g of dried anhydrous lithium chloride. The flask was vigorously agitated until two liquid layers were formed. The top layer, consisting of almost pure methyl borate, was separated in a micro separatory funnel. Further purification consisted of a distillation through a semimicro fractionating column, followed by a second lithium chloride separation, and finally a further fractional distillation. A boiling range of 67.5 to 68.5°C was observed, and a yield of 71 percent was obtained. The remaining methanol could be recycled for a further synthesis if required.

For comparison, the dilution curve for methyl borate is shown in Fig. 1. It is evident that despite the initially lower luminescence efficiency of the 2-phenyl-5-(4-biphenylyl)-1,3,4-oxadiazole (PBD) (3 g/lit), naphthalene (70 g/lit) scintillator, at C* concentrations greater than 0.03 g/ml this solution is superior to the methanol diluent. For methanol in a 20-ml cell, a net C^{14} counting rate of 7.39 ± 0.08 count/min over a background of 8.18 ± 0.05 count/min was the optimum counting rate obtained for contemporary carbon (*2*). For methyl borate with the same scintillator volume, we have realized a net C^{14} counting rate of 10.07 ± 0.08 count/min over a background of 4.26 ± 0.04 count/min. This improvement represents an extension in the range of the method of roughly 5000 years.

To date, six documented samples have been dated in our laboratories. The documentation was provided by the collector and is briefly summarized in Table 1. One sample (S-4) was dated by the methanol method because of the limited amount of sample available. The others were converted to trimethyl borate before counting. In each case, the unknown sample and the background dummy containing petrochemical carbon were alternated every 24 hours, thus bracketing each sample with two background counts. This procedure was facilitated by the use of two identical cells that had previously been carefully investigated for differences in radioactive content. The age assigned to any sample is the average age as determined by two 24-hour counts. The error assigned to any age is the statistical counting error. The results are summarized in Table 1.

Samples S-3 and S-4 have significance as part of a study by R. Lougeee of postglacial upthrusts on glacial seashores on either side of the Atlantic Ocean. According to N. R. Gadd, samples of wood from sample S-17 have previously been dated $11,050 \pm 400$ years by J. L. Kulp at Lamont Geological Observatory (sample L-190A), older than 30,840 years at Yale University (sample Y-242), and older than 40,000 years at the U.S. Geological Survey (sample W-189).

Samples S-6 and S-7 have been reported as greater than 38,000 years by the U.S. Geological Survey. Our results on samples S-6 and S-7 can be considered to be in good agreement with those obtained by other methods, but N. R. Gadd (*7*) suggests that geologic evidence would indicate that our sample S-17 should be much older than reported, and similar in age to samples S-6 and S-7.

Our experience with the afore-mentioned methods indicates that they are suitable for routine use in radiocarbon age estimates and offer certain attractive advantages in terms of simplicity of operation over other suggested scintillation techniques (*8*).

These methods also have sufficient accuracy to make them attractive in comparison to gas-counting techniques (*9*). The chemical syntheses of methyl borate are relatively simple. The electronic equipment is also of minimum complexity and avoids the use of anticoincidence equipment or refrigeration. However, in common with all other methods, problems of electronic drift and sample contamination may reduce the over-all precision below the statistically quoted limits. Increasing the scintillator volume would be expected to yield a higher precision but would necessitate the use of larger specimens and larger-scale syntheses.

Table 1. University of Manitoba radiocarbon dates.

Description	Sample No.	Age (yr)
Baie St. Catherine, Quebec. Driftwood from 13 to 20 ft below Mic Mac Terrace. Collected by R. Lougee, department of geography, Clark University.	S-4	3150 ± 130
Amherst, Nova Scotia. Stump from drowned forest in Bay of Fundy near Amherst. Collected by R. Lougee.	S-3	5300 ± 150
Missinaibi River, Ontario. Wood from a flat horizon of Missinaibi River area, from mouth of the Soweska River. Collected by O. L. Hughes, Geological Survey of Canada.	S-6	$29,000 \pm 1500$
Missinaibi River, Ontario. Peat from the same area as sample S-6. Collected by O. L. Hughes.	S-7	$38,500 \pm 3500$
St. Pierre-les-Becquets, Ontario. Wood. Collected by N. R. Gadd.	S-17	$20,200 \pm 800$
Edmonton, Alberta. Alberta wood from the upper till of the Edmonton district. Collected by C. P. Gravenor, department of geology, University of Alberta.	S-15	$21,600 \pm 900$

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Acetylrhodopsin

The photosensitive pigment of rod vision, rhodopsin, is a chromoprotein composed in equimolar parts of the protein opsin and the carotenoid retinene (*1*). During the course of an investigation of some of its chemical properties, a successful attempt was made to acetylate rhodopsin. The acetylated pigment possesses spectral and photosensitive properties essentially identical with those of rhodopsin. Once bleached, however, the modified protein does not regenerate in the presence of the neo-b isomer of retinene as does bleached rhodopsin (*2*). The object of this paper is to describe the preparation and some of the properties of acetylated rhodopsin (*3*).

Cattle rhodopsin was prepared by methods previously described (*4, 5*). It was purified further by passage through columns of mixed-bed, ion-exchange resins, which remove amino acids and peptides in addition to other impurities of low molecular weight (*6*).

The optical properties of rhodopsin provide a useful index of purity. The absorption spectrum in aqueous digitonin solution has maxima at about 498 and 350 mμ, associated with the carotenoid chromophore, and a sharp peak at 278

mμ because of the protein opsin (7). A minimum occurs at about 400 mμ. Since most impurities tend to raise the absorption at short wavelengths, the lower the ratios of the optical densities at 278 and 400 mμ to the density at 500 mμ (278/500 and 400/500 ratios), the purer in general is the preparation. In the best preparations, these indices have the following values: 400/500 ratio, 0.22 to 0.26; 278/500 ratio, 2.1.

The optical density at 500 mμ can be used also to determine the concentration of cattle rhodopsin, for its molar extinction is known to be 40,600 (*8*). The concentration of acetylrhodopsin was similarly determined, using, however, the extinction coefficient of rhodopsin. The coincidence of position and shape of the 500 mμ band of the two proteins is taken as sufficient evidence for the equivalence of their coefficients (see Fig. 1).

Rhodopsin was acetylated by the method of Fraenkel-Conrat (*9*). To solutions of rhodopsin in 1-percent digitonin, half-saturated with sodium acetate and chilled to 0°C, acetic anhydride (99 percent pure) was slowly added (3.0 ml/g of protein) during 1 hour. On the average, the rhodopsin solutions contained about 6 mg of protein in a final concentration of about 0.3 percent. After this treatment, the protein was dialyzed for 2 to 5 days at 4°C against large volumes of distilled water, previously adjusted to *p*H 7 with 1*M* sodium hydroxide. This procedure is known to acetylate free amino, sulfhydryl, and phenolic hydroxyl groups of proteins (*9, 10*).

The free amino groups of rhodopsin before and after acetylation were determined by the ninhydrin method (*11*), using alanine as standard. It should be pointed out that in this procedure the protein is denatured by heat. The percentage acetylation is based therefore on the free amino groups of the denatured and hence bleached protein—that is, denatured opsin—not on those of native rhodopsin.

Acetylation had no significant effect on the absorption spectrum of rhodopsin. Neither the positions of the absorption bands in the spectrum nor their relative optical densities were markedly altered. This is indicated in Table 1. The introduction of acetyl groups on the protein apparently does not disturb significantly the nature of the opsin-retinene relationship; it leaves retinene attached to acetylated opsin in such a way that the characteristic 500 mμ band of the chromophore remains unaltered (Fig. 1).

An average of 13 amino groups per molecule was found in denatured rhodopsin (Table 1). This is a small number for a protein of molecular weight about 40,000 (*1*) and may account in part for its insolubility in the absence of solubilizing agents. About 74 percent of the amino groups were acetylated by our procedure. This is the usual result obtained with this method (*10, 12*). No attempt has yet been made to prepare partially acetylated rhodopsins.

Although acetylation had no measurable effect on the absorption spectrum of rhodopsin, it abolished the capacity of rhodopsin to regenerate after bleaching. Ordinarily, bleached rhodopsin regenerates readily on the addition of neo-b retinene and incubation in the dark (*2*). The regeneration of acetylated rhodopsin after bleaching was tested immediately after acetylation but before dialysis. The solutions therefore contained about 2.5*M* sodium acetate; for this reason, the comparison solutions of rhodopsin were brought to the same salt concentration. It is known that the regeneration of rhodopsin is optimal at about *p*H 6.5 (*5*); but since this property might have been altered by acetylation, the acetylated preparations were tested over a range of *p*H from 4 to 7.5. In each case, rhodopsin and the acetylated pigment were bleached in orange (nonisomerizing) light, neo-b retinene in digitonin solution was added in excess, and the mixtures were incubated in the dark at 25°C until all changes were complete.

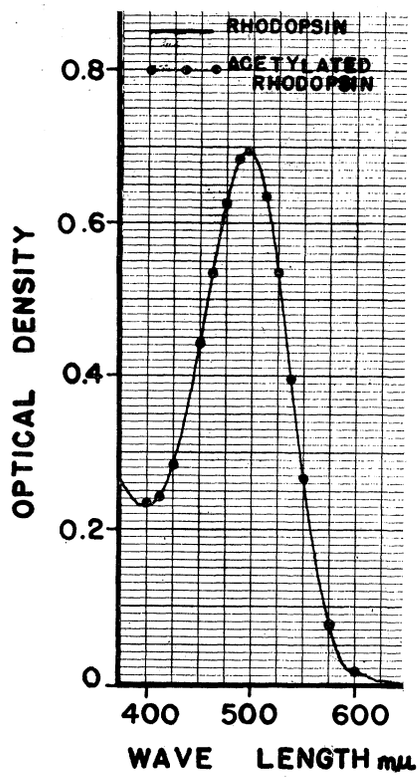

Fig. 1. Absorption spectra of rhodopsin and acetylated rhodopsin (22.5°C, *p*H 6.5).

Table 1. Extent of acetylation of free amino groups in rhodopsin.

Experiment No.	Preparation	Optical density ratio: 400 mμ / 500 mμ	Optical density ratio: 278 mμ / 500 mμ	Free amino groups per mole* (No.)	Acetylation of free amino groups (%)
1	Rhodopsin	0.330	2.83		
1	Acetylrhodopsin	0.290	2.71		
2	Rhodopsin	0.270	2.73		
2	Acetylrhodopsin	0.276	2.59		
3	Rhodopsin	0.273	2.44	14.5	
3	Acetylrhodopsin	0.266	2.38	3.9	73
4	Rhodopsin	0.266	2.55	11.1	
4	Acetylrhodopsin	0.283		2.8	74
5	Rhodopsin	0.245	2.90	11.5	
6	Rhodopsin	0.251	2.71	12.3	

* These determinations involve the denatured and hence bleached pigment, not native rhodopsin or acetylated rhodopsin.

The results of a typical experiment are as follows. At *p*H 6.5, the rhodopsin blank regenerates 83 percent; at *p*H 4.0, 5.0, 6.5, and 7.5, the acetylated rhodopsin regenerates 6.0, 7.0, 5.0, and 5.0 percent, respectively. It is apparent that under conditions in which rhodopsin regenerates 83 percent, acetylated rhodopsin indicates only a trace of regeneration, perhaps to be ascribed to traces of lightly acetylated or unacetylated pigment. Dialysis at 4°C against distilled water to remove the sodium acetate did not alter this relationship, although it did markedly decrease the regeneration of unacetylated rhodopsin.

Acetylated rhodopsin is very much less stable than the unacetylated pigment. When it is stored at 4°C in the dark, almost all the acetylated pigment is bleached within 2 weeks, though a similar rhodopsin solution is stable under the same conditions.

How can one explain that acetylation, though it does not affect the relationship between opsin and its chromophore in rhodopsin, prevents regeneration after bleaching? The native structure of rhodopsin, like that of other proteins, is probably maintained in part by a network of internal hydrogen bonds and also by the attachment of the prosthetic

group, for rhodopsin is much more stable than opsin (*13*). If, as seems likely, acetylation removes numbers of amino groups that formerly had been engaged in hydrogen bonding, this would loosen the structure of the protein, making it more susceptible to denaturation. Presumably, the structure in the neighborhood of the prosthetic group is still intact, as evidenced by the unchanged optical properties. The bleaching process, by dissociating the prosthetic group, removes a further stabilizing influence, and the liberated acetyl opsin readily denatures.

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Route of Elimination of Anthracene and 9-Methylanthracene Involving Protein Binding

The emphasis of current research on the mechanism of carcinogenesis has been on the binding of the carcinogen or its metabolites with tissue protein. The relationship between binding and carcinogenic potentiality has been studied by changing the chemical agent (for example, carcinogenic, weakly carcinogenic, and noncarcinogenic agents, azo dyes, polynuclear hydrocarbons, an aromatic amine, and an acridine), and the tissue (for example, liver of rat, mouse, guinea pig, rabbit, cotton rat, and chicken; and skin of mouse, hamster, rat, rabbit, and guinea pig).

From their correlations with azo dyes, Miller and Miller proposed a protein- or enzyme-deletion theory of carcinogenesis (*1, 2*). These authors considered not only the maximum value of protein binding but also the rates of increase and decrease of the maximum (*2*). Following the demonstration that the carcinogens 3,4-benzpyrene (*3*) and 1,2,5,6-dibenzanthracene (*4*) were bound to mouse-skin protein, three groups reported on the relationship between skin protein binding of hydrocarbons and the carcinogenic potential of the hydrocarbon-tissue combination. Moodie, Reid, and Wallick concluded that noncarcinogens are either not bound to protein at all or are much more weakly held than are carcinogens (*5*). Woodhouse (*6*) found no relationship between protein binding and carcinogenic potentiality. Heidelberger and Moldenhauer (*7*) found a positive relationship between the maximum value of protein binding and carcinogenic potential in seven out of eight instances.

It was established that the binding of 9,10-dimethyl-1,2-benzanthracene-9,10-C^{14} with mouse-skin protein was dependent on the dose of carcinogen per unit area of skin and related to the weight response of the treated skin (*8*). This study has now been extended to several hydrocarbons, each of which was applied in a series of graded doses (*9*). A significant difference was observed in the rates of elimination from mouse-skin protein between two trinuclear hydrocarbons and two tetranuclear hydrocarbons. Twenty-four hours after application to mouse skin, the protein binding of anthracene-9,10-C^{14} (a noncarcinogen) and of 9-methylanthracene-9,10-C^{14} (a noncarcinogen) (Tables 1 and 2) was shown to be much lower than the protein binding of 9,10-dimethyl-1,2-benzanthracene-9,10-C^{14} (*8*) (a potent carcinogen) and of 1,2-benzanthracene-9,10-C^{14} (*10*) (a weak carcinogen, *11*).

The relatively rapid elimination of anthracene-9,10-C^{14} and of 9-methylanthracene-9,10-C^{14} might have taken place through a transient protein-bound phase or might not have involved any appreciable protein binding. The previous observations (*8, 10*) with the series of graded doses showed binding to be dose-dependent and hence suggested that measurements made within 24 hours after the application of the highest dose would be necessary to detect a transient protein-bound phase.

The experimental procedure has been described (*8*). The data (Tables 1 and 2) demonstrate that the relatively rapid elimination of anthracene-9,10-C^{14} and 9-methylanthracene-9,10-C^{14} from mouse skin involve a transient protein-bound phase of at least the same order of magnitude as that observed with 9,10-dimethyl-1,2-benzanthracene-9,10-C^{14}. Thus, the maximum value of protein binding is independent of the carcinogenic activity and structure of the hydrocarbon. Data were obtained by feeding rats the five azo dyes 3′-methyl-4-dimethylaminoazobenzene, 4-dimethylaminoazobenzene, 4′methyl-4-dimethylaminoazobenzene, 3-methyl-4-monomethylaminoazobenzene, and 2-methyl-4-dimethylaminoazobenzene. The last three dyes were of low carcinogenic potentiality, and two of these three experiments did not support a positive relationship between carcinogenicity and extent of binding with liver protein (*2*). The results with the polynuclear hydrocarbons differ from the azo dye data (*2*); no positive relationship was established between the rate at which the maximum of protein binding was attained and carcinogenic potential. Also in contrast with the azo dyes—specifically the very weakly carcinogenic dyes 2-methyl-4-dimethylaminoazobenzene and 3-methyl-4-monomethylaminoazobenzene (*2*)—the protein binding of anthracene and of 9-methylanthracene receded faster from their observed maxima than the protein binding of 9,10-dimethyl-1,2-benzanthracene and 1,2-benzanthracene.

Thus there is no experimental demon-

Table 1. Incorporation of radioactivity by mouse skin following the application of anthracene-9,10-C^{14} (0.208-percent series). Each determination was made on the pooled tissues of four mice. Each dose, 104 μg (1860×10^3 count/min) was applied over 3.46 cm^2 of skin. The analyzed dose was 76.3 μg (1365×10^3 count/min) of hydrocarbon over 2.54 cm^2 of skin. Each result is the average of two determinations.

Time after application (hr)	Total activity of whole homogenate (count/min)	Specific activity (count/min mg)	
		Particulate protein	Supernatant protein
0.5	414,000	543	1150
17	62,800	205	667
24	42,100	76	157

Table 2. Incorporation of radioactivity by mouse skin following the application of 9-methylanthracene-9,10-C^{14} (0.225-percent series). Each determination was made on the pooled tissues of four mice. Each dose, 113 μg (1860×10^3 count/min) was applied over 3.46 cm^2 of skin. The analyzed dose was 83.7 μg (1365×10^3 count/min) of hydrocarbon over 2.54 cm^2 of skin. Each result is the average of two determinations.

Time after application (hr)	Total activity of whole homogenate (count/min)	Specific activity (count/min mg)	
		Particulate protein	Supernatant protein
0.5	1,490,000	1410	1600
17	31,400	35	50
24	18,400	30	*

* One determination yielded 43 count/min mg; the other was too low to measure.