A trifunctional $\beta$-oxidation protein, designated TFP, was purified to apparent homogeneity from oleate-induced mycelia of *Neurospora crassa*. 2-Enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-hydroxyacyl-CoA epimerase activities copurified in constant ratios with this protein when crude extracts were subjected to cation-exchange, dye-ligand, and adsorption chromatography. Trifunctionality was substantiated by coinciding enzyme activity ratios during the last two purification steps and additional chromatographic steps. The enzyme was shown to be a 365-kDa tetramer of subunits with a molecular mass of 93 kDa. Several lines of evidence suggest that these subunits are identical. Monospecific antibodies raised against the homogenous protein specifically precipitated the three enzymatic activities of TFP. Immunoblotting of fractions obtained after sucrose density centrifugation of a crude extract indicated that TFP was exclusively localized in glyoxysome-like microbodies. The $\beta$-oxidation system of *N. crassa* is structurally related to those of peroxisomes despite the presence of an acyl-CoA dehydrogenase rather than an acyl-CoA oxidase.

A mitochondrial 2-enoyl-CoA hydratase activity was separated from TFP and purified to apparent homogeneity. The absence of all other $\beta$-oxidation activities from mitochondria suggests that this organelle and its 2-enoyl-CoA hydratase are not involved in fatty acid degradation in *N. crassa*.

The $\beta$-oxidation pathways comprise the same chemical reactions in all fatty acid degrading organisms (1, 2). However, the proteins involved in their catalysis vary greatly. In eukaryotic cells $\beta$-oxidation has been detected in two different organelles, in mitochondria (3, 4) and/or in peroxisomes (glyoxysomes) (1, 5, 6). These different organelar systems are clearly distinguished by at least two features. They differ by the type of enzyme which catalyzes the first reaction of the $\beta$-oxidation cycle and, in addition, by the structural organization of the other enzymes (1, 2, 7-9). Whereas the mitochondrial $\beta$-oxidation cycle starts with an acyl-CoA dehydrogenase(s)-catalyzed reaction (10), the peroxisomal counterpart depends on an acyl-CoA oxidase (7, 11, 12). Moreover, in mitochondrial $\beta$-oxidation systems the individual enzymes are discrete and separable protein entities (13), while peroxisomal $\beta$-oxidation systems contain multifunctional proteins (2, 8). All these proteins comprise 2-enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities. In yeasts (2) and cucumber seeds (14) the multifunctional proteins, in addition, contain 3-hydroxyacyl-CoA epimerase. Very recently, it was reported that the multifunctional protein of rat peroxisomes also possesses a third activity, but this activity is an 2,3-enoyl-CoA isomerase rather than an epimerase (15).

Recently, we reported the existence of an unusual $\beta$-oxidation system in the filamentous fungus *Neurospora crassa* (16). It is not localized in mitochondria, although it contains an acyl-CoA dehydrogenase(s) rather than an acyl-CoA oxidase. Moreover, it is not associated with catalase, the classical marker of peroxisomes. Instead, all $\beta$-oxidation activities are housed in a different type of organelle which also contains the key enzymes of the glyoxylate cycle, i.e. isocitrate lyase and malate synthase. Thus, the $\beta$-oxidation system in *N. crassa* is neither typical for mitochondria nor peroxisomes, and it has yet to be established whether the atypical glyoxysome-like microbodies of *N. crassa* (16) can be assigned full membership in the microbody family.

In an attempt to determine at the molecular level the relatedness of the fatty acid-degradation system in catalase-free microbodies of *N. crassa* with those of mitochondria and peroxisomes we decided to study its enzymes more closely. This paper describes the isolation and characterization of a trifunctional protein from *N. crassa* which comprises three activities of its $\beta$-oxidation system. The presence of such a multifunctional protein indicates a great structural similarity of the $\beta$-oxidation system of *N. crassa* to the known peroxi- somal ones and clearly distinguishes it from the mitochondrial counterparts.

*N. crassa* contains a second 2-enoyl-CoA hydratase activity. It is a mitochondrial protein of M, 182,000 and appears not to be involved in fatty acid degradation.

**EXPERIMENTAL PROCEDURES AND RESULTS**

*Homogeneity, Molecular Mass, and Subunit Identity of the TFP—Several lines of evidence strongly support the conclu-

1 Portions of this paper (including "Experimental Procedures," part of "Results," Tables I-III, and Figs. 1, 2, and 8-13) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
and/or by interaction of the protein with the ampholytes. The purified protein eluted as a single component from reversed-phase HPLC (Fig. 4).

Amino-terminal sequence analysis of the TFP revealed no amino acid phenylthiohydantoins above background during five cycles of Edman degradation, indicating that the protein has a blocked amino terminus. In addition, limited proteolysis yielded only two major initial fragments and their molecular masses account for the molecular mass of approximately 93,000 Da of the undegraded protein subunit (55).

The native relative molecular mass of the purified TFP of N. crassa as determined by gel filtration chromatography on Sephacryl S-300 was 365,000 kDa (Fig. 5C), suggesting that this protein is a tetramer.

**Evidence for the Multifunctionality of the TFP**—Strictly parallel elution profiles for all three enzymatic activities, 2-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-hydroxyacyl-CoA epimerase, were obtained not only during the purification procedure (Fig. 1), but, in addition, with other chromatographic methods. These methods include gel filtration chromatography on Sephacryl S-300 (Fig. 5B) and affinity chromatography on NAD-agarose (Fig. 6). There was no evidence of any substantial change in the activity ratios, \( A_2/A_1 \) and \( A_1/A_3 \) (see Table I), during the last two steps of the purification.

A monoclonal rabbit antiserum was raised against the TFP. Double-immunodiffusion analysis (Fig. 7A) and immunoelectrophoresis (Fig. 7B) resulted in only one precipitin band.

**DISCUSSION**

Here we demonstrate that the \( \beta \)-oxidation system of catalase-free microbodies of oleate-induced mycelia from N. crassa contains a multifunctional protein (TFP) comprising 2-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-hydroxyacyl-CoA epimerase activities.

A standard procedure is established which makes it possible to obtain electrophoretically homogeneous protein after a more than 300-fold purification in about 27% overall yield

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**FIG. 3. Two-dimensional gel electrophoresis of the TFP.** I, IEF/SDS-PAGE system as described by O'Farrell (22). 30 \( \mu \)g of purified TFP was electrophoresed with a mixture of standard proteins as described under “Experimental Procedures.” The following standards were used: A, conalbumin (pl 7.1/78 kDa); B, carbonic anhydrase (pl 6.8/29 kDa); C, bovine serum albumin (pl 6.3/68 kDa); D, ovalbumin (pl 5.6/45 kDa). In addition, phosphorylase b (94 kDa) was used as molecular mass marker in the second dimension as indicated. II, NEPHGE/SDS-PAGE system as described by O'Farrell et al. (21). 3 \( \mu \)g of purified TFP was resolved under nonequilibrium conditions. The distance from the anode is indicated. Both gels were stained with Coomassie Blue.

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**FIG. 4. Reversed-phase HPLC of the TFP.** 370 \( \mu \)g of purified TFP in buffer A were injected on a 5-\( \mu \)m Vydac C\(_18\) column (4.6 \( \times \) 180 mm) at 0% solvent B. The flow rate was 1 ml/min and the programmed conditions are indicated. Absorbance was monitored at 214 nm. Solvent A was 0.1% trifluoroacetic acid in water and solvent B was 0.08% trifluoroacetic acid in 84% acetonitrile and 16% water.

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The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFP, trifunctional protein; IEF, isoelectric focusing; NEPHGE, nonequilibrium pH gradient electrophoresis; HPLC, high performance liquid chromatography; PTC, phenylthiocarbamoyl; PMSF, phenylmethanesulfonyl fluoride; TBS, Tris-buffered saline; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.
purified proteins were estimated by plotting log M, under "Experimental Procedures." was used to measure the void volume. The molecular masses of the proteins of rat peroxisomes (15) and cucumber glyoxysomes (26). were originally reported to possess only the first two activities. However, were recently found as third activities in the multifunctional enzymes required for the degradation of saturated and unsaturated fatty acids (data not shown). These findings suggest that the multifunctional β-oxidation systems in animals, plants, and eukaryotic microorganisms so far investigated at the molecular level (Table IV). Thus, it seems reasonable to conclude that those proteins are a characteristic structural feature of nonmitochondrial β-oxidation systems in eukaryotes. Interestingly, the only bacterial β-oxidation enzyme so far investigated at the molecular level also contains a multifunctional polypeptide (20). The structural relatedness of the TFP from N. crassa to those of other fungal species is strengthened by immunological cross-reactivity. For example, antibodies raised against either of the TFPs from N. crassa or C. tropicalis cross-reacted with both antigens (data not shown).

All five fungal multifunctional proteins comprise the three enzymatic activities, 2-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-hydroxyacyl-CoA epimerase (2). In contrast, the multifunctional proteins of higher eukaryotes, human (23), rat (25), and cucumber (26), were originally reported to possess only the first two activities. However, 3-enoyl-CoA epimerase and 3,2-enoyl-CoA isomerase activities were recently found as third activities in the multifunctional proteins of rat peroxisomes (15) and cucumber glyoxysomes (14), respectively.

These findings suggest that the multifunctional β-oxidation proteins of microorganisms fall into at least two subgroups. The rat trifunctional protein and perhaps other mammalian homologues on the one hand, and those of fungi on the other. To
which group the trifunctional protein of cucumber seeds might belong is still unclear. Judged by its native and subunit
molecular masses, the enzyme in the multifunctional pro-
teins of mammals, but its three enzymatic activities are the same as those of the fungal proteins. The existence of two
distinct subclasses of multifunctional \( \beta \)-oxidation proteins is supported by the primary sequences of these proteins of rat
and \( C. \) tropicalis. While the multifunctional protein of rat peroxisomes shows sequence similarity to the two
mitochondrial monofunctional counterparts, 2-enoyl-CoA hydratase (30) and L-3-hydroxyacyl-CoA dehydrogenase (31),
the sequence of the multifunctional protein of \( C. \) tropicalis exhibits no significant sequence similarity to either the multi-
fuctional protein of rat peroxisomes or to one of the mitochon-
drial \( \beta \)-oxidation proteins. These data indicate the po-
sibility that the phylogenetical relatedness of the multifunc-
tional proteins among each other and with the corresponding
mitochondrial counterparts is more complex than originally
suggested by the sequences of the rat enzymes. Clearly, se-
quence data of other multifunctional proteins are required to
gain a deeper insight into this interesting question.

In addition to the isolation of the TFP, a four-step pro-
cedure for the isolation of a second 2-enoyl-CoA hydratase from
\( N. \) crassa could be established which completely separated it
from the TFP during the first step. This protein was enriched
912-fold to apparent homogeneity. The enzyme was demon-
strated to be located in mitochondria. It was shown to have
properties, in particular molecular structure and heat stabil-
ity, very similar to the mitochondrial 2-enoyl-CoA hydratases
described from various mammalian sources (32-35). From the
absence of all \( \beta \)-oxidation activities it was concluded
that this second hydratase cannot be involved in fatty acid
degradation. Its function has yet to be established. This dual
distribution of 2-enoyl-CoA hydratase seems not to be unique
to \( N. \) crassa. There are reports in the literature describing two
2-enoyl-CoA hydratase activities, in microbodies as well as in
mitochondria, of other eukaryotic microorganisms (36, 37).
Thus, it seems not to be justified to take the occurrence of 2-
enoyl-CoA hydratase alone as an indication of fatty acid
degradation.

The occurrence of the TFP of \( N. \) crassa in functionally
atypical microbodies which lack catalase is of special interest
because this unusual \( \beta \)-oxidation system contains an acyl-
CoA dehydrogenase rather than an acyl-CoA oxidase (11).
This originally seemed to suggest a similarity to mitochondrial
\( \beta \)-oxidation systems, although it has yet to be established how
similar the acyl-CoA dehydrogenase(s) from \( N. \) crassa are
compared to the known mitochondrial ones. However, the
presence of the TFP clearly indicates that the \( \beta \)-oxidation
system of \( N. \) crassa is closely related to the known peroxisomal
\( \beta \)-oxidation systems. The different first enzyme in the non-
mitochondrial \( \beta \)-oxidation system of \( N. \) crassa compared to
the peroxisomal ones seems to reflect more the great func-
tional heterogeneity of microbodies rather than a \( \beta \)-oxidation
system which is structurally related to the mitochondrial ones.
This conclusion is further supported by the quaternary struc-
ture of the 3-oxoacyl-CoA thiolase from \( N. \) crassa (1, 2). It has
been found to be a dimeric protein like all known perox-
isolomal thiolases, whereas mitochondrial ones are tetramers.

The demonstration of this unusual nonmitochondrial \( \beta \)-
oxidation system raises the question of whether this system
is unique to \( N. \) crassa. Reports in the literature about the
occurrence of catalase-free microbodies in \( T. \) strainosa (38) and
\( E. \)uglena (39) as well as preliminary results from our
laboratory about \( \beta \)-oxidation systems in other filamentous
fungi (1, 2) suggest a wider distribution of such a type of
nonmitochondrial \( \beta \)-oxidation among eukaryotic microorga-
nisms.

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Oxidation System in the Filamentous Fungus N. crassa

High-performance liquid chromatography - Purified TF was chromatographed on a Kromasil C18 column with a linear gradient of 5 to 100% methanol in 1% acetic acid, at a flow rate of 1.5 ml/min.

Amino terminal sequence analysis - Manual Edman degradation and, additionally, use of an automated sequencer. Amino acid composition was determined for 45-50 peptide sequences as carried out described previously in (19).

Amino acid composition analysis - The quantitative determination of the amino acid composition of TF was carried out by means of a JEOL JLC-8A spectrophotometer. The N-terminal amino acid was determined described (42,43).

Molecular mass determination - Molecular sieving was performed with Sephadex G-100 superfine (1.4 x 100 cm) according to Andrews (44), using sodium bisulfite (45, 46) and in a standard method, as described in (47). The gel was stained with 0.1% comassie blue R-250 in methanol/acidic acid/water 15:3:1 and destained in methanol/acetic acid/water 4:1:9.

Two-dimensional gel electrophoresis - Two-dimensional gel electrophoresis of purified TF as described essentially in (48). Isoelectrofocusing was carried out on 12 cm gels of 3% acrylamide diameter 9.4 x 24 cm. Using a pH 4-7 gradient ampholines (3). Electrophoresis was carried out with reversed polarity and run at 400 V for 4 h. Handling of the second dimension was identical to that described by O'Farrell.

Affinity chromatography - The development of the affinity column that was applied was based essentially on a column of 0.9 x 4 cm column of DEAE-saprase type I, KL-Biochemica). The bound enzyme was eluted with 0.2 M acetic acid.

Immunoblotting method - Samples were subjected to SDS-PAGE, and the resolved proteins were then electrochemically transferred to nitrocellulose at 100 V for 1 h under a buffer containing 25% methanol. After transfer, some containing molecular mass standards were excised from the nitrocellulose sheet and cut into strips which were later electroblotting in buffer containing 30% methanol, 0.1% acetic acid, and 0.1% Tween 20, followed by a high concentration of methanol and sodium acetate, pH 6.8, and separated followed electrophoresis, and stained with Coomassie Brilliant Blue R-250. Two-dimensional gel electrophoresis was carried out with reversed polarity and run at 400 V for 4 h. Handling of the second dimension was identical to that described by O'Farrell.

Identification of the TF and the second 2-oxoacyl-CoA hydrolase among proteins of isolated organelles (protein staining of purified organelles with use of the monoclonal antibodies to the second 2-oxoacyl-CoA hydrolase) - The monoclonal antibodies to the second 2-oxoacyl-CoA hydrolase were prepared in mice. The result was confirmed by the separation of the second 2-oxoacyl-CoA hydrolase peaks by chromatography on hydroxyapatite and on gel filtration.

The hydroxylase activity could be inhibited by addition of a monoclonal rabbit antiserum raised against the purified protein (48). No immunological crossthreading between TF and this second 2-oxoacyl-CoA hydrolase was observed, although the antiserum against TF inhibited all three enzyme activities of TF, including its hydroxylase activity (49). Inhibition of the activities of TF occurred at the same equivalent point.

Identification of the TF and the second 2-oxoacyl-CoA hydrolase among proteins of isolated organelles (protein staining of purified organelles with use of the monoclonal antibodies to the second 2-oxoacyl-CoA hydrolase) - The monoclonal antibodies to the second 2-oxoacyl-CoA hydrolase were prepared in mice. The result was confirmed by the separation of the second 2-oxoacyl-CoA hydrolase peaks by chromatography on hydroxyapatite and on gel filtration.

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Fig. 8: SDS-PAGE of fractions from different purification steps of the mitochondrial 2-enoyl-CoA hydratase. The following fractions were analyzed on a 12% polyacrylamide slab gel: lane 1, crude extract (150 μg); lane 2, ammonium sulfate fractions (25 μg); lane 3, pool fractions (25 μg) from 0-1 M KCl eluate; lane 4, hydroxyapatite pool fractions (25 μg) from 0-1 M KCl eluate; lane 5, KCl gradient from Blue Sepharose CL-6B (15 μg); lane 6, matrix gel A pool fractions (15 μg) from a 0-1 M KCl eluate. The following molecular mass standards are indicated: phosphorylase b (M, 94,000); bovine serum albumin (M, 68,000); lactate dehydrogenase (M, 35,000); soybean trypsin inhibitor (M, 20,100).

Fig. 10: Determination of the subunit molecular masses of the purified 2-enoyl-CoA hydratase. The subunit molecular masses of the purified proteins were estimated by comparison of their migration to that of known protein standards during SDS-PAGE. The molecular mass standards (M) used for the analysis were 1: phosphorylase b (M, 94,000); 2: transferrin (M, 80,000); 3: bovine serum albumin (M, 68,000); 4: pyruvate kinase (M, 57,000); 5: glutamate dehydrogenase (M, 35,000); 6: lactate dehydrogenase (M, 33,000); 7: soybean trypsin inhibitor (M, 20,100); 8: myoglobin (M, 17,800). The relative mobility of the purified proteins expressed as N values is indicated.

Table 1: Purification of the trifunctional protein (2-enoyl-CoA hydratase, 1-3-hydroxyacyl-CoA dehydrogenase and 3-hydroxyacyl-CoA epimerase) from extracts of Neurospora crassa.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein extracts</th>
<th>2-enoyl-CoA hydratase</th>
<th>1-3-hydroxyacyl-CoA dehydrogenase</th>
<th>3-hydroxyacyl-CoA epimerase</th>
<th>Ratio of activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>U mg⁻¹</td>
<td>U mg⁻¹</td>
<td>U mg⁻¹</td>
<td>A/A₀</td>
</tr>
<tr>
<td>Crude extract</td>
<td>6810</td>
<td>760</td>
<td>131</td>
<td>0.06</td>
<td>1.36</td>
</tr>
<tr>
<td>Phosphoeluates</td>
<td>45</td>
<td>326</td>
<td>8</td>
<td>3.7</td>
<td>58.5</td>
</tr>
<tr>
<td>Blue Sepharose CL-6B eluates</td>
<td>7.3</td>
<td>177</td>
<td>26.3</td>
<td>1.5</td>
<td>0.05</td>
</tr>
<tr>
<td>Hydroxyapatite eluates</td>
<td>3.9</td>
<td>170</td>
<td>44.7</td>
<td>0.6</td>
<td>1.7</td>
</tr>
</tbody>
</table>

A/A₀ of activities for each enzyme activity was calculated as the ratio of the specific activity of the enzyme activity to the specific activity of the enzyme activity alone. The enzyme activities were measured at their optimal conditions.

Fig. 11: Heat stability of 2-enoyl-CoA hydratase activity of the mitochondrial 2-enoyl-CoA hydratase (A) and TFP (B). To compare the heat stability the purified enzymes were dialyzed with 50 mM Tris-HCl, pH 7.4, and heated at 57°C. Aliquots were taken at the times indicated to measure residual enzyme activity.
### TABLE II: Amino acid composition of TPV determined for the purified protein

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Number of Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic Acid</td>
<td>58.0%</td>
</tr>
<tr>
<td>Asparagine</td>
<td>48.2%</td>
</tr>
<tr>
<td>Threonine</td>
<td>40.0%</td>
</tr>
<tr>
<td>Serine</td>
<td>39.7%</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>37.0%</td>
</tr>
<tr>
<td>Glutamine</td>
<td>47.0%</td>
</tr>
<tr>
<td>Proline</td>
<td>83.0%</td>
</tr>
<tr>
<td>Glycine</td>
<td>92.9%</td>
</tr>
<tr>
<td>Alanine</td>
<td>92.0%</td>
</tr>
<tr>
<td>Valine</td>
<td>72.0%</td>
</tr>
<tr>
<td>Methionine</td>
<td>7.2%</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>45.1%</td>
</tr>
<tr>
<td>Leucine</td>
<td>59.4%</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>21.3%</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>43.3%</td>
</tr>
<tr>
<td>Histidine</td>
<td>61.1%</td>
</tr>
<tr>
<td>Spase</td>
<td>13.8%</td>
</tr>
<tr>
<td>Arginine</td>
<td>36.4%</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>M.O.</td>
</tr>
<tr>
<td>Cysteine</td>
<td>M.O.</td>
</tr>
</tbody>
</table>

Results are expressed as mol of amino acid per mol of TPV. The molecular mass of the enzyme was calculated to be 70,000.

### TABLE III: Purification of the mitochondrial 3-enoyl-CoA hydratase from extracts of Neurospora crassa

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>3-Enoyl-CoA hydratase activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>%</td>
<td>U/mg</td>
<td>%</td>
<td># fold</td>
</tr>
<tr>
<td>Total spec</td>
<td>3910</td>
<td>1018</td>
<td>0.26</td>
<td>100</td>
</tr>
<tr>
<td>Crude extract</td>
<td>41</td>
<td>334</td>
<td>5.5</td>
<td>21</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>3.1</td>
<td>245</td>
<td>60.5</td>
<td>329</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>0.9</td>
<td>173</td>
<td>192</td>
<td>721</td>
</tr>
<tr>
<td>Blue Sepharose CL-6B eluate</td>
<td>0.6</td>
<td>142</td>
<td>237</td>
<td>912</td>
</tr>
</tbody>
</table>

*Notas:* Gel Bad A, Klutet

14 g cells first grown on acetate (48 h) and subsequently shifted to acetate and succinate (24 h) as carbon sources were the starting materials for a typical preparation.

*Values are averages of those obtained after 24, 48, and 72 h hydrolysis

*Values extrapolated to zero time of hydrolysis

*Values extrapolated to 100% of hydrolysis

*Not determined