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Purification and Properties of the Fatty Acid Synthetase Complex from *Neurospora crassa*, and the Nature of the *fas*⁻ Mutation

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A procedure is described for the purification of the fatty acid synthetase complex (FAS) from *Neurospora crassa*. The enzyme complex has a molecular weight of 2.3×10^6, contains 6 mol of 4'-phosphopantetheine per mol, and on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate gives a single band, or a closely spaced doublet, which comigrates with standard myosin (molecular weight, 2×10^5). Since the slightly retarded component in the doublet accounts for all protein-bound 4'-phosphopantetheine, the complex appears to be made up of 11 to 12 equally sized subunits, 6 of which carry the acyl carrier protein function. In this unusual arrangement, notably the lack of the low-molecular-weight acyl carrier protein component seen in other FAS systems, as well as in its enzymatic properties, the *Neurospora* FAS complex is quite similar to the yeast enzyme. The FAS complex of a saturated fatty acid-requiring mutant, previously designated *cel*⁻, contains less than 2% of the 4'-phosphopantetheine prosthetic groups found in the wild-type complex. The leaky phenotype of this mutant, here designated *fas*⁻, is accounted for by a residual fatty acid synthesizing activity in its FAS complex, which is several-fold higher than expected from its residual content of 4'-phosphopanthetheine.

The enzymatic components of the soluble fatty acid synthetases (FAS) in bacteria and plants have been well characterized biochemically (20, 35), but it has been more difficult to dissect the multienzyme complexes found in other eucaryotes. Thus, although the yeast enzyme had been intensively studied biochemically for over a decade (11, 13, 18, 20, 30, 36), the elegant genetic and biochemical analysis by Schweizer and collaborators (9, 23-25, 31) very recently has radically changed the accepted picture of its subunit structure. We report here on a biochemical analysis of the FAS in *Neurospora crassa*, which confirms this re-evaluation of the structure of the fungal FAS complex. A mutant in *Neurospora*, originally designated *ol*⁻ for oleic acid requirer (20), was previously shown to actually require saturated fatty acid for growth by Henry and Keith (8), who also redesignated it as *cel*⁻, for fatty acid chain elongation negative. I now show that this mutant contains a defective FAS complex which is deficient in 4'-phosphopantetheine prosthetic groups, and suggest *fas*⁻, for fatty acid synthesis negative, as a more appropriate designation.

MATERIALS AND METHODS

Materials. The pantothenate-requiring strain *pan-2* and the saturated fatty acid-requiring strain *fas*⁻ (formerly *cel*⁻), originally from the Fungal Genetic Stock Center, Humbolt State College, Arcata, Calif., as well as a double mutant, *pan-2/fas*⁻, were kindly provided by S. Brody. Biochemicals and radiochemicals were obtained from commercial sources as follows: coenzyme A (CoA), malonyl CoA, acetyl CoA, butyryl CoA, caproyl CoA, lauryl CoA and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were from P/L Biochemicals; pantetheine, calcium pantothenate, dithiothreitol (DTT), phenylmethylsulfonyl fluoride, bovine serum albumin, cysteamine-hydrochloride and Tween 40 were from Sigma; phosphotransacetylase and acetyl phosphate were from Boehringer; [2-^{14}C]malonic acid (16.7 mCi/mmol); [^{3}H]acetic anhydride (500 mCi/mmol); and [1-^{14}C]pantothenic acid (3.72 mCi/mmol) were from New England Nuclear. [2-^{14}C]malonyl CoA and [^{3}H]acetyl CoA were synthesized by the procedures of Trams and Brady (32) and Simon and Shemin (28), respectively. Diacetyl cysteamine and *N*-acetylcysteamine (NAC) were prepared according to Martin et al. (15); acetoacetyl-NAC was prepared according to Lynen and Wieland (14). [^{3}H]CoA was prepared as previously described (4).

N. crassa was grown in liquid shaker culture at room temperature on Vogel medium (34) with 2% glucose as carbon source. Where indicated, further supplementation was with 0.04% Tween 40 as a source of saturated fatty acids and pantothenate, 0.27 μM; details are given below. Mycelia were harvested in late exponential growth phase, washed with distilled water, lyophilized, and finely powdered in a Waring

blender or Wiley mill.

Extraction and purification of FAS. Unless otherwise stated, all solutions contained 0.002 M EDTA (ethylenediaminetetraacetic acid) and 0.005 M DTT. Each gram of mycelial powder was extracted with magnetic stirring at 0 C for 30 min with 15 ml of 0.2 M potassium phosphate, pH 7, to which was added 0.1 ml of a fresh 10% phenylmethylsulfonyl fluoride solution in acetone. Insoluble material was removed by centrifugation for 30 min in a Sorval SS-34 rotor at 20,000 rpm to give the crude mycelial extract containing about 20 mg of protein/ml. The amount of protamine sulfate (usually about 0.05 ml of a 1.5% solution per ml of crude extract) which was required to precipitate nucleic acids without significant loss of FAS activity was determined on a small sample. After treating the bulk solution with the equivalent amount of protamine, and removing inactive material by centrifugation as above, the supernatant was further titrated with the protamine sulfate solution to precipitate the FAS activity (usually another 0.05 ml/ml of original crude extract). The yellowish, tough pellet obtained after centrifugation (SS-34, 10,000 rpm, 5 min) was suspended in about 1 ml of 0.3 M potassium phosphate per 50 ml of crude extract, and the mixture was vigorously extracted in a Teflon-glass homogenizer. Inactive material was removed by centrifugation (SS-34, 20,000 rpm, 1 h), and the yellowish, clear supernatant fluid was concentrated about fivefold on a Aminco centricone. Samples (0.5 ml or less) were centrifuged through an 11-ml 10 to 30% (wt/vol) sucrose gradient for 12 h at 40,000 rpm using a Beckman SW41 rotor. The gradients were prepared in 0.5 M potassium phosphate and, in addition to EDTA and DTT, also contained 5×10^{-6} M NADPH. Active fractions were pooled, concentrated as above, and applied in a volume of 0.2 ml or less per tube to a second set of sucrose density gradients identical to the first ones. After centrifugation and analysis pure FAS was collected, concentrated as above, and stored in liquid nitrogen in the gradient mixture.

Sodium dodecyl sulfate gel electrophoresis. The discontinuous procedure described by Neville (16) was used with 11% (wt/vol) total monomer and the pH 9.6 upper buffer. Discontinuous electrophoresis in the presence of urea was performed as described by Feit et al. (6). The Fairbanks system (5) was used for continuous electrophoresis. Samples were prepared for electrophoresis by heating in 10 mM DTT at 100 C for 2 min in the presence of 1% sodium dodecyl sulfate (SDS) at pH 9. Gels were fixed in 25% isopropanol-7.5% acetic acid overnight and stained in 0.25% Coomassie Blue in 50% methanol-7.5% acetic acid for 1 h, followed by electrophoretic destaining in 7.5% acetic acid.

For counting, stained gels were frozen on dry ice, and slices about 2 mm thick were cut manually with a razor blade. The slices were digested for 8 h at 50 C with 0.5 ml of 30% hydrogen peroxide in tightly capped scintillation vials which also contained a small plastic tube with a strip of filter paper soaked in ICN tissue solubilizer. Only trace amounts of radioactive carbon dioxide were found to be produced in the oxidation procedure. The digested samples were counted in 10 ml of the Patterson-Greene scintillation solvent (19).

Enzyme assays. The standard assay medium contained 0.1 M potassium phosphate, pH 7, 2.5 mM EDTA, 1 mM DTT, and 0.025% bovine serum albumin. FAS activity was assayed spectrophotometrically as the malonyl CoA-dependent oxidation of NADPH, essentially as described by Lynen (11), using a Gilford 240 automatic spectrophotometer. Acetyl CoA (0.01 μmol), 0.03 μmol of NADPH, and 0.5 to 5 mU of enzyme were added to 0.2 ml of standard assay medium. After recording a blank rate at 340 nm for 2 min, FAS was initiated by the addition of 0.015 μmol of malonyl CoA. β-ketoacyl reductase activity was measured as the oxidation of NADPH (0.03 μmol in 0.2 ml of standard assay medium), which was dependent on the addition of acetoacetyl-NAC (5 μmol).

Malonyl transacylase. [^{14}C]malonyl CoA, (0.01 μmol; specific activity, 1 mCi/mmol), 1.6 μmol of pantetheine, and 0.1 to 0.5 mU of enzyme were incubated in 0.2 ml of standard assay mix. After 5 min at 22 C, 1 ml of saturated sodium chloride in 0.1 N HCl and 1.2 ml of *n*-butanol were added, and the mixture was thoroughly agitated. The radioactivity in the butanol, corrected for the 80% recovery of authentic malonyl pantetheine in a single extraction, was a measure of the malonyl pantetheine formed in the reaction. Acetyl transacylase activity was determined in the identical manner, substituting 0.01 μmol of [^{3}H]acetyl CoA (8.4 mCi/mmol) as acyl donor and using 2 to 20 mU of enzyme/assay.

The activity of the β-ketoacyl synthetase component in the fatty acid synthetase was assayed as acetoacetyl-NAC-dependent $^{14}CO_2$ fixation, essentially as described by Lynen (13). Assay mixtures containing 10 μmol of tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.3, 0.5 μmol of DTT, 0.25 μmol of EDTA, 0.3 μmol of CoA, 6 μmol of acetoacetyl-NAC, 0.5 μmol of $H^{14}CO_3^-$ (specific activity, 2 mCi/mmol), and 50 to 3 mU of enzyme in a total volume of 0.1 ml were incubated at 37 C for 30 min. After the addition of 0.01 ml of 10 N HCl a 0.05-ml sample was dried on a small filter paper disk, and the nonvolatile radioactivity was determined by liquid scintillation counting as described (4). The condensing activity was also assayed by an adaptation of the malonyl CoA-CO_2 exchange reaction (4). Assays contained 10 μmol of tris(hydroxymethyl)aminomethane-hydrochloride, of pH 7.3, 0.5 μmol of DTT, 0.25 μmol of EDTA, 0.1 μmol of CoA, 0.03 μmol of malonyl CoA, 0.01 μmol of caproyl CoA, 0.5 μmol of $H^{14}CO_3$ (specific activity, 2 mCi/mmol), and 0.5 to 5 mU of enzyme in a total volume of 0.1 ml. After incubation at 37 C for 15 min, nonvolatile radioactivity was determined as above.

All enzymatic activities are given as international units. Overall fatty acid synthetase activity is measured as the rate of malonyl CoA utilization, i.e., as half the rate of NADPH oxidation.

Attempt to demonstrate 4'-phosphopantetheine transfer to *fas*$^-$ FAS in vitro. Purified *fas*$^-$ FAS (0.25 nmol) was incubated with 210 nmol of [^{3}H]-pantetheine-CoA (specific activity, 13×10^3 counts/min per nmol), 10 μmol of DTT, 15 μmol of $MgCl_2$,

and wild-type *Neurospora* crude extract (10 mg of protein) in a total volume of 0.6 ml. Two similar incubations, one without *fas*⁻ FAS, the other substituting heat-inactivated crude extract (100 C for 5 min), served as controls for nonspecific formation of acid-insoluble radioactivity. After incubation at 30 C for 30 min, 2 μmol of unlabeled carrier CoA was added to each incubation followed by 5 ml of 7.5% perchloric acid. The precipitated protein was recovered by centrifugation and resolubilized in 1 ml of a solution containing 1 mM CoA, 10 mM EDTA, and 5 mM DTT in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 9. After repeating this washing procedure three times, no acid-soluble radioactivity was detectable, and the acid-insoluble radioactivity was determined by counting a sample of the resolubilized protein.

Isolation of CoA from pan-2/fas⁻ grown on [^{14}C]pantothenate without FAS. The supernatant (250 ml), obtained after the second protamine sulfate precipitation of a crude mycelial extract, was made 5% (wt/vol) in perchloric acid, and the precipitate was removed by centrifugation. After neutralizing the supernatant fluid with 5 M potassium hydroxide, filtering, and lyophilizing, the residue was reduced, benzoylated, and extracted as described by Schweizer et al. (26). After alkaline debenzoylation in the presence of 100 mM mercaptoethanol, the CoA was finally purified by chromatography on diethylaminoethyl-cellulose as described (4).

Other analytical procedures. The molecular weight of *Neurospora* FAS was determined in a model E analytical centrifuge by the sedimentation-diffusion equilibrium method of Yphantis (37). Sulfhydryl groups were determined by the Ellman procedure (3). Phosphotransacetylase assays for CoA were performed as previously described (4). Protein was determined by the method of Lowry et al. (10) or the microburet (7) procedure. To measure the incorporation of [1-^{14}C]pantothenate into purified *Neurospora* FAS, peak tubes from the last gradients were pooled and exhaustively dialyzed against 0.5 M potassium phosphate (pH 6.5) to remove interfering sucrose and DTT. Protein was determined with the microburet procedure using bovine serum albumin as a standard; radioactivity was determined by precipitating duplicate samples onto glass filter paper disks, washing with trichloroacetic acid and ethanol-ether (1:1), drying, and counting in 2 ml of the Patterson-Greene (19) scintillation solvent without Triton X-100. Samples from the gradient were counted directly in 10 ml of the complete scintillation solvent. Absolute activities were determined with external and internal standard using a Packard 3320 liquid scintillation spectrometer.

RESULTS

Isolation of FAS. Table 1 summarizes the purification of wild-type and *fas*⁻ FAS, which relies on the fact that the synthetase may be precipitated with protamine sulfate. The enzyme cannot be prepared by the common procedures used for the yeast FAS complex (11). Ammonium sulfate fractionation gives poor recoveries without effective purification, and the *N. crassa* activity was not adsorbed to calcium phosphate gels or to hydroxyapatite even at ionic strength as low as 1 mM potassium phosphate. The enzyme adsorbed to diethylaminoethyl-cellulose at this low ionic strength, but no activity was recovered by elution with higher ionic strength buffers with or without added glycerol or sucrose. The enzyme could be pelleted at $10^5 \times g$, but yields on resuspension were very low, and final purification, therefore, had to rely on gradient centrifugations, although even this mild procedure gave considerable loss of FAS activity. Since high ionic strength seemed essential to maintain activity, the gradients were prepared in 0.5 M phosphate buffer containing saturating amounts of NADPH, which appeared to improve stability somewhat. Overall FAS activity was lost most rapidly, but the yield of β-ketoreductase activity was also rather low, particularly for the *fas*⁻ mutant. Depending on the time required for purification, the wild-type enzyme varied in specific activity from about 2,000 mU of FAS/mg of protein to values less than one-third of that, although SDS-gel electrophoresis of such preparations gave essentially identical patterns.

Partial activities of wild-type and fas⁻ Neurospora FAS. The activities (Table 2) of

TABLE 1. *Purification of FAS from wild-type and fas⁻ Neurospora*

Purification step	Wild type			*fas*⁻		
	Protein (mg)	Sp act (mU/mg)[a]	Yield (%)	Protein (mg)	Sp act (mU/mg)[b]	Yield (%)
Crude extract	8,100	8.5	100	3,850	13	100
Protamine sulfate precipitate	305	124	55	72	287	41
Final sucrose density gradient	4.6	1,800	12	1.7	2,700	9

[a] FAS activity.
[b] β-Keto reductase activity.

TABLE 2. *Specific enzymatic activities of purified FAS from wild-type and fas⁻ Neurospora*

Activity assayed	Wild type	*fas*⁻
Fatty acid synthesis	1,800[a]	15
β-Keto reductase	3,150	2,700
Malonyl transacylase	27,000	17,500
Acetyl transacylase	380	600
CO_2 fixation	1.6	ND[b]
CO_2 exchange:		
Butyryl CoA[c]	17	ND
Caproyl CoA[c]	68	ND
Lauryl CoA[c]	100	ND

[a] Values are given as milliunits per milligram of protein.

[b] ND, Not detectable.

[c] Chain length of saturated acyl CoA used in malonyl CoA-CO_2 exchange reaction.

the β-ketoacyl reductase and the malonyl and acetyl transferases compared to overall fatty acid synthetic activity for the *Neurospora* complex are similar to those reported for yeast (9, 11, 23). Table 2 also shows that these partial activities are intact in the *fas*⁻ mutant, although its overall fatty acid synthetic ability is only about 1% of that in the wild-type. Like the yeast enzyme, the *Neurospora* wild-type FAS complex catalyzes a slow fixation of $^{14}CO_2$ into malonyl NAC in the presence of acetoacetyl NAC and free CoA, at a rate of about 10^{-3} of that of overall fatty acid synthesis. This activity, however, is absent in the *fas*⁻ mutant. Since the product of this reaction is malonyl NAC (11), the overall fixation requires functional acetyl transacetylase, acyl carrier protein (ACP), and condensing activities; since the former is intact in the *fas*⁻ mutant, this finding would localize the lesion in one of the latter two components. The wild-type synthetase also catalyzes an acyl CoA-dependent malonyl CoA-CO_2 exchange reaction. Table 2 shows that the wild-type enzyme catalyzes this exchange with caproyl and lauryl CoA at a rate several-fold faster than that obtained with buryryl CoA. However, no CO_2-exchange activity can be demonstrated with any of these acyl CoA's in the mutant complex, showing that the mutation in *fas*⁻ renders its FAS incapable of elongating not only acetyl CoA but also longer acyl homologues.

Molecular weight and subunit composition of Neurospora FAS. As isolated after the second zonal centrifugation, the FAS complex was essentially pure by analytical ultracentrifugation, although SDS-gel electrophoresis sometimes showed a small amount of contaminating material. The synthetase gave a molecular weight of 2.28×10^6 by sedimentation-diffusion equilibrium, assuming a partial specific volume of 0.748, as reported for the yeast enzyme (21).

SDS-gel electrophoresis of either wild-type or *fas*⁻ FAS (Fig. 1) usually showed a single major band of 200,000 daltons which comigrated with standard myosin. Besides some presumably aggregated material which failed to enter the gel, small amounts of lower-molecular-weight peptides were usually present, but analysis of samples across the second zonal gradient showed that these were derived from contaminants which sedimented slightly faster than the FAS complex. When purified labeled FAS from *pan*-2 grown on [^{14}C]pantothenate was analyzed, the 200,000-dalton major band contained all the label. On one occasion, this major component appeared as two closely spaced bands of about equal staining intensity (Fig. 2) with radioactivity associated exclusively with the more slowly moving component. However, for reasons which remain unknown, the same sample rerun under presumably identical conditions failed to show this pattern. Omission of sample reduction, reduction at different temperature with or without urea present, and electrophoresis in a continuous or in a discontinuous SDS-urea system also failed to reproduce the pattern in Fig. 2. The major labeled component consistently migrated as a single band of molecular weight 200,000 in all subsequent preparations. Although the pattern in Fig. 2, therefore, could not be reproduced, the fact that on that occasion the labeled 4′-phosphopantetheine was exclusively found in the more slowly moving band indicates the presence of at least two different types of peptides in the complex, although they normally both comigrate with rabbit muscle myosin in SDS-gel electrophoresis. In yeast, two bands of molecular weight 185,000 and 180,000 are reported to be present, the former being labeled by [^{14}C]pantothenate. A complication in the yeast system was a varying degree of proteolytic transformation of that component to a band running with an apparent molecular weight of 177,000 (24). This was not observed in the *Neurospora* preparations which were extracted in the presence of phenylmethylsulfonyl fluoride.

Pantetheine content of Neurospora FAS. Figure 3a shows that protein, fatty acid-synthesizing activity, and ^{14}C radioactivity coincide on the gradient used as the last step to purify FAS from the *pan*-2-Fas⁺ mutant grown on [^{14}C]pantothenate.

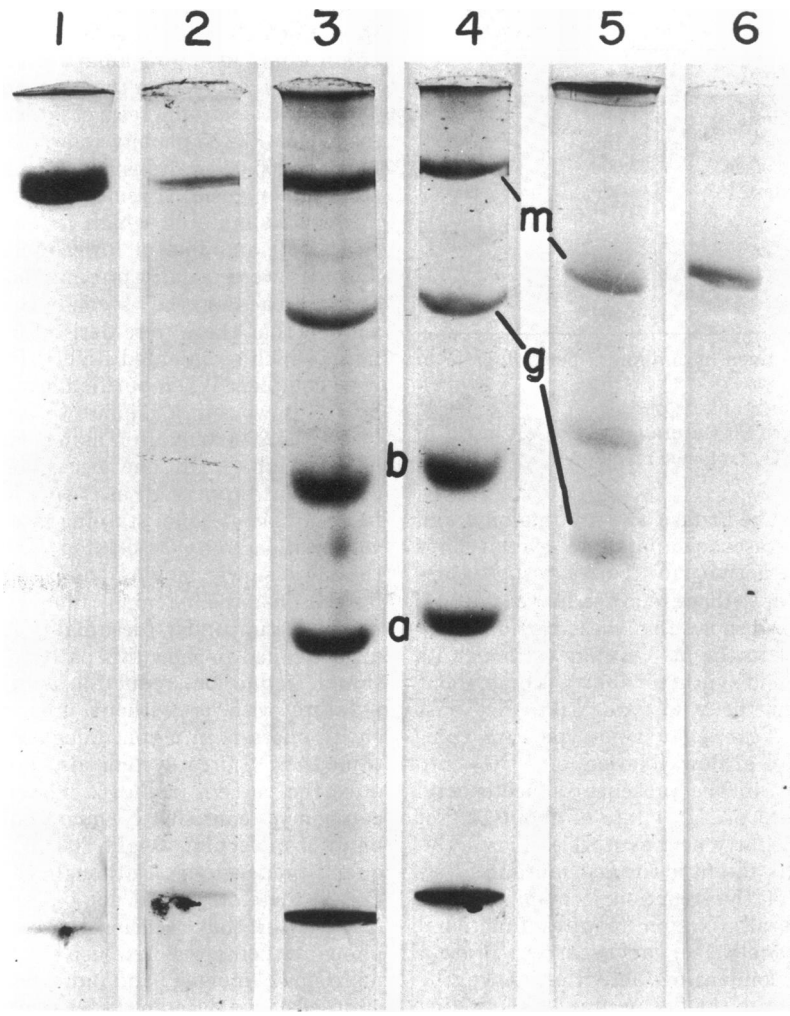

FIG. 1. *SDS-polyacrylamide gel electrophoresis of FAS from Neurospora (16). (1) pan-2/fas⁺ FAS, 40 μg; (2) same, 5 μg; (3) rabbit muscle myosin (m), Escherichia coli β-galactosidase (g), bovine serum albumin (b), and rabbit muscle actin (a), 10 μg each; (4) 2 plus 3; (5) myosin and β-galactosidase, 6 μg each; (6) pan-2/fas⁻ FAS, 10 μg. (1 to 4) were subjected to electrophoresis for 2.5 h at 1.5 mA per tube; (5 and 6) were subjected to electrophoresis for 14 h at 0.6 mA per tube.*

Determination of protein-bound radioactivity in the peak tubes gave a value of 2.65 nmol of pantothenate/mg of protein, equivalent to 6.05 nmol of 4′-phosphopantetheine per mol of 2.28×10^6-dalton enzyme. Thus, the *Neurospora* FAS complex appears to be composed of 11 to 12 peptides, with molecular weights of 2×10^6, 6 of which carry a 4′-phosphopantetheine prosthetic group.

Absence of protein-bound pantetheine in the fas⁻ mutant. Figure 3b shows that when the *pan-2/fas⁻* double mutant was grown on [^{14}C]-pantothenate, the β-ketoacyl reductase activity peak in the last gradient contained only traces of radioactivity, about 0.5% of that of the *fas⁺* wild type per mg of protein. Its sedimentation pattern on the sucrose gradient and its simple staining pattern on SDS-gel electrophoresis (Fig. 1) were otherwise the same as those obtained for the wild-type complex. In the absence of evidence for more extensive structural changes, the mutant FAS complex therefore appears to differ from that of the wild type simply by lacking the six 4′-phosphopantetheine prosthetic groups. This would be sufficient to account for the defect in condensing activity

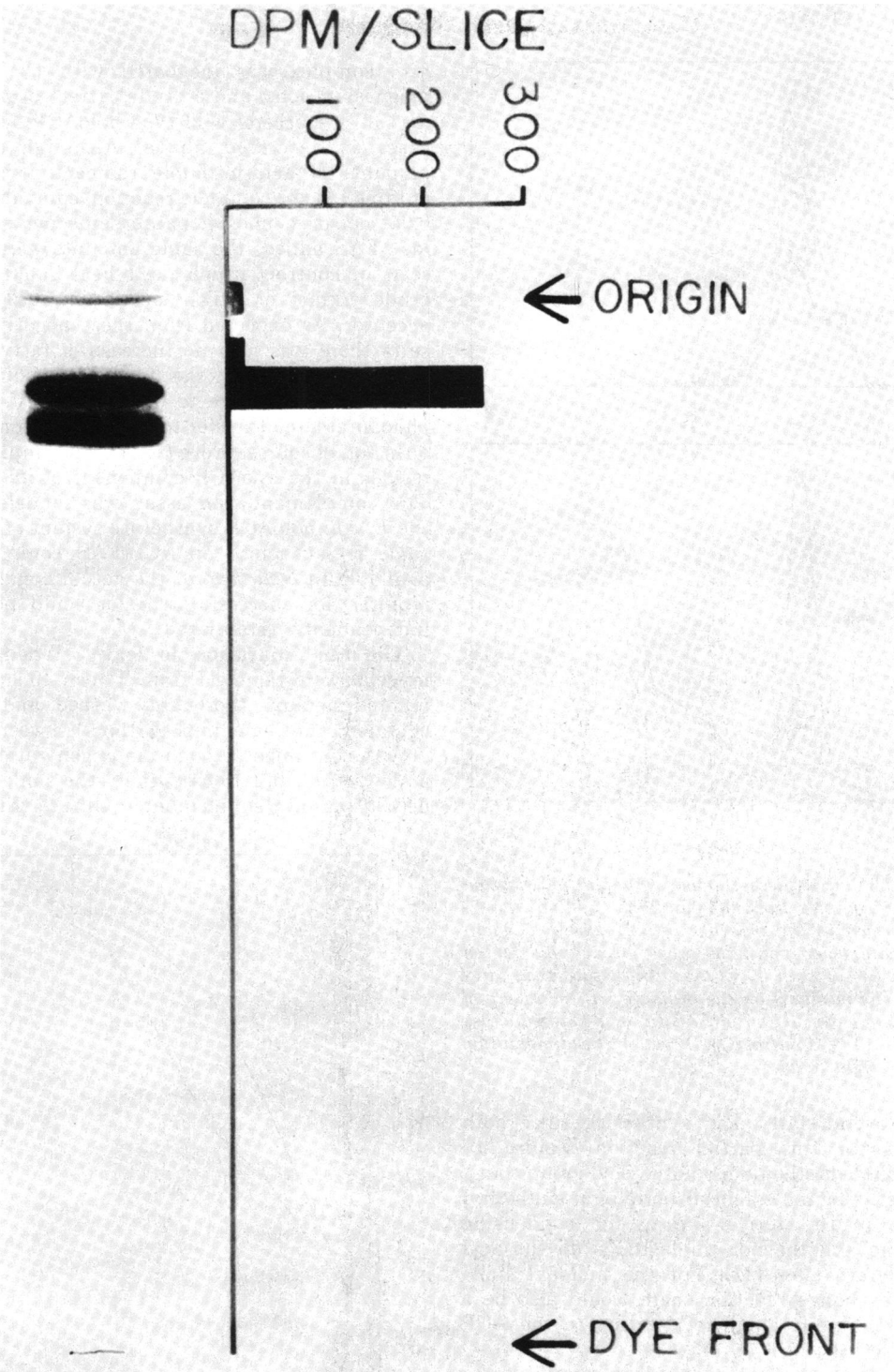

FIG. 2. *Distribution of ^{14}C radioactivity after SDS-gel electrophoretic separation of Neurospora FAS. Purified FAS (22 μg) (peak tube in Fig. 3a) from pan-2/fas^{+} grown in 0.27 μM [1-^{14}C]pantothenic acid was subjected to electrophoresis as described in the legend to Fig. 1 for 2.5 h at 1.5 mA. On this occasion the single band which is normally seen (Fig. 1) appeared as a doublet. The gel was stained, sliced, and counted as described in Materials and Methods. See Results.*

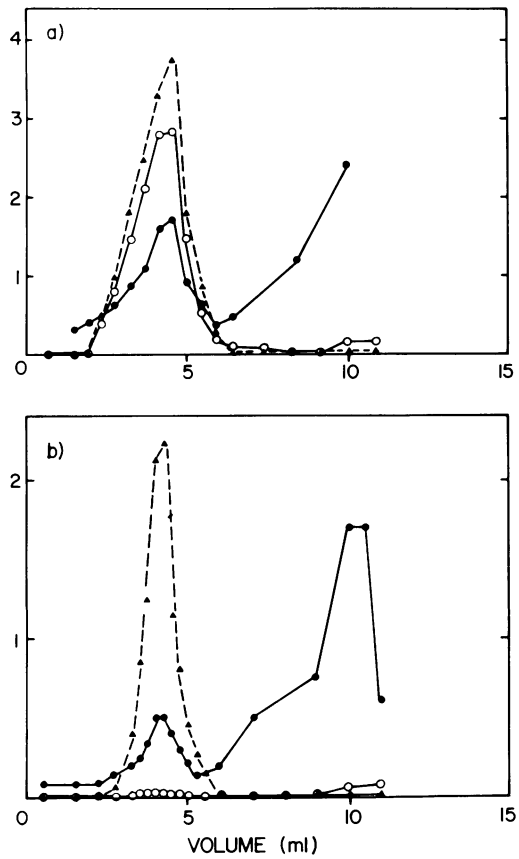

FIG. 3. *Final sucrose density gradient purification of FAS complex from* (a) *pan-2/fas*$^+$ and (b) pan-2/fas$^-$ *grown in the presence of 0.27 μm [1-*^{14}*C]pantothenate acid; pan-2/fas*$^-$ *was also supplemented with 0.04% Tween 40 as a source of saturated fatty acid. The numbers on the ordinate represent: mg of protein/ml (●); IU/ml of FAS* (a) *or β-ketoreductase* (b) (▲); $10^4 \times$ *dpm/ml* (○). *Direction of sedimentation is from right to left.*

(and overall fatty acid synthesis), since both assays for this partial reaction require 4'-phosphopantetheine-mediated acyl group transfer between the essential peripheral sulfhydryl group in the active site of the condensing enzyme and the non-thiol groups on the acyl transferase sites (11). For the malonyl CoA-CO_2 exchange reaction there would also be a requirement for malonyl binding to the ACP prosthetic group.

An attempt was made to determine if the defect in *fas*$^-$ could be assigned to an alteration of the apoACP portion of one of the FAS peptides or to a deficiency in a presumed holoACP synthetase (4). The purified *pan-2/fas*$^-$ complex was incubated with [^{3}H]CoA, magnesium ions, and a crude extract of *pan-2/fas*$^+$ as a source of wild-type holoACP synthetase, as described above. Although small amounts of acid-insoluble radioactivity were obtained in the complete reaction, equivalent to 0.05 mol of 4'-phosphopantetheine per mol of *fas*$^-$ FAS added, the same amounts were also seen in controls which used heat-inactivated crude extract or which lacked the *fas*$^-$ FAS acceptor. As expected from these negative results there was also no increase of fatty acid synthetic activity in the complete incubation mixture. This failure to demonstrate 4'-phosphopantetheine transfer to the mutant complex does not, of course, prove that the fas$^-$ mutation resides in the apoACP component of its FAS. The complementation assay was modeled on the *E. coli* holoACP synthetase system (4), but since no authentic apoACP-FAS complex is available in *Neurospora*, the actual conditions required for successful transfer could not be independently established.

The fas$^-$ mutation is leaky. When *fas*$^-$ mycelia were removed from Tween 40-supplemented medium, thoroughly washed, and reinoculated into medium without Tween 40, growth continued through many generations at a slower rate but to essentially the same final density as in a supplemented culture (Fig. 4).

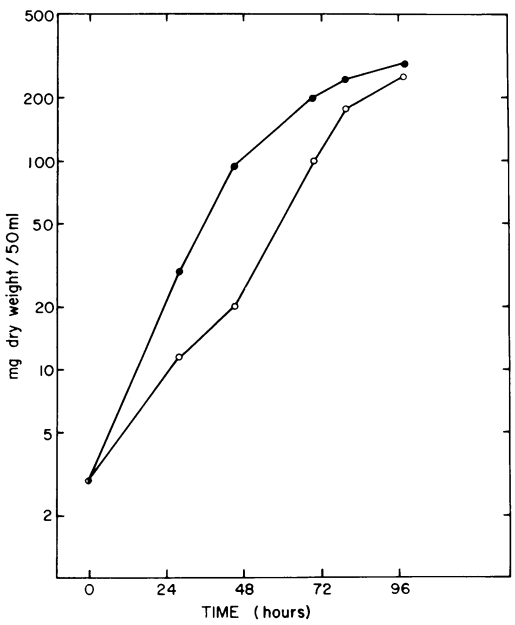

FIG. 4. *Growth of pan-2/fas*$^-$ *with* (●) *and without* (○) *0.04% Tween 40 supplementation.*

Due to the limitations of the assay, fatty acid synthesizing activity could not be demonstrated in crude extracts of such unsupplemented mycelia; however, assay of their purified FAS complex showed a very significant residual activity for overall fatty acid synthesis, about 7% of that found in the wild-type complex. Furthermore, when isolated from a large scale culture of the *pan*-2/*fas*$^-$ double mutant grown on [^{14}C]pantothenate without fatty acid supplement, the purified FAS complex also contained significant amounts of radioactivity, equivalent to 0.045 nmol of 4'-phosphopantetheine per mg of protein, or 1.8% of that obtained with the *pan*-2/*fas*$^+$ complex. To eliminate the possibility that a leaky *pan*-2 block might give an erroneously low estimate of protein-bound pantothenate, the mycelial CoA was isolated in this experiment as described above. Its specific radioactivity was found to be 3.5 mCi/mmol of phosphotransacetylase-reactive material, in good agreement with the manufacturer's value of 3.72 mCi/mmol for the pantothenic acid used to supplement the growth medium.

DISCUSSION

The data presented here show the *Neurospora* FAS to be quite similar to the yeast complex. The specific activities of overall fatty acid synthesis as well as the partial reactions tested here are roughly similar in the two complexes, although in both cases they vary considerably from preparation to preparation. The molecular weight of the yeast complex has been reported to be 2.2×10^6 by sedimentation velocity (18) and low-angle X-ray scattering (21), based on a measured partial specific volume of 0,748 ml/g. Using this same value in a sedimentation equilibrium analysis the identical molecular weight is obtained for the *Neurospora* complex.

In yeast complementation analysis of a large number of fatty acid auxotrophs shows two unlinked loci: *fas-1*, coding for the dehydratase (group V) and enoyl reductase activities (group II); and *fas-2*, coding for condensing (group VI) and β-ketoreductase activities (group VIII), as well as the ACP function (group VII) in the complex (9, 25). Each locus was originally assumed to be a closely linked gene cluster, but X-ray-induced mitotic recombination analysis showed *fas-1* to be a single gene of a length equivalent to a 200,000-dalton single polypeptide carrying the enzymatic activities for both the reductase and dehydratase component (31). SDS-gel electrophoretic analysis of the yeast complex showed two closely spaced bands of about equal intensity, corresponding to molecular weights of 185,000 and 180,000, respectively, with the 4'-phosphopantetheine group bond to the larger species (24). Since this prosthetic group was shown to be absent from all group VII mutants, it was concluded that the 185,000-dalton peptide band contained a multifunctional peptide coded for by the *fas-2* locus and that the 180,000-dalton band contained the multifunctional peptide coded for by the *fas-1* locus. No such genetic analysis is available in *Neurospora*, but our biochemical data is consistent with a similar subunit composition for its FAS complex, although minor analytical differences remain. Thus, the *Neurospora* complex also seems to contain a minimum of two kinds of peptides which, however, normally appear as a single band on SDS-polyacrylamide gel electrophoresis analysis, with a molecular weight very close to 200,000 since they comigrate with standard muscle myosin. The lower values for the yeast peptides were obtained by extrapolation in a different gel system, and a direct comparison will be necessary to determine if these are real differences. This is also true for the slight discrepancy in the number of 4'-phosphopantetheine groups per complex. Our value of six for the *Neurospora* complex is based on the specific radioactivity of commercial [1-^{14}C]pantothenic acid, which was found to be in good agreement with an independent determination of the specific activity of mycelial CoA. A value of 5 for the yeast enzyme was obtained by microbiological and chemical assays for pantethenate and β-alanine (27). The yeast enzyme has also been reported to contain 5 mol of flavine mononucleotide (18) and to bind 5 mol of malonyl groups at nonsulfhydryl malonyltransacylase sites (26).

The analysis of the *Neurospora* system confirms the important finding that the fungal ACP component is part of a very large polypeptide, rather than existing as the dissociable low-molecular-weight protein classically found in plant and bacterial systems (22). Previous reports on the isolation of such components from the yeast complex (36) are most likely attributed to proteolytic degradation. Very recently a similar situation has also been reported to pertain to the animal FAS complex (29). It thus appears that the subunits of about 200,000 dalton which were obtained by the reversible dissociation of the yeast enzyme (30) represented the actual multifunctional peptide monomers rather than being incompletely dis-

sociated oligomers, as concluded at the time.

The lesion in the FAS from the *fas*$^-$ mutant in *Neurospora* is seen to be similar to that in yeast mutants in complementation group VII, i.e., an absence of 4'-phosphopantetheine prosthetic groups. This accounts for its lack of overall fatty acid synthesis and its inability to catalyze the condensation partial reaction while retaining the other partial reactions assayed. The data does not allow for a conclusion whether this defect is due to a mutation in a holoACP synthetase enzyme or in the apoACP component of the FAS. No complementation could be seen in vitro, but this might be accounted for by experimental difficulties as discussed above. Furthermore, it is not known if transfer of the prosthetic group can occur after assembly of the complex or whether it must be accomplished at some previous stage. In the mammalian FAS complex an independent turnover of the 4'-phosphopantetheine (33) suggests that the prosthetic group can be removed from and reattached to the finished complex, but no such information is available for yeast or *Neurospora*. All pantetheineless mutants in yeast map in the *fas*-2 locus (24), suggesting a defect in the apoACP portion of that peptide, but the possibility exists that the holoACP synthetase function is part of the complex itself.

The growth of the *fas*$^-$ mutant without fatty acid supplementation (Fig. 4) was unexpected. Henry and Keith (8) used different culture conditions and apparently found a more pronounced fatty acid requirement for growth but noted that slow growth did occur without supplementation. This pronounced leakiness raised the possibility that *Neurospora* might have alternative pathways for de novo fatty acid synthesis independent of the FAS complex. However, the situation is complicated by the fact that a careful analysis of the purified FAS from unsupplemented *fas*$^-$ cells showed a considerable residual overall fatty acid synthetic ability, about 7% of that of the wild-type complex, and a low but significant content of radioactive 4'-phosphopantetheine groups, about 2% of that in the wild-type. These values are higher than those obtained with fatty acid-supplemented cultures; possibly, the slower growth without supplement allowed a residual holoACP-synthesizing system to transfer more prosthetic groups to the *fas*$^-$ complex. The question remains whether this residual fatty acid synthetic activity in unsupplemented *fas*$^-$ furnishes enough fatty acid to maintain the somewhat slower growth seen under those conditions. The doubling time for the wild-type on unsupplemented minimal medium is about 4 h; that of *fas*$^-$ (Fig. 4) is about 14 h. Furthermore, whereas the wild-type contains about 150 μmol of total fatty acid per g dry weight (1), unsupplemented *fas*$^-$ contains about 90 μmol per g dry weight, due to a 50% drop in its content of neutral lipids (S. Brody, personal communication). These differences would decrease the demands on *fas*$^-$ fatty acid synthesis in vivo to about one-fifth of that for the wild-type. Although this is still in excess of the 7% residual activity seen in vitro, the uncertainties of such comparisons are considerable, and at this time it does not seem warranted to postulate a second, hypothetical pathway for de novo fatty acid synthesis in *Neurospora*. FAS-independent microsomal (17) and mitochondrial (2) fatty acid elongating systems do occur in animals but appear incapable of de novo synthesis.

Returning to the structure of the *fas*$^-$ FAS, it is interesting to note its 7% residual overall fatty acid synthetic activity, compared to only 2% residual content of 4'-phosphopantetheine. Although this analysis needs to be confirmed, it would be compatible with a structure of the complex such that each 4'-phosphopantetheine "swinging arm" (13) of the ACP functions can interact with more than one set of the seven other functions which are necessary for overall fatty acid synthesis. This arrangement would also account for the extraordinary degree of intragenic complementation or cross-feeding which is seen in the yeast complex (25).

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