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THE RELATIONSHIP BETWEEN ETHYLENE AND THE SYNTHESIS OF RNA AND PROTEIN IN RIPENING APPLES

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Abstract—The stimulation by ethylene of the respiration of whole fruits and peel discs prepared from them is followed using Worcester Pearmain and Cox's Orange Pippin apples. RNA and protein synthesis in relation to ethylene stimulation is investigated in terms of incorporation of ^{14}C-uridine and ^{14}C-valine into RNA and protein fractions in the discs of peel. Application of ethylene (40–60 ppm) to the preclimacteric fruit induces increased ethylene production in the fruit; uridine incorporation into RNA rises to a peak followed by a peak in valine incorporation into protein, while a malate decarboxylating system develops in the ripening fruit.

INTRODUCTION

EVER since it was shown[1] that there was a net increase in 'protein' during the development of the respiration climacteric in apples, interest has centred on enzyme changes over this period. Hulme *et al.*[2] showed that the activity of NADP-dependent malic enzyme and pyruvate decarboxylase increased during the climacteric period. Since ethylene production by 'climacteric' fruit commences immediately before the respiration begins to rise, it was clearly of interest to see whether ethylene production induces enzyme synthesis here, especially since it has been suggested that ethylene induces the synthesis of enzymes in other plant phenomena.[3] Frenkel *et al.*[4] showed that increased incorporation of ^{14}C-phenylalanine into protein of whole pears occurs in the early stages of the climacteric. Furthermore, the protein synthesis inhibitor, cycloheximide, inhibits ripening and this inhibition is not relieved by the application of ethylene. Implicit in this work is a connection between ethylene, ripening and protein synthesis.

Hulme *et al.*[5] working with discs of apple peel have shown that a sequence of events occurs in discs taken from fruit as it passes through the climacteric which is closely paralleled by a similar sequence in discs taken from preclimacteric fruit 'aged' for periods of up to 48 hr. This sequence is: ethylene production, increase in respiration, increased incorporation of ^{14}C valine into protein and, as the respiration continues to rise to a peak, the development of a NADP-dependent malate decarboxylating system. Rhodes *et al.*[6] showed that the development of this activity was accelerated by exposing the discs to ethylene.

If enzyme synthesis is occurring, then, presumably, various species of RNA must be involved; Looney and Patterson[7] found an increase in RNA, probably ribosomal RNA, during the climacteric period in yellow transparent apples.

Recently Sacher and Salminen[8] found no evidence for the stimulation of RNA and

[1] A. C. HULME, Rept. Fd. Invest. Bd., London, 1936, page 128 (1937).
[2] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Proc. Roy. Soc. B* **158**, 514 (1963).
[3] L. W. MAPSON and A. C. HULME, *Progress in Phytochemistry*, Vol. 2, p. 342 (1970).
[4] C. FRENKEL, I. KLEIN and D. R. DILLEY, *Plant Physiol.* **43**, 1146 (1968).
[5] A. C. HULME, M. J. C. RHODES, T. GALLIARD and L. S. C. WOOLTORTON, *Plant Physiol.* **43**, 1154 (1968).
[6] M. J. C. RHODES, L. S. C. WOOLTORTON and A. C. HULME, *Qualitas Plant. Mater. Veg* **XIX** 1-3, 167 (1969).
[7] N. E. LOONEY and M. E. PATTERSON, *Phytochem.* **6**, 1517 (1967).
[8] J. A. SACHER and S. O. SALMINEN, *Plant Physiol.* **44**, 1371 (1969).

protein synthesis in slices of pre-climacteric avocado and banana (peel and pulp) tissue by applied ethylene (30 ppm).

We have examined the effect of ethylene (40–60 ppm) on the activity of malic enzyme (ME) and two other enzymes—pyruvate decarboxylase and alcohol dehydrogenase—involved in the malate decarboxylation system, and on the incorporation of ^{14}C uridine into RNA and ^{14}C-valine into protein in two varieties of apples during the progress of the respiration climacteric. Our results are reported here.

RESULTS

The respiration rate (CO_2-output) and ethylene production of whole fruits kept throughout in air and those kept in air following the application of 40–60 ppm of ethylene for 3 days for the two varieties of apples are shown in Figs. 1a and 2a. The Worcester Pearmains (W.P.) were more mature (respiration climacteric and production of ethylene sets in earlier) than the Cox's Orange Pippins (C.O.P.) at the commencement of the experiment. The ethylene application triggers off both the respiration climacteric and autonomous ethylene production.

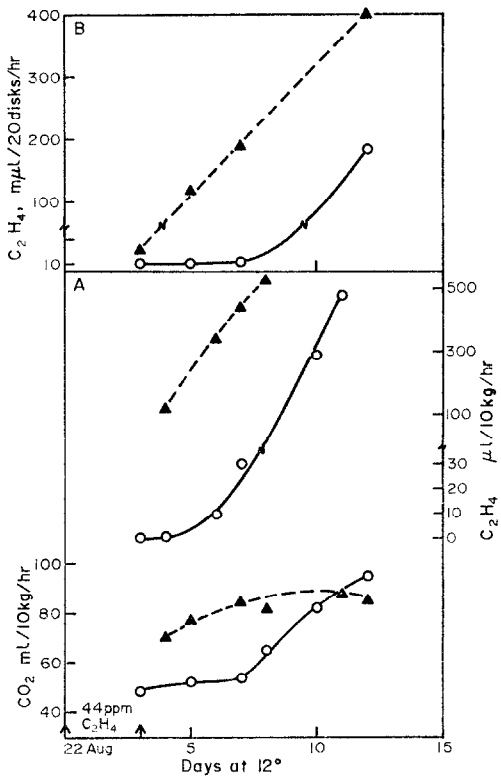

FIG. 1a. THE RATES OF RESPIRATION AND ETHYLENE PRODUCTION OF TWO SAMPLES OF PRECLIMACTERIC WORCESTER PEARMAIN APPLES STORED AT 12°, ONE STORED IN AIR (○——○) AND THE OTHER STORED IN THE PRESENCE OF 44 ppm OF ETHYLENE FOR 3 days AND THEN RETURNED TO AIR (▲——▲).

FIG. 1b. THE RATE OF ETHYLENE PRODUCTION BY DISCS OF PEEL TISSUE PREPARED FROM THE TWO SAMPLES OF APPLES.

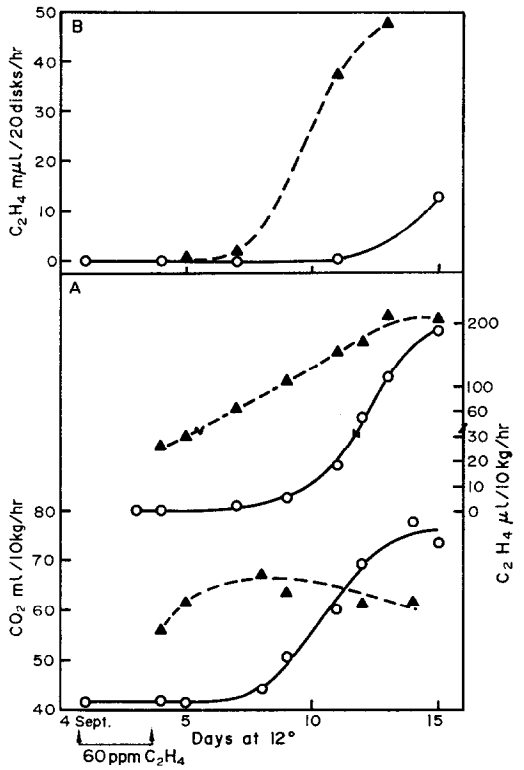

FIG. 2a. THE RATES OF RESPIRATION AND ETHYLENE PRODUCTION OF TWO SAMPLES OF PRECLIMACTERIC COX'S ORANGE PIPPIN APPLES STORED AT 12°, ONE STORED IN AIR (○——○) AND THE OTHER STORED IN THE PRESENCE OF 60 ppm ETHYLENE FROM THE SECOND TO THE FOURTH DAY OF STORAGE AND THEN RETURNED TO AIR (▲——▲).

FIG. 2b. THE RATE OF ETHYLENE PRODUCTION BY DISCS OF PEEL TISSUE PREPARED FROM THE TWO SAMPLES OF APPLES.

Evolution of ethylene by discs (Figs. 1b and 2b) from apples stored without ethylene treatment appears to lag behind the ethylene production of the fruit from which they have been excised. This does not mean, however, that very small amounts of ethylene do not appear *within the tissue* somewhat earlier: it almost certainly reflects the limits of detection of ethylene production by small quantities of tissue (the discs). Measurement of ethylene production by whole fruit was made daily whereas discs were only prepared at 4 stages of fruit development. These results do, however, indicate that the act of excision of tissue from the fruit does not cause an immediate burst of ethylene production, as is the case for some other tissues.[9]

The effect of adding malate (the malate effect) to discs of tissue taken from control and ethylene treated fruit at various stages in the climacteric are shown in Figs. 3a and 4a. The pattern followed the development of the climacteric in the whole fruit with the usual observation[2,5] that the full malate effect is not reached until after the peak of the respiration climacteric. Changes in the activities of malic enzyme (ME) pyruvate decarboxylase (PD)

[9] W. B. MACGLASSON, *Australian J. biol. Sci.* 22, 489 (1969).

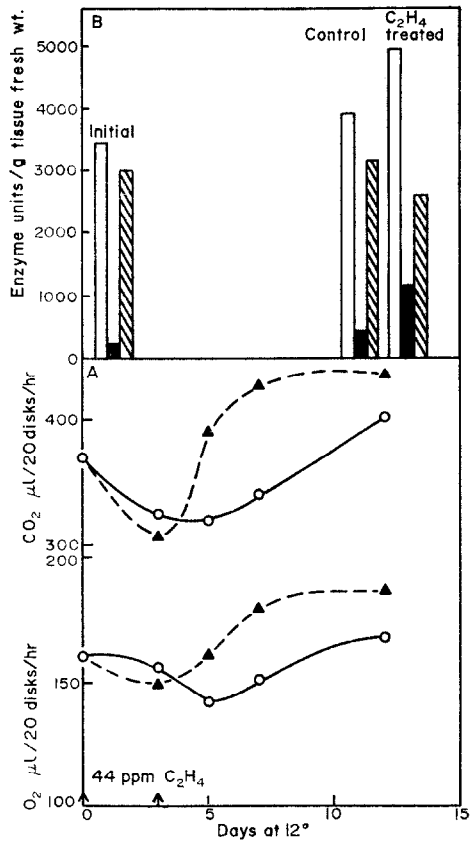

FIG. 3a. THE RATES OF O_2 UPTAKE AND CO_2 PRODUCTION IN THE PRESENCE OF ADDED MALATE OF DISCS OF PEEL TISSUE PREPARED FROM THE CONTROL AND ETHYLENE TREATED WORCESTER PEARMAIN APPLES.

FIG. 3b. THE ACTIVITIES OF ME, PD AND ADH IN EXTRACTS PREPARED FROM UNTREATED APPLES AT THE BEGINNING OF STORAGE AND FROM CONTROL AND ETHYLENE TREATED APPLES AFTER 12 days OF STORAGE.

and alcohol dehydrogenase (ADH) in the discs at the commencement of the experiment, and for both control and ethylene treated apples near the end of the storage period, are shown in Figs. 3b and 4b. ME and PD increase in the period and this increase is enhanced by the treatment with ethylene. Alcohol dehydrogenase activity shows little change in the control discs of Worcester Pearmain apples and only a small increase in the Cox's Orange Pippin tissue. Ethylene treatment, however, results in the activity of ADH being somewhat reduced towards the end of the storage period. All these results emphasise the difference in behaviour of control and ethylene-treated fruits.

The capacity for uptake of ^{14}C-uridine and of ^{14}C-valine into discs taken from fruits at the stages over the climacteric are shown in Table 1. In the more mature (when picked) Worcester Pearmain apples, ethylene caused a somewhat higher uptake of valine but no significant increase in uridine uptake in the early stages of the climacteric (see Figs. 1 and 2 for respiration changes in both sets of fruits). In the less mature Cox's Orange Pippin apples, ethylene has little effect on the uptake of valine until after the climacteric peak has been passed (Table 1 and Fig. 2a).

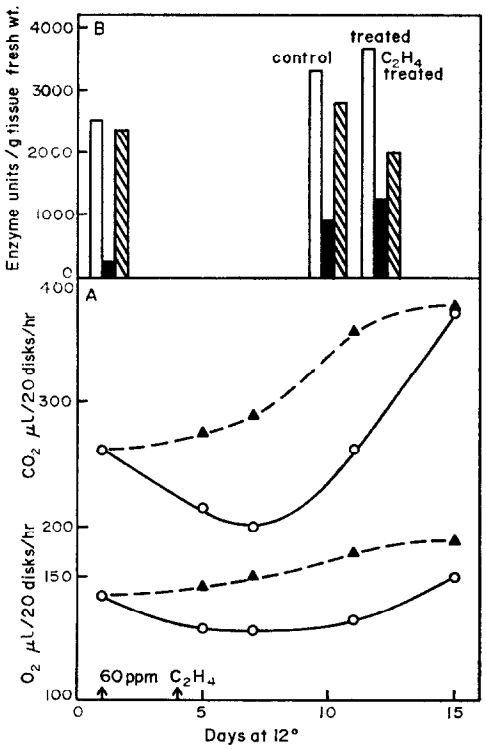

FIG. 4a. THE RATES OF O_2 UPTAKE AND CO_2 PRODUCTION IN THE PRESENCE OF ADDED MALATE OF DISCS OF PEEL TISSUE PREPARED FROM THE CONTROL AND ETHYLENE TREATED COX'S ORANGE PIPPIN APPLES.

FIG. 4b. THE ACTIVITIES OF ME, PD AND ADH IN EXTRACTS PREPARED FROM UNTREATED APPLES AT THE BEGINNING OF STORAGE AND FROM CONTROL AND ETHYLENE TREATED APPLES AFTER 11 days OF STORAGE.

TABLE 1. UPTAKE OF ^{14}C-VALINE AND ^{14}C-URIDINE AS A PERCENTAGE OF TOTAL COUNTS APPLIED (APPROX. 35,000 dis/min FOR VALINE AND 110,000 dis/min FOR URIDINE) TAKEN UP PER g/hr

Apple variety	Days at 12°	Control		Ethylene treated	
		Uptake valine	Uptake uridine	Uptake valine	Uptake uridine
Worcester Pearmain	0	11·5	23·8	—	—
	3	14·7	23·2	18·5	22·4
	5	13·5	25·5	19·0	26·8
	7	14·5	25·4	18·5	26·1
	12	15·4	25·4	19·3	25·4
Cox's Orange Pippin	1	16·6	24·9	—	—
	5	16·3	—	17·1	25·6
	7	15·7	23·6	15·0	25·1
	11	15·7	25·7	21·9	26·5
	15	19·2	—	23·1	—

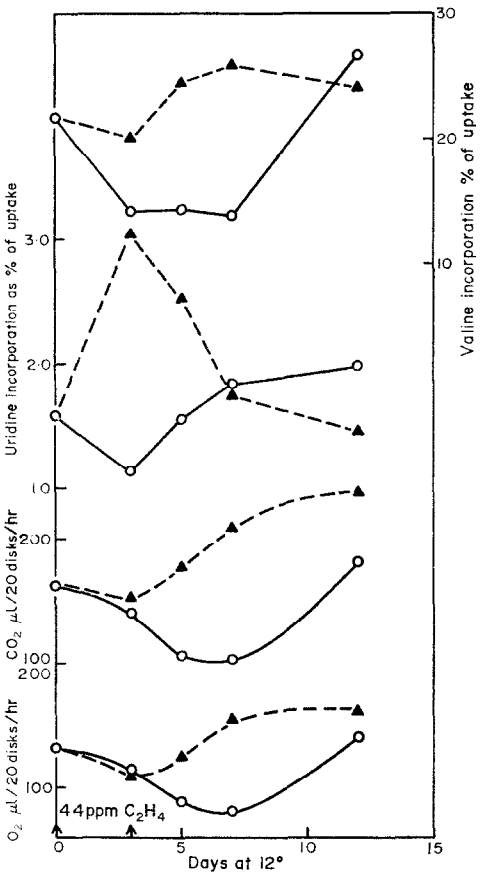

FIG. 5. THE RATES OF BASIC O_2 UPTAKE AND CO_2 OUTPUT, THE RATE OF INCORPORATION OF URIDINE INTO RNA AND THE RATE OF INCORPORATION OF VALINE INTO PROTEIN OF DISCS OF PEEL TISSUE PREPARED FROM CONTROL AND ETHYLENE TREATED WORCESTER PEARMAIN APPLES.

The basic respiration (O_2-uptake and CO_2-output) of discs of peel tissue taken from the fruit at various stages during the 'natural' and ethylene-induced climacteric (Figs. 5 and 6) follows the general pattern shown by the whole fruit in both series—the discs tend to lag behind the whole fruit in the more immature C.O.P. apples. (cf. Fig. 2a).

In the Worcester Pearmain apples, increased uridine *incorporation* into RNA precedes the increased respiration over the climacteric period in the discs (Fig. 5) and the increased decarboxylation of added malate (malate effect) (Figs. 3a and 4a). The increased incorporation of uridine is followed by an increased incorporation of valine into protein. The sequence, increased uridine incorporation followed by increased incorporation of valine during the early stages of the climacteric in whole fruit and discs and of the development of the malate effect is particularly striking in the ethylene-treated fruit (Fig. 5). With Cox's Orange Pippin apples where fewer determinations of uridine incorporation were made (Fig. 6) the picture for uridine is less clear and increased uridine and valine incorporation appear to occur almost simultaneously. Nevertheless, in both varieties ethylene treatment both accelerates

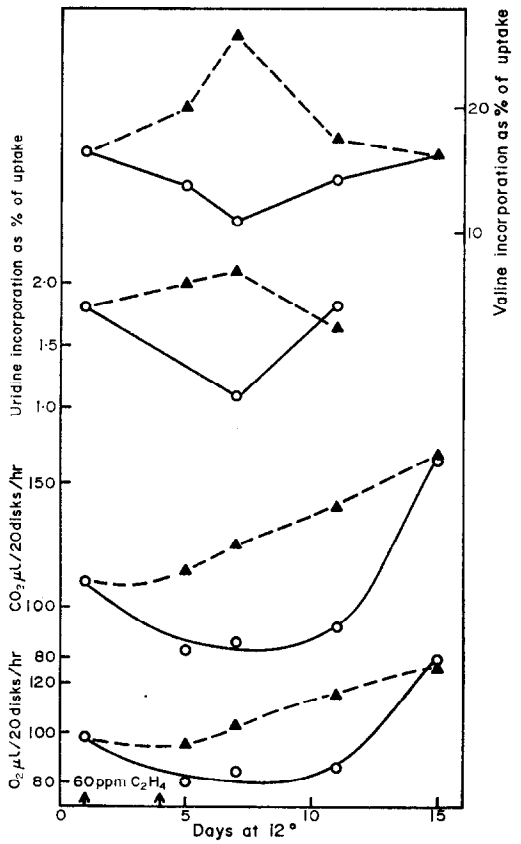

FIG. 6. THE RATES OF BASIC O_2 UPTAKE AND CO_2 OUTPUT, THE RATE OF INCORPORATION OF URIDINE INTO RNA AND THE RATE OF INCORPORATION OF VALINE INTO PROTEIN OF DISCS OF PEEL TISSUE PREPARED FROM CONTROL AND ETHYLENE TREATED COX'S ORANGE PIPPIN APPLES.

the onset of the respiration climacteric and leads to an enhancement of both valine and uridine incorporation compared with untreated fruit.

DISCUSSION

The results presented here agree with the suggestion of Hulme *et al.*[5] that ethylene initiates the onset of the respiration climacteric in apples by stimulating the synthesis of RNA and protein. The incorporation of uridine into RNA rises to a peak during the very early stages of the ethylene induced climacteric and this peak precedes a peak in incorporation of valine into protein. The peak in valine incorporation occurs just before the respiratory maximum. A similar sequence of events occurs during 24 hr ageing of discs of prelimacteric apples.[10] In this system the development of the malate effect is accelerated by application of ethylene to the discs during ageing. The present results support the suggestion that in the apple, ethylene whether endogenous or applied externally, induces an increase in the synthesis of the enzymes whose activity has been shown to increase at the time of the climacteric peak.

[10] M. J. C. RHODES, L. S. C. WOOLTORTON, T. GALLIARD and A. C. HULME, *Phytochem.* **7**, 1439 (1968).

It has been suggested[6] that the malate effect in apples involves the combined action of the enzymes ME, PD and ADH. In such a coupled reaction the $NADPH_2$ formed in the conversion of malate to pyruvate may be utilized by ADH in transforming acetaldehyde into ethanol. Ethanol formation from malate by apples was shown by Clijsters[11] and with ^{14}C malate by Rhodes *et al.*[6] This would explain the relatively small increase in O_2-uptake which accompanies the decarboxylation of malate added to the discs (Figs 3a and 4a). The activity of these enzyme systems have been shown to increase in the climacteric and it seems probable from our present results that this increase in activity is due to increased protein synthesis. It is puzzling, therefore, that Sacher and Salminen[8] could find no significant effect of ethylene on RNA and protein synthesis in aged tissue of the avocado and the banana.

EXPERIMENTAL

Pre-climacteric apples were picked from trees of the Worcester Pearmain and Cox's Orange Pippin (on Malling IX rootstocks) varieties growing in an orchard at the Burlingham Horticultural Station, Norfolk, on 22 August and 4 September 1969, respectively. For each variety the sample of fruit was divided into two batches and stored at 12° and their respiration and ethylene production monitored daily (Hulme *et al.*,[2] Galliard *et al.*[12]). In the case of the Worcester Pearmain fruit, one batch was treated immediately with 60 ppm of ethylene in air for 3 days and then returned to air. The other batch, which was maintained in air throughout, acted as the control fruit. With the Cox's Orange Pippin, the treated batch was equilibrated in air at 12° for 1 day before application of 44 ppm of ethylene for 3 days and then returned to air.

In both experiments, samples from the ethylene treated and control batches were taken at intervals during storage. On each occasion, discs of peel tissue were prepared and their basic respiration, malate decarboxylative activity and ethylene production measured as previously described.[10,12] The uptake and incorporation of U^{14}C valine into protein was measured as described by Rhodes *et al.*[10]

For the measurement of the uptake and incorporation into RNA of ^{14}C-2-uridine, 40 discs were incubated for 2 hr at 25° in 3 ml of a medium containing 0·05 M phosphate buffer, pH 4·5, 50 μg/ml chloramphenicol and 3 μc of ^{14}C-2-uridine (60·7 μc/mole). At the end of the incubation the discs were filtered, washed with 0·01M ^{12}C-uridine and then frozen in liquid nitrogen. The combined filtrate and washings were used for the determination of uptake as previously described.[10] The frozen discs were ground in a pestle and mortar in liquid nitrogen and the resulting fine powder was transferred to a polypropylene tube and extracted sequentially as follows: 20 ml 5% trichloroacetic acid (TCA) containing 0·01 M ^{12}C-uridine at 0°, 3 extractions with 20 ml 5% TCA at 0°, 2 extractions with 20 ml acetone, 20 ml acetone–ether (1 : 1) and finally with 20 ml ether. The extracted powder was dried *in vacuo* and counted by liquid scintillation techniques similar to those previously described for determining the incorporation of valine into protein.[10] More than 80 per cent of the counts in the TCA insoluble powder were solubilized by treatment with RNAase (Boehringer).

For the estimation of enzyme activity, 100 discs were homogenized for 1 min in an Ultraturrax Homogenizer in 30 ml of a medium containing 0·2 M HEPES, 0·001 M EDTA, 0·25 M sucrose, 0·001 M cysteine and 3% PVP (Kollidon 25) at pH 8·0. The homogenate was centrifuged at 1000 *g* for 10 min and the residue re-homogenized with a further 10 ml of medium. The residue was again separated by centrifugation and the two supernatants combined, clarified by centrifugation at 10,000 *g* for 20 min and made to 50 ml with distilled water. Samples of this extract were taken for the estimation of the activities of malic enzyme by the method of Hulme *et al.*,[2] of pyruvate decarboxylase as described by Bergmeyer[13] and of alcohol dehydrogenase by the procedure of Rhodes *et al.*[14]

[11] H. CLIJSTERS, *Physiol. Plantarum* **18,** 85 (1965).
[12] T. GALLIARD, M. J. C. RHODES, L. S. C. WOOLTORTON and A. C. HULME, *Phytochem.* **7,** 1465 (1968).
[13] H. U. BERGMEYER (ed.), *Methods of Enzymatic Analysis*, Academic Press, New York (1963).
[14] M. J. C. RHODES, L. S. C. WOOLTORTON and A. C. HULME (in preparation).