Anal. Calcd for $C_{20}H_{27}NO_2$: C, 69.66, H, 11.28; N, 5.80. Found: C, 69.83; H, 11.22; N, 5.77.

Preparation of 110 with $MgBr_2$-Et_2O. Procedure C: 974 mg (5.31 mmol) of **104**, 5.85 mmol of *sec*-BuLi, 8.82 μL (5.85 mmol) of TMEDA, 150 mL of THF, 5 min, 1.51 g (5.85 mmol) of $MgBr_2$-Et_2O was added with stirring at −60 °C for 15 min and then 712 μL (9.7 mmol) of acetone was added and with stirring for 20 min prior to workup. Recrystallization from dichloromethane/pentane afforded 724 mg (57%) of **110**: mp 77–79 °C, mixture mp 78–79 °C.

Metalation and Equilibration of 19. A solution of 201 mg (1.45 mmol) of **19** in 7 mL of THF was cooled to −78 °C and treated with 2.0 mL (2.8 mmol) of *n*-BuLi. The resulting solution was allowed to warm to ambient temperature for 15 min, after which 0.05 mL (0.3 mmol) of 2,2,6,6-tetramethylpiperidine was added. After 36 h, the mixture was cooled to 0 °C and treated with 0.1 mL (5 mmol) of D_2O. Extractive workup in the manner described for procedure A left a brown oil, which was distilled (Kugelrohr, 90–95 °C (0.2 mm)) to give 122 mg (60%) of a colorless liquid. Analysis of the ^1H NMR spectrum indicated a ca. 60:40 mixture of **117**:**118** respectively. The presence of **118** was indicated by the following resonances: δ 2.81 (d, J = 5 Hz, 3 H, NCH_3), 5.66 (br d, J = 10 Hz, 1 H, β-H), 5.95 (dt, J_1 = 10 Hz, J_2 ≈ 4 Hz, γ-H); mass spectrum (70 eV), isotope ratio m/e (relative intensity) 141 (8.3), 140 (68.4), 139 (42.1), 83 (49.8), 82 (90.1), 81 (60.0); calcd % d = 68% (for the mixture **117**, **118**).[38]

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Supplementary Material Available: Experimental details about compounds and the preparations of **22**, **25–27**, **29**, **30**, **32–35**, **37**, **39**, **40**, **43**, **44**, **47**, **53**, **64**, **65**, **67**, **70**, **71**, **75**, **78**, **79**, **83**, **84**, **88**, **89**, **92**, **94–97**, **100**, **101**, **103**, **105**, **108**, **106**, **111**, **112**, **114–116** (28 pages). Ordering information is given on any current masthead page.

(38) The amount of deuterium incorporation in a sample was determined by solving the following set of simultaneous equations: $d(I_{-1}) + (1-d)I_0 = a(I'_{-1})$; $d(I_0) + (1-d)I_{+1} = a(I'_0)$, where d = fraction of deuterated material in the sample, I_0 = relative intensity of the molecular ion peak in the starting material, I_{-1} = relative intensity of the M − 1 peak in the starting material, I_{+1} = relative intensity of the M + 1 peak in the starting material, I'_0 = relative intensity of the molecular ion peak in the deuterated sample, I'_{-1} = relative intensity of the M − 1 peak in the deuterated sample, and a = normalization coefficient. The process was repeated for another major peak in the spectrum, usually the acylium ion (M − NR_2). The results generally agreed within 1–2% with both peaks.

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Synthesis, Characterization, and Properties of Hexadecyl Silica: A Novel Hydrophobic Matrix for Protein Immobilization

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Abstract: A convenient and inexpensive synthesis of an insoluble protein carrier is described. Hexadecyl silica, prepared by simple heating of hexadecanol and silica gel, has been shown to be a suitable matrix for protein immobilization with total retention of biochemical activity and the possible use of the adsorbed proteins in continuous catalytic operations. Gels with various degrees of substitution were prepared, and the maximum ligand density attainable was found to be ca. 90 mg of lipid per g of adsorbent. The degree of substitution has been observed to be an important factor in the adsorptive properties of the carrier. The extent of adsorption of three arbitrarily chosen proteins was found to increase with increasing ligand density, reaching maximum values with gels of higher hydrophobicities. Protein adsorption was also found to be a function of matrix/protein ratio in a similar manner. The pH stability of the matrix was also investigated. Although the extent of cleavage of the ligand increased with increasing pH, no deleterious effect on the adsorptive properties of the support was observed.

In recent years, wide attention has been directed toward the application of enzymes in organic synthesis.[1] In fact, laboratory scale stereospecific preparation of a number of compounds has been realized with enzymes as chiral catalysts.[2] This will clearly provide a new dimension to the use of immobilized enzyme systems, the importance of which for industrial purposes has already been recognized.[3]

During the course of our studies on immobilized enzyme systems,[4-7] it became evident that hydrophobic matrices may be used for adsorption of a variety of proteins, including enzymes. More importantly, adsorption conditions with such hydrophobic gels did not appear to have an adverse effect on the native properties of the proteins examined. For example, adsorbed glutamate dehydrogenase, used as a model allosteric enzyme, underwent its normal heterotropic allosteric conformational transitions in response to its effectors (inhibitors and activators) to a similar extent as its free form.[4-6]

On the other hand, application of such gels to large-scale enzymatic transformations is hampered by (a) the number of reactions required for the preparation of the hydrophobic ligand (i.e., hexadecyl glycidyl ether), (b) the requirement of large

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volumes of dioxane for the subsequent attachment of the ligand to Sepharose which serves as the insoluble support, (c) the commercial price of Sepharose, and (d) the susceptibility of the adsorbent to microbial attack.

In view of these shortcomings, a hydrophobic inorganic support would provide an attractive alternative. The present paper describes the use of hexadecyl-substituted silica gel, prepared by simple heating of hexadecanol and silica gel, as a hydrophobic carrier for protein immobilization. Our results indicate that the matrix provides an inexpensive method for immobilization of enzymes with full retention of their catalytic activities.

Experimental Section

Materials. Silica gels (Art 7731 for TLC and Art 7729 for column chromatography) and Florisil (Art 12518) were purchased from Merck (Darmstadt, FRG). RNase A (5 × crystallized) and DNase I, both from bovine pancreas and protease from *Bacillus amyloliquefacieus*, were obtained from Sigma (St. Louis, MO). Alcohol dehydrogenase from Baker's yeast and pronase from *Streptomyces griseus* were supplied by Boehringer (Mannheim, FRG). All other chemicals and biochemicals were obtained from previously described sources.[4] [U-^{14}C]Palmitic acid (250 μCi/mmol) was purchased from The Radiochemical Centre, Amersham, England.

Synthesis of Hexadecyl Silicas. Hexadecanol[8] (8.0 g, 33 mmol) and 80.0 g of TLC silica gel in 500 mL of toluene were refluxed in a Dean-Stark apparatus for 18 h. The resulting white mass was then filtered and washed with refluxing toluene for 18 h in a Soxhlet in order to remove the noncovalently adsorbed alcohol. The derivatized gel was subsequently dried under vacuum to a constant weight. This adsorbent was used for immobilization of proteins reported here (vide infra). For carriers of different degrees of substitution, 3.0 g of silica was used in 19 mL of toluene and the amount of alcohol was varied from 30 mg to 1.2 g (see Figure 1). In the case of column chromatography silica gel or Florisil, 32.0 g (0.132 mol) of hexadecanol was used for 80.0 g of the support in 500 mL of toluene and the reflux time was increased to 48 h.

Determination of the Degree of Substitution. This was carried out by the use of [U-^{14}C]hexadecanol. Four grams of the radioactive adsorbent, prepared by the above procedure, was suspended in 25 mL of concentrated HCl and kept at 70 °C for 48 h. After repeated extractions with chloroform, the organic layer was dried over sodium sulfate and decolorized with a small amount of active charcoal, and the white solid obtained after evaporation of the solvent was assayed for ^{14}C content in a toluene-based scintillation liquid.[9]

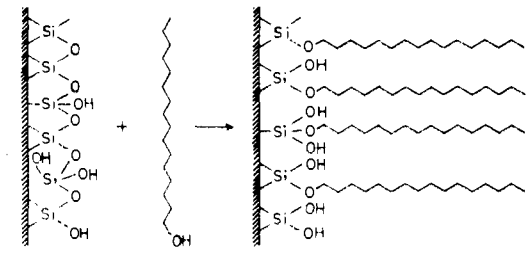
Determination of the Stability of Hexadecyl Silica at Various pH Values. This was performed by suspending 1.0 g of the radioactive adsorbent (21 mg of lipid/g of adsorbent) in 20 mL of a buffer containing glycine, succinate, and Mes, each at 5 mM concentration, and adjusted to different pH values (2–10). After stirring for 24 h, the suspension was filtered and washed twice with 10-mL portions of acetone and then 5 × 10 mL portions of ether. After the usual workup, each sample was assayed for ^{14}C content.

In a separate experiment, the adsorbent was suspended in 10 mM of Tris and 30 mM of calcium chloride, pH 8.0. This buffer was also used for studying continuous catalytic operation with immobilized trypsin (vide infra). After stirring for 24 h, the adsorbent was exhaustively washed with the buffer and the filtrate was repeatedly extracted with ether. The organic layer was then assayed for ^{14}C content as described above.

In a different set of experiments, samples of the adsorbent which had been treated with the above buffers (pH 2–10) for 24 h were subsequently tested for their adsorptive capacity toward pepsin and glutamate dehydrogenase.

Adsorption of Proteins to Hexadecyl Silica. One milligram of protein and 0.5 g of the adsorbent, in a final volume of 3.2 mL, were mixed for 15 min and the amount of the adsorbed protein was determined by centrifugation of the original suspension followed by resuspension-recentrifugation of the pellets in their respective buffers twice. In order to obtain a homogeneous suspension with highly substituted gels (≥62 mg of hexadecanol/g of adsorbent) which exhibited buoyant properties, it was found necessary to initially use a 25% ethanol-water solution followed by centrifugation and resuspension in the appropriate buffer. Percent adsorption was then determined spectrophotometrically as described earlier.[4] Tris, (5 mM, pH 7.5) was used for DNase and RNase, 5 mM Tris, pH 8.0 for protease, 0.03 M sodium pyrophosphate, pH 8.8 for alcohol dehydrogenase, and 0.001 N HCl for pronase. The buffers used for other proteins have been described previously.[4]

Scheme I



Enzyme Assays. Assays were performed by a 10 min initial binding of enzyme and the adsorbent and by following previously reported procedures.[4,5,10] For trypsin and α-chymotrypsin the hydrolytic reaction was initiated by the addition of their respective substrates. For glutamate dehydrogenase adsorption to the carrier was performed in the presence of α-ketoglutarate and ammonium chloride and the reduction process was initiated by the addition of NADH. In the case of alcohol dehydrogenase, NAD^+ was the last reactant added to initiate the oxidation reaction.

Continuous Catalytic Operation with Immobilized Trypsin. This was carried out by adsorbing 400 μg of the enzyme to 0.2 g of adsorbent and using a simple fluidized-bed reactor described earlier.[5] N^{α}-Benzoyl-L-arginine ethyl ester at 1 mM concentration was used as substrate, and the esterase reaction was performed at 22 °C.

Results and Discussion

In recent years, much attention has been directed toward the use of inorganic carriers for enzyme immobilization. In contrast to organic supports, these adsorbents are inexpensive, immune to bacterial degradation, and resistant to morphological changes caused by extreme variations in pH or pressure. In fact, immobilization of enzymes on a variety of inorganic supports has been achieved through (a) covalent attachment to derivatized supports,[11,12] (b) adsorption by inorganic bridge formation between enzyme and carrier,[11d] and (c) simple adsorption.[11d,12] In this regard, an assortment of support materials such as kaolinite, bentonite, various ceramics, calcium phosphate, carbon, molecular sieve, porous glass, and silica gel have been utilized as carriers.[11,12]

With the advent of ^{29}Si NMR spectroscopy, a new wealth of information on the surface structure of silica has recently appeared in the literature.[13] Furthermore, surface-functionalized silica gel has already found such diverse applications as phase-transfer catalysts,[14] in heterogeneous organic synthesis,[15] hydrophobic

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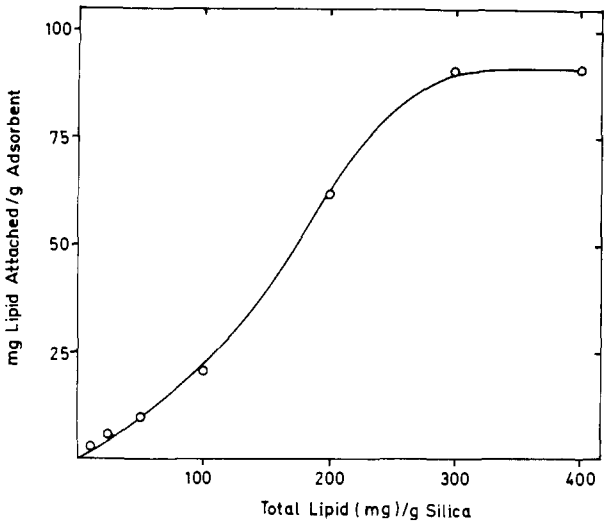

Figure 1. Dependence of the degree of substitution on the concentration of hexadecanol. Three grams of silica gel were used, and the amount of alcohol was varied from 30 mg to 1.2 g. For further details see the Experimental Section.

chromatography,[16] and resolution of chiral mixtures.[17]

Surface derivatization of silica gel may be achieved by the reaction of X-alkyl or X-aryltrialkoxysilanes,[14b] X-trialkylchlorosilane,[13a] and X-dialkyldichloro- and X-alkyltrichlorosilanes[13b] with the hydroxyl function of silica (X represents the desired functional group). Baverez and Bastick[18] have also reported that alcohols may react directly with the strained siloxane function of silica gel. Accordingly, a convenient synthetic scheme in which direct attachment of hexadecanol to silica gel is accomplished by simple heating provides an inorganic carrier with hydrophobic properties (Scheme I).

The surface hydrophobicity of such an adsorbent would clearly depend on the degree of substitution. This, in turn, depends on the concentration of hexadecanol and the reaction time. We found that at a fixed reaction time of 18 h the ligand density of the TLC silica gel used in this study increases with increasing concentration of hexadecanol and reaches a saturation level (0.37 mmol of hexadecanol/g of adsorbent) as shown in Figure 1. The values for the degrees of functionalization reported here appear to be typical of metal oxide derivatization.[14d] It is interesting to note that a high degree of substitution ($\geq$62 mg of lipid/g of adsorbent) results in gels with buoyant properties. Therefore, a homogeneous suspension of such gels could only be obtained by initially "wetting" the adsorbent with an organic–aqueous solvent system such as 25% ethanol–water which was used in this study. The adsorbent with a degree of substitution of 21 mg of lipid/g of adsorbent was used for all subsequent studies not only because of its ability to be "wetted" but also due to the fact that it showed near maximum capacity for protein adsorption (vide infra).

A number of arbitrarily chosen proteins were tested for adsorption onto the carrier in the form of suspension. Pepsin, trypsin, α-chymotrypsin, papain, protease, peroxidase, glucose oxidase, aldolase, lysozyme, alkaline phosphatase, DNase I, RNase, α-amylase, urease, glyceraldehyde-3-phosphate dehydrogenase, glutamate dehydrogenase, lactate dehydrogenase, alcohol dehydrogenase, bovine serum albumin, myoglobin, hemoglobin, γ-globulins, and cytochrome *c* showed 90–100% adsorption. Xanthine oxidase provided the only exception in that 40–50% of the protein was adsorbed.

A comparison of unsubstituted silica gel with the hydrophobic adsorbent prepared by the above procedure indicated that the latter has a larger capacity for protein immobilization. A number of

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Table I. Activities of Enzymes Immobilized on Hexadecyl Silica

enzyme	enzyme:adsorbent	reaction vol, mL	% activity
glutamate dehydrogenase	25 μg:4 mg	5.27	94
alcohol dehydrogenase	1 μg:10 mg	3.11	97
trypsin	0.1 mg:50 mg	5.1	100
α-chymotrypsin	100 μg:50 mg	7.25	100

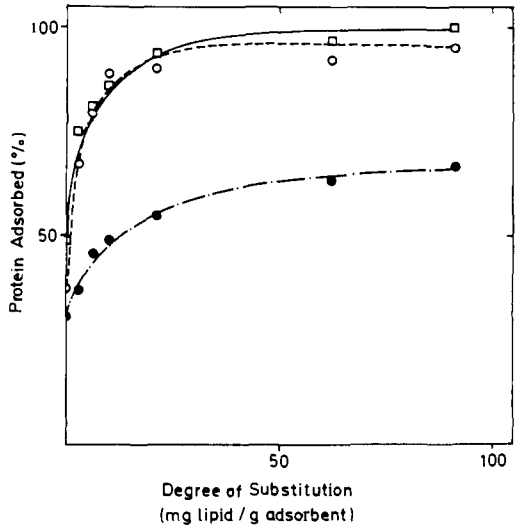

Figure 2. Dependence of protein adsorption on the degree of substitution. One milligram of enzyme and 0.5 g of the carrier of various degrees of substitution were mixed and the amount of the protein adsorbed was determined in each case. The proteins tested were pepsin (○), glutamte dehydrogenase (□), and xanthine oxidase (●).

the proteins mentioned above (peroxidase, glucose oxidase, alkaline phosphatase) showed very weak adsorption (<20%) to unsubstituted silica gel, and some (glutamate dehydrogenase, alcohol dehydrogenase, amyloglucosidase, aldolase, and pepsin) less than 50%. The difference may be ascribed to the presence of hydrophobic ligands which introduce a new parameter in addition to those (e.g., hydrogen bonding and formation of amine silicate bond[11e,13e]) responsible for protein adsorption to unsubstituted gels. Adsorption of proteins to the derivatized carrier involves interaction between hydrophobic side chains occurring on the surface or crevices of protein molecules and the long hydrophobic arms present on the exterior of the carrier.[4-6] Therefore, the hydrophobic arms stabilize binding by anchoring the protein molecules on the matrix and thus delaying protein desorption. This and the highly active immobilized enzyme preparations obtained with hexadecyl silica (see Table I) provide desirable conditions for continuous catalytic operation.

A comparison of the results presented in this article with those reported earlier[4,5] clearly indicates that hexadecyl silica is a more effective adsorbent than hexadecyl sepharose. For example, cytochrome *c* which is totally immobilized on hexadecyl silica irrespective of pH could only be adsorbed on hexadecyl sepharose at low pH, apparently due to conformational changes which lead to the exposure of its hydrophobic sites.[4] The enhanced affinity of hexadecyl silica for protein adsorption may be attributed not only to a higher ligand density on its surface but also to the intrinsic differences in hydrophilicity and physical morphology of the two gels.

The degree of adsorption of three typical proteins to gels containing different amounts of the hydrophobic ligand was found to vary according to Figure 2. Thus, under our experimental conditions, it is possible to obtain near total adsorption of pepsin and glutamate dehydrogenase only with gels of high degree of functionalization (i.e., >21 mg of hexadecanol/g of adsorbent). The adsorption of xanthine oxidase which showed poor binding affinity for the carrier (vide supra) was also found to depend on the degree of substitution (Figure 2). It is interesting to note that use of silica gel of larger particle size (column chromatography)

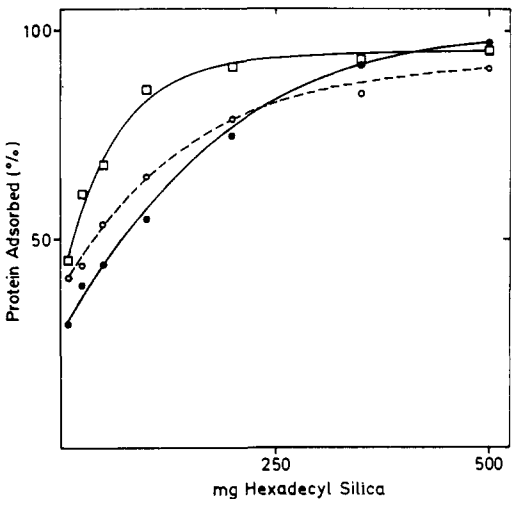

Figure 3. Adsorption of pepsin (○), glutamate dehydrogenase (□), and bovine serum albumin (●) to hexadecyl silica as a function of the concentration of the matrix.

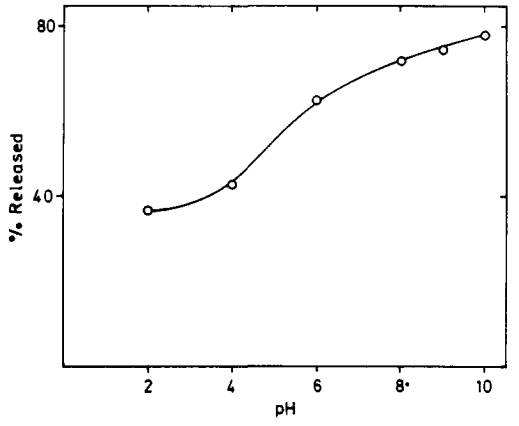

Figure 4. Stability of hexadecyl silica at different pH values. One gram of the radioactive adsorbent was suspended in 20 mL of buffer and after 24 h the amount of the ligand releases was assayed. Details are described under the Experimental Section.

or Florisil, both surface-saturated with the hydrophobic ligand, resulted in gels that showed only 27% and 38% binding for pepsin, respectively. This may be ascribed to the fact that the hydrophobic surface area provided by these derivatized carriers is not sufficient for adequate interaction to take place.

The effect of variation in matrix/protein ratio on the adsorption of three proteins which showed good affinity for the hydrophobic gel was also investigated. This was carried out by changing the amount of the adsorbent at a constant protein concentration. It is clear from Figure 3 that the percent adsorption increases with increasing amounts of the carrier in each case, with approximately 400 mg of the gel being required for optimal binding.

The pH stability of the adsorbent was investigated in the range of 2–10. It was found that prolonged treatment (24 h) caused release of the ligand in a manner depicted in Figure 4. Other derivatives of silica have also been reported to behave in a similar manner.[12a] It should be noted, however, that release of the ligand could only be observed when the treated adsorbent was repeatedly washed with organic solvents (see Experimental Section). On the other hand, no release could be detected on using buffers instead of organic solvents in the washing step. This may be attributed to a higher affinity of the cleaved hydrophobic ligand for the surface of silica gel than for the surrounding aqueous environment. In this manner, although the ligand is cleaved from the carrier, it remains tightly associated with the surface of the support. The cleaved but associated ligand appears to be capable of participating in hydrophobic interactions with apolar residues of proteins. In fact, no adverse effect on binding of glutamate dehydrogenase and pepsin to adsorbents which were pretreated with buffers of different pH values could be detected.

Percent activities of four immobilized enzymes in the form of suspension (as compared to their free form) are summarized in Table I. As indicated, these enzymes retained nearly full catalytic efficiency upon binding to the matrix. The present results are similar to those obtained with hexadecyl- and Triton X-100-substituted Sepharose 4B used as organic hydrophobic adsorbents for protein immobilization.[4-6]

The ability of immobilized trypsin for hydrolysis of N^{α}-benzoyl-L-arginine ethyl ester in a continuous operation was also investigated. The adsorbed enzyme was found to convert 277 mL of the reactant at 1 mM concentration to its products during a period of 7 h with 100% catalytic efficiency. On the other hand, no activity could be detected in the case of free enzyme after a period of 2.5 h.

Considering cost-effectiveness, simplicity of preparation, and physicochemical properties of the adsorbent, it may be stated that hexadecyl silica provides an excellent insoluble support for enzyme immobilization in the form of suspension. Preparations so obtained may prove useful in large-scale continuous enzymatic transformations.

Registry No. Glutamate dehydrogenase, 9029-12-3; alcohol dehydrogenase, 9031-72-5; trypsin, 9002-07-7; α-chymotrypsin, 9004-07-3; pepsin, 9001-75-6; papain, 9001-73-4; protease, 9001-92-7; peroxidase, 9003-99-0; glucose oxidase, 9001-37-0; aldolase, 9024-52-6; lysozyme, 9001-63-2; alkaline phosphatase, 9001-78-9; DNase I, 9003-98-9; RNase, 9001-99-4; α-amylase, 9000-90-2; urease, 9002-13-5; glyceraldehyde-3-phosphate dehydrogenase, 9001-50-7; lactate dehydrogenase, 9001-60-9; cytochrome *c*, 9007-43-6; hexadecanol, 36653-82-4.