyledon	Dicotyledon			
Order	Ulvales	Marchantiales	Filicales	Lycopodiales	Glumiflorae	Centro-spermae	Rosales		
Family	Ulvaceae				Gramineae	Chenopodiaceae	Leguminosae		
Genus and species	<i>Ulva lactuca</i>	<i>Lunularia cruciata</i>	<i>Pteridium aquilinum</i>	<i>Selaginella uncinata</i>	<i>Dactylis glomerata</i>	<i>Lolium perenne</i>	<i>Beta cicla</i>	<i>Trifolium subterraneum</i>	<i>Trifolium pratense</i>
Amide	5.59	5.52	5.12	5.31	5.07	5.22	5.28	5.39	5.40
Tyrosine	1.34	2.43	2.06	2.60	2.48	2.45	2.63	2.53	2.50
Diiodotyrosine	0.0	0.0	0.0	0.0	0.0	—	—	—	—
Tryptophan	1.01	1.70	1.14	1.43	1.84	1.84	1.50	1.75	1.70
Cyst(e)ine	2.2	1.31	1.14	1.22	1.40	1.44	1.45	1.15	1.25
Methionine	1.2	1.36	1.63	1.49	1.54	1.53	1.43	1.32	1.29
Arginine	—	—	15.3	—	14.6	14.0	12.8	—	14.9
Histidine	—	—	2.4	—	2.4	2.4	2.25	—	2.3
Lysine	—	—	6.45	—	6.1	6.0	6.7	—	6.5

Estimations corrected where necessary for carbohydrate impurities. Values in italics are for extracted protein preparations; those in ordinary type for whole protein preparations.

Amino-acids are given as percentage of total N.

Little work has been done with cryptogams other than unicellular organisms like yeasts and bacteria. Mazur & Clarke [1938] studied the amino-acid composition of the nitrogenous substances not extracted by alcohol from the tissues of various marine algae. In thalli from one specimen of *U. lactuca* they found that 5.5% of the remaining N was amide-N; and in this and another specimen, that 0.0 and 0.0% was tyrosine-N, 0.3 and 0.6% was tryptophan-N, 1.8 and 2.1% was cyst(e)ine-N, and 0.0 and 0.0% was methionine-N. Except for the amide and cyst(e)ine content, these results are very different from those reported in Table 1. Of the N remaining after alcohol extraction in tissues of *Osmunda claytoniana* (pteridophyte), the same authors found 0.6% cyst(e)ine-N, 1.1% methionine-N, 7.6% arginine-N, 3.7% histidine-N and 3.6% lysine-N. These results again differ considerably from the values in Table 1. Mazur & Clarke's analyses were actually analyses of the nitrogenous substances dissolved out by hot formic acid solution from the alcohol-extracted tissues, and it is not certain that the formic acid dissolved out unaltered protein.

The amounts of those amino-acids which were not estimated may exhibit different degrees of varia-

tions have been reported by Chibnall [1939], Lugg [1938 *a, b*; 1939] and Tristram [1939] for the whole proteins of the leaves and for preparations believed to be fairly representative, but in regard to age of tissue the sources of material included neither seedlings at the one extreme nor senescent plants at the other. Barrien & Wood [1939] and Hanson, Barrien & Wood [1941] have shown that the N:S ratios in their protein preparations from Sudan grass (*Andropogon sudanensis*) were higher for seedling leaves than for older leaves. Their S estimations, however, included any sulphate-S which may have been present. Reference has been made above to a claim by Smith & Wang [1941], of variation with season, but there is, as yet, no conclusive evidence that the composition of the whole leaf protein of any phanerogam varies markedly with changes in the conditions enumerated.

Phanerogam tissues contain at least two proteins of somewhat different composition. These have been tentatively referred to the fluid protoplasm and vacuole contents on the one hand, and to the granular inclusions like chloroplasts, chondriosomes and nuclei on the other [Lugg, 1938 *a, b*; 1939]; or to chloroplasts on the one hand, and the rest of the protein-containing morphological units on the

other [Barrien & Wood, 1939; Hanson *et al.* 1941]. Chibnall [1939] showed that vacuole protein may differ somewhat in composition from the proteins of the fluid and granular parts of the protoplasm.

In these somatic, photosynthesizing tissues two well-defined types of process occur—the ordinary living processes of the cell, and those processes intimately connected with photosynthesis—and it is possible that the physical and chemical properties and compositions of the proteins associated with these processes vary very little. Perhaps also the proportions of the different proteins differ little in different species, different climates, with different ages of tissue and so on.

It is generally agreed that present-day bryophytes, pteridophytes and spermatophytes represent increasing degrees of evolutionary development from a common ancestor; and there is no reason for supposing either that the basic living processes, or that the photosynthesizing processes, have changed radically, either in the gametophyte or the sporophyte generations of the plants. These considerations are in harmony with the observation recorded in this article, namely, that the 'whole' proteins of the gametophyte tissue of bryophytes, and of the sporophyte tissue of pteridophytes and spermatophytes, appear to be of similar composition. It is generally accepted that the Chlorophyceae (Thallophyte) are in the same main branch of evolutionary descent as the green land-plants [Bower, 1929; 1935; Fritsch, 1929]. It is likely that the *Ulva lactuca* thalli belong to both the sporophyte and the gametophyte generations of the plant [Föyn, 1929]. Possibly the individual proteins in the thalli of *U. lactuca* are similar to those of these other plants, but occur in very different proportions. The radical evolutionary step by which the tissues were transferred from a water to an air environment may have demanded modification of the composition and physical properties of individual proteins. It would be of interest to discover, from analyses of tissue proteins of sea phanerogams, whether the reverse step has required any modification of protein composition.

In sharp contrast to what appears to be the relative uniformity of composition of 'whole' proteins of the somatic photosynthesizing tissues of the bryophytes, pteridophytes and spermatophytes, is the non-uniformity of composition of proteins in tissues characteristic of the spermatophytes alone, namely, the seeds. Since the seedling depends for its early growth upon the seed proteins, these should correspond fairly closely in composition with the leaf proteins. This is sometimes not the case. True, satisfactory analyses have been made only of the proteins of angiosperm seeds, and these appear to

be elaborated in cells possessing $3n$ chromosome nuclei, but in regard to tissue protein composition no particular significance can be attached at present to chromosome number (cf. tissue protein composition of *Lunularia cruciata* possessing n chromosome nuclei, with those of the pteridophytes and spermatophytes possessing $2n$ chromosome nuclei). Leaf proteins appear to be of 'fibrous' type and are very readily denatured, whereas many seed proteins are of 'globular' type and can withstand prolonged periods of partial desiccation at relatively high temperatures. Possibly biological fitness for survival might otherwise be assured by fairly close correspondence of composition, whereas denatured seed proteins may be of little use to young seedlings; resistance to denaturation of seed proteins may be almost a prime criterion of fitness at the present stage of evolutionary development of the spermatophytes, an unfavourable composition of the proteins from the point of view of seedling nutrients being in comparison a small disadvantage.

The comprehensive analysis of a single protein preparation is laborious, and there are many species of pasture plants. The object of the investigation here described was to extend, if possible, the basis of the belief that for the purposes of animal nutrition studies, comprehensive analyses of pasture proteins might well be confined in the first instance to those of only a few species representative of the most important families of pasture plants. It is considered that the investigation has substantially broadened the basis for that belief.

SUMMARY

Partial analyses have been made of the 'whole' proteins of the somatic, photosynthesizing tissues of a variety of cryptogams (a thallophyte, *Ulva lactuca*; a bryophyte, *Lunularia cruciata*; and two pteridophytes, *Pteridium aquilinum* and *Selaginella uncinata*), and also of one more phanerogam (*Trifolium subterraneum*).

The apparent similarity of composition of tissue proteins of plants from the bryophytes upward through divisions exhibiting increasing degrees of evolutionary development, has been contrasted with the pronounced lack of uniformity in the compositions of seed proteins. An attempt has been made to interpret the findings in terms of hypothetical relationships between tissue function and environment, and protein composition and physical and chemical properties.

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A Simple Fluorescence Comparator and its Application to the Determination of Porphyrin

By C. RIMINGTON, *With an addendum by E. SCHUSTER, From the National Institute for Medical Research, London, N.W. 3*

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Several substances of biochemical interest are either themselves fluorescent in radiation of suitable wavelength, or are readily transformed quantitatively into fluorescent materials. Among these may be mentioned the porphyrins, stercobilin and related pigments, flavins, including vitamin B₂, certain chemotherapeutic drugs and aneurin.

Quantitative methods based on the measurement of fluorescence intensity have the advantages of being highly specific and extremely sensitive, enabling very small quantities of material to be determined with accuracy once they are suitably extracted. Such methods suffer, however, from the disadvantage of requiring, for accurate results, relatively expensive apparatus such as the combination of an ultraviolet source with the Zeiss or Pulfrich photometer or some type of photoelectric colorimeter.

For the purposes of a particular investigation it became necessary to carry out a number of quantitative determinations of porphyrin in single specimens of urine, each containing about 2 μ g. of porphyrin. Only measurements of fluorescence intensity were sufficiently sensitive, and in order to bring the method within the reach of the small medical or factory clinic an inexpensive fluorimeter or fluorescence comparator was essential which, acting very much like an ordinary colorimeter, would give results of sufficient accuracy for general purposes. It was realized that, with suitable adjustments, the instrument would serve for the determination of a number of fluorescent materials in addition to porphyrins, e.g. vitamins, drugs etc., as noted above. Its construction, and operation with porphyrin solutions, will be described in the

present paper, whilst other applications will be dealt with in subsequent communications.

Requirements of a fluorescence comparator

The essential requirements of a fluorescence comparator are:

- (1) An easily operated source of ultraviolet radiation with suitable filter to remove visible light.
- (2) An optical system to conduct the visible fluorescent light into the eyepiece. This should not be in the same axis as the ultraviolet source. The emergent fluorescence is collected in the present instrument at an angle of 55° to the ultraviolet beam and is reflected by a system of mirrors into the eyepiece, the optical path being approximately equal to the distance of normal vision, so that lenses may be dispensed with. A suitable filter is interposed, the transmission of which covers a suitably narrow band in the region in which the fluorescence lies. With porphyrins, Ilford spectral filter no. 607 (orange) transmitting 595–650 $m\mu$ is used.
- (3) A device for altering the intensity of one beam. In the present instance glass screens made by photographing a standard neutral-tinted wedge are used, and by adopting a reciprocating arrangement, rotation of a graduated knob brings about a gradual, but uniform, darkening of the field emanating from the standard. Matching is performed as in a colorimeter.

The construction and mechanical arrangements of the apparatus are described in the Addendum by Dr E. Schuster who has been responsible for the detailed design and who has kindly constructed the first four models produced.