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Changes in the activity of acetyl-CoA carboxylase during rape-seed formation

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During the formation of rape-seeds, lipid accumulated in the cotyledons from 16 days after pollination, rising to a plateau after 28 days. The accumulation of lipid was preceded by a marked rise in acetyl-CoA carboxylase activity, which declined rapidly, correlating with the decline in rate of lipid formation. Incubation of rape-seed extracts with avidin–agarose resulted in a decrease in acetyl-CoA carboxylase activity in the extract. Polyacrylamide-gel electrophoresis of polypeptides bound to avidin–agarose showed the presence of a polypeptide of M_r 225000. The intensity of this band increased during the period of increase of acetyl-CoA carboxylase activity in the seeds.

During seed formation, storage reserves are deposited for future mobilization and use by the germinating embryo. In many plant families the reserves are triacylglycerols deposited in oleosomes within the endosperm or cotyledons (Appelqvist, 1975). Numerous studies have demonstrated the rapid accumulation of lipid in maturing oil seeds and the differential timing and rates of synthesis of the component fatty acids (Gurr *et al.*, 1972; Ichihara & Noda, 1980; Pollard & Stumpf, 1980). Experiments *in vivo* to study the incorporation of labelled substrates into fatty acids have been widely employed (McMahon & Stumpf, 1966; Gurr *et al.*, 1974; Porra & Stumpf, 1976; Pollard & Stumpf, 1980), indicating increasing incorporation into triacylglycerols during the period of rapid accumulation of these reserves. A study of the activity of key enzymes in developing oil seeds will give information on the mechanisms whereby such enzymes are regulated during the developmental process.

We have measured the accumulation of lipid in developing rape-seeds (*Brassica napus*) and the activity of acetyl-CoA carboxylase, which catalyses the first step of fatty acid biosynthesis. This enzyme has been shown to catalyse a regulatory step in the synthesis of fatty acids in animals (Majerus *et al.*, 1968; Majerus & Kilburn, 1969; Geelen *et al.*, 1978; Lane *et al.*, 1979), micro-organisms (Mishina *et al.*, 1976; Horikawa *et al.*, 1980) and plants (Simcox *et al.*, 1979; Turnham & Northcote, 1982). In the present study we have shown that changes in the activity of acetyl-CoA carboxylase can be correlated with the accumulation of lipid in the cotyledons.

The structure of acetyl-CoA carboxylase from plant tissues is still unclear. The enzyme has been isolated from photosynthetic tissues (Kannangara & Stumpf, 1972; Mohan & Kekwick, 1980; Nikolau *et al.*, 1981) and non-photosynthetic tissues (Hatch & Stumpf, 1961; Heinstein & Stumpf, 1969; Brock & Kannangara, 1976; Egin-Buhler *et al.*, 1980). All the preparations of plant acetyl-CoA carboxylase described have been found to dissociate but the subunit structures obtained have differed depending on the preparation and the techniques employed. In the present study an attempt was made to identify the biotin-containing subunit of acetyl-CoA carboxylase from developing rape-seeds by using avidin–agarose.

Materials and methods

Rape-seed (*Brassica napus* var. Haplona) was provided by the Department of Applied Biology, University of Cambridge. $NaH^{14}CO_3$ (sp. radioactivity 2.05 GBq/mmol) was purchased from Amersham International, Amersham, Bucks., U.K. ATP, CoA, avidin–agarose and agarose (type X) were obtained from Sigma Chemical Co., Kingston-Upon-Thames, Surrey, U.K. All chemicals used were of A.R. quality wherever possible.

Acetyl-CoA was synthesized by acetylation of CoA using acetic anhydride (Simon & Shemin, 1953) and its concentration was determined by the method of Ramsay & Tubbs (1975).

Abbreviation used: SDS, sodium dodecyl sulphate.

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Growth of rape plants and pollination of flowers

Rape plants were grown in an indoor cabinet at 18–20°C with a 16h photo-period. Illumination was provided by fluorescent and tungsten lights.

Flowers were hand-pollinated and individually tagged. Seed pods were harvested from between 5 and 34 days after pollination.

Preparation of enzyme extracts and assay of acetyl-CoA carboxylase activity

Seeds were removed from the pods and enzyme extracts were prepared as described by Turnham & Northcote (1982). All subsequent manipulations were performed at 4°C. Seeds were homogenized in 100mM-Tris/HCl, pH 7.5, containing 20% glycerol, 0.2mM-phenylmethanesulphonyl fluoride and 15mM-mercaptoethanol (20ml of this extraction buffer/g of seeds), by using an MSE overhead homogenizer at full speed for 2min. Triton X-100 was added to a final concentration of 0.5% and the homogenate stirred for 10min, followed by centrifugation at 20000g for 10min in a Sorvall RC 2-B centrifuge. Proteins were precipitated from the supernatant by using $(NH_4)_2SO_4$ (80% saturation) and pelleted at 20000g. The pellet was redissolved in a small volume of homogenization medium. Acetyl-CoA carboxylase activity was determined by measuring the incorporation of $NaH^{14}CO_3$ into malonyl-CoA (Turnham & Northcote, 1982). The incubation mixture contained, in a total volume of 190μl, 10μmol of Tris/HCl, pH 8.7, 1.4μmol of $KHCO_3$, 0.1μmol of $NaH^{14}CO_3$ (205kBq), 0.04μmol of acetyl-CoA, 0.5μmol of ATP, 1.0μmol of $MgCl_2$ and up to 30μg of protein. All assays were performed using at least two different enzyme concentrations and were linear with respect to enzyme concentration and time under the conditions employed. Duplicate values did not deviate by more than 10% from the mean value.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) after precipitation with trichloroacetic acid, with bovine serum albumin as standard. Assays were performed on two samples of seeds at each time point. The values for each sample did not deviate by more than 10% from the mean value.

Extraction and estimation of lipid

Rape-seed lipids were extracted using chloroform/methanol (3:2, v/v), purified and measured as described previously (Turnham & Northcote, 1982).

Electron microscopy

Rape cotyledons were dissected out and fixed in 3% glutaraldehyde in 0.1M-phosphate buffer, pH 6.8, for 16h at 4°C, then post-fixed in 2% OsO_4 in buffer for 2h. The samples were dehydrated through an ethanol series and embedded in Araldite resin. Sections were cut on a Sorvall Porter Blum MT-2 ultramicrotome, stained with uranyl acetate (30min) and alkaline lead hydroxide (5–10min), and examined with an AEI EM6B microscope.

Incubation of enzyme extracts with avidin–agarose

Enzyme extracts were passed through a column (1cm × 8cm) of Sephadex G-25 equilibrated with extraction buffer at 4°C, to remove any $(NH_4)_2SO_4$ remaining after protein precipitation.

Samples of extracts were incubated with Agarose type X or avidin–agarose on a turntable at 4°C. The agarose gels were washed four times with extraction buffer before addition of enzyme. The incubations were optimized with respect to the volume of agarose gel used and the incubation time. At the end of the incubation the agarose was pelleted by centrifugation at 12000g for 2min in a Beckman microfuge and the supernatant was removed. The pellet was washed three times with 0.5M-KCl in extraction buffer, then three times with extraction buffer. Residual enzyme activity was measured in the supernatant after agarose or avidin–agarose incubation.

SDS/polyacrylamide-gel electrophoresis

Samples of seed extracts or agarose and avidin–agarose pellets were mixed with an equal volume of sample buffer [0.1M-dithiothreitol/0.08M-Tris/HCl (pH 6.8)/20% glycerol/0.0005% Bromophenol Blue/4% SDS (w/v)] and heated at 95°C for 10min. The samples were loaded on to 10% polyacrylamide gels with 5% stacking gels (Laemmli, 1970) and electrophoresed at 200V for 3–4h. Gels were stained with Coomassie Brilliant Blue R.

Results

Fresh weight and protein content of seeds during formation

The seed fresh weight increased rapidly from 5 days after pollination to a maximum at 24 days after pollination, after which it declined as the seeds began to dehydrate as they approached maturity (Fig. 1). The protein content per seed increased slowly from 5 to 16 days after pollination, followed by a rapid rise to a plateau after day 24 (Fig. 1).

Acetyl-CoA carboxylase activity and lipid deposition during seed formation

Fig. 2 shows the activity of acetyl-CoA carboxylase and the lipid content of the seeds during their development. Lipid began to accumulate 16 days after pollination and rose rapidly to a plateau after approx. 28 days, the final lipid content per seed being 62 times higher than the initial level. Acetyl-

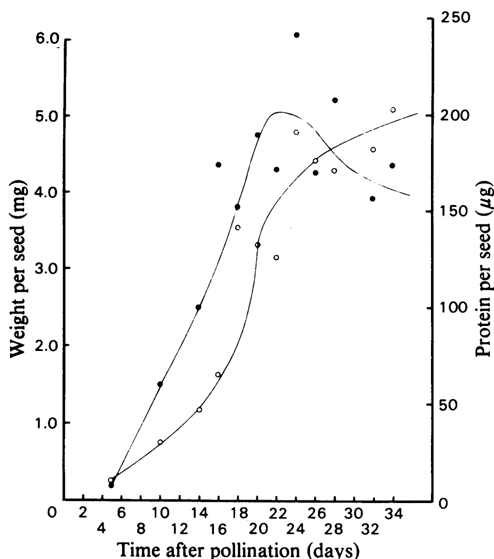


Fig. 1. *Fresh weight (●) and protein content (○) of developing rape-seeds*

Flowers were hand-pollinated and tagged and developing pods were excised at intervals. Seeds were dissected from the pods and weighed. Protein was determined by the method of Lowry *et al.* (1951) in seed homogenates after precipitation with trichloroacetic acid.

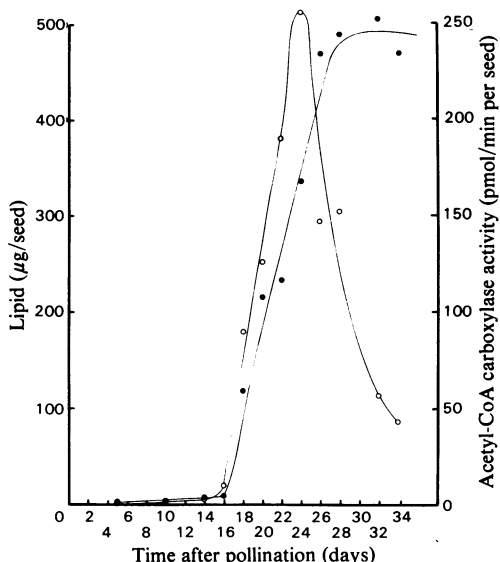


Fig. 2. *Changes in the activity of acetyl-CoA carboxylase (○) and lipid content (●) during seed formation measured per seed*

Acetyl-CoA carboxylase activity was assayed in the 20000 *g* supernatant of seed homogenates by measuring the acetyl-CoA-dependent fixation of $NaH^{14}CO_3$ into malonyl-CoA. Lipid was measured in chloroform/methanol extracts of the seeds.

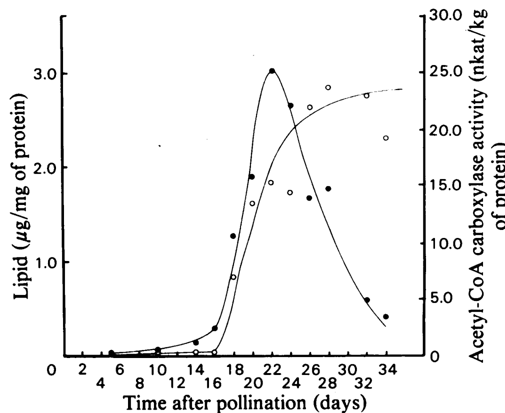


Fig. 3. *Acetyl-CoA carboxylase activity (●) and lipid content (○) of developing rape-seeds measured per kg of protein*

For further details see the caption to Fig. 2.

CoA carboxylase increased in activity from 14 days to a peak at 24 days after pollination, then dropped rapidly. The enzyme activity rose 64-fold between days 14 and 24, with the highest rate of increase occurring after day 16. However, as can be seen from Fig. 1, there is an increase in the protein content of the seeds concomitant with the increase in acetyl-CoA carboxylase activity. Therefore, to demonstrate a specific increase in the activity of the enzyme the results were calculated on the basis of specific activity (Fig. 3). A similar pattern of lipid deposition and enzyme activity was observed, except that the peak of enzyme activity appeared slightly earlier at 22 days after pollination. There was a 22-fold increase in enzyme activity between days 14 and 22. The results show a specific increase in acetyl-CoA carboxylase activity during rape-seed development.

Electron-microscopical studies reflected the pattern of lipid deposition seen in Figs. 2 and 3. After pollination (5 days) no storage lipid was present, but by day 17 a few isolated deposits were visible. Large numbers of lipid droplets lined the inside of the cell membrane at day 24 and at 32 days the cells were packed with storage lipid and storage protein (Plate 1).

Incubation of enzyme extracts with avidin–agarose

Optimization of incubation conditions. Samples of enzyme extracts prepared from developing rape-seeds were incubated with avidin–agarose or agarose to determine the optimum conditions for binding of acetyl-CoA carboxylase. Fig. 4 shows the enzyme activity remaining in the supernatant after incubation of extracts containing 200 µg of protein with

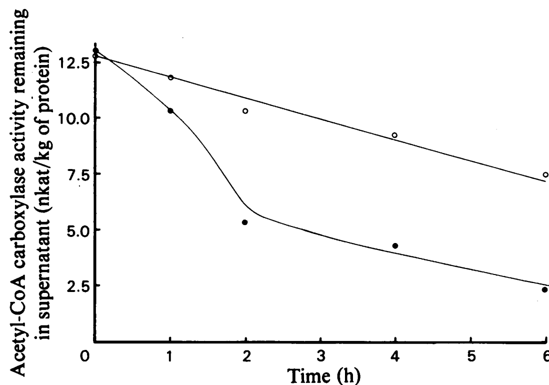


Fig. 4. *Acetyl-CoA carboxylase activity remaining after incubation of rape-seed extracts with avidin–agarose (●) or agarose (○) over a period of 6 h*

Seed extracts containing 200 μg of protein were mixed with 30 μl of avidin–agarose or 30 μl of agarose and incubated at 4°C with continuous mixing on a turntable. At various time intervals the avidin–agarose or agarose gel was pelleted by centrifugation and the activity of acetyl-CoA carboxylase in the supernatant was assayed (control samples incubated under similar conditions with addition of 30 μl of buffer but with neither agarose nor avidin–agarose showed an identical decline in supernatant enzyme activity as samples incubated with agarose).

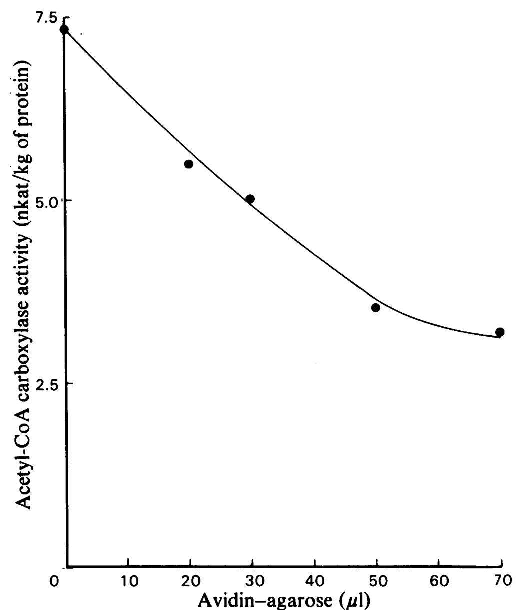


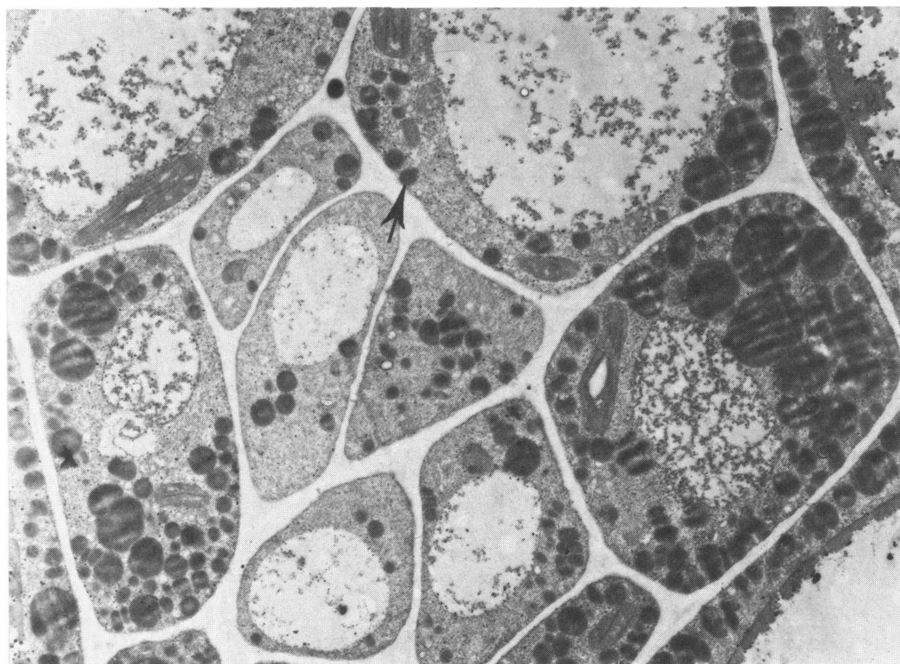
Fig. 5. *Acetyl-CoA carboxylase activity remaining after incubation of rape-seed extracts with increasing volumes of avidin–agarose*

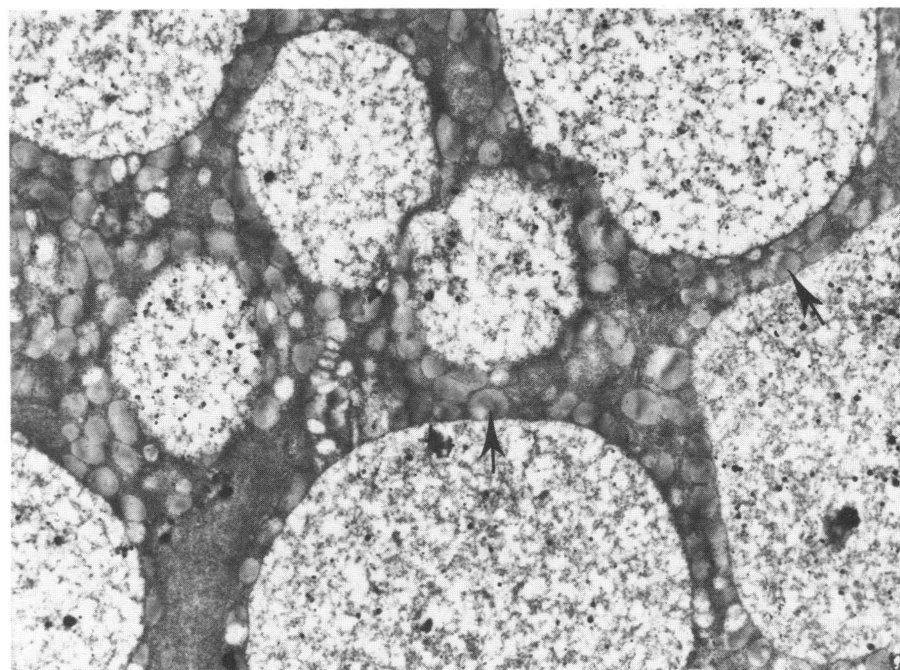
Samples of extract containing 200 μg of protein were incubated at 4°C for 2 h with up to 70 μl of avidin–agarose. The avidin–agarose was pelleted and acetyl-CoA carboxylase activity was assayed in the supernatant.

30 μl of agarose or avidin–agarose over a period of 6 h at 4°C. Control samples of enzyme extract were incubated under the same conditions without the addition of agarose. After 6 h there was a decrease of 42% in enzyme activity in the control (no gel) samples. An identical decrease in measurable activity over the same period was observed after incubation with unmodified agarose. This shows that no binding of enzyme was occurring to unmodified agarose, the loss of activity being due to other factors, such as the lability of the enzyme. As there was a constant loss of enzyme activity in the control samples, the binding of enzyme to avidin–agarose was calculated as a percentage of the initial acetyl-CoA carboxylase activity at the start of the incubation. After 6 h incubation with avidin–agarose the level of enzyme activity remaining in the supernatant was only 18% of the initial value, indicating that 40% of the enzyme had bound to avidin–agarose. However, after 2 h incubation, the binding of enzyme to avidin–agarose was 38%. Fig. 4 shows that after 2 h incubation the rate of loss of enzyme activity from the supernatant in the presence of avidin–agarose was the same as that in the unmodified agarose or with no gel. This indicates that enzyme binding was complete after approx. 2 h and the decrease in supernatant activity after this time was due to enzyme inactivation.

Fig. 5 shows the enzyme activity remaining in the supernatant after incubation of extracts containing 200 μg of protein with up to 70 μl of avidin–agarose for 2 h at 4°C. Increasing the volume of avidin–agarose between 0 and 50 μl resulted in a substantial decrease in enzyme activity remaining in the supernatant (53%) but a decrease of only a further 4% was observed on increasing the gel volume to 70 μl. In further experiments enzyme extracts containing approx. 300 μg of protein were incubated with 100 μl of avidin–agarose for 2.5 h at 4°C. Although only a maximum binding of approx. 50% occurred in these experiments, this should be comparable for all samples under the conditions used.

Incubation of extracts prepared from rape-seeds of different ages with avidin–agarose. Samples of extracts (300 μg of protein) prepared from rape-seeds at 10, 18, 22 and 34 days after pollination were incubated with 100 μl of avidin–agarose at 4°C for 2.5 h. For each extract there was a decrease in acetyl-CoA carboxylase activity remaining in the supernatant of 40–50% at the end of the incubation.





EXPLANATION OF PLATE 1

Rape-seed cotyledon (*a*) *24 days after pollination* (*magnification* × *6300*) *and* (*b*) *32 days after pollination* (× *10300*)
Both plates show lipid droplets (arrowed) and large protein-storage bodies.

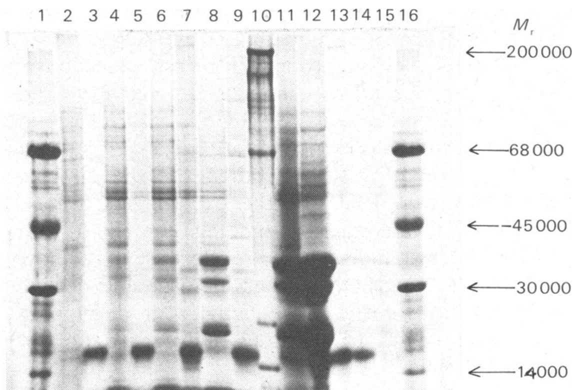


Fig. 6. *10% SDS/polyacrylamide-gel electrophoresis of rape-seed extracts and of avidin–agarose or agarose-bound proteins from seed extracts*

Samples of seed extracts were applied at a protein concentration of 80μg/track. Tracks 2, 4, 6 and 8 show seed proteins present at 10, 18, 22 and 34 days after pollination respectively. Samples of seed extracts containing 300μg of protein were incubated with 100μl of avidin–agarose at 4°C for 2.5h. The avidin–agarose was pelleted, washed three times with 0.5M-KCl in extraction buffer, then three times with buffer. The pellet was mixed with an equal volume of sample buffer and heated at 95°C for 10min. Tracks 3, 5, 7 and 9 show avidin–agarose-bound proteins from seed extracts prepared 10, 18, 22 and 34 days after pollination respectively. Track 11 shows the polypeptides present in an extract of rape-seeds of various ages and track 12 the polypeptides present in a partially purified preparation of acetyl-CoA carboxylase from this extract [11-fold purification of acetyl-CoA carboxylase by $(NH_4)_2SO_4$ fractionation]. Track 13 shows avidin–agarose-bound proteins from this partially purified preparation of acetyl-CoA carboxylase. Track 14 shows a sample of avidin–agarose heated at 95°C in sample buffer as for protein samples. Track 15 shows proteins bound to unmodified agarose after incubation with day 22 extract. Tracks 1 and 16 show marker-protein mixtures [bovine serum albumin (M_r 68000); ovalbumin (M_r 45000); carbonic anhydrase (M_r 30000); and lysozyme (M_r 14000)]. Track 10 shows a crude preparation of myosin from mouse muscle; the highest-molecular-weight band is myosin, M_r 200000.

The avidin–agarose pellet was washed, heated in sample buffer and the whole sample applied to a 10% SDS/polyacrylamide gel. Thus each track was loaded not with an equal amount of protein but with the total complement of proteins, which bound to avidin–agarose, present in an extract sample containing 300μg of total protein. The stained gel is shown in Fig. 6. The avidin–agarose pellet from day 10 showed only the presence of avidin (subunit M_r 18000) and one faintly staining band (M_r 62000). More bands were visible in the avidin–agarose pellets from days 18 and 22 particularly noticeable were two bands (M_r approx. 48000) and a single band of M_r 225000. This high-molecular-weight band was also present in the avidin–agarose-bound extract from day-34 seeds and in avidin–agarose-bound extract from a partially purified preparation of acetyl-CoA carboxylase from rape-seeds. It was not visible in any of the 20000g supernatants prepared from rape-seeds when applied at a protein concentration of 80μg/track. The band was not present in the agarose pellet after incubation of day-22 extract with unmodified agarose. There was an increase in the total amount of protein which bound to avidin–agarose in the day-22 extract. The intensity of the band at M_r 225000 was increased at day 22 compared with at day 18, but was lower at day 34.

Discussion

Acetyl-CoA carboxylase catalyses the first committed step in fatty acid biosynthesis. We have shown that in developing rape-seeds there was a marked rise in the specific activity of this enzyme, which preceded the accumulation of triacylglycerols within the cotyledons. A similar pattern of acetyl-CoA carboxylase activity and lipid deposition was reported by Simcox *et al.* (1979) in the endosperm of developing castor-oil seeds.

Acetyl-CoA carboxylase from plant tissues has been reported to be a very labile enzyme (Nielsen & Stumpf, 1976; Ebel & Hahlbrock, 1977; Mohan & Kekwick, 1980; Nikolau *et al.*, 1981). Acetyl-CoA carboxylase activity in extracts of developing rape-seeds declined by 42% in 6h at 4°C when incubated in the presence of 20% glycerol/0.2mM-phenylmethanesulphonyl fluoride/15mM-mercaptoethanol, thus limiting the usefulness of conventional techniques of protein separation in the purification of the enzyme.

Acetyl-CoA carboxylase has been purified from plant material, e.g. wheat germ (Hatch & Stumpf, 1961; Heinstein & Stumpf, 1969), barley embryos (Brock & Kannangara, 1976) and avocado plastids (Mohan & Kekwick, 1980). The molecular weight of the enzyme from these sources was estimated as 6.5×10^5. Several different subunit structures were observed in the enzyme preparations. The purified enzyme from wheat germ isolated by Heinstein & Stumpf (1969) dissociated into two components on ultracentrifugation but showed five bands on polyacrylamide-gel electrophoresis. Barley embryo acetyl-CoA carboxylase dissociated into three bands with molecular weights of 41000, 32000 and 21000

in polyacrylamide gels containing 8 M-urea (Brock & Kannangara, 1976). Three bands were also seen on SDS/polyacrylamide-gel electrophoresis of spinach chloroplast carboxylase (Mohan & Kekwick, 1980), although the molecular weights of these bands (47000, 57000 and 120000) were higher than those reported for barley embryos. Egin-Buhler *et al.* (1980) purified wheat-germ and parsley suspension culture acetyl-CoA carboxylases and showed them to have a similar subunit structure consisting of one large subunit (M_r 210000–240000) and one small subunit (M_r 98000–105000). The large subunit was shown to be the biotin-containing subunit of the enzyme by avidin-binding experiments.

We have attempted to identify the biotin-containing subunit of acetyl-CoA carboxylase by binding the enzyme from developing rape-seeds to avidin–agarose and removing non-specifically bound contaminants by salt washing. The strength of the avidin–biotin interaction (K_D = fM) precludes the elution of the bound enzyme in an active form. The enzyme was released from the avidin–agarose gel by boiling in sample buffer and the released polypeptides were analysed by SDS/polyacrylamide-gel electrophoresis. Preliminary optimization experiments showed that only approx. 50% of the enzyme could be removed from seed extracts by incubation with avidin–agarose and this proportion could not be increased by increasing the incubation time or the amount of avidin–agarose. The reasons for this are unknown. SDS/polyacrylamide electrophoresis revealed several differences in polypeptides bound to avidin–agarose from rape-seed extracts of different ages. Particularly noticeable was the presence of a polypeptide of M_r 225000, which was present in extracts of seeds at 18, 22 and 34 days after pollination but not in extracts of those after 10 days. This polypeptide was also prominent when a partially purified rape-seed extract [11-fold purification by $(NH_4)_2SO_4$ fractionation (results not shown)] was incubated with avidin–agarose. In this sample very few other bands were visible on the gel, although from other extracts, several polypeptides were bound by avidin–agarose. These may represent other biotin-containing polypeptides or may be contaminating species not removed by the salt-washing procedure used.

The presence of a polypeptide of M_r 225000, which binds to avidin–agarose, is in correlation with the findings of Egin-Buhler *et al.* (1980) that the biotin-containing subunit of acetyl-CoA carboxylase had an M_r of 210000–240000. Our results suggest that the 225000-M_r band from rape may represent the biotin-containing subunit of acetyl-CoA carboxylase. It is noteworthy that the intensity of this band, increases up to day 22 and then declines in the day 34 sample. Previous work on animal systems has shown that during fat synthesis, increases in acetyl-CoA carboxylase activity were due to increases in the amount of immunologically precipitable enzyme protein (Majerus & Kilburn, 1969). In *Candida* diminished activity and diminished synthesis of acetyl-CoA carboxylase in cells grown in the presence of fatty acid was due to a reduced level of mRNA coding for the enzyme (Horikawa *et al.*, 1980). Further experiments, using a system of avidin–agarose binding combined with SDS/polyacrylamide-gel electrophoresis, may show whether the rapid and marked increase and decline of acetyl-CoA carboxylase activity in developing rape seeds is due to synthesis and degradation of enzyme protein.

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