rated $(NH_4)_2SO_4$ and stirred 2.5 hours at room temperature. The precipitate was collected by centrifugation (12,000*g* for 30 min) and dissolved in 5 ml of TE buffer. After 30 min at room temperature, the solution was clarified by centrifugation (10,000*g* for 10 min) and filtered through a Millipore filter (0.45 μm). The solution was dialyzed against 0.5 liters of TE buffer overnight at 4°C. The hPRL (85% pure) was finally purified to homogeneity (>95%) by fast-performance liquid chromatography (FPLC) and DEAE fast-flow matrix essentially as described for the purification of hGH (7). After reduction, the purified hPRL shows a pronounced retardation in mobility by SDS-PAGE (as seen for hGH) suggesting that disulfide bonds have formed [S. Pollit and H. Zalkin, *J. Bacteriol.* **153**, 27 (1983)]. The concentrations of hPRL and hPRL mutants were determined by measuring absorbance at 280 nm and by using a calculated extinction coefficient of $\epsilon_{280}^{0.1\%} = 0.9$ [D. B. Wetlaufer, *Adv. Protein Chem.* **17**, 303 (1962)], which was adjusted accordingly when variants contained mutations in aromatic residues. Concentration values determined by absorbance agreed to within 10% of those determined by laser densitometry of proteins subjected to SDS-PAGE and stained with Coomassie blue for hGH (7, 8).

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Direct Gene Transfer into Mouse Muscle in Vivo

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RNA and DNA expression vectors containing genes for chloramphenicol acetyltransferase, luciferase, and β-galactosidase were separately injected into mouse skeletal muscle in vivo. Protein expression was readily detected in all cases, and no special delivery system was required for these effects. The extent of expression from both the RNA and DNA constructs was comparable to that obtained from fibroblasts transfected in vitro under optimal conditions. In situ cytochemical staining for β-galactosidase activity was localized to muscle cells following injection of the β-galactosidase DNA vector. After injection of the DNA luciferase expression vector, luciferase activity was present in the muscle for at least 2 months.

MOST EFFORTS TOWARD POSTNATAL gene therapy have relied on indirect means of introducing new genetic information into tissues: target cells are removed from the body, infected with viral vectors carrying the new genetic information, and then reimplanted into the body (*1*). For some applications, direct introduction of genes into tissues in vivo, without the use of viral vectors, would be useful. Direct in vivo gene transfer into postnatal animals has been achieved with formulations of DNA encapsulated in liposomes, DNA entrapped in proteoliposomes containing viral envelope receptor proteins (*2*), calcium phosphate–coprecipitated DNA (*3*), and DNA coupled to a polylysine-glycoprotein carrier complex (*4*). In vivo infectivity of cloned viral DNA sequences after direct intrahepatic injection with or without formation of calcium phosphate coprecipitates has also been described (*5*). With the use of cationic lipid vesicles (*6*), mRNA sequences containing elements that enhance stability can be efficiently translated in tissue culture cells (7) and in *Xenopus laevis* embryos (*8*). We now show that injection of pure RNA or DNA directly into mouse skeletal muscle results in significant expression of reporter genes within the muscle cells.

The quadricep muscles of mice were injected (*9*) with either 100 μg of pRSVCAT DNA plasmid (*10*) or 100 μg of βgCATβ-gA_n RNA (*7, 11, 12*). The RNA consists of the chloramphenicol acetyl transferase (CAT) coding sequences flanked by β-globin 5′ and 3′ untranslated sequences and a 3′ polyadenylate tract. CAT activity was readily detected in all four RNA injection sites 18 hours after injection and in all six DNA injection sites 48 hours after injection (Fig. 1). Extracts from two of the four RNA injection sites (Fig. 1, lanes 6 and 8) and from two of the six DNA injection sites (Fig. 1, lanes 11 and 20) contained amounts of CAT activity comparable to those obtained from fibroblasts transiently transfected with the corresponding constructs in vitro under optimal conditions (Fig. 1, lanes 9 and 10 and 21 to 24, respectively). The average total amount of CAT activity expressed in muscle was 960 pg for the RNA injections and 116 pg for the DNA injections. The variability in CAT activity recovered from different muscle sites probably represents variability inherent in the injection and extraction technique, because significant variability was observed when pure CAT protein or pRSVCAT-transfected fibroblasts were injected into the muscle sites and immediately excised for measurement of CAT activity. CAT activity was also recovered from abdominal muscle injected with the RNA or DNA CAT vectors (*13*), indicating that other muscles can take up and express polynucleotides.

The site of gene expression was determined for the pRS*Vlac-Z* DNA vector (*14*) expressing the *Escherichia coli* β-galactosidase gene (Fig. 2). Seven days after a single injection of 100 μg of pRS*Vlac-Z* DNA into individual quadricep muscles, the entire muscles were removed, and every fifth 15-μm cross section was histochemically stained for β-galactosidase activity. Approximately 60 (1.5%) of the ~4000 muscle cells that comprise the entire quadriceps and ~10 to 30% of the cells within the injection area were stained blue (Fig. 2, A and B). Positive β-galactosidase staining within some individual muscle cells was at least 1.2 mm deep on serial cross sections (Fig. 2, D to F), which may be the result of either transfection into multiple nuclei or the ability of cytoplasmic proteins expressed from one nucleus to be distributed widely within the muscle cell (*15*). Longitudinal sectioning also revealed β-galactosidase staining within muscle cells for at least 400 μm (Fig. 2C). Fainter blue staining often appeared in the bordering areas of cells adjacent to intensely stained cells. This most likely represents an artifact of the histochemical β-galactosidase

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stain, in which the reacted product diffuses before precipitating. An alternative hypothesis is that β-galactosidase can be transported from a transfected cell to an adjacent, untransfected cell.

A dose-response effect was observed when quadricep muscles were injected with various amounts of RNA (βgLucβgA$_n$) or DNA (pRSVL) constructs containing the firefly luciferase reporter gene (Fig. 3A). The injection of ten times more DNA resulted in luciferase activity increasing approximately tenfold from 33 pg of luciferase after the injection of 10 μg of DNA to 320 pg of luciferase after the injection of 100 μg of DNA. The injection of ten times more RNA also yielded approximately tenfold more luciferase. On the basis of the amount of DNA delivered, the efficiency of expression from the DNA vectors was similar in both transfected fibroblasts and injected muscles. Twenty micrograms of pRSVL DNA transfected into fibroblasts yielded a total of 120 pg of luciferase (6.0 pg of luciferase per microgram of DNA) (*16*), whereas 25 μg injected into muscle yielded an average of 116 pg of luciferase (4.6 pg of luciferase per microgram of DNA) (Fig. 3A). The expression from the RNA vectors was approximately sevenfold more efficient in transfected fibroblasts than in injected muscles. Twenty micrograms of βgLucβgA$_n$ RNA transfected into fibroblasts yielded a total of 450 pg of luciferase (*7, 16*), whereas 25 μg injected into muscle yielded 74 pg of luciferase (Fig. 3, A and B).

The time course of expression was also investigated (Fig. 3, B and C). Luciferase activity was assayed at various times after 100 μg of βgLucβgA$_n$ RNA (*7*) or 100 μg of pRSVL DNA (*17*) were injected. After RNA injection, the average luciferase activity reached a maximum of 74 pg at 18 hours and then decreased to 2 pg at 60 hours. In transfected fibroblasts, the luciferase activity was maximal at 8 hours. After DNA injection into muscle, substantial amounts of luciferase were present for at least 60 days.

The luciferase protein and the in vitro RNA transcript appear to have a half-life of less than 24 hours in muscle (Fig. 3B). Therefore, the persistence of luciferase activity for 60 days in muscle after pRSVL DNA injection is not likely to be due to the stability of luciferase protein or the stability of the in vivo RNA transcript. Southern (DNA) blot analysis of muscle DNA indicates that the foreign pRSVL DNA is present within the muscle tissue for at least 30 days (Fig. 4, lanes 6 to 9) and that the amount present at this time is similar to the amount of DNA present in muscle 2 and 15 days after injection (*13*). In muscle DNA digested with Bam HI (which cuts pRSVL once) (Fig. 4, lanes 6 to 9), the presence of a 5.6-kb band that corresponds to linearized pRSVL (Fig. 4, lane 2) suggests that the DNA is present either in a circular, extrachromosomal form or in large tandem repeats of the plasmid integrated into chromosome. In muscle DNA digested with Bgl II (which does not cut pRSVL), the presence of a band smaller than 10 kb (Fig. 4, lanes 12 and 13) and of the same size as the open, circular form of the plasmid pRSVL (Fig. 4, lane 1) implies that the DNA is present extrachromosomally in a circular form (*18*). Extrachromosomal DNA of muscle extracts was prepared by the method of Hirt, modified by Pauza and Galindo (*19*). The appear-

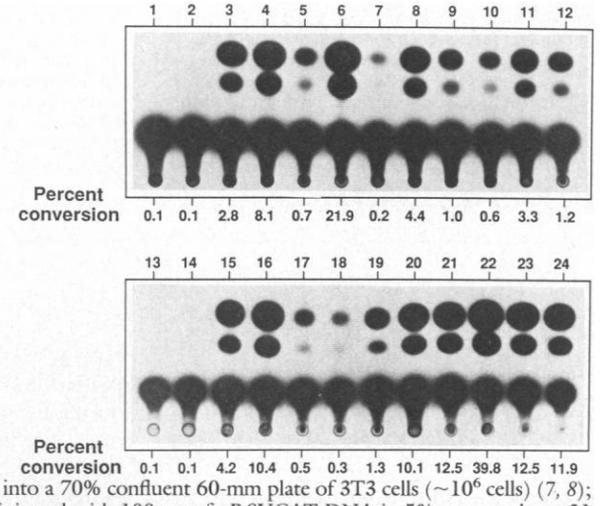

Fig. 1. Autoradiograms from two separate experiments showing CAT activity in extracts from quadricep muscles injected with pRSVCAT DNA or βgCATβgA$_n$ RNA. Lane numbers are above and percent chloramphenicol conversions are below each autoradiogram. Lanes 1 and 13, control fibroblasts; lanes 2 and 14, muscle injected with 5% sucrose solution; lanes 3 and 15, 0.005 unit of noninjected, purified CAT standard (Sigma); lanes 4 and 16, 0.05 unit of noninjected, purified CAT; lanes 5 to 8, muscle injected with 100 μg of βgCATβgA$_n$ RNA in 5% sucrose; lanes 9 and 10, 20 μg of βgCATβgA$_n$ RNA transfected with 60 μg of lipofectin reagent (*6*) into a 70% confluent 60-mm plate of 3T3 cells (~10^6 cells) (*7, 8*); lanes 11, 12, and 17 to 20, muscle injected with 100 μg of pRSVCAT DNA in 5% sucrose; lanes 21 and 22, 20 μg of pRSVCAT DNA transfected with 60 μg of lipofectin reagent into a 50% confluent 60-mm plate of 3T3 cells (~0.8 × 10^6 cells); lanes 23 and 24, 20 μg of pRSVCAT DNA transfected with calcium phosphate into a 50% confluent 60-mm plate of 3T3 cells as previously described (*25*). CAT activity was assayed 18 hours after all in vivo and in vitro RNA transfections and 48 hours after all DNA transfections. RNA and DNA polynucleotides were prepared as described (*26*) and muscle extracts were analyzed for CAT activity (*27, 28*). Similar results have been obtained in five separate experiments.

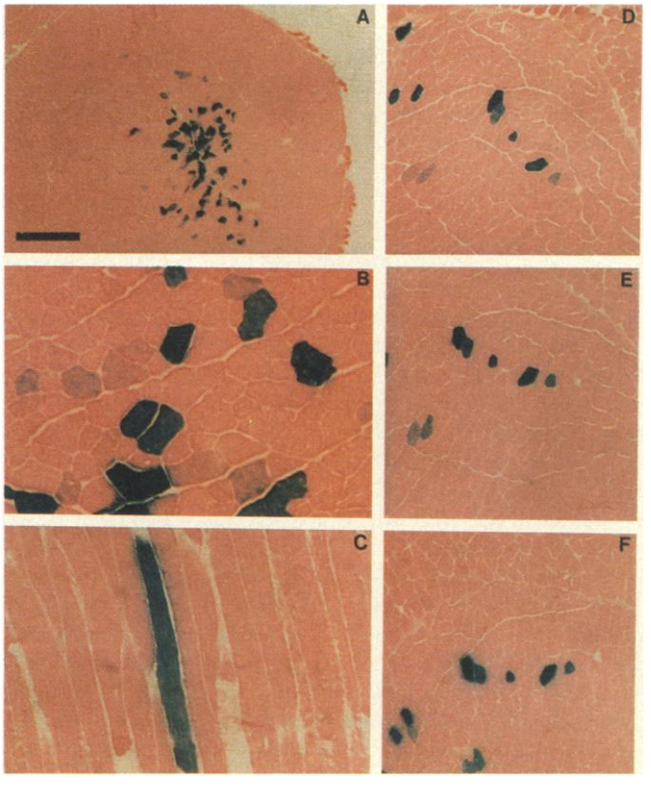

Fig. 2. In situ cytochemical staining of muscle cells for *E. coli* β-galactosidase activity. (**A** and **B**) Cross sections of a muscle injected with pRSV*lacZ* at ×25 and ×160 optical magnification, respectively. (**C**) A longitudinal section of another muscle injected with pRSV*lacZ*, ×160. (**D** to **F**) Serial cross sections of the same muscle that are 0.6 mm apart. Quadriceps muscles were injected once with 100 μg of pRSV*lacZ* DNA (*14*) in 20% sucrose, and the entire quadriceps were removed 7 days after the first injection. The muscle was frozen in liquid isopentane cooled with liquid N_2. Serial sections (15 μm) were sliced with a cryostat and placed immediately on gelatinized slides. The slices were fixed in 1.5% glutaraldehyde in phosphate-buffered saline for 10 min and stained 4 hours for β-galactosidase activity as described (*29*). The muscle was counterstained with eosin. Similar results have been obtained in 16 individual injection sites. Control muscle had no stained muscle cells. Scale bar: (A), 620 μm; (B and C), 100 μm; and (D to F), 260 μm.

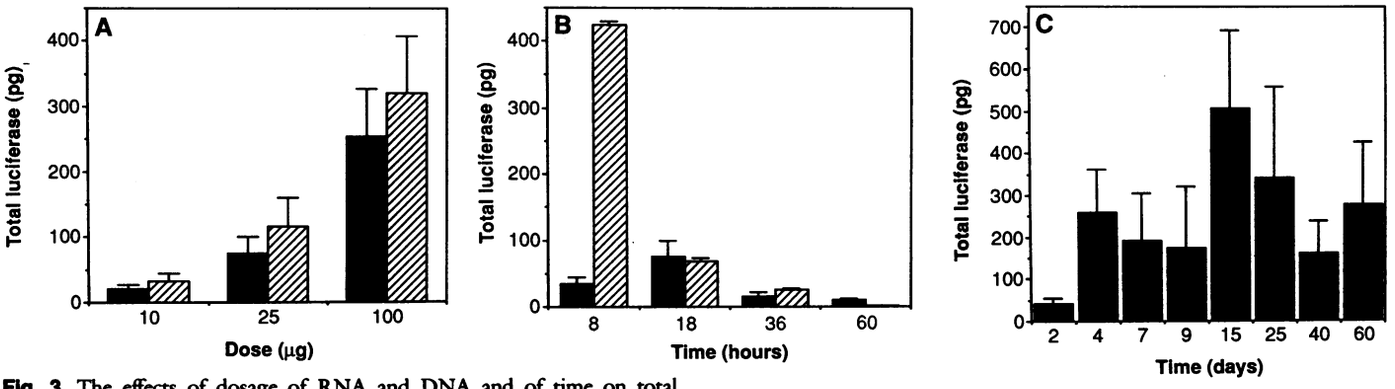

Fig. 3. The effects of dosage of RNA and DNA and of time on total luciferase extracted. Values indicate the average total luciferase activity ± SEM of muscle extracts from four to ten separate quadriceps that were injected or of fibroblast extracts from two to four separate lipofections. (**A**) Luciferase activity was measured 18 hours after the injection of varying amounts of βgLucβgA$_n$ RNA in 20% sucrose (solid bars) and 4 days after the injection of various amounts of pRSVL in 20% sucrose (striped bars). (**B**) Luciferase activity was assayed at various times after 20 μg of βgLucβgA$_n$ RNA were lipofected into 10^6 3T3 fibroblasts (striped bars) as previously described (*6, 7*), and after 100 μg of βgLucβgA$_n$ RNA in 20% sucrose was injected into quadriceps (solid bars). (**C**) Luciferase activity was assayed at various times after 100 μg of pRSVL DNA in 20% sucrose was injected intramuscularly. The RNA and DNA vectors were prepared as in Fig. 1, except that the lysis buffer was 100 m*M* potassium phosphate (*p*H 7.8), 1 m*M* DL-dithiothreitol, and 0.1% Triton X-100. An 87.5-μl portion of the 200-μl extract was analyzed for luciferase activity as described (*17*) with an LKB 1251 luminometer. Light units were converted to picograms of luciferase with a standard curve established by measuring the light units produced by purified firefly luciferase (Analytical Luminescence Laboratory) in control muscle extract. The RNA or DNA preparations did not contain any contaminating luciferase activity before injection. Control muscle injected with 20% sucrose had no detectable luciferase activity. Experiments were performed two or three times; the time points greater than 40 days were performed three times.

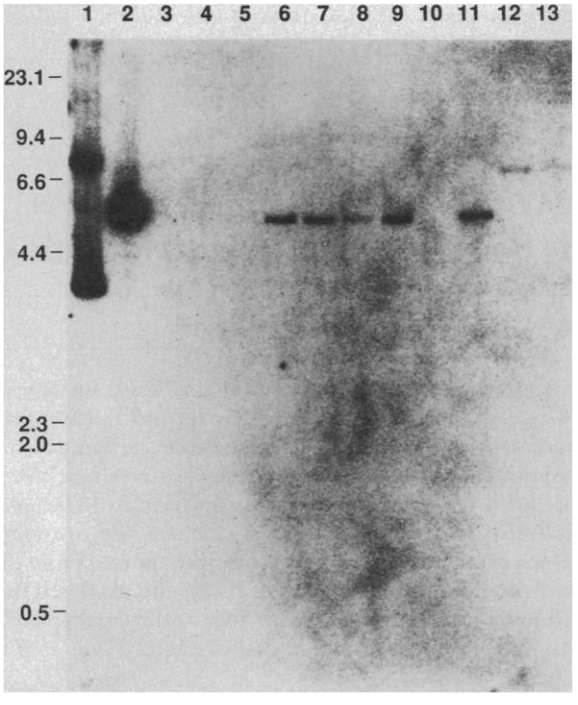

Fig. 4. Southern blot analysis of DNA from muscle injected 30 days previously with pRSVL. Lane 1, 0.05 ng of undigested pRSVL plasmid; lane 2, 0.05 ng of Bam HI–digested pRSVL; lane 3, empty; lane 4, Bam HI digest of Hirt supernatant from control muscle; lane 5, Bam HI digest of cellular DNA from control, uninjected muscle; lanes 6 and 7, Bam HI digest of Hirt supernatant from two different pools of pRSVL-injected muscles; lanes 8 and 9, Bam HI digest of cellular DNA from two different pools of pRSVL-injected muscle; lane 10, cellular DNA (as in lane 9) digested with Bam HI and Dpn I; lane 11, cellular DNA (as in lane 9) digested with Bam HI and Mbo I; lane 12, cellular DNA digested with Bgl II; and lane 13, Hirt supernatant digested with Bgl II. Preparations of muscle DNA were obtained from control, uninjected quadriceps or from quadriceps 30 days after injection with 100 μg of pRSVL in 20% sucrose. Two entire quadricep muscles from the same animal were pooled, minced into liquid N_2, and ground with a mortar and pestle. Total cellular DNA and Hirt supernatants were prepared as previously described (*21, 25*). Fifteen micrograms of the total cellular DNA or 10 μl of the 100-μl Hirt supernatant were digested, subjected to electrophoresis on a 1.0% agarose gel, transferred to Nytran (Scheicher & Schuell) with a vacublot apparatus (LKB), and hybridized with multiprimed ^{32}P-labeled luciferase probe (the Hind III–Bam HI fragment of pRSVL). After overnight hybridization, the final wash of the membrane was with 0.2× standard sodium citrate containing 0.5% SDS at 68°C. Kodak XAR5 film was exposed to the membrane for 45 hours at −70°C. Size markers (λ/Hind III) are shown on the left in kilobases.

ance of the pRSVL DNA in Hirt supernatants (Fig. 4, lanes 6, 7, and 13) and in bacteria rendered ampicillin-resistant after transformation with Hirt supernatants (*13*) also suggests that the DNA is present unintegrated. Although the majority of the exogenous DNA appears to be extrachromosomal, low levels of chromosomal integration cannot be definitively excluded. Overexposure of the blots has not revealed smears of hybridizing DNA that would represent plasmid DNA integrated at random sites. The sensitivity of the pRSVL DNA in muscle to Dpn I digestion (Fig. 4, lane 10) and its resistance to Mbo I digestion (Fig. 4, lane 11) suggests, as previously explained (*20*), that the DNA has not replicated within the muscle cells. Thus, the relatively stable expression of luciferase in muscle injected with pRSVL DNA is probably due to the persistence of injected DNA. Most of the DNA exists as a nonintegrated, circular form that does not replicate.

The mechanism of entry of these polynucleotides into the muscle cells is unknown. Polynucleotide expression has been obtained when the composition and volume of the injection fluid and the rate of injection were modified from the described protocol (*21*). Although we have detected low amounts of reporter enzyme in other tissues (liver, spleen, skin, lung, brain, and blood) injected with the RNA and DNA vectors, the levels in muscle were substantially greater. Muscle may be particularly suited to take up and express polynucleotides because of its structural features, such as its multinucleated cells, sarcoplasmic reticulum, and transverse tubule system, which contains extracellular fluid and penetrates deep into the muscle cell (*22, 23*). It is also possible that the polynucleotides enter damaged muscle cells, which then recover.

If direct transfer of genes into human muscle in situ also occurs, it may have several potential clinical applications. The effects of genetic diseases of muscle might be ameliorated by expression of the normal

gene within muscle cells (*24*). Muscle might also be a suitable tissue for the heterologous expression of a transgene that would modify disease states in which muscle is not primarily involved. The intracellular expression of genes encoding antigens may provide alternative approaches to vaccine development. The use of RNA and a tissue that can be repetitively accessed might be useful for a reversible type of gene transfer, administered much like conventional pharmaceutical treatments.

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23. Polynucleotides may enter the sarcoplasmic reticulum just as under certain in vitro conditions extracellular horseradish peroxidase can enter the sarcoplasmic reticulum via the transverse tubular system (*22*). The influx of positively charged calcium ions, which are in high concentration within the sarcoplasmic reticulum, into the cytoplasm during muscle contraction may mediate the entry of the negatively charged polynucleotides into the cytoplasm from the sarcoplasmic reticulum. However, current opinion is that under normal conditions there is no direct conduit between T tubules and sarcoplasmic reticulum.
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27. Muscle extracts were prepared by excising the entire quadriceps, mincing the muscle into a 1.5-ml microtube containing 200 μl of a lysis solution [20 m*M* tris-HCl (*p*H 7.4), 2 m*M* $MgCl_2$, and 0.1% Triton X-100], and grinding the muscle with a plastic pestle (Kontes) for 1 min. To ensure complete disruption of the muscle cells, we then placed the muscle tissue under 600 psi of N_2 in a bomb (Parr) at 4°C for 30 min before releasing the pressure. Fibroblasts were processed similarly after they were removed from the plates by treatment with trypsin, taken up into media with serum, washed twice with phosphate-buffered saline, and then the final cell pellet was suspended into 200 μl of lysis solution. Portions (75 μl) of the muscle and fibroblast extracts were assayed for CAT activity by incubating the reaction mixtures for 2 hours (*28*). The autoradiograms were made by exposing the thin-layer chromatography (TLC) plates, after spraying them with NEN Enhance, to Kodak XAR5 film for 54 hours at −70°C. Percent conversions were determined by measuring the radioactivity in the scraped TLC spots. Percent conversions were converted to picograms of CAT with the use of a standard log-log curve established by measuring the percent conversion produced by purified CAT (Sigma) and the conversion factor of 100,000 units of protein per milligram (Sigma).
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Is Soot Composed Predominantly of Carbon Clusters?

LAWRENCE B. EBERT

Soot generated from diesel fuel in a combustion tube is characterized by microanalysis, x-ray diffraction, chemical reactivity, and nuclear magnetic resonance to address the recent proposal of the significance of carbon clusters in soot. The data support a traditional model of soot as polynuclear aromatic compounds rather than as clusters of carbon atoms with minimal edge site density. The amounts of noncarbon atoms in the soot (hydrogen, oxygen, nitrogen, and sulfur) are commensurate with the edge density of the crystallites (2 by 2 nanometers) inferred from diffraction. The chemistry of soot, in being reduced by potassium metal and alkylated by alkyl iodides, is that known for aromatic compounds and not that anticipated for materials such as graphite, with a small fraction of carbon atoms on edges.

RECENTLY, IT WAS PROPOSED THAT the C_{60} carbon cluster might shed "a totally new and revealing light on several important aspects of carbon's chemical and physical properties that were quite unsuspected" [(*1*), p. 1139]. One of the outgrowths of this work was the prediction that "C_{60} should be a by-product of combustion and a key to the soot formation process" [(*1*), p. 1145]. Although there was no claim that the closed polyhedron C_{60} was a major component of soot, the idea was advanced that the known spherical morphology of soot could be interpreted as arising from open, spiraling, carbon clusters. Each spherical particle of soot would be a molecule.

There is no doubt that the proposal of carbon clusters is exciting and that the idea has captured the imagination of many. In the laser pyrolysis of carbonaceous substrates, however, two separate groups have failed to find C_{60} to be a dominant species (*2, 3*), and there is some disagreement over the interpretation of the experiment linking

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