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Purification and Characterization of Phytochrome from Oat Seedlings*

F. E. Mumford and E. L. Jenner

ABSTRACT: Phytochrome was extracted from etiolated oat seedlings and purified 750-fold by a four-step procedure involving chromatography on calcium phosphate, chromatography on Sephadex G-200, continuous electrophoresis on a free-flowing film, and finally gel filtration on Bio-Gel P-150. The product obtained was homogeneous to electrophoresis on cellulose polyacetate or acrylamide gel. Exclusion chromatography indicated that phytochrome had a molecular weight of about 60,000. The molecular extinction coefficients for the red-absorbing form maxima were ϵ_{280} 82,000, ϵ_{382} 26,000, and ϵ_{664} 76,000.

The action spectra of several light-induced morphological changes in plant development, including germination and the inception of flowering and of dormancy, suggest that they are all controlled by a single light-sensitive pigment which exists in two readily interconvertible forms (Borthwick *et al.*, 1952a,b; Hendricks *et al.*, 1956; Hendricks and Borthwick, 1959; Borthwick and Hendricks, 1960). The one form, P_R, has an absorption maximum at about 665 mμ; the other form, P_{FR}, has a maximum at about 725 mμ. These forms are interconverted by irradiation with light of the appropriate wavelength.

$$P_R \underset{\text{far-red light}}{\overset{\text{red light}}{\rightleftharpoons}} P_{FR}$$

This pigment, called phytochrome, was first detected in plant extracts by Butler *et al.* (1959) and was subsequently partially purified by Siegelman and Firer (1964). This paper describes the preparation and characterization of high-purity phytochrome from oat seedlings.

Experimental Section

Assays. The unit of phytochrome was previously defined as that quantity which dissolved in 1 ml of solution gave a ΔA_{665} (absorbancy P_R − absorbancy P_{FR}) of 1 in a 1-cm path (Mumford, 1966). For routine assay of column eluates, ΔA was estimated by use of a "Ratiospect" spectrophotometer with a built-in sample irradiator (Model R-2 Agricultural Specialty Co., Inc., Hyattsville, Md.). To obtain red light for sample irradiation the output of a Sylvania DFA T12 "Tru-Flector" projection lamp was passed through a 660-mμ Bausch and Lomb interference filter; far-red light was obtained by use of a 730-mμ interference filter. In column monitoring samples were irradiated for 0.5-min periods. However, in assays for final specific activity, 2.5-min irradiation periods were used. When the phytochrome concentration was greater than 40 units/l., the sample cup was only partially filled and the ΔA calculated by using the appropriate factor.

For reasons of convenience and sample preservation, protein was usually estimated by ultraviolet absorption at 280 mμ using the nomograph prepared by E. Adams (California Corp. for Biochemical Research, Los Angeles, Calif.) which is based on the extinction coefficients for enolase and nucleic acid given by Warburg and Christian (1942). Determination of the protein

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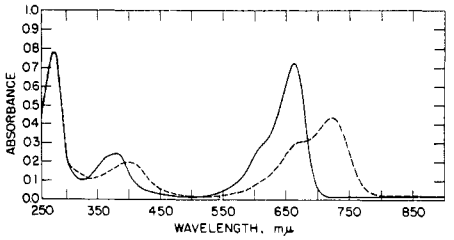

FIGURE 1: Absorption spectra of phytochrome in red absorbing form (———) and far-red form (- - -).

content of purified phytochrome by use of the Folin–Ciocalteu or biuret reagent (Layne, 1957), though, gave values which were only 67 and 65%, respectively, of those obtained by absorbance at 280 mμ. This discrepancy may be due to absorbance by the phytochrome chromophore in the 280-mμ region (Siegelman *et al.*, 1965).

We have defined specific activity as units of phytochrome activity per gram of protein. Specific activity was obtained, then, by using: sp act. = $(\Delta A_{665} \times 1000)$/(mg/ml of protein).

Spectra. Phytochrome spectra (Figure 1) were taken in a 5-cm path length cell in 0.005 M phosphate buffer, pH 7.8 (0.001 M EDTA), with a Cary Model 14 recording spectrophotometer fitted with a refrigerated sample holder. The far-red absorbing form of the pigment, P_{FR}, was obtained by irradiation for 5 min with light from a G.E. 6.6 A/T4Q/Cl-200W quartz–iodine lamp passed through a combination of a 660-mμ Bausch and Lomb interference filter and a Corning 3480 filter and then for 15 min to light passed through a Corning 3480 filter and a monochromator set at 600 mμ. The red-absorbing form, P_R, was generated by exposing the sample for 5 min to light from the quartz–iodine lamp passed through a combination of a 725-mμ interference filter and a Corning 2-62 filter and then for 30 min to light passed through the 2-62 Corning Filter and a monochromator set at 740 mμ.

Purification by Continuous Electrophoresis. Phytochrome was obtained from *Avena sativa* L.cv Garry (or Clintland) seedlings grown in vermiculite in complete darkness for 5 days at 25° (75% relative humidity). The extraction and preliminary purification on calcium phosphate and Sephadex G-200 columns followed the procedure of Siegelman and Firer (1964). This yielded material with a specific activity of 10–20 units/g of protein.

Electrophoretic purification was carried out in a support-free, flowing film (Hannig, 1961) using an "Elphor" Model FF apparatus (Brinkmann Instruments, Westbury, N. Y.). The buffer used in the separating chamber was 0.09 M Tris, 0.03 M acetic acid, and 0.001 M EDTA. This buffer had a pH of 8.3 (25°) and a specific resistivity of 940 ohms (0°). The electrode rinse buffer, circulated through the electrode chambers, was 0.06 M Tris and 0.09 M acetic acid, and had a pH of 4.9 (25°) and a specific resistivity of 560 (0°).

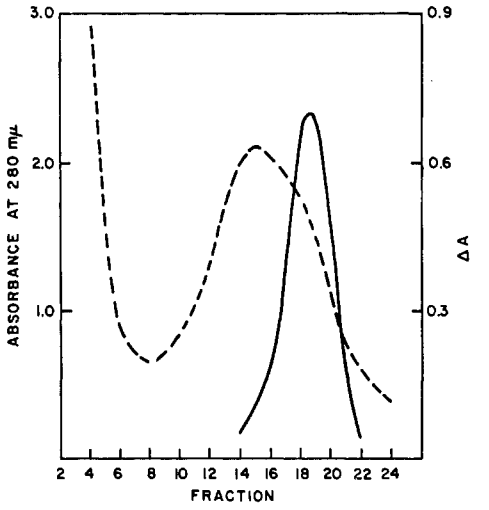

FIGURE 2: Fractionation of phytochrome in an "Elphor" Model FF electrophoresis apparatus. Protein concentration (A_{280}) is represented by the dashed line, and phytochrome concentration (ΔA) by the solid line.

In a representative experiment, the phytochrome for electrophoresis was precipitated by the addition of an equal volume of a 0.1 M sodium pyrophosphate solution saturated with ammonium sulfate. The precipitate was collected by centrifugation and redissolved in 25 ml of 0.005 M phosphate buffer, pH 7.8. The phytochrome solution was dialyzed at 4° against 2 l. of Tris buffer, pH 8.3, for 16 hr and then against 2 l. of fresh buffer for 6 hr. The dialyzed solution was clarified by centrifugation and assayed. The clear blue-green solution, 29.7 ml, contained 16.4 units of phytochrome with a specific activity of 17.5.

The "Elphor" FF was operated at 5° with a current of 160 ma and a total voltage drop of 2350 v. The buffer was introduced across the separating chamber at a rate of 106 ml/hr. This corresponds to 2.2 ml/hr in each of the 48 collecting tubes. The phytochrome solution was introduced at a rate of 1.7 ml/hr (54 mg of protein/hr) through a port 7 cm inside of the feeding edge and 19.5 cm from the cathode side. This position was directly above collecting tube 30. The separation required 17.5 hr. The pattern of separation is shown in Figure 2. The voltage drop in the center section of the separating chamber was found to be 45 v/cm by introducing the probes of a vacuum tube voltmeter into the appropriate collection ports. The phytochrome peak was displaced 11.4 cm laterally from the introduction point (tube 30). Since the linear flow rate on the table was 42 cm/hr this corresponded to an ionic migration rate of 11.1 cm/hr. The ionic mobility at 5° calculated from this is 7×10^{-5} cm/sec for phytochrome at pH 8.7 (the pH 25° of 8.3 here corrected to 5°).

The contents of tubes 18–20 were combined ("A") and treated with an equal volume of 0.1 M sodium pyrophosphate solution saturated with ammonium sulfate,

and the precipitate was collected by centrifugation. Tubes 17 and 21 were combined ("B") and worked up similarly. The phytochrome pellets were dissolved in 0.005 M phosphate buffer, pH 7.8, and assayed. Fraction A contained 7 units of phytochrome with a specific activity of 78; the yield from fraction B was 2.2 units with a specific activity of 25.

Fractionation by Bio-Gel P-150. Phytochrome purified by electrophoretic separation in the Elphor apparatus (12.7 units, sp act. 78 units/g of protein) was applied to a 2.5 × 100 cm Bio-Gel P-150 (Bio-Rad Laboratories, Richmond, Calif.) column in 3 ml of 0.005 M phosphate buffer, pH 7.8 (0.001 M EDTA). The sample was washed into the top of the Bio-Gel bed with 3 ml more of 0.005 M phosphate buffer, pH 7.8, and the column was then eluted with 0.25 M phosphate buffer, pH 7.8 (0.001 M EDTA). Fractions (6.5 ml each) were collected at 20-min intervals. The column was maintained at 4°. The phytochrome-containing fractions could be located by their blue color. A yellow band preceded the phytochrome. Fractions containing phytochrome were assayed for activity with a "Ratiospect" spectrophotometer and protein was determined by absorbance at 280 mμ (Figure 3A). Fractions 26–28 were combined and treated with an equal volume of 0.1 M sodium pyrophosphate solution saturated with ammonium sulfate (pH 8.5), and the precipitate which formed was collected by centrifugation for 10 min at 34,000*g*. The pellet obtained contained 6.96 units of phytochrome with a specific activity of 217 units/g of protein. Work-up of the side cuts in a similar manner gave another 3 units of phytochrome.

The heart cut was fractionated a second time through the same Bio-Gel P-150 column and the eluate collected and assayed as described (Figure 3B). Fractions were combined and worked up as indicated in Table I.

TABLE I: Final Fractionation of Phytochrome on Bio-Gel P-150.

Fractions Combined	Units/l.	Protein (mg/ml)	Sp[a] Act.	Total Units
25–28	522.0	1.38	378	2.6
23, 24, 29	244.0	0.81	300	1.2
22, 30	69.6	0.30	232	0.35

[a] Units of phytochrome per gram of protein.

"Sepraphore III" and Disc Electrophoresis. "Sepraphore III" cellulose polyacetate strips (Gelman Instrument Co., Ann Arbor, Mich.) were soaked overnight in sodium barbital buffer, pH 8.6, ionic strength 0.05. The strips were then blotted dry and 3-μl aliquots of phytochrome solutions containing 6 μg of protein/μl applied using the Gelman sample applicator. The strips were mounted in the electrophoresis chamber and subjected to a potential of 200 v for 65 min at 4°. Protein bands were located by staining the strips with 0.002% Nigrosin-WS in 5% acetic acid for 15 min and washing out excess dye.

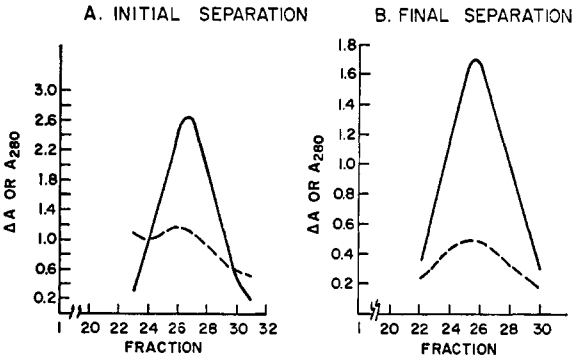

FIGURE 3: (A) Initial separation and (B) final separation of phytochrome on Bio-Gel P-150. Protein concentration (A_{280}) is represented by the dashed line and phytochrome concentration (ΔA) by the solid line.

Under these conditions phytochrome with a specific activity of 378 units/g of protein yielded one band at a point 13 mm from the origin. As the staining procedure could detect as little as 1 μg of protein, a purity of at least 90% is indicated for phytochrome with this specific activity. By way of comparison, phytochrome from the Elphor electrophoretic purification (sp act. 70–80 units/g of protein) showed, in addition to the phytochrome band, a heavy band 7 mm from the origin and a fainter band 2 mm from the origin.

Phytochrome with a specific activity of 378 units/g of protein was also assayed for homogeneity by the disc electrophoresis method of Ornstein and Davis as modified by Broome (1963). The gel was stained with Amidoschwartz and cleared electrically. A single protein band was present.

Amino Acid Analysis. Amino acids other than tryptophan were determined by the method of Moore and Stein (1963). Before analysis the solution of phytochrome (sp act. 378 units/g of protein) was dialyzed thoroughly against 0.005 M phosphate buffer, pH 7.8, to remove traces of ammonium sulfate carried over from previous ammonium sulfate precipitation steps. The dialyzed solution was spun at 34,000*g* for 60 min and the supernatant obtained was divided into four portions each of which contained 534 μg of protein (Folin–Ciocalteu determination). Each aliquot was mixed with an equal volume of concentrated hydrochloric acid and placed under vacuum in a sealed tube. The tubes were heated at 110° for 10, 20, 40, and 70 hr, respectively. Aliquots of hydrolysate equivalent to 356 μg of protein were then analyzed using a Beckman Model 120 B amino acid analyzer. The micromoles of amino acids obtained per milligram of protein hydrolyzed are shown in Table II. These experimental values were corrected where indicated

TABLE II: Amino Acid Composition of Phytochrome.

Amino Acid	μmoles of Amino Acid per Mg of Protein				Corrected μmoles of Amino Acid/mg of protein	Rel Ratio (methionine = 1)	μg of Amino Acids Recov[a] (−H_2O)
	10 Hr	20 Hr	40 Hr	70 Hr			
Aspartic acid	0.738	0.884	0.912	0.853	1.000	15.9	40.9
Threonine	0.293	0.403	0.388	0.382	0.403	6.4	14.5
Serine	0.534	0.626	0.616	0.555	0.675	10.7	20.9
Glutamic acid	0.736	0.864	0.889	0.445	0.91	14.5	42.4
Proline	0.494	0.484	0.516	0.453	0.610	9.7	21.1
Glycine	0.586	0.617	0.603	0.590	0.616	9.8	13.2
Alanine	0.755	0.847	0.759	0.795	0.847	13.5	21.4
Cystine	0.054	Trace	Trace	None			
Valine	0.336	0.521	0.588	0.598	0.598	9.5	21.1
Methionine	0.058	0.052	0.047	0.036	0.063	1.0	2.9
Isoleucine	0.230	0.392	0.384	0.428	0.428	6.8	17.2
Leucine	0.692	0.906	0.883	0.896	0.906	14.4	36.4
Tyrosine	0.185	0.222	0.209	0.158	0.304	4.8	17.6
Phenylalanine	0.324	0.410	0.397	0.384	0.417	6.6	21.8
Cysteic acid	0.075	0.160	0.187	0.187	0.187	3.0	10.1
Lysine	0.485	0.549	0.523	0.500	0.548	8.7	25.0
Histidine	0.238	0.244	0.257	0.236	0.288	4.6	14.0
Ammonia	1.079	1.123	1.059	1.166	1.11	17.6	6.3
Arginine	0.380	0.430	0.449	0.413	0.498	7.9	27.7
Tryptophan[b]					0.110	1.8	
						167.2	374.5

[a] Protein charged was 356 μg as determined by the Folin–Ciocalteu method. [b] Determined by the method of Spies and Chambers (1949).

by the method of Moore and Stein (1963). Tryptophan was estimated colorimetrically by the procedure of Spies and Chambers (1949). The relative ratio of the amino acids in phytochrome, based on methionine equal to one, and the micrograms of each amino acid recovered from the hydrolysis are also shown in Table II.

Glutamic acid is normally stable under the hydrolysis conditions employed, but in Table II it can be seen that marked destruction of this amino acid occurred when hydrolysis was extended more than 40 hr. This loss cannot be accounted for by a simple hydrolytic destruction of glutamic acid and must be due to a secondary reaction between glutamic acid and other hydrolysis products (possibly degradation products of the chromophore).

Amperometric Titration for SH Groups. Titration of the SH groups in phytochrome with silver ion was done by the method of Benesch and Benesch (1955). The titration was carried out in Tris buffer, pH 7.4, at 10–15° using a rotating platinum electrode *vs.* a standard calomel electrode. In duplicate runs with 1.2-mg (2×10^{-8} mole) samples of phytochrome, 0.21 and 0.23 ml of 0.001 M silver nitrate solution were required to reach the end point. Thus the samples contained between 2.1 and 2.3×10^{-7} mole of SH, respectively, or 10–11 SH groups/phytochrome molecule.

Emission Spectrographic Analysis for Metal Content. A 0.05% solution (3 ml) of phytochrome (sp act. 363) in 0.005 M phosphate buffer, pH 7.8, was dialyzed at 4° against 1 l. of 0.005 M phosphate buffer, pH 7.8, for a total of 72 hr. The dialysis buffer was continuously purged with nitrogen and was replaced each 24 hr. The dialyzed solution was spun at 34,000*g* for 20 min and the supernatant (3 ml containing 1.5 mg of protein) was submitted for emission spectrographic analysis for metals. Aliquots (3 ml) of each of the three dialysates and the small pellet obtained from the centrifugation were also analyzed. The only metals found at a significant level in the phytochrome sample were aluminum and copper which were present at between 0.0005–0.001 and 0.00005–0.0001 mg/mg of protein, respectively. Aluminum and copper were also found in the dialysates and pellet at about one-half the concentrations present in the protein solution.

Molecular Weight by Exclusion Chromatography. The estimation of the molecular weight was carried

out on Bio-Gel P-100, following a procedure similar to that of Andrews (1965). A column 2.5 × 45 cm was packed with Bio-Gel P-100 (150 mesh) which had previously been allowed to swell in 0.25 M phosphate buffer, pH 7.8 (0.001 M EDTA). The column was washed for 2 days with the 0.25 M buffer and then a solution (3 ml) containing phytochrome (2 mg) and three markers was applied to the column. The three markers were 2.5 mg of Blue Dextran 2000 (a polydextran of molecular weight approximately 2,000,000 obtained from Pharmacia Fine Chemicals, Piscataway, New Market, N. J.), 10 mg of egg albumin (Sigma Chemical Co., St. Louis, Mo), and 10 mg of bovine serum albumin (Sigma). The flow rate was about 23 ml/hr. Blue Dextran was assayed by its absorbance at 615 mμ, and the albumins by absorbance at 280 and 230 mμ. Phytochrome was determined by assay with the "Ratiospect." The V/V_0 values were as follows (V^0 is the elution volume of the Blue Dextran): bovine serum albumin, 1.08; phytochrome, 1.13; and egg albumin, 1.18. This indicates a molecular weight of approximately 55,000 for phytochrome (using molecular weights of 68,000 for bovine serum albumin and 44,000 for egg albumin). To check for possible effects of the ionic strength on the aggregation of phytochrome a determination was made at a phosphate concentration of only 0.05 M. The peak elution volume of phytochrome was unchanged. When phytochrome, egg albumin, and bovine serum albumin were separated on a Sephadex G-200 column, phytochrome also eluted between the two albumins with an indicated molecular weight of 62,000.

Results and Discussion

Electrophoretic fractionation of phytochrome, prepared by the Siegelman and Firer (1964) procedure (sp act. 10–20), on an "Elphor" Model FF apparatus resulted in a five- to tenfold increase in specific activity. The protein from the electrophoresis step was purified another fourfold by exclusion chromatography on Bio-Gel P-150. Phytochrome from the final Bio-Gel purification had a specific activity of 360–380 and was at least 90% pure as judged by electrophoresis on "Sepraphore III" cellulose polyacetate strips or disc electrophoresis on acrylamide gel. As is shown in Table III, phytochrome from the crude oats extract had a specific activity of 0.5 and thus the procedure employed (calcium phosphate chromatography, Sephadex G-200 chromatography, electrophoresis, and Bio-Gel P-150 chromatography) resulted in a 750-fold over-all purification on a protein basis.

Exclusion chromatography on Bio-Gel P-100 and Sephadex G-200 using egg albumin and bovine serum albumin as markers indicated a phytochrome molecular weight of 55,000–62,000. These values are considerably lower than the 90,000–150,000 reported by Siegelman and Firer (1964) by ultracentrifugation.[1]

The amino acid composition of phytochrome as determined by the method of Moore and Stein (1963) is given in Table II. Even though the protein hydrolyses were run in thoroughly evacuated sealed tubes, the cysteine (and/or cystine) present in the protein was apparently quickly converted to cysteic acid. Thus, cystine could be detected in the hydrolysates after a 10-hr hydrolysis period, but only cysteic acid was found on extended hydrolysis. Titration of phytochrome with silver ion using an amperometric procedure showed the presence of 10–11 SH groups/molecule in both P_R and P_{FR} (assuming a molecular weight of about 60,000). A maximum of 12 cysteine residues/60,000 mol wt unit was indicated by the amino acid analysis.

TABLE III: Yields and Purities during Phytochrome Purification.

Purification Step	Units of Phyto-chrome	Over-All Yield (%)	Sp Act.[a]
Crude extract from 25 kg of seedlings	240	100	0.5
After calcium phosphate chromatography	83	35	5.0
After Sephadex G-200 chromatography	48	20	10.0
After electrophoresis	22	9	75.0
After Bio-Gel P-150 chromatography	8	3	370.0

[a] Units of phytochrome per gram of protein. Protein concentrations based on absorbance at 280 mμ.

The absorption spectra of the two forms of phytochrome are shown in Figure 1. As it was estimated that the solution on which the spectra were taken contained 0.114 mg/ml of protein (Folin–Ciocalteu assay), the phytochrome concentration (assuming a molecular weight of 60,000) would be 1.9×10^{-6} M. The molecular extinction coefficients for the maxima in P_R and P_{FR}, then, would be as follows: P_R $\epsilon_{280} = 82{,}000$, $\epsilon_{382} = 26{,}000$, $\epsilon_{664} = 76{,}000$; P_{FR} $\epsilon_{280} = 82{,}000$, $\epsilon_{400} = 21{,}000$, $\epsilon_{724} = 46{,}000$.

Emission spectrographic analysis of highly purified phytochrome for metals showed the presence of only copper and aluminum in significant quantities. Thus, 1 mole of phytochrome yielded a maximum of 0.1 mole of copper and 2 moles of aluminum. It would appear, therefore, that copper is present only as a contaminant; aluminum, however, cannot be ruled out as a possible phytochrome constituent.

[1] The sedimentation behavior of phytochrome in the ultracentrifuge is currently being investigated by Dr. L. L. Anderson of this laboratory. Equilibrium experiments indicate a molecular weight of approximately 60,000 with evidence of some aggregation to higher molecular weight species. These studies will be the subject of a separate publication.

Acknowledgments

We wish to thank Dr. H. W. Siegelman for helpful discussions on calcium phosphate and Sephadex G-200 chromatography. We are indebted to Dr. D. W. Krause for the amino acid determinations and to Dr. J. C. Rowell for the absorption spectra shown in Figure 1. We also wish to acknowledge the technical assistance of Mr. E. M. Riley and Mr. P. McCarthy.

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Studies on Bile Acids. Some Observations on the Intracellular Localization of Major Bile Acids in Rat Liver*

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ABSTRACT: The subcellular distribution of major bile acids in rat liver has been studied by the application of recently developed gas–liquid partition chromatographic methods. The relative concentrations of several bile acids in rat portal blood and liver homogenate resembled each other very closely except for chenodeoxycholic acid. The concentration of chenodeoxycholic acid in liver is significantly higher than in portal blood.

The cytoplasmic compartment accounts for approximately 70% of the bile acids with more than 50% for each individual bile acid studied. The ratio of cholic/deoxycholic in each subcellular fraction revealed the existence of a relatively larger proportion of deoxycholic acid in the mitochondrial and microsomal fractions compared to that in the cytoplasmic fraction (1.7 and 1.3 *vs.* 5.1). Since the enzymes concerned with hydroxylation (7α-hydroxylase) and conjugation are located in microsomes and partly in the mitochondria, there seems to be a relationship between localization of bile acids in these subcellular particles and their functional role.

The primary bile acids, cholic[1] and chenodeoxycholic, are known to be principal end products of cholesterol catabolism, in which the liver occupies a focal position (Bergström *et al.*, 1960). In the rat, deoxycholic, a secondary bile acid generated by bacterial action in the gut, upon reaching the liver *via* the enterohepatic circulation, is rapidly hydroxylated to cholic acid by a 7α-hydroxylating system (Bergström *et al.*, 1953a,b).

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† Trainee, National Heart Institute Training Program, HE-5399, U. S. Public Health Service.

[1] The systematic nomenclature of bile acids referred to in this report by trivial names are as follows: lithocholic acid, 3α-hydroxy-5β-cholanoic acid; hyodeoxycholic acid, 3α,6α-dihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; α-muricholic acid, 3α,6β,7α-trihydroxy-5β-cholanoic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanoic acid.