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PHOTOSYNTHETIC INDUCTION PHENOMENA IN SPINACH CHLOROPLASTS IN RELATION TO THE NATURE OF THE ISOLATING MEDIUM

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SUMMARY

1. Measurements were made of photosynthetic CO_2 fixation and O_2 evolution by spinach chloroplasts isolated in sorbitol media containing 2-(*N*-morpholino)ethanesulphonate (MES).

2. The chloroplasts isolated in MES–sorbitol media exhibited induction phenomena which were similar to those shown by chloroplasts isolated in orthophosphate–sugar mixtures. Added ribose 5-phosphate shortened the lags which preceded the attainment of maximal rates of CO_2 fixation and O_2 evolution. O_2 evolution reached its maximum rate almost immediately in the presence of 3-phosphoglycerate. Induction periods were shortened by pre-illumination of the parent tissue prior to separation of the chloroplasts.

3. In the absence of added substrate (other than CO_2) lags exhibited by chloroplasts isolated in MES–sorbitol were shorter than those observed with chloroplasts prepared in orthophosphate–sorbitol. These shorter lags could be extended by briefly exposing the chloroplasts to sugar media containing orthophosphate, malate or acetate or to Tris–NaCl.

4. The results are discussed in relation to photosynthetic induction phenomena and current methods of chloroplast isolation.

INTRODUCTION

Since the first demonstration of CO_2 fixation by isolated chloroplasts[1], modifications of conventional methods of separation have led to successive increases in rate[2]. The use of sugars in preference to saline was sufficient (when combined with brief grinding and rapid centrifugation) to raise rates of fixation from the region of 3–6 μmoles/mg chlorophyll per h to 20–40 (refs. 3–5). Subsequently, and especially with actively growing summer spinach, higher rates were obtained with an absolute maximum of just over 100[6]. Recently, still greater rates (155 μmoles/mg per h) have been reported by JENSEN AND BASSHAM[7] and for the first time CO_2 fixation by isolated chloroplasts has surpassed the average performance (80–120) and approached the maximum value (about 300) achieved by whole leaves.

Abbreviations: MES, 2-(*N*-morpholino)ethanesulphonate; HEPES, *N*-2-hydroxyethy piperazine-*N'*-2-ethanesulphonate.

In our own work[4,5,8,9] we have consistently observed an initial lag or induction period in CO_2 fixation similar to that first observed by BAMBERGER AND GIBBS[10] (see also ref. 2). The lag could be diminished by the addition of certain carbon-cycle intermediates[2,5,6,8] in substrate, or catalytic quantities[5,8] and of these ribose 5-phosphate was the most effective. In the absence of added substrate (other than CO_2) the lag was more prolonged (sometimes in excess of 10 min in chloroplasts prepared from dark-stored leaves) and the maximum rate then only rarely exceeded 60 % of that obtained in the presence of ribose 5-phosphate. By contrast, JENSEN AND BASSHAM[7] obtained their highest rate in the absence of added sugar phosphate and their progress curve showed no initial lag or induction period. In essentials, their method of isolation[7] seems to differ from ours[3,5] only in the substitution of 0.05 M 2-(*N*-morpholino)-ethanesulphonate (MES)[11] at pH 6.1 for 0.1 M orthophosphate at pH 6.8. In order to establish the cause of the differences apparently imposed by this procedure we have investigated the kinetics of photosynthesis shown by chloroplasts prepared and assayed according to JENSEN AND BASSHAM[7] and the effects of exposing these chloroplasts to other media (such as orthophosphate–sorbitol[3]) for brief periods (5 min at 0°) prior to assay.

In many experiments we have taken advantage of the fact that under certain well-defined conditions (*e.g.* in the presence of CO_2 or CO_2 *plus* ribose 5-phosphate) the kinetics of O_2 evolution parallel those of CO_2 fixation[12]. O_2 evolution may be continuously recorded using an oxygen electrode[12] and is therefore more convenient for this purpose than the determination of ^{14}C in individual samples.

MATERIALS AND METHODS

Isolation of chloroplasts

Spinach was purchased in local markets and stored in the dark at 5° until required. Prior to isolation of chloroplasts the leaf midribs were removed and 50 g of lamina were usually illuminated in water at 20° at an intensity of 1000 ft. candles. The laminae were then chilled in ice-water and macerated in a motor driven blendor for 3–5 sec in 200 ml of the appropriate grinding medium. After filtration through muslin[3,7] the homogenates were rapidly centrifuged (from rest to 4000 × *g* to rest in 90 sec (refs. 3, 7)) and the pellets resuspended or washed as indicated below.

Grinding media

(*I*) *Phosphate–sugar* (*Fig. 1*). Leaves were blended in a solution containing 0.33 M sucrose, 0.1 M orthophosphate at pH 6.8 and small quantities[3] of $MgCl_2$, NaCl and sodium isoascorbate. Pellets were resuspended in a similar solution from which the phosphate was omitted and the sucrose increased to 0.45 M (ref. 3).

(*II*) *MES–sorbitol*[7] (*Figs. 2–9*). Solution contained 0.05 M MES adjusted to pH 6.1 with NaOH, 0.33 M sorbitol, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.5 mM KH_2PO_4 and 2 mM sodium isoascorbate. Pellets were resuspended in a similar solution buffered by *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate (HEPES) at pH 6.7.

Washing media

In a number of experiments (Figs. 3–5) chloroplasts initially isolated in MES–sorbitol were resuspended for 5 min at 0° in one of the following solutions prior to

a second centrifugation and resuspension in HEPES–NaOH as in (*II*) above: (a) in the experiments illustrated in Fig. 3 (Curves 1 and 2), Fig. 4 (Curves 1 and 2), Fig. 5 (Curves 1 and 2), 0.33 M sorbitol, 0.1 M MES–NaOH at pH 6.1; (b) in Fig. 3 (Curves 3 and 4), 0.33 M sorbitol, 0.1 M orthophosphate at pH 6.1; (c) in Fig. 4 (Curves 3 and 4), 0.33 M sorbitol containing either 0.1 M acetate or 0.1 M malate at pH 6.1; (d) in Fig. 5 (Curves 3 and 4), 0.35 M NaCl, 0.1 M Tris–HCl at pH 7.5.

Assay procedure

O_2 evolution

Chloroplast suspensions containing 162 μg chlorophyll in a total volume of 0.2 ml resuspending solution were mixed with 1.6 ml of a solution similar to the resuspending solution except that it did not contain NaCl and was buffered to pH 7.6 by HEPES–NaOH. In addition it also contained 18 μmoles Na_2CO_3 and 7.5 μmoles inorganic pyrophosphate (*cf.* ref. 7). The reaction mixture chloroplast suspension was tipped into a Rank oxygen electrode cell and incubated in darkness for 3 min to allow equilibration to 20° prior to illumination. (For further details see ref. 12.) The experiments were run under aerobic conditions so that in the figures relating to O_2 evolution the zero point on the O_2 scale is, in absolute terms, in the region of 0.28 μmole of O_2 per ml of solution and does not indicate anaerobic conditions.

CO_2 fixation

Reaction mixtures in the experiment relating to Fig. 1 contained: chloroplasts prepared in the orthophosphate sugar medium (84 μg chlorophyll), reduced glutathione (1 μmole), sucrose (30 μmoles), $MnCl_2$ (0.5 μmole), $MgCl_2$ (0.25 μmole), EDTA (0.5 μmole), KH_2PO_4 (0.5 μmole), tricine–NaOH (pH 7.5) (7.5 μmoles), Na_2CO_3 (1.75 μmole containing 50 μC ^{14}C) and 1.2 μmoles $NaHCO_3$ in a final volume of 0.3 ml. The reaction mixtures were incubated in the dark at 20° for 3 min, then illuminated at a light intensity in excess of 2000 ft. candles. CO_2 fixation was determined as previous described[6].

RESULTS

Kinetics of photosynthesis by chloroplasts isolated in orthophosphate–sugar media

CO_2 fixation by spinach chloroplasts isolated in media containing 0.33 M sucrose (or equimolar glucose, fructose or sorbitol), 0.1 M orthophosphate at pH 6.8 and small quantities of $MgCl_2$ and NaCl[3,5] is not linear with time[6] (see also GIBBS[2]). There is an initial lag or induction period which may be shortened by the addition of certain carbon-cycle intermediates. Fig. 1 is typical and resembles corresponding time courses obtained with chloroplasts from other species (especially peas[5]) and spinach chloroplasts prepared in other media such as Tris–NaCl[2,6,10]. It should be noted that the reaction mixture used in this and previously reported experiments[3–6,8,9] did not contain large quantities of orthophosphate since this was omitted entirely from the medium in which the chloroplasts were resuspended. The reaction mixtures (see legend to Fig. 1) contained only small quantities of orthophosphate and the pH was maintained by tricine (Tris gave initially similar kinetics). It will be seen (Fig. 1) that the addition of a substrate quantity of ribose 5-phosphate shortened the lag and increased the maximum rate observed in the presence of CO_2 alone. Addition of

equimolar 3-phosphoglycerate gave virtually the same rate as ribose 5-phosphate but after a longer lag. This contrasts with the effect of this compound on O_2 evolution where its addition eliminates the lag[12]. The progress curves for O_2 evolution in the presence of CO_2 alone, and in the presence of CO_2 and added ribose 5-phosphate parallel those of fixation[12].

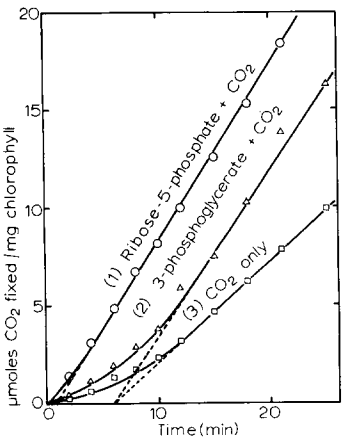


Fig. 1. Kinetics of CO_2 fixation by chloroplasts isolated in orthophosphate–sugar media. In addition to other reactants (see METHODS) the reaction mixtures contained, in (1) ribose 5-phosphate (2 μmoles), in (2) 3-phosphoglycerate (2 μmoles) and in (3) no further additions.

Induction phenomena in O_2 evolution by chloroplasts isolated in MES–sorbitol

Fig. 2 shows the polarographic records of O_2 evolution by spinach chloroplasts prepared in MES–sorbitol[7] (see METHODS) and assayed in mixtures similar to those employed by JENSEN AND BASSHAM[7]. It illustrates the effect of preilluminating the parent tissue and the effect of adding ribose 5-phosphate. Each curve shows an initial lag before the maximum rate was reached. The longest lag was with chloroplasts from dark-stored leaves to which the CO_2 was added as the sole substrate. This lag could be shortened by pre-illumination of the leaf prior to separation of the chloroplasts or by the addition of ribose 5-phosphate. The shortest lag was obtained by a combination of pre-illumination and addition of ribose 5-phosphate. It will be seen that the addition of ribose 5-phosphate affected the lag but not the maximum rate, whereas in Fig. 1 both rate and lag were affected. The lags observed with CO_2 alone were considerably shorter than those previously recorded[12] for chloroplasts prepared in orthophosphate–sugar media and assayed in mixtures[3,5,12] similar to those used in Fig. 1.

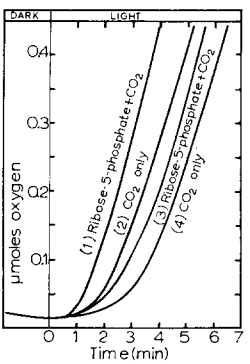
There was no appreciable lag when 3-phosphoglycerate was substituted for ribose 5-phosphate and CO_2 (*cf.* ref. 12).

Changes in kinetics following exposure to orthophosphate–sorbitol and other media

When chloroplasts isolated in MES–sorbitol were washed in MES–sorbitol their subsequent kinetic behaviour was unchanged (*cf.* Fig. 3, Curves 1 and 2 with Fig. 2). However, exposure for 5 min at 0° to orthophosphate–sorbitol was sufficient to modify the subsequent kinetics (Fig. 3) so that they now more closely resembled those obtained with chloroplasts prepared in orthophosphate–sugar media (see Fig. 1 and

ref. 12). In each case, the kinetics of CO_2 fixation (not shown) were similar to those of O_2 evolution.

The maximum rate obtained in the presence of ribose 5-phosphate was largely unchanged by the exposure to orthophosphate–sorbitol but with CO_2 as the sole added substrate the maximum rate was depressed and the lag lengthened. Essentially similar effects were produced by exposure to orthophosphate– sorbitol at pH's 6.8 and 7.5. The conditions of exposure (5 min at 0°) were intended to simulate the degree



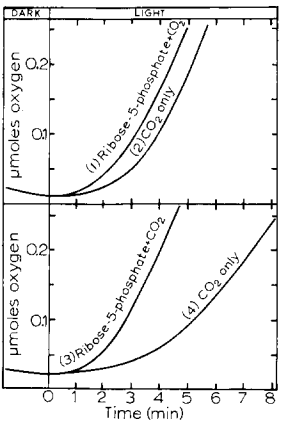
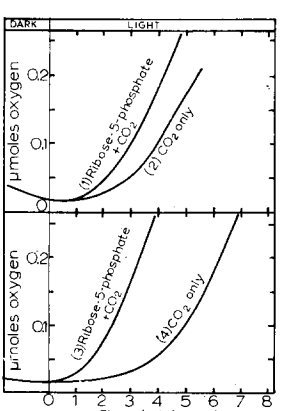


Fig. 2. Polarographic records of O_2 evolution by chloroplasts isolated in MES–sorbitol. For reaction mixtures see METHODS. In addition, (1) and (3) contained ribose 5-phosphate (12 μmoles). In (1) and (2) the chloroplasts were separated from leaves which had been pre-illuminated. In (3) and (4) the leaves were taken from the dark.

Fig. 3. Polarographic records of O_2 evolution by chloroplasts washed in MES–sorbitol (1 and 2) or orthophosphate–sorbitol (3 and 4). In addition to other reactants (see METHODS) ribose 5-phosphate (12 μmoles) and bicarbonate in (1) and (3), bicarbonate only in (2) and (4).



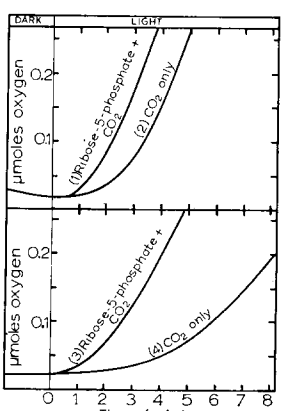


Fig. 4. Polarographic records of O_2 evolution by chloroplasts washed in MES–sorbitol (1 and 2) or malate–sorbitol (3 and 4). Conditions similar to those in Fig. 3 (see METHODS). Ribose 5-phosphate (12 μmoles) and CO_2 in (1) and (3), CO_2 only in (2) and (4). Evolution following exposure to acetate–sorbitol (not shown) was identical to that following exposure to malate–sorbitol.

Fig. 5. Polarographic records of O_2 evolution by chloroplasts washed in MES–sorbitol (1 and 2) or Tris–NaCl (3 and 4). Other conditions as for Fig. 4.

of exposure to similar media during the isolation procedure normally adopted[5] but extension of the duration of the wash did not accentuate the effect appreciably. Fig. 4 shows that an essentially identical response could be induced by exposure to sorbitol media in which orthophosphate was replaced by equimolar acetate or malate. In Fig. 5 an entirely different medium was used (Tris–NaCl as used by GIBBS[2]) but again the effect was to prolong the lag and depress the maximum rate when CO_2 was used as the only added substrate.

Absence of induction phenomena after pre-illumination of the chloroplasts

It has been previously demonstrated that if photosynthetic CO_2 fixation[5] or O_2 evolution[12] (by chloroplasts isolated in orthophosphate–sugar media) is interrupted by a brief dark interval, there is little or no lag when illumination is resumed. This, of course, is also a feature of induction phenomena by intact organisms[13]. In JENSEN AND BASSHAM's experiments[7], chloroplasts were preincubated for 3 min in the light prior to the addition of $^{14}CO_2$ so that their time scale does not relate to the onset of illumination but only to the period of measurement.

We have confirmed with their preparations that their assay gives initially linear kinetics for both CO_2 fixation and O_2 evolution but that if measurements are started at the onset of illumination, rather than at the time of addition of CO_2-bicarbonate, then lags are observed (*cf.* Fig. 2).

Rates of O_2 evolution by chloroplasts isolated in MES–sorbitol

During the course of this work chloroplasts were frequently isolated from winter-grown spinach using methods and solutions which were similar, in essentials, to those employed by JENSEN AND BASSHAM[7]. Table I summarises results from a representative sample of 18 experiments which involved measurements of O_2 evolution in the presence and absence of ribose 5-phosphate. The values are not directly comparable because the determinations (which were incidental to the main purpose of the experiments) were made at different times, sometimes as much as 4 h, after isolation. They are included here to give some indication of the rates of evolution which may be anticipated in routine work with spinach which is not of the best quality. In our past experience (see *e.g.* ref. 6) median rates of CO_2 fixation by chloroplasts isolated from field-grown spinach in the summer could be as much as 3 times as high as those from winter-grown spinach, purchased in the local market (Covent Garden). We should

TABLE I

RATES OF O_2 EVOLUTION BY CHLOROPLASTS ISOLATED IN MES–SORBITOL

Chloroplasts were isolated and assayed in solutions similar to those employed by JENSEN AND BASSHAM[7] (see METHODS). It should be noted that the rates of O_2 evolution are not corrected for the increased O_2 uptake observed immediately after illumination[12] and could therefore have been associated with rates of CO_2 fixation which were at least 50% higher.

Added substrate	*Number of determinations*	*Rate (μmoles O_2 per mg chlorophyll per h)*			
		Min.	*Max.*	*Mean*	*Median*
CO_2 only	22	11	89	44	40–50
Ribose 5-phosphate (12 μmoles) *plus* CO_2	19	16	79	49	40–50

therefore anticipate rates of O_2 evolution, in the summer, of the same order as those already reported for CO_2 fixation[6,7,12].

DISCUSSION

We have found that spinach chloroplasts, isolated according to the technique of JENSEN AND BASSHAM[7], exhibit photosynthetic induction phenomena which are broadly similar to those previously recorded for chloroplasts isolated in Tris–NaCl[2,10] and orthophosphate–sugar mixtures[5,6,8,12]. Maximal rates of CO_2 fixation and O_2 evolution were not attained immediately upon illumination but only after an initial lag. This lag could be diminished by pre-illumination of the parent tissue prior to separation of the chloroplasts. In the presence of substrate concentrations of ribose 5-phosphate the initial lag was normally diminished by 40–60 sec (occasionally less) but there was little stimulation of the maximum rate. In this respect the results differ quantitatively from those obtained with chloroplasts prepared in the other media, where added ribose 5-phosphate usually had a more marked effect on the lag and where the rate with CO_2 alone rarely exceeded 60 % of that seen in the presence of ribose 5-phosphate. In the presence of 3-phosphoglycerate the lag in O_2 evolution could be almost entirely eliminated as before[12].

The demonstration of induction periods does not necessarily conflict with the observation of JENSEN AND BASSHAM[7]. These workers preincubated their chloroplasts in the light for 3 min prior to the addition of $^{14}CO_2$. In our experience this would have been sufficient to disguise any short induction period which might otherwise have been recorded.

The significance of the earlier observations has been discussed elsewhere and it seems clear that the present work would not conflict with the conclusions which have been already drawn[5,8,9,12]. Progress curves for CO_2 fixation and O_2 evolution by isolated chloroplasts frequently resemble those shown by whole tissues (see *e.g.* ref. 13) and by chloroplasts within the leaf[14]. They are affected in a similar fashion by temperature[9], by light intensity[8] and by pre-illumination[5,12]. We continue to subscribe, therefore, to the view that a common causal feature of many of these phenomena is an initial substrate deficiency which is overcome by autocatalytic acceleration as photosynthetic products are fed back into the cycle (*cf.* ref. 15). This would account for the fact that a number of cycle intermediates shorten the lags when added either in substrate or catalytic quantities[2,5,6,8,10]. Of these perhaps the most interesting is 3-phosphoglycerate which at least under some experimental conditions (Fig. 1) gives a longer lag than ribose 5-phosphate for CO_2 fixation and a much shorter lag, or no lag, for O_2 evolution. The similarity of the maximum rates of fixation supported by ribose 5-phosphate and 3-phosphoglycerate would not support the view[7] that chloroplasts prepared in orthophosphate–sugar media necessarily lack the ability to regenerate the CO_2 acceptor from the products of fixation.

We have recognised, however[5], as have others (see *e.g.* ref. 2) that the *in vitro* lags may reflect changes imposed during isolation and indeed the present work provides direct evidence that the subsequent kinetic behaviour may be modified by the nature of a washing medium (see also ref. 2). Thus chloroplasts isolated in MES–sorbitol[7] gave much smaller lags, in the presence of CO_2 as the sole added substrate, than did those isolated in orthophosphate–sorbitol. A brief exposure to orthophosphate–sor-

bitol was then sufficient to bring about an extension of these lags (Fig. 3) so that the kinetic behaviour resembled that of chloroplasts initially isolated in orthophosphate media (Fig. 1). Evidently the modified kinetics were not induced merely by the resuspension procedure, or by ageing, since they were not induced in controls resuspended in MES–sorbitol (Curves 1 and 2 in Figs. 3–5). It seems equally clear that the effect of orthophosphate–sorbitol washing is not specific since essentially the same responses followed exposure to Tris–NaCl (Fig. 5) or to sorbitol media in which the orthophosphate was replaced by malate or acetate (Fig. 4). It is by no means certain, of course, that these treatments affect the photosynthetic mechanism in the same way and indeed the modified kinetics could be consistent with inhibition of a dark reaction, depletion of intermediates, or even an increased permeability of the chloroplast envelope. It is possible that the response to orthophosphate–sorbitol may be partly attributable to the small quantities of orthophosphate carried through, with the chloroplasts, from the washing medium to the reaction mixtures. Such a response would be of interest because of the possibility of metabolic control and it has been explored in the following paper[16].

The rates of photosynthesis by chloroplasts isolated and assayed according to JENSEN AND BASSHAM's technique[7] during this work were good but not exceptional. However, the work was carried out entirely with winter-grown spinach, often long stored and of uncertain history. While we might well anticipate much higher rates from summer spinach this may nevertheless underline the probability that in searching for better performance by isolated chloroplasts we are unlikely, except for partial reactions, to improve on the rates achieved by the parent tissue.

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REFERENCES

1 D. I. ARNON, M. B. ALLEN AND F. R. WHATLEY, *Nature*, 174 (1954) 394.
2 M. GIBBS, E. S. BAMBERGER, P. W. ELLYARD AND R. G. EVERSON, *Proc. NATO Inst. Advan. Study, Biochem. of the Chloroplast, Aberystwyth, 1965*, Vol. 2, Academic Press, London, 1967, p. 3.
3 D. A. WALKER, *Biochem. J.*, 92 (1964) 22c.
4 D. A. WALKER, *Plant Physiol.*, 40 (1965) 1157.
5 D. A. WALKER, *Proc. NATO Inst. Advan. Study, Biochem. of the Chloroplast, Aberystwyth, 1965*, Vol. 2, Academic Press, London, 1967, p. 53.
6 C. BUCKE, D. A. WALKER AND C. W. BALDRY, *Biochem. J.*, 101 (1966) 636.
7 R. G. JENSEN AND J. A. BASSHAM, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1905.
8 C. W. BALDRY, D. A. WALKER AND C. BUCKE, *Biochem. J.*, 101 (1966) 642.
9 C. W. BALDRY, C. BUCKE AND D. A. WALKER, *Biochim. Biophys. Acta*, 126 (1966) 207.
10 M. GIBBS AND E. S. BAMBERGER, *Plant Physiol.*, 37 (1962) 63.
11 N. E. GOOD, G. D. WINGET, W. WINTER, T. N. CONNOLLY, S. IZAWA AND R. M. M. SINGH, *Biochemistry*, 5 (1966) 467.
12 D. A. WALKER AND R. HILL, *Biochim. Biophys. Acta*, 131 (1967) 330.
13 E. D. MCALISTER, *Smithsonian Inst. Misc. Collections*, 95 (1937) 1.
14 U. W. HEBER AND K. A. SANTARIUS, *Biochim. Biophys. Acta*, 109 (1965) 390.
15 M. J. SELWYN, *Biochim. Biophys. Acta*, 126 (1966) 214.
16 W. COCKBURN, C. W. BALDRY AND D. A. WALKER, *Biochim. Biophys. Acta*, 143 (1967) 614.