Fluorescence and Bioluminescence of Bacterial Luciferase Intermediates†

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ABSTRACT: An intermediate in the luciferase-catalyzed bioluminescent oxidation of $FMNH_2$, isolated and purified by chromatography at −20°, was postulated to be an oxygenated reduced flavine-luciferase. Maintained and studied at −20 to −30°, this material exhibits a relatively weak fluorescence emission peaking at about 505 nm when excited at 370 nm. It may comprise more than one species. Upon continued exposure to light at 370 nm, the intensity of this fluorescence increases, often by a factor of 5 or more, and its emission spectrum is blue shifted to a maximum at about 485 nm. Upon warming this fluorescence is lost and the fluorescence of flavine mononucleotide appears. If warming is carried out in the presence of a long chain aldehyde, bioluminescence occurs, with the appearance of a similar amount of flavine fluorescence. The bioluminescence yield is about the same with irradiated and nonirradiated samples. The bioluminescence emission spectrum corresponds exactly to the fluorescence emission spectrum of the intermediate formed by irradiation, implicating the latter as being structurally close to the emitting species in bioluminescence.

Bacterial luciferase catalyzes a reaction in which the concomitant oxidation of long chain aldehyde and reduced flavine mononucleotide results in the emission of light with a reasonably high quantum yield (Hastings and Gibson, 1963). The turnover of the enzyme is very slow, due, as previously shown, to the occurrence of an intermediate (designated II) with a half-life of the order of 10–20 sec at 20°, and stable for days or longer at liquid nitrogen temperatures (Hastings et al., 1964). Its isolation was recently achieved using low temperature (−20°) chromatography (Hastings et al., 1973). Its molecular structure is postulated to correspond to a reduced flavine hydroperoxide, bound to enzyme (E).

$$E + FMNH_2 \leftrightarrows \underset{I}{E\text{-}FMNH_2} \xrightarrow{O_2} \underset{II}{E\text{-}FMNH\text{-}OOH} \overset{ald}{\rightleftarrows} \underset{IIA}{ald\text{-}E\text{-}FMNH\text{-}OOH}$$

In the proposed reaction sequence the intermediate can combine reversibly with aldehyde (ald) to form an intermediate designated IIA. Both II and IIA break down upon warming: II gives FMN, H_2O_2, and free luciferase, while IIA yields bioluminescence, FMN, luciferase, and acid (Shimomura et al., 1972; Dunn et al., 1973; Vigny and Michelson, 1974; Hastings and Balny, 1975).

Between −20 and −30° the intermediate (II) exhibits fluorescence peaking in the blue at 505 nm. Upon warming, this fluorescence is lost and the fluorescence of FMN (λ_{max} 525 nm) appears; if the warming is carried out in the presence of aldehyde, bioluminescence is emitted during this reaction. In our study of these fluorescence phenomena, we have found evidence indicating that what has been referred to as a single intermediate may comprise two or more. We also report observations of an unusual photochemical conversion of an intermediate from one form to another, both active for bioluminescence, but differing in fluorescence properties.

Materials and Methods

The luciferase was isolated from the luminous bacterium *Beneckea harveyi* (previously referred to as strain MAV) (Reichelt and Baumann, 1973; Hastings et al., 1969) and purified by procedures described previously (Baldwin et al., 1975). Intermediate II was prepared by mixing luciferase (>90% pure, 0.8 ml, 10 mg ml^{-1}) and $FMNH_2$ (0.2 ml, 2 m*M*) at 5° in 50% ethylene glycol–phosphate buffer (pH 7.0) (Douzou, 1973, 1974). After 5 sec the temperature was quickly lowered to below −20° by mixing with 1 ml of the same buffer at −45° and holding in a dewar at −45°. The material was then applied to a Sephadex LH-20 column (2.5 × 20 cm), precooled at −25°, and chromatographed at this temperature (Balny et al., 1975). The fractions were assayed for bioluminescence activity by injecting a small (100 μl) aliquot into phosphate buffer (pH 7.0) with aldehyde (50 μ*M* octanal) at 23°. Activity is expressed as the initial maximum intensity. The active fractions were stored between −30 and −45° prior to use, which was usually within 24 hr.

Bioluminescence was measured using a photometer (Mitchell and Hastings, 1971) calibrated with a liquid standard (Hastings and Weber, 1963). Absorbance was measured in the Aminco Chance DW-2 spectrophotometer and fluorescence was measured in the Aminco-Bowman spectrophotofluorometer, both modified to permit temperature control of the sample down to as low as −65° (Maurel et al., 1974). The fluorescence spectra were recorded at −30° using 2-mm slits, giving a half-bandwidth of about 24 nm. The data are uncorrected for the exciting lamp intensity profile, efficiency of the monochromators, and the photomultiplier sensitivity. For the present experiments this is not a problem, since it is the spectral differences between inter-

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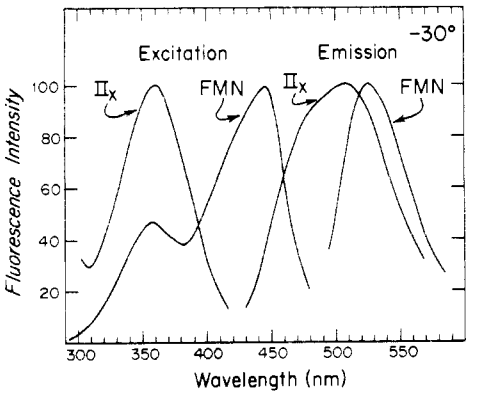

FIGURE 1: Excitation and emission spectra of intermediate(s), II_x, measured at −30° in 50% ethylene glycol and 0.1 *M* phosphate buffer (pH 7.0) just after elution from Sephadex LH-20 at −25°. For comparison the normalized excitation and emission spectra for FMN are given under the same conditions. The excitation spectra for II_x and FMN emission were monitored at 485 and at 525 nm, respectively. The emission spectra for II_x and FMN were obtained via excitation at 370 and 450 nm, respectively.

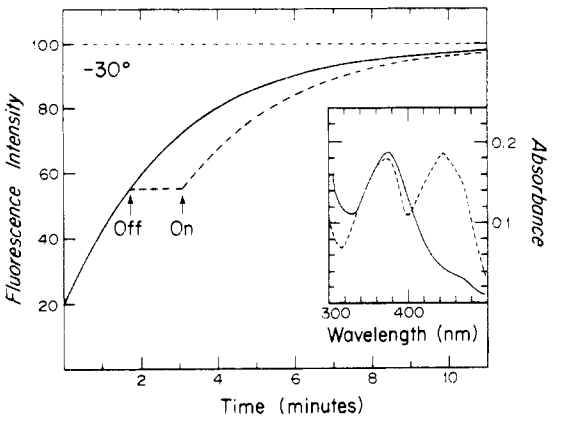

FIGURE 2: Effect of irradiation of II_x by light at 370 nm upon the intensity of the fluorescence emission at 485 nm, as a function of time. Also shown (- - -) is an experiment in which irradiation was stopped for a time and then resumed. Inset: Visible absorbance spectrum measured at −25° of one of the preparations of II used in these experiments, both before (—) and after (- - -) warming to 23° to form the product FMN. The absorbance at 280 nm was about 1.2. Based on the absorption of the product FMN, the concentration of the intermediate was 15 μM.

mediates and products which are of primary concern (along with comparisons of fluorescence and bioluminescence emission spectra). For comparison we provide recordings from this same instrument of the excitation and emission spectra of FMN.

Other procedures used are described in previous publications (Hastings et al., 1973; Hastings and Balny, 1975).

Results

The excitation and emission spectra (at −30°) of the oxygenated reduced flavine–luciferase intermediate (here designated II_x) are shown in Figure 1. The inset of Figure 2 shows that II_x has an absorbance peak which closely matches the excitation spectrum. A shoulder in the emission spectrum, however, suggests the presence of at least two components, one with its maximum emission in the vicinity of 485 nm and the second with a maximum about 505 nm, but not identical with that of oxidized flavine mononucleotide (maximum, ~525 nm). Excitation and emission spectra for a sample of FMN measured under the same conditions are presented on the same graph to facilitate comparison.

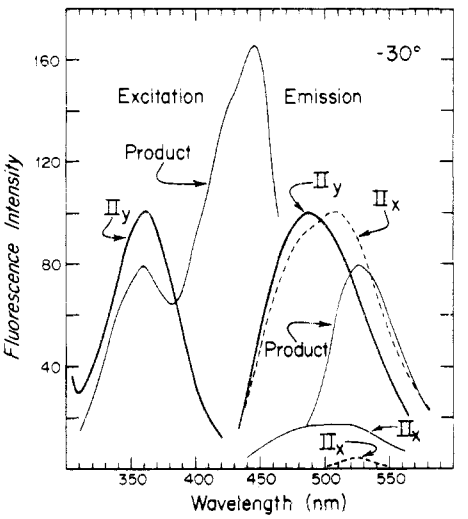

FIGURE 3: Excitation and emission spectra for the intermediate(s) II_y (—), formed after irradiation of II_x in the cuvet for 15 min (λ_{exc}, 370 nm; λ_{emis}, 485 nm). The spectrum of II_x from Figure 1 is shown again at its correct relative maximum intensity (about 18%) and also normalized (- - -) to facilitate comparison with II_y. (The excitation spectrum of II_x is identical with that of II_y.) The fluorescence emission spectrum of the product (actually measured by excitation at 450 nm) is plotted normalized to excitation at 375 nm in order to permit a comparison of relative fluorescence yields of II_x, II_y, and product (FMN). The excitation spectrum of the product is also shown. Finally, the amount of apparent FMN fluorescence (excited at 450 nm but based on 375 nm) which was measured in the initial II_x preparation is shown as a dashed line whose maximum is about 4% at 525 nm. This increased to about 6% following irradiation.

As the measurements are being carried out, however, an unusual phenomenon occurs. With continued irradiation at 370 nm at −30° the fluorescence emission at 485 nm increases dramatically (Figure 2).[1] The increase is dependent upon continued irradiation, and the product formed does not decay (rapidly) in the dark (as shown by the dashed line). The full action spectrum for this increase in fluorescence has not been determined, but 370 nm does appear to be the most effective wavelength. An increase of more than fivefold may occur within 10 min, while the emission spectrum shifts to the blue, with a final maximum in the vicinity of 485 nm (Figure 3). For purposes of discussion we refer to the sample prior to irradiation as II_x, and that afterwards as II_y, but it should be understood that the symbols II_x and II_y are not intended to refer to molecular species, but only to the samples.

As illustrated in Figures 1 and 3, no differences were observed in the excitation spectra for II_x and II_y; both correspond closely to the absorbance of II_x in the region of 370 nm (Figure 2, inset). Changes in the absorbance spectrum following irradiation were thus not expected, and none were observed. To examine for such possible light-induced absorbance changes, irradiation of II_x was carried out at −30°, directly in the cuvet in the spectrophotometer employing a xenon source with a broad band pass filter transparent between 340 and 450 nm.

Warming to 23° results in the breakdown of the intermediate(s), yielding one FMN per luciferase (Hastings and

[1] The fluorescence changes shown in Figures 2 and 3 are attributed to the material which is directly in the light beam. At the temperatures used diffusion and convection are much reduced, so unirradiated material from the rest of the cuvet does not interfere. Stirring and a longer time of irradiation were used (as in Table I) in order to convert all of the material in the cuvet.

Table I: Effect of Aldehyde and Irradiation at −30° upon the Fluorescence and Bioluminescence Capacity of the Intermediate, and the Amount of FMN Formed.[a]

Type and Sequence of Treatments (Fluorescence Intensities)	Bioluminescence (T quanta sec^{-1})	Fluorescence of Product (25°)
II_x (6.2)	1.4	23
	1.5	19
		21
II_x (4) $\xrightarrow{h\nu}$ II_y (28)	0.9	18
	1.2	24
	1.3	
II_x (4.2) $\xrightarrow{h\nu}$ II_y (27) $\xrightarrow{ald}$ II_yA (25)	1.0	24
	1.2	23
II_x (5) $\xrightarrow{ald}$ II_xA (6) $\xrightarrow{h\nu}$ IIA_y (27)	0.4	
	1.6	23
	1.2	

[a] Intermediate II was prepared and purified by Sephadex chromatography at −25°. Samples, of about 0.35 ml (referred to as II_x), were transferred at −30° to a fluorescence cuvet, and maintained at that temperature. With excitation at 370 nm, the maximum fluorescence intensity in the 480–510-nm region was recorded, and is noted in parentheses. Sequential treatments by irradiation and aldehyde addition at −30° resulted in changes in fluorescence intensity as noted; following the treatment, 0.1-ml aliquots were removed and assayed for bioluminescence by rapid injection into 2 ml of 0.01 *M* phosphate buffer (pH 7) with 50 μM octanal at 23°. Bioluminescence is given as the initial maximum intensity; the apparent first-order rate constant for the decay was the same (k = 0.02 sec^{-1}) in all samples; the photon yield can be obtained by multiplying by 50. The irradiation treatments (designated by $h\nu$) were carried out in the sample compartment of the spectrophotofluorimeter with the excitation light for 30 min, with stirring every 3 min. Aldehyde (1 μl of 7 m*M* octanal in the ethylene glycol–phosphate buffer) was added to the sample with stirring at −30° giving a final aldehyde concentration of 20 μM. The determinations of the fluorescence of the FMN product were carried out in a separate series of experiments in which each entire sample was allowed to warm for 6 min in the cuvet itself after which the fluorescence emission spectrum and intensity were recorded. Fluorescence intensities in arbitrary units.

Balny, 1975). The amount of FMN formed (Table I) was the same whether starting with II_x or II_y, and warming in the presence of aldehyde also does not alter the yield of FMN. The fluorescence excitation and emission spectra of the product (Figure 3), previously shown to be FMN (Hastings and Balny, 1975), are indeed identical with those of authentic FMN (Figure 1).

FMN fluorescence was routinely measured by excitation at 450 nm in order to distinguish it from the intermediate(s). In plotting (Figure 3) the intensity of the FMN emission is based on excitation at 370 nm in order to compare its fluorescence intensity with that of II_y. The fact that the apparent fluorescence intensity of FMN is lower than II_y is attributed to the lower sensitivity of the phototube at 525 nm than at 485 nm. It is estimated that the quantum yield of fluorescence of II_y and FMN are actually about the same.[2]

[2] In our first report on the isolation of the oxygenated reduced flavine-luciferase intermediate (designated II), we reported that its fluorescence yield was about one-third that of free FMN. From Figure 3 it is evident that this estimate could have been based on material which had been subjected to irradiation for only a short while. Unirradiated II has an apparent yield (based on peak intensities) of perhaps 20–25% that of FMN, while after irradiation the fluorescence quantum yield of II is about the same as FMN.

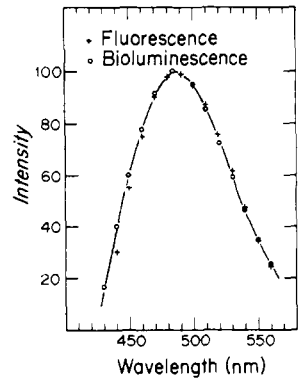

FIGURE 4: Emission spectrum of bioluminescence (O), measured directly from the cuvet during warming of II_y in the presence of aldehyde, plotted together with the fluorescence emission spectrum of II_y (x) taken from Figure 3. Ordinate, intensity of bioluminescence and fluorescence, normalized at the peak.

The bioluminescence potential of II_x and II_y were also found to be about the same, assayed by injection into buffer at 23° with aldehyde (Table I). Although there was a considerable variability in the determinations, it was certainly clear that the bioluminescence of II_y was not increased like its fluorescence, by a factor of 5. The addition of aldehyde at −30° prior to irradiation also had no major effect upon either the increase in fluorescence or the bioluminescence potential.

When the intermediate, irradiated or not, was allowed to warm up in the presence of aldehyde directly in the cuvet of the spectrophotofluorimeter, bioluminescence occurred. Warming was carried out with a programmed temperature control device, the temperature of the material in the cuvet being measured all the while with a thermocouple. The bioluminescence emission began to occur at about −20° and rose to a peak around 4°, decaying thereafter. During this period the fluorescence emission of the intermediate declined, while that of the product FMN at 525 nm appeared. The intensity[3] and duration of the bioluminescence emission were fully adequate to permit determinations of the emission spectrum of bioluminescence. Spectrally it appears to be identical with the fluorescence of II_y (Figure 4).

Discussion

The effect of light on II_x is unusual. Assuming, for the moment, that the subscripts x and y can be used to designate molecular species, one can write

$$II_x(\phi) \xrightarrow{h\nu} II_y(5\phi)$$

to represent a photochemical conversion in which the product has a fivefold higher fluorescence quantum yield (ϕ), with no detected alteration in the absorption spectrum of the compound. Such a photochemical conversion might involve different forms of the same chromophore having different degrees of solvent or protein microenvironmental relaxation, or conformational isomers of the reduced flavine nucleus. Alternatively, the different forms might involve normal intermediates in the bioluminescence pathway, such as the 1a,4a-dioxetane and the 4a-hydroperoxide suggested by Orf and Dolphin (1974). Thus in the scheme below, II_x

[3] 50% ethylene glycol does not decrease the quantum yield of bioluminescence at 0° as is the case at 25° (Hastings and Balny, 1975).

and II_y are represented as sequential intermediates in the normal reaction

$$FMNH_2 + E \rightleftharpoons I \xrightarrow{O_2} II_x \xrightarrow{(h\nu)} II_y \xrightarrow{ald} IIA \rightarrow \rightarrow \rightarrow E + FMN + \text{acid} + \text{light}$$

where E is luciferase, I is the reduced flavine–enzyme complex, and II the oxygenated reduced flavine–enzyme intermediate in its several forms. The several arrows are used to indicate that there are certainly several steps between IIA and product. Alternatively, it could be assumed that the two intermediates are sequential, but that II_y occurs prior to II_x.

$$\rightarrow I \xrightarrow{O_2} II_y \xrightarrow{(h\nu)} II_x \xrightarrow{ald} IIA \rightarrow \rightarrow \rightarrow \text{products}$$

In this scheme the initial mixture would include II_y and II_x, and light would drive the equilibrium between these two to the left.

If instead the photochemical conversion involves a conformational isomer, or something of that nature, it could be represented in the following way

$$\begin{array}{ccc} \rightarrow I \xrightarrow{O_2} II_x & \xrightarrow{ald} & II_xA \rightarrow \rightarrow \rightarrow \text{products} \\ \downarrow h\nu & & \downarrow h\nu \\ II_y & \xrightarrow{ald} & II_yA \rightarrow \rightarrow \rightarrow \text{products} \end{array}$$

Such a scheme is evidently somewhat cumbersome but may be more useful in terms of the observations now available. If the emission spectrum of the bioluminescence were different, depending on whether the starting material prior to warming was II_xA or II_yA, then this scheme could accommodate the observation. But such is not the case: the bioluminescence spectra are the same.

The structure of II is of special interest because its fluorescence emission spectrum after irradiation actually matches quite exactly the bioluminescence emission spectrum. In spite of this spectral similarity, it seems almost certain that II (either before or after irradiation) cannot be identical with the emitting species, for subsequent reaction with aldehyde is required to populate the excited state. Nevertheless, the molecular structure of II may be of direct relevance to the spectral properties of the emitting species.

Although an energy transfer step may be involved in some cases,[4] the specific emitter in a chemi- or bioluminescent reaction is generally an intermediate or product whose excited state is populated during the reaction. Although oxidized flavine (FMN) is a product of the bacterial bioluminescence reaction, and is also a highly fluorescent compound (quantum yield ~0.3; Weber and Teale (1957)), it has become evident that it cannot be the emitter. Free flavine in aqueous solution has a fluorescence emission peaking at 525 nm, while bacterial luminescence both in vivo and in vitro emits well to the blue with λ_{max} typically in the vicinity of 495 nm (Hastings et al., 1965; Seliger and Morton, 1969).[5] The possibility that the emission shift could be attributed to effects of binding to enzyme and/or polarity of the enzyme site has been considered but is not supported by the evidence (Mitchell and Hastings, 1969). In particular, it has recently been found that oxidized FMN when bound to luciferase is actually nonfluorescent (Baldwin et al., 1975). Unless the site where free FMN binds the apoenzyme is different from the one involved in the reaction, this finding essentially eliminates FMN itself as the emitter.

It has also been proposed that the 1-*N*-protonated form of oxidized FMN is the emitting species (Eley et al., 1970). The only evidence in support of this is that its fluorescence spectrum is reasonably close to that of the bioluminescence. However, the fact that an actual intermediate in the reaction has a fluorescence identical with the bioluminescence suggests that this should be viewed as an even better clue to the structure of the emitter. Thus, although our intermediate cannot *itself* be the emitter, its structure, postulated to involve a substitution at the 4a position, may be closely related. The fluorescence of such substituted flavines does, in fact, resemble that of our intermediate (Ghisla et al., 1974).

Acknowledgments

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[4] In the chemiluminescence of small synthetic dioxetanes, such as tetramethyldioxetane, the excited product has a very low quantum yield of emission, but can efficiently transfer its energy to brightly fluorescent acceptors (Turro et al., 1974; Wilson, 1975). Similar processes occur in certain coelenterates, where the in vivo light emission involves intermolecular energy transfer (Morin and Hastings, 1971).

[5] When appropriate corrections are applied to the spectral data presented in this publication (Figures 1, 3, and 5) the emissions peaking at about 485 nm shift about 10 nm to the red and correspond very closely to the published corrected spectra for the emission of bioluminescence (Hastings et al., 1965; Seliger and Morton, 1968).

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Microsomal Monooxygenation of the Carcinostatic 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea. Synthesis and Identification of Cis and Trans Monohydroxylated Products†

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ABSTRACT: Liver microsomal hydroxylation of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea was shown to occur on the cyclohexyl ring at positions 3 and 4. Four metabolites were isolated by selective solvent extraction and purified by high-pressure liquid chromatography. *cis*-4-, *trans*-4-, *cis*-3-, and *trans*-3-OH derivatives of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea were synthesized and their chromatographic, mass spectral, and nuclear magnetic resonance characteristics matched those of the metabolites. The position of ring hydroxylation and the identity of each geometric isomer were established by nuclear magnetic resonance using a shift reagent in conjunction with spin decoupling techniques. Microsomes from rats pretreated with phenobarbital showed a sixfold increase in hydroxylation rate (19.8 vs. 3.3 nmol per mg per min). The induction was quite selective for cis-4 hydroxylation (19-fold); however, induction of trans-4 (threefold), cis-3 (threefold), and trans-3 (twofold) hydroxylation did occur. Quantitatively the *cis*-4-hydroxy metabolite was 67% of the total product by phenobarbital-induced microsomes and 21% for normal microsomes. Microsomes from animals pretreated with 3-methylcholanthrene gave about the same rate and product distribution that normal microsomes gave. A mixture of 80% carbon monoxide–20% oxygen inhibited formation of all four hydroxy metabolites with the inhibition ranging from 55 to 78%.

Nitrosoureas including CCNU[1] are undergoing intensive clinical evaluation and are highly promising for use as chemotherapeautic agents for brain tumors (Walker, 1973), Hodgkins disease (Selawry and Hansen, 1972), and lung cancer (Takita and Brugarolas, 1973). Carter et al. (1972) have stated that these lipid soluble compounds are among the most promising antineoplastic agents to emerge from the Chemotherapy Program of the National Cancer Institute. They distribute widely to tissues and have a short plasma half-life (Oliverio et al., 1970). Montgomery et al. (1967) and Reed et al. (1975) have shown that these compounds also have relatively short half-lives in buffers. We have begun an extensive study of the metabolism of CCNU in an effort to understand its mode of action as an antitumor agent and to understand its toxic action. Until recently, little was known about the metabolism of CCNU when it was found to be hydroxylated in the liver as an initial metabolic step (May et al., 1974). According to the prediction of Hansch et al. (1972), the introduction of a hydrophilic group into CCNU should make it a more potent less toxic drug.

Liver microsomes catalyze an NADPH and cytochrome P-450 dependent hydroxylation of the cyclohexyl group of a number of compounds (Ullrich, 1969; Diehl et al., 1970; McMahon et al., 1965; McMahon and Sullivan, 1966). We reported earlier that CCNU is hydroxylated by a microsomal system that requires NADPH and oxygen, that is inhibited by carbon monoxide and is inducible by pretreatment of animals with phenobarbital. Further evidence for the involvement of cytochrome P-450 was obtained when CCNU was found to bind to microsomes and produce a type I difference spectrum. The spectrally determined dissociation constant for the P-450–CCNU complex was 4×10^{-5} *M* (May et al., 1974).

The present paper deals with the isolation and identifica-

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[1] Abbreviations used are: CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; *cis*-4-OH-CCNU, 1-(2-chloroethyl)-3-(*cis*-4-hydroxycyclohexyl)-1-nitrosourea; *trans*-4-OH-CCNU, 1-(2-chloroethyl)-3-(*trans*-4-hydroxycyclohexyl)-1-nitrosourea; *cis*-3-OH-CCNU, 1-(2-chloroethyl)-3-(*cis*-3-hydroxycyclohexyl)-1-nitrosourea; *trans*-3-OH-CCNU, 1-(2-chloroethyl)-3(*trans*-3-hydroxycyclohexyl)-1-nitrosourea; [*cyclohexyl*-1-^{14}C]CCNU, [^{14}C]CCNU labeled in its cyclohexyl moiety; i-CCNU, 1-(2-chloroethyl)-3-cyclohexyl-3-nitrosourea; Eu(dpm)$_3$, tris(dipivaloylmethanato)europium(III).