Monograph on Nickel-Volume 3

7/31/74

Monograph on NICKEL VOL III
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VOLUME 3

GRAS MONOGRAPH SERIES

NICKEL

(COPIES OF ARTICLES CITED BUT NOT USED IN MONOGRAPH SUMMARY)

prepared for

THE FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION AND WELFARE

JULY 31, 1974

This publication was prepared under Contract Number FDA 72-100 with the Public Health Service, Food and Drug Administration, Department of Health, Education, and Welfare

prepared by

Tracor Jitco, Inc.

Ni. Amer. Industr. Hyg. Ass. J. 27:202-5 (1966)

Nickel

(Except the Carbonyls)

Ni

Significant Physical Properties

Nickel is a hard, ductile, magnetic metal having a silver-white color.

Atomic weight:	58.71
Ignition temperature:	None[1]
Explosive limits:	None[1]
Specific gravity:	8.90 (water = 1)
Solubility:	Insoluble in water; soluble in acids
Melting point:	1455°C
Boiling point:	2900°C

I. Hygienic Standards

A. Recommended maximal atmospheric concentrations — Tentative (8 hours): 1.0 milligram of the metal and its soluble salts per cubic meter of air.[2] This value appears to be based on the carcinogenic action of nickel and its salts.[3] The insoluble oxide and sulfide also have produced cancer in animals.[4]

B. Short exposure tolerance: No data available.

C. Atmospheric concentration immediately hazardous to life: No data available.

The Committee wishes to acknowledge the assistance of the Industrial Hygiene and Clinical Toxicology Committee of I.M.A. in preparation of the medical information section of this Guide.

II. Toxic Properties

A. Inhalation: Nickel and/or its compounds are carcinogenic and toxic. A significant increase in cancer of the lung and sinuses has occurred among employees in refineries in England, Norway and Canada.[3,5,6,7] In England, this has been attributed, in part, to nickel carbonyl used in the process. One authority feels that the action of the carbonyl is due to its ability to deposit finely divided nickel in the body.[8] However, the carbonyl process was not used in the refineries in Canada and Norway. Few other effects have been reported among users of nickel but invariably these have been clouded by concomitant exposure to other materials. Operations from which

effects have been reported include polishing,[9] electroplating,[10] nickel-cadmium battery manufacturing[11] and welding.[12] In the latter report which involved exposure to nickel fumes, ozone and possibly trichloroethylene decomposition products, the slow recovery and persistence of residual symptoms were reminiscent of the symptoms following acute exposure to nickel carbonyl.[13] Two unpublished cases of severe but transient pneumonitis involved exposure to nickel fumes. In one, after-the-fact-sampling showed exposure to 0.26 mg nickel/m³ for six hours.[14]

Rats and guinea pigs exposed to 15 mg/m³ of powdered nickel developed malignant pulmonary neoplasms.[15] Nickel and nickel oxide caused acute and chronic intoxication in animals, manifested by pathological changes in the lungs, pneumonia and hyperplasia of lymphoid tissue.[16] Nickel dust, 0.19 micron, inhaled for ten minutes per day at 30 to 50 mg/m³ for 30 days caused a toxic effect on the respiratory organs.[17]

B. Skin contact: Dermatitis — the familiar nickel itch — is the most common effect produced in humans. It is a sensitization reaction.[18] Nickel and its salts are not absorbed through the skin in amounts sufficient to cause intoxication.

Eye contact: This does not present any problem peculiar to nickel.

Ingestion: The metal and less soluble compounds have a low order of oral toxicity; the soluble salts, high. In higher doses, the latter cause giddiness and nausea.[19] Small doses of nickel sulfate in water, 0.1 to 0.5 mg/kg daily for 161 days, caused severe damage to the myocardium and liver parenchyma.[20] In humans, myocardial infarction is accompanied by a significant increase in serum nickel. Whether this is cause or effect is not established.[21]

E. Other: Nickel salts are highly toxic by intravenous or subcutaneous administration.[18] Metallic nickel, the oxide and the sulfide are reported to induce cancer on injection; the sulfate did not.[4,22]

III. Industrial Hygiene Practice

A. Industrial uses: Nickel is used in numerous high temperature corrosion resistant alloys, in electroplating and in the production of catalysts. A relatively new use in this country is in the production of nickel-cadmium batteries.

B. Evaluation of exposure:

1. *Air sampling:* The sample for dust, mist or fume may be collected by any one of the standard procedures — filter paper, electrostatic precipitator or impinger.
2. *Biological materials:* No relationship has been established between degree of exposure and quantity of nickel excreted or contained in the body organs (except for the carbonyl). The "normal" concentration of nickel in the urine is reported to have a range of 0.76 to 1.1 micrograms per 100 ml; in the blood, 3.0 μg per 100 gm.[23,24]
3. *Analytical methods:* The spectrographic method is preferred for biological materials and air samples.[24] There is no entirely satisfactory chemical method for the quantitative determination of small amounts of nickel, although several "oxime" methods have been used.[18] Of these, the use of alpha-furildioxime and of dimethylglyoxime, either alone or followed by conversion to the diethyl-dithiocarbamate complex have been reported successful.[23,25,26]

C. Hazards and their recommended control: In spite of its relatively high toxicity, industrial experience with nickel has been good. It may be, of course, that cases of poisoning have been overlooked. Experience indicates

a difference in effect between the fumes of a low nickel alloy, such as stainless steel for example, and the high nickel alloys. For control of the immediate effect, particular attention should be paid to the fumes and fine dust of the high nickel materials. The long-range carcinogenic potential should be remembered for all nickel compounds.

1. *Inhalation:* Welding, grinding, plasma arc and other operations releasing fumes or dusts should be provided with local exhaust ventilation to maintain concentrations below 1.0 mg nickel/m^3, preferably much below. For temporary or intermittent operations where ventilation is not practicable, an airline respirator should be worn. Where occasional respiratory protection may be needed in addition to ventilation, a respirator may be worn which is designed for protection against fumes and dusts of material having a TLV not less than 0.1 mg/m^3, *i.e.*, "not significantly more toxic than lead." Respirators should be fitted properly to the wearer according to the manufacturer's instructions and should be approved by the U. S. Bureau of Mines.
2. *Skin contact:* Contact with solutions of nickel salts should be avoided by appropriate handling procedures or protective clothing. When contact does occur, the solution should be washed from the skin at once. If sensitization occurs, avoidance of contact with all nickel may be required.
3. *Eye contact:* Nickel does not appear to present any special problem; however, contact should be avoided for the same reason noted in the preceding paragraph. Face shields or goggles should be worn if eye contact with the solution is a possibility.
4. *Ingestion:* This should not be a problem, but containers of solutions should be clearly labelled.
5. *Fire and explosion:* Metallic nickel less than 100 microns in size does not ignite.[1]

IV. Medical Information

A. EMERGENCY TREATMENT: Eyes splashed with solutions of nickel salts should be flushed with water immediately and then irrigated for 15 minutes. If ingestion of soluble compounds occurs gastic lavage may be indicated. Inhalation of very fine dust may cause acute pneumonitis and/or pulmonary edema. The use of 4, sodium diethyl-dithiocarbamate (Dithiocarb) has been recommended. Furthermore, the patient should be immobilized for 24 to 48 hours and some form of oxygen administration should be available in the event pulmonary edema occurs.

B. Special Procedures: Individuals having chronic exposure to nickel dust, even though they wear appropriate respiratory protection, should have annual 14" x 17" x-ray pictures of the chest.

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federal register

THURSDAY, MARCH 28, 1974

WASHINGTON, D.C.

Volume 39 ■ Number 61

PART II

ENVIRONMENTAL PROTECTION AGENCY

■

ELECTROPLATING POINT SOURCE CATEGORY; COPPER, NICKEL, CHROMIUM AND ZINC ON FERROUS AND NONFERROUS MATERIALS SUBCATEGORY

Effluent Guidelines and Standards

Title 40—Protection of the Environment

CHAPTER I—ENVIRONMENTAL PROTECTION AGENCY

SCHAPTER N—EFFLUENT GUIDELINES AND STANDARDS

PART 413—ELECTROPLATING POINT SOURCE CATEGORY

Subpart A—Copper, Nickel, Chromium, and Zinc on Ferrous and Nonferrous Materials Subcategory

On October 5, 1973, notice was published in the FEDERAL REGISTER (38 FR 27694), that the Environmental Protection Agency (EPA or Agency) was proposing effluent limitations guidelines for existing sources and standards of performance and pretreatment standards for new sources within the electroplating of copper, nickel, chromium, and zinc on ferrous and nonferrous materials subcategory of the electroplating category of point sources.

The purpose of this notice is to establish final effluent limitations guidelines for existing sources and standards of performance and pretreatment standards for new sources in the electroplating category of point sources, by amending 40 CFR Chapter I, Subchapter N, to add a new Part 413. This final rulemaking is promulgated pursuant to sections 301, 304 (b) and (c), 306 (b) and (c) and 307(c) of the Federal Water Pollution Control Act, as amended (the Act); 33 U.S.C. 1251, 1311, 1314 (b) and (c), 1316 (b) and (c) and 1317(c); 86 Stat. 816 et seq.; Pub. L. 92-500. Regulations regarding cooling water intake structures for all 'egories of point sources under section (b) of the Act will be promulgated in CFR 402.

In addition, the EPA is simultaneously proposing a separate provision which appears in the proposed rules section of this Part II of the FEDERAL REGISTER, 39 FR 11515, stating the application of the limitations and standards set forth below to users of publicly owned treatment works which are subject to pretreatment standards under section 307 (b) of the Act. The basis of that proposed regulation is set forth in the associated notice of proposed rulemaking.

The legal basis, methodology and factual conclusions which support promulgation of this regulation were set forth in substantial detail in the notice of public review procedures published August 6, 1973 (38 FR 21202) and in the notice of proposed rulemaking for the electroplating of copper, nickel, chromium, and zinc on ferrous and nonferrous materials subcategory. In addition, the regulations as proposed were supported by two other documents: (1) The document entitled "Development Document for Proposed Effluent Limitations Guidelines and New Source Performance Standards for the Copper, Nickel, Chromium, and Zinc Segment of the Electroplating Point Source Category" (August, 1973) and (2) the document entitled "Economic Analysis of Proposed Effluent Guidelines for the Electroplating Point Source Category ›pper, Nickel, Chromium, and Zinc)" ptember, 1973). Both of these docu-ments were made available to the public and circulated to interested persons at approximately the time of publication of the notice of proposed rulemaking.

Interested persons were invited to participate in the rulemaking by submitting written comments within 30 days from the date of publication. Prior public participation, in the form of solicited comments and responses from the States, Federal agencies, and other interested parties, was described in the preamble to the proposed regulation. The EPA has considered carefully all of the comments received, and a discussion of these comments with the Agency's response thereto follows.

(a) *Summary of comments.* The following responded to the request for written comments contained in the preamble to the proposed regulation; C. E. Mange Development Laboratory, Welch Allyn Inc., Platers Supply Company, General Motors Corporation, Colorado Department of Public Health. Municipality of Metropolitan Seattle, MacDermid, Inc., County Sanitation Districts of Los Angeles County, Sybron Corporation, Van Der Horst Corporation of America, U.S. Department of Health, Education, and Welfare, Environmental Resources Defense Council, Commonwealth of Massachusetts, Pratt and Whitney Division of United Aircraft Corporation, Columbus Industrial Association. Keeler Brass Company, Lancy Laboratories, National Association of Metal Finishers, American Electroplaters' Society, Chrome-Rite Company, Masters Electro-Plating Association, Association of Home Appliance Manufacturers, Oxy Metal Finishing Corporation, Metal Finishing Suppliers' Association, Chromium, Inc., E. I. DuPont de Nemours, Inc., General Electric Company, Raytheon Company, Ford Motor Company, Whirlpool Corporation, Western Electric, Scientific Control Laboratories, Inc., Electronic Industries Association, The Bendix Corporation, U.S. Department of Commerce, Eastman Kodak Company, TRW, Inc., Burndy Corporation, Jamestown Electroplating Works, Inc., Douglas and Lomason Company, Revere Copper and Brass, Inc., Metropolitan Sanitary District of Greater Chicago, State of New York Department of Environmental Conservation, Sargent and Company, Digital Equipment Corporation, AMP, Inc., Products Finishing, Wald Manufacturing Company, Inc., United States Water Resources Council, S. K. Williams Company, Manufacturing Jewelers and Silversmiths of America, Inc., U.S. Department of the Interior, True Temper Corporation, Dallas Water Utilities, Texas Instruments, Inc., Institute of Printed Circuits, Caterpillar Tractor Company, State of Connecticut Department of Environmental Protection, State of Michigan Department of Natural Resources, and API Industries, Inc.

(1) Approximately one-third of the comments received stated that the use of milligrams per square meter plated as the basis for effluent limitations is impractical, uneconomical, or difficult to apply. Alternate bases for the guidelines were suggested by several commenters.

While it may be difficult or inconvenient for some platers to determine their production in terms of square meters plated, it is not impossible. Section 308 of the "Act" authorizes the Administrator to require the owner or operator of any point source to establish and maintain records to determine compliance with effluent limitations. Paragraphs (b) of §§ 413.12 and 413.15 have been added to this regulation to provide the plater flexibility in complying with recordkeeping and production reporting requirements imposed by the guidelines.

The effluent limitations applicable to a particular discharger may be determined by multiplying the effluent limitations in paragraph (a) of § 413.12 for existing sources by the production expressed in sq m or sq ft. The product of this calculation has dimensions of mg or lb. An effluent factor of 200 liters per sq m (5 gallons per sq ft) is the water use for rinsing typical of plants in the electroplating industry. Individual dischargers may decide that further water conservation is necessary or desirable to meet effluent limitations based on the best practicable control technology currently available.

(2) Several comments stated that the effluent limitations as proposed would impose a severe economic hardship on the small plater.

If small shops employ chemical treatment with good water use, they should meet the guidelines as proposed. Hence, an exemption or relaxation of the standards based on size is warranted only from an economic or administrative standpoint and not on the basis of technical feasibility of waste treatment. After an analysis of additional data supplied by the industry and a re-evaluation of the economic impact of the guidelines, it was determined that the minimum cost for effective physical-chemical waste treatment may create an economic burden on the very small plater. On the basis of this further analysis, the best practicable control technology currently available for shops plating less than 33 sq m per hour or having a total installed current capacity less than 2000 amperes was determined to be cyanide destruction, if any, equalization and pH adjustment. The final regulation reflects the additional analysis and the revision of the best practicable control technology currently available to alleviate the economic impact of the Guidelines on the small plater.

(3) Several commenters suggested that the waste water control and treatment techniques suggested in the Development Document are not proven and are not completely reliable.

Numerous techniques to reduce the discharge of pollutants are discussed in the Development Document. All techniques are not applicable to every plater, but each is successfully demonstrated in the industry.

(4) Comments were received that process modifications such as eliminating copper plating by increasing the thickness of nickel or substituting trivalent chromium baths for hexavalent baths are not practical.

The regulations are based on proven treatment technology with emphasis on water conservation. Such process modifications are suggested as alternate methods to meet the guidelines under certain circumstances. No claim is made that such modifications are practical for every plater.

(5) Several comments indicated that the proposed guidelines would create severe adverse economic effects on the electroplating industry in general.

As discussed in section "E", the economic impact of the final guidelines is expected to be significantly lower than that of the proposed guidelines. This is due to a significant reduction in the number of plants to be affected by the guidelines and to reduced requirements for very small operations (less than 33 square meters plated per hour, or less than 2000 amperes installed current capacity).

Because pretreatment requirements are being proposed as a separate package, the guidelines now being promulgated will affect only about 25 percent of the industry, the remaining 75 percent being plants that discharge to municipal treatment systems. On this basis and with consideration of the reduced requirement for very small operations the economic analysis indicates that fewer than 100 closures are expected as a result of the 1977 requirements, as compared to an estimated more than 500 for the guidelines as proposed. These plants would represent fewer than 600 employees and less than 2.0 percent of the total independent plating capacity. For 1983, fewer than 80 additional closures are expected, representing less than 250 employees and less than 1.0 percent of the total independent plating capacity.

Although most of these closures would occur in semiurban areas, it still has been estimated that about half of the affected employees should find re-employment in the electroplating industry while the remainder would have to seek re-employment in other industries.

(6) Commenters suggested that the proposed effluent limitations would impose a severe technological hardship on the industry and that the effluent limitations are too stringent.

The effluent limitations are being achieved by numerous platers and can be met by many others by only minor changes in operation. The baseline technology for the electroplating industry, chemical treatment, has been practiced by the industry for over 25 years. By controlling the volume of waste water generated and employing a well-designed and well-operated chemical treatment system, platers can meet the guidelines as promulgated.

(7) Numerous comments indicated that solid waste disposal and groundwater contamination problems may be created by the proposed guidelines.

It is recognized that the removal of metals from waste water by chemical precipitation methods produces a sludge which requires adequate precautions to prevent contamination of surface and subsurface waters. It is expected that the guidelines will focus attention on the problem of solids disposal so that satisfactory solutions will be found. Chemical precipitation with solids separation is widely accepted as the best practicable control technology for this industry. The sludge produced should be disposed of in an acceptable landfill.

Soluble metal salts produced by certain concentration or recovery techniques such as evaporation pose a greater environmental hazard than the metallic hydroxides resulting from chemical treatment. An environmentally acceptable solution to this problem, which is not peculiar to the electroplating industry, is expected prior to the deadline for application of the best available technology economically achievable in 1983.

(8) Several comments were received that the proposed effluent limitations guidelines are not consistent with the interim guidelines for metal finishing. The industry generally agrees with the interim guidelines effluent concentrations and with the concept of chemical treatment of waste waters as the best practicable control technology currently available.

The essential difference between the interim guidelines concentrations and the proposed effluent limitations guidelines is that the latter place a limit on the absolute amount of pollutants discharged to surface waters. Restrictions on concentration alone do not achieve the same result in terms of limiting the total amount of pollutants discharged.

The interim effluent concentrations were developed to be broadly applicable to metal finishing operations. The effluent limitations guidelines were developed for copper, nickel, chromium and zinc electroplating operations and are based on a program of extensive technical data analysis to satisfy the detailed requirements of sections 301, 304(b), 306, and 307 of the Act. The results of the study to develop effluent limitations guidelines show conclusively that the interim guidelines concentrations and the effluent limitations guidelines are consistent and complementary.

(9) A comment was received that § 413.15 of the proposed regulation was not clear as to whether all existing electroplaters or only new sources would be required to pretreat to the standards of performance.

The pretreatment standards proposed in § 413.15 apply to new sources. Section 413.16 of the regulation promulgated herein states the applicability of standards of performance as pretreatment standards for new sources.

(10) Several commenters stated that EPA should establish a separate category of effluent limitations for manufacturers which employ electroplating as a part of the overall manufacturing process.

The effluent limitations guidelines and standards of performance for the electroplating category of sources are applicable only to those portions of a manufacturing process which include an electroplating operation. When applied to captive facilities, such as those operated by aircraft engine manufacturers, only the electroplating operations described in § 413.10 are subject to the effluent limitations and standards of performance promulgated herein. Separate effluent limitations guidelines and standards of performance for the metal fabricating industries will be developed by EPA.

(11) The U.S. Department of Commerce stated that the concentration of total suspended solids upon which the limitations are based appears unrealistically low and the limitation should be revised upward.

The data presented in section IX of the Development Document for the proposed regulations show that numerous plants surveyed are achieving 15 mg/l or less total suspended solids and that an exemplary plant achieved a monthly average total suspended solids figure of 15 mg/l or less for a period of twelve consecutive months. Verification effluent sampling and analysis conducted by the EPA contractor showed an average of 22 mg/l total suspended solids in the discharge from exemplary plants based on short sampling periods. As a result of further data analysis, EPA concluded that the monthly average effluent limitations for total suspended solids should be revised upward both for existing and for new sources.

(12) A number of commenters observed that the levels specified as best practicable control technology currently available in the proposed guidelines were not achievable.

The Agency re-examined the assumptions on which the proposed guidelines were based. These assumptions included a typical water use of 80 liters/sq m of plated area, and an effluent concentration for each of the parameters controlled of 0.5 mg/l, except for cyanide, hexavalent chromium and total suspended solids, which were set at 0.05 mg/l, 0.05 mg/l and 15 mg/l respectively. While the specified concentrations were achieved by a number of the exemplary plants reviewed by the contractor, only one such plant was able to achieve the concentrations while simultaneously maintaining water usage at or below the 80 l/sq m, figure. Accordingly, the Agency believed that it would be inappropriate to establish best practicable control technology currently available solely on the basis of the performance of a single plant. With a revised water usage of 160 l/sq m, the limitations have been achieved by two plants directly studied by the contractor; moreover, industry data indicate that eight additional plants achieve this level of control. Thus, this level of performance can, with greater certainty, be said to constitute best practicable control technology currently available, and the final guidelines have been revised to reflect these findings.

(b) *Revision of the proposed regulation prior to promulgation.* As a result of public comments and continuing review and evaluation of the proposed regula-

tion by the EPA, the following changes have been made in the regulation:

(1) The best practicable control technology for small platers (less than 33 square meters surface plated per hour or less than 2000 amperes installed current capacity) has been redefined in § 413.12 (c) to be cyanide destruction, if any, equalization and pH adjustment to alleviate the economic impact of the guidelines on the segment of the industry most severely affected by the proposed regulations.

(2) The monthly average effluent limitations and standards of performance for total suspended solids have been revised upward to reflect the conclusions of further technical analysis.

(3) Paragraph (b) of §§ 413.12 and 413.15 have been added to require that point source dischargers maintain production records to determine compliance with the effluent limitations pursuant to section 308 of the Act.

(4) Section 304(b)(1)(B) of the Act provides for "guidelines" to implement the uniform national standards of section 301(b)(1)(A). Thus Congress recognized that some flexibility was necessary in order to take into account the complexity of the industrial world with respect to the practicability of pollution control technology. In conformity with the Congressional intent and in recognition of the possible failure of these regulations to account for all factors bearing on the practicability of control technology, it was concluded that some provision was needed to authorize flexibility in the strict application of the limitations contained in the regulation where required by special circumstances applicable to individual dischargers. Accordingly, a provision allowing flexibility in the application of the limitations representing best practicable control technology currently available has been added to each subpart, to account for special circumstances that may not have been adequately accounted for when these regulations were developed.

(5) The limitations for best practicable control technology currently available and new source performance standards have been revised to reflect a more liberal and realistic water use factor. See section IX of the Development Document for illustrations of operations and methods of applications.

(c) *Economic impact.* The economic impact of the final guidelines is expected to be significantly lower than that of the proposed guidelines because of three factors: (1) The estimate of the total number of independent plants affected by the guidelines has been reduced by eliminating plants in SIC 3471 that do not use copper, nickel, chromium or zinc in their operations, (2) plants discharging to municipal systems, approximately three-fourths of the industry, are not affected by the guidelines now being promulgated and (3) the requirements in 1977 for low volume plants (less than 33 square meters plated per hour) have been relaxed.

In light of these changes, the revised economic impact indicates that less than 100 independent operations could close as a result of the 1977 guidelines. These plants would represent fewer than 600 employees and less than 2.0 percent of the total independent plating capacity. This impact is substantially lower than the estimate of more than 500 expected closures associated with the proposed guidelines. The revised analysis for 1983 indicates fewer than 80 additional closures are expected representing about 250 employees and less than 1.0 percent of the total independent plating capacity.

Price increases for 1977 are still expected to be in the range of 15 percent for those plants not connected to municipal treatment systems. Additional increases of up to 8 percent are expected for 1983.

The potential impact cited above should have only minimal impact on overall industry viability. The total potential capacity loss for 1977 and 1983 is less than 3.0 percent. This should be replaced easily by existing plants and new plants. Moreover, although the final guidelines will affect only direct dischargers so that most of the closures will occur in semi-urban areas, it still has been estimated that half of the affected employees would be able to find re-employment in the electroplating industry.

As in the earlier analysis, it has been concluded that some captive shops could be affected by the guidelines. However, as with the independent shops, the impact on captive shops should be reduced significantly because the guidelines will apply only to direct dischargers and because the requirements for low volume operations in 1977 have been relaxed.

(d) *Cost-benefit analysis.* The detrimental effects of the constituents of waste waters now discharged by point sources within the copper, nickel, chromium, and zinc on ferrous and nonferrous materials segment of the electroplating point source category are discussed in section VI of the report entitled "Development Document for Effluent Limitations Guidelines for the Copper, Nickel, Chromium, and Zinc on Ferrous and Nonferrous Materials Manufacturing Segment of the Electroplating Point Source Category" February, 1974). It is not feasible to quantify in economic terms, particularly on a national basis, the costs resulting from the discharge of these pollutants to our Nation's waterways. Nevertheless, as indicated in section VI, the pollutants discharged have substantial and damaging impacts on the quality of water and therefore on its capacity to support healthy populations of wildlife, fish and other aquatic wildlife and on its suitability for industrial, recreational and drinking water supply uses.

The total cost of implementing the effluent limitations guidelines includes the direct capital and operating costs of the pollution control technology employed to achieve compliance and the indirect economic and environmental costs identified in Section VIII and in the supplementary report entitled "Economic Analysis of Proposed Effluent Guidelines Electroplating Industry" (September, 1973). Implementing the effluent limitations guidelines will substantially reduce the environmental harm which would otherwise be attributable to the continued discharge of polluted waste waters from existing and newly constructed plants in the electroplating industry. The Agency believes that the benefits of thus reducing the pollutants discharged justify the associated costs which, though substantial in absolute terms, represent a relatively small percentage of the total capital investment in the industry.

(e) *Solid waste control.* Solid waste control must be considered. The waterborne wastes from the electroplating industry may contain a considerable volume of metals in various forms as a part of the suspended solids pollutant. Best practicable control technology and best available control technology as they are known today, require disposal of the pollutants removed from waste waters in this industry in the form of solid wastes and liquid concentrates. In some cases these are nonhazardous substances requiring only minimal custodial care. However, some constituents may be hazardous and may require special consideration. In order to ensure long term protection of the environment from these hazardous or harmful constituents, special consideration of disposal sites must be made. All landfill sites where such hazardous wastes are disposed should be selected so as to prevent horizontal and vertical migration of these contaminants to ground or surface waters. In cases where geologic conditions may not reasonably ensure this, adequate precautions (e.g., impervious liners) should be taken to ensure long term protection to the environment from hazardous materials. Where appropriate the location of solid hazardous materials disposal sites should be permanently recorded in the appropriate office of the legal jurisdiction in which the site is located.

(f) Publication of information on processes, procedures, or operating methods which result in the elimination or reduction of the discharge of pollutants.

In conformance with the requirements of section 304(c), a manual entitled, "Development Document for Effluent Limitations Guidelines and New Source Performance Standards for the Copper, Nickel, Chromium, and Zinc Segment of the Electroplating Point Source Category," has been published and is available for purchase from the Government Printing Office, Washington, D.C. 20401 for a nominal fee.

(g) *Final rulemaking.* In consideration of the foregoing, 40 CFR Chapter I, Subchapter N is hereby amended by adding a new Part 413, Electroplating Point Source Category, to read as set forth below.

This final regulation is promulgated as set forth below and shall be effective May 28, 1974.

Dated: March 20, 1974.

JOHN QUARLES,
Acting Aministrator.

Sec.
413.10 Applicability; description of the electroplating of copper, nickel, chromium, and zinc on ferrous and nonferrous materials subcategory.

Sec.
413.11 Specialized definitions.
413.12 Effluent limitations guidelines representing the degree of effluent reduction attainable by the application of the best practicable control technology currently available.
413.13 Effluent limitations guidelines representing the degree of effluent reduction attainable by the application of the best available technology economically achievable.
413.14 Reserved.
413.15 Standards of performance for new sources.
413.16 Pretreatment standards for new sources.

AUTHORITY: Secs. 301, 304 (b) and (c), 306 (b) and (c) and 307(c), Federal Water Pollution Control Act, as amended (33 U.S.C. 1251, 1311, 1314 (b) and (c), 1316 (b) and (c) and 1317(c)); 86 Stat. 816 et seq.; Pub. L. 92-500.

Subpart A—Electroplating of Copper, Nickel, Chromium, and Zinc on Ferrous and Nonferrous Materials Subcategory

§ 413.10 Applicability; description of the electroplating of copper, nickel, chromium, and zinc on ferrous and nonferrous materials subcategory.

The provisions of this subpart are applicable to discharges of pollutants resulting from the process in which a ferrous or nonferrous base material is rack or barrel electoplated with copper, nickel, chromium, zinc, or any combination thereof.

§ 413.11 Specialized definitions.

For the purpose of this subpart:

(a) Except as provided below, the general definitions, abbreviations and methods of analysis set forth in 40 CFR Part 136 shall apply to this subpart.

(b) The term "sq m" shall mean the area plated with copper, nickel, chromium, zinc, or treated by chromating expressed in square meters.

(c) The term "sq ft" shall mean the area plated with copper, nickel, chromium, zinc, or treated by chromating expressed in square feet.

(d) The term "operation" shall mean any step in the plating process in which copper, nickel, chromium, or zinc metal or chromate is deposited on a base material following by a rinse.

(e) The term "CN,A" shall mean those cyanides amenable to chlorination as described in 1972 Annual Book of ASTM Standards, 1972, Standard D 2036-72, Method B, page 553.

§ 413.12 Effluent limitations guidelines representing the degree of effluent reduction attainable by the application of the best practicable control technology currently available.

In establishing the limitations set forth in this section, EPA took into account all information it was able to collect, develop and solicit with respect to factors (such as age and size of plant, raw materials, manufacturing processes, products produced, treatment technology available, energy requirements and costs) which can affect the industry subcategorization and effluent levels established. It is, however, possible that data which would affect these limitations have not been available and, as a result, these limitations should be adjusted for certain plants in this industry. An individual discharger or other interested person may submit evidence to the Regional Administrator (or to the State, if the State has the authority to issue NPDES permits) that factors relating to the equipment or facilities involved, the process applied, or other such factors related to such discharger are fundamentally different from the factors considered in the establishment of the guidelines. On the basis of such evidence or other available information, the Regional Administrator (or the State) will make a written finding that such factors are or are not fundamentally different for that facility compared to those specified in the Development Document. If such fundamentally different factors are found to exist, the Regional Administrator or the State shall establish for the discharger effluent limitations in the NPDES permit either more or less stringent than the limitations established herein, to the extent dictated by such fundamentally different factors. Such limitations must be approved by the Administrator of the Environmental Protection Agency. The Administrator may approve or disapprove such limitations, specify other limitations, or initiate proceedings to revise these regulations.

(a) The following limitations establish the quantity or quality of pollutants or pollutant properties, controlled by this section, which may be discharged by a point source subject to the provisions of this subpart after application of the best practicable control technology currently available:

Effluent characteristic	Effluent limitations: Maximum for any 1 day	Effluent limitations: Average of daily values for 30 consecutive days shall not exceed—
	Metric units (milligrams per square meters per operation)	
Cu	160	80
Ni	160	80
CrVI	16	8
Cr, total	160	80
Zn	160	80
CN,A	16	8
CN, total	160	80
TSS	4,800	3,200
pH	Within the range 6.0 to 9.5.	
	English units (pounds per million square feet per operation)	
Cu	32.7	16.4
Ni	32.7	16.4
CrVI	3.3	1.6
Cr, total	32.7	16.4
Zn	32.7	16.4
CN,A	3.3	1.6
CN, total	32.7	16.4
TSS	982.0	654.0
pH	Within the range 6.0 to 9.5.	

(b) Pursuant to section 308 of the Act, point sources subject to the provisions of this subpart shall maintain records of production expressed in sq m or sq ft as defined in § 413.11 for the purpose of determining compliance with the effluent limitations in § 413.12(a) of this subpart. For the purpose of complying with the requirements of this paragraph, a discharger may establish a correlation between area plated and another parameter, such as ampere-hours used in plating.

(c) Application of the factors listed in section 304(b) requires variation from the effluent limitations set forth in § 413.12(a) for any point source subject to such effluent limitations with a production less than 33 square meters per hour or an installed direct current capacity less than 2000 amperes. For such sources, the best practicable control technology currently available consists of cyanide destruction, if any, equalization and pH adjustment to a range of 6.0 to 9.0 prior to discharge.

§ 413.13 Effluent limitations guidelines representing the degree of effluent reduction attainable by the application of the best available technology economically achievable.

(a) The following limitations establish the quantity or quality of pollutants or pollutant properties, which may be discharged by a point source subject to the provisions of this subpart after application of the best available technology economically achievable: there shall be no discharge of process waste water pollutants to navigable waters.

(b) Application of the factors listed in section 304(b) of the Act may require variation from the effluent limitation set forth in this section for any point source subject to such effluent limitation with a production less than 120 sq m per hour. If variation is determined to be necessary for any such source, such source shall be subject to effluent limitations no less stringent than those required by the standards of performance for new sources set forth in § 413.15.

§ 413.14 [Reserved]

§ 413.15 Standards of performance for new sources.

(a) The following standards of performance establish the quantity or quality of pollutants or pollutant properties, controlled by this section, which may be discharged by a new source subject to the provisions of this subpart:

Effluent characteristic	Effluent limitations: Maximum for any 1 day	Effluent limitations: Average of daily values for 30 consecutive days shall not exceed—
	Metric units (milligrams per square meters per operation)	
Cu	80	40
Ni	80	40
CrVI	8	4
Cr, total	80	40
Zn	80	40
CN,A	8	4
CN, total	80	40
TSS	2,400	1,600
pH	Within the range 6.0 to 9.5.	
	English units (pounds per million square feet per operation)	
Cu	16.4	8.2
Ni	16.4	8.2
CrVI	1.6	.8
Cr, total	16.4	4.2
Zn	16.4	4.2
CN,A	1.6	.8
CN, total	16.2	4.7
TSS	491.0	327.0
pH	Within the range 6.0 to 9.5.	

(b) Pursuant to section 308 of the Act, point sources subject to the provisions of this subpart shall maintain records of production expressed in sq m or sq ft as defined in § 413.11 for the purpose of determining compliance with the effluent limitations in § 413.15(a) of this subpart. For the purpose of complying with the requirements of this paragraph, a discharger may establish a correlation between area plated and another parameter, such as ampere-hours used in plating.

§ 413.16 Pretreatment standards for new sources.

The pretreatment standards under section 307(c) of the Act for a source within the electroplating of copper, nickel, chromium and zinc on ferrous and nonferrous materials subcategory, which is a user of a publicly owned treatment works (and which would be a new source subject to section 306 of the Act, if it were to discharge pollutants to the navigable waters), shall be the standard set forth in 40 CFR Part 128, except that, for the purpose of this section, 40 CFR 128.133 shall be amended to read as follows:

In addition to the prohibitions set forth in 40 CFR 128.131, the pretreatment standard for incompatible pollutants introduced into a publicly owned treatment works shall be the standard of performance for new sources specified in 40 CFR 413.15; provided that, if the publicly owned treatment works which receives the pollutants is committed, in its NPDES permit, to remove a specified percentage of any incompatible pollutant, the pretreatment standard applicable to users of such treatment works shall, except in the case of standards providing for no discharge of pollutants, be correspondingly reduced in stringency for that pollutant.

[FR Doc.74-7066 Filed 3-27-74;8:45 am]

ENVIRONMENTAL PROTECTION AGENCY

[40 CFR Part 413]

ELECTROPLATING POINT SOURCE CATEGORY

Subpart A—Copper, Nickel, Chromium and Zinc on Ferrous and Nonferrous Materials Subcategory

PROPOSED APPLICATION OF EFFLUENT LIMITATIONS GUIDELINES FOR EXISTING SOURCES TO PRETREATMENT STANDARDS FOR INCOMPATIBLE POLLUTANTS

Notice is hereby given pursuant to sections 301, 304 and 307(b) of the Federal Water Pollution Control Act, as amended (the Act) 33 U.S.C. 1251, 1311, 1314 and 1317(b); 86 Stat. 816 et seq.; Pub. L. 92-500, that the proposed regulation set forth below concerns the application of effluent limitations guidelines for existing sources to pretreatment standards for incompatible pollutants. The proposal will amend 40 CFR Part 413—Electroplating Point Source Category, establishing for each subcategory therein the extent of application of effluent limitations guidelines to existing sources which discharge to publicly owned treatment works. The regulation is intended to be complementary to the general regulation for pretreatment standards set forth at 40 CFR 128. The general regulation was proposed July 19, 1973 (38 FR 19236), and published in final form on November 8, 1973 (38 FR 30982).

The proposed regulation is also intended to supplement a final regulation being simultaneously promulgated by the Environmental Protection Agency (EPA or Agency) which provides effluent limitations guidelines for existing sources and standards of performance and pretreatment standards for new sources within the copper, nickel, chromium and zinc on ferrous and nonferrous materials subcategory of the electroplating point source category. The latter regulation applies to the portion of a discharge which is directed to the navigable waters. The regulation proposed below applies to users of publicly owned treatment works which fall within the description of the point source category to which the guidelines and standards (40 CFR Part 413) promulgated simultaneously apply. However, the proposed regulation applies to the introduction of incompatible pollutants which are directed into a publicly owned treatment works, rather than to discharges of pollutants to navigable waters.

The general pretreatment standard divides pollutants discharged by users of publicly owned treatment works into two broad categories: "Compatible" and "incompatible." Compatible pollutants are generally not subject to pretreatment standards. (See 40 CFR 128.110 (State or local law) and 40 CFR 128.131 (Prohibited wastes) for requirements which may be applicable to compatible pollutants.) Incompatible pollutants are subject to pretreatment standards as provided in 40 CFR 128.133, which provides as follows:

In addition to the prohibitions set forth in § 128.131, the pretreatment standard for incompatible pollutants introduced into a publicly owned treatment works by a major contributing industry not subject to section 307 (c) of the Act shall be, for sources within the corresponding industrial or commercial category, that established by promulgated effluent limitations guidelines defining best practicable control technology currently available pursuant to sections 301(b) and 304(b) of the Act; *Provided*, That, if the publicly owned treatment works which receives the pollutants is committed, in its NPDES permit, to remove a specified percentage of any incompatible pollutant, the pretreatment standard applicable to users of such treatment works shall be correspondingly reduced for that pollutant; and provided further that when the effluent limitations guidelines for each industry are promulgated, a separate provision will be proposed concerning the application of such guidelines to pretreatment.

The regulation proposed below is intended to implement that portion of § 128.133, above, requiring that a separate provision be made stating the application to pretreatment standards of effluent limitations guidelines based upon best practicable control technology currently available.

Questions were raised during the public comment period on the proposed general pretreatment standard (40 CFR Part 128) about the propriety of applying a standard based upon best practicable control technology currently available to all plants subject to pretreatment standards. In general, EPA believes the analysis supporting the effluent limitations guidelines is appropriate to support the application of those standards to users of publicly owned treatment works. However, to ensure that those standards are appropriate in all cases, EPA now seeks additional comments focusing upon the application of effluent limitations guidelines to users of publicly owned treatment works.

Section 413.15 of the proposed regulation for point sources within the copper, nickel, chromium and zinc on ferrous and nonferrous materials subcategory (October 5, 1973; 38 FR 27699), contained the proposed pretreatment standard for new sources. The regulation promulgated simultaneously herewith contains § 413.16 which states the applicability of standards of performance for purposes of pretreatment standard for new sources.

A preliminary Development Document was made available to the public at approximately the time of publication of the notice of proposed rulemaking, and the final Development Document, entitled "Development Document for Effluent Limitations Guidelines and New Source Performance Standards for the Copper, Nickel, Chromium and Zinc Segment of the Electroplating Point Source Category," is now being published. The economic analysis report, entitled "Economic Analysis of Proposed Effluent Guidelines for the Electroplating Point Source Category (Copper, Nickel, Chromium, and Zinc)" (September, 1973), was made available at the time of proposal. Copies of the final Development Document and economic analysis report will continue to be maintained for inspection and copying during the comment period at the EPA Information Center, Room 227, West Tower, Waterside Mall, 401 M Street, SW., Washington, D.C. Copies will also be available for inspection at EPA regional offices and at State water pollution control agency offices. Copies of the Development Document may be purchased from the Superintendent of Documents, Government Printing Office, Washington, D.C. 20402. Copies of the economic analysis report will be available for purchase through the National Technical Information Service, Springfield, Virginia 22151.

On June 14, 1973, the Agency published procedures designed to insure that, when certain major standards, regulations, and guidelines are proposed, an explanation of their basis, purpose and environmental effects is made available to the public (38 FR 15653), the procedures are applicable to major standards, regulations and guidelines which are proposed on or after December 31, 1973, and which either prescribe national standards of environmental quality or require national emission, effluent or performance standards or limitations.

The Agency determined to implement these procedures in order to insure that the public was provided with background information to assist it in commenting on the merits of a proposed action. In brief, the procedures call for the Agency to make public the information available to it delineating the major environmental effects of a proposed action, to discuss the pertinent nonenvironmental factors affecting the decision, and to explain the viable options available to it and the reasons for the option selected.

The procedures contemplate publication of this information in the FEDERAL REGISTER, where this is practicable. They provide, however, that where such publication is impracticable because of the length of these materials, the material may be made available in an alternate format.

The Development Document referred to above contains information available to the Agency concerning the major environmental effects of the regulation proposed below. The information includes: (1) The identification of pollutants present in waste waters resulting from the electroplating of copper, nickel, chromium or zinc, the characteristics of these pollutants, and the degree of pollutant reduction obtainable through implementation of the proposed standard; and (2) the anticipated effects on other aspects of the environment (including air, subsurface waters, solid waste disposal and land use, and noise) of the treatment technologies available to meet the standard proposed.

The Development Document and the economic analysis report referred to above also contain information available to the Agency regarding the estimated cost and energy consumption implications of those treatment technologies and the potential effects of those costs on the price of electroplating. The two reports

exceed, in the aggregate, 100 pages in length and contain a substantial number of charts, diagrams and tables. It is clearly impracticable to publish the material contained in these documents in the FEDERAL REGISTER. To the extent possible, significant aspects of the material have been presented in summary form in the preamble to the proposed regulation containing effluent limitations guidelines, new source performance standards and pretreatment standards for new sources within the electroplating category (38 FR 27694; October 5, 1973). Additional discussion is contained in the analysis of public comments on the proposed regulation and the Agency's response to those comments. This discussion appears in the preamble to the promulgated regulation (40 CFR Part 413) which currently is being published in the rules and regulations section of this Part II of the FEDERAL REGISTER (39 FR 11510).

The options available to the Agency in establishing the level of pollutant reduction obtainable through the best practicable control technology currently available, and the reasons for the particular level of reduction selected are discussed in the documents described above. In applying the effluent limitations guidelines to pretreatment standards for the introduction of incompatible pollutants into municipal systems by existing sources in the copper, nickel, chromium and zinc on ferrous and nonferrous materials subcategory, the Agency has, essentially, three options. The first is to declare that the guidelines do not apply. The second is to apply the guidelines unchanged. The third is to modify the guidelines to reflect: (1) Differences between direct dischargers and plants utilizing municipal systems which affect the practicability of the latter employing the technology available to achieve the effluent limitations guidelines; or (2) characteristics of the relevant pollutants which require higher levels of reduction (or permit less stringent levels) in order to insure that the pollutants do not interfere with the treatment works or pass through them untreated.

As described in the Development Document, the process waste waters from the electroplating subcategory contain high concentrations of heavy metals and cyanide which could interfere with the operation of publicly owned treatment works, pass through such works untreated or inadequately treated or otherwise be incompatible with such treatment works. In the opinion of the EPA, these process waste waters should be treated to the level required by the application of the best practicable control technology currently available before discharge of these materials to publicly owned treatment works.

Interested persons may participate in this rulemaking by submitting written comments in triplicate to the EPA Information Center, Environmental Protection Agency, Washington, D.C. 20460, Attention: Mr. Philip B. Wisman. Comments on all aspects of the proposed regulations are solicited. In the event comments are in the nature of criticisms as to the adequacy of data which is available, or which may be relied upon by the Agency, comments should identify and, if possible, provide any additional data which may be available and should indicate why such data is essential to the development of the regulations. In the event comments address the approach taken by the Agency in establishing pretreatment standards for existing sources, EPA solicits suggestions as to what alternative approach should be taken and why and how this alternative better satisfies the detailed requirements of sections 301, 304 and 307(b) of the Act.

A copy of all public comments will be available for inspection and copying at the EPA Information Center, Room 227, West Tower, Waterside Mall, 401 M Street SW., Washington, D.C. 20460. The EPA information regulation, 40 CFR 2, provides that a reasonable fee may be charged for copying.

In consideration of the foregoing, it is hereby proposed that 40 CFR Part 413 be amended to add § 413.14. All comments received on or before April 29, 1974, will be considered.

Dated: March 20, 1974.

JOHN QUARLES,
Acting Administrator.

Part 413 is proposed to be amended by adding § 413.14 to Subpart A as follows:

§ 413.14 Pretreatment standards for existing sources.

For the purpose of pretreatment standards for incompatible pollutants established under 40 CFR 128.133, the effluent limitations guidelines set forth in 40 CFR 413.12 above shall apply and, subject to the provisions of 40 CFR Part 128 concerning pretreatment, process waste water from this subcategory may not be introduced into a publicly owned treatment works, except in compliance with such limitations.

[FR Doc.74-7084 Filed 2-27-74;8:45 am]

Jour. Dairy Sci.
32(10)877-880 (1949)

NICKEL IN COWS' MILK.[1]

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This paper is the fifth in a series on the mineral elements of cows' milk (2, 3, 4, 5). Previous investigators (1, 7, 8, 9, 14, 15) are almost unanimous in agreement that nickel, unlike cobalt and certain other trace elements, is not present in milk. Only one group (1) reports its presence (in evaporated milk); the others, with one exception (8), do not make any positive statement about its absence—it simply is not listed among those elements revealed by spectrographic analysis of milk ash. In the exceptional case referred to above (8), the authors state that "no evidence has been obtained of any of the following elements in pure milk". There follows a list of 34 elements in which both cobalt and nickel appear, together with the comment that "the complete absence of cobalt is somewhat unexpected, for it is an element with active biological properities". Cobalt has since been found in cows' milk by several investigators (4, 6, 12, 13), and since cobalt and nickel are so closely related in the periodic system, it has been thought worth while to include nickel in the series of elements under investigation here. The objective has been two-fold: (a) to determine whether nickel is naturally present in milk, and (b) to find out whether, if present, the amount of it can be increased by feeding a nickel compound to cows.

EXPERIMENTAL

The procedure was similar to that described in an earlier paper (2). The work was carried on during two winter feeding seasons, eight cows being used in 1948 as in earlier work, and six in 1949. The four breeds represented in each sub-group of cows in 1948 were the Ayrshire, Guernsey, Holstein and milking Shorthorn. In 1949 the last three mentioned were used. Each breed pair was quite closely matched with respect to age and stage of lactation. The supplement fed was nickel (ous) chloride ($NiCl_2 \cdot 6H_2O$) in approximate daily amount of 500 mg., which is the equivalent of 145 mg. of elemental nickel. One group received the supplement during December and January in 1947–48; the other group received it during February and March. In 1949 the feeding trial was started a month later, so that one group received the supplement during January and February and the other during March and April. The reason for repeating the trials will appear in a moment.

Composite 2-day milk samples of 2 l. each were taken from each cow once a month. Nickel was determined in triplicate on 500-ml. portions of each sample by the method of Alexander *et al.* (1). This method, when used in conjunction with a quartz spectrophotometer, working with standard solutions of nickel chloride and known amounts of added nickel, gave good results. Results the

Received for publication June 16, 1949.
[1] Contribution No. 727 of the Massachusetts Agricultural Experiment Station.

first year were obtained with the aid of a grating type of spectrophotometer and were not uniform. Admittedly the grating type of instrument is not as delicate as the quartz type, and it was therefore deemed advisable to repeat the work a second season using an instrument[2] that would more adequately resolve the very minute differences involved. In addition, as a preliminary to work of the second season, each step in the analytical procedure was subjected to rigorous inquiry in order to refine technique wherever possible.

RESULTS

The values obtained are summarized in the accompanying tables, table 1 containing the results for 1948, and table 2 those for 1949. In the 1948 results, al-

TABLE 1

Effect on nickel content of the milk of feeding cows nickel chloride (season of 1947–48)
(γ of nickel/l. of milk)

Month	Cows on control ration					Cows receiving supplemental nickel				
	1st half of the season									
	[a]A232	G639	H447	S74	Av.	A205	G629	H444	S62	Av.
December	12.8	20.2	10.6	1.6	11.3	8.8	22.6	20.0	21.1	18.1
January	10.0	5.2	1.2	2.8	4.8	3.2	2.8	0	25.0	7.8
Av.—1st half	11.4	12.7	5.9	2.2	8.1	6.0	12.7	10.0	23.1	13.0
	2nd half of the season									
	A205	G629	H444	S62	Av.	A232	G639	H447	S74	Av.
February	1.2	0.5	10.4	0	3.0	6.4	0.8	0.6	6.0	3.5
March	1.4	1.4	0.8	0.3	1.0	1.8	1.8	20.4	1.6	6.4
Av. 2nd half	1.3	1.0	5.6	0.2	2.0	4.1	1.3	10.5	3.8	4.9
Av.—entire season	6.4	6.9	5.8	1.2	5.1	5.1	7.0	10.3	13.5	9.0

[a] The initial letter prefixed to each cow's number indicates the breed.

though the trend was toward a higher concentration of nickel in the milk when the supplement was fed (9.0γ/l. on the average, in contrast to 5.1γ), as already noted the individual values were entirely lacking in uniformity (5 of the 16 possible comparisons were in the reverse direction) and because of the wide variations the difference in the general averages (3.9γ/l.) was not significant statistically. It therefore was decided to repeat the work, and it will be noted from table 2 that the results were even more inconclusive than in 1948. The cows showed a slightly higher average amount of nickel in their milk when not receiving the supplement than they did when they received it; there was even greater variability in the individual results; and the small average difference was statistically not sig-

[2] The instrument used in this later work was a Model DU Beckman spectrophotometer. Measurements were made at a wave length of 385μ, using Corning filter #9863 (red purple Corex A).

nificant. The values for the 1949 season are in general of a much lower magnitude than those for 1948. This is attributed to refinements of technique and method and to the use of a more precise spectrophotometer.

Obviously these inconclusive and conflicting results in 2 successive years mean one of two things; either cows do not secrete supplemental nickel uniformly into their milk as they do cobalt (4), or else natural milk does not contain nickel at all, in which case the widely varying amounts found represent varying amounts of nickel dissolved from the milking machine. It was decided to either eliminate or confirm this latter possibility.

TABLE 2

Effect on nickel content of the milk of feeding cows nickel chloride. (season of 1949)
(γ of nickel/l. of milk)

Month	Cows on control ration				Cows receiving supplemental nickel			
	1st half of the trial							
	G692	H567	S74	Av.	G686	H444	S68	Av.
January	0.48	1.36	0.48	0.77	0.20	1.12	0.40	0.57
February	0.52	1.04	1.76	1.11	0.96	0.56	0.48	0.67
Av.—1st half	0.50	1.20	1.12	0.94	0.58	0.84	0.44	0.62
	2nd half of the trial							
	G686	H444	S68	Av.	G692	H567	S74	Av.
March	0.24	2.24	1.12	1.20	1.40	2.20	0.88	1.49
April	0.32	0.72	1.54	0.86	0.24	0.72	0.66	0.54
Av.—2nd half	0.28	1.48	1.33	1.03	0.82	1.46	0.77	1.02
Av.—entire season	0.39	1.34	1.23	0.99	0.70	1.15	0.61	0.82

At the conclusion of the feeding trial in April, 1949, composite samples for two milkings from each sub-group of cows were obtained without any contact with metal by milking by hand directly into 2-l. glass jars after the fore-milk had been first drawn off and discarded. These samples when carried through the usual analytical procedure were found to contain not even a slight trace of nickel.

The conclusion from this final phase of the investigation hardly needs stating, but it raises an interesting question. Here are two elements—cobalt and nickel—side by side in the periodic system; their atomic weights and atomic numbers are respectively: 58.9 and 58.7, 27 and 28. The only known difference in structure is a difference of one electron in the "M" orbit; cobalt has 15 electrons in this orbit, while nickel has 16. In the past few years it has been shown by numerous investigators that cobalt has a remarkable biological significance and within a year it has been proven to be a constituent of vitamin B_{12} (11). Nickel on the other hand is not known to be essential to biological systems, and here is further evidence of the same sort; viz. that either it is not absorbed into the blood stream or else the mammary gland excludes it while permitting cobalt to pass into

the milk. A similar situation with respect to iron and manganese was noted and commented on in an earlier paper of this series (5).

SUMMARY

Nickelous chloride was fed as a supplement (500 mg. daily) to the rations of six cows for a period of 2 months by the double reversal method and the milk was analyzed for nickel. Although varying amounts of nickel were found in the numerous milk samples taken, it was shown that when the milk was kept from contact with metal by milking directly into glass jars, nickel was not present.

Therefore it was concluded that nickel is not a constituent of natural milk and that the varying amounts found in the course of the investigation came from the milking machine.

The difference in this respect beween cobalt and nickel, two closely related elements, is discussed briefly.

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SHORT-TERM CULTURE OF LEUCOCYTES IN NICKEL HYPERSENSITIVITY

Acta Dermato-Venereol. 42(5): 412-417 (1962)

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The present investigation concerns the effect of nickel on leucocytes from patients hypersensitive to nickel. Our study is based on previous reports of cellular changes induced *in vitro* by several immunologic reactions. Thus Hayashi and Ono (13) found cytologic signs of cell death in monocytes from hypersensitive rabbits when the cells were brought into contact with the actual antigen protein. Black (4) reported that leucocytes from atopic patients showed toxic effects when atopic allergens were added. In delayed hypersensitivity of the tuberculin type the cell-damaging effects of antigen have been demonstrated (10, 22). In auto-immune diseases serum from afflicted individuals may affect cells from healthy organs (1, 2, 5, 15, 20).

In contact hypersensitivity, however, it has not been possible to demonstrate cell damage *in vitro* by the addition of appropriate antigen to cells from hypersensitive persons (9, 3) or by the exposure of skin cells to allergen before cultivation (8). Studies have so far been made only of skin cells from patients with hypersensitivity of the contact type. As lymphocytes are considered to be of primary importance for transmission of contact hypersensitivity (17, 12), we believed it of special importance to investigate the sensitivity of lymphocyte-monocyte type cells when exposed to contact allergens. We have studied a system where mitoses of lymphocytoid-monocytoid cells (14) were induced in blood by addition of phytohemagglutinin (19), the mitotic activity of the cells was used as an indicator of their vitality. The occurrence of mitoses in blood from nickel-hypersensitive and non-hypersensitive subjects at different nickel concentrations in the culture fluid was investigated.

Material and methods

Subjects

The patients with nickel hypersensitivity were fifteen women, 23 to 57 years old. All had a typical history of allergic eczematous contact dermatitis due to nickel and all had strongly positive patch tests with 5 % nickel sulfate in aqueous solution. Twelve of the patients were also tested intracutaneously (7) with 1, 0.1, 0.01 and 0.001 m*M* nickel chloride in physiologic saline. The volume injected in the intracutaneous test was 0.05 ml. An infiltration of more than 5 mm in diameter was considered as a positive reaction. One out of the twelve patients tested showed a positive reaction to the 0.01 m*M* nickel chloride solution, three reacted to the 0.1 m*M* solution and five patients reacted to the 1 m*M* solution.

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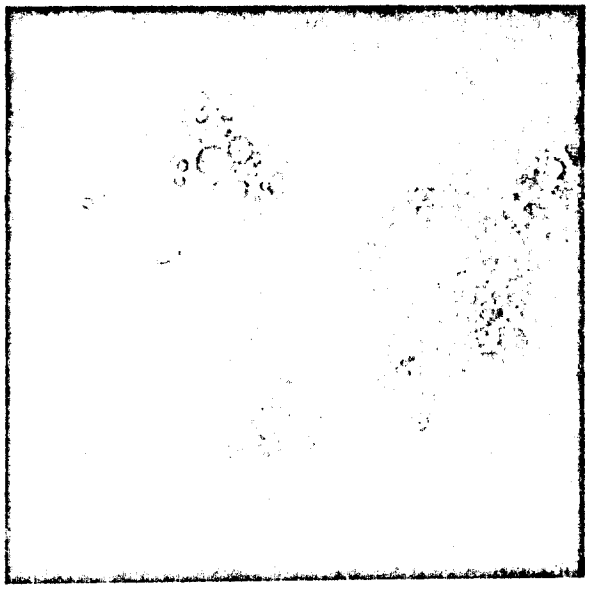

Fig. 1. Squashed cells not treated with distilled water but fixed and stained as described in the text. The picture shows one granulocyte, one mononuclear cell without nucleolus, two mononuclear cells with nucleoli and one mitotic cell.

The normal persons were ten females and five males, 17 to 72 years old. Epicutaneous patch testing with 5 % nickel sulfate were negative in all. Six of the normal subjects were tested with the above-mentioned solution of nickel chloride intracutaneously. None showed positive reactions.

Culturing of leucocytes

Ten-millilitre venous blood samples were drawn into heparinised syringes and transferred to tubes containing 2.5 ml of 6 % dextran[1] (16). The tubes were then inclined at an angle of 45° for 30—45 minutes. After this time the aggregated erythrocytes had sedimented and the supernatant could be pipetted off. The supernatant was then centrifuged at 160 *g* for 8 minutes. The sedimented cells were suspended to a final concentration of 1—2 million cells per ml in a culture medium of the following composition:

Parker 199, normal dilution (21)	7 ml
Autologous serum	3 ml
Bacto-Phytohemagglutinin (Difco) (one vial in 5 ml Ringer solution)	0.02 ml
Heparin (Vitrum) (5 % aqueous solution without preservative)	1 drip

In the first twenty experiments (ten normal subjects and ten nickel-hypersensitive patients) each cell suspension sample was distributed into ten Pyrex tubes.

[1] Macrodex from Pharmacia AB, Uppsala.

Table 1. *Number of individuals showing mitoses of blood cells in culture fluids with varying nickel concentration.*

Nickel concentration	Control subjects		Nickel-hypersensitive subjects	
	Number of subjects studied	Number of subjects with mitoses	Number of subjects studied	Number of subjects with mitoses
1 m*M*	15	0	15	0
0.3 m*M*	5	1	5	2
0.1 m*M*	15	15	15	15
0.01 m*M*	10	10	10	10
0.001 m*M*	10	10	10	10

Nickel chloride in Parker 199 was added to the cell suspensions (1 : 10). The final concentration of nickel thus became: 1 m*M* in tubes 1 and 2, 0.1 m*M* in tubes 3 and 4, 0.01 m*M* in tubes 5 and 6, 0.001 m*M* in tubes 7 and 8. To tubes 9 and 10, which served as controls, Parker 199 without nickel was added. In the last ten experiments (five normal subjects and five nickel-hypersensitive patients) each sample of cell suspension was divided into seven Pyrex tubes. Nickel chloride in Parker 199 was added to the cell suspensions (1 : 10) so that the final concentration of nickel became: 1 m*M* in tube 1, 0.3 m*M* in tubes 2 and 3, 0.1 m*M* in tubes 4 and 5. To tubes 6 and 7, which served as controls, Parker 199 without nickel was added. The tubes, sealed with rubber stoppers, were incubated at 37° C for 72 hours, after which colchicin[1] in physiologic saline was added to the final concentration of 0.04 μg/ml of colchicin. After further incubation for 3 hours at 37° C the cell suspensions were diluted with three times their own volume of distilled water. They were then left for 7 minutes and afterwards centrifuged at 670 *g* for 5 minutes. A fixing solution was added about ten times the volume of the sediment consisting of 60 % acetic acid in 0.1 *N* hydrochloric acid. After some minutes the fixing solution was decanted and the remaining cell-containing fluid was diluted with an equal volume of 2 % orcein in 60 % acetic acid. The cells were thoroughly mixed in the staining fluid by repeated pipetting in a Pasteur pipette. The mixture was then placed on a slide and squashed. Immediately after squashing the specimens were examined for the presence of mitoses with a × 40 phase-contrast objective and a × 10 ocular. Several mitoses could generally be found per minute. If no mitoses were found in 10 minutes, they were considered to be absent.

Results

Large mononuclear cell forms were always present in samples where mitoses were found. The mononuclear cells had a round nucleus with one or more nucleoli and the chromatin was finely dispersed.

The effect of nickel on the cells from normal subjects and from nickel-hypersensitive patients was the same (Table 1). It was regularly found that 1 m*M* nickel in the cell culture fluid prevented mitoses and the mononuclear cell forms described were always absent in this nickel concentration. In specimens with

[1] Colcemid, Ciba.

0.1, 0.01 and 0.001 m*M*, as well as in specimens without nickel, mitoses were regularly present. Individual variations in the number of mitoses and in the cytologic picture were found. As 1 m*M* but not 0.1 m*M* nickel inhibited mitoses, we tested in ten additional experiments (five normal subjects and five nickel-hypersensitive patients) also the effect of 0.3 m*M* nickel. It was found that the large mononuclear cell forms described earlier occurred in a concentration of 0.3 m*M*, but in lower numbers than in specimens with 0.1 m*M* nickel. As in the first twenty experiments 1 m*M* inhibited mitoses while 0.1 m*M* had no anti-mitotic effect. In one of the five normal subjects a few mitoses were found in the specimens with 0.3 m*M* nickel and in two of the five nickel-hypersensitive patients mitoses were found at the same concentration.

Discussion

Leucocytes from nickel-hypersensitive persons showed inhibition of mitoses in the same nickel concentrations as cells from normal persons. It is interesting that 0.1 m*M* and 0.01 m*M* nickel chloride solutions, when injected intradermally, could induce inflammation in the skin of hypersensitive persons without influencing the mitotic capacity of their leucocytes *in vitro*.

Our results are in agreement with those of Everett *et al.* (9) and Bizzozero and Depaoli (3), who were unable to demonstrate a specific cell damage of contact antigens *in vitro* using skin tissue culture. If a specimen contained cells of different sensitivity as regards the hapten — *i. e.* belonging to different clones (6) sensitive cells might be inhibited, while non-sensitive cells could give rise to mitoses. As the number of mitoses was not determined in the different specimens, a sample from a hypersensitive person with inhibition of mitoses in some cells could not be differentiated from a sample from a normal person without any mitotic inhibition. Another possible explanation of our negative results is that an injurious effect of the allergen on a sensitive cell does not prevent mitosis. It is more likely, however, that in contact hypersensitivity, under our experimental conditions, the allergen has no specific effect on cells from the hypersensitive individuals. This, in turn, might depend on an adequate, complete, protein antigen (18) not having been formed or on the antibody not having been activated (11). It is also possible that neither the cells studied by Everett *et al.* nor our type of cells are the proper target cells for the contact allergen.

SUMMARY

The occurrence of mitoses in leucocytes from normal and from nickel-hypersensitive subjects was investigated in short-term cultures in the presence of 1, 0.3, 0.1, 0.01 or 0.001 m*M* nickel in the cultures.

Leucocytes from normal subjects or from nickel-hypersensitive subjects did not undergo mitoses in such cell cultures when the concentration of nickel in the culture fluid was 1 m*M*. Mitoses were found in one out of five normal and in two out of five nickel-hypersensitive subjects at a nickel concentration of 0.3 m*M* in the culture fluid. Mitoses were regularly found when the nickel concentration was 0.1 m*M*. This concentration was found to be sufficiently high to induce inflammation at the injection site in four out of twelve intracutaneously tested, nickel-hypersensitive patients.

The results show that the mitotic capacity of leucocytes from normal and from nickel-hypersensitive subjects is influenced to the same degree by nickel ions *in vitro*.

RÉSUMÉ

On a étudié la fréquence des mitoses dans les leucocytes de sujets normaux et de sujets hypersensibles au nickel, au moyen de cultures à court terme, en présence de 1, 0,3, 0,1, 0,01 et 0,001 m*M* de nickel dans les cultures.

Les leucocytes des sujets normaux et des sujets hypersensibles au nickel, ne présentaient aucune mitose dans ces cultures de cellules, lorsque la concentration en nickel était de 1 m*M* dans le liquide de culture. On a trouvé des mitoses dans un cas sur cinq chez les sujets normaux, et dans deux cas sur cinq chez les sujets hypersensibles au nickel, lorsque la concentration en nickel du liquide de culture était de 0,3 m*M*. On a trouvé régulièrement des mitoses lorsque la concentration en nickel était de 0,1 m*M*. On a observé que cette concentration était suffisamment élevée, pour provoquer une réaction inflammatoire à la place de l'injection, chez quatre des douze patients hypersensibles au nickel après un test intracutané.

Ces résultats démontrent que le pouvoir mitotique des leucocytes provenant de personnes normales et de sujets hypersensibles au nickel, est influencé au même degré par les ions nickel.

ZUSAMMENFASSUNG

Es wurde das Vorkommen von Mitosen in Leukocyten von normalen und von nickelüberempfindlichen Personen in Kurzzeit-Kulturen in Gegenwart von 1, 0,3, 0,1, 0,01 oder 0,001 m*M* Nickel untersucht.

Bei einer Konzentration von 1 m*M* Nickel im Kulturmedium fanden in Leukocyten von normalen und nickelüberempfindlichen Personen keine Mitosen statt. Sie wurden aber festgestellt bei einer Nickelkonzentration in der Kulturflüssigkeit von 0,3 m*M*, und zwar bei einer von 5 normalen und bei 2 von 5 nickelüberempfindlichen Personen. Regelmässig fanden sich Mitosen, wenn die Nickelkonzentration 0,1 m*M* betrug. Diese Konzentration war ausreichend, um bei 4 von 12 intracutan getesteten nickelüberempfindlichen Patienten eine entzündliche Reaktion an der Injektionsstelle zu induzieren.

Die Ergebnisse zeigen, dass die mitotische Kapazität von Leukocyten normaler und nickelüberempfindlicher Personen durch Nickelionen in vitro in gleichem Masse beeinflusst wird.

RESUMEN

Ha sido investigada la producción de mitosis en leucocitos de individuos normales y con hipersensibilidad al níquel, en cultivos a corto plazo, en presencia de 1, 0,3, 0,1, 0,01 y 0,001 m*M* de níquel.

Los leucocitos de individuos normales o hipersensibles al níquel no tenían mitosis en tales cultivos celulares cuando la concentración de níquel en el líquido de cultivo era de 1 m*M*. Se encontraron mitosis en uno de cinco individuos normales y en dos de cinco individuos hipersensibles al níquel, cuando la concentración de níquel en el líquido de cultivo era de 0,3 m*M*. Las mitosis se hallaban de un modo regular cuando la concentración de níquel era de 0,1 m*M*. Ha podido comprobarse que esta concentración es suficientemente alta para producir inflamación en el sitio de la inyección en cuatro de doce pacientes hipersensibles al níquel inyectados intracutáneamente.

Estos resultados demuestran que la capacidad mitótica de leucocitos, tanto de individuos normales como hipersensibles al níquel, es influida en el mismo grado por los iones de níquel *«in vitro»*.

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Archives of Environmental Health
25(1)23(1972)

Trace Elements in Soil and Plants and Antral Cancer

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The trace metal content of plant material used for snuff was found to be closely related to the soil content. The contamination of preparations does not seem to be a factor. Not all snuff users get cancer of the maxillary antrum. It may be that people using plant material for snuff from certain soils high in nickel and chromium would be a high-risk population.

THE concept that the pathologic characteristics of cancer may be related to soil has been suggested by Wynne-Griffith[1] and Stocks and Davies,[2] among others. Cancer of the esophagus and of the liver is significantly frequent in the Bantu of southern Africa, and rare in the United States,[3] to suggest that these cancers may be related to food and soil. Stocks[4] and Stocks and Davies[5] demonstrated that marked differences existed in the geographical distribution of certain cancers, and several investigations have shown a distinct relationship between soil types and specific cancers.[1,2]

Submitted for publication Sept 10, 1971; accepted Nov 10.

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Carcinoma of the maxillary antrum occurs infrequently in the United States[6]; however, in the Bantu of southern Africa, it is the commonest respiratory tract neoplasm.[7,8] The relationship between cancer of the maxillary antrum in the Bantu and the use of snuff has been documented by Keen[7] and Higginson and Oettle.[3]

The Bantu people of southern Africa prepare snuff by mixing the ash of many different plants with powdered tobacco leaves. The result is a very potent snuff which is extensively used in tribal areas.

Three of the commonest types of snuff have been studied. These are (1) the aloe snuff, which is prepared by adding the ash of dried leaves of the *Aloe marlothii* to powdered tobacco; (2) the amaranthus snuff, in which the ash of the plant *Amaranthus spinosum*, commonly known as "pigweed," is added; and (3) ubhoco snuff, in which the ash of the incinerated roots of *Turbina oblongata*, a member of the Convolvulacea family, is added.

In a previous publication,[9] the analyses of trace elements of the plant ingredients showed that chromium was significantly raised in the *Turbina* snuff and that the nickel content was also raised. The plant material analyzed for trace metal content is illustrated in the Figure. As a result of these investigations it was decided to discover the source of the trace elements.

Materials and Methods

Agricultural field workers in Swaziland collected the snuff and plant material. Soil samples from the same plant and snuff source area were obtained later and analyzed by atomic absorption spectrophotometry.

Determination of Acid-Soluble Metals in Soil

The extracting reagent used was concentrated nitric acid diluted with distilled water in a 1:10 volume-to-volume ratio. One gram of the soil sample was placed in a 250-ml metal free beaker (rinsed with nitric acid). To the sample of soil, 100 ml of nitric acid solution was added, and this mixture was placed on a hot plate, boiled for 30 minutes, and stirred occasionally. The material was removed, cooled, and filtered through filter paper. The filtrate was evaporated to a small volume (5 to 10 ml) on a hot plate, removed, and allowed to cool. The extracted material then was placed in a 25-ml glass-stoppered graduated cylinder with nitric acid solution and adjusted to a volume of 25 ml. The solution was aspirated into the atomic absorption spectrophotometer using appropriate standards for the determination of zinc, lead, chromium, and nickel.

Results

The trace metal content found in soil and the ratio of plant ash to soil are shown in the Table. In the soil where the *Turbina oblongata* plant grew, chromium was found to be present in a concentration of 6,000 ppm. The amount of chromium in the soil samples in which the aloe and amaranthus plants grew was also raised. The nickel content of the soil in which *Turbina* grew was also high, but the zinc content of the soil in which the same plant grew was low.

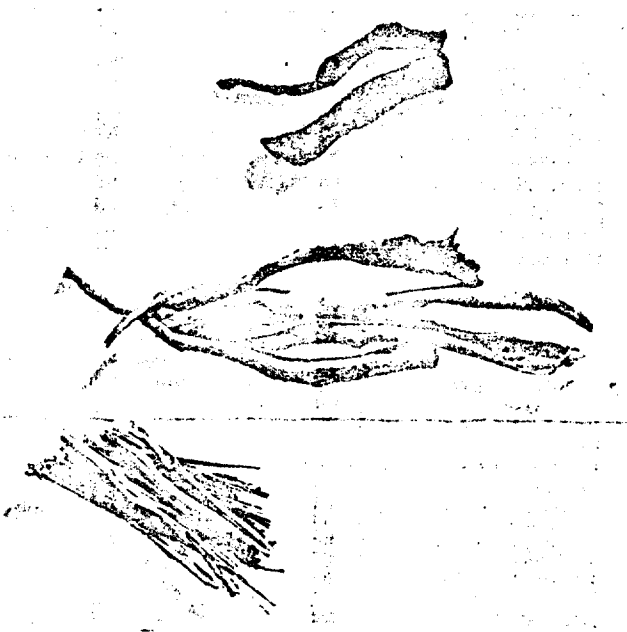
Comment

The analysis of soil correlated well with the plant chromium content. The zinc content in the plant ash was much higher than in soil. The plants are sometimes ashed on zinc sheets and, in the processing of the ash, further zinc might be introduced. On the other hand, it has been shown that plants grown on deficient soils concentrate zinc and absorb the maximum amount. It may be that not only is the *Turbina* plant capable of growing in a chromium soil but that it also has the ability to absorb more chromium. Some plants may selectively avoid absorption of certain trace metals.

Chromium is a recognized carcinogen, particularly for the nasal mucosa, and the evidence suggests that it may be a factor in the high incidence of antral cancer in the South African Bantu.

In view of the fact that the trace elements chromium and nickel have been demonstrated in the soil, a possible preventive measure

Plant material used for making snuff obtained from known soils. Top, Turbina oblongata root (ubhoco snuff). Center, Aloe mariothii plant (aloe snuff). Bottom, Stems of Amaranthus spinosum plant (amaranthus snuff).



Soil Analysis				
Element	Type	Soil	Plant*	Plant-Soil Ratio
Chromium	Aloe	475	17	0.04
	Amaranthus	100	38	0.38
	Turbina oblongata	6,000	259	0.04
Nickel	Aloe	18	112	6.0
	Amaranthus	9	58	6.0
	Turbina oblongata	226	94	0.4
Zinc	Aloe	200	129	0.6
	Amaranthus	225	968	4.3
	Turbina oblongata	50	164	3.5

* Plant ash content (in parts per million) calculated in natural state as received.

would be to advise people to use ingredients for the manufacture of snuff only from plants grown in soil which is relatively free of chromium.

It is probable that other plants used as foodstuffs, such as mealies, sorghum, and vegetables, may be contaminated with chromium. Ingestion of these substances could be related to other types of cancer, such as carcinoma of the liver and esophagus, which have a high incidence in southern Africa. The use of the same chemical carcinogen may produce different lesions depending on the site of entry and the surface contact. This may also apply to the higher incidence of oral cancer since many people use the snuff orally, as it is said to help toothache and headache. It is suggested that more attention should be focussed on soil and plants, from the point of view of trace elements, in the hope that other possible oncogenic factors may be established.

Conclusion

In societies in which members grow their own food and make their own snuff from local plant ingredients, it is relatively easy to trace the source of food and snuff ingredients. Previous investigations have shown that certain snuffs contain relatively large amounts of chromium and nickel as trace elements. The source of these metallic elements has been traced to the soil in which the plants grow. There appears to be a selectivity for trace elements in some of the plants. The results indicate that further investigations along these lines should be carried out in other parts of southern Africa.

This study was supported by grant 5P1OE500159 from the Public Health Service.

David W. Yaeger performed the trace metal analyses.

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[CANCER RESEARCH 30, 48–50, January 1970]

Nickel Carbonyl Inhibition of RNA Synthesis by a Chromatin-RNA Polymerase Complex from Hepatic Nuclei[1]

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SUMMARY

Administration of nickel carbonyl to rats inhibited RNA synthesis by a chromatin-RNA polymerase complex prepared from lysed hepatic nuclei. Rats were killed 6 hr after i.v. administration of $Ni(CO)_4$ in LD_{50} dosage (2.2 mg Ni/100 g body weight). The rate of RNA synthesis by the chromatin-RNA polymerase complex from $Ni(CO)_4$-treated rats averaged 0.16 (S.E. = ±0.02, N = 18) μmole cytidine triphosphate-^{3}H/10 min/g DNA (controls = 0.33 ± 0.03, N = 18, $p < 0.001$). The molar ratio of Ni to DNA nucleotides in the chromatin-RNA polymerase complex from $Ni(CO)_4$-treated rats averaged 0.046, and the concentration of nickel in the final assay mixtures averaged 3.4×10^{-6} M. *In vitro* additions of $Ni(CO)_4$ or $NiCl_2$ in final concentrations of 1×10^{-5} M to assay mixtures containing chromatin-RNA polymerase complex from control rats did *not* inhibit the incorporation of cytidine triphosphate-^{3}H into RNA. This study shows that *in vivo* administration of $Ni(CO)_4$ to rats produces inhibition of RNA synthesis which persists after disruption of hepatic nuclei and excludes inhibition owing to impaired transport of RNA precursors across the nuclear membrane.

INTRODUCTION

Previous investigations have demonstrated 75% inhibition of orotic acid-^{14}C incorporation into liver RNA (1) and 60% inhibition of RNA polymerase activity in intact hepatic nuclei (14) of rats killed 24 hr after injection of nickel carbonyl in LD_{50} dosage. These studies suggested that $Ni(CO)_4$ may block RNA synthesis directly by preventing transcription of the genome, or, alternatively, that $Ni(CO)_4$ may act indirectly by impairing transport of RNA precursors into the nucleus. In an attempt to localize the site of the toxic action of $Ni(CO)_4$, we have undertaken the present study of the effect of $Ni(CO)_4$ upon RNA synthesis by a chromatin-RNA polymerase complex [Weiss' "aggregate enzyme" (17)] prepared from lysed hepatic nuclei.

[1]This investigation was supported by U. S. Atomic Energy Commission Grant AT(30-1)4051, American Cancer Society Grant E-374C, and USPHS Research Grant CA 11250-01 from the National Cancer Institute.

Received April 1, 1969; accepted May 7, 1969.

MATERIALS AND METHODS

The experimental animals were 36 male rats of the Sprague-Dawley strain weighing 180 to 200 g. Three treated and three untreated rats were included in each day's experiment. The rats were fasted for 24 hr before sacrifice. The treated rats received $Ni(CO)_4$ in LD_{50} dosage [5 μl $Ni(CO)_4$/100 g body weight, equivalent to 2.2 mg Ni/100 g] 6 hr before sacrifice. The $Ni(CO)_4$ was injected into a tail vein by means of a 10-μl syringe. The untreated rats served as controls. After the rats were decapitated and exsanguinated, the livers were excised and washed in cold 0.9% (w/v) NaCl solution.

Chromatin-RNA polymerase complex ("aggregate enzyme") was prepared from isolated hepatic nuclei by an adaptation of Weiss' method (17). A 20% (w/v) liver homogenate was prepared in 0.25 M sucrose solution containing 0.001 M $MgCl_2$ with a Blaessig glass homogenizer. The entire homogenate of each liver was strained through gauze and centrifuged at 600 × *g* for 6 min at 4°. The pellet was washed twice with 20 ml of homogenizing solution and once with 0.25 M sucrose solution. The sediment was inspected by phase microscopy to verify that the nuclei were intact and virtually free of cytoplasm. The nuclear pellet was suspended in 20 ml 0.05 M Tris buffer, pH 7.4, and allowed to stand for 10 min at 4°. The lysed nuclei were centrifuged at 10,000 × *g* for 10 min, and the pellet was resuspended to a final volume of 4 ml in 0.05 M Tris buffer. One ml of 2 M KCl solution was added to the suspension dropwise with constant stirring. Within 3 min, a gelatinous precipitate developed, which was sedimented by centrifugation at 900 × *g* for 5 min at 4°. The precipitate was washed twice with 20 ml of 0.05 M Tris buffer containing 0.4 M KCl. This preparation ("chromatin-RNA polymerase complex") was finally suspended in 1 ml of 0.05 M Tris buffer, pH 8.1, by homogenization with a Teflon-glass homogenizer.

Samples (0.1 ml) of the suspension of chromatin-RNA polymerase complex were taken for assay of RNA synthesis as described by Sunderman and Esfahani (14). Cytidine triphosphate-^{3}H (specific activity, 1.4 Ci/mmole) was used as the labeled nucleotide. The radioactivity incorporated into RNA was measured in a Beckman CPM-100 liquid scintillation spectrometer. Duplicate aliquots of each sample were counted within an error of ±2%. The counting efficiency of each sample was determined by addition of an internal standard containing 550,000 dpm of CTP-^{3}H. RNA synthesis was negligible if ATP, UTP, or GTP were omitted from the

reaction mixture. The DNA content of each reaction mixture was determined by the diphenylamine method of Burton (2). The rate of RNA synthesis was expressed in units which are equal to 1 μmole CTP-^{3}H incorporated into RNA/10 min/g DNA in the reaction mixture (17).

In order to measure the nickel content of the chromatin-RNA polymerase complex, 3 rats were given i.v. injections of $^{63}Ni(CO)_4$ (specific activity, 191.5 μCi/mmole) in dosage of 5 μl/100 g body weight. The distribution of ^{63}Ni at each step of the isolation procedure was determined as described by Sunderman and Selin (16), and the results were expressed as a percentage of the total radioactivity present in the liver homogenate.

In vitro effects of $Ni(CO)_4$ or $NiCl_2$ were studied with suspensions of chromatin-RNA polymerase complex from livers of 9 untreated rats. RNA synthesis was measured in the presence of 1×10^5 M $Ni(CO)_4$ or $NiCl_2$ and the results were expressed as percentages of the rates of RNA synthesis in paired control samples.

RESULTS

The effect of $Ni(CO)_4$ administration *in vivo* upon RNA synthesis by chromatin-RNA polymerase complex from lysed hepatic nuclei is presented in Table 1. Under the experimental conditions $Ni(CO)_4$ produced an average of 52% inhibition of RNA synthesis by the chromatin-RNA polymerase complex ($p < 0.001$).

Table 1

Effect of $Ni(CO)_4$ upon RNA synthesis by chromatin-RNA polymerase complex from hepatic nuclei

	No. of rats	RNA synthesis (μmole CTP-^{3}H/10 min/g DNA) Mean ± S.E.	p
Controls	18	0.33 ± 0.03	
$Ni(CO)_4$[a]	18	0.16 ± 0.02	<0.001

[a]$Ni(CO)_4$ (2.2 mg Ni/100 g, i.v.) 6 hr before sacrifice.

The partition of ^{63}Ni during preparation of the chromatin-RNA polymerase complex is described in Table 2. The livers contained 0.69% (range, 0.60 to 0.77%) of the injected ^{63}Ni. The final homogenized suspension of chromatin-RNA polymerase contained an average of 6.6% (range, 6.2 to 7.1%) of the ^{63}Ni present in the liver homogenate. On the basis of the specific activity of the injected $^{63}Ni(CO)_4$, the calculated concentration of Ni in the 2-ml suspension of chromatin-RNA polymerase complex was 1.7×10^{-5} M, and the concentration of Ni in the final assay mixture was 3.4×10^{-6} M. The mean concentration of DNA in the suspension of chromatin-RNA polymerase complex from nickel carbonyl-treated rats was 245 μg/2 ml. Assuming the average molecular weight of the DNA nucleotides to be 327, the mean molar ratio of Ni to DNA nucleotides in the chromatin-RNA polymerase complex was 0.046, equivalent to 1 mole Ni/22 moles nucleotide bases.

Table 2

Partition of ^{63}Ni during preparation of chromatin-RNA polymerase complex from hepatic nuclei

Preparations (n = 3)	% liver ^{63}Ni Mean	Range
Initial nuclear pellet	31.1	26.1–39.0
Final nuclear pellet (3 X resedimented)	19.6	14.7–27.7
Initial chromatin-RNA polymerase pellet	16.5	11.9–23.8
Final chromatin-RNA polymerase suspension (3 X resedimented)	6.6[a]	6.2– 7.1

[a]Molar ratio of Ni to DNA nucleotides = 0.046

Nickel carbonyl and $NiCl_2$ were added to assay mixtures *in vitro* in concentrations which were 3 times greater than that produced by *in vivo* administration of $Ni(CO)_4$. In Table 3, RNA synthesis by chromatin-RNA polymerase complex from untreated rats after *in vitro* additions of the nickel compounds to assay mixtures are expressed as percentages of the paired control values. Under the experimental conditions, the *in vitro* additions of $Ni(CO)_4$ or $NiCl_2$ did not significantly affect RNA synthesis by the chromatin-RNA polymerase complex.

Table 3

RNA synthesis by chromatin-RNA polymerase complex after in vitro additions of $Ni(CO)_4$ or $NiCl_2$

	Ni concentration in assay mixture (M)	No. of rat livers	RNA synthesis (% paired controls) Mean ± S.E.	p
Ni(CO) *in vitro*	1×10^{-5}	9	95 ± 9	>0.9
$NiCl_2$ *in vitro*	1×10^{-5}	9	104 ± 6	>0.9
[$Ni(CO)_4$ *in vivo*	3.4×10^{-6}	18	48 ± 6	<0.001]

DISCUSSION

The evidence which has implicated $Ni(CO)_4$ as a carcinogen has been summarized in a recent publication (13), and the pathological and biochemical alterations which develop in rats after exposures to $Ni(CO)_4$ have been described in previous reports from our laboratory (5–7, 10–12, 15). Recent studies with $^{63}Ni(CO)_4$ and $Ni(^{14}CO)_4$ have partially elucidated the metabolism and excretion of $Ni(CO)_4$ in rats (8, 16). The evidence for an inhibitory effect of $Ni(CO)_4$ upon synthesis of hepatic RNA includes (*a*) inhibition of cortisone induction of hepatic tryptophan pyrrolase (11), (*b*) inhibition of orotic acid-^{14}C incorporation *in vivo* into liver RNA (1), and (*c*) inhibition of RNA polymerase activity *in vitro* in intact liver nuclei (14). The present study has shown that the inhibition of RNA synthesis persists after disruption of the hepatic nuclei, and excludes inhibition owing to impaired transport of RNA precursors across the nuclear membrane. The present study has also shown that the inhibition of RNA synthesis is not attributable merely to the

presence of nickel in the assay mixture, because inhibition was not observed after *in vitro* additions of $Ni(CO)_4$ or $NiCl_2$ in concentrations (1×10^{-5} M) greater than that produced by injection of $Ni(CO)_4$ *in vivo*. This is important because Novello and Stirpe (9) have reported 12 to 21% inhibition of RNA polymerase activity in intact hepatic nuclei after the addition of $NiCl_2$ to assay mixtures at a concentration of 1×10^{-3} M.

As the next step in our investigation of the site of the inhibitory effect of $Ni(CO)_4$ upon synthesis of hepatic RNA, attempts are being made to measure the template activity of hepatic chromatin and DNA from $Ni(CO)_4$-treated rats for transcription by RNA polymerase from livers of control rats and *vice versa*. With the methods which have recently been developed for the purification of soluble RNA polymerase from rat liver (3, 4), it should now be possible to determine whether $Ni(CO)_4$ inhibition of RNA synthesis results from nickel binding to chromatin or DNA or whether it results from an interaction of nickel with DNA-dependent RNA polymerase.

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Ann. Inst. Pasteur (Paris) 53(4): 371-378, 1934

Research on the Physiological Importance of Nickel and Cobalt [1]

By: Gabriel Bertrand and Hirosi Nakamura

When it was recognized by one of us in collaboration with Macheboeuf that human and animal organs normally contained very small quantities of nickel and cobalt (2), the question was raised of whether these metals were mere impurities of the living material or whether they filled some kind of physiological role, comparable, for example, to those of iron and zinc.

Experiments were then undertaken in several directions at the same time. Contrary to all expectation, those which gave the first results were those which, having for the object the research of a special physiologic function of nickel and cobalt, enabled the demonstration of the existence of a relationship between the two metals, the pancreas and insulin (3).

While this developed from the first experiments, we were concerned with studying by the method of synthetic feeding, whether nickel and cobalt intervened, at least in an overall way, in the nutritive processes and growth phenomena. In spite of the extreme smallness of the amounts of nickel and cobalt, on the order of a few hundredths of a millogram per kilogram of live bodyweight with which we were concerned, we were encouraged in this work by the results obtained during the preceding years using the method of studying zinc and iron (4).

The principle of this method will be recalled; mice of the same breed were separated from their mother at the age of three weeks, --in other words, at the time when it was possible to wean them--and divided into two lots. Those of the first lot were fed with a mixture of organic materials and

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[1] An extract of this article was published in Comptes rendus de l'Academie des Sciences, 185, 1927, p. 321.

minerals carefully purified; those of the second with the same mixture to which was added a very small known quantity of the metals used in the experiment. The differences manifested were noted, and we redid the experiment a certain number of times so as to obtain good averages.

The organic substances (starch, lactose, casein, etc.) and minerals (sodium chloride, phosphates, etc.) were freed of all traces of nickel and cobalt by the same procedures as those previously used to rid zinc and iron of these substances; but in order to have the greatest certainty, the number of purifications was multiplied even more. Therefore, each portion of starch was treated up to 12 times by the acidulated water of hydrochloric acid (1): by washing with pure water, by ammoniacal water, again with pure water and finally with alcohol, this represents about twenty successive treatments, each comprising several hours of machine agitation in the presence of the chosen liquid, at least a whole day of rest and decantation. As can be seen, it not only was necessary for us to take great care but also to use a lot of time to prepare the stock of the substances necessary for feeding our animals.

Our experiments were carried out on 35 mice coming from 5 litters. Six died of starvation because they were not accustomed to the artificial diet to which we subjected them. Therefore, 29 remained and were divided into as many series as there were litters.

The formula for the food mixture, fashioned into little loaves of bread in baking is the same as in the previous experiment except that they always

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[1] Since iron is more abundant than nickel and cobalt in natural substances, it is more difficult to eliminate and since it shows a recognizable reaction--with the aide of ammonium sulfocyanate--much more sensitive, we kept up the purification until the treated substance no longer gave any trace of an iron reaction. (see Gab. Bertrand and B. Soc. Chem. Biol. 7, 1925, p. 933).

contained zinc and iron. We only added to it for the mice of the second lot 0.0025 g nickel chloride and 0.001 g of cobalt sulfate per kilogram (1). (see page 376 for the table of results obtained).

In summary, we had 14 mice fed without nickel nor cobalt whose average survival was 19.7 days, and 15 mice having received nickel and cobalt whose average survival was 23.1 days.

Further, we found that the mice fed with the nutritive mixture with nickel and cobalt added had fixed a part of these metals in their tissues. Having emptied their digestive system and analyzed the results, we found on the average:

In Mice Fed	Nickel per		Cobalt per	
	mouse	kg.-sec.	mouse	kg.-sec.
	mg	mg	mg	mg
without nickel or cobalt	0.0004	0.047	0.00035	0.025
with nickel and cobalt	0.0027	0.198	0.00085	0.065

These results directly demonstrate the intervention of nickel and cobalt in the totality of nutritive phenomena. It will be noted, not without interest, that a few tenths of a milligram of these two metals added to food suffices to increase the survival of the animals in the experiment by about 17%; also it must be noted that only a part (close to a tenth) has been retained by the organism.

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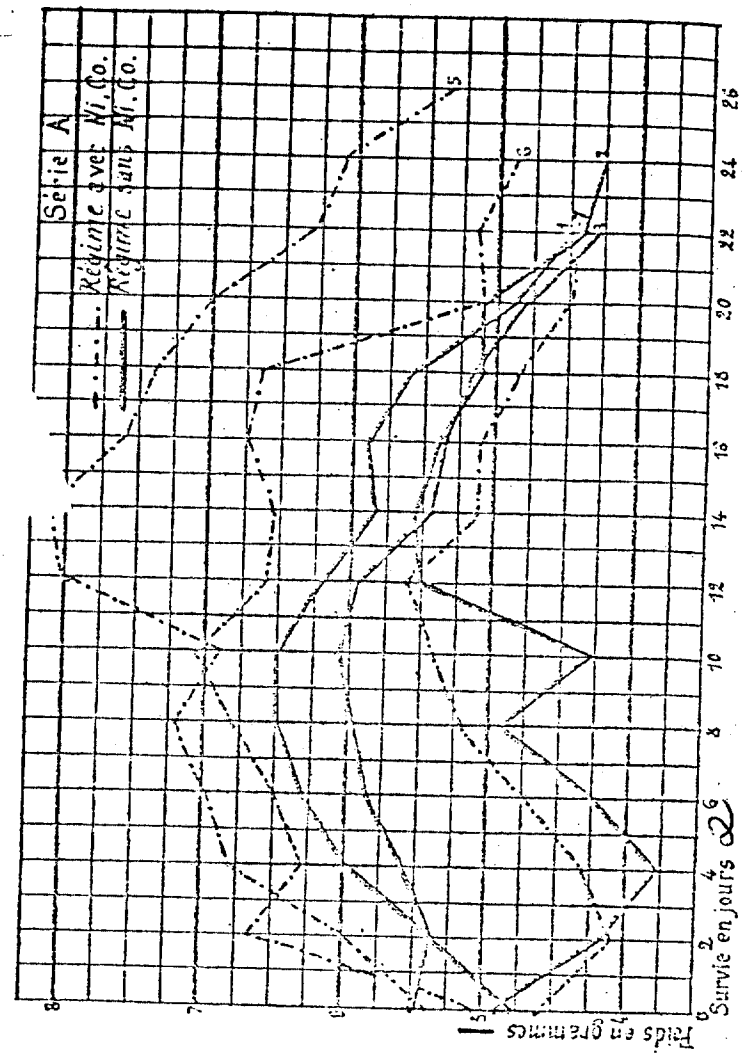
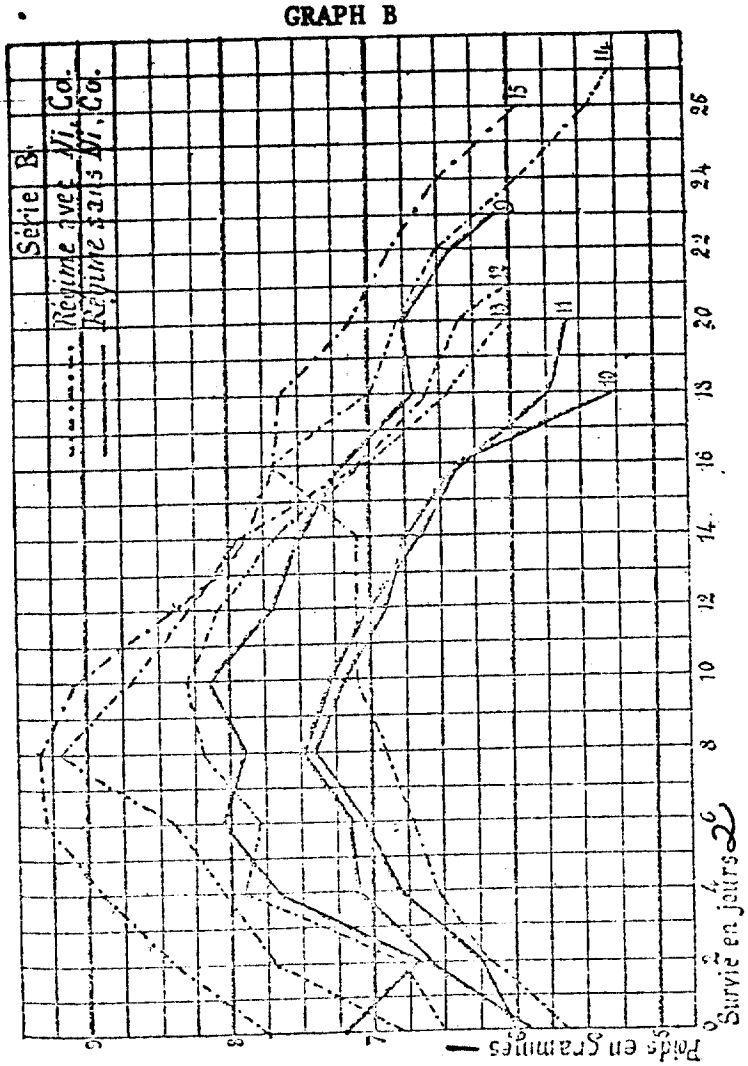
[1] In basing this on the analyses of vegetable food and especially cereals. [Gab. Bertrand and Mokragnatz. Bull Soc. Chim. (4), 37, 1925, p. 554].

GRAPH A



1--Weight in grams
2--Survival in days
__ . __ . __ . __ . Diet with Ni, Co
_____ Diet without Ni, Co

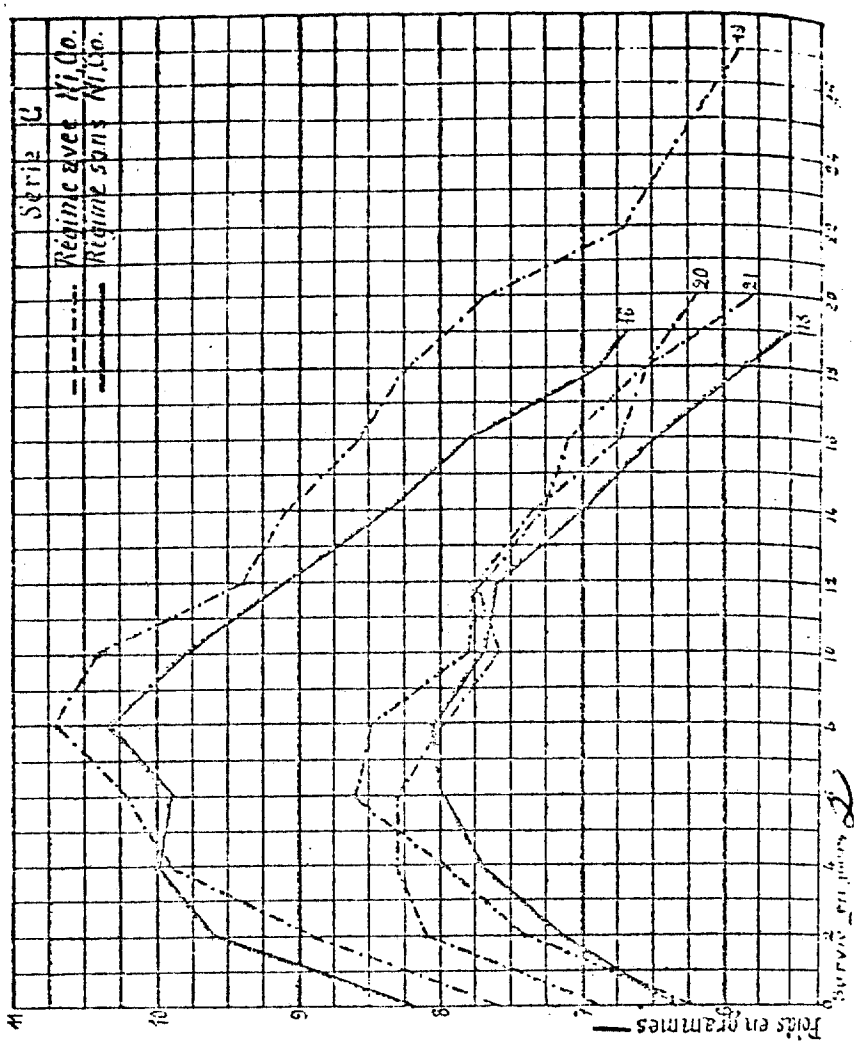
GRAPH B



1--Weight in grams
2--Survival in days

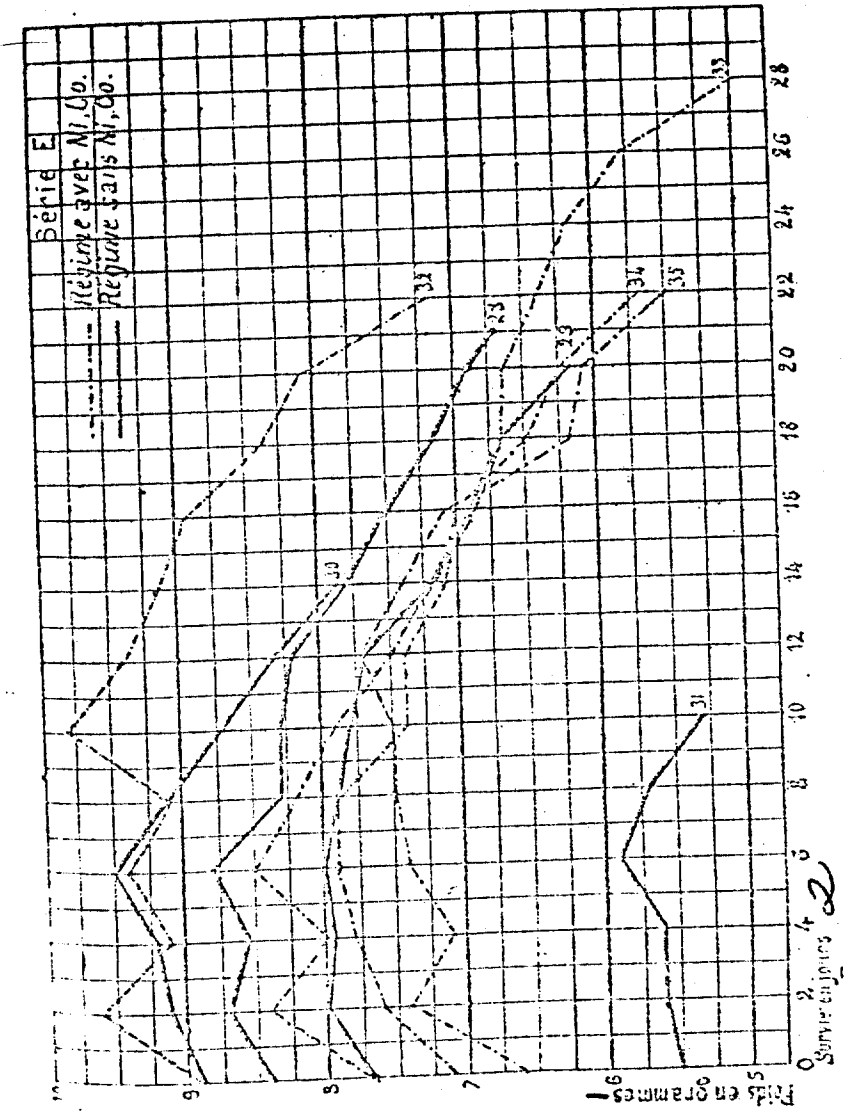
— · — · — · — . Diet with Ni, Co
———————— Diet without Ni, Co

GRAPH C



1--Weight in grams
2--Survival in days
__ · __ · __ · __ · Diet with Ni, Co
_____ Diet without Ni, Co

GRAPH E



1--Weight in grams
2--Survival in days

— · — · — · — · Diet with Ni, Co
——————— Diet without Ni, Co

TABLE

		1 Poids des souris en grammes		4 Aliment consommé en grammes	5 Survie en jours
		2 Au début	3 A la mort		
6 Série A. — *Débutant le 9 août 1926 avec des souris blanches nées le 19 juillet 1926 (âgées de vingt et un jours).*					
7 Sans nickel ni cobalt.	N° 1 (femelle)	5,50	4,50	22,2	22
	N° 2 (femelle)	4,95	4,25	22,3	24
	N° 3 (mâle)	4,80	4,30	18,9	22
8 Avec nickel et cobalt.	N° 5 (mâle)	5,40	5,35	29,7	26
	N° 6 (mâle)	5,05	4,90	26,1	24
	N° 7 (femelle)	4,60	3,95	15,9	22
(Graphique A.)					
9 Série B. — *Débutant le 15 juillet 1926 avec des souris blanches nées le 24 juin 1928 (âgées de vingt et un jours).*					
7 Sans nickel ni cobalt.	N° 9 (mâle)	7,20	6,10	27,5	23
	N° 10 (femelle)	6,00	5,30	21,3	18
	N° 11 (mâle)	5,90	5,60	23,4	20
8 Avec nickel et cobalt.	N° 12 (femelle)	6,80	6,00	26,5	21
	N° 13 (femelle)	6,50	6,05	25,0	20
	N° 14 (femelle)	5,65	5,30	28,2	27
	N° 15 (mâle)	7,75	5,95	23,3	26
(Graphique B.)					
10 Série C. — *Débutant le 9 août 1926 avec des souris blanches nées le 19 juillet 1926 (âgées de vingt et un jours).*					
7 Sans nickel ni cobalt.	N° 16 (mâle)	8,20	6,70	32,5	19
	N° 18 (femelle)	6,35	5,50	27,8	19
8 Avec nickel et cobalt.	N° 19 (femelle)	7,60	5,90	40,6	27
	N° 20 (femelle)	6,90	6,20	26,6	20
	N° 21 (mâle)	6,25	5,80	32,9	20
(Graphique C.)					
11 Série D. — *Débutant le 9 août 1926 avec des souris blanches nées le 19 juillet 1926 (âgées de vingt et un jours).*					
7 Sans nickel ni cobalt.	N° 22 (mâle)	7,65	6,90	30,2	24
	N° 24 (femelle)	6,95	5,60	24,4	20
8 Avec nickel et cobalt.	N° 25 (femelle)	7,20	5,90	30,5	20
12 Série E. — *Débutant le 11 décembre 1926 avec des souris blanches nées le 20 novembre 1926 (âgées de vingt et un jours).*					
7 Sans nickel ni cobalt.	N° 28 (mâle)	8,40	6,70	32,8	21
	N° 29 (femelle)	7,70	6,20	26,0	20
	N° 30 (mâle)	8,90	7,80	28,9	14
	N° 31 (mâle)	5,50	5,30	13,2	10
8 Avec nickel et cobalt.	N° 32 (femelle)	8,40	7,20	40,6	22
	N° 33 (mâle)	7,70	5,00	37,7	23
	N° 34 (mâle)	7,10	5,70	27,9	22
	N° 35 (femelle)	6,60	5,50	28,6	22
(Graphique E.)					

1--Weight of the mice in grams
2--At the beginning
3--At death
4--Food consumed in grams
5--Survival in days
6--Series A--Beginning August 9, 1926 with white mice born July 19, 1926, (age, 21 days)
7--Without nickel or cobalt
8--With nickel and cobalt
9--Series B--Beginning July 15, 1926, with white mice born on June 24, 1928, (age, 21 days)
10--Series C--Beginning August 9, 1926, with the white mice born July 19, 1926 (age, 21 days)
11--Series D--Beginning August 9, 1926, with white mice born July 19, 1926, (age, 21 days)
12--Series E--Beginning on December 11, 1926, with white mice born on November 20, 1926, (age, 21 days)

Ann. Inst. Pasteur (Paris)
53(4): 371-378, 1934

RECHERCHES SUR L'IMPORTANCE PHYSIOLOGIQUE DU NICKEL ET DU COBALT (1)

par MM. Gabriel BERTRAND et Hirosi NAKAMURA.

Lorsqu'il a été reconnu par l'un de nous en collaboration avec Machebœuf que les organes de l'homme et des animaux renferment normalement de très petites quantités de nickel et de cobalt (2), la question s'est posée de savoir si ces métaux étaient de simples impuretés de la matière vivante ou bien s'ils remplissaient un rôle physiologique quelconque, comparable, par exemple, à ceux du fer et du zinc.

Des expériences furent alors poussées dans plusieurs directions à la fois. Contrairement à toute attente, celles qui donnèrent les premiers résultats furent celles qui, ayant pour objet de rechercher une fonction physiologique spéciale du nickel et du cobalt, permirent de démontrer l'existence d'une relation entre les deux métaux, le pancréas et l'insuline (3).

Tandis que se développaient ces premières expériences, nous étions occupés à rechercher, par la méthode d'alimentation synthétique, si le nickel et le cobalt intervenaient, au moins d'une manière globale, dans les processus nutritifs et les phénomènes de croissance. Malgré l'extrême petitesse des proportions de nickel et de cobalt, de l'ordre de quelques centièmes de milligramme par kilogramme de matière vivante, avec lesquelles nous avions affaire, nous étions encouragés dans cette voie par les résultats obtenus au cours des années précédentes en appliquant la méthode à l'étude du zinc et du fer (4).

(1) Un extrait de ce mémoire a été publié dans les *Comptes rendus de l'Académie des Sciences*, **185**, 1927, p. 321.

(2) Gab. Bertrand et Machebœuf. *B. Soc. Chim.* (4), **37**, 1925, p. 934, et **39**, 1926, p. 942.

(3) Gab. Bertrand et Machebœuf. *B. Soc. Chim.* (4), **39**, 1926, p. 1646, et **41**, 1927, p. 1213.

(4) Gab. Bertrand et B. Benzon. Ces *Annales*, **38**, 1924, p. 405 et p. 449, et *B. Soc. chim. biol.*, **6**, 1924, p. 203 et 394; Gab. Bertrand et H. Nakamura. Ces *Annales*, **39**, 1925, p. 698, et **39**, 1925, p. 708; *B. Soc. chim. biol.*, **7**, 1925, p. 942

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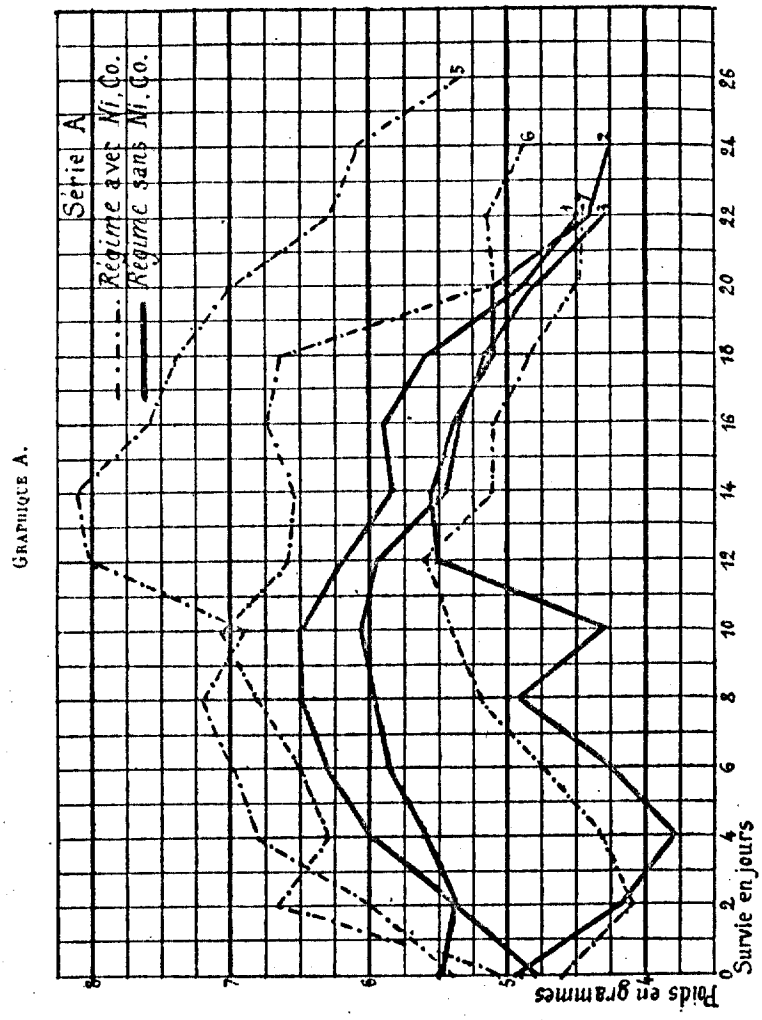
On se rappelle le principe de cette méthode : des souris d'une même portée sont séparées de leur mère à l'âge de trois



Graphique A.

semaines, c'est-à-dire au moment où il est possible de les sevrer, et partagées en deux lots. Celles du premier lot sont nourries avec un mélange de substances organiques et minérales minutieusement purifiées, celles du second avec le même

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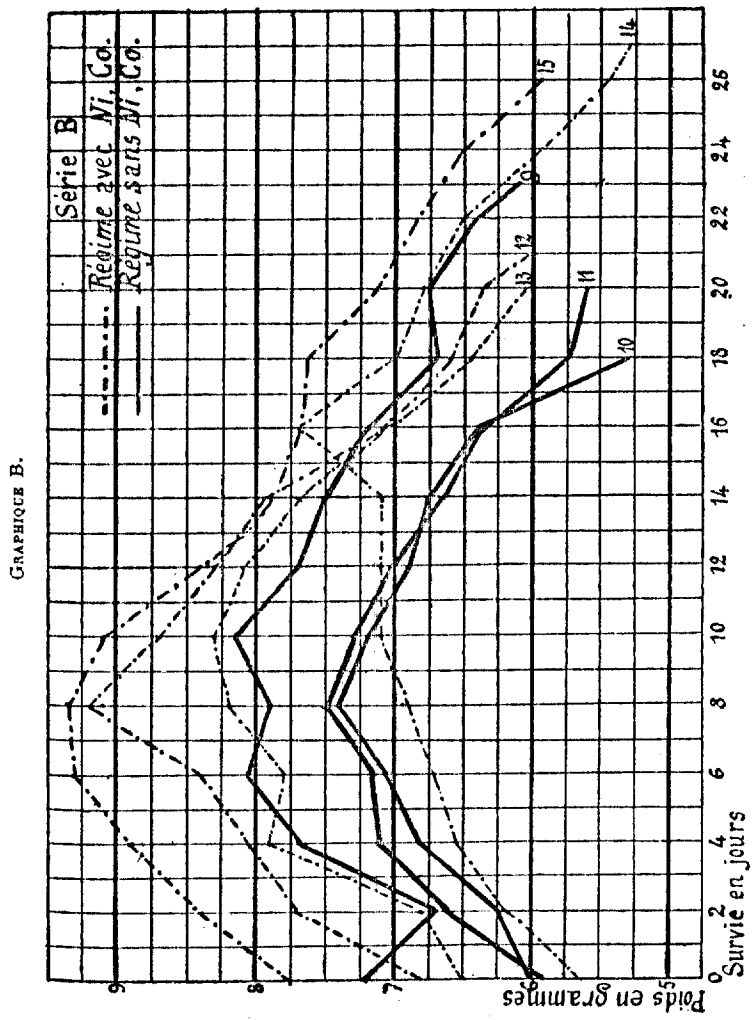
mélange additionné d'une quantité connue et très petite des métaux en expérience. On note les différences qui peuvent se

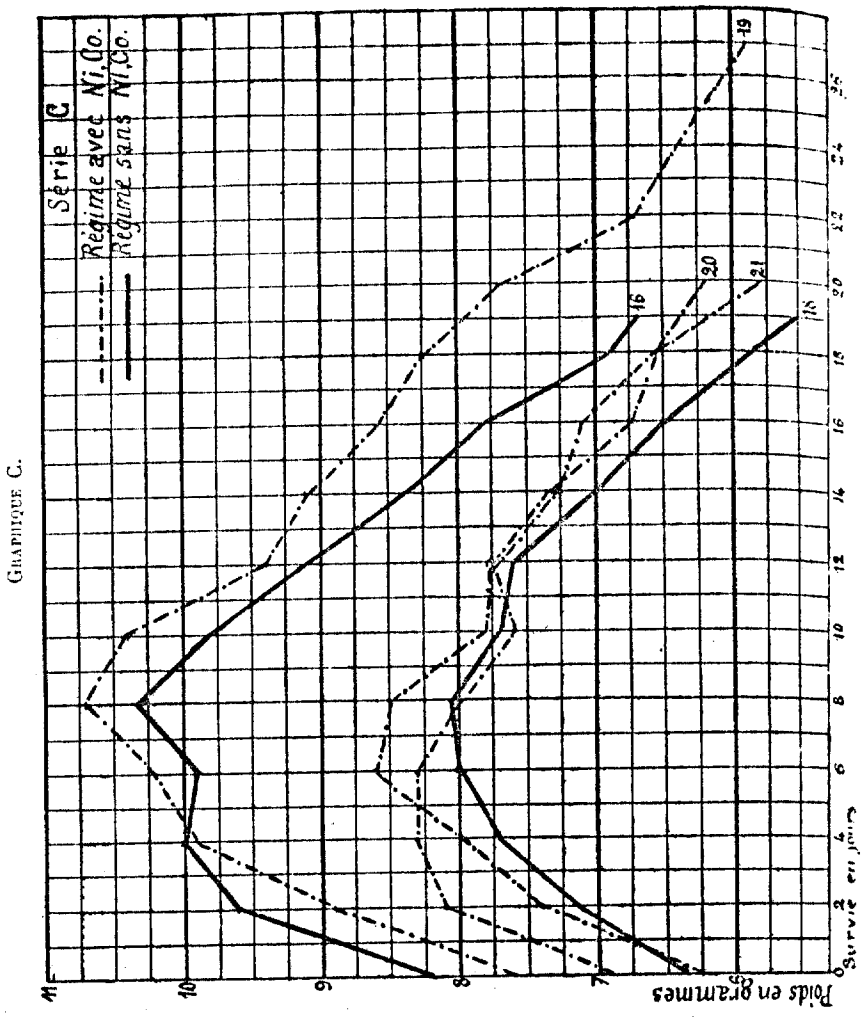


GRAPHIQUE B.

manifester et l'on renouvelle l'expérience un certain nombre de fois, de manière à obtenir de bonnes moyennes.

Les substances organiques (fécule, lactose, caséine, etc.) et minérales (chlorure de sodium, phosphates, etc.) ont été débar-

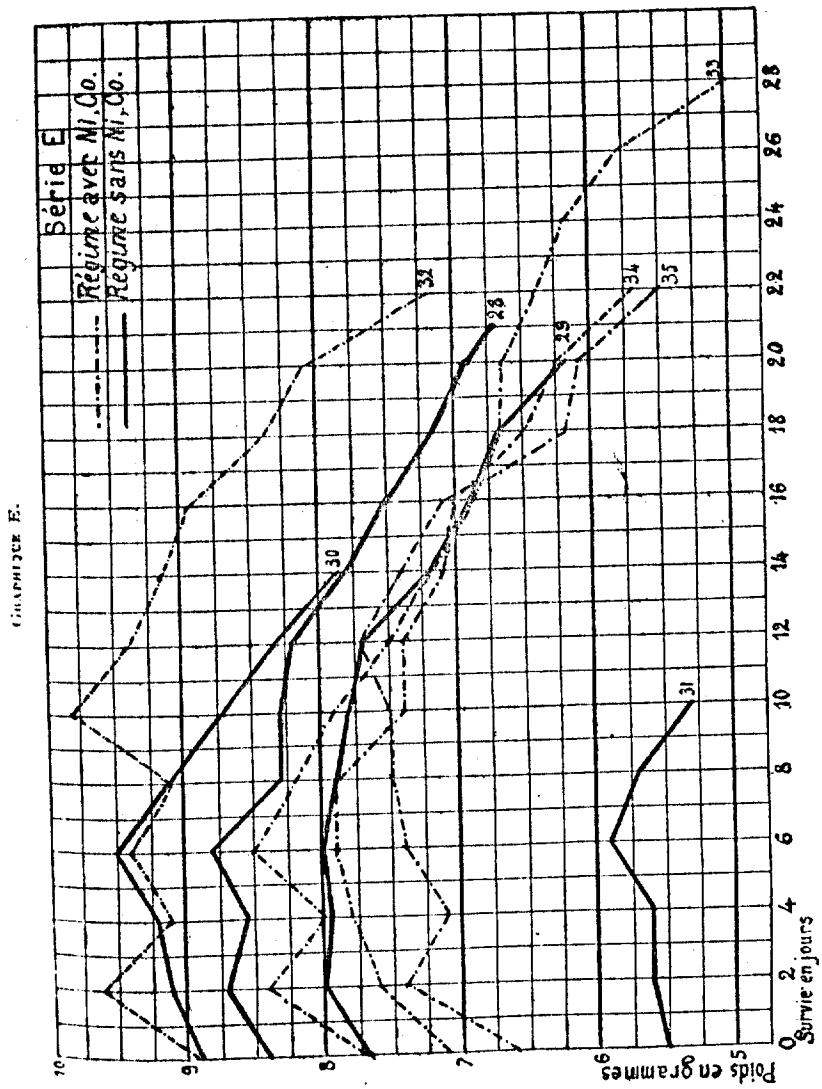
rassées de toute trace de nickel et de cobalt par les mêmes procédés que ceux utilisés antérieurement pour obtenir ces



GRAPHIQUE C.

substances exemptes de zinc et de fer; mais, afin d'avoir le maximum de garantie, les purifications ont été multipliées un plus grand nombre de fois encore. C'est ainsi que chaque portion de fécule a été traitée jusqu'à douze fois par l'eau acidulée

d'acide chlorhydrique (1) ; avec les lavages à l'eau pure, à l'eau



(1) Comme le fer est plus abondant que le nickel et le cobalt dans les produits naturels, qu'il est aussi difficile à éliminer et qu'il présente une réaction de reconnaissance — à l'aide du sulfocyanate d'ammonium — beaucoup plus sensible, nous avons poursuivi la purification jusqu'à ce que la substance traitée ne donnât plus trace de réaction du fer. (Voir Gab. Bertrand et H. Nakamura. Ces *Annales*, 39, 1925, p. 698, et *B. Soc. chim. biol.*, 7, 1925, p. 933.)

		Poids des souris en grammes		Aliment consommé en grammes	Survie en jours
		Au début	A la mort		
Série A. — *Débutant le 9 août 1926 avec des souris blanches nées le 19 juillet 1926 (âgées de vingt et un jours).*					
Sans nickel ni cobalt.	N° 1 (femelle)	5,50	4,50	22,2	22
	N° 2 (femelle)	4,95	4,25	22,3	24
	N° 3 (mâle)	4,80	4,30	18,9	22
Avec nickel et cobalt.	N° 5 (mâle)	5,40	5,35	29,7	26
	N° 6 (mâle)	5,05	4,90	26,1	24
	N° 7 (femelle)	4,60	3,95	15,9	22
(Graphique A.)					
Série B. — *Débutant le 15 juillet 1926 avec des souris blanches nées le 24 juin 1926 (âgées de vingt et un jours).*					
Sans nickel ni cobalt.	N° 9 (mâle)	7,20	6,10	27,5	23
	N° 10 (femelle)	6,00	5,30	21,3	18
	N° 11 (mâle)	5,90	5,60	23,4	20
Avec nickel et cobalt.	N° 12 (femelle)	6,80	6,00	26,5	21
	N° 13 (femelle)	6,50	6,05	25,0	20
	N° 14 (femelle)	5,65	5,30	28,2	27
	N° 15 (mâle)	7,75	5,95	28,3	26
(Graphique B.)					
Série C. — *Débutant le 9 août 1926 avec des souris blanches nées le 19 juillet 1926 (âgées de vingt et un jours).*					
Sans nickel ni cobalt.	N° 16 (mâle)	8,20	6,70	32,5	19
	N° 18 (femelle)	6,35	5,50	27,8	19
Avec nickel et cobalt.	N° 19 (femelle)	7,60	5,90	40,6	27
	N° 20 (femelle)	6,90	6,20	26,6	20
	N° 21 (mâle)	6,25	5,80	32,9	20
(Graphique C.)					
Série D. — *Débutant le 9 août 1926 avec des souris blanches nées le 19 juillet 1926 (âgées de vingt et un jours).*					
Sans nickel ni cobalt.	N° 22 (mâle)	7,65	6,90	30,2	24
	N° 24 (femelle)	6,95	5,60	24,4	20
Avec nickel et cobalt.	N° 25 (femelle)	7,20	5,90	30,5	20
Série E. — *Débutant le 11 décembre 1926 avec des souris blanches nées le 20 novembre 1926 (âgées de vingt et un jours).*					
Sans nickel ni cobalt.	N° 28 (mâle)	8,40	6,70	32,8	21
	N° 29 (femelle)	7,10	6,20	26,0	20
	N° 30 (mâle)	8,90	7,80	28,9	14
	N° 31 (mâle)	5,50	5,30	13,2	10
Avec nickel et cobalt.	N° 32 (femelle)	8,40	7,20	40,6	22
	N° 33 (mâle)	7,70	5,00	37,7	28
	N° 34 (mâle)	7,10	5,70	27,9	22
	N° 35 (femelle)	6,60	5,50	28,6	22
(Graphique E.)					

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ammoniacale, de nouveau à l'eau pure et enfin à l'alcool, cela représente une vingtaine environ de traitements successifs, comprenant chacun plusieurs heures d'agitation à la machine en présence du liquide choisi, une journée au moins de repos et une décantation. Comme on peut s'en rendre compte, il nous a fallu non seulement beaucoup de soin, mais beaucoup de temps pour préparer le stock de substances nécessaires à l'alimentation de nos animaux.

Nos expériences ont porté sur 35 souris provenant de cinq portées. 6 souris sont mortes d'inanition, par défaut d'accoutumance au régime artificiel auquel nous les soumettions. Il en est donc resté 29, divisées en autant de séries qu'il y en avait de portées.

La formule du mélange alimentaire, façonné en petit pains cuits au four, est restée la même que dans les expériences antérieures, à cela près qu'elle comportait toujours du zinc et du fer. On lui a seulement ajouté, pour les souris du second lot, 0 gr. 0025 de chlorure de nickel et 0 gr. 001 de sulfate de cobalt par kilogramme (1). (Voir à la page 376 le tableau des résultats obtenus.)

En résumé, nous avons eu 14 souris alimentées sans nickel ni cobalt dont la survie moyenne a été dix-neuf jours sept dixièmes, et 15 souris ayant reçu du nickel et du cobalt dont la survie moyenne a été de vingt-trois jours un dixième.

Nous avons constaté, en outre, que les souris nourries avec le mélange nutritif additionné de nickel et de cobalt avaient fixé une partie de ces métaux dans leurs tissus. Les ayant vidées de leur tube digestif et analysées, nous avons trouvé, en moyenne :

CHEZ LES SOURIS ALIMENTÉES	NICKEL PAR		COBALT PAR	
	souris	kg.-sec.	souris	kg.-sec.
	milligr.	milligr.	milligr.	milligr.
Sans nickel ni cobalt	0,0004	0,047	0,00035	0,025
Avec nickel et cobalt	0,0027	0,198	0,00085	0,065

(1) En se basant sur des analyses d'aliments végétaux et notamment de céréales [Gab. BERTRAND et MOKRAGNATZ. *Bull. Soc. chim.* (4), 37, 1925, p. 551].

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Ces résultats démontrent, d'une manière directe, l'intervention du nickel et du cobalt dans l'ensemble des phénomènes nutritifs. On ne remarquera pas sans intérêt qu'il a suffi de quelques centièmes de milligramme de ces deux métaux ajoutés aux aliments pour augmenter la survie des animaux en expérience de 17 p. 100 environ; encore faut-il noter qu'une partie seulement (à peu près un dixième) avait été retenue par l'organisme.

Compt. Rend. Acad. Sci. 183(1) 5-8 (1926)

Biological Chemistry--Influence of Nickel and Cobalt on the Action Exerted by Insulin in Dogs.

By: Gabriel Bertrand and M. Machebœuf

The experiments which we recently published (1) showed that certain preparations of insulin act more intensely on glycemia in rabbits when they are injected at the same time as small quantities of nickel and cobalt than when they are injected without these metals.

Before drawing the likely conclusions from these experiments and especially before generalizing their scope to humans, it seemed necessary to us to repeat them on higher animals, not excusively adapted to herbivorous diets.

We had more than just these theoretical reasons for using a new species. When, with the aim of following the hypoglycemic effect of insulin, we punctured the heart five or six times on the same day and each time removed 2 to 3 cm^3 of blood, we hesistate to use the same rabbit for a second experiment without letting it rest for a long enough time. In practice, as many subjects should be used as there are experiments; in other words, more or less running the risk of meeting up with the individual differences which appear from time to time in physiological research.

By choosing a dog for the experiments which we are describing today, we were able, in contrast, to perform each series on the same animal; a few days of rest sufficed between injections; the agreement of the results obtained with insulin alone at the beginning and the end of each series, proved to us that the sensitivity of the animals to insulin actually remained the same during the course of the experiments.

We used the insulin preparation that had been used in series I of our experiments on rabbits. This preparation was injected after preliminary

(1) Comptes rendus, 182, 1926, p. 1504.

attempts on a first animal, in a dose of three clinical units to a 9.2 kg dog, and a dose of five clinical units to 13.3 kg dog.

The solutions of nickel and cobalt were the same as in previous experiments; the amounts injected are indicated on the graphs. These also mention the dates on which the injections were given.

As can be easily verified by comparing the results which we give today with those which we have already published, nickel and cobalt behave essentially the same in relation to insulin in rabbits and dogs: there is an increase of the activity of the hypoglycemic substance. In the dog as in the rabbit, it seems that cobalt had a greater influence than nickel and also that the two metals together slow down, while at the same time prolonging the effect of insulin.

Following from these results, must nickel and cobalt be considered as being coenzymes of insulin in the way that manganese is the coenzyme of laccase[1]. Are these two metals part of the insulin molecule? And too, are there two substances in insulin, or more concisely in insulin preparations, of which one depends on the action of nickel and the other on that of cobalt?

To answer the first question, it is necessary to be able to make the insulin act in vitro, which still has not been done. It may be that nickel and cobalt only intervene indirectly through the intermediary of a specific organ.

As for the other questions, it is probably going to be simple to answer them soon. J.J. Abel just announced that he had succeeded in obtaining

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[1] In the case of laccase, manganese, playing the role which one of us first characterized by the expression coferment (or codiastase or also coenzyme) (Gabriel Bertrand, Comptes rendus, 124, 1897, p. 1355) and later by active complement (Revue générale des Sciences, 16, 1905, p. 451), is the part of the catalytic system which causes the considered chemical action.

insulin in the crystallized state [2]. When a sufficient quantity of this precious substance will have been prepared, a little of it may be sacrificed for the study of nickel and cobalt.

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[2] Proc. Nat. Acad. Sci., 12, 1925, p. 132. From an extract found in Chemical Abstracts (20, 1926, p. 1494) which does not give the element composition of crystallized insulin.

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13306

3393 7

COMPTES RENDUS

DES SÉANCES

DE L'ACADÉMIE DES SCIENCES.

183 (1) 5-8 (1926)

SÉANCE DU LUNDI 5 JUILLET 1926.

PRÉSIDENCE DE M. CHARLES LALLEMAND.

MÉMOIRES ET COMMUNICATIONS

DES MEMBRES ET DES CORRESPONDANTS DE L'ACADÉMIE.

M. le MINISTRE DE L'INSTRUCTION PUBLIQUE ET DES BEAUX-ARTS adresse ampliation du décret, en date du 29 juin 1926, qui porte approbation de l'élection que l'Académie a faite de M. PIERRE WEISS pour occuper la place de Membre non résidant vacante par le décès de M. *G. Gouy*.

Il est donné lecture de ce décret.

M. le PRÉSIDENT souhaite la bienvenue à M. FARID BOULAD BEY, membre de l'Institut d'Égypte, qui assiste à la séance.

CHIMIE BIOLOGIQUE. — *Influence du nickel et du cobalt sur l'action exercée par l'insuline chez le chien.* Note de MM. GABRIEL BERTRAND et M. MÂCHEBŒUF.

Les expériences que nous avons publiées récemment (1) ont montré que certaines préparations d'insuline agissent d'une manière plus intense sur la

(1) *Comptes rendus*, 182, 1926, p. 1504.

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glycémie du lapin lorsqu'on les injecte en même temps que de petites quantités de nickel et de cobalt que lorsqu'on les injecte sans addition des mêmes métaux.

Avant de tirer de ces expériences les conséquences qu'elles comportent et surtout d'en généraliser la portée à l'homme, il nous a paru nécessaire de les répéter sur un animal plus élevé en organisation et adapté à un régime non exclusivement herbivore.

Nous n'avions d'ailleurs pas que ces raisons théoriques pour opérer sur une nouvelle espèce. Lorsque, dans le but de suivre l'action hypoglycémiante de l'insuline, on a ponctionné le cœur à cinq ou six reprises dans la même journée et prélevé chaque fois 2^{cm^3} à 3^{cm^3} de sang, on hésite à se servir du même lapin pour une seconde expérience sans l'avoir laissé reposer assez longtemps. Pratiquement, on doit donc recourir à autant d'individus qu'il y a d'expériences, c'est-à-dire courir plus ou moins le risque de rencontrer une de ces différences individuelles qui apparaissent de temps en temps dans les recherches physiologiques.

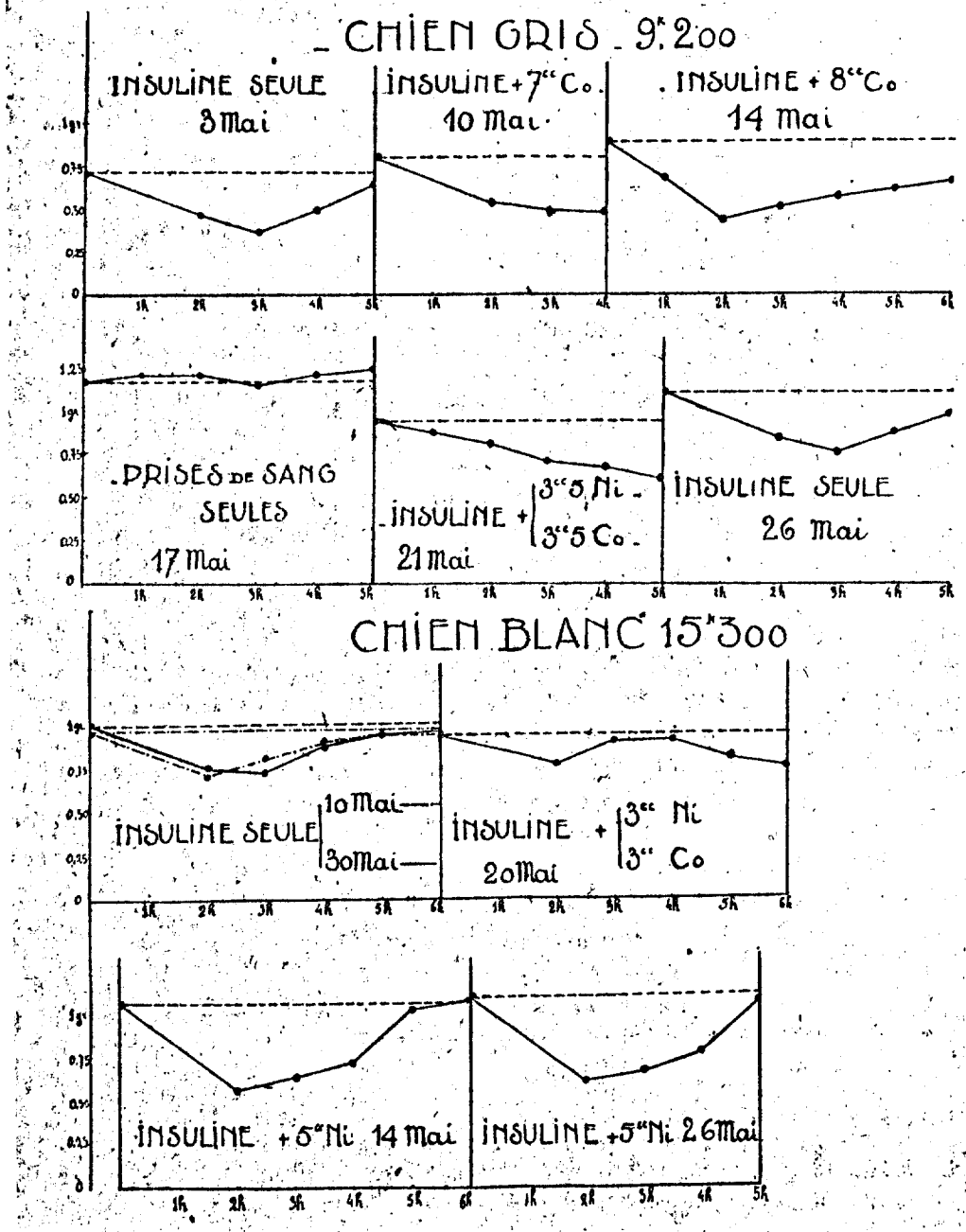
En choisissant le chien pour les expériences que nous décrivons aujourd'hui, nous avons pu, au contraire, effectuer chaque série sur le même animal; quelques jours de repos ont suffi entre les injections; la concordance des résultats obtenus avec l'insuline seule, au commencement et à la fin de chaque série, nous a prouvé, en effet que la sensibilité des animaux à l'insuline était restée la même au cours des expériences.

Nous avons employé la préparation d'insuline qui avait servi dans la série 1 de nos expériences sur le lapin. Cette préparation a été injectée, après essais préliminaires sur un premier animal, à la dose de trois unités cliniques au chien de $9^{kg},2$ et à la dose de cinq unités cliniques au chien de $13^{kg},3$.

Les solutions de nickel et de cobalt ont été les mêmes que dans les expériences antérieures; les doses injectées sont indiquées sur les graphiques. Ceux-ci mentionnent en outre les dates auxquelles ont été effectuées les injections.

Comme on peut aisément le constater en comparant les résultats que nous donnons aujourd'hui avec ceux que nous avons déjà publiés, le nickel et le cobalt se comportent sensiblement de la même manière vis-à-vis de l'insuline chez le lapin et chez le chien : il y a augmentation d'activité de la substance hypoglycémiante. Chez le chien comme chez le lapin, il semble que le cobalt ait une influence plus grande que le nickel et aussi que les deux métaux réunis ralentissent tout en la prolongeant l'action de l'insuline.

Doit-on, en conséquence de ces résultats, considérer le nickel et le cobalt



comme étant les coferments de l'insuline au même titre que le manganèse

est le coferment de la laccase (¹)? Ces deux métaux font-ils partie de la molécule de l'insuline? Et même, y a-t-il deux substances dans l'insuline, ou plus exactement dans les préparations d'insuline, dont l'une dépend de l'action du nickel et l'autre de celle du cobalt?

Pour répondre à la première question, il faudrait pouvoir faire agir l'insuline *in vitro*, ce à quoi l'on n'est pas encore parvenu. Il se pourrait que le nickel et le cobalt n'interviennent que d'une manière indirecte, par l'intermédiaire d'un organe déterminé.

Quant aux autres questions, il va probablement être facile d'y répondre bientôt. J.-J. Abel vient d'annoncer qu'il avait réussi à obtenir l'insuline à l'état cristallisé (²). Lorsqu'une quantité suffisante de cette précieuse substance aura été préparée, il pourra en être sacrifié un peu pour y rechercher le nickel et le cobalt.

M. Ch. Moureu s'exprime en ces termes :

J'ai l'honneur de présenter à l'Académie, au nom de notre confrère M. G. Urbain, en mon nom personnel, et à celui de MM. A. Lepape, J. Bardet et de nos autres collaborateurs, un mémoire que vient de publier les *Annales de l'Institut d'Hydrologie et de Climatologie* (t. 3, n° 3, 1925) sur l'*Analyse des eaux thermales de Vichy*.

Ce long et important travail inaugure les recherches d'ordre analytique que l'Institut d'Hydrologie a pour mission d'exécuter sur les sources hydrominérales françaises. Il comprend des mesures faites sur place, aux sources mêmes, et des déterminations faites à nos laboratoires à Paris. Six des sources les plus chaudes de Vichy ont été examinées; les recherches les plus approfondies ont porté sur les trois sources chaudes principales (Grande Grille, Chomel, Hôpital) de notre grande station thermale.

La composition de ces eaux n'a fait l'objet d'aucun travail complet depuis les célèbres recherches de Willm, qui datent de 1881.

(¹) Dans le cas de la laccase, le manganèse, jouant le rôle que l'un de nous a d'abord caractérisé par l'expression coferment (ou codiastase, ou aussi coenzyme) (Gabriel Bertrand, *Comptes rendus*, 124, 1897, p. 1355), et plus tard de complémentaire active (*Revue générale des Sciences*, 16, 1905, p. 451), est la partie du système catalytique qui provoque l'action chimique considérée.

(²) *Proc. Nat. Acad. Sci.*, 12, 1926, p. 132. D'après un extrait contenu dans *Chemical Abstracts* (20, 1926, p. 1494), qui ne donne pas la composition élémentaire de l'insuline cristallisée.

Comptes Rendus 182:1504 (1926)

Biological Chemistry--Influence of Nickel and Cobalt on the Effect Exerted by Insulin in Rabbits

By: Gabriel Bertrand and M. Macheboeuf[1]

As we have recently reported, the pancreas is one of the organs richest in nickel and in cobalt. Furthermore, the insulin substances which are taken from it are richer still; this is why for equal weights, a few hundred times more nickel and cobalt can be found in these substances than can be found in the glands from which it is extracted[2].

Related to these results of the role played by manganese in the oxydasic effect of laccase [3], we studied whether nickel and cobalt intervened in some way on the hypoglycemic effect of insulin.

After some preliminary experiments, we prepared with sea water made isotonic, some solutions containing 0.1 mg/cc of either nickel or cobalt (in the form of sulfate or chloride); then we compared the hypoglycemic effect of insulin in the absence and presence of a known proportion of each of these metallic solutions or their mixture. Further, before being able to measure, if it had taken place, the effect of the metals on glycemia, we tested the mineral solutions without insulin. Let us immediately mention that at amounts (of 0.05 to 0.01 mg/kg of animal) with which we ordinarily experimented, the two metals were without appreciable effects.

The animals used had been fasted since the evening (last meal at 15h). The solutions were injected into them under the skin of the abdomen, immediately after a first blood sampling. This was taken directly from the heart. The process was continued in the same way, generally every hour as is indicated in the graphs.

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(1) Meeting of June 14, 1920.

(2) Comptes Rendes, 182, 1926, p. 1305.

(3) Gabriel Bertrand, Comptes Rendus, 124, 1897, p. 1032 and 1055.

In the experiments on the rabbits which we report today, the weight of the animals was between 1500 and 2000 g and as close as possible in each series.

The insulin used was in amounts of 2/3 of a clinical unit per 1500 g of animal in the first two experiments and 2/3 of a clinical unit per 2000 g of animal in the third.

As for the metallic solutions, they were injected, except for the contrary indications in the graphs, in an amount of 1 cm^3 per 1500 g of animal in every series. For the experiments with nickel and cobalt, 1 cm^3 of a mixture of equal parts of the two solutions was injected in place of 1 cm^3 of each of them.

Finally, note that with the exception of the experiments in the first series, the others were done twice, in other words on two animals at a time. The graphs, then, give the average of the results obtained.

The influence of nickel and cobalt was different according to the preparations of insulin. This should be expected since these preparations had various amounts of these metals. This was the case in one of the preparations for which we found:

	Experimental Sample		Proportions in Thousandths of mg per 100 units	
	clinical	organic	Ni	Co
Commercial preparation in lactosed powder	1000	880 mg	6	60

furnished almost exactly the same results on the rabbit in the presence and in the absence of two metals, tried in usual amounts of 0.06 mg per kilogram of animal. When we repeated the experiments with this preparation tripling or sextupling the amount of metal, there was a decrease, then a cessation of the hypoglycemic effect of insulin, at least during the four hours following the injections. This can be seen on the graphs which summarize the results of the second series of experiments.

With other preparations, by contrast, nickel and cobalt increased in a significant proportion the quantity of sugar destroyed. It can be judged at first glance by comparing the included areas, on each of the graphs of series I and III, between the right of the constant ordinate equal to initial glycemia and the curve of the amounts of blood sugar 4 to 5 hours following the injection.

A difference is noted in this remarkable activation of insulin by nickel and cobalt according to whether the metals are added alone or together.

Added alone, they do not sensitively modify the speed of the hypoglycemic action, they intensify and prolong it. The two metals then behave nearly the same. All the same, it seemed that with the preparations which we used, cobalt was a little more active than nickel.

If both metals are added to insulin at the same time, half of each one, there must then be a superimposition of the effect previously obtained. To the contrary, the hypoglycemia curve most often shows a different aspect: the speed of the destruction of sugar is slowed down while its duration is prolonged more than when the metals are added separately. It is as if the mixture of nickel and cobalt moderates the effect of insulin, regularizing it and making it more durable. This can be seen well by examining the graphs of first series of experiments.

The facts which we just described include theoretical and practical results which we propose to examine in new experiments, destined to complete the preceding ones. Today, we will content ourselves, with bringing out the possibility of qualitative and quantitative improvement of insulin preparations by the addition in proper proportions of nickel and cobalt.

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Comptes Rendus 182: 1504 (1926)

CHIMIE BIOLOGIQUE. — *Influence du nickel et du cobalt sur l'action exercée par l'insuline, chez le lapin.* Note [1] de MM. **Gabriel Bertrand** et **M. Mâcheboeuf.**

Comme nous l'avons communiqué récemment, le pancréas est un des organes les plus riches en nickel et en cobalt. En outre, les préparations d'insuline qu'on en tire sont encore plus riches : c'est ainsi qu'à poids égal on peut trouver des centaines de fois plus de nickel et de cobalt dans ces préparations que dans les glandes d'où elles ont été extraites [2].

Rapprochant ces résultats du rôle joué par le manganèse dans l'action oxydasique de la laccase [3], nous avons cherché si le nickel et le cobalt intervenaient d'une manière quelconque dans l'action hypoglycémiante de l'insuline.

Après quelques essais d'orientation, nous avons préparé, avec de l'eau de mer rendue isotonique, des solutions renfermant $0^{mg},1$ par centimètre cube soit de nickel, soit de cobalt (à l'état de sulfate ou de chlorure), puis nous avons comparé l'action hypoglycémiante de l'insuline en l'absence et en présence d'une proportion déterminée de chacune de ces solutions métalliques ou de leur mélange. En outre, afin de pouvoir tenir compte, s'il y avait lieu, de l'action des métaux sur la glycémie, nous avons essayé les solutions minérales sans insuline. Mentionnons tout de suite qu'aux doses (de $0^{mg},05$ à $0^{mg},01$ par kilogramme d'animal) auxquelles nous les avons ordinairement expérimentés, les deux métaux étaient sans effet appréciable.

Les animaux utilisés étaient à jeun depuis la veille (dernier repas à 15^h). On leur injectait les solutions sous la peau de l'abdomen, immédiatement après une première prise de sang. Celle-ci était faite directement dans le cœur. Il en était de même des suivantes, répétées généralement d'heure en heure, comme il est indiqué sur les graphiques.

Dans les expériences sur les lapins que nous rapportons aujourd'hui, les poids des animaux étaient compris entre 1500 et 2000^g et aussi voisins que possible dans chaque série.

L'insuline a été employée à la dose de $\frac{2}{5}$ d'unité clinique par 1500^g

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(1) Séance du 14 juin 1926.

(2) *Comptes rendus*, 182, 1926, p. 1305.

(3) Gabriel Bertrand, *Comptes rendus*, 124, 1897, p. 1032 et 1355.

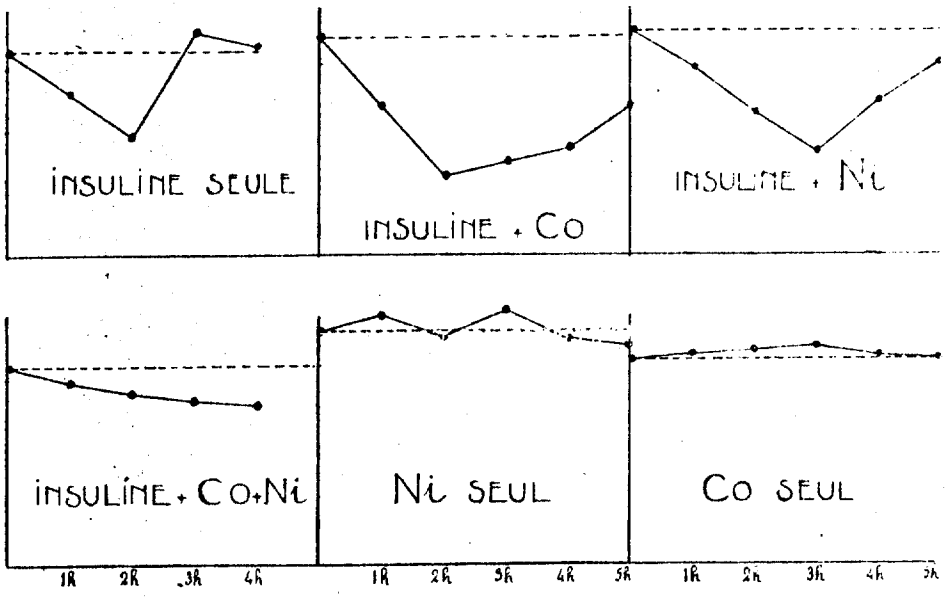
INSULINE SEULE
INSULINE + Co
INSULINE + Ni
INSULINE + Co+Ni
Ni SEUL
Co SEUL
1h 2h 3h 4h
1h 2h 3h 4h 5h
1h 2h 3h 4h 5h

Fig. 1.

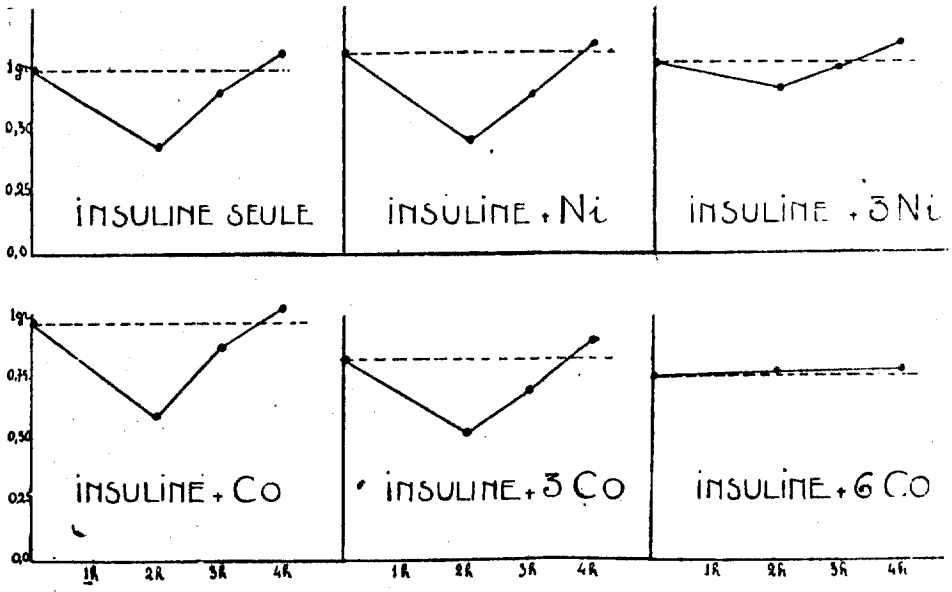
1g
0,50
0,25
0,0
INSULINE SEULE
INSULINE + Ni
INSULINE + 3 Ni
INSULINE + Co
INSULINE + 3 Co
INSULINE + 6 Co
1h 2h 3h 4h

Fig. 2.

d'animal dans les deux premières séries d'expériences et de $\frac{2}{3}$ d'unité clinique par 2000^{g} d'animal dans la troisième.

Quant aux solutions métalliques, on les a injectées, sauf indications con-

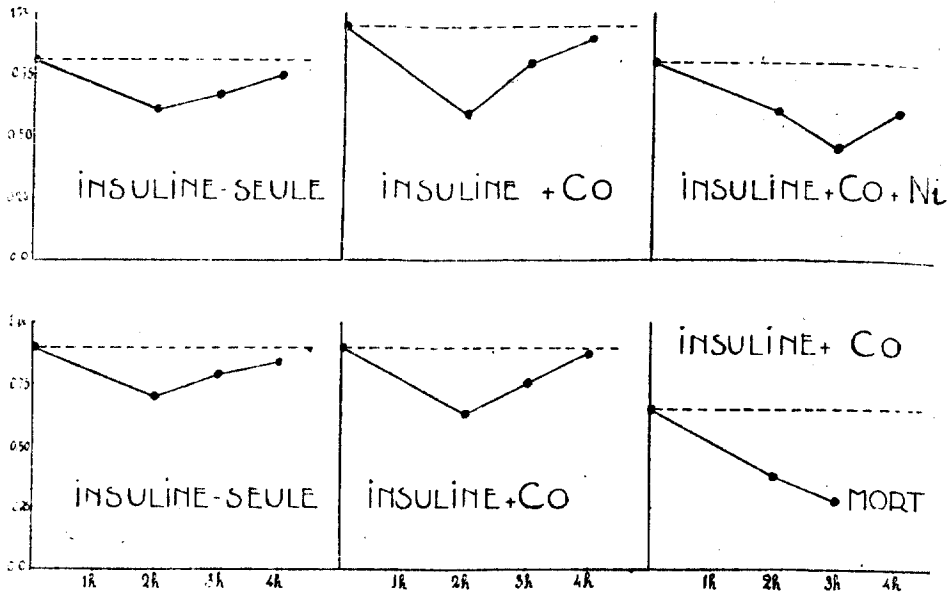

Fig. 3.

traires mentionnées sur les graphiques, à la dose de 1$^{cm^3}$ par 1500^{g} d'animal dans toutes les séries. Pour les expériences avec le nickel et le cobalt réunis, on a injecté 1$^{cm^3}$ du mélange à parties égales des deux solutions au lieu de 1$^{cm^3}$ de chacune d'elles.

Notons enfin qu'à l'exception des expériences de la première série, les autres ont été faites en double, c'est-à-dire sur deux animaux à la fois. Les graphiques donnent alors la moyenne des résultats obtenus.

L'influence du nickel et du cobalt a été différente suivant les préparations d'insuline. Il fallait d'ailleurs s'y attendre, puisque ces préparations offraient des teneurs variables en métaux. C'est ainsi qu'une des préparations dans laquelle nous avions trouvé :

	Prise d'essai en		Proportions en millièmes de milligramme pour 100 unités.	
	unités cliniques.	matières organiques.	Ni.	Co.
Préparation commerciale en poudre lactosée....	100	880^{mg}	6	60

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a fourni presque exactement les mêmes résultats sur le lapin en présence et en l'absence des deux métaux, essayés à la dose ordinaire de $0^{mg},06$ par kilogramme d'animal. Lorsque nous avons répété les expériences avec cette préparation en triplant et en sextuplant la dose de métal, il y a eu diminution, puis arrêt de l'action hypoglycémiante de l'insuline, au moins pendant les quatre heures qui ont suivi les injections. C'est ce qu'on voit sur les graphiques du tableau qui résume les résultats de la deuxième série d'expériences.

Avec d'autres préparations, au contraire, le nickel et le cobalt ont augmenté dans une proportion importante la quantité de sucre détruit. On en jugera au premier coup d'œil en comparant les surfaces comprises, sur chacun des graphiques des séries 1 et 3, entre la droite d'ordonnée constante égale à la glycémie initiale et la courbe des teneurs en sucre sanguin pendant les 4 à 5 heures qui suivent l'injection.

Une différence est à noter dans cette activation remarquable de l'insuline par le nickel et le cobalt suivant que les métaux sont ajoutés seuls ou associés.

Ajoutés seuls, ils ne modifient pas d'une manière sensible la vitesse de l'action hypoglycémiante, ils l'intensifient et la prolongent. Les deux métaux se comportent alors à peu près l'un comme l'autre. Tout au plus semble-t-il, avec les préparations dont nous nous sommes servis, que le cobalt soit un peu plus actif que le nickel.

Si l'on ajoute à la fois les deux métaux à l'insuline, moitié l'un et moitié l'autre, il devrait donc y avoir superposition de l'effet obtenu avec les précédents. Au contraire, la courbe d'hypoglycémie présente le plus souvent alors une autre allure : la vitesse de la destruction du sucre est ralentie tandis que sa durée est prolongée plus longuement que par les métaux ajoutés séparément. C'est comme si le mélange de nickel et de cobalt amortissait l'action de l'insuline, la régularisait et la rendait plus durable. On se rend très bien compte de cet effet en examinant les graphiques de la première série d'expériences.

Les faits que nous venons d'exposer comportent des conséquences théoriques et pratiques que nous nous proposons d'examiner à la suite de nouvelles expériences, destinées à compléter les précédentes. Nous nous contenterons, pour aujourd'hui, de faire ressortir la possibilité d'amélioration qualitative et quantitative des préparations d'insuline par l'addition, en proportions convenables, de nickel et de cobalt.

Comptes Rendus 183: 257 (1926)

Chemotherapy--Nickel, Cobalt and Diabetes

By: Gabriel Bertrand and M. Mâcheboeuf

In making known the results of our experiments on the distribution of nickel and cobalt in the organs of humans and animals, we drew attention to the relatively high amount in the pancreas (1).

Following this, guided by a whole series of research on the role of manganese and other elements in biological processes, we succeeded in discovering an influence of these new elements in living material on certain insulin preparations (2).

As diabetes or, more exactly certain forms of diabetes, depend on either an anatomic or perhaps simply a functional alteration of the pancreas, it was not illogical to study whether using small quantities of nickel and cobalt introduced into the general circulation, a cure or at least an improvement of this illness could be obtained.

We began this research more than a year ago, first with nickel or cobalt alone, then with the two metals together. We used for this saline solutions for which we gave the formulas in describing our physiological experiments (3). These solutions which may be added in various proportions to egg whites, to horse serum or human serum without producing the least coagulation or the least cloudiness, are very well-tolerated locally, even in subcutaneous injections: they cause no pain and are easily absorbed. At the beginning of the treatment, as it is still being done today, we inject 5 cm^3 of solution every two days: after 4 to 5 injections, we continue with 10 cm^3 injections every two days.

Even with these very small amounts (we note that there is only 0.1 mg of the metals under consideration per cubic centimeter), ordinarily under the influence of the first injections, there is observed a more or less clear

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(1) Comptes rendus, 182, 1926, p. 1305.

(2) Comptes rendus, 182, 1926, p. 1504, and 183, 1926, p. 5.

(3) It must be sterilized in the cold, by filtration with a porcelain bouqie.

increase of the quantity of sugar eliminated in 24 hours, this increase of glycosuria being accompanied by a more significant glycemia and polyuria.

Then, there appeared striking differences between the cases of diabetes:

In some (5 children out of 5 treated, adults and old people afflicted with a consumptive type), there was no improvement, and in view of the actual condition, it was thought preferable to suspend the treatment.

On the other hand, in other cases, we found partial or complete return to diuresis of normal volume, with corresponding decrease of thirst, a progressive decrease sometimes leading to a more or less stable suppression of urinary sugar, an increased tolerance for glucides, sometimes, but not always, a lowering of the glycemia.

One aspect of the new treatment which we describe is particularly interesting to think about: that is the administration of nickel and cobalt, not by hypodermic injection, but by mouth. It seems that given the diffusibility of the two metals where they are found in solution, the distribution throughout the organism must also be effected by the second route as well as the first. The first experiments which we did in this vein seemed to well justify the theoretical conjecture.

Let us finally note the case of an old diabetic, subjected without interruption for two years to insulin treatment (two injections of 20 clinical units each day) in whom we obtained a greater tolerance for glucides and a reduction of 25% of the quantity of injected insulin by the simple oral absorption of 10 g of the mixed solution of nickel and cobalt each day an hour before the main meal.

All these experiments are encouraging, but we know we can only consider

them as preliminary. We would have preferred before publishing the results, to increase their number and organize them systematically, to examine carefully the differences between nickle and cobalt used alone or together, to study whether there are types of diabetes particularly sensitive to the effect of one or the other of these metals, etc.. But it would have been necessary for this to use much time and material which we lack. Non-complicated cases of diabetes are not ordinarily hospitalized; but without hospitalization, in other words, without effective surveillance, it is very difficult to be sure of the effects of the treatment. We thought that it would be better under these conditions, to bring our first observations to the attention of physiologist and doctors and thus to encourage an increase in research.

Several specialists having all the desirable authority have already been made aware of these experiments; we will let them publish the results of their personal observations (1).

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(1) See below, p. 326, article by F. Rathery and Miss L. Levina.

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Comptes Rendus 183: 257 (1926)

ACADÉMIE DES SCIENCES.

SÉANCE DU LUNDI 26 JUILLET 1926.

PRÉSIDENCE DE M. CHARLES LALLEMAND.

MÉMOIRES ET COMMUNICATIONS
DES MEMBRES ET DES CORRESPONDANTS DE L'ACADÉMIE.

CHIMIOTHÉRAPIE. — *Nickel, cobalt et diabète.* Note de MM. GABRIEL BERTRAND et M. MÂCHEBŒUF.

En faisant connaître les résultats de nos expériences sur la distribution du nickel et du cobalt dans les organes de l'homme et des animaux, nous avons attiré l'attention sur la teneur relativement élevée du pancréas (1).

Dans la suite, guidés par toute une série de recherches sur le rôle du manganèse et d'autres éléments dans les processus biologiques, nous avons réussi à découvrir une influence des deux nouveaux éléments de la matière vivante sur certaines préparations d'insuline (2).

Comme le diabète ou, plus exactement, certaines formes de diabète dépendent d'une altération soit anatomique, soit peut-être simplement fonctionnelle du pancréas, il n'était pas illogique de rechercher si, à l'aide de petites quantités de nickel et de cobalt, introduites dans la circulation générale, on pourrait obtenir la guérison ou tout au moins l'amélioration de certains cas de la maladie.

Nous avons commencé cette recherche il y a plus d'une année, d'abord avec le nickel ou le cobalt seuls, puis avec les deux métaux réunis. Nous avons utilisé pour cela les solutions salines dont nous avons donné les formules en décrivant nos expériences physiologiques (3). Ces solutions, qui

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(1) *Comptes rendus*, 182, 1926, p. 1305.
(2) *Comptes rendus*, 182, 1926, p. 1504, et 183, 1926, p. 5.
(3) Elles doivent être stérilisées à froid, par filtration à la bougie de porcelaine.

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peuvent être ajoutées en proportions diverses à du blanc d'œuf, à du sérum de cheval ou à du sérum humain sans produire la moindre coagulation ni le moindre louche, sont très bien supportées localement, même en injections sous-cutanées : elles ne provoquent aucune douleur et se résorbent avec facilité. Au début du traitement, tel qu'il est encore expérimenté aujourd'hui, on injecte 5^{cm^3} de solution tous les deux jours; après 4 à 5 injections, on continue à raison de 10^{cm^3} tous les deux jours.

Même avec ces très petites doses (nous rappelons qu'il y a seulement $0^{mg},1$ des métaux considérés par centimètre cube), on observe ordinairement, sous l'influence des premières injections, une augmentation plus ou moins nette de la quantité de sucre éliminé en 24 heures, cette augmentation de la glycosurie pouvant être accompagnée d'une plus grande glycémie et d'une plus grande polyurie.

Puis apparaissent des différences frappantes entre les cas de diabète :

Dans les uns (5 enfants sur 5 traités, adultes et vieillards atteints de diabète du type consomptif), il n'y a pas eu d'amélioration et, dans l'état actuel de la question, on a cru préférable de suspendre le traitement;

Dans les autres cas, au contraire, on a constaté un retour partiel ou complet de la diurèse au volume normal, avec disparition concomitante de la soif, une diminution progressive allant parfois jusqu'à la suppression plus ou moins durable du sucre urinaire, une tolérance augmentée vis-à-vis des glucides, quelquefois, mais pas toujours, un abaissement de la glycémie.

Une perspective du nouveau traitement que nous indiquons est particulièrement intéressante à envisager : c'est l'administration du nickel et du cobalt, non plus par injection hypodermique, mais par la bouche. Il semble, en effet, étant donnée la diffusibilité des deux métaux, tels qu'ils se trouvent en solution, que la pénétration dans l'organisme doive être aussi assurée par la seconde voie que par la précédente. Les premières expériences que nous avons faites dans ce sens semblent bien justifier la prévision théorique.

Signalons enfin le cas d'un diabétique ancien, soumis depuis deux années sans interruption au traitement par l'insuline (deux injections de 20 unités cliniques chaque jour), chez lequel nous avons obtenu une plus grande tolérance aux glucides et une réduction de 25 pour 100 de la quantité d'insuline injectée par simple absorption buccale de 10^{g} de solution mixte de nickel et de cobalt, chaque jour, une heure avant le principal repas.

Toutes ces expériences sont encourageantes, mais nous ne saurions les

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considérer qu'au titre de préliminaires. Nous aurions préféré, avant d'en publier les résultats, les multiplier et les systématiser, examiner avec soin les différences que peuvent présenter le nickel et le cobalt, employés seuls ou associés, rechercher même s'il n'y a pas des cas de diabète particulièrement sensibles à l'action de l'un ou de l'autre de ces métaux, etc. Mais il aurait fallu pour cela disposer de beaucoup de temps et de moyens qui nous manquent. On n'a pas l'habitude d'hospitaliser les cas de diabète non compliqués; or, sans hospitalisation, c'est-à-dire sans une surveillance effective, on ne peut que très difficilement s'assurer des effets d'un traitement. Nous avons pensé qu'il valait mieux, dans ces conditions, porter nos premières observations à la connaissance des physiologistes et des médecins, et favoriser ainsi la multiplication des recherches.

Plusieurs spécialistes, ayant toute l'autorité désirable, ont déjà été mis au courant de ces expériences; nous leur laisserons publier les résultats de leurs observations personnelles [1].

HYDRAULIQUE. — *Détermination expérimentale du coefficient de débit des tuyères fonctionnant en écoulement libre.* Note [2] de MM. **Rateau, Leroux** et **Bourgeat**.

A la suggestion de M. Rateau, et suivant un programme arrêté par lui, des expériences ont été entreprises en vue de la détermination précise du coefficient m de débit de tuyères, fonctionnant en écoulement libre, défini par $q = m\omega\sqrt{2gh}$, où ω est la section de sortie.

Les tuyères étudiées, dont les profils ont été dessinés dans l'idée d'atteindre les plus forts coefficients possibles avec des lignes simples, sont de deux types; les figures 1 et 2 indiquent leurs formes et leurs dimensions relatives en fonction du diamètre de sortie pris comme unité. Il a été construit 6 tuyères en bronze, calibrées au $\frac{1}{100}$ de millimètre, 4 du type A, de diamètres 1, 2, 4 et 8^{cm}, et 2 du type B, de diamètres 2 et 4^{cm}. Pour les deux tuyères de 2^{cm}, l'ajutage cylindrique, de 0,2 diamètre de longueur, est amovible; on a pu ainsi opérer avec ou sans lui.

Les expériences ont consisté à alimenter chaque tuyère sous des charges variant, en progression géométrique, entre 0,1 et 8^{m}, et à mesurer, avec

(1) Voir ci-dessous, p. 326, la Note de M. F. Rathery et Mlle L. Levina.

(2) Séance du 19 juillet 1926.

Ann. Inst. Pasteur 47:451-454, 1931

On the Toxicity of Aluminum Compared to that of Iron, Nickel and Other Metals

by Gabriel Bertrand and P. Serbescu

The usage of aluminum cooking utensils and artificial leavens, called "chemical leavens" or "baking powders" containing aluminum-sodium alums are causes for the introduction of aluminum into the human organism. These usages are increasingly widespread. It is therefore not surprising that during the last few years, fears of their chronic harmful effects have been expressed; it has even been suggested that daily ingestion of minimal quantities of aluminum favors the development of cancer.

Our actual knowledge unfortunately does not enable us to say to what degree these fears may be unfounded or confirmed.

A fair number of researchers has studied the effects of several aluminum compounds on laboratory animals and in a few cases, even determined the lethal doses (1); but it is not possible to deduce from the publication of their results what would happen in man as a result of the artificial introduction of small amounts in food products.

It seems to us that we would already have an initial idea of the overall harmfulness of aluminum to the human organism by measuring the toxicity of this metal, not in relation to the various species of animals, but in comparison to the toxicity of other metals and in particular metals which are currently

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(1) See especially: E. Smith, Aluminum compounds in food, New York, 1928; E. MacCollum, O. Rask and E. Becker (J. Biol. Chem. 77, 1928, p. 753); V. Myers and J. Moll (Ibid. 78, 1928, p. 60); V. Myers and D. Morrison (Ibid. p. 615); F. Underhil, F. Peterman and A. Sperandeo (Am. J. Physiol., 90, 1929, p. 76); K. MacKensie (Biochem. J., 24, 1930, p. 1433). An analysis of previous work can be found in these publications.

used, such as copper or nickel in the manufacture of kitchen utensils or which are in contact with food products, such as iron and zinc.

The degree of toxicity is a very contingent measurement: not only does it vary greatly with the method of administration, but it also depends, all other things being equal, on the time encompassed between the beginning of poisoning and the moment of death, considered as the criterion.

In the experiments whose results we are today publishing, we injected under the abdominal skin an equal weight of the metals being compared, and it was the duration of survival after the injection which served as the measure of toxicity.

The metals were weighed in the crystallized sulfate state, except for trivalent iron which was used in the chloride form.(1) They were dissolved to a concentration of 1 gram in 1 liter of redistilled water and injected into guinea pigs in a constant dose of 100 mg per kilogram of live bodyweight. The solutions were injected far under the skin so as to avoid losses of liquid at the point of entry of the needle after the injection. Finally, to compensate for the differences in sensitivity among the guinea pigs, we did each experiment five times for each metal.

We observed during the course of the preliminary experiments that when large doses are used, larger than what we chose, death frequently occurred before the complete absorption of the injected liquid; this happened several

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(1) See especially: E. Smith, Aluminum compounds in food, New York, 1928; E. MacCollum, O. Rask and E. Becker (J. Biol. Chem. 77, 1928, p. 753); V. Myers and J. Moll (Ibid. 78, 1928, p. 60); V. Myers and D. Morrison (Ibid. p. 615); F. Underhill, F. Peterman and A. Sperandeo (Am. J. Physiol., 90, 1929, p. 76); K. MacKensie (Biochem, J., 24, 1930, p. 1933). An analysis of pervious work can be found in these publications.

times with an amount of 100 mg per kilogram, especially in the case of copper. As a result, the animals were always autopsied immediately after death and when a little of the metallic solution remained at the point of inoculation, the experiment was begun again. When, in contrast, lower doses were used, the individual differences were accentuated; we could no longer foresee even approximately, the duration of survival following the injection and observation of the animal up until the time of death became difficult.

Here, arranged in the order of the decreasing toxicity of the metals studied, are the results which we obtained:

We obtained the same differences in toxicity among copper, nickel and aluminum using rabbits, also given subcutaneous injections in doses of 100 mg per kilogram live bodyweight.

When, in contrast, we used lower doses, the individual differences were accentuated at the same time as the duration of survival was lengthened.

Also, these results show that aluminum is ranked next to iron and is as a result, much less toxic than nickel and copper, used as aluminum is in the manufacture of kitchen utensils.

In the absence of contrary evidence, it seems that the fears of harmful effects of minimal quantities of alumium artificially introduced into food products, are not founded, at least as long as the quantities remain on the order of those which exist in the normal state in plant and animal tissues (1).

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(1) See principally: E. Smith (loc. cit.): F. Underhill, F. Peterman, Gnors and Krausse (Am. J. Physiol. 90, 1929, p. 72); O. Winter and O. Bird (J. Am. Chem. Soc., 51, 1929, p. 2964); O. Rask and E. MacCollum (J. Md. Acad. Sci., 1, 1930, p. 38); I. Tourtelotte and O. Rask (Ind. Eng. Chem., Anal. edit., 3, 1931, p. 97); G. Bertrand and G. Levy; (C.R., 192, 1931, p. 525).

TABLE 1

1 Métaux étudiés		2 Sels utilisés		7 Poids molec. / Poids atom.	8 Poids des cobayes en grammes	9 Durée de survie en heures	
3 Noms	4 Poids atom.	5 Formules	6 Poids molec.			10 Individuelles	11 moyennes
12 Cuivre	63,57	$SO^4Cu, 5H^2O$	250,25	3,94	300	0,50	0,52
					292	0,58	
					297	1,00	
					280	0,47	
					320	0,44	
Nickel	58,69	$SO^4Ni, 7H^2O$	281,62	4,80	290	0,50	1,00
					320	1,10	
					357	1,10	
					348	0,35	
					375	1,15	
Cadmium	112,41	$SO^4Cd, 8H^2O$	352,59	3,14	672	1,36	1,19
					717	1,36	
					607	1,25	
					372	1,05	
					315	0,50	
Cobalt	58,94	$SO^4Co, 7H^2O$	281,87	4,78	320	1,51	2,17
					281	2,52	
					287	2,37	
					277	2,53	
					375	1,10	
Zinc	65,38	$SO^4Zn, 7H^2O$	288,31	4,41	405	3,00	3,01
					310	3,50	
					297	2,13	
					323	3,47	
					270	2,15	
Manganèse	54,93	$SO^4Mn, 4H^2O$	223,49	4,07	302	16,15	7,14
					277	5,30	
					329	5,14	
					312	5,00	
					380	4,00	
13 Fer trivalent	55,84	$FeCl^3$	162,22	2,90	312	5,20	7,51
					397	5,50	
					358	12,00	
					375	7,35	
					362	8,50	
Aluminium	26,97	$SO^4)^3Al^2, 18H^2O$	666,41	12,35	285	7,05	8,34
					342	7,50	
					312	10,47	
					310	10,48	
					277	6,20	
14 Fer bivalent	55,84	$SO^4Fe, 7H^2O$	278,71	4,99	329	6,00	8,44
					372	9,00	
					392	5,26	
					296	12,00	
					247	5,00	
					282	6,15	

1-Metals studied
2-Salts used
3-Names
4-Atomic weights
5-Formulas
6-Molecular weights
7-$\frac{\text{Molecular weight}}{\text{Atomic weight}}$
8-Weight of the guinea pigs in grams
9-Survival duration in hours
10-Individual
11-Average
12-Copper
13-Trivalent iron
14-Bivalent iron

1 NOMS DES MÉTAUX ÉTUDIÉS	2 POIDS des lapins en grammes	3 SURVIES individuelles en heures	4 SURVIES moyennes en heures
5 Cuivre	2 100 2 100 1 650 2 150	0,55 0,52 0,55 0,50	0,53
Nickel	2 350 2 360 2 300 2 700 1 970	1,10 2,05 0,35 1,00 0,35	1,11
Aluminium	1 820 1 830 2 150 2 100 1 950	7,05 6,30 7,53 8,53 10,35	8,09

1-Names of Metals studied
2-Weight of the rabbits in grams
3-Individual survivals in hours
4-Average survivals in hours
5-Copper

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Ann. Inst. Pasteur
47: 451-454 (1931)

SUR LA TOXICITÉ DE L'ALUMINIUM COMPARÉE A CELLE DU FER, DU NICKEL ET D'AUTRES MÉTAUX

par MM. Gabriel BERTRAND et P. SERBESCU.

L'emploi des ustensiles de cuisine en aluminium et celui des levains artificiels, dits « levures chimiques » ou « baking powders », contenant de l'alun alumino-sodique, sont des causes d'introduction de l'aluminium dans l'organisme humain. Ces emplois se généralisent de plus en plus. Aussi n'est-il pas étonnant qu'on ait formulé à leur sujet, dans ces dernières années, des craintes d'actions nocives chroniques; on a même été jusqu'à prétendre que l'ingestion journalière de minimes quantités d'aluminium favorisait le développement du cancer.

Nos connaissances actuelles ne nous permettent malheureusement pas de dire dans quelle mesure de telles craintes peuvent être soit infirmées soit confirmées.

D'assez nombreux expérimentateurs ont bien étudié les effets de plusieurs combinaisons aluminiques sur les animaux de laboratoire et même déterminé, dans quelques cas, les doses léthales (1); mais il n'est pas possible de se rendre compte, d'après la publication de leurs résultats, de ce que pourraient produire chez l'homme les petites proportions d'aluminium introduites artificiellement dans les substances alimentaires.

Il nous a paru que l'on aurait déjà une première idée de la nocivité globale de l'aluminium pour l'organisme humain en mesurant la toxicité de ce métal, non par rapport à diverses espèces animales, mais comparativement à la toxicité d'autres métaux et, en particulier, de métaux d'usage courant employés, comme le cuivre et le nickel, dans la fabrication des ustensiles

(1) Voir notamment : E. Smith, Aluminium compounds in food, New-York, 1928; E. Mac Collum, O. Rask et E. Becker (*Journ. Biol. Chem.*, 77, 1928, p. 753); V. Myers et J. Moll (*Ibid.* 78, 1928, p. 605); V. Myers et D. Morrison (*Ibid.* p. 615); F. Underhill, F. Peterman et A. Sperandeo (*Amer. Journ. Physiol.*, 90, 1929, p. 76); K. Mackensie (*Bioch. Journ.*, 24, 1930, p. 1433). On trouvera dans ces publications l'analyse des travaux antérieurs.

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de cuisine, ou souvent mis en contact, comme le fer et le zinc, avec des substances alimentaires.

La mesure de la toxicité est une mesure très contingente : non seulement elle varie beaucoup avec le mode d'administration, mais elle dépend encore, toutes choses égales, d'ailleurs, du temps compris entre le début de l'intoxication et le moment de la mort, considéré comme critérium.

Dans les expériences dont nous faisons connaître aujourd'hui les résultats, il a été injecté sous la peau de l'abdomen un même poids de chacun des métaux mis en comparaison et c'est la durée de la survie après l'injection qui sert de mesure à la toxicité.

Les métaux ont été pesés à l'état de sulfates cristallisés, sauf le fer trivalent qui a été pris sous la forme de chlorure (1). Ils ont été dissous à la concentration de 1 gramme dans 1 litre d'eau redistillée et injectés au cobaye, à la dose constante de 100 milligrammes par kilogramme de poids vif. Les solutions ont été poussées loin sous la peau, de manière à éviter des pertes de liquide par le point d'entrée de l'aiguille, après l'injection. Enfin, pour compenser les différences de sensibilité entre les cobayes, nous avons effectué cinq fois l'expérience pour chaque métal.

Nous avons observé, au cours d'expériences préliminaires, que lorsqu'on utilise des doses fortes, supérieures à celle que nous avons choisie, la mort survient fréquemment avant l'absorption complète du liquide injecté; cela s'est même produit quelques fois avec la dose de 100 milligrammes par kilogramme, notamment dans le cas du cuivre. En conséquence, les animaux ont toujours été autopsiés aussitôt après la mort, et lorsqu'il restait un peu de solution métallique au point d'inoculation, l'expérience était recommencée. Quand, au contraire, on utilise des doses inférieures, les différences individuelles s'accentuent, on ne peut plus prévoir, au moins approximativement, la durée de survie consécutive à l'injection, et la surveillance des animaux jusqu'à l'heure de la mort devient difficile.

Voici, rassemblés d'après l'ordre de toxicité décroissante des métaux étudiés, les résultats que nous avons obtenus :

(1) En solution officinale dont le titre a été déterminé par un dosage de sesquioxyde de fer.

TABLEAU I.

MÉTAUX ÉTUDIÉS		SELS UTILISÉS		POIDS MOLÉC. / POIDS ATOM.	POIDS des cobayes en grammes	DURÉE de survie en heures	
Noms	Poids atom.	Formules	Poids moléc.			individuelles	moyennes
Cuivre	63,57	$SO^4Cu, 5H^2O$	250,25	3,94	300 292 297 280 329	0,50 0,58 1,00 0,47 0,44	0,52
Nickel	58,69	$SO^4Ni, 7H^2O$	281,62	4,80	290 320 357 348 375	0,50 1,10 1,10 0,35 1,15	1,00
Cadmium	112,41	$SO^4Cd, 8H^2O$	352,59	3,14	672 717 607 372 315	1,36 1,36 1,25 1,05 0,50	1,19
Cobalt	58,94	$SO^4Co, 7H^2O$	281,87	4,78	320 281 287 377 375	1,51 2,52 2,37 2,55 1,10	2,17
Zinc.	66,38	$SO^4Zn, 7H^2O$	288,31	4,41	405 310 297 328 270	3,00 3,50 2,13 3,47 2,15	3,01
Manganèse . . .	54,93	$SO^4Mn, 4H^2O$	223,49	4,07	302 277 329 312 380	16,15 5,39 5,14 5,00 4,00	7,14
Fer trivalent . .	55,84	$FeCl^3$	162,22	2,90	312 307 308 375 362	5,20 5,30 12,00 7,35 8,50	7,51
Aluminium . . .	26,97	$(SO^4)^3Al^2, 18H^2O$	666,41	12,35	285 342 342 310 277	7,05 7,50 10,47 10,48 6,20	8,34
Fer bivalent. . .	55,84	$SO^4Fe, 7H^2O$	278,77	4,99	329 372 302 296 297 282	6,00 9,00 5,26 12,00 5,00 6,15	8,44

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Nous avons obtenu les mêmes différences de toxicité entre le cuivre, le nickel et l'aluminium en opérant sur les lapins aussi par injection sous-cutanée et à la dose de 100 milligrammes par kilogramme de poids vif.

NOMS DES MÉTAUX ÉTUDIÉS	POIDS des lapins en grammes	SURVIES individuelles en heures	SURVIES moyennes en heures
Cuivre	2 100	0,55	0,53
	2 100	0,52	
	1 650	0,55	
	2 150	0,50	
Nickel	2 350	1,40	1,11
	2 360	2,05	
	2 300	0,35	
	2 700	1,00	
	1 970	0,35	
Aluminium	1 820	7,05	8,09
	1 830	6,30	
	2 150	7,53	
	2 100	8,45	
	1 950	10,35	

Quand, au contraire, on utilise des doses inférieures, les différences individuelles s'accentuent en même temps que les durées de survie s'allongent.

Tous ces résultats montrent que l'aluminium se range à côté du fer, qu'il est, par conséquent, beaucoup moins toxique que le nickel et le cuivre, employés comme lui dans la fabrication des ustensiles de cuisine.

En l'absence de preuve contraire, il semble donc que les craintes d'actions nocives de minimes quantités d'aluminium, introduites artificiellement dans les substances alimentaires, ne soient pas fondées, du moins tant que ces quantités resteront de l'ordre de grandeur de celles qui existent à l'état normal dans les tissus des plantes et des animaux (1).

(1) Voir principalement : E. Smith (*loc. cit.*); F. Underhill, F. Peterman, Gnors et Krause (*Amer. Journ. Physiol*, 90, 1929, p. 72); O. Winter et O. Bird (*Journ. Amer. Chem. Soc.*, 51, 1929, p. 2964); O. Rask et E. Mac Collum (*J. Maryland Acad. Sc.*, 1, 1930, p. 38); I. Tourtelotte et O. Rask (*Ind. and Eng. Chem., Anal., edit.*, 3 1931, p. 97); G. Bertrand et Mme G. Lévy (*C. R.*, 192, 1931, p. 525).

J. Pharm. Chim. 29:410-413, 1939

Comparative Toxicities of Cobalt and Nickel

by

F. Caujolle (1)

The close parallelism which exists from the chemical standpoint between cobalt and nickel explains why these two metals have occupied common stages in the biological studies which have been done on them.

Cobalt and nickel were first of all both considered as very toxic and shared with copper the reputation of being very dangerous; then when Galippe reduced to correct proportions the toxicity attributed to copper, the toxicological study of cobalt and especially of nickel was taken up again. The modern works of Laborde and Riche (12) of Dzerchgowsky and his co-workers on nickel of Chittenden and Norris on cobalt and of Le Goff (14) on both metals have a common general conclusion: "neither cobalt nor nickel are as terrible as the first observations had indicated."

While completely accepting this conclusion, we are not relieved of the necessity of specifying its range. To this end, we are first of all going to recall the experimental data which our co-workers and ourselves have put together (3 to 8, 13).

1-In animals cobalt is always less toxic than nickel; without being very aggressive, it causes more easily than cobalt very clear signs of acute intoxication.

a) In Batrachians-It is consistently observed that tadpoles of frogs die much more quickly in $\frac{M}{1,000}$ or $\frac{M}{10,000}$ solutions of nickel chloride than in the corresponding solutions of cobalt chloride.

(1) Work presented in the March 1, 1939 meeting of the Pharmacy Society of Paris.

b) In Reptiles-Young grass snakes of essentially equal weights were put into tanks containing $\frac{M}{1,000}$, $\frac{M}{10,000}$, $\frac{M}{100,000}$ solutions of nickel and cobalt chloride. At the end of the same time, the percentage of dead subjects was consistently higher with nickel than with cobalt (unedited experiments done in collaboration with Chinonis).

c) In Mammals-With Lafitte (7) and with Canal (5), we have clearly established that the lethal dose may be as low as 20 and 10 mg/kg for nickel while it remains higher than 30 mg/kg for cobalt; these amounts are calculated from the effects resulting from the intravenous injections of chlorides done on dogs under anesthesia. With Argaud, we verified that the histological lesions of the liver and the kidneys were more marked under the effects of nickel than under the effect of equivalent quantities of cobalt (work in progress).

2-In plants

a) In liverworts-For the offshoots of Lunularia cruciata, the lethal dose in Marchal's milieu is 3 p. 10,000 for nickel chloride and 4 p. 10,000 for cobalt chloride (quantities expressed in g of metal), (5, 7, 9); under the influence of the same salts at 2 p. 10,000 the development of offshoots takes place, but the young tissues are much more profoundly altered in the solution of nickel salt than in that of cobalt (Chalaud). Similar results with Fegatella conica.

b) In phanogameric plants-The works done under the direction of Chalaud at the Science Faculty at Toulouse, enabled us to observe in a general way, the alleged toxic effects caused by nickel and cobalt salts on the germination of various Gramineae or Leguminous plants: Triticum, Phaseolus vulgaris and Pisum sativum (3, 13); in general in equivalent doses, the salts of nickel were more toxic than those of cobalt.

3-The salivary and pancreatic amylases were activated by a very large amount of chlorides added in the usual concentration to the milieu; in equal amounts, cobalt chloride is more activating than nickel chloride (1, 3, 8, 13).

There is no doubt that without being very toxic metals, nickel and cobalt cannot be considered as harmless to the entirety of organisms. We think that the lethal doses indicated by Riche and Laborde, at least for administration by the intravenous route of nickel sulfate must be remarked.

The effect of very small amounts of nickel or cobalt salts is not well known. It is probable that a chronic poisoning as occurs with lead poisoning cannot occur with nickel and cobalt because these metals are too unaggressive and especially because their urinary elimination takes place well; but in every case, the possibilities of this chronic poisoning have never up to now been put to the proof of experimentation.

On the other hand, from the pharmacodynamic viewpoint, it is deceptive to underestimate the rapid effects of sufficiently high amounts of nickel and cobalt on the liver and kidney parenchyma, signs which are evidence of a clear toxicity. These data are important to keep in mind, because the natural biological dispositions of these metals are numerous, interesting and in many respects, open up the way for the conception of new therapeutic uses.

Another idea which becomes clear from all the reported facts: nickel is slightly but clearly more toxic than cobalt. In a recent work, Bertrand and Serbescu (2) corroborated this conclusion. This seems to present a double practical interest: on the one hand, the presence of cobalt in nickel plate poses no particular danger; on the other hand, in all food industries where substitution is possible without affecting the yield, nickel should be replaced as the hydrogenation catalyst by cobalt (4).

J. Pharm. Chim. 29: 410-413 (1939)

Toxicités comparées du cobalt et du nickel ;

par F. CAUJOLLE (1).

Le parallélisme étroit qui existe au point de vue chimique entre le cobalt et le nickel explique pourquoi ces deux métaux ont connu des étapes communes dans les études biologiques qui leur ont été consacrées.

Cobalt et nickel furent d'abord considérés tous deux comme très toxiques et partagèrent avec le cuivre la réputation d'être très dangereux ; puis lorsque GALIPPE eut réduit à de justes proportions la toxicité attribuée au cuivre, l'étude toxicologique du cobalt et surtout celle du nickel furent reprises. Les travaux modernes de LABORDE et RICHE [12], de DZERCHGOWSKY et de ses collaborateurs pour le nickel, de CHITTENDEN et NORRIS pour le cobalt, de LE GOFF [14] pour les deux métaux ont une conclusion générale commune : « ni le cobalt, ni le nickel ne sont aussi redoutables que les observations premières l'avaient indiqué ».

Tout en acceptant cette conclusion, nous n'en réservons pas moins la nécessité de préciser sa portée. A cette fin, nous allons tout d'abord rappeler les données expérimentales que nos collaborateurs et nous-mêmes avons pu réunir (3 à 8, 13).

1° Dans la série animale le cobalt est toujours moins toxique que le nickel ; sans être très agressif le nickel détermine toujours plus aisément que le cobalt, des signes très nets d'intoxication aiguë.

a) *Chez les Batraciens.* — Il est constant d'observer que des têtards de crapaud périssent plus vite dans des solution $\frac{M}{1.000}$ ou $\frac{M}{10.000}$ de chlorure de nickel que dans les solutions correspondantes de chlorure de cobalt.

b) *Chez les Reptiles.* — De jeunes couleuvres, de poids sensiblement égal, sont réparties dans des bocaux conte-

(1) Travail présenté à la séance du 1er mars 1939 de la Société de Pharmacie de Paris.

nant des solutions $\frac{M}{1.000}$, $\frac{M}{10.000}$, $\frac{M}{100.000}$ de chlorures de nickel et de cobalt. Au bout d'un même temps le pourcentage des sujets morts est régulièrement plus élevé avec le nickel qu'avec le cobalt (expériences inédites, faites en collaboration avec CHINONIS).

c) *Chez les Mammifères.* — Avec LAFITTE [7] et avec CANAL [5], nous avons nettement établi que la dose mortelle pour le chien pouvait s'abaisser à 20 et même 10 mg. par kilog pour le nickel, tandis qu'elle reste très supérieure à 30 mg. par kilog pour le cobalt ; ces doses sont calculées d'après les effets résultant d'injections intraveineuses des chlorures pratiquées sur des chiens sous anesthésie. Avec ARGAUD, nous avons vérifié que les lésions histologiques du foie et du rein étaient plus accusées sous l'effet du nickel que sous l'effet de quantités équivalentes de cobalt (travaux en cours).

2° Dans la série végétale.

a) *Chez les Hépatiques.* — Pour les propagules de Lunularia cruciata la dose mortelle sur milieu de MARCHAL est de 3 p. 10.000 pour le chlorure de nickel et de 4 p. 10.000 pour le chlorure de cobalt (quantités exprimées en g. de métal), (5, 7, 9) ; sous l'influence des mêmes sels à 2 p. 10.000 le développement des propagules se réalise, mais les tissus jeunes sont beaucoup plus profondément altérés dans la solution du sel de nickel que dans celle du sel de cobalt (CHALAUD). Résultats analogues avec Fegatella conica.

b) *Chez les Phanérogames.* — Les travaux effectués sous la direction de CHALAUD, à la Faculté des Sciences de Toulouse, nous ont permis d'observer, d'une manière générale, les effets toxiques accusés déterminés par les sels de nickel et ceux de cobalt sur les germinations de diverses Graminées ou Légumineuses : Triticum, Phaseolus vulgaris et Pisum sativum (3, 13) ; en général, à doses équivalentes, les sels de nickel sont plus toxiques que ceux de cobalt.

3° Les amylases salivaire et pancréatique sont activées par un très grand nombre de chlorures ajoutés à concentration convenable dans les milieux ; à doses égales, le chlorure de cobalt est plus activant que le chlorure de nickel (1, 3, 8, 13).

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Il n'est pas douteux que sans être des métaux très toxiques le nickel et le cobalt ne sauraient être considérés comme inoffensifs pour l'économie. Nous pensons que les doses léthales indiquées par RICHE et LABORDE, du moins pour l'administration par voie intraveineuse du sulfate de nickel, doivent être relevées.

L'effet de très petites doses de sels de nickel ou de cobalt est mal connu. Il est probable qu'une intoxication chronique affectant l'allure du saturnisme ne saurait être réalisée avec le nickel et le cobalt parce que ces métaux sont trop peu agressifs et surtout parce que leur élimination urinaire s'effectue bien ; mais en tous cas les possibilités de cette intoxication chronique n'ont jamais été mises jusqu'ici à l'épreuve de l'expérience.

Par contre, du point de vue pharmacodynamique, il est illusoire de mésestimer les effets rapides de doses suffisamment élevées de nickel et de cobalt sur les parenchymes du foie et du rein, signatures évidentes d'une toxicité nette. Cette donnée est importante à retenir, parce que les aptitudes biologiques de ces deux métaux sont nombreuses, intéressantes et ouvrent la voie, en bien des points, à des conceptions thérapeutiques nouvelles.

⁂

Une autre notion se dégage de l'ensemble des faits rapportés : le nickel est légèrement mais nettement plus toxique que le cobalt. Dans un travail récent, BERTRAND et SERBESCU [2] corroborent cette conclusion. Elle nous parait présenter un double intérêt pratique : d'une part, la présence du cobalt dans la vaisselle de nickel ne pré-

sente aucun danger particulier ; d'autre part, dans toutes les industries alimentaires, où la substitution est possible sans inconvénient pour le rendement, le nickel doit être remplacé comme catalyseur d'hydrogénation par le cobalt [4].

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J. Pharm. Chim. 29:391-409, 1939

Contribution to the Toxicological Study of Nickel

By: F. Caujolle and G. Canal[1]

The toxicological history of nickel shows a fairly great similarity to that of cobalt. Formerly considered very toxic by Gmelin (8) and by Kobert (10), nickel has ceased appearing so terrible when it presents itself to chemists and biologists in the form of defined and pure compounds--probably the presence of arsenic in the old preparations explained the severity and the clinical form of the poisonings reported.

Since 1850, the development of the industrial production of nickel opened to this metal very diverse fields of application by virtue of its outstanding physical and mechanical qualities; since then, the study of nickel has elicited a large number of works: Azary (1) and A. Stuart (14) concluded the existence of a greater toxicity for nickel and its usual derivatives; on the other hand, Dzerchgowsky and his co-workers (7), Laborde and Riche (11, 12, 13) affirmed their innocuousness. From the very numerous articles published of which we will cite only the most characteristic, it seems certain that nickel is infinitely less toxic than lead and that it is unable to create a chronic poisoning by the effect of accumulation of very small amounts which taken alone are incapable of bringing about toxic phenomena. The hygiene problem raised by the usage of nickel in the food industry seems to be resolved: nickel may be considered as a practically non-toxic metal under normal conditions of application actually in use.

A recent work of G. Bertrand and Serbescu (2) having for its object the comparison of the toxicity of common metals, gave very significant indications; by the method of average survivals, the metals were classified

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[1] Work presented at the March 1, 1939 meeting of the Pharmacy Society of Paris.

in the order of increasing toxicity for guinea pigs (hypodermic route) as follows: Iron (II), Al, Fe (III), Mn, Zn, Co, Cd, Ni, Cu.

If these conclusions confirm the research of hygienists in showing that nickel is of an aggressivity very close to that of copper although slightly less in comparison with the pharmacodynamics, the problem of the toxicity of nickel and its derivatives remains unsolved (4,5). May nickel be admitted without reservation into the therapeutic arsenal where the hypodermic route or especially the intravenous route tend to become predominant; is it capable of causing serious disorders in the higher vertebrates? Such are the questions which seem to us to merit complementary experimentation, of which we propose first of all to exactly describe the techniques.

II. Experimental Techniques

Physiological Technique--Because of the elimination reflexes which start in motion in the dog with the administration of nickel salts by means of an esophageal probe, we used exclusively the intravenous route: the anesthesitized subjects received in the central tip of the saphena known quantities of the aqueous solution of nickel salts, isotonic solutions or isotonized to sodium chloride.

The techniques used to study the biliary or urinary elimination of nickel are identical to those previously used in similar studies on cobalt which have already been described (3, 4, 6). The subjects were sacrificed by electrocution.

Chemical Technique--The study and the quantitative determination of nickel were performed using the dimethylglyoxime reaction of Tschugaeff, for which G. Bertrand, Mokragantz, and Macheboeuf have outlined the conditions of application.

After the destruction of the organic matter by calcination at low temperature, the nickel is recovered by draining off the hydrochloric acid

to which 5 p. 100 nitric acid was added; the drained liquids are concentrated in a water-bath, then a great excess of ammonia is added, ferric hydrate precipitates (1); it is separated and carefully washed with ammonia.. The ammoniacal liquid and the washing waters of ferric hydrate[1] are added to a large amount of warm water and then maintained for an hour and a half at boiling: a precipitate is formed containing all the nickel; this precipitate is redissolved in a hydrochloric milieux; from the solution obtained, nickel is precipitated to the state of pure sulfide; the sulfide oxidized by a mesh muffle is dissolved in diluted hydrochloric acid, the nickel chloride formed is treated by an excess of an alcoholic solution of dimethylglyoxime of 2 p. 100, a precipitate is obtained in a slightly ammoniacal milieu, it is filtered on Schott's crucible, dried at 100-102°, then weighed.

All this detail will be found very useful in this technique, which gave excellent results in previous publications (4, 5).

None of the various other methods which we tried gave us as satisfactory results; in particular it is good in view of the determination of small quantities of nickel to use colorimetric methods, actually described, with only the greatest reservations.

III. Experimentation

The techniques which we just described are adapted well to the study of acute poisoning from the nickel salts; they enable the following of the course

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[1] Generally, ferric hydrate precipitates without bringing any nickel, one can be assured of this; if traces of nickel were brought, they can be recovered by calcinating the hydrate and reprecipitating the ammonia hydrochloric solution of ferric oxide obtained. It seems that the presence of organic matter intervenes in the attraction of nickel by $Fe(OH)_2$.

of the different phases of this poisoning, the elimination of nickel; finally after autopsy, they enable the precise identification of the elective localizations of this metal.

Our experiments were done on two dogs who received several injections, in the form of aqueous solutions of nickel chloride, of 7.45 to 44.9 mg of metallic nickel per kilogram; all these injections were very slowly administered into the saphena.

Dog I.-- $\overset{o}{+}$, 17 kg. Examination before the experiment: --azotemia 0.37 g p. 1.000; urine containing neither sugar, nor reducers, nor albumin. Anesthesia: morphine and a sleep-inducing drug. Injection in a half-hour of 340 mg of Ni-metal diluted in 200 cm^3 of physiologic serum.

Rapid reactions: transitory acceleration of the pulse and the respiratory rate; violent intestinal spasms bringing about the expulsion of material; all these phenomena seemed to get better, when the cardiac rhythm suddenly became irregular, actual stoppages appearing, death suddenly occurring from cardiac syncope a quarter of an hour after the end of the injection.

Autopsy: the liver, kidneys, spleen were greatly congested; the pancreas was studded with small hemorrhagic areas; the bladder was nearly empty.

The poisoning brutally struck a young, seemingly very healthy subject; the fixation of nickel was very rapid because in the blood obtained by puncture of the vessels immediately following death, the amount of nickel was slightly less than 0.5 mg p. 100. The emonctories didn't have time to function usefully; the bile contained in the gall bladder contained only minute traces of nickel; the few cubic centimeters of urine collected by

puncture of the bladder just before death also contained only insignificant quantities of nickel. Table 1 indicates the localization of nickel in the various viscera examined.

Table 1

Organ	Total Wt. in g.	Total Ni in mg.	Ni in mg p. 100
Heart	116	3.5	3.0
Kidney	90	5.4	6.0
Liver	412	2.3	5.5
Tongue	70	1.9	1.3
Stomach + its contents	257	2.0	4.6
Lungs (Pieces)	(44)	Clear presence	(less than 0.5)
Pancreas	21.5	minute	traces
Spleen	38	1.4	0.5
Brain	70	minute	traces

Dog II.--19 kg. ♂. Examination before the experiment; azotemia 0.40 g p. 1000; albuminous urine, no sugar reducers. Anesthesia: morphine and a sleep-inducing drug. A choledochal fistula was created. Four injections at intervals of (?) hours, representing a total of 852 mg of metallic nickel, diluted in more than a half-liter of physiologic serum.

Each injection accelerated the cardiac and pulmonary rhythm this acceleration was transient; excretion of serosanguineous fecal matter after the first injection; urine scanty and quickly bloody.

Autopsy: congested kidneys, liver and spleen studded with hemorrhagic areas of varying extent. The bladder was empty and the intestinal contents were serosanguineous.

Tables II and III indicate quantities of nickel contained in the viscera and excreted.

Table 2

Amount of Ni (in mg. p. 100) in carotid blood: 1.66 (puncture done immediately before sacrifice).

Organ	Total Wt. in g.	Total Ni in mg.	Ni in mg. p. 100
Heart	168	1.67	1.0
Kidney	106	5.1	4.8
Liver	738	47.2	6.4
Tongue	75	3.1	4.1
Stomach and its contents	290	15.4	5.3
Lungs (pieces)	(180)	"	2.9
Pancreas	39	1.1	2.8
Spleen	70	0.27	0.4
Suprarenals			
Brain	76	Minute	traces
Cranial bone	(76)	slight	traces
Masticatory muscles	(52)	"	0.6

Table 3

Excreta	Quantity Collected	Total Ni in mg.
Urine (puncture)	32.5 cm^3	0.10
Bile from fistula	less than 1 cm^3	0.10
Bladder and gall bladder	28.5 g	0.60
Fecal matter	about 100 g.	1.3

Dog III--16.5 kg. ♂. Examination before the experiment: azotemia 0.80 g p. 1000; urine very slightly albuminous, no reducing sugars. Anesthesia: morphine and sleep-inducing drug. A choledochal fistula was created. In four injections (total duration: 35 minutes), the subject received 455 mg of metallic Ni diluted in one-half liter of serum; the first injection preceded the second by an hour and contained only 65 mg of metallic Ni; the second, third, and fourth injections were of equal amounts and succeeded each other in intervals of two hours.

The subject who had reacted violently to the first injection, tolerated the following injections more easily, the symptoms of poisoning being those already described for subjects I and II.

Autopsy revealed voluminous liver and kidneys, very congested purplish-blue color; some ecchymotic spots were distributed over the small ansa. The bladder contained about 45 cm^3 of very albuminous urine.

Tables IV and V indicate the quantities of nickel contained in the viscera and the excreta.

Table 4

Amount of Ni (in mg p. 100 g) of carotid blood: 1.05 (puncture done just before sacrifice).

Organ	Total Wt. in g.	Total Ni in mg.	Ni in mg. p. 100 g.
Heart	120	2.5	2.1
Kidneys	120	5.6	5.6
Liver	620	42	6.7
Pancreas	52	0.8	2.5
Spleen	50	2.0	4.0
Brain	80	Minute traces	
Muscular mass from the paw	(107)	0.51	4.8

Table 5

Excreta	Quantity Collected	Total Ni in mg.
Urine (autopsy)	about 50 g	29.4
Bile from fistula	22 g	0.46
Bladder and gall bladder	11.5 cm^3	0.40
Fecal matter	40 cm^3	0.5

Dog IV--17.5 kg. ♂. Examination before the experiment: azotemia 0.40 g p. 1000; urine very slightly albuminous, no reducing sugars. Anesthesia: morphine and chloralose, A choledochal fistula was created, the animal was shocked by the operation which was much longer than usual (anatomic malformation of the ductus choleductus). Preparation was made for the measurement of the carotid pressure. Because of the condition of the subject, the nickel was administered only in reduced doses: three injections 1½ to 2 hours apart of equal strength representing a total of 130 mg of metallic Ni diluted in 150 cm^3 of physiologic serum.

Each injection caused a transient acceleration of the cardiac and respiratory rates (see graphs 1 to 4) and brought about elimination rapidly taking the appearance of diarrhea.

Autopsy revealed the usual lesions. Table VI indicates the distribution of nickel in the viscera.

Table 6

Organ	Total Wt. in g.	Total Ni in mg.	Ni in mg p. 100 g.
Heart	145	0.6	0.4
Kidney	73	2.6	3.5
Liver	505	(abundant)	(abundant)
Spleen	44	0.5	about 1
Brain	79	0	0
Stomach	40	0.8	2.0

The cephalorhachidian fluid contained no trace of nickel; the amount of nickel in the blood at the end of the experiment could not be determined because of an accident in handling.

The passage of nickel was clearly observed in the urine, but the impossibility of placing a urethral probe prevented the obtaining of rigorous results; the urine had to be removed by puncture of the bladder and was done only in a very incomplete manner; 21 cm^3 of bile was collected and contained a total of 0.20 g of nickel. In the fecal material, the passage of nickel was very reduced, only the diarrhea excreted after the third injection clearly contained any of it.

Dog V--Young male of 18 kg; slight traces of albumin, no reducing sugars in the urine. Anesthesia: morphine and chloralose. Preparation was made for the graphic measurement of the carotid pressure and a biliary fistula was created. A urethral probe was put in place. The shocked animal had to be warmed.

A first injection was done containing 122 mg of metallic Ni; an hour-and-a-half later the second injection containing a double quantity of metallic nickel caused death. The experiment, so brutally terminated, presented only a very relative interest to the study of the localization of the metal because of its certainly very elevated concentration in the blood at the time of death; only the eliminations were retained for study.

Table 7

Excreta	Quantity Collected	Total Ni in mg.
Bile from fistula	2 cm^3	0
Bladder and gall bladder	48 g	traces
Urine from probe	11.5 cm^3	1.42
Bladder urine	2.6 cm^3	1.82

The graphs recording the carotid pressure (graphs 5 to 7) offer the possibility of studying in a very precise fashion the mechanism of the death, and they constitute important data which will be interpreted later.

Dog VI--Young male of 9 kg. Azotemia 0.21 g p. 1000, albuminous urine, no reducing sugars. Anesthesia: morphine and chloralose. A choledochal fistula was created. In 15 minutes a single injection containing 88.9 mg of metallic Ni or about 10 mg per kilogram was given. The subject reacted violently, the number of heartbeats exceeded 96 to 156 per minute, the respiratory rate momentarily reached the clearest paroxysmal dyspnea; the gastrointestinal reaction was brutal, the fecal matter excreted rapidly became diarrheal, but was never bloody; vomiting after multiple efforts 3 hours after the injection. The animal was very agitated and had to receive a second dose of chloralose. It succumbed 8 hours after the end of the injection.

At autopsy, the liver and kidneys presented the typical appearance already described: on the purplish-blue bottom of the hyperhemic tissues, some hemorrhagic areas broke away.

Table 8

Amount of Ni (in mg p. 100 g) of carotid blood: less than 1 mg (puncture done immediately following death)

Organ	Total Wt. in mg.	Total Ni in mg.	Ni i mg p. 100
Kidney	55	1.8	3.2
Liver	201	20.0	6.84
Stomach and its contents	250	0.94	0.35
Pancreas	38	0	0
Cranial bone	60	0	0
Small intestine and its contents	74	0.81	1.1
Cephalorhachidian fluid	12 cm^3	Slight traces	

Table 9

Excreta	Quantity Collected	Total Ni in mg.
Bile from fistula	4.2 cm^3	Very slight traces
Bladder and gall bladder	9 g	traces
Urine	35 cm^3	23.7
Fecal matter	about 200 g	traces
Vomited matter	about 100 g	Very slight traces

Tables 8 and 9 indicate the localization of the nickel and its distribution in the excreta.

The urine excreted at the end of the experiment was deep brown, strongly albuminous and bloody.

Dog VII--15 kg +. Albuminous urine but containing no reducing sugar. Anesthesia with chloralose; a choledochal fistula was created and a stationary probe was placed in the bladder. In three injections, spaced three hours apart and given slowly, two times taking 10 to 20 minutes, the subject

received 345 mg of metallic Ni. The usual reactions were manifested; the fecal material was plainly bloody, and it also caused an abundant and very mucous and bloody secretion of saliva. Death occurred suddenly after the third injection, respiratory movements stopping first.

Autopsy enabled the verification of the partial atrophy of a kidney; the typical lesions were found again. The bladder was empty.

Table 10

Amount of Ni (in mg p. 100 g) of carotid blood sampled at death:2.51

Organ	Total Wt. in g.	Total Ni in mg	Ni in mg p. 100 g
Kidney	97	3.7	3.75
Liver	465	15.81	3.40
Brain	73	Very slight traces	
Suprarenals	not weighed	Very slight traces	
Salivary glands	not weighed	slight traces	

Table 11

Excreta	Quantity Collected	Total Ni in mg
Bile from fistula	1.1 cm^3	1.0
Bladder and gall bladder	18 g	0.5
Urine	28 cm^3	21
Saliva	5 cm^3	Very clear traces

Urine became mahagony colored at the end of the experiment.

Dog VIII--25 kg ♂. Bladder empty at the start. Anesthesia with chloralose. Biliary fistula was created, the biliary secretion was first of all minimal, but it didn't take long for it to become abundant. A stationery probe was placed in the bladder. Pulse at 120, The subject which appeared robust, received two injections containing a total of about 420 mg of Ni-metal; it tolerated the first injection well, but succombed

shortly after the second which was not completed. The reactions to the first injection were marked by an acceleration of the pulse which reached 144 and transient slowing of the respiratory rate, no gastro-intestinal reaction. The reactions to the second injection revealed: the respiratory rate notably slowed down, then the respiratory movements stopped while the heart continued to beat; the subject was then given artificial respiration; the pulse fell to 40, then went up to 62 after a camphor injection; this improvement was of short duration--the subject, deeply poisoned, died.

The subject whose physiological integrity at the beginning of the experiment was very doubtful, eliminated about 1 mg of nickel in the bile (amount of Ni 2.3 mg per 100 g) and 1.34 mg of nickel in the urine (amount of Ni 0.7 mg p. 100)g); the urine always reduced, was bloody at the end of the experiment.

Dog IX--23 kg, ♂, afflicted with undiagnosed dermatosis. Azotemia 0.32 g p. 1000; slight traces of albumin, no reducing sugars in the urine. Anesthesia with chloralose. Biliary fistula, easily created yielded abundantly; a stationary probe placed in the bladder also gave a significant supply. The very vigorous subject, reacted to each injection with a increase in the respiratory rate, by a sometimes significant secretion of foam, by an increase of the intensity of the color of the erythematous plagues which it carries. He received a total of 570 mg of metallic Ni in 4 spaced injections. The fourth injection was given after laparatomy into a branch of the mesenteric vein, so as to observe whether this direct route of transport caused an increase in the biliary elimination of nickel; the subject lost very little blood, nevertheless, after this fourth injection, the pulse fell from 140 to 44, the animal became chilled, an injection of camphor oil was necessary the adynamic phase disappeared. The subject was electrocuted an hour after the end of the fourth injection.

Tables 12 and 13 indicate the localizations and eliminations observed.

Table 12

The blood sampled immediately before electrocution contained 0.96 mg of metallic Ni.p. 100 g.

Organ	Total Wt. in g	Total Ni in mg	Ni in mg p. 100 g
Kidney	160	6.27	3.92
Liver	653	40.62	6.22
Brain	81	Slight traces	
Heart	244	1.40	0.58
Lung	67	total wt. not determined	2.29
Pancreas	43	traces	
Suprarenals	not weighed	traces	
Stomach and duodenum	300	2.13	0.71

Table 13

Excreta	Quantity Collected	Ni in mg p. 100 g
Bile from fistula	22.1 cm^3	1.00
Bladder and gall bladder	33 g	Very slight precipitate
Urine from probe and bladder urine	570 cm^3	98.13

About 20 to 30 cm^3 of urine were lost in the last phases of excretion, in other words, at the time when the urine contained the most nickel; it must then be admitted that the urinary elimination of nickel was higher than about 115 to 125 mg; the urine first pale yellow, became mahogany-brown, then brownish-black without being bloody.

Experiments I to IX gave comparable results with respect to the localizations; by contrast, the amounts of observed eliminations didn't seem to permit conclusions of a general nature: two complementary experiments were especially directed towards the study of the eliminations.

Dog X--15 kg, ♂, albuminuria and hyperazotemia. Anesthesia: choralose. A stationary probe was placed in the bladder and a fistula made in the hepatic duct. The subject received in a single injection 150 mg of metallic Ni. In seven hours, he eliminated: 20 cm^3 of bile containing 0.15 mg Ni;--a froth containing traces of nickel--about 300 cm^3 of urine containing a total of more than 60 mg of nickel; urine elimination showed two distinct phases: 1--in the two hours following the injection, urine was pale yellow and contained only traces of nickel, 2--from the third to the fourth hour, the urine took on a deep color going from brownish-yellow to mahogany, then to brownish-black, the urine was then full of nickel, the amount of the metal attained 10 mg p. 100 g of urine.

Dog XI--18 kg, ♂, Non-albuminous urine, azotemia 0.39 g p. 1000, anesthesia: choralose. A choledochal fistula was created and a stationary probe placed in the bladder. The subject received 455.0 mg of metallic Ni in two injections. In two hours, he eliminated: 10.1 cm^3 of bile containing 0.89 mg of Ni--abundant, slightly bloody forth containing traces of nickel;--about 400 cm^3 of urine containing 98 mg of nickel; the urine excreted took the appearance previously described: at the time of the greatest elimination, the urine was strongly colored--it was never bloody, but was albuminous from the fifth hour.

IV. Elimination of Nickel

The amounts which we administered to the subjects in the experiment varied from 7 to 45 mg of metallic per kilogram of animal; the doses were generally divided; in all circumstances, the reactions of the subject always narrowly considered, enabled us to obtain acute experimental poisoning; it was only exceptionally and with hypersensitive subjects that lethal doses were reached.

Under the conditions of our experiments, the organic defense reflexes had the possibility of acting since we avoided overpowering the organism with an initial massive dose and sometimes we started in motion many of these reflexes, by maintaining our doses at sufficiently high thresholds. These conditions favorable to the study of the routes of elimination of nickel were also done under different anesthetics (morphine and sleep-inducer, morphine and chloralose, chloralose) so as to eventually reveal the individual influences of these substances whose effects on the secretions were clearly polarized: morphine diminished the glandular activities, chloralose clearly played the role of a choleretic. The amounts of anesthesia necessarily varied with the subject: for chloralose; for example, we used 0.10 g to 0.20 g per kilogram.

The conclusions which arise from our experiments are the following:

1--Even under the influence of chloralose, the biliary elimination of nickel is always minimal (4);

2--On the other hand, the urinary elimination is generally very great, even in subjects suspected to have renal lesions, then very often albuminuric;

3--Under the sialagogic effect attributed to nickel, small quantities of the metal can be seen to pass into the froth excreted;

4--The feces may contain nickel, either dumped out by the glands of the intestinal tract or brought by hemorrhage.

V. Localization of Nickel

The works of Riche and Laborde resulted in the following data: nickel was refound principally in the liver and the nerve tissues (12), the kidneys contained less than the liver and the nerve tissue (13). Our experiments did not completely confirm this data.

To better show the elective localizations, we calculated the relationship R of the concentrations of nickel in various organs to the concentration of nickel in the blood at the time of sacrifice. This method introduced

by R. Fabre in experimental toxicology has the advantage of attenuating errors of interpretation related to the variable form of blood circulation in the various organs to be compared--it constitutes a certain improvement in the method of absolute comparisons and remains in substance the method of choice until the time when we will be able to make direct measurements of the blood in the organs.

Organs	Values Limited by R
Liver	1.2 to 11
Kidneys	1.5 to 12
Brain	1
Heart	0.5 to 6
Pancreas and Spleen	1 to 4

The following conclusions arise from our experiments:

1--The localization of nickel takes place selectively at the emunctory level, some show a high efficacy (kidney) or lesser (liver).

2--The fixation of nickel by cerebral matter is very reduced, at least in the case of transport in the form of nickel chloride; it is hardly probable that the substitution of sulfate (used by Laborde and Riche) for chloride could change this result; on the other hand, the usage of liposoluble salts which we actually studied enabled us to envisage the problem in a different light.

3--The heart always fixes appreciable quantities of nickel.

4--The endocrine glands retained small quantities of the metal, but not enough so that a selective fixation could be attributed to them.

VI. Toxicity

The results of our experiments lead us to attribute to nickel a fairly high toxicity. It will be remembered that death could be caused fairly rapidly with doses on the order of 10 to 20 mg of metal per kilogram

of animal; these lethal doses were less than those indicated by Laborde and Riche (5).

The subjects with which the experiment was conducted all experienced vigorous defense reactions: spasms, vomiting, elimination, generally diarrheal, spewing of froth--diarrhea and froth, sometimes bloody. The affliction of the respiratory nerve centers is clear (dyspnea nearly constant) and that of the cardiac center is also characteristic. The terminal lesions of the myocardium may take on a subacute character, which graphs 1 to 7 clearly show, graph 7 in particular, is completely characteristic of the relatively severe fall in blood pressure under the effect of a sudden giving way of the myocardium.

All the emunctories collaborate in the elimination of nickel; but their efficacy seems rapidly struck down by the effect of the metal, which reveals itself to be very aggressive to the point of blocking almost entirely the biliary eliminations, which is so effective with other metals. The vicarious role of the salivary glands and the glands of the digestive tract, a role whose useful effect remains very limited, is one of the clearest physiological proofs of the aggressivity of nickel.

The comparative study of the toxicity of nickel and cobalt will be the object of our next article.

Graph 1--Dog IV.

Significant, but transient elevation of blood pressure with slight increase of the differential.

Graphs 2, 3, 4--Dog IV.

Progressive fall in pressure with the decrease of the differential (2 and 3); increasingly clear dimunition of the force of the systolic contraction with arrythmia (4).

Graphs 5, 6, 7

Graph 5 shows the elevation of blood pressure and the slowing of the respiratory rate caused by the injection of an aqueous solution of nickel chloride.

Graph 6 translates the fatigue of the myocardium under the toxic effect of nickel.

Graph 7 shows the collapse of the differential and the increasingly serious slowing of the respiratory rate ending up with respiratory syncope which is immediately associated with cardiac syncope.

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J. Pharm. Chim.
29: 391-409
(1939)

Contribution à l'étude toxicologique du nickel ;

par MM. F. Caujolle et G. Canal (1).

I. — Introduction.

L'histoire toxicologique du nickel présente avec celle du cobalt une assez grande analogie. Jadis considéré comme très toxique par Gmelin [8] et par Kobert [10], le nickel cessa de paraître aussi redoutable lorsqu'il se présenta aux chimistes et aux biologistes sous forme de combinaisons définies et pures, — vraisemblablement la présence d'arsenic dans les préparations anciennes explique et la gravité et la forme clinique des intoxications rapportées.

Dès 1850, le développement de la production industrielle du nickel ouvre à ce métal les champs d'application très divers que ses brillantes qualités physiques et mécaniques faisaient pressentir ; dès lors l'étude de la toxicité du nickel sollicite un grand nombre de travaux : — Azary [1] et A. Stuart [14] concluent à l'existence d'une toxicité élevée pour le nickel et ses dérivés usuels ; — par contre Dzerchgowsky et ses collaborateurs [7], Laborde et Riche [11, 12, 13] affirment leur innocuité. Des très nombreux mémoires publiés, dont nous ne citons que les plus caractéristiques, il paraît certain que le nickel est infiniment moins toxique que le plomb et qu'il est inapte à créer une intoxication chronique par effet d'accumulation de doses très petites et incapables, prises isolément, de déclencher des phénomènes toxiques. Le problème d'hygiène posé par l'utilisation du nickel dans le matériel des industries alimentaires paraît résolu : le nickel peut être considéré comme un métal pratiquement non toxique, dans les conditions normales d'application actuellement en usage.

Un travail récent de G. Bertrand et Serbescu [2] ayant pour objet de comparer la toxicité des métaux

(1) Travail présenté à la séance du 1er mars 1939 de la Société de Pharmacie de Paris.

usuels, donne des indications très significatives ; par la méthode des survies moyennes, les métaux se classent par ordre de toxicité croissante pour le cobaye (voie hypodermique) de la manière suivante : Fer (II), Al, Fe (III), Mn, Zn, Co, Cd, Ni, Cu.

Si ces conclusions confirment les recherches des hygiénistes en montrant que le nickel est d'une agressivité très voisine de celle du cuivre, bien que légèrement inférieure, au regard de la pharmacodynamie le problème de la toxicité du nickel et de ses dérivés reste entier (4,5). Le nickel peut-il être admis sans réserve dans l'arsenal thérapeutique, où la voie hypodermique et surtout intraveineuse tendent à devenir prépondérante ; est-il capable de déterminer chez les vertébrés supérieurs des désordres graves ? Telles sont les questions qui nous paraissaient mériter une expérimentation complémentaire, dont nous proposons tout d'abord de définir exactement la technique.

II. — Techniques expérimentales.

Technique physiologique. — En raison des réflexes d'évacuation que déclenche chez le chien l'administration de sels de nickel au moyen de la sonde oesophagienne, nous avons utilisé exclusivement la voie intraveineuse : les sujets, anesthésiés, recevaient dans le bout central de la saphène des quantités connues de solutions aqueuses de sels de nickel, solutions isotoniques ou isotonisées au chlorure de sodium.

Les techniques utilisées pour étudier les éliminations biliaire ou urinaire du nickel sont identiques à celles employées lors des études similaires faites sur le cobalt, elles ont déjà été décrites (3, 4, 6). Les sujets sont sacrifiés par électrocution.

Technique chimique. — La recherche et le dosage du nickel ont été effectués grâce à la réaction à la diméthylglyoxime de Tschugaeff, dont, G. Bertrand, Mokragnatz et Machebœuf ont précisé les conditions d'application.

Après destruction de la matière organique par calci-

nation à basse température, le nickel est repris par épuisement à l'acide chlorhydrique additionné de 5 p. 100 d'acide nitrique ; les liqueurs d'épuisement sont concentrées au bain-marie, puis additionnées d'un grand excès d'ammoniaque, l'hydrate ferrique (1) précipite, on le sépare, on le lave soigneusement à l'ammoniaque. La liqueur ammoniacale et les eaux de lavage de l'hydrate ferrique sont additionnées d'un excès d'eau de chaux puis maintenues une heure et demie à l'ébullition : il se forme un précipité contenant tout le nickel ; ce précipité est redissous en milieu chlorhydrique, de la solution obtenue le nickel est précipité à l'état de sulfure pur ; le sulfure oxydé par le grillage au moufle est dissous dans de l'acide chlorhydrique dilué, le chlorure de nickel formé est traité par un excès d'une solution alcoolique de diméthylglyoxime à 2 p. 100, on achève la précipitation en milieu légèrement ammoniacal, on filtre sur creuset de SCHOTT, on sèche à 100-102°, on pèse.

On trouvera toutes précisions utiles sur cette technique, qui donne d'excellents résultats, dans des publications antérieures [4, 5].

Aucune des diverses autres méthodes que nous avons essayées, ne nous a donné des résultats aussi satisfaisants ; en particulier il est bon, en vue du dosage de petites quantités de nickel, de n'utiliser les méthodes colorimétriques, jusqu'ici décrites, qu'avec la plus grande réserve.

III. — Expérimentation.

Les techniques que nous venons de décrire s'adaptent bien à l'étude de l'intoxication aigüe par les sels de nickel ; elles permettent de suivre au cours des différentes phases de cette intoxication l'élimination du nickel ; enfin après autopsie, elles permettent de préciser les localisations électives du métal.

(1) Généralement l'hydrate ferrique précipite sans entraîner de nickel, on s'en assure ; si des traces de nickel ont été entraînées, on les récupère en calcinant l'hydrate et reprécipitant par l'ammoniaque la solution chlorhydrique de l'oxyde ferrique obtenu. Il semble que la présence de matières organiques intervienne dans l'entraînement du nickel par $Fe(OH)_3$.

Nos expériences ont porté sur 2 chiens, qui ont reçu en plusieurs injections, sous forme de solutions aqueuses de chlorure de nickel, de 7,45 à 44,9 mg. de nickel-métal par kilog ; toutes les injections ont été poussées dans la saphène très lentement.

Chien. — ♀, 17 kg. Examen avant l'expérience : — azotémie 0,37 p. 1.000 ; urines ne contenant ni sucres réducteurs, ni albumine. Anesthésie : morphine et somnifène. Injection en demi-heure de 340 mg. de Ni-métal dilués dans 200 cm³ de sérum physiologique.

Réactions rapides : accélération transitoire du pouls et du rythme respiratoire, spasmes intestinaux violents déterminant l'expulsion de matières ; tous ces phénomènes paraissent s'amender, lorsque brusquement le rythme cardiaque devient irrégulier, de véritables arrêts apparaissent, la mort survient par syncope cardiaque un quart d'heure après la fin de l'injection.

Autopsie : le foie, les reins, la rate sont fortement congestionnés, le pancréas est parsemé de petits foyers hémorragiques ; la vessie est à peu près vide.

L'intoxication a frappé brutalement un sujet jeune, paraissant très sain ; la fixation du nickel a été très rapide, car sur le sang obtenu par ponction des vaisseaux immédiatement après la mort le taux du nickel est légèrement inférieur à 0,5 mg. p. 100. Les émonctoires n'ont pas eu le temps de fonctionner utilement : la bile contenue dans la vésicule ne contient que des traces infimes de nickel ; les quelques centimètres cubes d'urine recueillis par ponction de la vessie peu avant la mort ne renferment également que des quantités insignifiantes de nickel.

Le tableau I indique la localisation du nickel sur les divers viscères examinés.

TABLEAU I.

Organe	Poids total en g.	Ni total en mg.	Ni en mg. p. 100
—	—	—	—
Cœur	116	3,5	3,0
Rein	90	5,4	6,0
Foie	412	2,3	5,5
Langue	70	1,9	1,3
Estomac et son contenu	257	2,0	4,6
Poumons (fragments)	(44)	Présence nette (moins de 0,5)	
Pancréas	21,5	Traces infimes	
Rate	38	1,4	0,5
Cerveau	70	Traces infimes	

Chien II. — 19 kg. ♂. Examen avant l'expérience : azotémie 0,40 g. p. 1000 ; urines albumineuses, pas de sucres réducteurs. Anesthésie : morphine et somnifène. On institue une fistule cholédocienne. 4 injections, à intervalles de 1 heures, représentant au total 852 mg. de Ni-métal, dilués dans plus de demi-litre de sérum physiologique.

Chaque injection accélère les rythmes cardiaque et pulmonaire, cette accélération est passagère ; émissions de matières fécales séro-sanguinolentes dès la première injection ; urines peu abondantes et rapidement sanglantes.

Autopsie : rein congestionné, foie et rate semés de taches hémorragiques d'étendue variable. La vessie est vide, le contenu intestinal est muco-sanguinolent.

Les tableaux II et III indiquent les quantités de nickel contenues dans les viscères et les excreta :

TABLEAU II.

Teneur en Ni (en mg. p. 100) du sang carotidien : 1,66 (ponction réalisée immédiatement avant le sacrifice).

Organe	Poids total en g.	Ni total en mg.	Ni en mg. p. 100
—	—	—	—
Cœur	168	1,67	1,0
Rein	106	5,1	4,8
Foie	738	47,2	6,4
Langue	75	3,1	4,1
Estomac et son contenu	290	15,4	5,3
Poumons (fragments)	(180)	»	2,9
Pancréas	39	1,1	2,8
Rate	70	0,27	0,4
Surrénales			
Cerveau	76	Traces infimes	
Os du crane	(76)	Traces légères	
Muscles masticateurs	(52)	»	0,6

TABLEAU III.

Excreta	Quantité recueillie	Ni total en mg.
—	—	—
Urines (ponction)	32,5 cm³	0,10
Bile de la fistule	moins de 1 cm³	0,10
Vésicule et bile vésiculaire	28,5 g.	0,60
Matières fécales	environ 100 g.	1,3

Chien III. — 16,5 kg. ♂. Examen avant l'expérience : azotémie 0,80 g. p. 1000 ; urine très légèrement albumineuse, pas

de sucres réducteurs. Anesthésie : morphine et somnifène. On institue une fistule cholédocienne. En 4 injections (durée totale : 35 minutes), le sujet reçoit 455 mg. de Ni-métal, dilués dans un demi-litre de sérum ; la première injection précède la 2e d'une heure et n'apporte que 65 mg. de Ni-métal ; les 2e, 3e et 4e injections sont d'égale importance et se succèdent à intervalle de 2 heures.

Le sujet qui a réagi violemment à la première injection supporte les injections suivantes plus facilement, les signes de l'intoxication sont ceux déjà décrits pour les sujets I et II.

L'autopsie découvre un foie et des reins volumineux, très congestionnés, de couleur violacée ; des taches ecchymotiques sont disséminées sur les anses grêles. La vessie renferme 45 cm³ d'urines très albumineuses.

Les tableaux 4 et 0 indiquent les quantités de nickel contenues dans les viscères et les excreta.

Tableau IV.

Teneur en Ni (en mg. p. 100 g.) du sang carotidien : 1,05 (ponction réalisée peu avant le sacrifice).

Organe	Poids total en g.	Ni total en mg.	Ni en mg. p. 100 g.
Cœur	120	2,5	2,1
Rein	120	5,6	5,6
Foie	620	42	6,7
Pancréas	32	0,8	2,5
Rate	50	2,0	4,0
Cerveau	80	Traces infimes	
Masse musculaire de la patte	(107)	0,51	4,8

TABLEAU V.

Excreta	Quantité recueillie	Ni total en mg.
Urines (autopsie)	environ 50 g.	29,5
Bile de la fistule	22 g.	0,46
Vésicule et bile vésiculaire	11,5 cm³	0,40
Matières fécales	40 cm³	0,5

Chien IV. — 17,5 kg. ♂. Examen avant l'expérience : azotémie 0,40 g. p. 1000 ; urine très légèrement albumineuse, pas de sucres réducteurs. Anesthésie : morphine et chloralose. On institue une fistule cholédocienne, l'animal est très shocké, par l'acte opératoire qui a été plus long que de coutume (malformation anatomique du cholédoque). On prépare l'en-

registrement de la pression carotidienne. En raison de l'état du sujet, le nickel n'est administré qu'à doses réduites : trois injections, espacées de 1 h. 1/2 à 2 heures d'importance égale, représentant au total 130 mg. de Ni-métal dilués dans 150 cm³ de sérum physiologique.

Chaque injection détermine une accélération transitoire des rythmes cardiaque (voir graphique 1 à 4) et respiratoire, et sécrétenche des évacuations prenant rapidement une apparence diarrhéiforme.

L'autopsie révèle les lésions habituelles.

Le tableau 6 indique la répartition du nickel dans les viscères.

TABLEAU VI.

Organe	Poids total en g.	Ni total en mg.	Ni en mg. p. 100 g.
—	—	—	—
Cœur	145	0,6	0,4
Rein	73	2,6	3,5
Foie	505	(abondant)	(abondant)
Rate	44	0,5	env. 1
Cerveau	79	0	0
Estomac	40	0,8	2,0

Le liquide céphalo-rachidien ne contenait pas trace de nickel ; la teneur du nickel dans le sang en fin d'expérience n'a pu être déterminée par suite d'un accident de manipulation.

Le passage du nickel a été nettement observé dans l'urine, mais l'impossibilité de placer une sonde urétrale à demeure n'a pas permis d'obtenir des résultats rigoureux, l'urine devait être prélevée par ponction de la vessie et ne le fut que de façon très incomplète. 21 cm³ de bile ont été recueillis, ils contenaient au total environ 0,20 g. de nickel. Dans les matières fécales le passage du nickel a été très réduit, seule la diarrhée émise après la 3e injection en contenait nettement.

Chien V. — Jeune mâle de 18 kg. ; traces légères d'albumine, pas de sucres réducteurs dans l'urine. Anesthésie : morphine et chloralose. On prépare l'enregistrement graphique de la pression carotidienne et on constitue une fistule biliaire. On place à demeure une sonde urétrale. L'animal shocké doit être réchauffé.

On pratique une première injection contenant 122 mg. de Ni-métal ; une heure et demi après une seconde injection contenant une quantité double de Ni-métal détermine la mort. L'expérience aussi brutalement terminée ne présente qu'un intérêt très relatif pour l'étude de la localisation du métal, en raison de sa concentration sûrement très élevée dans le

sang au moment de la mort ; seules les éliminations seront retenues.

TABLEAU VII.

Excreta	Quantité recueillie	Ni total en mg.
—	—	—
Bile de fistule	2 cm³	0
Vésicule et bile vésiculaire	48 g.	traces
Urines de sondage	11,5 cm³	1,42
Urine vésiculaire	2,6 cm³	1,82

Les graphiques d'enregistrement de la pression carotidienne (graphiques 5 à 7) offrent la possibilité d'étudier de façon très précise le mécanisme de la mort, ils constituent un document important qui sera interprété plus loin.

Chien VI. — Jeune mâle de 9 kg. Azotémie 0 g.,21 p. 1000, urines albumineuses, pas de sucres réducteurs. Anesthésie : morphine et chloralose. On institue une fistule cholédocienne. En 15 minutes on pratique une injection unique contenant 88,9 mg. de Ni-métal, soit environ 10 mg. par kilog. Le sujet réagit violemment, le nombre de pulsations cardiaques passe de 96 à 156 par minute, le rythme respiratoire atteint par moment la dyspnée paroxystique la plus nette ; la défense gastro-intestinale est brutale, les matières fécales émises deviennent rapidement diarrhéiques, mais ne sont jamais sanguinolentes ; vomissements après de multiples efforts 3 heures après l'injection. L'animal très agité, doit recevoir une seconde dose de chloralose. Il succombe 8 heures après la fin de l'injection.

A l'autopsie, le foie et le rein présentent l'aspect typique déjà décrit : sur le fond violacé des tissus hyperhémiés se détachent de rares foyers hémorragiques.

TABLEAU VIII.

Teneur en Ni (en mg. p. 100 g.) du sang carotidien : moins de 1 mg. (ponction réalisée immédiatement après la mort).

Organe	Poids total en mg.	Ni total en mg.	Ni en mg. p. 100
—	—	—	—
Rein	55	1,8	3,2
Foie	201	20,0	6,84
Estomac et son contenu	250	0,94	0,35
Pancréas	38	0	0
Os cranien	60	0	0
Intestin grêle et son contenu	74	0,81	1,1
Liquide céphalo-rachidien	12 cm³	Traces légères	

TABLEAU IX.

Excreta	Quantité recueillie	Ni total en mg.
Bile de fistule	4,2 cm³	Traces très légères
Vésicule et bile vésiculaire	9 g.	Traces
Urines	35 cm³	23,7
Matières fécales	env. 200 g.	Traces
Matières vomies	env. 100 g.	Traces très légères

Les tableaux VIII et IX indiquent la localisation du nickel et sa diffusion dans les excreta.

Les urines émises en fin d'expérience étaient brun foncé, fortement albumineuses et sanglantes.

Chien VII. — 15 kg. ♀ Urines albumineuses mais ne contenant aucun sucre réducteur. Anesthésie au chloralose ; une fistule cholédocienne est instituée et une sonde est placée à demeure dans la vessie. En trois injections, espacées de trois heures et poussées lentement en deux temps couvrant de 10 à 20 minutes, le sujet reçoit 345 mg. de Ni-métal. Les réactions habituelles se manifestent ; les émissions fécales sont franchement sanglantes, de même il s'établit une sécrétion abondante de salive très muqueuse et sanguinolente. La mort survient peu après la troisième injection, les mouvements respiratoires se sont arrêtés les premiers.

L'autopsie permet de constater l'atrophie partielle d'un rein ; les lésions typiques se retrouvent. La vessie est vide.

TABLEAU X.

Teneur en Ni (en mg. p. 100 g.) du sang carotidien prélevé à la mort : 2,51.

Organe	Poids total en g.	Ni total en mg.	Ni en mg. p. 100 g.
Rein	97	3,7	3,75
Foie	465	15,81	3,40
Cerveau	73	Traces très légères	
Surrénales	non pesées	Traces très légères	
Glandes salivaires	non pesées	Traces légères	

TABLEAU XI.

Excreta	Quantité recueillie	Ni total en mg.
Bile de fistule	1,1 cm³	1,0
Vésicule et bile vésiculaire	18 g.	0,5
Urines	28 cm³	21
Salive	5 cm³	Traces très nettes

Les urines sont devenues acajou en fin d'expérience.

Chien VIII. — 25 kg. ♂. Vessie vide au départ. Anesthésie au chloralose. On institue une fistule biliaire, la sécrétion biliaire d'abord minime ne tarde pas à devenir abondante. Une sonde à demeure est installée dans la vessie. Pouls à 120. Le sujet, qui paraît robuste, reçoit deux injections contenant au total environ 420 mg. de Ni-métal ; il supporte bien la première injection, mais succombe peu après la seconde qui n'a pas été achevée. Les réactions à la première injection ont été marquées par une accélération du pouls qui atteint 144 et un ralentissement passager du rtyhme respiratoire, aucune défense gastro-intestinale. Les réactions à la seconde injection sont accusées : le rythme respiratoire se ralentit notablement, puis les mouvements respiratoires cessent, tandis que le cœur continue à battre ; le sujet est alors mis en respiration artificielle ; le pouls, tombé à 40, se ranime à 62 après une injection d'huile camphrée, cette amélioration est de brève durée, le sujet, profondément intoxiqué, meurt.

Le sujet, dont l'intégrité physiologique en début d'expérience était fort douteuse, élimine environ 1 mg. de nickel par la bile (teneur en Ni 2,3 mg. p. 100 g.) et 1,34 mg. de nickel par les urines (teneur en Ni 0,7 mg. p. 100 g). ; ces urines, toujours réduites, étaient sanguinolentes en fin d'expérience.

Chien IX. — 23 kg. ♂, atteint d'une dermatose de nature non diagnostiquée. Azotémie 0,32 g. p. 1000 ; traces légères d'albumine, pas de sucres réducteurs dans les urines. Anesthésie au chloralose. Une fistule biliaire, facilement instituée, débite avec abondance ; une sonde à demeure placée dans la vessie donne également un important débit. Le sujet, très vigoureux, réagit à chaque injection par une augmentation du rythme respiratoire, par une sécrétion parfois importante de bave, par l'augmentation d'intensité de la couleur des plaques érythèmateuses dont il est porteur. Il reçoit au total 570 mg. de Ni-métal, en 4 injections espacées. La 4e injection est pratiquée, après laparatomie dans une branche de la veine mésentérique, afin d'observer si cette voie d'apport direct au foie provoque une augmentation de l'élimination biliaire du nickel ; le sujet perd très peu de sang, néanmoins après cette 4e injection, le pouls tombe de 140 à 44, l'animal se refroidit, une injection d'huile camphrée est nécessaire, la phase d'adynamie disparait. Le sujet est électrocuté une heure après la fin de la 4e injection.

Les tableaux 12 et 13 indiquent les localisations et éliminations observées.

TABLEAU XII.

Le sang prélevé immédiatement avant l'électrocution contient 0,06 mg. de Ni-métal p. 100 g.

Organe	Poids total en g.	Ni total en mg.	Ni en mg. p. 100 g.
Rein	160	6,27	3,92
Foie	653	40,62	6,22
Cerveau	81	Traces légères	
Cœur	244	1,40	0,58
Poumon	67	poids total non déterminé.	2,29
Pancréas	43	Traces	
Surrénales	non pesées	Traces	
Estomac et duodénum	300	2,13	0,71

Tableau XIII.

Excreta	Quantité recueillie	Ni en mg. p. 100g.
Bile de fistule	22,1 cm³	1,00
Vésicule et bile vésiculaire	33 g.	Très léger précipité
Urines de sondage et urines vésiculaires	570 cm³	98,13

Environ 20 à 30 cm³ d'urine ont été perdus dans les dernières phases de l'émission, c'est-à-dire au moment où les urines étaient les plus chargées en nickel, on peut donc admettre que l'élimination urinaire du nickel s'est élevée aux environ de 115 à 125 mg. ; les urines, primitivement jaune paille, sont devenues brun-acajou, puis brun-noir, sans être sanguinolentes.

Les expériences I à IX ont donné des résultats comparables en ce qui concerne les localisations ; par contre les taux d'élimination observés ne paraissent pas permettre des conclusions d'ordre général : deux expériences complémentaires ont été spécialement orientées vers l'étude des éliminations.

Chien X. — 15 kg. ♂. Albuminurique et hyperazotémique. Anesthésie : chloralose. On place une sonde à demeure dans la vessie et on pratique une fistule du canal hépatique. Le sujet reçoit en une injection unique 150 mg. de Ni-métal. En sept heures, il élimine : — 20 cm³ de bile contenant 0,15 mg. de Ni, — une bave contenant des traces de nickel, — environ 300 cm³ d'urines contenant au total plus de 60 mg. de nickel ; l'élimination urinaire a présenté deux phases distinctes : 1° dans les deux heures qui suivent l'injection, l'urine est

jaune paille et ne contient que des traces de nickel, 2° de la 3° à la 4° heure, l'urine prend une couleur foncée allant du brun jaune à l'acajou, puis au brun noir, l'urine est alors fortement chargée de nickel, le taux du métal pouvant atteindre 10 mg. p. 100 g. d'urine.

Chien XI. — 18 kg. ♂. Urine non albumineuse, azotémie 0,39 g. p. 1000. Anesthésie : chloralose. On institue une fistule cholédocienne et on place une sonde à demeure dans la vessie. Le sujet reçoit 455,0 mg. de Ni-métal en 2 injections. En dix heures il élimine : —10,1 cm³ de bile contenant 0.89 mg. de Ni — une bave peu abondante, légèrement sanguinolente, contenant des traces de nickel, — environ 400 cm³ d'urine contenant 98 mg. de nickel ; la sécrétion urinaire a revêtu l'apparence signalée précédemment : aux moments de l'élimination la plus active, l'urine a été fortement colorée, — elle n'a jamais été sanguinolente, mais fut albumineuse dès la 5° heure.

IV. — Elimination du nickel.

Les doses que nous avons administrées aux sujets en expérience varient de 7 à 45 mg. de Ni-métal par kilog d'animal ; ces doses ont été généralement fragmentées, en toutes circonstances les réactions du sujet, toujours étroitement considérées, nous ont permis de réaliser l'intoxication expérimentale aiguë ; ce n'est qu'exceptionnellement et avec des sujets hypersensibles que les doses léthales ont été atteintes.

Dans les conditions de nos expériences, les réflexes de la défense organique avaient la possibilité d'entrer en jeu puisque nous évitions d'accabler l'organisme par un apport initial massif et que par ailleurs nous déclenchions la plénitude de ces réflexes en maintenant nos apports à des seuils suffisamment élevés. Ces conditions favorables à l'étude des voies d'élimination du nickel ont été également réalisées sous des anesthésiques différents (morphine + somnifène, morphine + chloralose, chloralose) de façon à révéler éventuellement l'influence propre de ces substances, dont les effets sur les sécrétions sont nettement polarisés : la morphine diminuant les activités glandulaires, le chloralose jouant le rôle d'un cholérétique net. Les doses d'anesthésiques sont néces-

sairement variables avec le sujet : pour le chloralose par exemple, nous utilisons de 0,10 g à 0,20 g. par kilog.

Les conclusions qui se dégagent de nos expériences sont les suivantes :

1° Même sous l'influence du chloralose, l'élimination biliaire du nickel est toujours minime (4) ;

2° Par contre l'élimination urinaire est généralement très active, même chez des sujets suspects de lésions rénales, puisque très souvent albuminuriques ;

3° Sous l'effet sialagogue du au nickel, on peut voir passer de petites quantités du métal dans la bave excrétée ;

4° Les fécès peuvent contenir du nickel, soit déversé par les glandes du tractus intestinal, soit apporté par hémorragie.

V. — Localisation du nickel.

Des travaux de Riche et Laborde résultent les données suivantes: le nickel se retrouve principalement dans le foie et dans les tissus nerveux [12], les reins sont plus pauvres que le foie et les tissus nerveux [13]. Nos expériences ne confirment pas complètement ces données.

Pour bien mettre en relief les localisations électives, nous avons calculé le rapport R des concentrations du nickel dans les divers organes à la concentration du nickel dans le sang au moment du sacrifice. Cette méthode, introduite par R. Fabre en toxicologie expérimentale, a l'avantage d'atténuer les erreurs d'interprétation liées à l'état d'irrigation sanguine variable des divers organes à comparer, — elle constitue une amélioration certaine de la méthode des comparaisons absolues et demeurera en la matière la méthode de choix jusqu'au jour où sera permis le dosage direct du sang dans les organes.

Organes	Valeurs limites de R
Foie	1,2 à 11
Reins	1,5 à 12
Cerveau	1
Cœur	0,5 à 6
Pancréas et Rate	1 à 4

Les conclusions qui se dégagent de nos expériences sont les suivantes :

1° La localisation du nickel s'effectue électivement au niveau des émonctoires, que ceux-ci présentent une efficacité élevée (rein) ou restreinte (foie).

2° La fixation du nickel par la matière cérébrale est très réduite, du moins dans le cas d'apport sous forme de chlorure de nickel ; il est peu probable que la substitution du sulfate (employé par LABORDE et RICHE) au chlorure puisse changer ce résultat ; par contre l'utilisation de sels liposolubles, que nous étudions actuellement, permettra d'envisager le problème sous une autre incidence.

3° Le cœur fixe toujours des quantités appréciables de nickel.

4° Les glandes endocrines retiennent de petites quantités de métal, mais sans qu'il soit permis d'indiquer une fixation élective à leur niveau.

VI — Toxicité.

Les résultats de nos expériences conduisent à attribuer au nickel une toxicité assez élevée. On retiendra que la mort a pu être déterminée assez rapidement avec des doses de l'ordre de 10 à 20 mg. de métal par kilog d'animal ; ces doses léthales sont moindres que celles indiquées par LABORDE et RICHE [5].

Les sujets avec lesquels l'expérience a pu être poursuivie ont tous accusé d'énergiques réactions de défense : spasmes, vomissements ; évacuations généralement diarrhéiques, rejet de bave, — diarrhée et bave parfois sanglantes. L'atteinte des centres nerveux respiratoires est nette (dyspnée à peu près constante), celle du centre cardiaque est tout aussi caractéristique. Les lésions terminales du myocarde peuvent prendre un caractère suraigü, que les graphiques 1 à 7 mettent nettement en évidence, le graphique 7 en particulier est tout à fait caractéristique de l'effondrement relativement brutal de

la pression sanguine, sous l'effet d'un fléchissement brusque du myocarde.

Tous les émonctoires collaborent à l'évacuation du nickel ; mais leur efficacité paraît rapidement sidérée par l'effet du métal, qui se révèle très agressif au point de bloquer presque entièrement l'élimination biliaire, si active pour d'autres métaux. Le rôle de vicariance des glandes salivaires et des glandes du tractus digestif, rôle dont l'effet utile demeure très limité, est une des preuves physiologiques les plus nettes de l'agressivité du nickel.

L'étude comparée de la toxicité du cobalt et du nickel fera l'objet d'un prochain mémoire.

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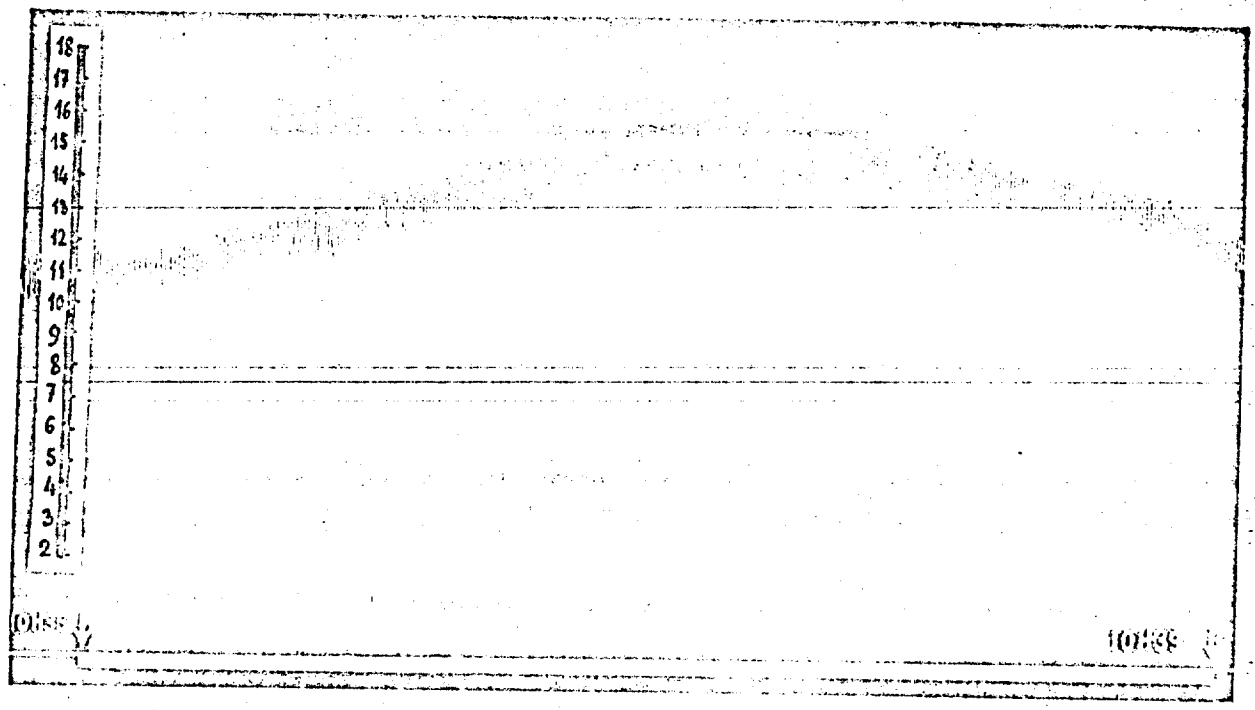
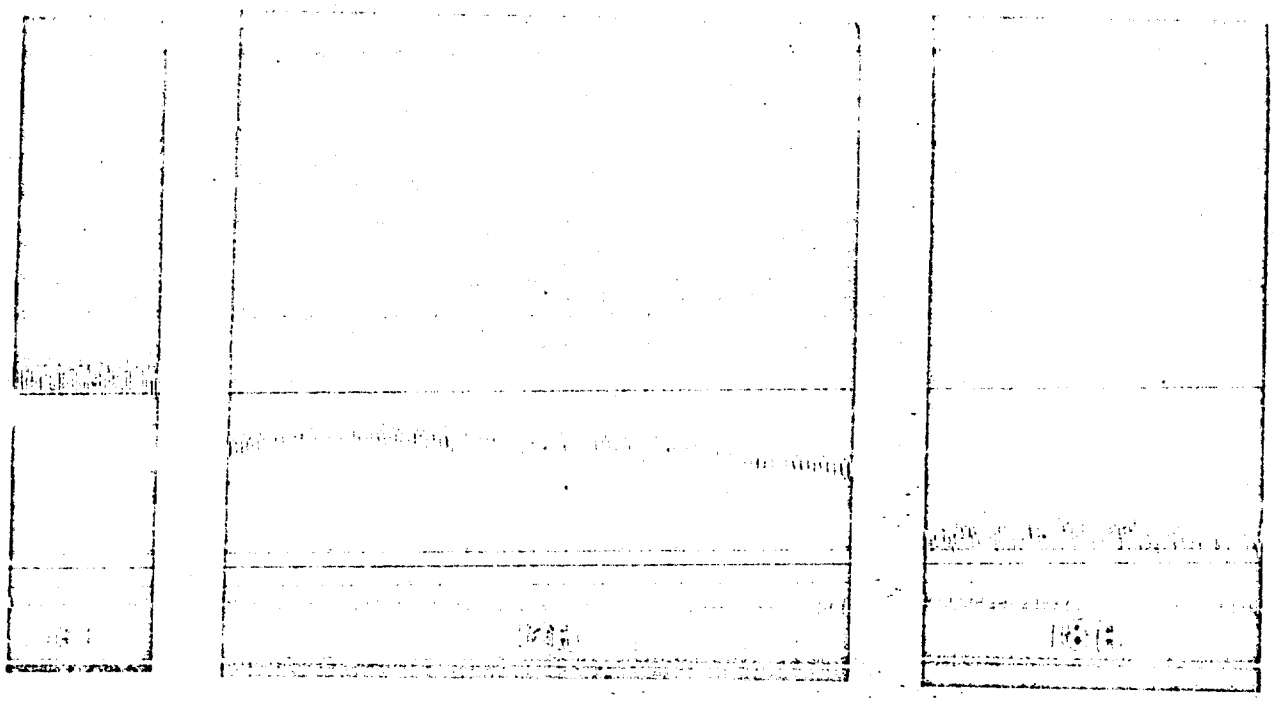
CHIEN IV.



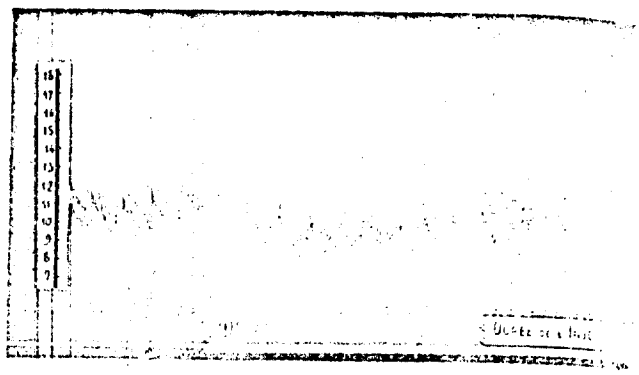
Graphique 1.

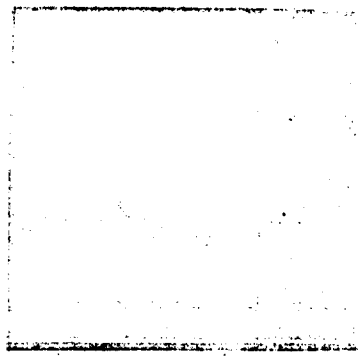
Elévation notable mais transitoire de la pression sanguine, avec augmentation légère de la différentielle.



GRAPHIQUE 2. GRAPHIQUE 3. GRAPHIQUE 4.

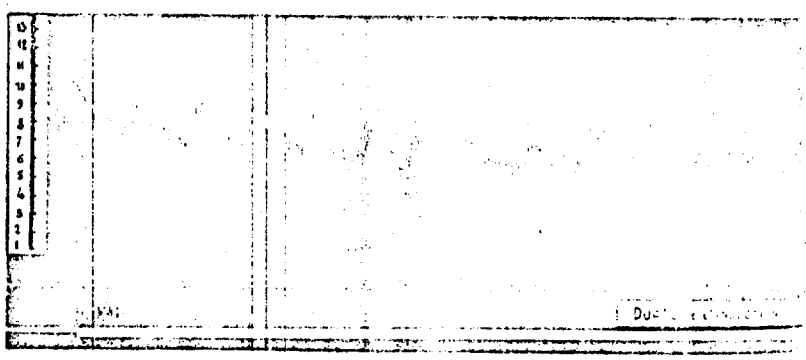
Chute progressive de la pression avec diminution de la différentielle (2 et 3) ; épuisement de plus en plus net de la force de contraction systolique avec arythmie (4).

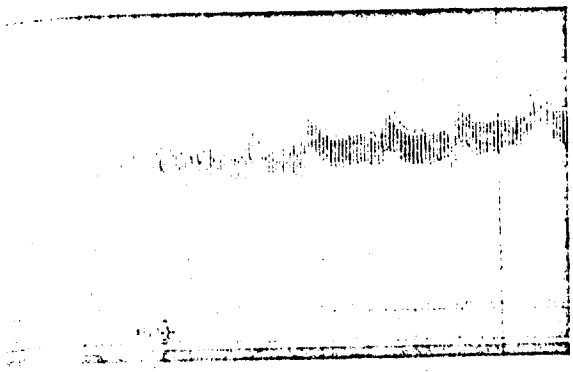




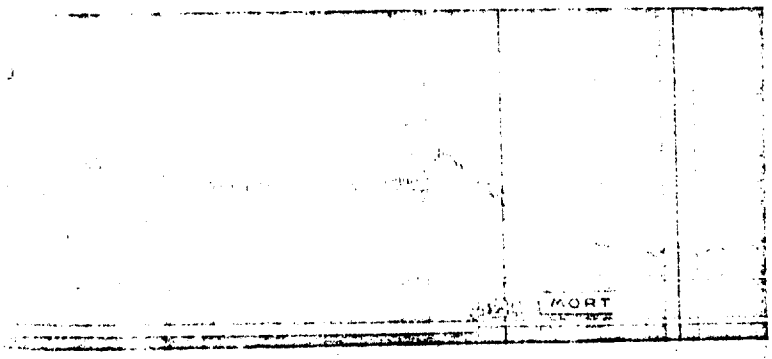
Le graphique 5 montre l'élévation de la pression sanguine et le ralentissement du rythme respiratoire déterminés par l'injection d'une solution aqueuse de chlorure de nickel.

Le graphique 6 traduit la fatigue du myocarde sous l'effet toxique du nickel.





Le graphique 7 accuse l'affaissement de la différentielle et le ralentissement de plus en plus accusé du rythme respiratoire, aboutissant à la syncope respiratoire, à laquelle s'associe immédiatement la syncope cardiaque.



Stain Technol. 37(5) 325-6 (1962)

Demonstration of Nickel in Skin by Means of Dimethylglyoxime

When nickel salts are applied topically to skin, the presence of nickel can be detected on the surface and beneath the surface as it is absorbed through the various layers. In serial sections cut in a plane either parallel or perpendicular to the skin surface, nickel can be observed histochemically by a modified method adapted from a colorimetric reaction described by Feigl (1954). Nickel can be located in the section as red-colored acicular or needle-like crystals formed by complexing of the metal with dimethylglyoxime (DMG). The complex is very insoluble in water and will not migrate within the tissue. DMG will form insoluble complexes with nickel at trace-amount levels as low as 0.1 ppm.

Histochemical Procedure

1. Freshly cut frozen sections are placed on clean glass slides without the use of water or other vehicle in order to prevent leaching or relocation of the nickel.
2. With the tissue side down, the slide is placed over an open beaker containing concentrated NH_4OH for 10 sec. The ammonia vapors render the tissue alkaline, which is necessary for complexing.
3. The slide is turned over and 1 drop of 1.0% dimethylglyoxime (Eastman Organic Chemicals, No. 98) in 95% ethanol is placed on the section for 30 sec.
4. The section is washed gently with 70% ethanol to remove the excess dimethylglyoxime reagent.

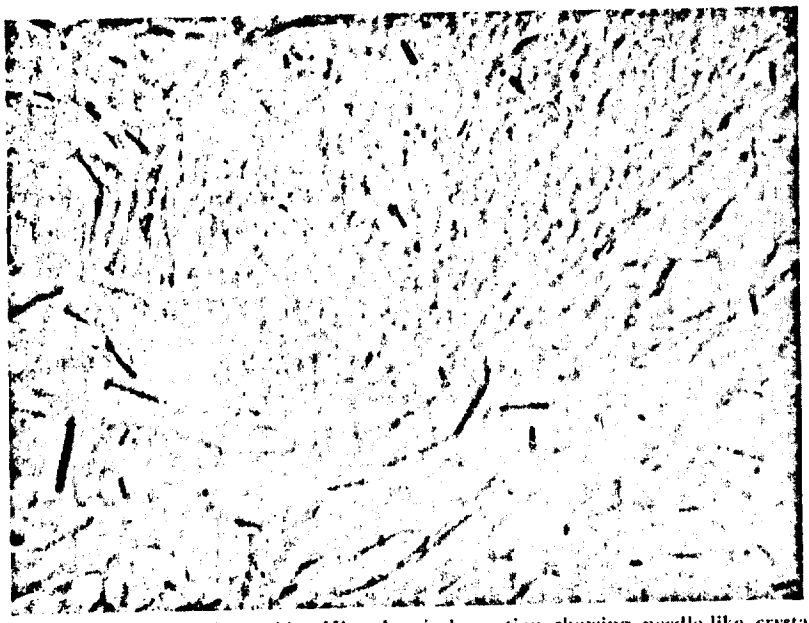


FIG. 1. Frozen section of rat skin. Histochemical reaction showing needle-like crystals of nickel-dimethylglyoxime complex in the dermis. × 450.

5. Several drops of 95% ethanol are placed on the section, followed by several drops of absolute ethanol, each for 2–5 sec.
6. Several drops of xylene are used to clear for 5 sec.
7. Mounting is done in Canada balsam with a No. 1 thickness cover glass.

Results. Sections examined with a standard light microscope show sufficient contrast of the red needles without the use of a background stain. However, superior contrast is achieved with the use of an interference microscope. With this microscope, the following conditions produce optimum contrast: (a) double focus interference lens system; (b) polychromatic light source with no filters; (c) analyzer setting at 212°; and (d) medium opening of the iris diaphragm. Any magnification of 100 to 1000 with either microscope is satisfactory.

Needle-like crystals of the Ni-DMG complex are shown in the dermis following the topical application of nickel chloride on rat skin (figure 1).

Caution. Impure dimethylglyoxime reagent causes fading of the nickel-complex color.—B. R. CHOMAN, *Research and Development Division, Lever Brothers Company, Edgewater, New Jersey.*

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Dissertation Absts. 16(9)1570(1956)

CHEMISTRY, BIOLOGICAL

A SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF SUBMICROGRAM AMOUNTS OF NICKEL IN HUMAN BLOOD

(Publication No. 17,602)

Maxwell Lewis Cluett, Ph.D.
University of Virginia, 1956

A method has been developed for the determination of nickel in human blood and may, of course, easily be extended to other biological materials.

Blood is drawn into a sterilized hypodermic syringe equipped with a special platinum-ruthenium alloy needle. Sodium heparin is added as an anticoagulating agent. The sample is wet-ashed with nitric acid in an open Pyrex vessel on a hot-plate at 330°C. The mineral constituents are converted to chlorides by evaporation to dryness from strong hydrochloric acid solutions. Iron(III), copper and lead interfere in the spectrophotometric procedure for nickel and hence must be removed. Iron and copper are separated by an ion exchange procedure, based on the selective adsorption of these metal ions on Dowex-1 anion exchange resin from 7.0-7.5 molar hydrochloric acid solution. Following the ion exchange procedure, lead ions are separated by their selective "adsorption" on calcium carbonate from neutral or alkaline solution. The average recovery from solutions containing known microgram amounts of nickel is 100 ± 4 per cent when passed through the Dowex-1 column, and 98 ± 5 per cent, from similar solutions contaminated with lead, upon passing through the calcium carbonate column. The spectrophotometric procedure is based upon the extraction from alkaline solution of the complex formed between nickel and diethyldithiocarbamate. Isoamyl alcohol is the extracting solvent and the absorbance is measured at 325 mμ in a 1-cm. cell. The procedure has an absolute sensitivity of 0.0048 γ of nickel per cm.^{2} for an absorbance of 0.001 unit. The gram atom absorptivity is 37,000. The standard deviation in determining 0.5 microgram of nickel is 4.0 per cent.

The isolation, separation and spectrophotometric procedures were used as an analytical method for the determination of nickel in human whole blood samples and one plasma sample. Ten-milliliter samples were analyzed and the nickel concentration in whole blood was found to range from 0.020 to 0.090 part per million; the average concentration in blood from eight patients was 0.041 part per million. The concentration of nickel in the plasma sample was 0.012 part per million.

The application of photoelectric spectrophotometry in trace analysis is reviewed. Two methods are available, depending on the choice of references for setting the zero and 100 instrument readings. One method is specifically applicable for trace analysis when solutions sufficiently concentrated to permit optimum conditions are not available. An experimental working curve and the standard deviation of each method have been determined for our procedure for traces of nickel.

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Analyst 88: 125-30 (1963)

An Improved Chromatographic Method for determining Trace Elements in Foodstuffs

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The quantitative paper-chromatographic determination of copper, cobalt, nickel, molybdenum and manganese in foodstuffs is described. The extraction of the elements after ashing the material has been investigated. The elements are separated as their chlorides on slotted chromatography paper, and are identified colorimetrically. The concentrations are determined by reflectance measurements on the papers, and the results are calculated with the aid of prepared standard graphs. As little as 0·05 μg of the elements can be determined.

The application of paper chromatography to separating and determining trace elements in foodstuffs has recently been reviewed and extended by some investigators.[1,2,3,4] The elements are separated and then determined colorimetrically[1,2] or by visual comparison with known standards on the papers after spraying with suitable reagents.[2] The determination has also been carried out by automatic densitometry. This investigation was undertaken in an endeavour to apply chromatography on Whatman No. 1 (type CRL/1) paper, as used by Hunt, North and Wells[1] and Duffield,[2] to the routine determination of trace elements in foodstuffs. During this work several modifications were introduced, and the determinations were carried out by a simplified technique with use of an E.E.L. mark III Reflectometer Head (Evans Electroselenium Ltd.). The elements determined are copper, cobalt, nickel, manganese and molybdenum.

EXPERIMENTAL

The ashing and leaching of various types of analytical samples came under close investigation. The different ash contents cause some difficulty, since a large volume of solvent is required for extracting the elements from material having a high ash content before they can be applied on the paper. Cobalt, nickel and molybdenum, normally present at low concentrations, would not be detected in these circumstances. Coulson, Davies and Luna[1] used a wet-ashing technique; they then filtered and extracted with dithizone to concentrate the elements. This proved time-consuming, and ashing with concentrated nitric acid was then investigated.[5] This gives an extremely good ash, but a tendency to spatter makes it of doubtful value in work on trace elements; it also necessitates the conversion of the nitrates formed to the chlorides. Webb, Stevens and Hallas[3] used an extraction technique based on the fact that the chlorides of the elements are soluble in 1 per cent. hydrochloric acid in acetone; it could not, however, be incorporated in the proposed technique, owing to the small volumes required for spotting on the papers. The main interfering elements encountered in large amounts are calcium, magnesium, iron, potassium and silicates. In the proposed method, centrifugation is used to remove silicates and other ashing debris, and all evaporations are carried out in the original ashing dishes. This procedure requires the minimum of equipment, and, after ashing overnight, the medium can be prepared for chromatography in only 6 hours. The final precipitate is dissolved in a minimum of 2 ml of diluted hydrochloric acid (1 + 1).

The two solvent systems used are—

1. For separating copper, cobalt, nickel and manganese, ethyl methyl ketone, concentrated hydrochloric acid and water (15:3:2); approximately 30 minutes are required for development. When much copper and iron are present tailing of the copper takes place, but can be prevented by changing the solvent ratio to 15:3·5:1·5.

2. For separating molybdenum and zinc, n-butanol and 4 N hydrochloric acid (15 + 6); 2 hours are required for development.

The determination of copper, cobalt and nickel with rubeanic acid follows that laid down by Hunt, North and Wells[1] and used by Duffield.[2] This allows the use of a graded distribution in the standards from 0·05 to 1·0 μg, in steps of 0·1 μg. Amounts of cobalt less than 0·05 μg are best determined with 2-nitroso-1-naphthol, which, according to Bellucci,[6] is more sensitive than the 1-nitroso-2-naphthol used by Coulson, Davies and Luna.[4] The greater sensitivity of 2-nitroso-1-naphthol was not confirmed by Yoe and Barton,[7] but in this investigation it gave a more intense colour on the paper. Manganese is determined in the same solvent and developed with formaldoxime, as described by Webb, Stevens and Hallas.[3] Time is a critical factor in these analyses, particularly in the determination of manganese, the colour of which tends to fade rather quickly in air. The times laid down in the proposed method are essential for reproducibility and for comparison with standards.

The separation of molybdenum and zinc is best carried out in solvent mixture 2. The zinc travels as a band, which shows up well with the Zincon reagent or 1-nitroso-2-naphthol 4-sulphonic acid. It is not possible to determine zinc quantitatively on paper, since the band produced is so concentrated that even with millimicrogram amounts it leads to erroneous comparisons with standards. It is best determined colorimetrically after the spots have been eluted from the paper. Molybdenum is determined with dithiol as indicator, which is normally used in conjunction with thioglycollic acid in 1 per cent. w/v sodium hydroxide solution.[8] This was found to be impracticable, as the high concentration of iron present forms a purple thioglycollate complex that tends to mask and interfere with the normal development of the molybdenum - dithiol complex. Bingley[8] reported that the presence of iron increased the sensitivity of the molybdenum - dithiol complex, but he again incorporated the thioglycollic acid. He also reported that a concentration of 4 N hydrochloric acid was necessary. In the proposed method the dithiol is dissolved in ether and sprayed directly on the dry paper after chromatography. After the paper has again been dried and then sprayed with N hydrochloric acid, iron shows as a greyish spot that does not interfere with the colour-density determination of molybdenum; hydrochloric acid helps to fix and intensify the green dithiol complex.

When visual analysis is applied to a sample, standards must be run at the same time. This is time-consuming and, when more than 50 p.p.m. of maganese are present, it can lead to large errors because of the dilution factors involved. These errors can be avoided by employing reflectance measurements with an E.E.L. mark III Reflectometer Head and Unigalvo type 20. The head is used without the diffusing glass or lens, and, by selecting appropriate filters for the colours developed on the paper, the percentage reflectance can be measured. By this means standard charts are prepared of concentration (0·05 to 0·5 μg) against percentage reflectance. For the reflectance measurements masks are used that limit the reading to the area of the spot. Brass plates with slits (*a*) 13 mm × 20 mm and (*b*) 13 mm × 15 mm were made to fit the head, 13 mm being the width of each strip of the CRL chromatography paper. Mask (*a*) is used for molybdenum, manganese and nickel, and mask (*b*) for copper and cobalt.

METHOD

APPARATUS—

Vitreosil basins.
Centrifuge tubes, 15 ml.
Stoppered glass tubes.

REAGENT—

Hydrochloric acid, diluted (1 + 1).

PREPARATION OF SAMPLE—

Ash 20 g of the sample overnight at 550° C in a vitreosil basin. Allow to cool, and add 5 ml of diluted hydrochloric acid (1 + 1). Evaporate on a steam-bath, and heat on a hot-plate for 30 minutes. Repeat the hydrochloric acid addition and evaporation, and then add 5 ml of hot diluted hydrochloric acid (1 + 1), heat, and transfer to a 15-ml centrifuge tube, washing in all debris with not more than 5 ml of diluted hydrochloric acid (1 + 1). Place the tube in a boiling-water bath for 30 minutes, spin in a centrifuge, and then return the supernatant liquid to the vitreosil basin. To the precipitate in the tube add 5 ml of diluted hydrochloric acid (1 + 1), and again place in a boiling-water bath for 30 minutes;

then spin in a centrifuge, and add the washings to the original supernatant liquid. Repeat the washing. Evaporate the combined solutions to dryness on a steam-bath, and dissolve the residue in 2 ml of diluted hydrochloric acid (1 + 1); if necessary, heat gently to dissolve. Transfer the solution to a stoppered glass tube. Prepare (1 + 4), (1 + 9) and (1 + 19) dilutions of portions from the stoppered glass tube, and use 0·01 ml of these solutions for spotting on the paper.

Paper chromatography

Apparatus—

Petri dishes, 5 inch—For covering the beakers.

Capillaries—Calibrated to deliver 0·01 or 0·02 ml.

Whatman No. 1 chromatography paper (type CRL/1)—This paper allows the application of 10 samples at a time.

A swivel mounted cradle on which the paper can be mounted for spraying purposes.

E.E.L. mark III Reflectometer Head.

E.E.L. Unigalvo type 20.

Procedure—

Apply 10-μl spots of sample solution on the paper, and dry in a current of warm air to give a spot 1 cm in diameter on the baseline, which is approximately 1·5 cm from the bottom of the paper. Join the ends of the paper to form a cylinder, and place in a 500-ml beaker containing 20 ml of the appropriate solvent; cover with a Petri dish, and develop by an ascending-solvent technique.

Determination of copper, cobalt and nickel

Reagents—

Solvent mixture—Ethyl methyl ketone, concentrated hydrochloric acid and water in the ratio 15:3:2.

Rubeanic acid solution—A 0·1 per cent. w/v solution in ethanol.

2-Nitroso-1-naphthol solution—Dissolve 0·05 g of the reagent in 10 ml of water and 0·5 ml of N sodium hydroxide with heating, and then dilute to 100 ml with water.

Ammonia solution, sp.gr. 0·88.

Procedure—

Apply the sample as described above, and stand the paper in the beaker containing 20 ml of the solvent. Allow the solvent to diffuse to the top of the strips (30 minutes). Remove the paper, and dry in air for 5 minutes. Stand the dried paper in ammonia vapour for 10 minutes to neutralise, dry in a current of warm air, and then spray both sides with the rubeanic acid solution. The spots denoting copper, cobalt, and nickel can be read immediately; nickel (purple-blue, R_F 0·07), cobalt (orange-yellow, R_F 0·27) and copper (olive-green, R_F 0·48).

Determination of manganese

Reagents—

Solvent mixture—Ethyl methyl ketone, concentrated hydrochloric acid and water in the ratio 15:3:2.

Formaldoxime—Dissolve 20 g of paraformaldehyde and 55 g of hydroxylamine sulphate in boiling water, and dilute to 100 ml. Dilute 1 ml of this solution to 10 ml with water.

Ammonia solution, sp.gr. 0·88.

Sodium hydroxide, N.

Procedure—

Run the chromatogram as for copper, cobalt and nickel. Dry the paper in air for 5 minutes, and neutralise in an atmosphere of ammonia for 10 minutes; dry, and spray with formaldoxime. Replace the paper in ammonia vapour for 5 minutes, then spray with N sodium hydroxide to fix the complex, and dry in a current of warm air. When the paper is dry, replace it in ammonia vapour for 5 minutes, and then read immediately. The colour can be re-intensified in ammonia vapour should it fade during reading. Manganese is denoted by a reddish brown colour (R_F 0·15). The time sequence is extremely important for this element.

DETERMINATION OF MOLYBDENUM

REAGENTS—

Solvent mixture—n-Butanol - 4 N hydrochloric acid mixture (15 + 6).
Dithiol solution—A 1 per cent. w/v solution in ether, prepared once a week.
1-Nitroso-2-naphthol-4-sulphonic acid solution, 0·5 per cent. w/v in ethanol.

PROCEDURE—

Apply the samples as stated previously, and develop in the butanol - hydrochloric acid solvent (2 hours). Remove the paper, and, for determining molybdenum, dry in a current of warm air. Spray with dithiol solution, dry, and spray with N hydrochloric acid. Molybdenum is denoted by an apple-green colour (R_F 0·55).

The presence of zinc may be detected by running a paper as for molybdenum, neutralising in ammonia vapour for 5 minutes, and spraying with 1-nitroso-2-naphthol-4-sulphonic acid solution. At levels as low as 0·1 μg on the paper, zinc appears as an orange band (R_F 0·8

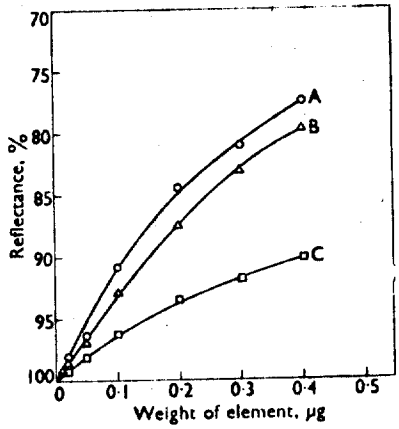

Fig. 1. Calibration curves: curve A, cobalt; curve B, nickel; curve C, copper

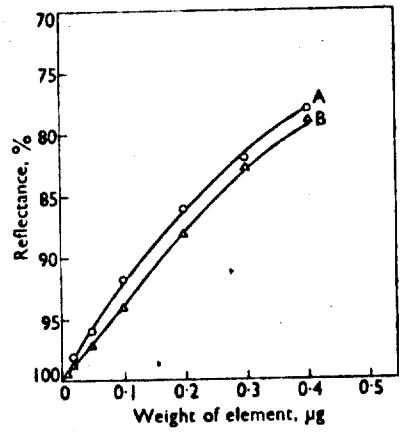

Fig. 2. Calibration curves: curve A, molybdenum; curve B, manganese

PREPARATION OF STANDARDS

Prepare a mixed synthetic standard containing copper, cobalt, nickel, molybdenum, manganese and zinc. Dilute to the required concentrations, evaporate the solutions, and

carry out the extraction procedure described for the ashed material. Finally, dissolve in 2-ml portions of dilute hydrochloric acid (1 + 1) so that 10-μl portions contain 0·05, 0·1, 0·2, 0·3, 0·4 or 0·5 μg of the elements.

Run chromatograms of the standards, and read the reflectance with the red filter for copper, nickel and molybdenum and the blue filter for cobalt and manganese. Prepare standard curves of percentage reflectance against concentration (see Figs. 1 and 2).

TABLE I

ANALYSIS OF CEREALS

Cereal	Copper found		Cobalt found		Nickel found		Manganese* found		Molybdenum found	
	A, p.p.m.	B, p.p.m.	A, p.p.m.	B, p.p.m.	A, p.p.m.	B, p.p.m.	A, p.p.m.	B, p.p.p.	A, p.p.m.	B, p.p.m.
Oats ..	3·25	3·1	<0·05	0·02	1·5	2·9	62	54	0·33	0·38
Barley ..	5·0	6·5	<0·05	0·01	0·25	0·28	27	30	0·15	0·16
Oats ..	6·25	6·2	<0·05	0·05	6·75	7·2	90	98	0·35	0·44
Barley ..	6·75	7·2	<0·05	0·01	0·2	0·11	17	19	0·2	0·18
Oats ..	3·8	4·2	<0·05	0·01	0·15	0·17	40	33	0·25	0·44
Barley ..	4·2	4·1	<0·05	0·01	0·3	0·30	17	17	0·35	0·68
Oats ..	2·25	2·3	<0·05	0·03	0·7	1·10	44	32	0·28	0·33
Barley ..	3·25	2·8	<0·05	0·02	0·25	0·15	15	14	0·4	0·47

A = Chromatographic analysis. B = Spectrographic analysis.

* Manganese, which is present in large amount, requires a dilution of (1 + 19) to bring the final spot within the range of the reflectometer. Cobalt, molybdenum and nickel, present in only small amounts, are placed on the paper in 20-μl spots; this brings the reading on the reflectometer to a more acceptable range.

TABLE II

RECOVERY OF ELEMENTS FROM A SAMPLE OF OATS

Mean of three results

	Copper		Cobalt		Nickel		Manganese		Molybdenum	
Amount present in original material, μg	5·2	5·2	—	—	0·1	0·1	21	21	0·3	0·3
Amount added, μg ..	4·0	8·0	<0·01	<0·02	0·17	0·34	13	26	0·44	0·88
Total amount found, μg	8·6	15	<0·05	<0·05	0·4	0·6	35	51	0·6	0·9

TABLE III

CHROMATOGRAPHIC ANALYSIS OF FOODSTUFFS

Sample	Ash content, %	Copper found, p.p.m.	Cobalt found, p.p.m.	Nickel found, p.p.m.	Molybdenum found, p.p.m.	Manganese found, p.p.m.
Heavy hog fattening ration	7	22	<0·15	<0·15	<0·15	108
Sow and weaner mix ..	7·3	75	<0·1	<0·1	<0·1	78
	7·2	67	<0·15	<0·15	<0·15	81
Fishmeal	12·6	2	<0·15	<0·15	<0·15	11
	12·8	3	<0·15	<0·15	<0·15	11
Meat and bone meal ..	42	—	<0·5	<0·5	<0·5	17

DISCUSSION OF METHOD

Table I shows the results obtained on cereals by the proposed method. These results compare favourably with those by arc spectrography carried out by a separate investigator at the Johnstown Castle Soils Laboratory, Co. Wexford. The ashing and extraction techniques used in the latter method were those of Mitchell[9] and Farmer.[10] The value of the method is obvious when it is considered that 10 samples can be analysed by one operator for these five elements in 2 days, a minimum of space, equipment and chemicals being necessary. When determining manganese it is advisable to include a known standard on the paper for reading, together with the unknowns, as a check that the development procedure

has been carried out properly. A high iron content in the original sample will tend to inhibit the separation of molybdenum, but, if the paper is left for at least 15 minutes in the solvent after completion of the run, a definite separation is obtained.

Table II shows recoveries carried out on a sample of oats expressed as the mean of three determinations. In the analysis of foodstuffs with a high ash content (see Table III) a large volume of diluted hydrochloric acid (1 + 1) is required for dissolving the extracted elements before they are placed on the papers. For meat and bone meal, which has a 42 per cent. ash, 10 ml of acid are required to dissolve the elements from 10 g of material. This raised the background limit of detection from 0·05 to 0·5 p.p.m.

The reflectance measurement operates within a range of 0·01 to 0·5 μg with a short deflection on the scale. Above 0·5 μg the curve levels off owing to supersaturation of the spot with the element. This can be a source of error when reading from the chart, as a change in reflectance of 1 division on the scale can amount to 0·02 μg for nickel and 0·03 μg for copper. This is not a serious error with an undiluted solution, but for manganese, which requires a dilution of (1 + 19) when present at the 100 p.p.m. level, the error can be ± 6 per cent.

We thank Mr. Garret A. Fleming, M.Sc., for spectrographic analysis and helpful discussions, Mr. J. M. Dunne for technical assistance and Mr. T. Kendrick for preparing the graphs.

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First received *January 19th*, 1962
Amended, *September 28th*, 1962

THE BRITISH JOURNAL
OF
DERMATOLOGY

MARCH 1964

76(3) 99-109

STUDIES ON THE BINDING OF PROTEIN BY NICKEL. WITH SPECIAL REFERENCE TO ITS ROLE IN NICKEL SENSITIVITY.

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THE mechanism of nickel dermatitis is generally considered to be allergic. According to the Landsteiner and Chase hypothesis (1941), nickel would be required to act as a hapten, combining with a protein to form a specific antigen and leading to the formation of a specific antibody. Reaction between the nickel-protein antigen and the corresponding antibody would then instigate a series of events culminating in the clinically observed dermatitis. A number of haptens which apparently act in this way have been studied and their mode of combination with protein elucidated.

No such investigations have been carried out in the case of nickel; further, nickel dermatitis is in some ways atypical of the group of contact dermatoses into which it is generally placed. As in other forms of contact dermatitis, no antibodies have been demonstrated in nickel-sensitive patients, but the literature contains no references to the successful experimental sensitization of either humans or laboratory animals. It was, therefore, considered of importance to carry out a preliminary study of the chemistry of nickel-protein complexes, in order to establish whether nickel could, in fact, behave as a hapten and initiate an allergic response of the type postulated by Landsteiner and Chase.

The investigation of a metallo-protein complex, with reference to the nature and number of binding sites occupied by the metal, requires a protein of known molecular weight and high purity. These considerations indicated the use of bovine serum albumin, in the main, for this study.

INVESTIGATION.

The following protein preparations were used in the experiments to be described:

(i) Crystalline bovine serum albumin, molecular weight 69,000 (Armour Pharmaceutical Co., Ltd., Eastbourne, Sussex).
(ii) Human serum γ-globulin for immunology (Virus Reference Centre, Colindale).
(iii) Gelatine powder and casein, soluble (Hopkins and Williams, Chadwell Heath, Essex).

(iv) Pseudoglobulin prepared from human serum by salting out with ammonium sulphate (Rocha e Silva *et al.*, 1949).

(v) Cyanide-kerateine and sulphide-kerateine prepared according to Goddard and Michaelis (1934).

Dialysis was performed in $\frac{18}{32}$ inch flat width Visking Nojax tubing (Hudes Merchandising Co., Ltd., London). The buffers employed were sodium cacodylate (British Drug Houses, Ltd., Poole, Dorset), acetate, and metaborate (Hopkins and Williams). All other chemicals were of Analar grade (Hopkins and Williams). The nickel salt used was nickel sulphate, $NiSO_4.7H_2O$.

Equilibrium dialysis.—The chief experimental method used was equilibrium dialysis (Hughes and Klotz, 1956). In this procedure the nickel and protein solutions are initially separated by a semipermeable membrane. The free metal ions will diffuse through the membrane until at equilibrium their concentration on either side is the same; metal bound to the protein, however, is effectively removed from the system. Knowing the initial concentration of nickel and its final concentration in free solution, it is possible to calculate how much is bound to the protein, by the equations

$$r = M_P/P_r \quad . \quad . \quad . \quad . \quad . \quad . \quad \text{(i)}$$

$$M_P = M_r - V_r(M) \quad . \quad . \quad . \quad . \quad . \quad \text{(ii)}$$

in which r is the number of atoms of nickel bound per molecule of protein, M_P is the number of moles of protein-bound metal, P_r the total number of moles of protein per tube, (M) the concentration of free metal, V_r the total volume of solution and M_r the total amount of metal in the system.

As a rule the protein solution was placed inside a dialysis bag, made by knotting each end of a strip of Visking Nojax tubing, and placed inside a stoppered test-tube containing the metal solution; occasionally it was more convenient to have the protein externally and the nickel inside the bag.

Before each experiment the dialysis tubing was boiled for a total of 4 hours in several changes of distilled water to remove contaminants. It was then soaked in distilled water for 3 hours, and overnight in the buffer to be used in the subsequent experiment. All dialysis experiments were done at room temperature, the tubes with their enclosed bags being gently agitated by a rotary shaker to hasten equilibrium.

Determination of nickel.—Nickel was estimated at first polarographically by means of a commercial pen-recording apparatus using a dropping mercury electrode. Owing to repeated instrumental failure, a colorimetric method was substituted and nearly all the analyses were done using the latter technique.

To 0·1 ml. of nickel solution was added 0·9 ml. of ammonia solution (specific gravity 0·880), 9 ml. of water and 0·1 ml. of bromine water. The mixture was then shaken and 0·5 ml. of 1% dimethylglyoxime in ethanol was added. The mixture was again shaken and allowed to stand for 30 minutes. The resulting red-brown colour was then estimated in a "Spekker" photoelectric colorimeter using No. 5 Ilford filters (λ max. = 550 mμ). Beer's Law was obeyed within the limits of 10-800 μg./ml. In a series of 40 preliminary estimations the technique was found to have an error of $\pm 1{\cdot}6\%$.

Modification of proteins.—Samples of bovine serum albumin were treated by various methods to block amino, carboxyl, imidazolyl, phenolic, aliphatic hydroxyl and thiol groups (Frankael Conrat *et al.*, 1949). In addition amino groups were removed by the Van Slyke method (Pearse, 1953, and Wells, 1956). The dinitrofluorobenzene method of Sanger (1945) was also used to block ϵ-amino groups of lysine residues.

The following preliminary experiments were conducted.

(i) A series of tubes was set up with water inside the dialysis membrane and nickel solution in the outer compartment. Tubes were removed and the contents assayed

at hourly intervals over five hours in order to ascertain the time required for dialysis to reach equilibrium.

(ii) A series of tubes was set up in which the nickel concentration in the outer compartment was varied in the range 0·43–27·3 μmoles/ml. These were assayed internally and externally after equilibrium had been reached in order to determine the percentage recovery of the dialysis procedure.

To ascertain the effect of the concentration of nickel on the degree of protein binding, 2 ml. aliquots of 4% bovine serum albumin in cacodylate buffer (pH 7·0), were placed inside dialysis bags and dialysed against 2 ml. of nickel solution at a number of different concentrations, usually 6–10, ranging from 100 to 1600 μg./ml. (1·7–27·3 μmoles/ml.). After equilibrium had been reached (3 hours) the external (protein free) solution was analysed for nickel. Similar experiments were also carried out, mostly at pH 7·0, using human γ-globulins, casein, gelatine, pseudoglobulins and kerateines.

To determine what part, if any, the Gibbs-Donnan membrane phenomenon played in these experiments, several equilibrium dialysis experiments with bovine serum albumin and nickel were performed, as above, except that sodium chloride was added to a final concentration of 1 *M* (Hughes and Klotz, 1956).

The effect of pH on nickel binding was investigated. Dialysis systems of nickel and bovine serum albumin were set up at pH 3·5 and 5·0 (acetate buffer), 5·0 and 7·0 (cacodylate buffer) and 7·0 and 9·0 (borax buffer). A range of nickel concentrations was used in each case.

The nickel-binding capacity of denatured protein was studied by equilibrium dialysis, using bovine serum albumin previously treated with urea, heat or ultraviolet radiation.

Procedures used to denature the albumin were as follows.

(i) Solutions of bovine serum albumin were mixed with an equal volume of 12 *M* urea solution. After standing for 2 hours, the urea was removed by dialysis against water.

(ii) Bovine serum albumin solutions were kept at 50° C. for 20 minutes.

(iii) Bovine serum albumin solutions in quartz cuvettes were exposed to a medium-pressure quartz mercury vapour arc for 2 hours. Precautions were taken to ensure that the heat from the lamp was negligible by keeping the lamp at a distance such that there was no rise in temperature of the solution.

After each of the above treatments the protein was used in the usual type of equilibrium dialysis experiment against nickel, the latter usually being placed inside the dialysis bag, instead of outside, and the protein externally.

The effect of blocking or removing various reactive groups was studied by taking ml. samples of modified bovine serum albumin as a 4% solution in cacodylate buffer pH 7·0, and dialysing against a number of concentrations of nickel in the manner eviously described. Because of changes in the physical properties of the modified otein solutions, it was sometimes more convenient to have the nickel within the dialysis bag, the protein outside it. The metal-protein binding was compared with ntrol samples of bovine serum albumin that had been treated in all respects similarly to the modified samples except that the modifying reagent was omitted. This was done to ensure that observed changes in metal-protein binding were not due to denaturation arising out of the manipulative procedures.

The stability of the nickel-protein complex was investigated directly. Nickel-protein preparations obtained by dialysis were subjected either to prolonged paper electrophoresis at pH 7·0, or washing in four changes of 400 ml. of cacodylate buffer (pH 7·0). The electrophoretic strips were stained for protein with 0·2% Ponceau S in 5% acetic acid and for nickel by spraying with a saturated solution of dimethylglyoxime in ethanol or a similar solution of dithio-oxamide. In the experiments where nickel-protein complexes were washed, the nickel remaining bound was estimated by a standard qualitative dimethylglyoxime "spot test" (sensitive to one part in 10^6).

RESULTS.

With the dialysis method used, equilibrium was attained at three hours and there was no significant uptake of nickel by the dialysis bags. The recovery of nickel in three experiments was 98·7 % which is of the same order of accuracy as with the colorimetric method.

The curve obtained at pH 7·0 by plotting atoms of nickel bound per molecule of protein *versus* final concentration of free nickel is shown in Fig. 1. This curve was not sensibly altered by performing dialysis in the presence of 1 M NaCl; so that errors due to the Gibbs-Donnan membrane effect are negligible.

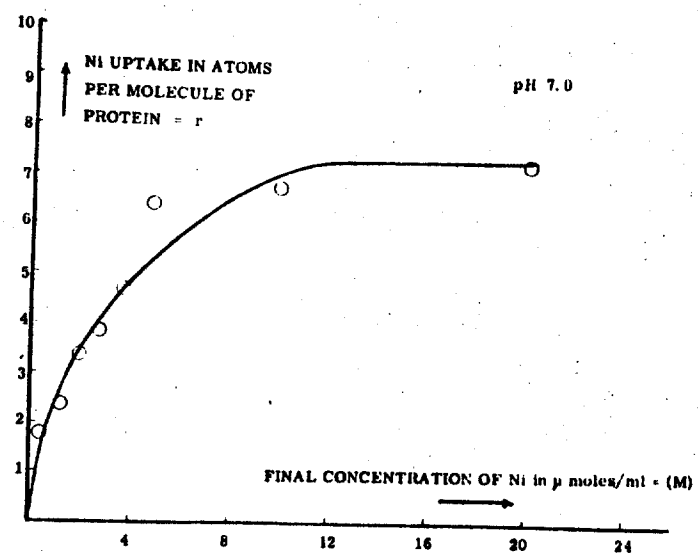

FIG. 1.—Uptake of nickel by bovine serum albumin at pH 7·0 as obtained by equilibrium dialysis of various concentrations of Ni^{++} with constant protein concentration (4·0%), cacodylate buffer. Ionic strength = 0·1.

small. If more than two or three individual experimental values failed to fall within ±2 % of a "best curve" through the points, as selected by the "least squares" method from a semi-logarithmic plot, the results of the experiment were rejected.

The effect of varying pH on binding is shown by comparing Fig. 1 with Figs. 2 and 3. At pH 3·5 no binding could be demonstrated, whilst at pH 5·0 some occurred. This was greater at pH 7·0 and even more so at pH 9. Experiments at pH 9 sometimes gave points that were considerably scattered, probably due to the precipitation of nickel hydroxide which interferred with diffusion through the dialysis membrane. The results of the experiments with γ-globulins, pseudoglobulins and kerateine showed that these proteins bound nickel. However, only the results of the experiments with γ-globulins, for which a value of

150,000 as a mean molecular weight was taken, were suitable for a quantitative and graphical representation (Figs. 4 and 8). The results with pseudoglobulins and kerateine were not reproducible; this was almost certainly due to denaturation and degradation.

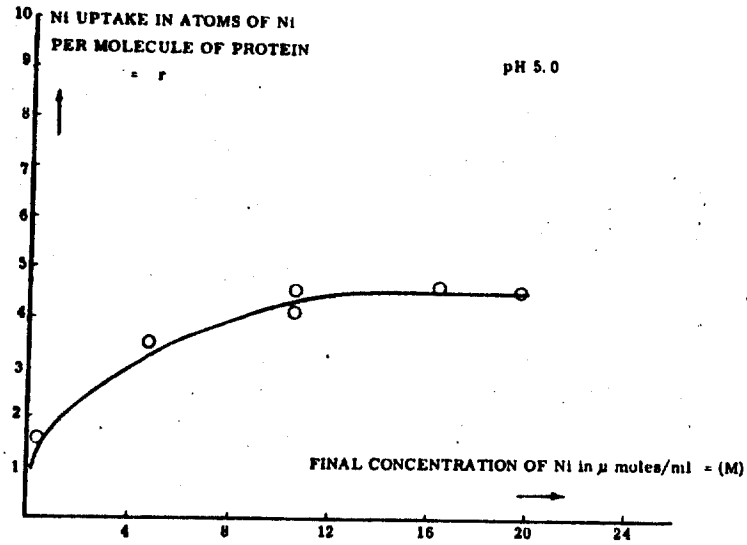

FIG. 2.—Uptake of nickel by bovine serum albumin at pH 5·0, acetate buffer. Ionic strength = 0·1.

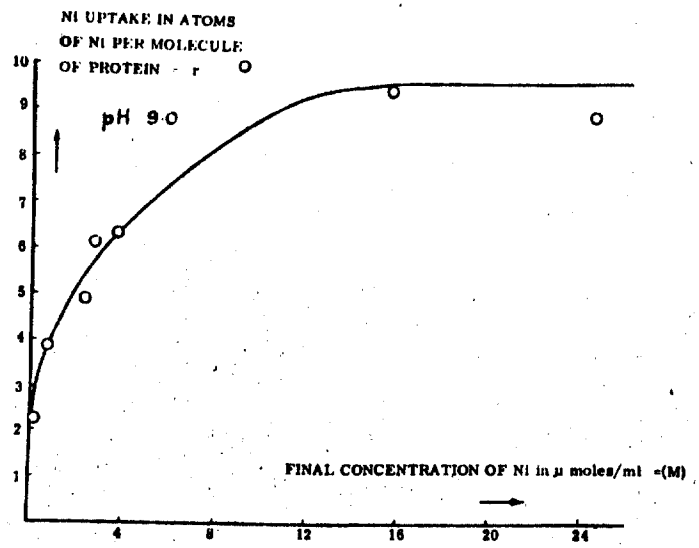

FIG. 3.—Uptake of nickel by bovine serum albumin at pH 9·0, metaborate buffer. Ionic strength = 0·1.

The result of the experiments in which bovine serum albumin was deliberately denatured was invariably a large increase of nickel binding. This also obtained in other denatured proteins such as soluble casein and gelatine powder, in which the high binding values obtained were of the same order as those found in denatured bovine serum albumin (Table I).

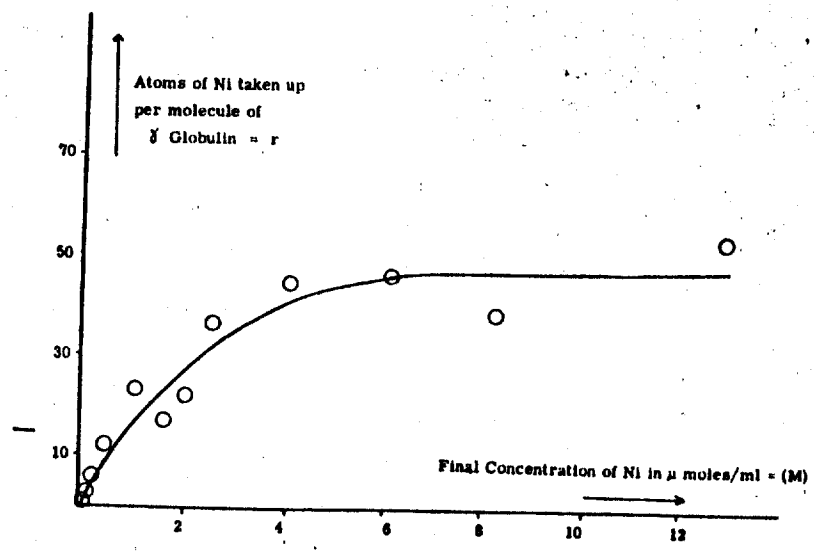

FIG. 4.—Uptake of nickel by γ-globulin at pH 7·0, cacodylate buffer. Ionic strength = 0·1.

TABLE I.—*Values for Nickel Uptake by Native and Denatured Proteins at pH 7·0*

Protein.	Uptake in mg. Ni/g. protein.	Uptake in atoms of Ni per molecule of protein.	Final concentration of free Ni in μ moles/ml.
Native :			
Bovine serum albumin (B.S.A.)	9·56	7	8·53
γ globulin	35·88	44·4	8·87
Serum proteins	22·52	—	8·55
Denatured :			
B.S.A. (UV)	34·4	25·2	8·3
B.S.A. (6*M* urea)	23·2	17	6·89
B.S.A. (heat)	31·1	22·8	6·55
Gelatine	76·1	—	4·17
Casein	79·7	—	3·5
Pseudo globulin	22·8	—	1·14
SH kerateine	8·7	—	6·5
S–S kerateine	40·8	—	6·65

The results of blocking individual reactive groups in bovine serum albumin are shown in Table II. Blocking of thiol, aliphatic hydroxyl, phenolic and imidazolyl groups generally had no effect. However, occasionally increased binding occurred which may be accounted for by incidental denaturation of the bovine

TABLE II.—*Uptake of Nickel at pH 7 on to Bovine Serum Albumin and Other Proteins in which Various Reactive Groups have been Modified.*

Group modified.	Uptake in mg. Ni/g. protein.	Uptake in atoms of Ni per molecule of protein.	Final concentration of free Ni in μ moles/ml.
Bovine serum albumin :			
Amino (Van Slyke)	2·56	1·88	6·2
Amino (Acetylation)	2·24	1·6	7·2
Amino (DNFB)	24·4	17·9	4·23
Carboxyl (methylation)	0	0	7·5
Thiol (oxidation)	9·56	7·0	8·53
Imidazole (*p*-diazobenzenesulphonic)	11·16	8·2	7·84
Phenolic (*p*-diazobenzenesulphonic)	12·35	9·05	7·6
Aliphatic hydroxyl (H_2SO_4)	27·84	20·4	4·9
Gelatine :			
Amino (acetylation)	3·86	—	6·15
Casein :			
Amino (acetylation)	7·64	—	5·17

serum albumin. On the other hand, methylation of carboxyl groups regularly abolished binding completely. Acetylation of amino groups or their removal by the Van Slyke method, reduced binding to a varying extent; usually this fell to about 25% of the control. Reaction of ϵ-amino groups with Sanger reagent (dinitrofluorobenzene) gave an increase of binding; again this may be interpreted in terms of the denaturation accompanying the chemical manipulation of the albumin.

In some experiments with kerateine, attempts were made to see what change in nickel-protein binding would occur if the thiol groups were oxidized. Solutions of kerateine, shown by the nitroprusside test to have many freely reacting thiol groups, were treated by small additions of hydrogen peroxide until tests for thiols were negative. These were then compared by equilibrium dialysis with controls. The values obtained were highly scattered in distribution, but there was clearly no reduction in nickel binding caused by oxidation.

The experiments on the stability of the nickel-albumin complex clearly indicate that this metallo-protein complex is of low stability. In the electrophoresis experiments the nickel and protein dissociated during the course of the experiment. In the case of the dialysis experiment, no nickel could be detected in combination with the protein at zero free concentration of nickel.

DISCUSSION.

In order to estimate the total number of available binding sites from the data shown in Figs. 1–3, it is convenient to use a double reciprocal plot (Figs. 5–7). Extrapolation to zero on the latter plots gives figures of 5, 9 and 10 for the number of available sites per molecule on bovine serum albumin at pH values of 5·0, 7·0 and 9·0 respectively. In the case of human γ-globulins, a value of

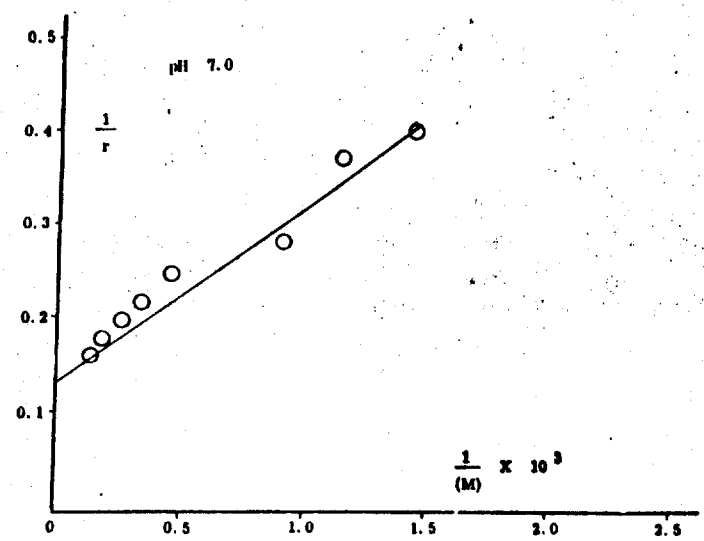

FIG. 5.—Uptake of nickel by bovine serum albumin. Double reciprocal plot of Fig. 1; where r = uptake of nickel in atoms per molecule of protein, (M) = final concentration of unbound nickel in μ moles/ml. From the following expression:

$$\frac{1}{r} = \frac{1}{kn} \cdot \frac{1}{(M)} + \frac{1}{n}$$

it follows that the intercept with the ordinate scale gives $1/n$, where n = number of *available* binding sites. Value obtained is approximately 9 available binding sites for nickel per molecule of protein.

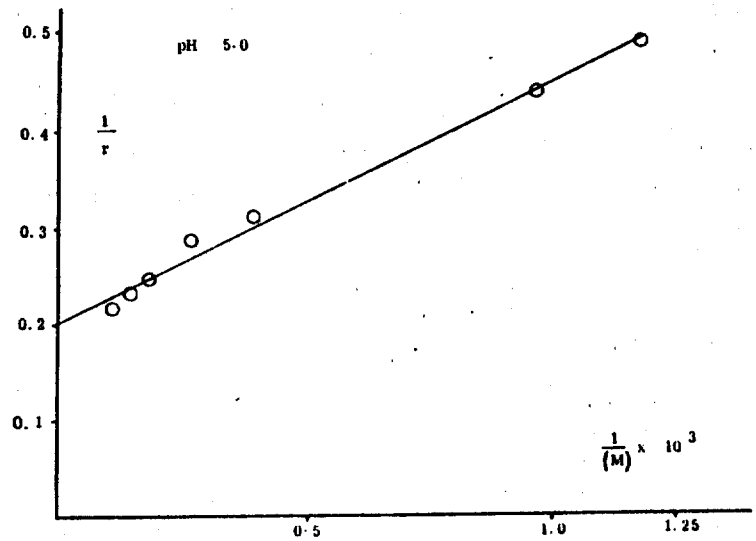

FIG. 6.—Uptake of nickel by bovine serum albumin at pH 5·0, double reciprocal plot of Fig. 2, $n = 5$.

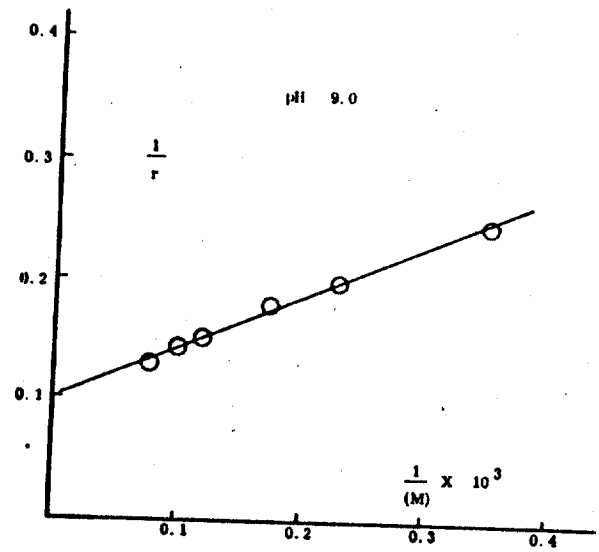

FIG. 7.—Uptake of nickel by bovine serum albumin at pH 9·0, double reciprocal plot of Fig. 3, $n = 10$.

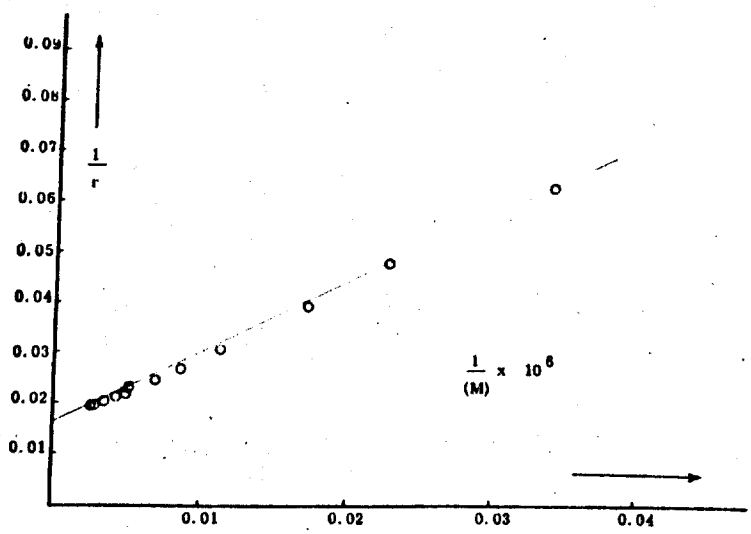

FIG. 8.—Uptake of nickel by γ-globulin, double reciprocal plot of Fig. 4, $n = 66$ (assuming an homogeneous protein with M.W. = 150,000).

about 66 is obtained ; this figure, however, is of doubtful significance since the protein preparation is grossly unhomogeneous.

These figures may be used to obtain an estimate of the "intrinsic binding constant" for the reaction between nickel and albumin from the equation

$$\frac{1}{r} = \frac{1}{nk\,(M)} + \frac{1}{n} \qquad \text{(Hughes and Klotz, 1956),}$$

where r = atoms of nickel bound per molecule of protein,
n = number of available binding sites,
k = intrinsic binding constant,
(M) = concentration of free nickel.

The values obtained fall in the range 10^2 to 10^3. This implies a very low stabilit for the nickel-albumin complex, which strongly supports the evidence obtaine from the electrophoresis and dialysis experiments on the complex.

Further confirmation of the nature and stability of the nickel-albumin comple is given by the results of the experiments using chemically modified albumi It was demonstrated that nickel was linked to the protein *via* the amino an carboxyl groups, the presence of both being required for reaction to occu . Albert (1950, 1952) has shown that complexes between nickel and free amin acids *via* these groups are of low stability ($10^4 < k < 10^8$). Because of the greatly increased effects of steric hindrance in the reactions with albumin compared with those obtaining with free amino acids, the values of the " intrinsic stability constant " reported here, for bovine serum albumin, are of the order of magnitude to be anticipated from Albert's measurements.

Eisen and Tabachnic (1958) stated that hapten-protein combination, to form an antigen, must be through a stable bond (e.g. covalent). For example, in the case of mercury, which is a known allergic sensitizing agent, the stability of the complex with albumin is very high (k = approximately 10^{20}) (Edsall *et al.*, 1954 ; Edloch *et al.*, 1953). The results reported here demonstrate clearly that nickel does not combine with the proteins studied in this manner. The relatively unstable complex between nickel and albumin would, therefore, be unlikely to behave as an antigen. A similar conclusion was reached by Everall, Truter and Truter (1954) on different grounds.

It is therefore concluded that an hapten allergic mechanism is most unlikely to obtain in nickel dermatitis, and it is clear that further work will be required in order to elucidate its true aetiology.

Summary.

The reaction between nickel and proteins was studied using the technique of equilibrium dialysis. Crystalline bovine serum albumen was used in most of the experiments. Human γ-globulin, crude commercial casein and gelatine, pseudoglobulin and kerateine were also used.

The nickel-protein complex was shown to be of low stability, the quantity of nickel bound by the protein being dependent upon the concentration of free nickel ions, the pH, and on the particular protein employed.

Experiments in which reactive groups were selectively modified suggested that the sites of binding of the nickel ions to the protein were primarily carboxyl and amino groups.

As quantitative studies of the reaction kinetics indicated that the nickel-protein complex is of low stability, it is considered unlikely that nickel behaves as an hapten capable of initiating an allergic response.

I am grateful to Dr. I. A Magnus for his advice and encouragement during the course of this work, and to Dr. W. L. Gent, Mr. P. D. Mier, and Dr. A. Tickner for their valuable suggestions. I gratefully acknowledge a generous grant towards this work from Mond Nickel.

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Brit. J. Cancer
20(4) 886-895 (1966)

STRAIN DIFFERENCES IN THE RESPONSE OF RATS TO THE INJECTION OF NICKEL SULPHIDE

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It has been shown by Gilman and Ruckerbauer (1962) and Gilman (1962) that the intramuscular injection of nickel sulphide into either Hooded or Fischer rats produces a high incidence of tumours, many of which are well differentiated rhabdomyosarcomata. Thus of two groups of Hooded rats receiving injections of refinery dust with a nickel sulphide (Ni_3S_2) content of 57·0%, 70–71% developed tumours with an average latent period of 160–170 days; and of Fischer rats with injected nickel sulphide, 89% developed tumours within an average of 150 days.

By contrast, Payne (1964) found that Bethesda Black rats were relatively resistant to the carcinogenic action of intramuscularly implanted nickel sulphide. The final proportion of animals that developed tumours was high (74%), but the latent period was longer than for the Hooded and Fischer rats examined by Gilman; the first tumours were produced after 15 months, the average latent period being 17·3 months. These tumours were described as rapidly growing, anaplastic sarcomata.

As part of an investigation into the cause of this difference in response, a study has been made of the short- and long-term reactions of the muscle of the three strains—Hooded, Fischer and Bethesda Black—to powdered nickel sulphide. The results obtained indicated that the relative resistance of the Bethesda Black rats may be related to the intense and prolonged phagocytic response to the powdered carcinogen which was observed in this strain.

MATERIALS AND METHODS

Male and female rats, of the closed colonies of Hooded, Fischer and Bethesda Black strains of the Department of Histology, Ontario Veterinary College, were used. The animals were approximately 2–3 months old at the start of the experiments.

One tenth of a ml. of a suspension of nickel sulphide (Ni_3S_2) in aqueous penicillin G procaine ("Ayercillin"), containing 100 mg. Ni_3S_2/ml., was injected intramuscularly into each gastrocnemius of every rat.

The first experiment was designed to assess the relative sensitivities of Hooded and Bethesda Black rats to the carcinogenic action of the nickel compound, under the experimental conditions used. Fifteen male and 15 female Hooded, and 15 male and 12 female Bethesda Black rats were used. Those animals which developed tumours during the experiment were killed for autopsy when visible tumours appeared. One sample of each sex of the Bethesda Black rats, without apparent tumours, was killed for examination 4½ and 10 months after injection,

and the rats remaining after 14 months were autopsied whether or not they had developed tumours.

In the second experiment, a comparison was made of the short-term response of the muscle tissue of the three strains to the injected nickel sulphide. Five males and 10 females of each of the Hooded and Bethesda Black strains, and 10 males and 10 females of the Fischer strain, were used. Samples of both males and females were killed for examination at fortnightly intervals after the injection of the suspension.

Tissues for histological examination were fixed in Zenker-acetic, embedded in paraffin wax, and sectioned at 8 μ. The sections were stained with carmalum-aniline blue-orange G.

RESULTS

First experiment

The Bethesda Black rats were less susceptible than the Hooded to the carcinogenic action of the nickel sulphide. As shown in Table I, tumours were produced in all the Hooded, but in only 14 of the 27 Bethesda Black rats. Further, although almost all the Hooded rats developed tumours at the site of injection in both legs, in the Bethesda Black only one leg was affected, and the average latent period was approximately twice as long.

Although in both strains most of the tumours contained rhabdomyosarcomatous elements, in the Bethesda Black these were usually poorly differentiated, and mixed with fibro-sarcomata, reticulum cell sarcomata, or both. Some of the tumours in both strains contained phagocytes, with ingested nickel sulphide, scattered among the abnormal muscle cells.

In the 2 Bethesda Black rats examined 4½ months after injection, and showing no sign of tumour formation, the nickel sulphide was contained in a grey soft tissue mass lying on the surface of the leg muscles; the sulphide could not be separated from the tissue. Nickel sulphide was still present in the tissue of tumour-free Bethesda Black rats killed up to 14 months after injection. The soft tissue masses (Fig. 1) containing the sulphide were smaller than at 4½ months, and were often scattered as small patches in the connective tissue overlying the muscle, which appeared normal to the naked eye.

Histological examination of these masses showed that most of the nickel was within phagocytes (Fig. 2). These were sometimes embedded in a loose network of thick connective tissue bundles, and, in the rats killed after 14 months' exposure to the sulphide, were often associated with large numbers of lymphocytes (Fig. 2); in approximately half the animals, variable numbers of plasma cells were also seen at the peripheries of the phagocyte aggregates.

In a few of the Bethesda Black rats exposed to nickel sulphide for 14 months, the powder was also present as masses, with associated necrotic tissue, in spaces lined with foreign body giant cells. In one rat there was a region of early fibrosarcoma within the phagocyte mass.

The muscle fibres nearest to the phagocyte aggregates, from which they were usually separated by variable amounts of fairly dense connective tissue, were somewhat narrower than the mature muscle further away, and many of them showed abnormalities in the direction of their fibrils; some were "ring fibres" (Fig. 3) and in others the fibrils formed interlacing networks (Fig. 4). Similar

TABLE I.—*Tumour Production in Hooded and Bethesda Black Rats in Response to Intramuscular Injection of Nickel Sulphide*

Strain	Sex	Proportion of rats with tumours	Number of tumours	Period for development of visible tumour (weeks)	Number of tumours containing: Rhabdomyosarcoma [2] Poor	Fair	Good	Very good	Fibrosarcoma	Reticulum cell sarcoma	Other components
Hooded	♂	15/15	28	17–26	4	6	17	0	4	1	0
	♀	13/13	26	16–22	2	7	15	2	1	2	1 (Haemangio-sarcoma)
Bethesda Black	♂	7/13[1]	7	23–57	4	2	0	1	3	4	1 (Lympho-sarcoma)
	♀	7/10	7	19–56	1	4	0	0	3	3	0

[1] The total numbers of male and female Bethesda Black rats are those at the conclusion of the experiment (14 months after injection).
2 rats of each sex, killed at 4½ and 10 months after injection, were tumour-free.
[2] Degree of differentiation.

appearances were seen in groups of fibres, or individual fibres, at some distance from the deposit of nickel sulphide. A few fibres showing Zenker's degeneration were also present.

In the Hooded and Bethesda Black rats in which tumours were present, and from which normal muscle adjacent to the tumours was examined histologically, no ring fibres were seen in this tissue.

In approximately half of the Bethesda Black rats killed 14 months after injection, the popliteal, inguinal or lumbar lymph nodes contained macroscopically visible deposits of nickel sulphide; histologically, these were seen to consist of phagocytes with ingested sulphide. Some lymph nodes also contained aggregates of similar cells devoid of the powder (Fig. 5). Deposits of sulphide were not seen in the lymph nodes of tumour-bearing rats of either strain.

In the 2 Hooded rats which developed tumours in one leg only, no sulphide could be seen at the injection site in the unaffected leg.

Second experiment

The main results are summarized in Table II.

TABLE II

Histological findings	Strain	Time in weeks: 2	4	6	8	10
Ni_3S_2 loose in muscle	Fischer	+	−	−	−	−
	Hooded	+	−	−	−	−
	Bethesda Black	+	−	−	−	−
Ni_3S_2 in sac with necrotic tissue	Fischer	+	+++	+++	++	−
	Hooded	+	+++	++	++	(+)
	Bethesda Black	+	+++	(+)	−	−
Ni_3S_2 in phagocytes at injection site	Fischer	−	(+)	(+)	+	+
	Hooded	−	(+)	+	−	+
	Bethesda Black	−	+	++	+++	+++
Ni_3S_2 in phagocytes in lymph nodes	Fischer	−	(+)	+	(+)	(+)
	Hooded	−	+	++	+	−
	Bethesda Black	−	+	+++	+++	++
Abnormal myoblasts and muscle straps present	Fischer	−	−	+	−	−
	Hooded	−	−	−	−	−
	Bethesda Black	−	−	−	−	−

Two weeks after the injection of powdered nickel sulphide, the injection site appeared macroscopically similar in the three strains. The powder was partially localized in one or more sacs within the muscle tissue, and between the tissue planes, although some was still loose on the surface of the muscle. No sulphide could be seen with the naked eye in the regional lymph nodes.

Histological examination of the site showed that all the tissue immediately in contact with the nickel sulphide was dead. In all three strains, there was a surrounding zone where little or no particulate sulphide could be seen, but many muscle fibres were necrotic and were replaced by a collection of cells forming the "muscle cell tubes" of Waldeyer (Fig. 6). In some of the necrotic fibres, the peripheral cytoplasm appeared healthy, and had formed multinucleated masses and straps (Fig. 7). In the Hooded and Fischer rats, in contrast to the Bethesda Black, there was much muscle regeneration in this zone; many myoblasts, some in mitosis, and muscle straps were seen.

After 4 weeks' exposure to nickel sulphide, the three strains showed a localization of the powder in one or more soft tissue masses, approximately 5 × 2 mm., usually lying between the heads of the gastrocnemius or on the surface of this muscle. In many of these masses, the powder lay in the centre of a sac containing necrotic tissue; the wall of the sac apparently contained nickel sulphide inextricably bound up with the tissue. Some sulphide was present in the local and lumbar lymph nodes of the Hooded and Bethesda Black rats, and a small amount was seen in the lumbar nodes of the Fischer rats.

Histologically, the tissue immediately adjacent to the deposits of nickel sulphide was necrotic. In the Hooded rats, this region was surrounded by a zone of intense regenerative activity, with accompanying degeneration; multinucleated masses and muscle straps could be seen, and there were a few mitotic figures. In one of these rats, some granulation tissue, with phagocytes containing nickel sulphide, was also present.

In the Bethesda Black rats, the granulation tissue surrounding the necrotic zone was more abundant, and consisted of a loose network of dense connective tissue bands containing pockets of phagocytes laden with nickel sulphide. The granulation tissue was bordered by a thin band of immature muscle fibres and

EXPLANATION OF PLATES

FIG. 1. Bethesda Black rat, 57 weeks after injection. Site of implantation. Phagocyte aggregates in loose connective tissue. ×37.

FIG. 2. Part of one aggregate shown in Fig. 1. Granules of nickel sulphide are seen in pale phagocytes. Many lymphocytes and some plasma cells are also present. ×460.

FIG. 3. Bethesda Black rat, 57 weeks after injection. Muscle near nickel sulphide deposit. Ring fibres are present; the annular portions of most of these show cross striations. ×460.

FIG. 4. Bethesda Black rat, 57 weeks after injection. Muscle near nickel sulphide deposit. Aberrant fibril bundles in a muscle fibre. ×760.

FIG. 5. Bethesda Black rat, 57 weeks after injection. Lymph node. Aggregates of large pale-staining cells, devoid of nickel sulphide. ×460.

FIG. 6. Bethesda Black rat, 2 weeks after injection. Muscle near implantation site. The fibres are degenerating, with the formation of "muscle cell tubes". ×460.

FIG. 7. Fischer rat, 2 weeks after injection. Muscle near injection site. The central parts of the fibres have broken down, and the surviving peripheral regions have formed multinucleated masses and straps. ×460.

FIG. 8. Bethesda Black rat, 8 weeks after injection. Mature muscle near implantation site. One fibre shows longitudinal splitting into component fibril bundles. ×460.

FIG. 9.—Bethesda Black rat, 6 weeks after injection. Site of implantation. Almost all the sulphide is associated with large, pale-staining cells, most of which look healthy. ×690.

FIG. 10. Hooded rat, 6 weeks after injection. Site of implantation. A few phagocytic cells are present. Some nickel sulphide is associated with these cells, many of which are degenerating. Free nickel sulphide is also present. ×690.

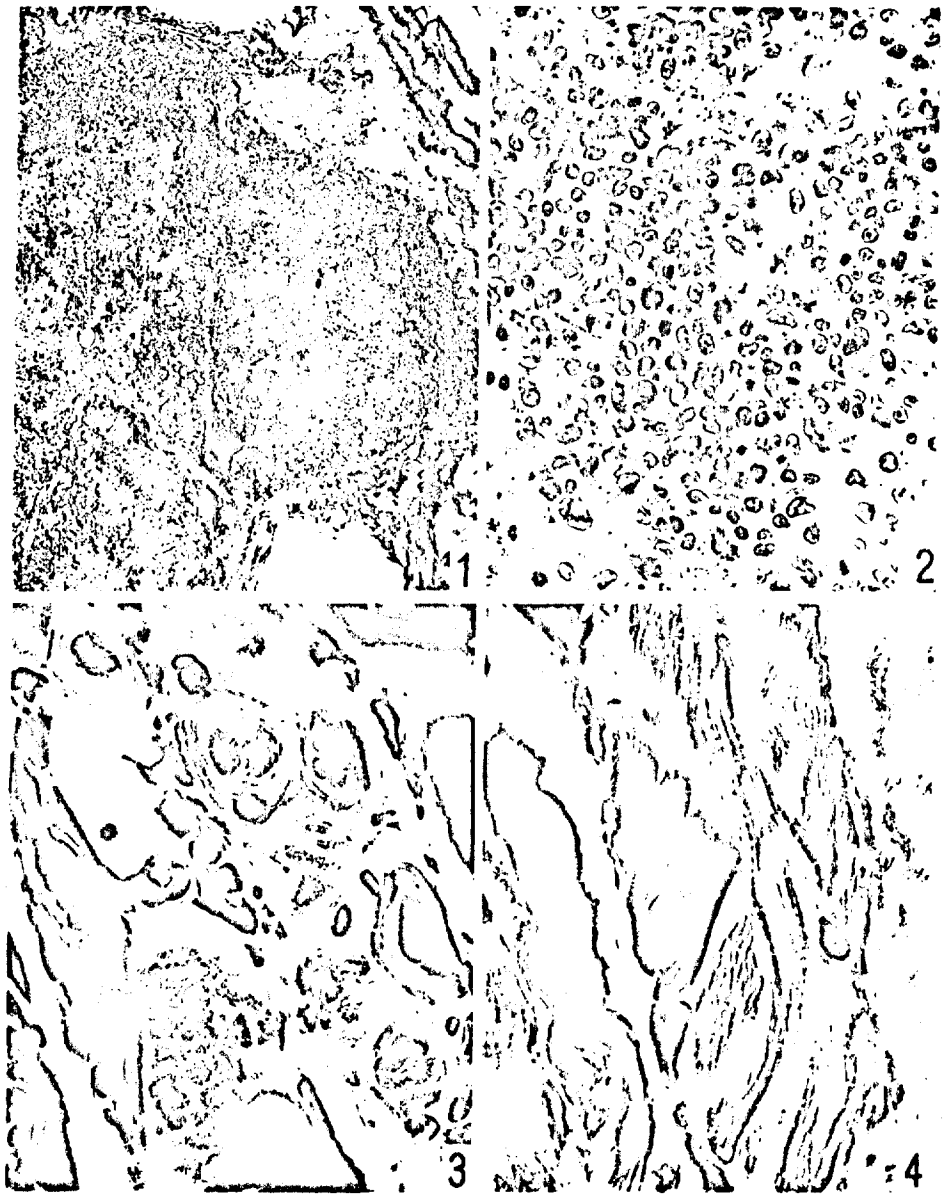
FIG. 11.—Fischer rat, 6 weeks after injection. Site of implantation. Many extravasated erythrocytes are present, and much of the nickel sulphide is free. Many of the cells are degenerating or dead. ×690.

FIG. 12.—Bethesda Black rat, 10 weeks after injection. Lymph node. A focus of sulphide-laden phagocytes, some of which are degenerating. ×460.

FIG. 13.—Bethesda Black rat, 10 weeks after injection. Site of implantation. Most of the nickel sulphide is within pale-staining phagocytic cells, enmeshed in connective tissue. Bands of lymphocytes are present at the periphery of the aggregate of phagocytes. ×460.

FIG. 14.—Hooded rat, 10 weeks after injection. Site of implantation. Abnormal, probably malignant muscle straps in dense connective tissue, with a few sulphide-containing phagocytes. ×460.

FIG. 15.—Fischer rat, 10 weeks after injection. Site of implantation. Abnormal myoblasts in dense connective tissue. Nickel sulphide is present, both free and associated with cells. ×460.



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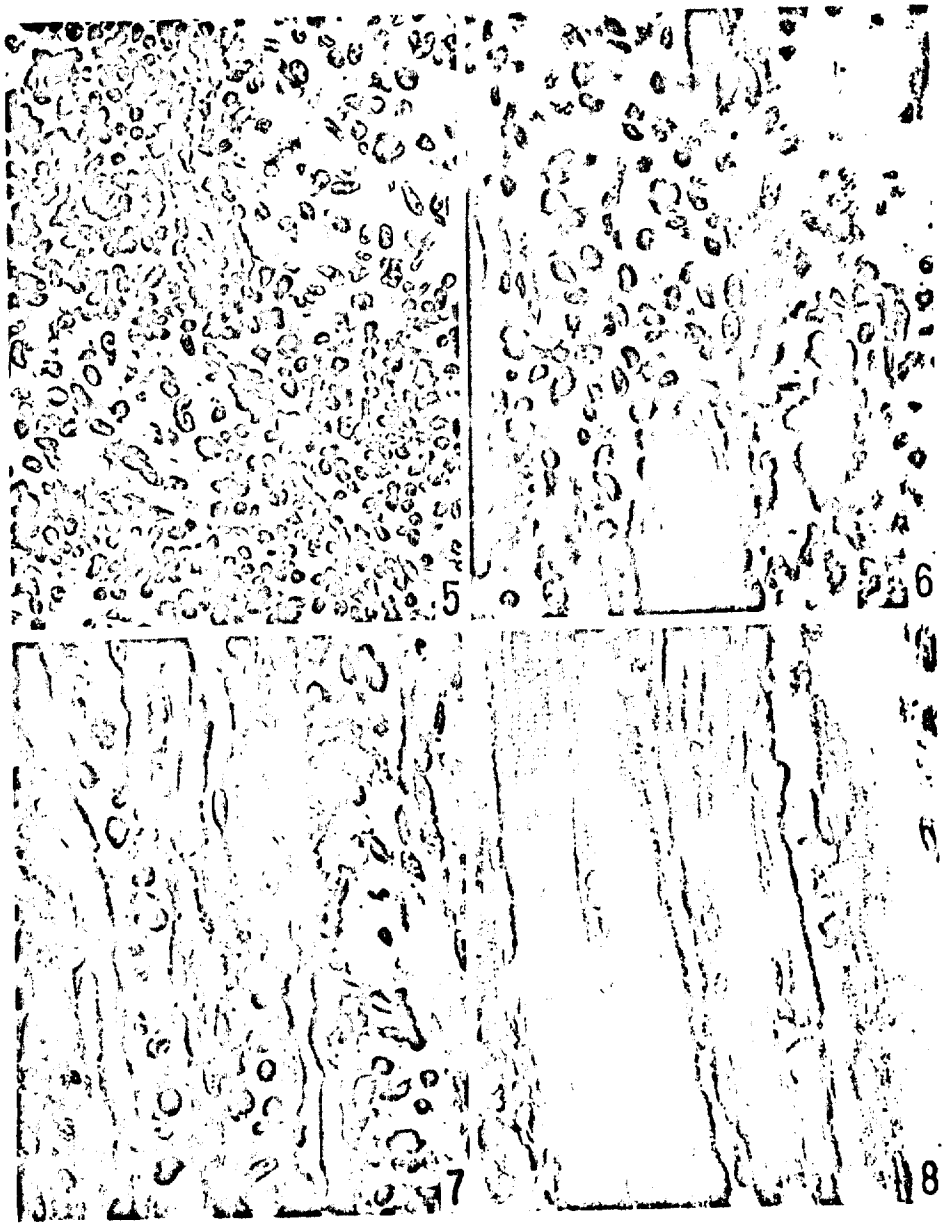

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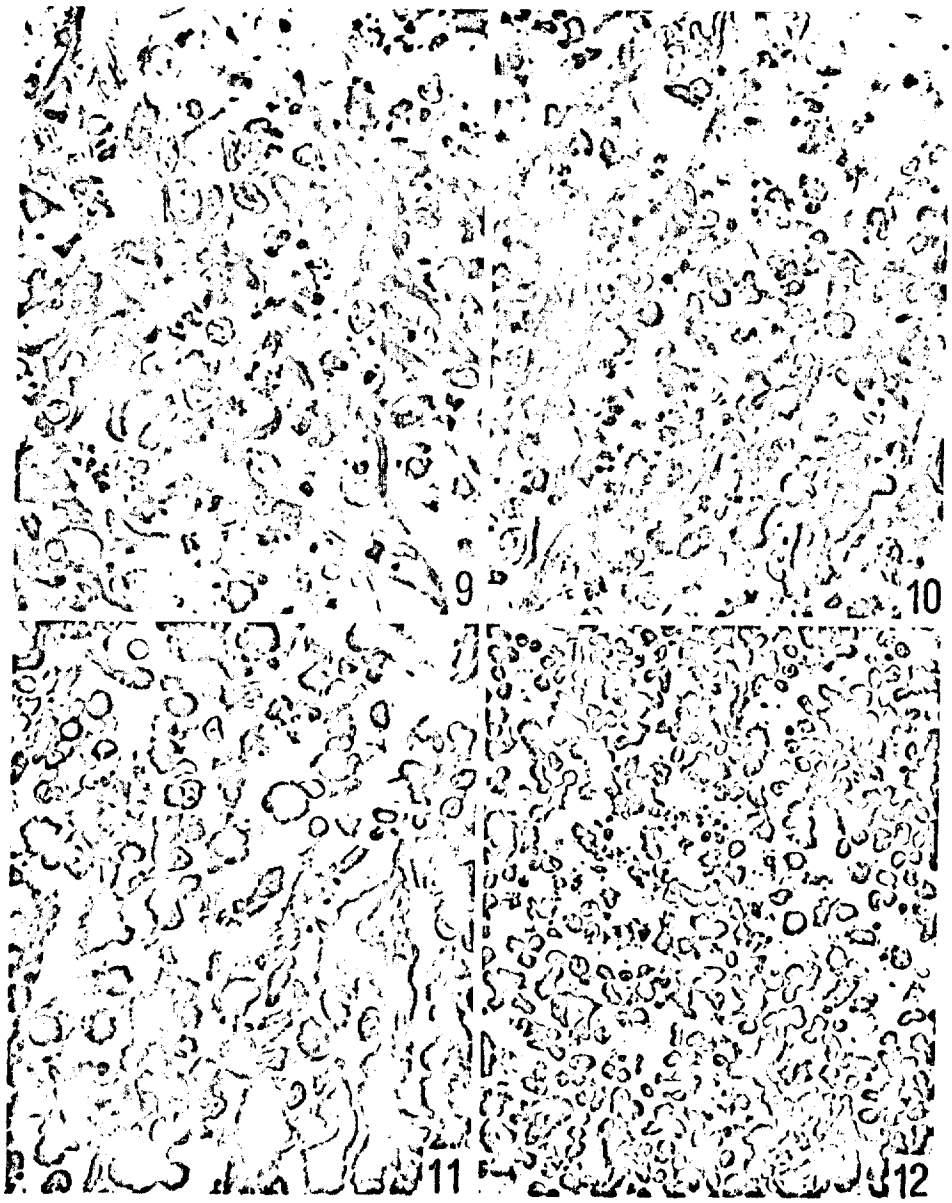

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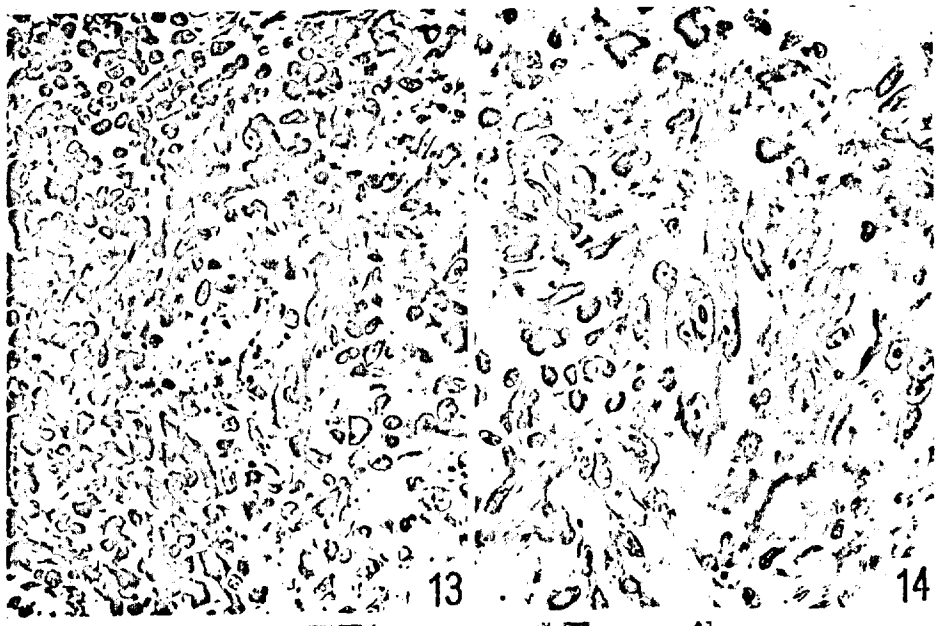

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muscle straps, scattered among which were a few phagocytes containing sulphide or yellow-brown pigment.

In the Fischer rats, the necrotic region containing the nickel sulphide was surrounded either by granulation tissue containing some phagocytic cells or by regenerating muscle, including muscle straps and immature striated fibres. Some mitoses were seen in this tissue.

In all three strains, the mature muscle surrounding the reaction zone showed histological evidence of sublethal damage, including sub-sarcolemmal "rowing" of nuclei and a longitudinal splitting of fibres into smaller bundles of fibrils. These features persisted throughout the early stages of the response to the injection (Fig. 8).

The nickel sulphide in the lymph nodes was contained within groups of phagocytes.

In one of the male Fischer rats killed at this stage, the kidneys had rough surfaces and were found on histological examination to contain regions of proximal convoluted tubular necrosis.

By the sixth week after injection there were macroscopic differences in the tissue response at the site, between the Bethesda Black and the other two strains. In the Hooded and Fischer rats, the nickel sulphide lay in a sac between the heads of the gastrocnemius, and could be scraped out as a loose paste mixed with necrotic tissue; some sulphide was also mixed with the tissue of the wall of the sac. By contrast, in 2 of the 3 Bethesda Black rats the nickel sulphide could not be removed from the tissue in which it was localized and to which it gave a grey colour. The bands of mixed tissue and sulphide lay in loose connective tissue on the surface of the muscle, or between its heads.

At this stage, nickel sulphide could be seen in the popliteal and lumbar lymph nodes of the three strains, but the amount was greatest in the Bethesda Black rats.

Histologically, the sulphide at the injection site in 2 of the Bethesda Black rats was contained within phagocytic cells, which formed a large mass subdivided into smaller aggregates by vascular connective tissue (Fig. 9); some plasma cells and a few lymphocytes were seen at the periphery of the mass of phagocytes. In one rat, in which some loose sulphide was also present, this was enclosed in a cavity lined by multinucleated giant cells. The phagocyte aggregates contained variable amounts of connective tissue, and were usually clearly demarcated from the surrounding muscle. The muscle fibres closest to the reaction zone were immature, and the mature muscle further away showed signs of sublethal damage.

In the Bethesda Black rat in which the sulphide had been contained in a sac, and in one Hooded rat, the cellular response to the nickel sulphide was qualitatively similar but quantitatively less than in most of the Bethesda Black rats; much of the sulphide was still free, either as individual particles lying among the cells or as clumps surrounded by necrotic tissue (Fig. 10). In these and in the other Hooded rats, some haemorrhagic, vascular granulation tissue was present; this contained some muscle straps and myoblasts, and was surrounded by a zone of muscle regeneration with some mitosis. Some degenerating muscle was also seen in the reaction zone, which was not clearly demarcated from the surrounding mature muscle.

In the Fischer rats, there was still less of an active response to the carcinogen, which was surrounded by necrotic tissue and a cellular, haemorrhagic exudate (Fig. 1). The regenerating muscle bordering this zone was not sharply defined

from it, and some of the muscle fibres formed were abnormal, having an irregular shape and large nuclei.

As in the Bethesda Black rats, the mature muscle at some distance from the injection site in the Hooded and Fischer rats showed "rowing" of nuclei and longitudinal splitting. The differences in the amounts of nickel sulphide seen macroscopically in the lymph nodes were related to the size of the aggregates of sulphide-laden phagocytes seen microscopically.

The tissue response of the Bethesda Black rats killed 8 weeks after injection differed little, either macroscopically or histologically, from that seen in most of the animals of this strain killed at 6 weeks. In the Hooded and Fischer rats the macroscopic response was the same as at 6 weeks. Histologically, there was an increase in the amount of dense connective tissue surrounding the nickel sulphide deposit in the Hooded rats, and a slight increase in the cellular response in the Fischer. In both these strains, phagocytes containing nickel sulphide were intermingled with regenerating muscle, of which a number of straps appeared abnormal, with variable diameter and nuclear size.

At this stage, the popliteal and lumbar lymph nodes of the Bethesda Black and Hooded rats contained nickel sulphide, but there was relatively little in the lymph nodes of the Fischer rats. The histological changes in the nodes were as at earlier stages, but some of the phagocytes were necrotic.

The kidneys of both the male Fischer rats killed at this stage had rough surfaces, and showed histological evidence of proximal tubular necrosis. In the other animals, the kidneys appeared normal both macroscopically and microscopically.

By the tenth week, there appeared to be less nickel sulphide in the draining lymph nodes in all the strains (Fig. 12). In the Bethesda Black rats, the appearance of the injection site was unchanged (Fig. 13), except that one animal showed the first unequivocal "ring fibre" seen in the series; this was among the mature muscle close to the mass of phagocytes. In other rats of this strain, some of the regenerating muscle formed an interweaving pattern; some regenerating fibres were seen between neighbouring phagocyte aggregates.

In the Hooded rats killed at this stage some loose nickel sulphide still remained within the localizing mass, but an increased proportion was intimately mixed with apparently healthy tissue. Histological examination showed that, surrounding a small region of necrosis and loose sulphide, there was a region of dense, often vascular connective tissue containing various cell types, including phagocytes laden with nickel sulphide, and abnormal regenerating muscle tissue. In many places, the appearance of the muscle straps suggested the beginning of tumour formation (Fig. 14).

In the Fischer rats killed at this time, most of the nickel sulphide was still loose in thin-walled sacs between the heads of the gastrocnemius or between this and other muscles. Histologically, some necrotic material was still present around the carcinogen; some mature but necrotic muscle fibres were recognizable in this zone. The surrounding tissue consisted of variable amounts of dense, sometimes vascular connective tissue containing a mixture of cells including phagocytes with engulfed nickel sulphide, fibroblasts and myoblasts and muscle straps, many of which appeared abnormal (Fig. 15). More phagocytes were present than in the Fischer rats exposed for shorter periods to the carcinogen, but they were still much fewer than in the Bethesda Black rats from 6 weeks onward.

The male Fischer rats killed at this stage again showed, in contrast to the

other animals, a patchy necrosis of the proximal convoluted tubules of the kidneys.

DISCUSSION

The first of the experiments reported here confirms the relative resistance of the Bethesda Black strain of rats to the carcinogenic action of powdered nickel sulphide, although the latent period before the development of a tumour in a rat of this strain was shorter than in Payne's (1964) experiments. Since the experiment was ended after 14 months, it is not possible to say what the final tumour incidence would have been had these rats been exposed to the carcinogen for the same period as those in Payne's study.

The most striking difference among the three strains of rats in the early response to the injection of powdered nickel sulphide was the massive phagocytic invasion of the site in the resistant strain ; these phagocytes ingested the carcinogen without apparent damage. In the Hooded rats, some cellular reaction was seen from the sixth week, but it was much less than in the Bethesda Black rats, and the phagocytes sometimes appeared to be killed by the ingested sulphide ; much of the powder was still free, either in a mass surrounded by necrotic and reactive tissue, or among the reactive tissue cells. In the Fischer rats the phagocytic response was still less, and was delayed until a time when abnormal regenerating muscle straps were present.

It is not clear what proportion of the phagocytes arose from the " muscle cell tubes " seen in the tissue within 2 weeks of the injection of the nickel sulphide, and what proportion had invaded the tissue from elsewhere. It seems probable (Field, 1960) that some at least of the phagocytic cells in such tubes are derived from muscle tissue. In the Bethesda Black rats, in the early stages, the formation of muscle straps played less part in the repair process than in the other two strains, and it is possible that in this strain the cells of the tubes differentiated into phagocytes rather than muscle cells. Some mitoses were seen in the mass of phagocytes, but were too few to account for the great number of cells present ; it seems probable, therefore, that these cells arose from both intra- and extra-muscular sources.

The localization of the nickel sulphide within the phagocytes would probably reduce the concentration of nickel compounds available for action on the regenerating muscle at the periphery of the phagocyte aggregates, both as a direct result of intracellular localization and through transportation of the powder to the regional lymph nodes. At all stages of the early reaction, the lymph nodes of the Bethesda Black rats contained larger accumulations of sulphide-laden phagocytes than did those of either of the other strains. A further observation in support of this suggestion is that in the Fischer rats, in which the phagocytic response was least, continuing muscle necrosis could be seen as late as the tenth week after injection. The renal tubular damage seen in some of the male Fischer rats could possibly have been a toxic necrosis, due to the absorption of relatively more soluble nickel from the site in this strain.

The mode of formation of the muscle fibres with aberrant fibrils, in the Bethesda Black rats exposed for periods of 10 weeks and more to nickel sulphide, is not clear. The histological evidence indicates that they might have arisen either from the intertwining developing fibres seen in some areas of muscle regeneration or from mature muscle which had been only partially damaged. A feature of the mature muscle surrounding the phagocyte masses in the early stages of the reaction to

nickel sulphide was the number of fibres showing "rowing" of nuclei, a phenomenon which, according to Mason (1960), may represent a sublethal reaction to injury. There were also areas in which muscle fibres appeared to have been split longitudinally into component bundles of fibrils. Berthrong and Griffith (1961) have suggested that formation of ring fibres may result from the occurrence of muscle regeneration in the absence of a sarcolemma; it is possible that, in this way, the ring fibres arose from an aberrant development of the peripheral region of the fibres showing nuclear rowing, while fibres with interweaving fibrils resulted from a reconstitution from the fibril bundles of a split fibre.

In those Bethesda Black rats which developed tumours, most of these were rhabdomyosarcomata, as in the Hooded rats in the present investigation and in the Hooded and Fischer rats studied by Gilman (1962). They were, however, less well differentiated than those in the Hooded rats, and were often mixed with fibrosarcomata and reticulum cell sarcomata. In Payne's experiments (1964), the Bethesda Black rats developed tumours which were described as "rapidly growing, anaplastic sarcomata"; it is possible that these, and the mixed tumours produced in the present experiments, were derived from the phagocytic tissue formed in response to the nickel sulphide in this strain. In the present series, an early fibrosarcoma was seen in the centre of a phagocyte mass in one of the Bethesda Black rats exposed for 14 months to the carcinogen.

Two interesting features of these phagocyte aggregates were their persistence for at least 14 months and their frequent association with plasma cells and, particularly in the later stage, with large numbers of lymphocytes. There is an increasing amount of evidence (e.g. Fagraeus, 1948; Halpern, 1959; Fishman, 1961; Harris, 1966) that the reaction of an antigen with macrophages is necessary for the subsequent induction of antibody production by lymphocytes. It is possible that the nickel sulphide combines either with necrotic muscle protein before ingestion by the phagocytes, or with intracellular material in these cells, to form foreign proteins which are then able to induce antibody formation by the surrounding lymphocytes. The existence of such an antibody would be expected to reduce the availability of nickel compounds in a form suitable for action on muscle, and also, if it were an antibody to a nickel-muscle protein complex, to cause the destruction of muscle fibres which subsequently took up sublethal quantities of nickel.

SUMMARY

The responses of rats of the Hooded, Fischer and Bethesda Black strains to intramuscular implants of powdered nickel sulphide have been compared. In confirmation of Payne's results, it has been found that the Bethesda Black strain is more resistant than the Hooded to the carcinogenic action of this compound.

A distinctive feature of the response of the Bethesda Black rats to the nickel sulphide was the development at the site of injection of large masses of phagocytic cells, which engulfed the powder without undergoing obvious damage. These phagocyte aggregates persisted for at least 14 months, and some were surrounded in the later stages by lymphocytes. Some of the muscle fibres near the phagocytes showed great variability in the orientation of their fibrils.

It is suggested that the ingestion of the nickel sulphide by the phagocytes may reduce the concentration of nickel compounds available for action on the muscle tissue to a subcarcinogenic level, both by the intracellular localization of the com-

pound and by its transport to the regional lymph nodes. The possibility that the association of lymphocytes with the sulphide-containing phagocytes might result in the development of an immune response is also considered.

This work was supported partly by a Sir Henry Wellcome Travelling Fellowship, and partly by a grant from the National Cancer Institute of Canada. I should like to thank Dr. J. P. W. Gilman for his generous provision of facilities at his laboratory, and am also grateful to Dame Honor Fell, F.R.S. and Dr. A. Glücksmann, for their helpful comments on the manuscript. Some of the autopsies and histological preparations were made by Miss Winifred Smith; the photographs were taken by Mr. M. Applin.

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CAN JOURNAL OF PHYSIOLOGY
3, No. 4, October 1967. Printed in U.S.A.

Effects of metal ions and sulfhydryl inhibitors on glucose metabolism by adipose tissue[1]

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DIXIT, PADMAKAR K., AND ARNOLD LAZAROW. *Effects of metal ions and sulfhydryl inhibitors on glucose metabolism by adipose tissue.* Am. J. Physiol. 213(4): 849–856. 1967.—Effect of divalent metal ions and sulfhydryl compounds on the metabolism of glucose by rat adipose tissue (epididymal fat pad) has been studied with carbon 14-labeled glucose. Cobalt and nickel ions enhanced glucose uptake, its oxidation to carbon dioxide and incorporation into fat-pad lipids, thus simulating the action of insulin. In addition nickel ion increased glucose incorporation into glycogen. Cobalt did not have this effect. In vitro addition of insulin antiserum did not influence the action of cobalt, as was the case with insulin. Cobalt, like insulin, enhanced the oxidation of C-1 glucose, but not of C-6 glucose. Sulfhydryl inhibitors varied in their effect; at certain concentrations *p*-hydroxymercuribenzoate, arsenite, and cadmium increased the oxidation of C-1 glucose to carbon dioxide; however, at higher concentrations they either inhibited or did not affect glucose metabolism. N-ethylmaleimide (NEM) and iodoacetate, at all concentrations tested, inhibited or had no effect on the metabolism of glucose. Prior treatment of the fat pad with NEM effectively blocked both insulin and cobalt action on the fat pad.

cobalt, nickel; zinc; *p*-hydroxymercuribenzoate; arsenite; iodoacetate; N-ethylmaleimide; cadmium; glucose-1-^{14}C and -6-^{14}C oxidation; glycogen synthesis; glucose uptake

ALLOXAN ADDITION IN VITRO increased the permeability of toadfish (*Opsanus tau*) islet tissue to mannitol-^{14}C. Preincubation of the islet tissue with glutathione, cysteine, or 2,3-dimercaptopropanol (BAL), protected against this permeability change (27, 28). In fact BAL (a dithiol compound), when added in vitro reversed the permeability effect of alloxan, whereas glutathione and cysteine (monothiol compounds) did not do so (11). When used alone, none of the monothiol (sulfhydryl) inhibitors (iodoacetate and *p*-hydroxymercuribenzoate) affected the in vitro permeability of islet tissue to mannitol. By contrast, the dithiol inhibitors (arsenite and cadmium) increased islet permeability (26).

It is suggested (4, 11) that cell membrane sulfhydryl groups play a role in the permeability of the pancreatic β-cell. Divalent metals such as cobalt, zinc, and ferrous ion likewise protected rats against the diabetogenic effects of alloxan (12). Zinc also protected toadfish islets against the in vitro permeability effects of alloxan (11). Since insulin influences glucose utilization in adipose tissue (31) by controlling the entrance of glucose into the cell (2), we felt it would be of interest to study the effect of metal ions and sulfhydryl compounds on glucose metabolism.

METHODS AND MATERIALS

Glucose utilization was determined by measuring its oxidation to carbon dioxide and its incorporation into lipids and glycogen. In a few instances the disappearance of glucose from the medium was also determined. The technique used was similar to that employed in the insulin-bioassay procedure (24).

Young Holtzman rats, weighing between 240 and 260 g, were decapitated by guillotine. The epididymal fat pad was removed and cut into six pieces, weighing approximately 50 mg each, and placed in six separate vials. The incubation medium consisted of 1 ml bicarbonate buffer (pH 7.4) containing 1 mg glucose, 2 mg gelatin, and 0.2 μc of glucose-1-^{14}C (6, 7). The amount of labeled glucose in each vial was approximately 10–15 μg. In those experiments where metabolism of glucose-1-^{14}C and glucose-6-^{14}C were compared, the specific activities were approximately the same. In a few experiments the metabolism of glucose was studied in the presence of crystalline serum bovine albumin (Armour or Sigma) at a concentration of 40 mg/ml medium. The ionic concentration (mmoles/liter) of the medium was as follows: Na^+ = 143; HCO_3^- = 40; K^+ = 5; Ca^{++} = 1; Cl^- = 111 (13). One piece of the fat-pad tissue from each rat was incubated in buffer medium without other additions, a second was incubated with 500 μU insulin/ml; the remaining pieces were incubated in various concentrations of one of the

Received for publication 13 March 1967.
[1]This investigation was supported by Public Health Service Research and Training Grants AM 5127 and AM 6517 from the National Institute of Arthritis and Metabolic Diseases.

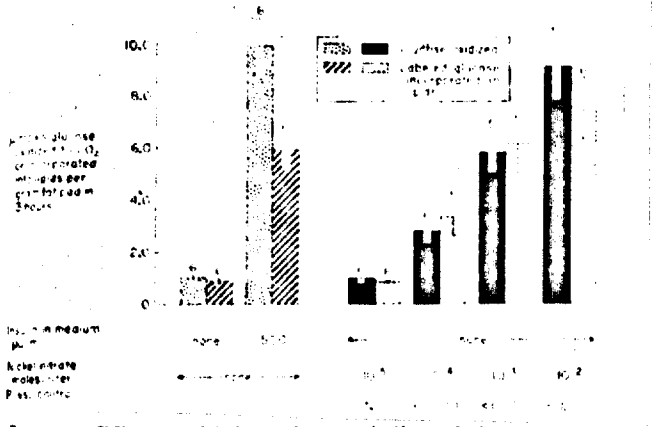

FIG. 3. Effect of nickel on the metabolism of glucose-1-^{14}C by rat epididymal fat pad. Other details as in Fig. 1.

the initial and final concentrations in the medium were determined by the colorimetric method of Nelson (23). In one experiment the effect of preincubation of fat pad in NEM (10^{-3} M) was studied.

RESULTS

The in vitro effect of divalent metals on glucose metabolism by epididymal fat pad. Figure 1 compares the effect of cobalt ion with that obtained with insulin (500 μU/ml). At a Co^{++} ion concentration of 10^{-2} M in the medium, the glucose-1-^{14}C oxidation was increased fivefold (from 0.70 for the control to 3.50 μmoles/g tissue per 3 hr for the experimental). At 10^{-3} M, 10^{-4} M, and 10^{-5} M concentrations, the glucose oxidized was 2.78, 1.51, and 0.75 μmoles, respectively. By contrast, insulin increased glucose oxidation eightfold (5.74 μmoles of glucose).

Cobalt likewise stimulated glucose-1-^{14}C incorporation into fat-pad lipids (Fig. 1); at a concentration of 10^{-2} M, it was increased threefold (from 1.0 to 3.0 μmoles/g fat pad). Insulin increased approximately fourfold the incorporation of glucose into lipids.

Figure 2 compares the effect of cobalt ion (10^{-2} M) in the presence of gelatin and albumin; both glucose oxidation and glucose incorporation into lipids were equally enhanced. Insulin, however, exerted a greater stimulatory effect in the presence of albumin than in gelatin ($P = < 0.05$). Cobalt did not affect glycogen synthesis; whereas insulin markedly increased glucose incorporation into glycogen in the presence of both gelatin and albumin.

Nickel likewise stimulates glucose oxidation and incorporation into lipids; the effect was most pronounced at a concentration of 10^{-2} M (Fig. 3).

Figure 4 compares the effect of nickel in the presence of albumin and gelatin; both glucose oxidation and incorporation into lipids were enhanced to a greater extent in the presence of gelatin than in albumin ($P = < 0.001$). In contrast the glucose oxidation by insulin was significantly greater in albumin than in gelatin ($P = < 0.0001$). Nickel, in contrast to cobalt, enhanced incorporation of glucose into glycogen (Fig. 4), but this effect is much less than that of insulin.

Zinc likewise increased glucose oxidation at all the concentrations tested (10^{-2} M to 10^{-5} M) (Fig. 5). Divalent ferrous ion and trivalent chromium ion had no significant effect on glucose oxidation at all concentrations tested (10^{-2} M to 10^{-5} M) (Fig. 10).

The cobalt-induced stimulation of glucose oxidation was not blocked by the addition in vitro of anti-insulin serum. By contrast, equivalent amounts of AIS-GP completely blocked the effect of insulin (Fig. 6).

Fat pads removed from rats injected with AIS-GP exhibited a threefold increase in glucose oxidation in the presence of cobalt ions; this is the same as that observed in the controls injected with NGPS (Fig. 7). However, fat pads from AIS-GP injected animals showed

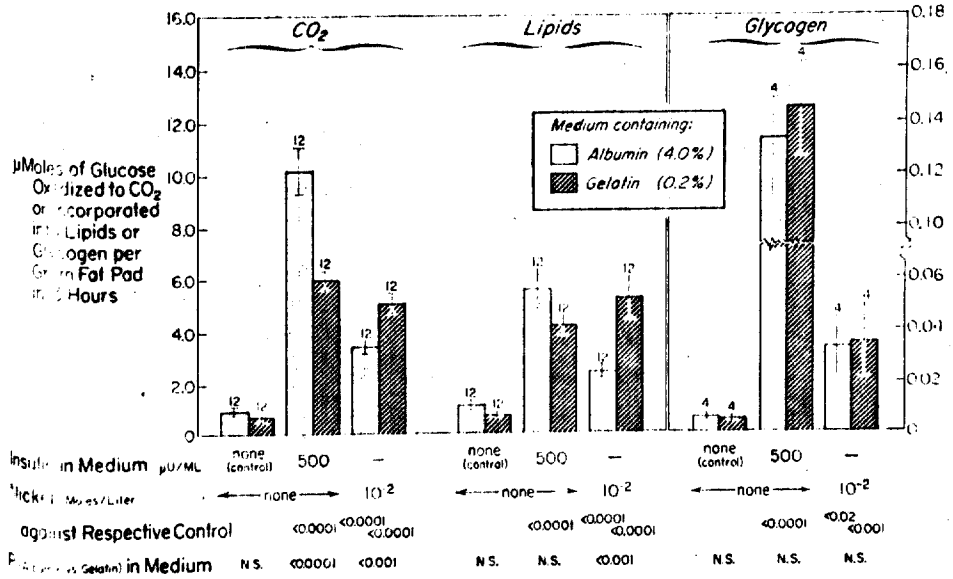

FIG. 4. A comparative effect of nickel (10^{-2} M) on glucose metabolism by rat epididymal fat pad in media containing 4.0% albumin or 0.2% gelatin. Other details as in Fig. 1.

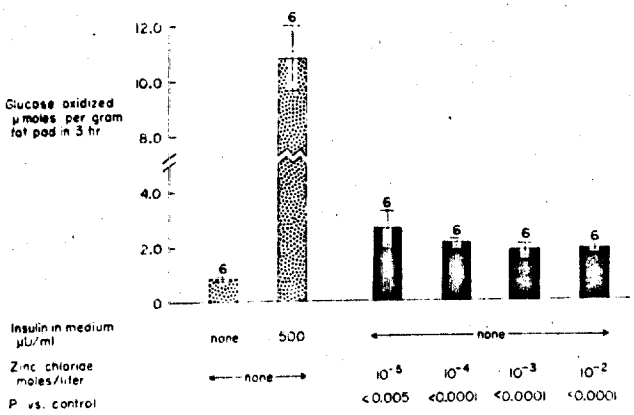

FIG. 5. Effect of zinc on glucose-1-^{14}C metabolism by rat epididymal fat pad. Other details as in Fig. 1.

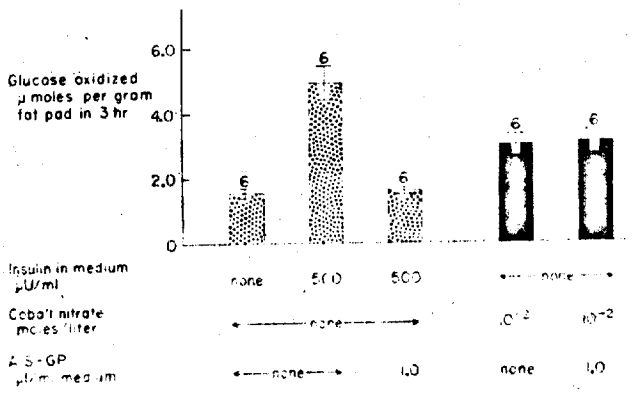

FIG. 6. Effect of adding anti-insulin serum (AIS-GP) to the medium containing cobalt on the oxidation of glucose by the rat epididymal fat pad. Other details as in Fig. 1.

a diminished response to insulin; glucose oxidation was increased only twofold for the AIS-GP injected rats as compared to a sevenfold increase for the NGPS injected controls.

In vitro effect of sulfhydryl and dithiol inhibitors. Figure 8 shows that although *p*-hydroxymercuribenzoate (PHMB) at a concentration of 10^{-4} M significantly increased glucose oxidation ($P = <0.0001$), this agent had no significant stimulatory effect at higher (10^{-3} M) or lower (10^{-5} M) concentrations. At very high concentrations (10^{-2} M) PHMB produced a significant inhibition of glucose oxidation. PHMB stimulated incorporation of glucose into lipids in the presence of both gelatin and albumin, but it did not affect glycogen synthesis (results not illustrated).

Figure 9 shows that PHMB, cobalt, and nickel significantly enhanced glucose utilization (as measured by the disappearance from the medium) in the presence of both albumin and gelatin.

Sodium arsenite, cadmium, iodoacetate (IA), and N-ethylmaleimide (NEM) had either a stimulatory or inhibitory effect on the glucose metabolism depending upon the concentration (10^{-2} M to 10^{-5} M) (Fig. 10).

Effect of cobalt on the oxidation of C-1- and C-6-labeled glucose. The effect of insulin and cobalt on the metabolism of both C-1- and C-6-labeled glucose substrates are compared in Fig. 11. Insulin and cobalt increased oxidation of C-1 glucose; however, neither influenced $^{14}CO_2$ production from C-6 glucose. Both insulin and cobalt stimulated the incorporation of C-1 and C-6 glucose into lipids. Cobalt differed from insulin in that it had no effect on incorporation of glucose into glycogen; whereas insulin increased the incorporation of both C-1 and C-6-labeled glucose into glycogen.

Effect of preincubation with N-ethylmaleimide. Figure 12 shows that pretreatment of the fat pad with N-ethylmaleimide depresses the glucose oxidation to values below the basal levels and that this effect was not reversed by either insulin or cobalt.

DISCUSSION

Addition of insulin to the medium markedly enhances the metabolism of glucose by the epididymal fat pad.

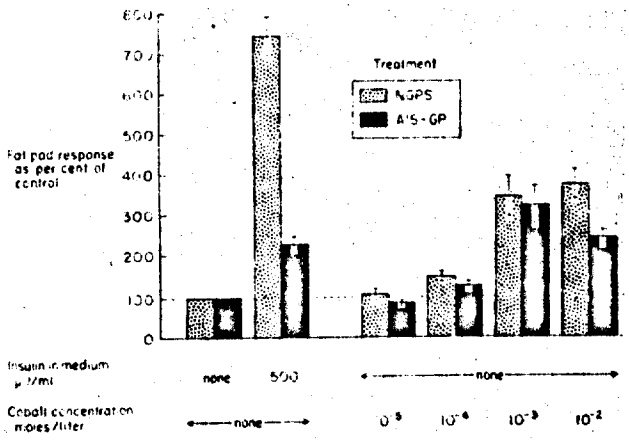

FIG. 7. Effect of cobalt (added to the medium) on the oxidation of glucose by fat pads removed from rats injected with either anti-insulin (AIS-GP) or normal guinea pig (NGPS) serum. Each bar represents the mean value of six observations. Other details as in Fig. 1.

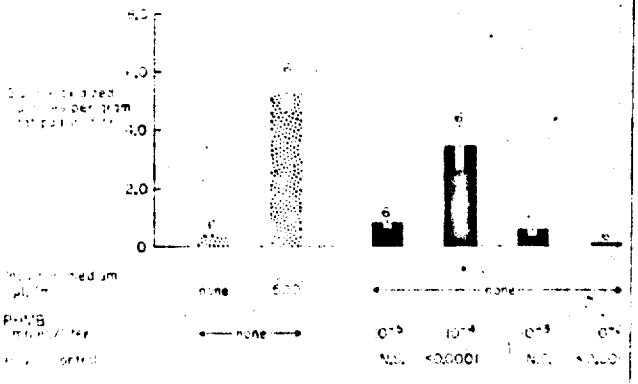

FIG. 8. Effect of *p*-hydroxymercuribenzoate (PHMB) on the oxidation of glucose by rat epididymal fat pad. Other details as in Fig. 1.

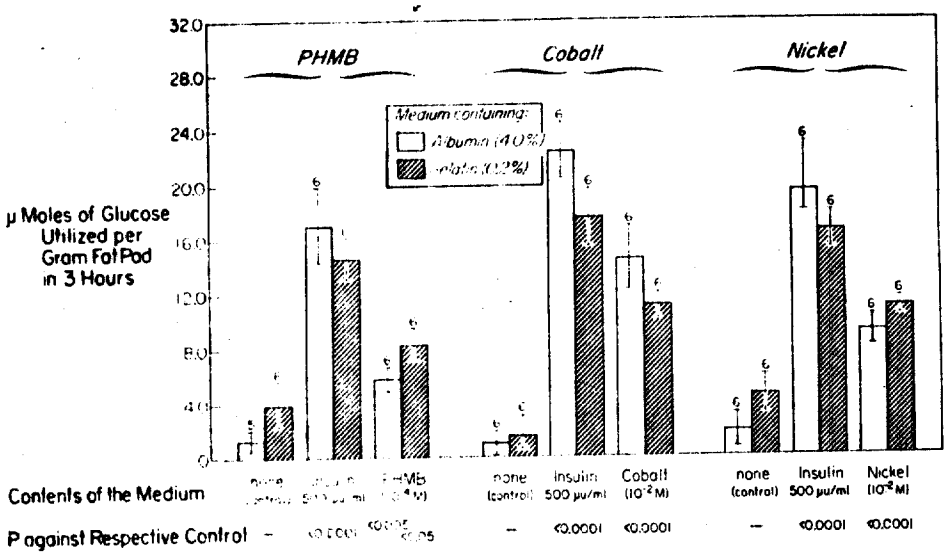

FIG. 9. Effect of *p*-hydroxymercuribenzoate, cobalt, and nickel on the uptake of glucose by fat-pad tissue in albumin- (4.0%) and gelatin- (0.2%) containing media. No labeled glucose was added. For each observation, fat-pad pieces from two rats were used.

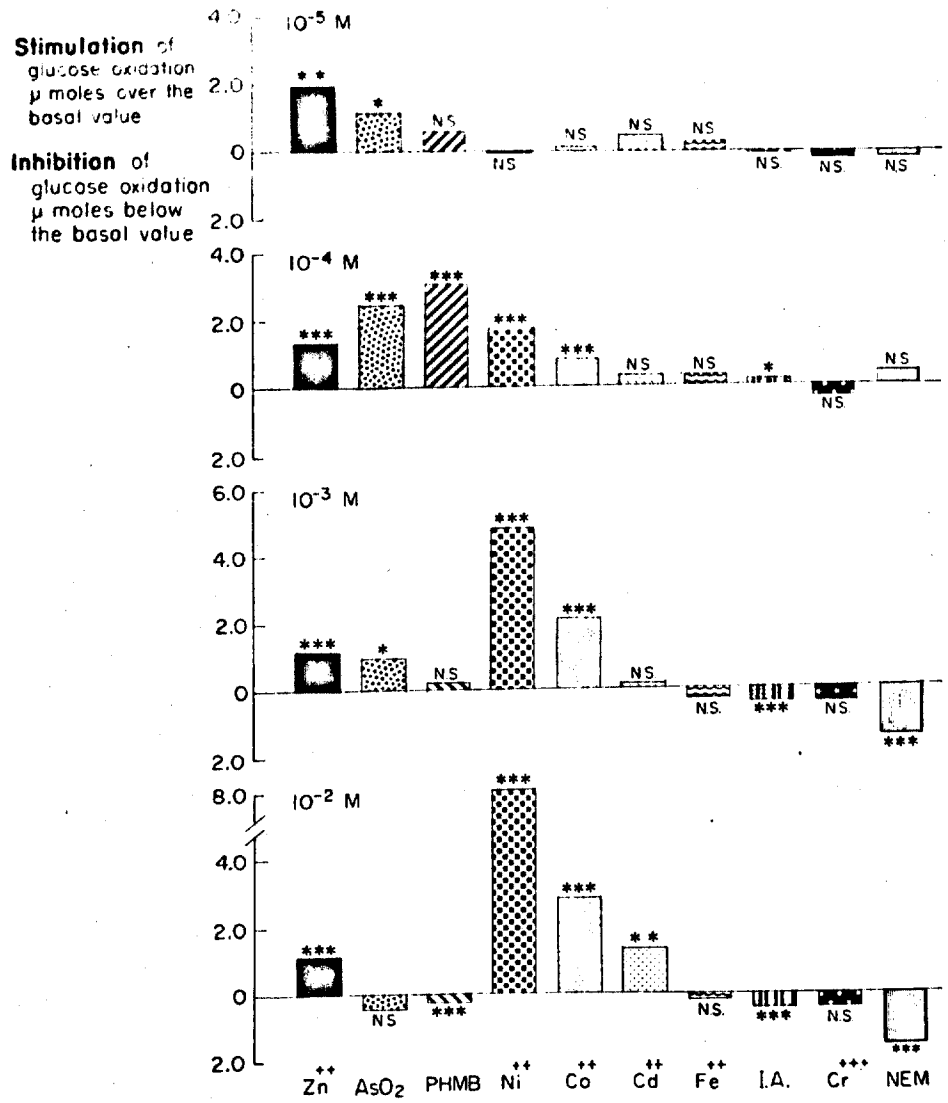

FIG. 10. Comparison of the effect of various metals, sulfhydryl and dithiol inhibitors added in vitro on the oxidation of glucose by the rat epididymal fat pad. Each bar represents a mean of six observations. * = the observed stimulation or inhibition had a P value < 0.05, ** $P = < 0.001$, *** $P = < 0.0001$.

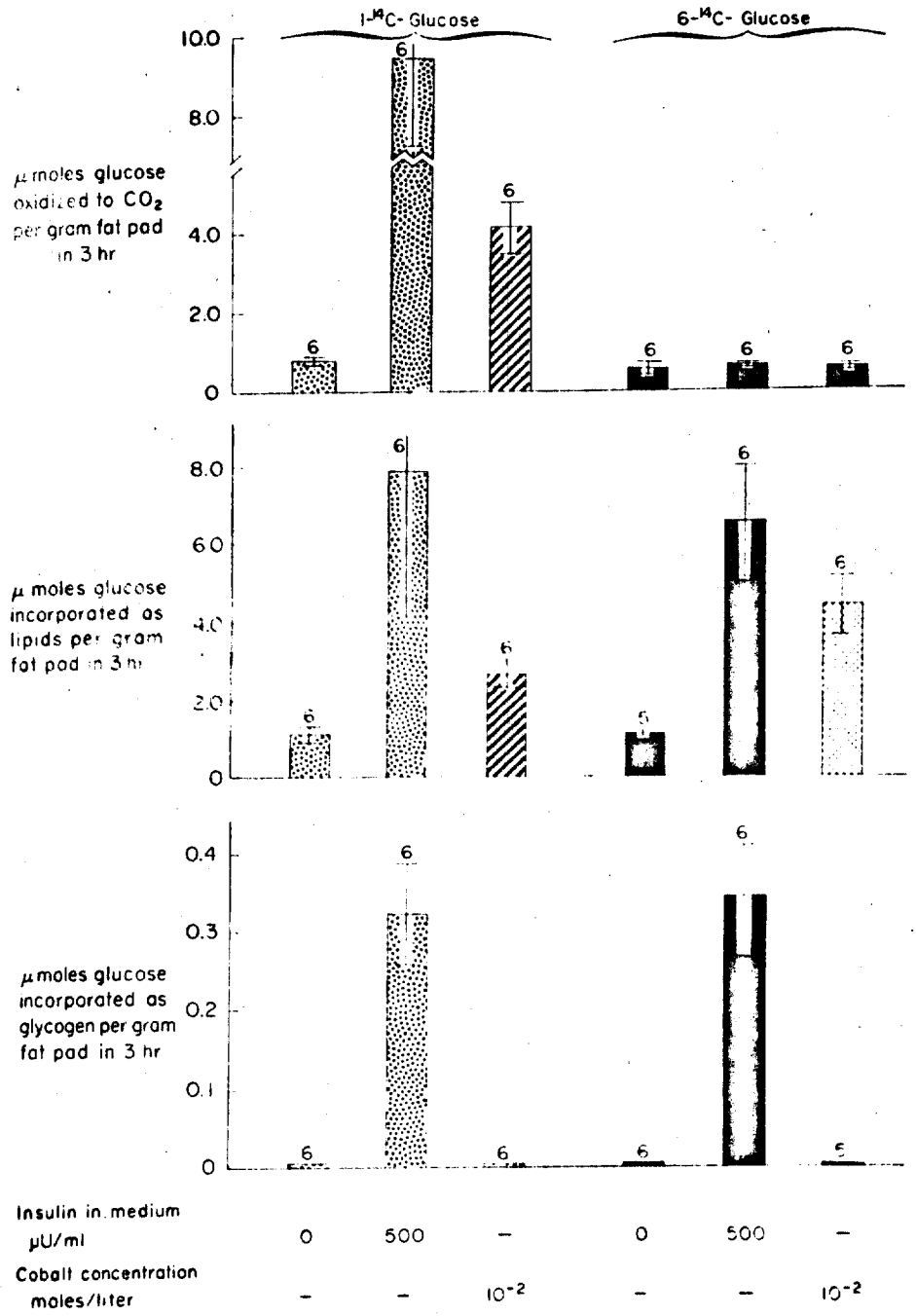

FIG. 11. Comparison of the effects of insulin and cobalt on the metabolism of glucose-1-^{14}C and -6-^{14}C by the rat epididymal fat pad. Other details as in Fig. 1.

This response has been used in the assay of this hormone (18). Insulin increases the entrance of glucose into the cell through its presumed control of cell membrane permeability or hexokinase stimulation (17). This would facilitate the subsequent oxidation of glucose or its conversion to fat and glycogen. Although other hormones—growth hormone (32), adrenocorticotropin (ACTH), epinephrine (16), oxytocin (21), and unrelated substances—ribonucleic acid, adenylic acid, adenosine (9), royal jelly (8), and ethylenediaminetetraacetic acid (EDTA) (5, 14) likewise stimulate the in vitro oxidation of glucose by the epididymal fat pad; unlike insulin, none of these agents, with the exception of EDTA, has any effect on glycogen synthesis. The present study demonstrated that certain divalent metals and some sulfhydryl inhibitors stimulate glucose oxidation by the epididymal fat pad. Cobalt and nickel ions are more effective at higher (10^{-2} M) than at lower (10^{-4} M) concentrations; whereas PHMB was stimulatory at 10^{-4} M and inhibitory at 10^{-2} M. These agents likewise stimulated the conversion of glucose into lipids. Although Co^{++} had no effect on incorporation into

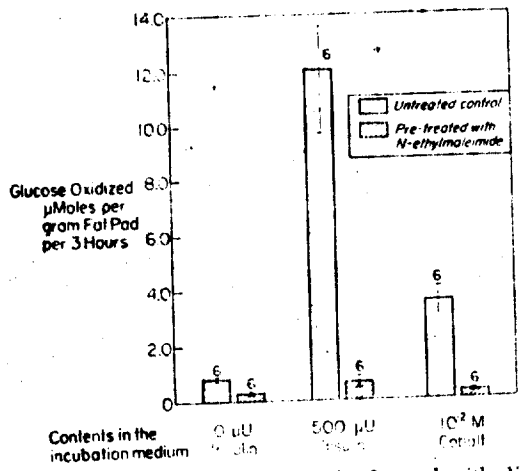

FIG. 12. Effect of preincubation of the fat pad with *N*-ethylmaleimide (10^{-3} M) on the glucose metabolism in the presence of insulin or cobalt. In this experiment one fat pad was preincubated in NEM at room temp for 15 min while the opposite fat pad was incubated in buffer. Following incubation, both the fat pads were removed, rinsed in three changes of buffer, and each cut into three pieces. The three pieces were incubated in 1 ml media containing no insulin, 500 µunits insulin, and cobalt (10^{-2} M). Other details as in Fig. 1.

glycogen, Ni^{++} stimulated glucose incorporation (Fig. 4). Since the oxidation of glucose to carbon dioxide in the presence of cobalt was not affected by the in vitro addition of insulin antibody, it is unlikely that cobalt exerts its effect through an indirect action on "residual" insulin that may be present in the fat pad.

According to Leonards and Landau (13), increased concentration of glucose in the medium causes enhanced oxidation of both C-1 and C-6 glucose, stimulates the synthesis of both fatty acids and glycerol, but has no effect on glycogen synthesis. By contrast, whereas insulin addition stimulates the oxidation of C-1 glucose, it does not have a corresponding effect on C-6-glucose oxidation; although it increases the incorporation of both C-1- and C-6-labeled glucose into glycogen and lipids. In contrast to insulin and oxytocin (which selectively stimulate oxidation of C-1 glucose), adrenaline, ACTH (16), and growth hormone (32) have a greater effect on C-6- than on C-1-glucose oxidation. Our studies indicate that cobalt (a divalent ion) selectively favors the oxidation of C-1 glucose (Fig. 11) but it has no effect on incorporation into glycogen unlike insulin and nickel. Although insulin stimulates glucose uptake and prevents lipolysis at both high- and low-protein levels in the media, the other hormones studied (ACTH, adrenaline, and noradrenaline) inhibited glucose utilization and accentuated triglyceride hydrolysis when the protein content in the incubation medium was low (0.2% albumin), but not when it was high (4.0% albumin) (2). Since cobalt, nickel, and PHMB are equally effective in 0.2% gelatin and 4.0% albumin, it seems unlikely that they would have a lipolytic action. Further, we did not find any evidence of increased free fatty acid liberation in the medium as determined by the thiobarbituric-acid test (3) following incubation in the presence of cobalt and PHMB.

Our studies suggest that cobalt and nickel may influence glucose transport into the adipose cell. It has been reported that Co^{++} (and other ions) inhibits oxidation via the tricarboxylic acid cycle, possibly by chelating with the sulfhydryl groups of dihydrolipoic acid on the mitochondrial surface (29). This would result in an apparent increase in the hexosemonophosphate shunt. However, we do not know whether the added cobalt enters the cell in sufficient concentrations to affect the intracellular enzyme(s), and thus inhibit glucose metabolism via the tricarboxylic acid cycle.

Trivalent chromium (in concentrations from 10^{-5} to 10^{-2} M) did not affect glucose metabolism by the normal epididymal fat pad. Mertz and co-workers (19, 20) reported that whereas trace amounts of trivalent chromium (0.01 µg) did not have any significant effect on the glucose uptake by fat pads obtained from chromium-deficient rat, chromium potentiated the insulin effect on these fat pads.

Our studies indicate that although some sulfhydryl inhibitors, PHMB (10^{-4} M), arsenite (10^{-3} to 10^{-5} M) and cadmium (10^{-2} M), significantly increased the oxidation of glucose-1-^{14}C to carbon dioxide, others, N-ethylmaleimide and iodoacetate, either had no effect or they inhibited glucose oxidation, depending upon the concentration used. (Ball (1) and Lynn (15) have likewise reported that sulfhydryl inhibitors have an insulin-like effect on the fat pad. However, the studies of Whitney and co-workers (30) indicate that pretreatment of the fat pads with PHMB and NEM abolishes the insulin effect on glucose uptake.) Thiol groups presumably play a role at the active site of glucose entry into the cell (10) and insulin perhaps reacts with a tissue receptor through the formation of a sulfur-sulfur bond (between the insulin disulfide and the receptor thiol). Also, it was postulated that pretreatment of adipose tissue with the sulfhydryl inhibitors (iodoacetate and NEM) neutralized the glucose transporting action of insulin without affecting the binding of insulin to the fat pad (22); perhaps a sulfhydryl-dependent system responsible for the transport of glucose into the cell, which is normally activated by insulin, is blocked by the inhibitors. We have found that prior treatment of the fat pad with NEM (10^{-3} M) completely inhibited the effect of cobalt (Fig. 12). This observation is consistent with the hypothesis that receptor-thiol groups control the entry of glucose into the cell.

Our finding that some sulfhydryl inhibitors enhance glucose oxidation by the fat pad at certain concentrations (e.g., PHMB at 10^{-4} M), whereas others have the opposite effect, indicates that the various sulfhydryl inhibitors differ in their sites of action. NEM and iodoacetate may act only at the cell surface while PHMB may block specific intracellular reactions as well.

We thank Mrs. Skaidra Ogrins and Mrs. Dianne Renalls for their excellent technical assistance.

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CANCER OF THE LUNG AND NOSE IN NICKEL WORKERS

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(RECEIVED FOR PUBLICATION FEBRUARY 4, 1958)

In 1949 the Minister of Pensions and National Insurance of Great Britain prescribed cancer of the lung and cancer of the nose as industrial diseases, when they occurred among certain classes of workers engaged in the refining of nickel. The published epidemiological evidence regarding the hazard is, however, scanty. It consists only of a series of case reports (Amor, 1939; Løken, 1950) and a statement of the total number of cases which occurred between 1923 and 1950 and were reported to the Ministry (Chief Inspector of Factories, 1952). The present investigation was, therefore, undertaken to obtain evidence relating to (1) the magnitude of the risk, and (2) the belief that the risk had been eliminated from the refinery concerned between 1920 and 1924.

The data for the investigation were obtained from the records of death held by the medical officers of health of the districts in which the workers at the refinery lived. These records included copies of the death certificates of all persons dying in the districts and also of the certificates of persons resident in the districts, who had died elsewhere. Since the majority of the workers live in the Pontardawe Rural District of the County of Glamorganshire and a few others live in the County Borough of Swansea and in two other neighbouring districts, it was possible to obtain information about the causes of death of nearly all—if not of all—the workers who died during employment at the refinery and of a large proportion of the retired workers who had worked at the refinery when they had last been employed. These data can be compared with the national data for the different causes of death during the same period or with similar data for the same districts, but relating to men in other occupations. In the present study, it was thought better to use the other data from the same districts, when they were numerically adequate, as by this means it was possible to eliminate differences in the incidence of the two diseases due to non-industrial local factors.

The Data

Data for the 15,247 men over the age of 15 years who were resident in the four selected districts and who died in the period 1948-56 are shown in Table 1. The numbers of deaths are shown separately for three causes, five occupational groups, and 13 age groups. The causes have been classified as cancer of the lung, cancer of the nose, or " other causes " according to the diagnosis given on the death certificate. Where two or more causes were referred to the death has been classified to the underlying cause, in accordance with international usage—except in 10 instances in which a primary cancer of the lung was given as an associated disease, when the death was classified as if it had been attributable to the cancer. The numbers of cases in each occupational and age group in which the international usage was varied are shown in Table 1. The five occupational groups consist of nickel, steel, and colliery workers, men employed in certain " other selected occupations ", and those in all other occupations. The men in " other selected occupations " were employed at an oil refinery or were described as aluminium, copper, spelter or " patent fuel " workers; they were studied separately because it was thought possible that one or other of these industries might also carry a specific risk of lung cancer.

The death records for earlier periods were available in the offices of the medical officers of health for only two of the four areas studied; one of them was, however, the area in which the refinery is situated and in which the majority of the nickel workers resided. Data for these two areas for the period 1938-47 are shown in Table 2: the corresponding data for the period 1948-56 (included in Table 1) are also shown in Table 2 for comparison. The data are shown in the same way as in Table 1, save that, because of the smaller numbers, they are given in larger age groups.

TABLE 1

NUMBER OF DEATHS FROM CANCER OF LUNG AND OF NOSE AND OTHER CAUSES AMONG MEN RESIDENT IN FOUR LOCAL AUTHORITY AREAS IN SOUTH WALES (1948-1956) SUBDIVIDED BY AGE AND NATURE OF LAST EMPLOYMENT

Age at Death (years)	Number of Deaths among Men Employed in:																		
	Nickel Industry			Other Selected Occupations*			Steel Industry			Coal-mining			All Other Occupations			All Occupations			
	Cancer of Lung	Cancer of Nose	Other Causes	Cancer of Lung	Cancer of Nose	Other Causes	Cancer of Lung	Cancer of Nose	Other Causes	Cancer of Lung	Cancer of Nose	Other Causes	Cancer of Lung	Cancer of Nose	Other Causes	Cancer of Lung	Cancer of Nose	Other Causes	All Causes
15–	0	0	1	0	0	2	0	0	12	0	0	16	0	0	141	0	0	171	171
25–	0	0	4	0	0	23	0	0	15	0	0	47	5	0	214	5	0	299	304
35–	0	0	6	1	0	17	2	0	63	2	0	104	14	0	373	19	0	564	583
45–	1	0	3	6	0	34	10	1	64	4	0	99	40	0	365	61	1	565	627
50–	11	1	16	8	0	54	15†	0	105	13	0	158	68	0	510	115	1	844	960
55–	11	1	22	9	0	67	17†	0	158	8	0	201	87	5	778	132	6	1,226	1,364
60–	15	2	13	18	0	79	32	0	233	16†	0	336	106‡	0	1,041	187	2	1,702	1,891
65–	8	7	28	7	1	86	28†	0	318	10	1	399	94	0	1,328	147	9	2,159	2,315
70–	2	1	19	4	0	85	10	0	352	12‡	1	470	59‡	1	1,378	87	3	2,303	2,393
75–	0	1	21	1	0	78	5	0	361	6	0	463	23	0	1,314	35	1	2,237	2,273
80–	0	0	6	0	0	47	2	1	251	1	0	302	6	0	916	9	1	1,526	1,536
85 and over	0	0	0	0	0	33	0	1	119	0	0	133	1	0	525	1	1	810	812
Not stated	0	0	0	0	0	1	0	0	4	1	0	1	0	0	11	1	0	17	18
All ages: 15 and over	48	13	139	54	1	606	121	3	2,055	73	2	2,729	503	6	8,894	799	25	14,423	15,247

*Men employed in aluminium, copper, spelter, and "patent fuel" works and in oil refineries.
†Including one case (‡two cases) in which primary lung cancer was referred to as an associated condition and not as the direct cause of death.

Cancer of the Lung

From the data shown in Table 1 it is, of course, impossible to calculate death rates, since the numbers of men employed in the different occupations are not known. If, however, the experience of one of the occupational groups is used as a standard, it is possible to estimate the numbers of deaths expected to be due to lung cancer in the other groups. The most suitable group to use in this way is the group of men employed in "all other occupations". The

TABLE 2

NUMBERS OF DEATHS FROM CANCER OF LUNG AND OF NOSE AND OTHER CAUSES AMONG MEN RESIDENT IN TWO LOCAL AUTHORITY AREAS IN SOUTH WALES (1938-47 AND 1948-56) SUBDIVIDED BY AGE AND NATURE OF LAST EMPLOYMENT

Period	Age at Death (years)	Number of Deaths among Men Employed in:																		
		Nickel Industry			Other Selected Occupations*			Steel Industry			Coal-mining			All Other Occupations			All Occupations			
		Cancer of Lung	Cancer of Nose	Other Causes	Cancer of Lung	Cancer of Nose	Other Causes	Cancer of Lung	Cancer of Nose	Other Causes	Cancer of Lung	Cancer of Nose	Other Causes	Cancer of Lung	Cancer of Nose	Other Causes	Cancer of Lung	Cancer of Nose	Other Causes	All Causes
1938 to 1947	15–	0	0	2	0	0	0	0	0	17	0	0	28	0	0	62	0	0	109	109
	25–	0	0	2	0	0	6	0	0	39	0	0	48	0	0	82	0	0	177	177
	35–	0	0	7	0	0	6	2	0	53	1	0	69	0	0	94	3	0	229	232
	45–	14	2	23	0	0	8	9	0	77	1	0	154	9	0	189	33	2	451	486
	55–	16	12	36	0	0	12	7	0	176	3	0	271	13	1	390	39	13	885	937
	65–	6	2	20	0	0	8	4†	1	241	2	0	311	5	1	455	17	4	1,035	1,056
	75 and over	0	0	2	0	0	5	0	0	201	1	1	190	2	0	428	3	1	826	830
	Not stated	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	1	1
	All ages 15 years and over	36	16	92	0	0	46	22	1	804	8	1	1,071	29	2	1,700	95	20	3,713	3,828
1948 to 1956	15–	0	0	0	0	0	1	0	0	3	0	0	4	0	0	28	0	0	36	36
	25–	0	0	2	0	0	2	0	0	3	0	0	14	1	0	43	1	0	64	65
	35–	0	0	4	0	0	2	1	0	21	1	0	47	2	0	78	4	0	152	156
	45–	9	1	12	3	0	17	10†	0	55	6	0	117	18	0	161	46	1	362	409
	55–	21	3	24	2	0	12	13	0	151	8†	0	220	30	1	353	74	4	760	838
	65–	9	8	37	0	0	21	18†	0	256	8‡	2	347	31†	0	533	66	10	1,194	1,270
	75 and over	0	1	18	0	0	3	3	1	308	0	0	334	6	0	589	9	2	1,252	1,263
	Not stated	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—
	All ages 15 years and over	39	13	97	5	0	58	45	1	797	23	2	1,083	88	1	1,785	200	17	3,820	4,037

*Men employed in aluminium, copper, spelter, and "patent fuel" works and in oil refineries.
†Including one case (‡two cases) in which primary lung cancer was referred to as an associated condition and not as the direct cause of death.

number of deaths is large and the types of occupation are numerous and varied; the total mortality of the group is, therefore, unlikely to have been influenced to any great extent by any single occupational factor. The number of deaths expected to be due to lung cancer in the other occupational groups may then be estimated by assuming that the ratio between the number due to lung cancer and the number due to " other causes ", that is, all causes, other than cancer of the lung or nose, should be the same, at each age, as in the standard group. For example, there were 87 deaths due to lung cancer and 778 deaths due to " other causes " among men aged 55-59 years employed in " all other occupations ". There were 22 deaths due to " other causes " among men of the same ages employed in the nickel industry and it is, therefore, estimated that the expected number of deaths from lung cancer among the nickel workers is $\frac{87}{778} \times 22$, *i.e.*, 2·46. The number actually observed was 11. The results obtained by calculating the expected numbers for each of the age groups studied and summing for all ages are shown in Table 3*. Among the nickel workers the number of lung cancer deaths observed was nearly five times the number expected. Among the steel workers and the workers in the group of " other selected occupations ", the observed numbers were close to, but slightly higher than, the expected numbers; among the colliery workers the observed number was substantially less.

TABLE 3

COMPARISON BETWEEN OBSERVED AND EXPECTED NUMBERS OF DEATHS FROM CANCER OF LUNG IN FOUR OCCUPATIONAL GROUPS

No. of Deaths	Nickel Workers	Men in Other Selected Occupations	Steel Workers	Colliery Workers
Expected from the experience of men in " all other occupations "	9·88	39·12	110·97	152·16
Observed	48	54	121	73
Observed as % of expected	486	138	109	48

In this investigation, which is essentially a study of proportional mortality, a number of alternative explanations of the results are possible. The proportion of lung cancer deaths among the standard population may be abnormally low, the number of deaths from " other causes " among the nickel workers may be abnormally few, or the deaths from lung cancer may be excessive. The first explanation can be dismissed, since the proportion of lung cancer deaths in the standard population was very close to that which would have been expected on the basis of the mortality data for the whole country. From the national data it can be calculated that 493 deaths from lung cancer would be expected to have accompanied the 8,894 deaths from causes other than cancer of the lung and nose which occurred at the ages shown in Table 1; in fact, the number of lung cancer cases was 503 and the number in which death was certified as due to lung cancer was 499, which is almost identical with the number expected. Moreover, it may be noted that the experience of the standard population leads to estimates for the expected numbers of lung cancer deaths among two of the other groups of " non-nickel " workers which are reasonably close to the numbers observed. For the second explanation to be valid, it would be necessary for the mortality among nickel workers from all " other causes " to be only one-fifth of the national mortality—and this is certainly unreasonable. From knowledge of the mortality of industrial workers in general it is, on the contrary, more likely that by virtue of their social status, the total " other cause " mortality of the nickel workers was somewhat higher than that in the non-industrial sections of the community.

It is extremely unlikely that any substantial proportion of the deaths attributed to lung cancer were falsely diagnosed as a result of a bias in favour of diagnosing lung cancer among nickel workers. For if this had been so, it must be presumed that there would have been a deficiency of deaths attributed to those other causes which could most easily be confused with cancer of the lung, namely, other types of cancer and other respiratory diseases. In fact, of the 139 deaths among nickel workers during the period 1948-56, which were not attributed to cancer of the lung or cancer of the nose, 25 were attributed to other types of cancer and 21 to other respiratory diseases. Data for the causes of death, other than what was required to place them in one of the other categories shown in Tables 1 and 2, were not extracted for the non-nickel workers; but an estimate of the expected proportions attributable to " other causes " and to " other respiratory disease " can be obtained from the national mortality data for men of the same ages in England and Wales during the same period. The estimated numbers are 22 and 25 out of 139 respectively. The number of lung cancer deaths among the nickel workers might also have been artificially increased if there had been a specific tendency to describe as nickel workers men who had died of lung cancer and who, after employment at the refinery, had taken up some other occupation. Evidence that a bias of this type may, in the case of coal-miners,

*The few men whose ages at death were not known were presumed to have been distributed among the age groups in the same proportions as the men whose ages were known.

contribute to the excess mortality which is recorded on death certificates for this occupation has been obtained by Heasman, Liddell, and Reid (1958). It is, however, unlikely that such a factor should have biased the present results to an important extent, since the excess mortality has been practically constant since 1938 (see Table 4) and has shown no tendency to increase since the disease was prescribed in 1949. It is concluded, therefore, that the only reasonable interpretation of the results is that the high proportion of lung cancer deaths among nickel workers was due to an excess mortality from the disease and that, in the period 1948-56, the risk of dying from the disease was approximately five times " normal ".

Not all the workers appear to have been equally at risk. Of the 48 nickel workers whose deaths were attributed to lung cancer during this period, 28, or 58%, were described as having been employed directly on the process (that is, " processman " or " process worker "); on the other hand, the proportion of men whose deaths were attributed to causes other than cancer of the lung or nose and who were similarly described was somewhat smaller—54 out of 139 or 39%. The numbers of deaths from lung cancer expected among the process and the non-process workers are estimated to have been 3·93 and 5·95 respectively, so that on this basis the risk among the process workers appears to have been approximately double that among the other employees (28 observed to 3·93 expected compared with 20 observed to 5·95 expected, ratios of 7·1 to 1 and 3·4 to 1). Some of the non-process workers are, however, likely to have been employed as process workers at some time previously or to have had some closer contact with the specific process (for example, gatekeeper, labourer, and fitter). The relative risks to these two groups cannot, therefore, be satisfactorily measured from the present data and it is possible that the real difference is much greater.

TABLE 4

COMPARISON BETWEEN OBSERVED AND EXPECTED NUMBERS OF DEATHS FROM CANCER OF LUNG IN FOUR OCCUPATIONAL GROUPS IN 1938-47 AND 1948-56

Period	No. of Deaths	Nickel Workers	Men in Other Selected Occupations	Steel Workers	Colliery Workers
1938-47	Expected from the experience of men in "all other occupations"	2·61	0·89	13·12	20·67
	Observed	36	0	22	8
	Observed as % of expected	1,379	—	168	39
1948-56	Expected from the experience of men in "all other occupations"	5·86	4·27	37·62	56·89
	Observed	39	5	45	23
	Observed as % of expected	666	117	119	40

Lung Cancer in Other Special Occupations

In contrast to the nickel workers, some part of the discrepancy between the observed and expected numbers of lung cancer deaths among the colliery workers may be attributed to an abnormal death rate from " other causes ". According to the preliminary data on occupational mortality in England and Wales in 1950 (Registrar General, 1954), the death rate from all causes other than lung cancer among coal-miners aged 20-64 years was 20% above the average for the whole country. If, therefore, the numbers of deaths from " other causes " among the coal-miners are reduced by one-sixth (20/120), the expected number of lung cancer deaths becomes 126·80, and, in this case, the observed number is 58% of the expected. The mines in the area studied are, however, anthracite mines and anthracite miners in South Wales have suffered a higher mortality than other miners. In 1930-32, for example, the standardized mortality ratio for all coal workers below ground aged 20-65 years was 105, but it was 143 for the workers below ground in the anthracite mines of South Wales (Registrar General, 1938). If therefore, this difference has persisted in more recent periods the expected number of lung cancer deaths among miners should be reduced still further. The mortality from lung cancer among coal-miners in Britain has consistently been recorded as lower than that for all men and according to the occupational mortality data for 1950 was, at ages 20-64 years, 69% of the general rate. The present method of investigation would, therefore, appear to have provided a reasonably accurate estimate of mineworkers' mortality from this disease.

Lung cancer mortality among the five groups of workers included in " other selected occupations " will be discussed in another report.

Nasal Cancer

In the study of nasal cancer it is not possible to use quite the same technique, since the disease is normally very rare and the number of deaths shown in Table 1 due to nasal cancer among the men in " all other occupations " is very small. A more reliable estimate of the expected number of deaths might be obtained by using the whole group of non-nickel workers as a standard. This is vitiated, however, by the fact that the risk among the nickel workers appears to have been relatively so great that the inclusion of a small number of deaths of ex-nickel workers among the deaths of men who were last employed in some other occupation has

artificially increased the mortality from nasal cancer among the other workers in the area. Thus, in the four areas studied there were 12 deaths from nasal cancer during the period 1948-56 among men who were not nickel workers at the time of their death. The relatives of 10 of them were traced and it was learnt that four had been employed at the nickel refinery at some time. Similarly, in the two areas for which information was available back to 1938, four deaths from cancer of the nose were found to have occurred during the period 1938-47 among men who were not nickel workers at the time of death. Relatives of all these men were traced and it was discovered that three of them had previously been nickel workers. In all, therefore, at least seven out of the 16 non-nickel workers resident in the area who died of nasal cancer may at some time have been exposed to the particular hazard which is being studied.

In these circumstances, it is preferable to use the national data to provide an estimate of the expected mortality. Detailed figures have been published for the numbers of deaths attributed to a group of upper respiratory cancers consisting of cancer of the nose, nasal cavities, middle ear and accessory sinuses for each year since 1950, and figures for the total numbers of these cancers by sex have been published for the years 1940-49. Since the crude male death rate has been practically constant since 1942 (varying only from 5 to 6 per million), and since the deaths attributed to nasal cancer in Tables 1 and 2 include all the deaths which would have been classified by the Registrar General in this category—and no others—the national data provide a reasonable basis for comparison with the data collected in the present investigation. If, therefore, the whole male population of England and Wales is taken as the standard population and the same method of estimation is used as was used previously, estimates of the expected numbers of deaths from nasal cancer can be obtained. The results are shown in Table 5. From Table 5 it appears that the mortality from nasal cancer among the nickel workers during the period 1948-56 has been about 150 times higher than normal.

TABLE 5

COMPARISON BETWEEN OBSERVED AND EXPECTED NUMBERS OF DEATHS FROM CANCER OF NOSE AMONG NICKEL AND NON-NICKEL WORKERS

Population	No. of Deaths from Cancer of Nose		Ratio of Numbers Observed and Expected
	Observed	Expected	
Nickel workers:			
Four areas, 1948-56	13	0·082	159:1
Two areas, 1938-47	16	0·066	242:1
All areas available, 1938-56	29	0·148	196:1
Non-nickel workers:			
Four areas, 1948-56	12*	8·279	—
Two areas, 1938-47	4*	2·132	—
All areas available	16*	10·411	—

*Including several deaths among men with a history of previous employment in the nickel refinery: four in the period 1948-56 and three in 1938-47.

As previously with lung cancer, the risk does not appear to have been distributed equally among all the workers. Of the 29 nickel workers whose deaths were attributed to nasal cancer during the whole period 1938-56, 19, or 66%, were described as having been last employed before death directly on the process; the proportion of men who were similarly described and whose deaths were attributed to causes other than cancer of the lung or nose was 99 out of 231, or 43%. The numbers of deaths from nasal cancer expected among these two groups of workers are estimated to have been 0·064 and 0·084, so that on this basis the risk among the process workers appears to have been approximately two and a half times that among the other employees (19 observed to 0·064 expected compared with 10 observed to 0·084 expected, ratios of approximately 300 to 1 and 120 to 1). It must, however, be stressed again that the present data are unsatisfactory for estimating the relative risks among different occupational groups within the industry and the real difference may have been very much greater.

The total number of deaths registered among non-nickel workers during the whole period 1938-56 is somewhat larger than the expected number (16 against 10·4), but seven of these deaths occurred in men who are known to have been nickel workers previously. If these seven deaths are excluded the agreement is close and it may be concluded that the estimates of the numbers of deaths expected are reasonably accurate.

Secular Changes in Risk from Cancer of the Lung and Nose

To test the extent to which the risks have altered it is necessary to examine data collected over a long period of years since the induction period before the appearance of cancer is generally long. In the present case, data adequate for this purpose are available for only two of the four areas which were originally studied; these have been shown in Table 2. The men who were employed in " all other occupations " and were resident in these two areas have been used as the standards and the expected numbers of deaths attributable to cancer of the lung have been calculated by the technique described previously (working, however, with 10-year instead of five-year age groups, because of the smaller numbers). The results obtained are shown in Table 4. From these figures it would appear that

the specific risk of lung cancer in the industry has decreased from about 14 times " normal " to about seven times " normal ". This change, however, cannot be regarded as evidence of a decrease in risk among the nickel workers since the " normal " risk of lung cancer increased substantially during the period in question. In fact the excess over expectation of lung cancer deaths among the nickel workers was 3·3 per year during 1938-47 and 3·7 per year during 1948-56. Similarly, if the national data are used to estimate the numbers of deaths from nasal cancer expected in the two periods, it appears that the average excesses were 1·6 and 1·4 deaths per year. Clearly cases are still occurring and there has not as yet been any substantial change in their number.

On the other hand, from the data in Table 6, it is clear that the distribution of the ages at death has changed for both types of cancer. The change is particularly striking for cancer of the nose, for which the average age at death has increased by 6·2 years from 59·6 to 65·8 years. For cancer of the lung the increase is less marked, but it is still appreciable (an average increase of 3·3 years from 57·0 to 60·3 years). These changes may be regarded as evidence that the greatest risk occurred at some time in the past; for, in this situation, the average age of the men who suffered the heaviest exposure, and who may be presumed to suffer the heaviest specific mortality, must be expected to increase steadily with the passage of time. Further, for neither type of cancer is there any evidence of an increased mortality among men who were aged less than 50 years during the period 1948-56. It is clear, therefore, that there is no present evidence of any excess risk among men who, by virtue of their age, must have begun to be employed at the refinery after 1924. This is substantiated by Morgan (1958) who has made a detailed study of the occupational histories of the employees known to the industry to have died of cancer. He has found that none of those who started to work at the refinery after 1924 has died of cancer of the nose and only six of them have died of cancer of the lung. An estimate based on the national mortality rates for lung cancer suggested that if the risk had been entirely eliminated the expected number of lung cancer deaths would have been nine.

TABLE 6

AGE DISTRIBUTION OF DEATHS FROM CANCER OF LUNG AND OF NOSE AMONG NICKEL WORKERS IN 1938-47 AND 1948-56

Age (years)	Cancer of Lung		Cancer of Nose	
	1938–47	1948–56	1938–47	1948–56
Under 45	0	0	0	0
45–	4	1	0	0
50–	10	8	2	1
55–	10	7	7	1
60–	6	14	5	2
65–	3	7	2	7
70–	3	2	0	1
75 or more	0	0	0	1
All ages	36	39	16	13

Total Number of Respiratory Cancer Deaths Reported

The deaths from cancer reported in Tables 1 and 2 must be presumed to include a large proportion of the deaths due to cancer of the lung and nose which have been reported previously as occurring among nickel workers (Chief Inspector of Factories, 1952). The deaths in the Chief Inspector's report had, however, all occurred before the end of 1950 so that they do not include the 33 deaths from lung cancer and the 12 deaths from nasal cancer found in the present inquiry to have occurred among nickel workers and ex-nickel workers during the six years 1951-56. The total numbers of deaths now known to have occurred from cancer of the lung and nose during the period 1923 to 1956 are, therefore, at least 126 and 62 respectively.

In contrast, it may be noted that, in the present inquiry, only one nickel worker was found to have died from cancer of the larynx during the whole period 1938-56, and no other similar death has been reported for any earlier period.

Summary

Cancer of the lung and cancer of the nose have been prescribed as industrial diseases when they occur among certain classes of nickel workers. There is, however, no published evidence to show the size of the risks nor the extent to which they have been eliminated.

Data were collected from the records of deaths held by the medical officers of health for the districts in which workers at a nickel refinery lived. From the ratio of deaths attributed to lung cancer to deaths from causes other than cancer of the lung and nose among the other men resident in the same area an estimate was made of the number of lung cancer deaths which would be expected to have occurred among men employed in four specified occupational groups. During the years 1948-56, there were 139 deaths from " other causes " among nickel workers and 48 from lung cancer; the expected number of lung cancer deaths among them was 9·9. The excess did not appear to be due to a bias in favour of diagnosing lung cancer among nickel workers, and it is concluded that during 1948-56 the risk of nickel workers dying from the disease was approximately five times " normal ".

During 1948-56, 13 deaths among nickel workers were due to cancer of the nose. An estimate based upon the national mortality data suggested that the expected number of deaths was less than 0·1 and that the nickel workers' risk of dying from the disease was approximately 150 times "normal".

Comparison of the data for 1938-47 with those for 1948-56 provided no evidence of any substantial change in the annual number of deaths due to these specific diseases. There was, however, an increase in the average age at which death occurred and no excess in mortality was apparent at ages under 50. It is, therefore, reasonable to believe that the hazard has been largely or completely removed in the last 35 years.

The total numbers of deaths from these two diseases which are now known to have occurred among British nickel workers are: cancer of the lung, 126; cancer of the nose, 62.

I am most grateful to Dr. G. M. Davies, Dr. E. B. Meyrick, and Dr. H. R. Stubbins, medical officers of health, for making their records available for examination and for the assistance given by them and by their staffs. I am also very grateful for the assistance of the health visitors, who succeeded in obtaining occupational histories from such a high proportion of those non-nickel workers who had died of cancer of the nose, and to Miss K. Jones and Miss J. Parrish who extracted the greater part of the data.

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CHROM 3447

A thin-layer chromatographic method for the determination of trace metals in cereal

The advantages of using thin-layer chromatography as opposed to paper chromatography for the separation of metal ions have been discussed before[1]. A shorter development time and sharper delineation of the spots are the most obvious advantages. It has also been shown in earlier papers that diffuse reflectance spectroscopy can lend itself well to quantitative determination of trace metals separated on thin-layer chromatograms.[1,2] Rubeanic acid and pyridine-2-aldehyde-2-quinolylhydrazone (PAQH) have been investigated extensively as chromatographic spraying reagents[1-5] for cobalt, copper and nickel. In this study a combination of these techniques has been adopted for the determination of traces of cobalt, copper and nickel in oats.

Experimental

Preparation of samples and standards. An improved ashing technique proposed by CONOLLY AND MAGUIRE[5] has been used. This involves ashing of a 20 g sample for 12 h at 550°. After cooling, 5 ml of dilute (1:1) hydrochloric acid are added and the mixture is evaporated on a steam bath and heated on a hot plate for 30 min. The hydrochloric acid treatment is repeated followed by the addition of 5 ml of hot dilute (1:1) hydrochloric acid. Silicates and other debris are centrifuged after the system has been heated in boiling water for 30 min. The residue is washed twice with 5 ml of hot (1:1) hydrochloric acid and the original supernatant liquid and washings are combined and evaporated to dryness. This residue is dissolved in 2 ml of (1:1) hydrochloric acid and kept for analysis.

Appropriate dilution series of synthetic copper, cobalt and nickel mixtures (1:1) in hydrochloric acid are prepared.

Doubly distilled water, reagent grade chloride salts and hydrochloric acid are used.

Chromatographic procedure. The chromatoplates were coated with a Desaga thin-layer chromatography applicator; the layer thickness was adjusted to 0.25 mm. After coating with Merck Silica Gel G, the plates were heated at 110° for 1 h and stored in a dry cabinet over silica gel adsorbent. The spots were applied with a 10 μl Hamilton microsyringe and then chromatographed in a preconditioned chromatography chamber. The solvent system used for chromatographic separation was 0.5 % concentrated hydrochloric acid in acetone.[1] Standards were developed simultaneously with the sample. The plates were then dried in air for 5 min and exposed to ammonia vapour for 10 min to neutralize. After 5 min drying at 110° in a convection oven the plates were sprayed with either a 0.1 % w/v rubeanic acid in ethanol solution or with a 0.03 % w/v ethanolic solution of PAQH until the plate appeared transparent. (For more details see Results and discussion.) Drying of the plates at 110° for 5 min is recommended for best results.[1,2]

Quantitative evaluation. The cells and technique used in preparing the separated components for quantitative analysis have been described previously.[6] Adsorbent from the same plate serves as a reference standard. The 30 mg or 20 mg samples removed from the plates for analysis of the spots are weighed to ± 0.1 mg on an Oertling auto-

matic balance and are ground in a small agate mortar for two periods of 1 min each to insure homogeneity of the sample. Samples that are not analyzed immediately are stored in a desiccator over silica gel. A Beckman D.U. spectrophotometer with a standard diffuse reflectance attachment is used for the photometric measurements. The wavelength settings are determined from spectra recorded previously.[1,2] For rubeanic acid complexes in this study they were 630 nm for nickel, 465 nm for cobalt and 580 nm for copper. For the PAQH complexes they were 508 nm for cobalt and 490 nm for nickel. For calibration curves the reflectance values R or the values of the Kubelka-Munk function $(1-R)^2/2R$ are plotted *versus* concentration.[1,2]

Results and discussion

Separation and detection. The separation on silica gel occurred in the order $Ni^{2+} < Co^{2+} < Cu^{2+}$. Complete resolution was achieved within 10–20 min. The separation time is about one-half the time required to separate the ions on cellulose and about one-tenth the time needed on alumina.[1]

The visual detection limits for the rubeanic acid complexes were found to be about 0.03 μg per spot (0.5 cm in diameter) for nickel and copper and 0.05 μg per spot for cobalt. The instrumental detection limits for 20 mg samples taken at an accuracy level of 50 % were 0.05 μg for nickel and copper and 0.1 μg for cobalt.

For sample spots applied in 5 μl amounts, no difficulty was encountered in detecting nickel and copper as rubeanic acid complexes. The cobalt however was present in such small amounts that either larger samples had to be applied on the chromatogram or a different spray reagent had to be used. Previous investigations had shown that with a 0.03 % PAQH solution as a spray reagent the detection limit for cobalt could be improved by a factor of ten (0.01 μg per spot at a 50 % accuracy level).[2] The plate was prepared in the same manner as for rubeanic acid. Colored zones for copper and iron (including iron impurities from the chromatographic adsorbent) did not interfere with the cobalt spot. Faint spots for large quantities of zinc and manganese, as well as the reagent background, faded completely upon heating the plate for 5 min at 110° after spraying. If nickel is present in very low concentrations, one can also use the PAQH complex for its detection. Spraying of the nickel spot with a 1.0 N sodium hydroxide solution is recommended; the detection limit is 0.01 μg per spot.[2]

Quantitative evaluation. Linear Kubelka-Munk plots extending up to concentrations of 10 μg per spot for rubeanic acid complexes and up to 1 μg per spot for PAQH complexes have already been reported.[1,2]

Four 20 g samples from the same batch of oats were prepared for analysis (see Preparation of samples and standards). Nickel and copper were determined as rubeanic acid complexes; cobalt as PAQH complex. The results are reported in Table I.

The recovery of metals from a sample of oats was tested by adding known amounts of the metal to the original sample (see Table II).

CONOLLY AND MAGUIRE[5] have reported fairly good agreement of data obtained by a similar chromatographic technique and a spectrographic technique. The main advantages of this method in comparison with a spectrographic method are better accuracy (between 2 and 5 % for samples in a medium concentration range[1]) and shorter time required for analysis. The two methods are comparable in sensitivity. From Table I it can be seen that metal concentrations of around 0.02 and 0.03 p.p.m.

TABLE I

ANALYSIS OF OATS*

Amount of metal found (p.p.m.)	*Sample No.*				*Average and standard deviation (p.p.m)*	*Relative standard deviation (%)*
	1	*2*	*3*	*4*		
Cobalt	0.03	0.02	0.02	0.03	$0.02_5 \pm 0.00_5$	± 20
Copper	3.45	3.68	3.50	3.27	3.48 ± 0.17	± 4.9
Nickel	0.96	1.05	0.90	0.99	$0.98 \pm 0.06_3$	± 6.4

* The value for each sample is the mean obtained from four spots, developed simultaneously.

TABLE II

RECOVERY OF METALS FROM A SAMPLE OF OATS*

	Cobalt		*Copper*		*Nickel*	
Amount present in original samples (p.p.m.)	0.03	0.03	3.48	3.48	0.96	0.96
Amount added (p.p.m.)	0.05	0.10	3.0	6.0	1.0	3.0
Total amount found (p.p.m.)	0.07	0.15	6.69	9.98	2.08	4.12

* Mean of four results.

can be measured with an accuracy of ± 20 %. This method should also be applicable to other cereals and to a wide range of foodstuffs.

Acknowledgements

Miss CHARALYNE FREEMAN is to be thanked for technical assistance.

This work was supported by a grant from the National Research Council of Canada.

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Received February 12th, 1968

Amer. Soc. Brew. Chem. Proc. 172-176 (1966)

Analysis of Trace Metals in Beer by Atomic Absorption Spectrophotometry

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THERE HAS BEEN interest for many years in the brewing industry in the analysis of trace metals in beer. Colorimetric methods are necessarily cumbersome, subject to interferences, and not available for all metals. Electrochemical methods are not possible at the low levels at which most metals are present in beer. It is not surprising, then, that as flame analysis techniques were developed they were adopted for use by brewing analysts. Stone *et al.* (6) used flame emission photometry to analyze for sodium, potassium, and calcium in beer and water, and this method is now generally considered standard for these metals in laboratories which have the necessary equipment available. Flame photometry is not sensitive enough, however, to detect metals present in beer which are weaker emitters than the alkali metals and which are present in comparatively small amounts. Hudson (2) applied a spectrographic technique to the analysis of metals in brewing materials, and Steiner and Oliver (5) reported on the spectrochemical determination of trace metals in beer. Such methods, although capable of great sensitivity, require extensive sample preparation, and are not well suited for large numbers of routine analyses.

Atomic absorption is a relatively new technique, pioneered in New Zealand and Australia a decade ago. It is only in the last few years that commercial instruments have been available in North America. Atomic absorption spectrophotometry superficially resembles flame photometry, in that the sample is introduced into a flame to atomize it. Light from a lamp having cathode made of the element to be [illegible] is passed through the flame [illegible] photodetector. Atoms of the [illegible] which are unexcited, or in their ground state, will absorb a portion of the light from the lamp, and this absorption is directly related to the amount of the element present in the flame. Because the lamp is emitting only at certain discrete wave lengths and the element in the sample is absorbing only at those wave lengths, the method is generally completely free from the many spectral interferences which plague emission flame photometry. Also, in general, even in a flame fewer than 1% of the atoms of a metal present are excited, while the rest remain in the ground state. Atomic absorption, then, is inherently capable of great sensitivity.

Experimental

The spectrophotometer used for these determinations is a Perkin-Elmer Model 303, which is a double-beam instrument employing a grating monochromator, and an air-acetylene premix burner with a wide-slot (0.025-in.) head. Lamps for all elements are hollow cathode, except sodium and potassium, for which vapor lamps are used.

Three materials have been analyzed to date. These are beer, wort, and yeast. Beer presents no problems, but in wort the levels of many of the metals are so low that an extraction step must be used. It has been found that by the use of a chelating agent, ammonium pyrrolidine dithiocarbamate (APDC), and a water-immiscible organic solvent, methyl isobutyl ketone (MIBK), many of the heavy metals can be quantitatively extracted from a large volume of aqueous solution and concentrated in the organic phase (4). In all analyses reported, the following methods were used for sample preparation.

Beer. For most elements only decarbonation is required. For analysis of calcium or magnesium, dilute decarbonated beer 1:100 with distilled water.

Wort. To one liter of wort add 30 ml. of a 1% aqueous solution of APDC. Add 250 ml. MIBK and shake mechanically for 15 min. Break the emulsion by centrifuging, separate the layers, and analyze the upper (MIBK) layer.

Yeast. Wash several times with distilled water to remove beer. Remove as much water as possible by vacuum filtration. Split the sample and dry one portion at 105°C. to find the moisture content. Ash the other moiety at 700°C., moisten the ash thoroughly with conc. HCl, and dilute with distilled water to a volume sufficient for the analyses. Filter to remove ash. Calculate the metals as ppm. in yeast, dry basis.

A standard solution is run with each set of analyses. These standards are made by an appropriate dilution of a stock solution in the liquid to be tested; that is, when beer is being analyzed the standard is made up in beer. This is necessary because the flow rate through the burner capillary tube is affected by the solution being used. Stock solutions are made by dissolving reagent grade salts of the metals in distilled water to give metal concentrations of 500 or 1,000 ppm. Because all the metals discussed give straight-line curves when run with known amounts present, it is possible to calculate levels in unknowns by a direct-ratio comparison with the standard. It is not workable to have a standard calibration curve, because any of a number of variables, such as a slightly different acetylene pressure or a deterioration of the cathode lamp, will change the slope. Running a freshly made standard with each set of samples obviates any difficulty.

Sensitivities and Detection Limits

The metals discussed here fall naturally into several groups by sensitivity. The group for which the method is the least sensitive consists of iron, copper, cobalt, and nickel. Figure 1 shows typical curves for these metals in beer. These curves were run with the instrument set for maximum sensitivity. Below 1% absorption it is difficult to get reproducible results, so the lower limit for accurate analysis would be that at which the curve intersects the 1% absorption line. The detection limits can be lowered considerably by the MIBK-APDC extraction used for wort, as all these metals chelate easily. Cobalt is rather different from the others in that it has several absorption peaks which can be used for analysis, and rather than having one most sensitive wave length, different ones may be better as

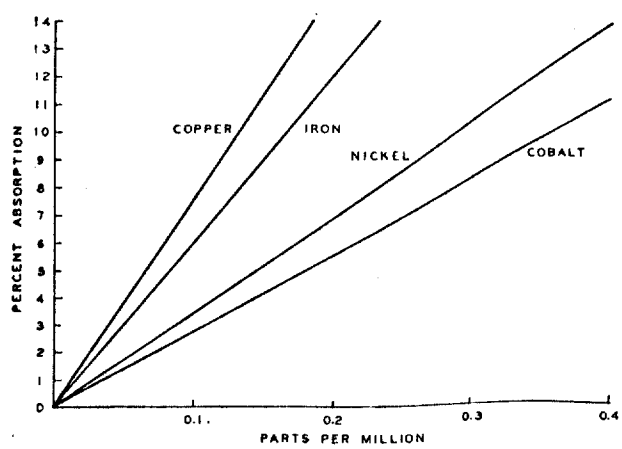

Fig. 1. Typical analysis curves for trace metals in beer run at the following wave lengths (mmu): copper, 325; iron, 248; nickel, 232; and cobalt, 241.

conditions of solution change (1).

The second group consists of zinc, manganese, and chromium. Figure 2 shows typical analysis curves for these metals in beer. In this case the instrument was not set for the greatest sensitivity. The method is so sensitive for these metals that greater accuracy is achieved by running at a low sensitivity setting, thus getting less needle fluctuation on the instrument and greater reproducibility. Only when the levels of these metals are very low is it necessary to use the scale expansion device. On low sensitivity settings it is necessary to convert per cent absorption read from the instrument into the logarithmic absorbance to obtain straight-line plots.

Magnesium is, of all metals tested, the one for which the method is the most sensitive. Figure 3 shows a typical magnesium curve. This curve was run with the instrument at the lowest sensitivity setting, so it can be seen that the analysis for this element can easily be made at parts-per-billion levels. When beer is being tested for magnesium it is necessary to dilute it 1 to 100 with distilled water.

The alkali metals can be run very easily by atomic absorption. However, like magnesium, they absorb very strongly and must in general be analyzed for by some method which decreases the sensitivity. One such method is to use a wave length other than the most strongly absorbing. Potassium can be satisfactorily run at 404 rather than 767 mmu, and sodium at 330 in lead

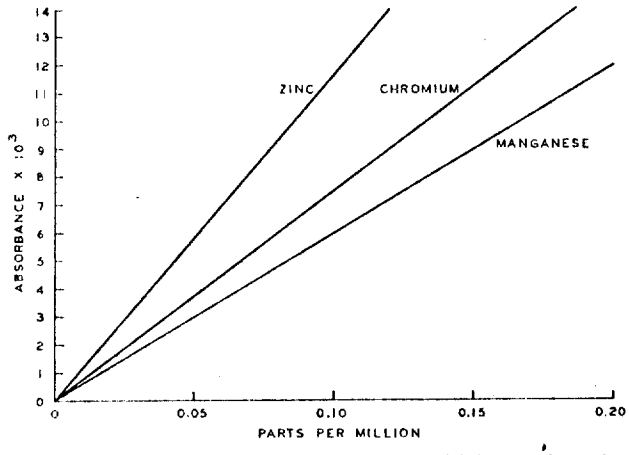

Fig. 2. Typical analysis curves at low sensitivity settings for metals in beer run at the following wave lengths (mmu): zinc, 214; manganese, 279; and chromium, 358.

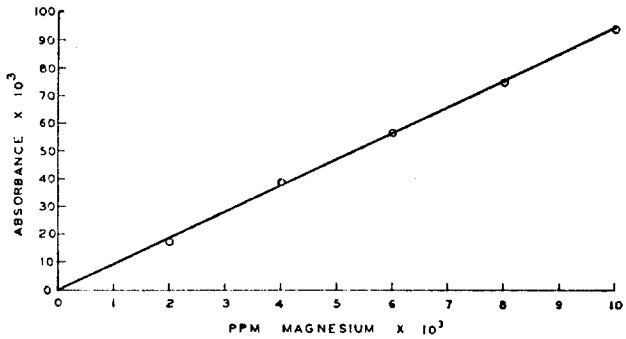

Fig. 3. Typical curve for magnesium run at low sensitivity settings at 285 mmu.

of 589 mmu. No satisfactory secondary wave length has been found for calcium, however, and this element only, of all here discussed, is highly susceptible to chemical interferences. These metals can be just as easily determined by other methods, such as flame photometry, thus avoiding dilution errors, and so will not be further discussed here.

An attempt was made to analyze for lead and tin. The detection limits for these metals, however, is sufficiently high so that none could be found in any of the beers tested. A great deal of work by instrumentation people is going into the development of better burners, improved lamps, and other accessories; in the near future not only lead and tin but also such metals as aluminum and arsenic may have detection limits low enough so that beer can be analyzed for them.

Discussion of Results

Tables I and II show analyses for 12 canned and 16 bottled beers. All beers were brewed in the United States. Chromium was found in two samples of bottled beer, at levels of 0.03 and 0.06 ppm. In all other samples it was below the detection limit, and so is omitted from the tables. In the case of cobalt, it is obvious that those beers in which it was found have had the cobalt added as a foam stabilizer. Iron, as would be expected, is somewhat higher in the canned than in the bottled samples. The difference in metal pick-up between cans and bottles is more apparent with nickel, and especially so with zinc. These metals are not thought to be detrimental to the beer, but the pick-up of metal is interesting in that it shows that cans do contribute ions to the beer.

TABLE I
Parts per Million of Several Trace Metals in 12 Canned Beers

Sample	Co[a]	Cu	Fe	Mg	Mn	Ni	Zn
1	*	0.16	0.07	75	0.05	0.15	1.30
2	*	0.25	0.11	79	0.06	0.22	1.69
3	*	0.24	0.08	100	0.09	0.18	0.25
4	*	0.15	0.17	91	0.09	0.07	0.04
5	*	0.18	0.08	85	0.07	0.11	1.74
6	1.05	0.20	0.38	158	0.05	0.08	3.02
7	*	0.10	0.19	93	0.05	0.07	0.77
8	*	0.13	0.11	174	0.07	0.03	0.09
9	*	0.13	0.19	135	0.06	0.07	0.15
10	*	0.21	0.15	131	0.05	0.06	0.09
11	*	0.10	0.20	115	0.02	0.03	0.23
12	*	0.06	0.14	133	0.22	0.03	0.36
Average		0.16	0.16	114	0.07	0.09	0.81

[a]*Below detection limits.

TABLE II
Parts per Million of Several Trace Metals in 16 Bottled Beers

Sample	Co[a]	Cu	Fe	Mg	Mn	Ni	Zn
1	*	0.15	0.04	87	0.07	0.09	*
2	*	0.13	0.23	91	0.05	*	*
3	*	0.28	0.13	113	0.05	*	*
4	*	0.19	0.04	96	0.04	*	*
5	0.61	0.19	0.09	113	0.03	*	*
6	*	0.20	0.09	83	0.02	*	*
7	*	0.26	0.09	102	0.03	*	*
8	*	0.37	0.06	90	0.04	*	*
9	*	0.22	0.08	70	0.04	*	*
10	*	0.65	0.11	89	0.06	*	*
11	*	0.32	0.10	72	0.04	*	*
12	*	0.14	0.09	76	0.04	*	*
13	*	0.15	0.11	70	0.04	*	*
14	*	0.41	0.08	75	0.05	*	*
15	*	0.04	0.06	79	0.04	*	*
16	0.98	0.08	0.15	96	0.11	*	*
Average		0.24	0.10	88	0.05		

[a]*Below detection limits.

TABLE III

Parts per Million of Several Trace Metals in a Typical Lager Wort and in a Brewers' Yeast *(Saccharomyces carlsbergensis)*

Sample	Co[a]	Cr[a]	Cu	Fe	Mg	Mn	Ni	Zn[a]
Lager wort	*	*	0.08	0.07	148	0.10	0.01	*
Yeast (dry basis)	2.8	*	8.5	55	2870	3.5	7.1	62

[a]*Below detection limits.

Table III gives some data on wort and yeast. At present, work is continuing along these lines. Plans include adding metals before fermentation and checking whether the yeast picks them up, and determining rate of pick-up and possibly the effect of the metals on the yeast. Insufficient data have been collected as yet to be presented at this time.

Summary

Atomic absorption is a valuable analytical tool for the analysis of trace metals. Although the method is relatively new, there is a rapidly growing body of literature in the field. Slavin (3) has compiled a literature review of applications in the food industry which contains some 34 references. The method is rapid and accurate. It is possible with this technique to analyze for trace metals in beer, wort, and yeast. Some typical analyses have been presented and discussed.

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J. Path 97(2) 375-382 (1969)

ELECTRON-MICROSCOPE INVESTIGATION OF EXPERIMENTAL RHABDOMYOSARCOMA

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PLATES CLVI–CLXI

RHABDOMYOSARCOMAS are highly malignant tumours of striated muscle and may arise at any age, but most of the reported cases have been in infants and children. Rhabdomyosarcoma of the head and neck is really a tumour of childhood: in 170 cases, Dito and Batsakis (1962) found that almost 80 per cent. were children aged 12 yr or less. Orbital tumours (a third of the cases) were commonest, followed by intra-oral, intranasal and pharyngeal rhabdomyosarcomas, and then tumours arising in the soft tissue of the temple, face and neck. The middle ear, mastoid (Friedmann *et al.*, 1965), and air sinuses were less commonly affected, and the region of the salivary glands and mandible only rarely.

Histologically, the several varieties of rhabdomyosarcoma mimic the stages of development of striated muscle. Horn and Enterline's classification of embryonal, alveolar and pleomorphic types has been widely accepted. The present authors have, however, adopted the classification of Ashton and Morgan who, in a recent study of 34 cases of orbital rhabdomyosarcoma (Ashton and Morgan, 1965), distinguished three main types according to the optical microscopic appearance: (I) embryonal sarcoma, (II) non-striated embryonal rhabdomyosarcoma and (III) striated embryonal rhabdomyosarcoma.

Electron-microscope studies have been hampered by the rarity of this tumour, although we were the first to examine and describe the fine structure of a rhabdomyosarcoma of the ear (Friedmann *et al.*); more recently Overbeck (1967) gave a clear description of the fine structure of the cells of sarcoma botryoides of the vagina in a 17-mth-old girl.

The present investigations were based on a study of the fine structure of seventeen experimentally produced rhabdomyosarcomas with the aim of finding some additional features that might help in the diagnosis and classification of rhabdomyosarcoma. Another reason for this investigation was the fact that the fine structure of malignant mesenchymatous tumours induced by the local injection of various metals such as nickel had not been studied.

MATERIALS AND METHOD

Fifty-five female Sprague-Dawley rats weighing *c.* 200–250 g were given a single injection of nickel sulphide or nickel sponge (spectrographically pure nickel, Johnson Matthey). The nickel sulphide was sterilised in a hot-air oven at 71°C for 1 hr; 100 mg was suspended in 1 ml of sterile physiological saline solution immediately before the injection and 0·2 ml of the suspension was injected into the right gastrocnemius muscle with a serum-II needle.

Received 28 Nov. 1967; *accepted* 11 July 1968.

The rats were examined at weekly intervals and the time taken to produce a tumour about the size of a pea was noted. The rats were killed when the tumours had reached a size of 3–4 cm across, which usually took another 4–5 wk. A complete necropsy was performed on these animals and portions of the tumour and any secondaries were taken and placed in 4 per cent. formaldehyde in saline, Bouin's fluid, 3 per cent. glutaraldehyde in Sorensen's buffer (*p*H 7·4), and 1 per cent. osmium tetroxide in buffered ice-cold Dextran sucrose solution. Specimens of some of the tumours were implanted through a small skin incision into the medial group of thigh muscles of other rats. The material fixed in formol-saline and Bouin's fluid was processed in paraffin wax and sections (5–8 μm thick) were stained with haematoxylin and eosin, phosphotungstic acid-haematoxylin (PTAH), Heidenhain's haematoxylin and Van Gieson. The material in osmium tetroxide and glutaraldehyde was embedded in Araldite. Sections were cut at 1–2 μm on a Huxley microtome for phase-contrast study and at 50–80 nm for electron microscopy on an LKB Ultrotome. Sections were contrasted with uranyl acetate and lead citrate, 10 min. in each, and studied under the Siemens Elmiskop I at magnifications of $\times$2000–40,000.

TABLE I

Details of seventeen rhabdomyosarcomas in female Sprague-Dawley rats

Tumour no.	Animal no.	Injected material	Induction period (days)	Type of rhabdomyoblast	Histological type	Comment
1	RH 1/12	Ni_3S_2	205	III	II	
2	RH 1/16	Ni_3S_2	157	III	III	
3	RH 2/1	Ni_3S_2	174	III	II	Secondaries in regional lymph-glands
4	RH 2/2	Ni_3S_2	229	III	III	
5	RH 2/10	Ni_3S_2	195	III	III	
6	RH 2/12	Ni sponge	223	I	I	Embryonal sarcoma
7	RH 7/3	Ni_3S_2	157	III	III	
8	RH 16/1	Ni sponge	150	III	III	Necrotic
9	RH 17/1	Ni_3S_2	160	II	II	Necrotic
10	RH 17/2	Ni_3S_2	182	III	III	
11	RH 17/3	Ni_3S_2	182	II	I	Non-striated embryonal rhabdomyosarcoma
12	RH 18/1	Ni sponge	125	III	III	
13	RH 28/3	Ni sponge	240	III	III	
14	RH 30/1	Ni_3S_2	190	III	III	Multiple secondaries
15	RH 32/1	Ni_3S_2	224	III	III	
16	RH 35/1	Ni sponge	224	III	III	
17	RH 35/2	Ni sponge	238	III	III	

RESULTS

Seventeen out of fifty-five treated rats developed primary neoplasms (table I): 11 out of 30 animals after an injection of nickel sulphide (Ni_3S_2), 6 out of 25 animals after an injection of pure nickel. The average period between the injection of nickel and the first appearance of the tumour was *c.* 190 days (range 125–240 days). By this time the effect of any injury sustained during the injection had disappeared and the tumour could be felt as a hard lump about the size of a small pea in the musculature; usually a further 4–5 wk elapsed before the animal was killed. Macroscopically these tumours have a fairly well-defined capsule and display a lobulated structure of the tumour tissue, which tends to bulge when cut as though under considerable pressure. The

EM STUDY OF EXPERIMENTAL RHABDOMYOSARCOMA

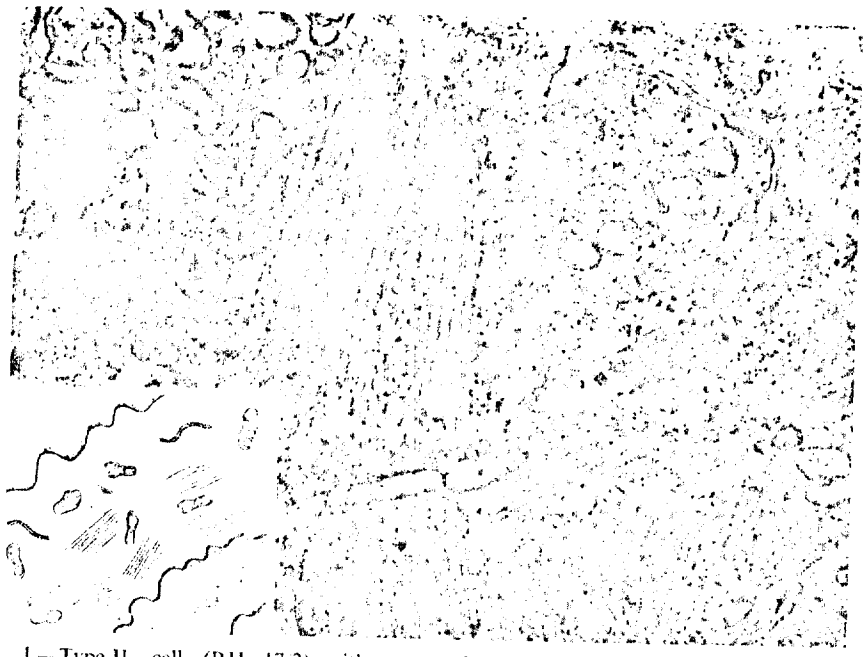

FIG. 1.—Type-II cell (RH 17/2) with scattered groups of short myofilaments, myosin (10–12 nm) and actin (5–7 nm). ×40,000.

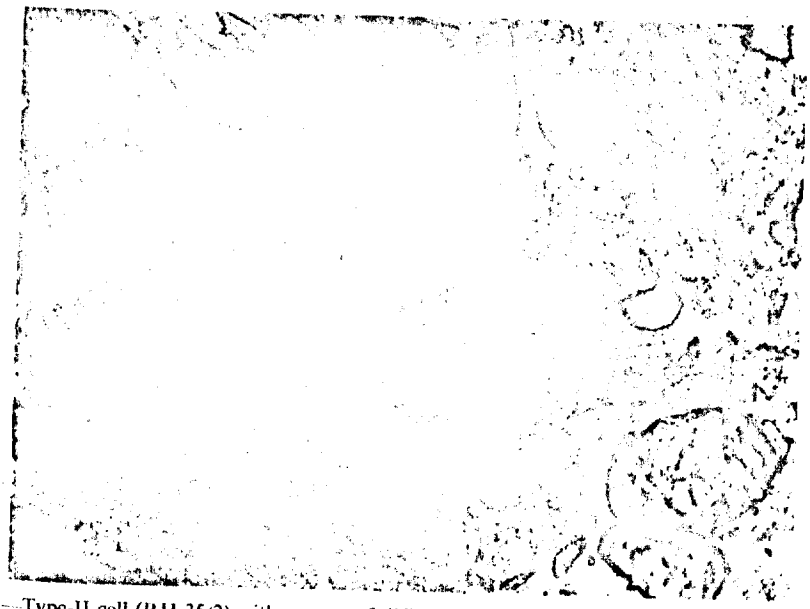

FIG. 2.—Type-II cell (RH 35/2) with groups of differentiating myofilaments, some cut across. Note swollen mitochondria with disrupted cristae. ×20,000.

EM STUDY OF EXPERIMENTAL RHABDOMYOSARCOMA

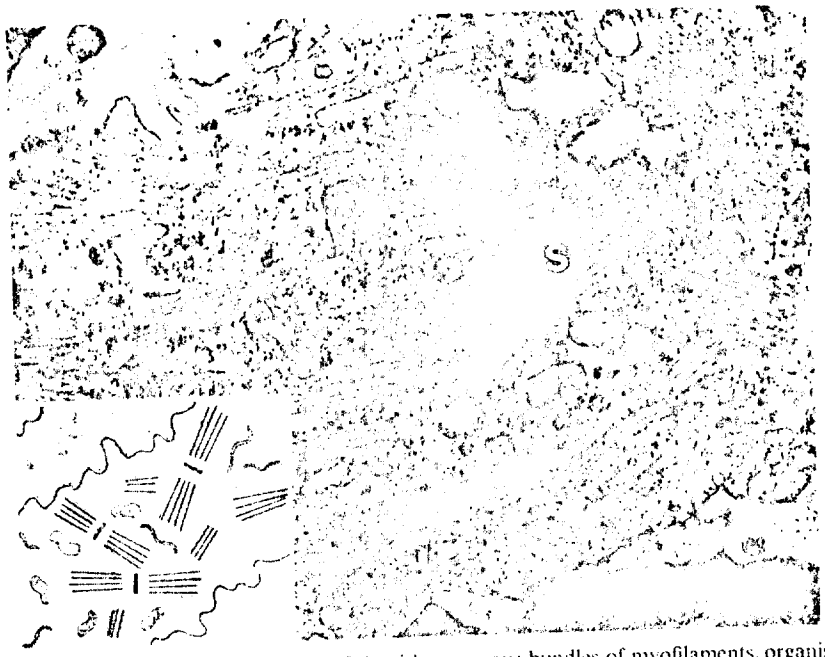

FIG. 3.—Cytoplasm of type-III cell (RH 18 1) with numerous bundles of myofilaments, organising to form sarcomeres (S) in the centre, with M-band and H-band, but no Z-bands. × 31,500.

FIG. 4.—Type-III cell (RH 2 1) showing myofilaments and dark irregularly disrupted Z-bands. × 30,000.

EM STUDY OF EXPERIMENTAL RHABDOMYOSARCOMA

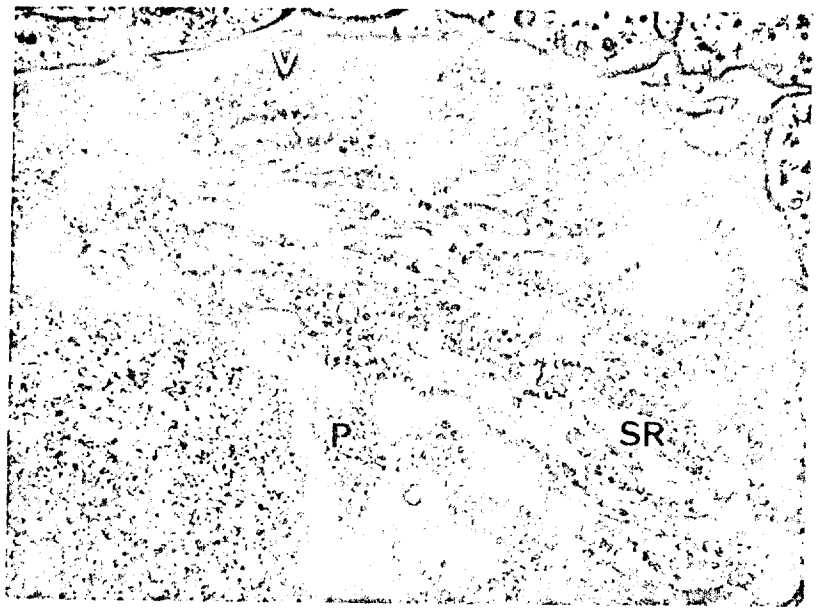

FIG. 5.—Part of type-IV cell (RH 32 1) with nucleus and cytoplasm containing myofilaments and scattered dark patches of Z-band material. Note polyribosomes (P), pinocytic vacuoles (V) and branching sarcoplasmic reticulum (SR). ×20,000.

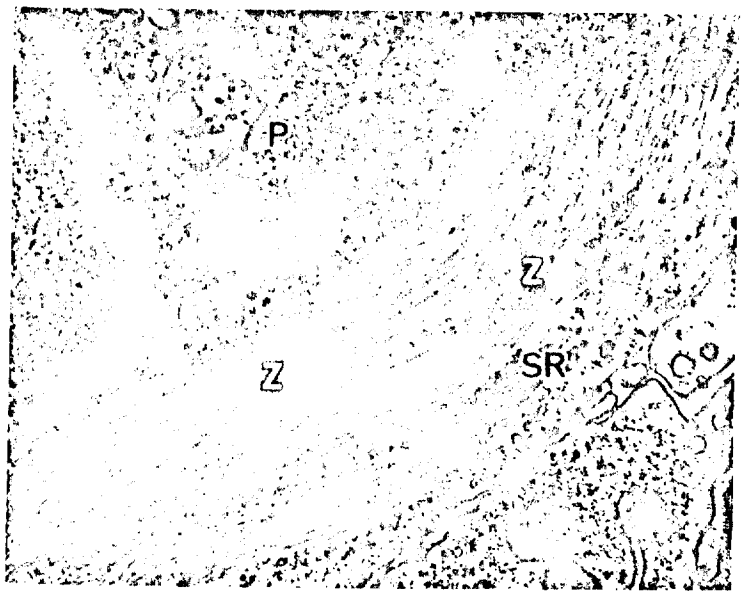

FIG. 6.—Part of cell (type-IV as in fig. 5) showing well-differentiated myofibrils running parallel to the surface of nucleus and cell. Note irregular Z-bands (Z) and tubules of sarcoplasmic reticulum (SR). ×20,000.

central portion is often somewhat degenerated and cells taken from this area show fatty degeneration or some necrosis. The tumour is usually highly vascular and during removal may bleed excessively from the large veins running over its surface. In one or two instances sudden increase in the size of the tumour was due to haemorrhage within it.

There were secondary deposits in only two rats. One of these rats showed multiple secondary deposits in the lungs and mediastinal lymph-nodes (RH 30/1) and the second a more regional or local spread to the inguinal lymph-nodes and into the abdominal cavity (RH 2/1) (table I).

The tumours were studied by optical microscopy, phase-contrast microscopy and electron microscopy.

Histology

With the optical microscope it is of course possible to examine a large area of the tumour and assess the general pattern and the variability of structure within each tumour.

The sections stained with Heidenhain's haematoxylin and Van Gieson are helpful in showing cross-striation and in distinguishing normal muscle and collagenous tissue from the tumour tissue. Any staining method, however, that has a stage where the stain is differentiated may lead to a certain patchiness of the cross-striation, and though such a technique is excellent for demonstrating normal muscle fibres, it leaves much to be desired when applied to tumour tissue.

Sections stained with PTAH show a less patchy appearance, but although it was possible to pick out longitudinal or circular fibrils, it was often more difficult to find cross-striation in the tumour cells.

Sections 1–2 μm in thickness of osmium-fixed tissues were examined by phase contrast to provide a suitable area for electron microscopy, and have also proved useful in the histological classification of the tumours. The areas selected for electron microscopy were representative of the tumour as seen with the optical microscope, but it has often been necessary to cut a large number of blocks in order to reduce the sampling error.

As table II shows, most of the tumours were striated embryonal rhabdomyosarcomas.

TABLE II

Histological types of 17 experimental tumours (Ashton and Morgan's classification)

Histological type	Number of tumours
I—Embryonal sarcoma	2
II—Non-striated embryonal rhabdomyosarcoma	3
III—Striated embryonal rhabdomyosarcoma	12

The growth is usually composed of round or spindle cells embedded in an oedematous fibrous stroma. The characteristic feature lies in the differentiation of the embryonal myoblasts which, through various stages, develop into long muscle fibres displaying the characteristic striation; the striated cells may be localised in certain areas of the tumour. It is difficult to follow the various stages of differentiation with the optical microscope, or to ascertain the order of

development of the characteristic myofilaments and of their differentiation into myofibrils: for this purpose electron microscopy is essential.

Electron microscopy

According to the degree of differentiation it is possible to distinguish *four types of rhabdomyoblasts* by examination with the electron microscope.

Type I. Round or polygonal cells with small but irregularly shaped nuclei, only small amounts of endoplasmic reticulum, but large numbers of ribosomes usually grouped together to form polyribosomes scattered throughout the cytoplasm. Sometimes these cells may have a small number of myofilaments, of apparently no fixed length, localised in the perinuclear zone (fig. 8). The cells do not appear to have a basement-membrane. Tumours wholly composed of these cells would be classified as embryonal sarcomas.

Type II. Usually spindle-shaped or polygonal cells with large irregular nuclei, which contain actin and myosin filaments of standard length. The filaments, single or in loose bundles, are irregularly distributed in the cytoplasm or may form whorl-like foci of differentiation (figs. 1 and 2). There are large numbers of ribosomes in the cytoplasm. There are no Z-bands or other signs of myofibrillar differentiation. Tumours composed mainly of such cells would be classified as non-striated embryonal rhabdomyosarcoma.

Type III. Large cells {strap cells, tadpole cells, spider cells (Cappell, 1964) under the optical microscope} contain both actin and myosin filaments of standard length arranged as sheaves or forming sarcomeres irregularly distributed in the cytoplasm (fig. 3). Although myosin filaments are present there is only an occasional M-band to be seen. Z-band material, on the other hand, may occur in some abundance irregularly distributed in the cytoplasm or attached to bundles of myofilaments (fig. 4). The nuclei are large and of irregular size and shape. Mitochondria are swollen and show loss of cristae. Tumours composed of these cells would be classified as non-striated embryonal rhabdomyosarcoma.

Type IV. Large cells of varying size and shape and containing organised actin and myosin filaments arranged to form sarcomeres which link up to form myofibrils. The Z-bands are prominent but are positioned at various levels so that they do not seem to form continuous bands with the Z-bands of neighbouring myofibrils. There are also M-bands and the lighter H-bands, and some of these cells may closely resemble normal muscle fibres, but can usually be distinguished from them by their irregular and bizarre nuclei. They also have a well-defined basement-membrane (figs. 5–7). Tumours containing such cells will show striation under the optical microscope: striated embryonal rhabdomyosarcoma.

Fig. 8 in contrast shows the bland features of undifferentiated cells of anaplastic embryonal sarcomas, which are less common in experimentally produced malignant tumours of muscle tissue, but more common in transplanted neoplasms.

A less familiar structure or organelle was observed in the differentiated rhabdomyoblasts of some tumours, consisting of a system of tubules and cisternae forming a regular latticework amidst the differentiated myofibrils. In agreement with Hagopian and Spiro (1967) we have called this structure the " fenestrated sarcoplasmic reticulum " (figs. 9–12), which is discussed below.

The various stages of differentiation of the rhabdomyoblasts may be described as follows: slender filaments localised in the perinuclear zone appear in the early undifferentiated mesenchymatous cells. Subsequently short myofilaments develop, are irregularly distributed in the cytoplasm and have a tendency to be arranged in foci or bundles. On further differentiation sarcomeres are formed, which link up to form myofibrils showing, on further maturation, the characteristic pattern of Z-bands and M-bands, although not as regularly as in normal muscle.

Undifferentiated cells contain abundant supplies of ribosomes often in groups as polyribosomes constituting the storage protein from which myofilaments are formed. The darkly stained Z-band material appears at a later stage and seems to be formed at any point of the cytoplasm before it is incorporated between two sarcomeres.

The arrangement and distribution of the myofibrils are relevant to the demonstration of cross-striation with the optical microscope. Thus, cells with myofibrils running longitudinally and parallel to the surface of the cell and nucleus might be more readily recognised than cells of type II or III containing incomplete myofibrils or cells in which the myofibrils are distributed at random.

Discussion

In May 1932 a Parliamentary question drew attention to a number of cases of nasal cancer that had occurred among workers in a nickel refinery at Clydach in South Wales. It was later demonstrated (Hueper, 1955) that it was possible to produce malignant mesenchymatous tumours in rats and rabbits by the injection of nickel and other heavy metals and that the sulphides of nickel and cobalt tended to be more sarcogenic than the oxides of these metals (Gilman, 1962). Heath and Daniel (1964) suggested that it was nickel rather than the sulphide radical that was the cause. It seemed to make little difference, except perhaps in time, whether the nickel compound was introduced into the muscle by direct injection, by inclusion in perfusion chambers or as chips or discs (Gilman and Herchen, 1963).

In our series 55 female Sprague-Dawley rats were given 0·2 mg of nickel sulphide or nickel sponge intramuscularly, and 17 rhabdomyosarcomas developed at the site of injection, eleven after nickel sulphide and six after nickel sponge. There was no appreciable macroscopic or microscopic difference between the tumours produced by either method; the tumours had a fairly well-defined capsule and lobulated structure.

With the electron microscope four types of cells could be distinguished according to the presence and distribution in the cell of actin and myosin filaments and their organisation into sarcomeres and myofibrils. Most tumours

were composed of more than one type of cell; this follows closely the findings of Corbeil (1967) in tissue culture.

The important point is that the electron micrographs provide proof of the presence of actin filaments (*c.* 5–7 nm), myosin filaments (*c.* 10–12 nm), and also of Z-band material—regularly or irregularly distributed and orientated. In this way electron microscopy is useful in the diagnosis and classification of malignant mesenchymatous tumours.

The development of organised myofilaments in experimental rhabdomyosarcoma accords with the theory now generally favoured about the origin of these tumours from mesenchymatous tissue, which has retained the potential of differentiation to striped muscle.

An electron-microscope study of developing chick skeletal muscle by Fischman (1967) has focused our attention on the organisation of myofibrillar proteins into characteristic, cross-striated myofibrils. His observations on the developing leg muscle of 12-day-old chick embryos compare well with our findings in neoplastic cells of experimental rhabdomyosarcoma.

The undifferentiated cells contain an abundance of scattered ribosomes, which provide the storage protein for the formation of the actin and myosin filaments. These in turn become organised into bundles and sarcomeres leading ultimately to the formation of myofibrils. It is interesting that in a study of the ultrastructure of the frog muscle spindle, Andersson-Cedergren and Karlsson (1967) have seen myofilaments in close contact with ribosome aggregates.

An unusual structure or organelle, the fenestrated sarcoplasmic reticulum, merits attention. It is composed of a system of tubules and cisternae and was described in detail by Hagopian and Spiro (1967) in the femoral muscle of the cockroach in which it forms a fenestrated envelope on each myofibril. Newcomb (1967) described a similar structure, which he called the " tubular complex ", in the root tip cells of *Phaseolus vulgaris* L., the French or kidney bean. Both structures resemble the " fenestrated sarcoplasmic reticulum " we have found in differentiated rhabdomyoblasts of experimentally produced rhabdomyosarcoma.

It is not clear at present whether such structures may be found only under special conditions, e.g., where protein storage occurs (Newcomb). In other words, the structures might be associated with protein synthesis and play a role in the accumulation, storage and utilisation of protein in plant cells or in muscle tissue. No such structures, however, seem to have been observed in the developing normal muscle (Fischman). This structure or organelle obviously merits further investigation.

Regenerative or degenerative processes may occur in the rhabdomyoblast at any stage of its differentiation: in a few transplanted tumours we found considerable regenerative and degenerative changes leading to a breakdown of the myofibrils and myofilaments into ill-defined filamentous structures.

The myofibrils first lost their striation and broke up into smaller portions. They lost their parallel orientation and were dispersed as single filaments running through the cytoplasm. Subsequently with the break-up of the filaments the rhabdomyoblasts became indistinguishable from other undifferentiated cells.

An oncological result of our primarily morphological studies may be of some interest. The incidence of tumours was comparatively low: about 31 per cent. against 70–71 per cent. in hooded rats and 80 per cent. in Fischer rats (Gilman, 1962; Gilman and Ruckerbauer, 1962). Payne (1964) found that Bethesda rats were somewhat resistant to the carcinogenic action of intramuscularly implanted nickel sulphide. The final proportion of animals that developed tumours was higher than in our series, 74 per cent., but the latent period was longer than that for the hooded, Fischer and Sprague-Dawley rats: the first tumours in Payne's experiments were produced after 15 mth, the average latent period being 17·3 mth against only 6–7 mth in our series. Daniel (1966) compared the responses of rats of the hooded, Fischer and Bethesda Black strains to intramuscular implants of powdered nickel sulphide and found that the Bethesda strain is more resistant than the hooded to the carcinogenic action of this compound: 14 out of 23 as against 28 out of 28. It is suggested by Daniel that this might be due to the development at the site of injection of large masses of phagocytic cells, which engulfed the powder without undergoing obvious damage. Thus the concentration of nickel compounds available for action on the muscle tissue is reduced to a subcarcinogenic level by the intracellular localisation of the compound and by its transport to the regional lymph-glands.

The sex of the animal does not seem to make any difference. We have studied only female rats, but there seems to be no reason to suggest that the apparent resistance of the Sprague-Dawley rat to the carcinogenic effect of nickel is sex-linked.

Comparative investigations of different strains are in progress together with a comparative electron-microscope study of the effect of other heavy metals (in co-operation with Mr J. C. Heath, Strangeways Research Laboratory) with the object of elucidating some of the interesting problems encountered in the course of the present investigations.

Summary

We have studied the ultrastructure of seventeen rhabdomyosarcomas produced in female Sprague-Dawley rats by the intramuscular injection of nickel or nickel sulphide. Details are given of a cytological classification of the rhabdomyoblast into four types, based on the absence or presence of actin and myosin filaments, their distribution and ultimate organisation into sarcomeres and myofibrils.

An unusual structure or organelle, the fenestrated sarcoplasmic reticulum, is described; it has been previously noted in some plants and the synchronous muscles of insects. It forms a regular lattice-work of tubules and cisternae enveloping the myofibril and may play some role in the synthesis of protein and of the myofilaments. The observations reported may assist in the diagnosis and classification of rhabdomyosarcoma in man.

A comparative resistance of Sprague-Dawley rats for the oncogenic activity of nickel has been noted.

The work has been aided by grants from the British Empire Cancer Campaign for Research and from the Central Research Fund of The University of London which are gratefully acknowledged. Thanks are due to Mr G. A. Jolly for technical assistance and to Mrs J. A. Hooker for secretarial help.

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Addendum

Since this paper was accepted for publication, Ishikawa (1968) has described the formation of elaborate networks of T-system tubules in 11-day chick embryo breast-muscle cells cultured for 1–11 days; some of the illustrations resemble those of the fenestrated sarcoplasmic reticulum in the present paper.

An electron-microscope study of an embryonal rhabdomyosarcoma has been reported by Toker (1968), and our attention has been drawn to a paper by Rubin and Davison (1962) on three cases of rhabdomyosarcoma of the ear.

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BIOCHIMICA ET BIOPHYSICA ACTA 163(3):331-338(1968)

BBA 75190

THE MECHANISM OF THE PARTIAL INHIBITION OF FERMENTATION IN YEAST BY NICKEL IONS

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(Received June 27th, 1968)

SUMMARY

Nickel inhibits fermentation following its transport into a non-exchangeable compartment in the cell. Extracellular pH influences the inhibition by affecting the transport. Once inside the cell, the Ni^{2+} inhibition can be modified by a number of factors such as flushing with gas, the presence of alcohol and acetaldehyde, the glucose concentration and the presence of O_2. These modifications can be explained on the assumption that the predominant effect of Ni^{2+} is to inhibit the enzyme, alcohol dehydrogenase. Changes in absorption at 345 mμ (related to the NADH level) support this suggestion. Purified alcohol dehydrogenase is highly sensitive to Ni^{2+}.

INTRODUCTION

Many heavy metal cations can inhibit the fermentation of glucose by yeast cells[1] but several different mechanisms may be involved. For example, uranyl ion inhibits by blocking the transport of sugar into the cell[2]. The inhibitory sites, in this case, are phosphoryl and carboxyl groups located on the outer surface of the cell, presumably on the plasma membrane. Virtually all of the uranyl ion taken up by the cell is bound to these sites, and it can be competitively displaced by other extracellular cations resulting in a complete reversal of the inhibitory effect. In contrast, metals reactive with sulfhydryl groups, such as mercury, silver and copper, penetrate the membrane rapidly. They not only interfere with membrane functions but react with internal sulfhydryl groups resulting in inhibition of many enzymic functions[3].

Among the metal ions that have been investigated, Ni^{2+} is of special interest because it inhibits at relatively low concentrations, but only a part of the fermentation (usually about 60%) can be blocked even at exceedingly high concentrations of the metal[1,4,5]. Because Ni^{2+} can competitively displace UO_2^{2+} from the membrane sites of yeast, it has been assumed that the two cations inhibit by the same mechanism, a blockage of sugar transport, but that Ni^{2+} produces only a partial inhibition because it introduces a smaller configurational change at the membrane sites[6].

Recently it has been demonstrated that in addition to binding to surface groups, Ni^{2+} can be transported into the cell[7]. This finding suggests that inhibitory effects of

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the metal may be due to an inhibition of glycolytic enzymes within the cell rather than of sugar transport systems on the outer surface of the membrane. In the present study, exposure of the cells to Ni^{2+} was followed by appropriate washing to remove surface-bound metal. The inhibitory effects reported are therefore related only to Ni^{2+} that was transported into a non-exchangeable pool. The predominant effect of Ni^{2+} on metabolism seems to be due to inhibition of the enzyme, alcohol dehydrogenase.

METHODS

Commercial fresh bakers' yeast (Standard Brands, Inc.) was starved under aeration for 12 h and washed three times with distilled water. The washed cells were stored at 4° and used for experiments for up to 6 days. Before each experiment the were again washed 3 times and the amount of cells was measured by a cytocrit method using Wintrobe tubes. No correction was made for interstitial water (amounting to about 22 %) trapped between the packed cells[8].

Divalent cations taken up by yeast cells can be divided into two fractions. The first represents cations bound to anion sites on the surface of the cell. This component is exchangeable and can be rapidly displaced by adding competing cations or, in the case of radioactive isotopes, by adding non-radioactive isotopes of the same metal[9]. The second represents cations that penetrate the membrane or are transported into the cell. This component can be displaced only very slowly, if at all, by other cations or by exchange of non-radioactive isotope of the same cation[10]. Nickel binds reversibly to the surface sites[6] but the present paper is concerned only with the effect of Ni^{2+} that has been transported into the interior of the cell as a non-exchangeable component[7]. In many experiments, therefore, the cells were allowed to take up Ni^{2+} and then washed free of external Ni^{2+} before the effects on metabolism were determined.

The CO_2 and O_2 consumption were determined by standard Warburg manometry. No buffers were used because they form complexes with Ni^{2+}. Parallel systems were set up to follow the changes in pH. In non-Warburg experiments in which Ni^{2+} uptake and sugar consumption were measured, the pH was maintained at a constant level with a pH-Stat (Radiometer) using NaOH. The small amounts of Na^+ added had no effect on Ni^{2+} uptake or on metabolism.

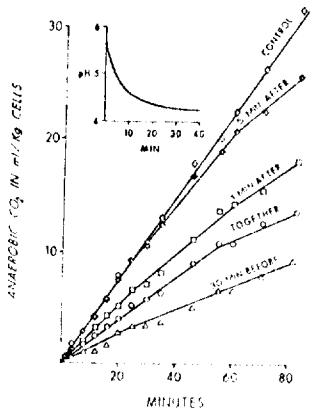
Glucose was determined by the glucose oxidase method as modified by WASHKO AND RICE[11].

Alcohol dehydrogenase from yeast was purchased from Boehringer Mannheim Company. Enzymatic activity was determined by measuring the decrease in light absorption of NADH at 366 mμ after addition of acetaldehyde[12]. The decrease in absorption was recorded on a double-beam spectrophotometer (Unicam).

RESULTS

Nickel added to yeast under anaerobic conditions with glucose as substrate caused a partial inhibition of CO_2 production but the magnitude of this effect was dependent on the time and order of addition of nickel and of glucose (Fig. 1). The inhibition was greatest (70 %) when the cells were exposed to Ni^{2+} for 30 min before

glucose was added and minimal when the Ni^{2+} was added 5 min after the glucose. In the latter case no inhibition was evident until after 40 min. A possible contributing factor to this behavior is the change in the pH of the medium that occurs after the addition of glucose (insert of Fig. 1). The pH fell from an initial value of 5.6 to 4.3 in 30 min. The reduction in pH did not influence the rate of CO_2 production in the control (Fig. 1) but it had a remarkable effect on the inhibition by Ni^{2+} (Fig. 2).



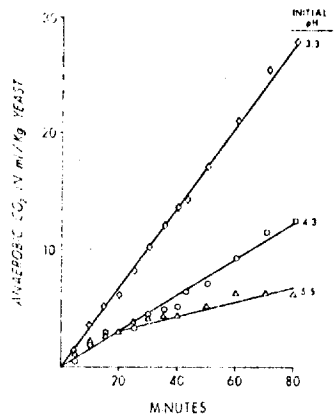


Fig. 1. Influence of the time of addition of nickel and of glucose on anaerobic CO_2 production. Inset: change in pH on addition of glucose. 5 mg yeast per flask, 5 mM glucose, 0.5 mM $NiCl_2$, gas phase N_2, temperature 28.2°.

Fig. 2. Influence of initial pH of the medium on CO_2 production in the presence of nickel. 5 mg yeast per flask, 5 mM glucose, 0.5 mM $NiCl_2$, gas phase N_2, temperature 28.2°. Initial pH adjusted to 3.3, 4.3 and 5.5 with diluted HCl.

At pH 5.5 the inhibition was considerable (compare with control in Fig. 1), but at pH 4.3, the inhibition was significantly reduced, and at pH 3.3 the rate of fermentation was virtually the same as that in a control without Ni^{2+}. A similar dependence on pH was also found by measuring the effects of Ni^{2+} on glucose utilization, measured in the same experiment.

If Ni^{2+} inhibits by blocking sugar transport at the cell membrane, then the variations demonstrated in Figs. 1 and 2 would presumably be due to differences in the amount of Ni^{2+} binding to the ligands of the cell surface. Even if the Ni^{2+} concentration was raised to 100 mM (compared to 0.5 mM used in the experiment of Fig. 2), no inhibition was evident at pH 3.3. Yet the surface groups would presumably be saturated at such high concentrations[6]. The other possible explanation is based on the pH dependence of Ni^{2+} transport, which shows marked inhibition at pH 3.3 (ref. 7). To differentiate clearly between the effects of surface-bound Ni^{2+} and transported Ni^{2+}, advantage was taken of the observation that pretreatment of yeast with phosphate *plus* glucose leads to a remarkably enhanced transport of divalent cations[10] including Ni^{2+} (ref. 7). During pretreatment the cells absorbed Ni^{2+} and they were then washed free of external Ni^{2+} before testing for inhibitory effects at different values of pH. The inhibition was about 70 % and it was not influenced by an external pH even as low as 2.5 (Fig. 3). These results are in sharp contrast to those of the experiment in which the exposure to Ni^{2+} occurred at the different pH's (Fig. 2). It

seems evident, therefore, that the inhibitory sites themselves are not accessible to extracellular pH, but that the process by which Ni^{2+} reaches the sites, the transport, is the pH-dependent process.

The first insight into the mechanism of fermentation inhibition by non-exchangeable Ni^{2+} came from an unexpected observation. Anaerobic sugar consumption of Ni^{2+}-treated cells was measured in a parallel experiment in a Warburg apparatus and in an erlenmeyer flask, the latter being used in order to take repeated samples.

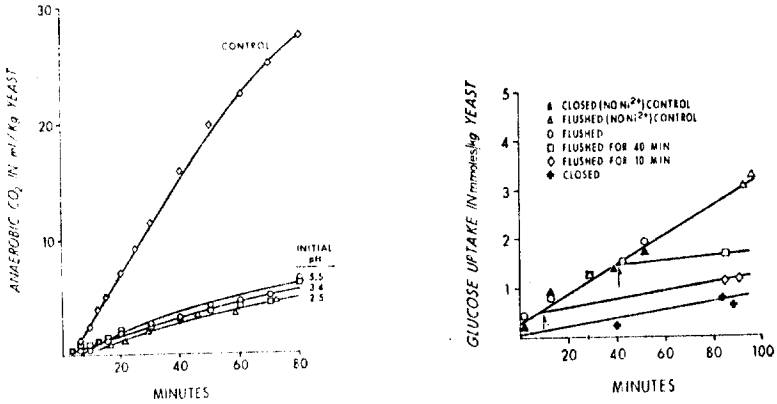


Fig. 3. The effect of pH on the CO_2 production of cells preloaded with nickel. Preloading conditions: 1% yeast, 10 mM KH_2PO_4, 0.1 mM glucose, 5 mM $NiCl_2$, 1 h, 37°. Washing procedure: 2 times distilled water, 2 times 0.1 mM succinic–tartaric acid buffer (pH 5) and again 2 times distilled water. The starting pH was adjusted with diluted HCl to 2.5, 3.4 and 5.5. 10 mg yeast per flask, 5 mM glucose, gas phase N_2, temperature 28.2°.

Fig. 4. Effect of flushing the flask with N_2 on the inhibition of glucose uptake by Ni^{2+}. Yeast, 5 mg per flask; 0.5 mM glucose, 0.5 mM $NiCl_2$, temperature 28.2°. Arrows show time at which flushing ceased and the vessels were closed.

No inhibition was observed in the erlenmeyer but the usual 60% inhibition was observed in the Warburg flask. The only difference was the means of maintaining anaerobiosis. The Warburg flask was filled with N_2 prior to the experiment and then closed, whereas the erlenmeyer flask was flushed continuously with N_2. The effect was not due to impurities in the gas because substitution of CO_2 for N_2 gave the same result. Furthermore, the same reversal of inhibition could be demonstrated by flushing with air.

An experiment demonstrating the reversal of inhibition due to flushing with gas (N_2) is shown in Fig. 4. Warburg flasks were used and sugar consumption was measured, each point representing one flask. In the absence of Ni^{2+}, the rate of sugar consumption was the same in flasks that were initially filled with N_2 and then closed just before sugar was added and in flasks in which N_2 flow was maintained continuously. In the Ni^{2+}-treated cells, however, large differences were observed. In the closed flask the inhibition was 73%, whereas in the flasks with continuous N_2 flow, no significant inhibition was observed. In flasks that were flushed for 10–40 min after the addition of glucose, the inhibition apparently started soon after the flow of N_2 was halted.

The effect of flushing cannot be attributed to differences in the uptake of Ni^{2+}

because the inhibition reappeared as soon as the flushing was terminated. It therefore seemed probable that the reversal of inhibition was due to removal of a volatile product of metabolism. Three products of fermentation are volatile: CO_2, acetaldehyde and ethanol. CO_2 seemed unlikely because the phenomenon of reversal was the same with pure N_2 or with pure CO_2. On the other hand, acetaldehyde and ethanol had large effects on the inhibition by Ni^{2+} (Fig. 5). Neither substance at the concentrations used had much effect in the absence of Ni^{2+}, but in its presence, acetaldehyde almost

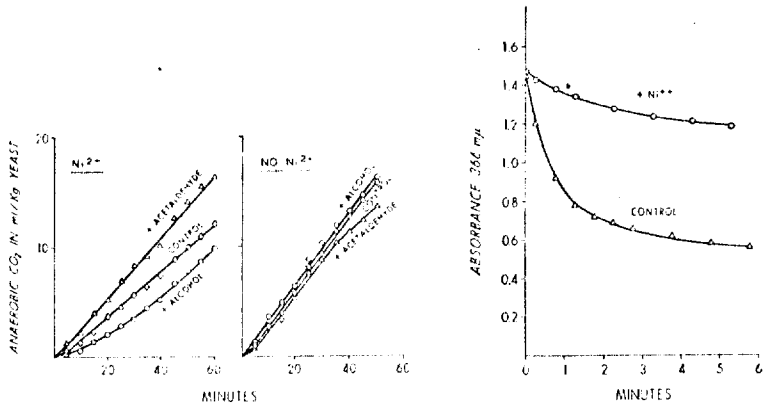


Fig. 5. Influence of alcohol or acetaldehyde on the CO_2 production of yeast cells preloaded with nickel. 5 mg yeast per flask, 5 mM glucose, 0.1% alcohol or acetaldehyde, gas phase N_2, temperature 28.2°. Preloading conditions: 0.5% yeast, 5 mM glucose, 1 mM $NiCl_2$, 1 h, 37°.

Fig. 6. Effect of Ni^{2+} on the enzymatic activity of alcohol dehydrogenase from yeast determined by the decrease in absorbance of NADH at 366 mμ. 1.5 ml 100 mM Tris (pH 8.6), 0.02 ml alcohol dehydrogenase (0.3 mg/ml), 0.08 ml NADH (20 mg/ml), 0.02 ml acetaldehyde 0.5%, 0.3 ml 5 mM $NiCl_2$, water to 3.9 ml.

reversed the inhibition, whereas alcohol intensified the inhibition. It seemed likely, therefore, that the effect of flushing with gas might be due to the continuous removal of alcohol.

The reciprocal effects of acetaldehyde and alcohol upon the Ni^{2+} inhibition suggested that the site of action was the enzyme, alcohol dehydrogenase which catalyzes the equilibrium between the two substrates. Indeed, the purified enzyme is very susceptible to inhibition by Ni^{2+} (Fig. 6). At 0.5 mM (the same concentration used in the experiments on cells), the inhibition was about 85%. Unfortunately, however, such results indicate only that the enzyme is a potential target for Ni^{2+} in the intact cell but not that it is the target or that other enzymes might not also be targets.

Further evidence for the localization of the Ni^{2+} effect was obtained by studies at different glucose concentrations under aerobic conditions. With low glucose concentrations, virtually all of the pyruvate produced by the glycolytic chain feeds into the Krebs cycle *via* pyruvate oxidase. Oxidation *via* this pathway is complete so that the R.Q. is 1.0. If, however, the glucose concentration is high, the production of pyruvate exceeds the capacity of the oxidase. It "spills over" into the pyruvate decarboxylase reaction leading to the production of "extra" CO_2 (R.Q. is above 1.0)

and of alcohol. The latter substance accumulates because oxidation *via* the two-carbon pathway cannot keep pace with production[13,14].

In view of the above properties of the yeast metabolic systems, the assumption that Ni^{2+} inhibits alcohol dehydrogenase leads to the prediction that aerobic metabolism of glucose should be insensitive to Ni^{2+}, provided the substrate concentration is sufficiently low that the R.Q. is 1.0. On the other hand, aerobic metabolism should be sensitive to Ni^{2+} only at glucose concentrations sufficiently high to produce "spillover" through the pyruvate decarboxylase and alcohol dehydrogenase (R.Q. over 1.0). Indeed, the predicted results were experimentally verified (Fig. 7). With a glucose concentration of 1 mM, the CO_2 production and O_2 consumption were equal (R.Q. = 1.0) and Ni^{2+} had no inhibitory effect. With a glucose concentration of 5 mM, CO_2 production was increased to a greater extent than the O_2 consumption (R.Q. 1.2). Both the "extra" CO_2 and "extra" O_2 were inhibited by Ni^{2+} so that the levels of CO_2 production and O_2 consumption were reduced to equality at a rate equal to that found at 1 mM glucose. Thus only the "spillover" through decarboxylase and alcohol dehydrogenase was inhibited by Ni^{2+}.

Because of the site of inhibition of Ni^{2+}, the degree of inhibition depends on the conditions and method of measurement. For example, from the data of Fig. 7, the inhibition of O_2 consumption or CO_2 production is essentially zero at 1 mM glucose, but at 5 mM glucose the inhibition of O_2 consumption is 59 %, that of CO_2 production is 67 %, and that of aerobic fermentation is virtually 100 %. On the other hand, the inhibition of anaerobic fermentation was 25 % at 1 mM glucose and 76 % at 5 mM glucose in an experiment parallel to that of Fig. 7.

Oxidation of NADH to NAD^+ is associated with the alcohol dehydrogenase reaction. The levels of NADH and of NAD^+ that are bound by the 1,3-glyceraldehyde phosphate dehydrogenase can be measured by spectrophotometric methods using extinction at 345 mμ. In yeast cells changes in the levels of NADH associated with activities of alcohol dehydrogenase can be determined[15–19]. After uptake of Ni^{2+}, the

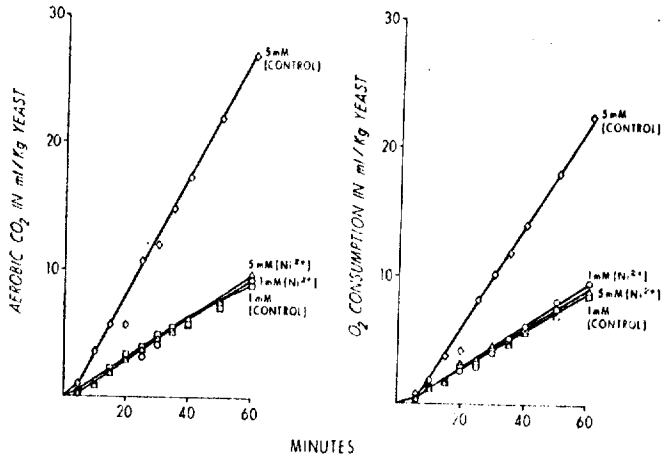


Fig. 7. CO_2 production and O_2 consumption of yeast cells loaded with nickel as in Fig. 3 at high and low glucose concentrations. 5 mg yeast per flask, 1 mM and 5 mM glucose, gas phase air, temperature 28.2°.

normal oscillations at 345 mμ upon addition of glucose are eliminated and the rise in extinction is delayed. The final value of the extinction at 345 mμ is considerably higher than in the control suggesting an elevated level of NADH. These results support the suggestion that Ni^{2+} inhibits at the level of alcohol dehydrogenase, reducing the rate of oxidation of NADH.

DISCUSSION

The inhibitory effects of Ni^{2+} reported in this paper concern transport of the metal into the cell[7] rather than its binding to surface anions[6]. Thus the effects are observed in cells preloaded with Ni^{2+} after the surface-bound Ni^{2+} has been washed away. In such preloaded cells, the inhibition is independent of extracellular pH but it is dependent on other factors such as flushing with gas (N_2, CO_2, or air), the addition of alcohol and acetaldehyde, the presence of O_2, and the concentration of glucose. The pattern can be readily understood on the basis that Ni^{2+} inhibits the enzyme, alcohol dehydrogenase, thereby reducing the supply of NAD^+, essential for the phosphoglyceraldehyde dehydrogenase reaction. Flushing would remove alcohol, thereby allowing the reaction acetaldehyde-to-alcohol to proceed more rapidly. This would increase the supply of NAD^+ and increase the rate of fermentation. The same effect is achieved by the addition of acetaldehyde. Addition of alcohol, on the other hand, would back up the reaction, reduce the production of NAD^+, and increase the inhibition.

In the presence of O_2, alcohol dehydrogenase is not involved in sugar metabolism provided the concentration of substrate is low. As expected, Ni^{2+} has no inhibitory effect. When the sugar concentration is raised to a higher level so that "spillover" through the decarboxylase reaction occurs along with the formation of alcohol[13,14], then Ni^{2+} inhibits the extra metabolism associated with the "spillover". These experiments indicate that none of the glycolytic reactions between glucose and pyruvate and none of the decarboxylic acid cycle and respiratory reactions are inhibited by Ni^{2+}. They do point to the alcoholic dehydrogenase as the most likely site. Changes in the level of NADH in cells loaded with Ni^{2+} also are consistent with this mechanism. Finally, purified alcohol dehydrogenase is very sensitive to Ni^{2+}.

Ni^{2+} has been of considerable interest among the metal ions because, even in high concentrations, it only partially inhibits glucose metabolism[1] and glucose uptake[5,6]. An explanation has been presented in terms of configurational changes in the binding groups (phosphoryl groups) of the outer surface of the cell membrane[6]. On the basis of the present study, another explanation is given based on transport into the cell and subsequent inhibition of the enzyme, alcohol dehydrogenase. Even the complete inhibition of this enzyme would allow CO_2 production and glucose uptake to proceed at a reduced rate *via* the third mode of fermentation[20] with NAD^+ produced by the conversion of dihydroxyacetone phosphate to α-glycerophosphate by the enzyme glycerophosphate dehydrogenase with glycerol as one of the final end products.

ACKNOWLEDGEMENTS

This work was supported in part by the U.S. Atomic Energy Commission under contract with The University of Rochester Atomic Energy Project and has been assigned Report No. UR-49-957.

While at The University of Rochester, the senior author was supported during this study by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft, Bad Godesberg, Germany.

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Proc. Can. Cancer Res. Conf.
6: 209-23 (1964)

Muscle Tumourigenesis*

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PRIMARY rhabdomyosarcomas involving adult skeletal muscle are considered rare in experimental animals and in man,[33] particularly if incidence is compared with the proportion of total body tissue that is of muscle origin.[26] However, the actual frequency with which rhabdomyosarcomas occur is probably in excess of that indicated in the literature, because unequivocable cross striations in redifferentiating rather than in residual muscle cells, are often not demonstrable in immature types of myoblastic tumours.[2, 21] Furthermore, until recently the alveolar type of rhabdomyosarcoma must frequently have been classified as either a carcinoma or reticulum cell sarcoma.[5, 18, 28]

In experimental oncogenesis, rhabdomyosarcomas appear relatively frequently amongst sarcomas resulting from exposure of skeletal muscle to various tumourigenic stimuli, including polycylic hydrocarbons,[29, 31] X-ray followed by trauma[13] and implantation with certain compounds of cobalt, cadmium and nickel.[6, 16, 17]

Properties of Metal Carcinogens

Rats are generally noted for their susceptibility to the induction of a wide variety of sarcomas through chemical and physical means[30] and particularly for the readiness with which they develop various types of mesenchymal tumours on exposure to many relatively inert macromolecular substances.[1, 23] Such sarcomas generally range in incidence between 25 per cent and 45 per cent and develop from the connective tissue encapsulating the implant, following a prolonged latent period.[25] Carcinogenic metallurgical compounds show a marked affinity to striated muscle, inducing a preponderance of highly malignant rhabdomyosarcomas in the rat. The length of the latent period involved varies with both the compound and strain of rat, but tends to be considerably shorter than with the so-called "physical carcinogens".[8] Tissue preference, high tumour incidence and a relatively short latent period make certain metal compounds most useful experimental tools for studies on the mechanism of muscle tumourigenesis; a value enhanced by their chemical simplicity, low solubility and local action. The induction of rhabdomyosarcomas with N_3S_2 in millipore diffusion chambers† has demonstrated that

* This study was supported in part by a grant in aid of research from the National Cancer Institute of Canada.

† Two millipore T.W. filters (11 mm diam.; 0.45μ $\pm$ 0.02 pore size) separated by a lucite ring.

direct contact between carcinogenic metal particles and cells is not essential to muscle tumourigenesis. Likewise the physical shape or size of metal implant does not appear to affect either the rate of induction or the proportion of muscle tumours induced[8] (Table 1).

TABLE 1. *Effect of Physical Shape of Implant on Tumour Response*

Type of implant	Tumour rats / Rats on exp.	Average latent period (days)	Tumour distribution Rhabdo.	Fibro.	No histol.
Diffusion Chamber + 10 mg Ni_3S_2	14/17	305*	9	3	2
Ni_3S_2 Disc (11 mm diam.)	14/17	148	12	2	—
Ni_3S_2 Chips (0.3 – 0.5 mm)	5/7	149	4	—	1
Ni_3S_2 Powder – 10 mg (5μ size)	20/20	167	15	2	3
Ni_3S_2 Powder – 10 mg (2μ size)	19/20	170	15	3	1
Diffusion Chamber (Empty)	1/19*	460*	—	1	—
Fe_2O_3 Disc (11 mm diam.)	0/20	—	—	—	—

* Significant difference (1% level).

Species and Strain Differences

In the rat, nickel, cobalt and cadmium have all been reported to exhibit selective preference for striated muscle cells during the processes of local tumourigenesis, with nickel compounds being particularly consistent in this regard.[7, 16, 17, 24]

Different strains of rats show considerable variation in their response to tumourigenic nickel compounds, particularly in regard to length of latent period, while species differences in response are even more marked. Thus, in mice, nickel compounds induce relatively few sarcomas that are clearly of striated-muscle origin; the majority are either fibrosarcomas or poorly differentiated mesenchymal tumours of uncertain histological class (Figs. 1 and 2). Table 2 shows NiO and Ni_3S_2 to be equally tumourigenic to mice, while the former compound (NiO) is far less active in rats. Cobalt fails to induce tumours at all in mice[7, 14] while on the basis of our limited trials, the rabbit appears to be refractory to Ni_3S_2 tumourigenesis. However, Hueper[22] has reported the induction of fibrosarcomas in rabbits with powdered metalic nickel. We have

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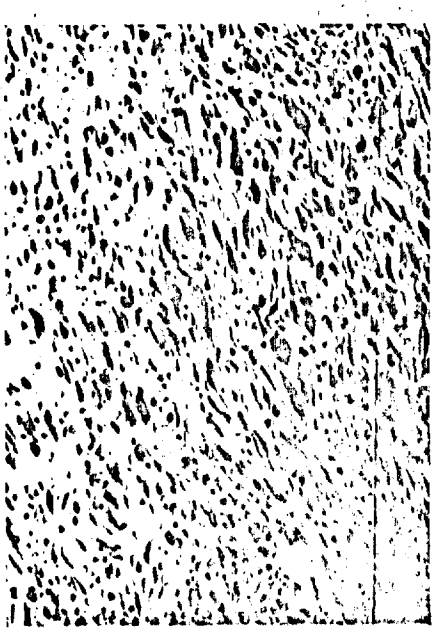
TABLE 2. *Tumour Response to Different Nickel Compounds Within and Between Species*

Species	Comp.	No. on Expt.	% Tum. Bearers	A.L.P.* (days)	A.P.T.† (days)	Tumour Distribution				
						Rhabdo.	Mes. T.‡	Fibro.	Others§	No histol.
Mice (Swiss)	Ni_3S_2	45	60	251	132	2	3	14	2	6
	NiO	50	66	287	78	5	7	16	1	4
Rats (Wistar)	Ni_3S_2	30	83	150	55	19	2	0	1	3
	NiO	32	65	302	83	14	2	4	0	2

* A.L.P. = Average of Latent Periods in days from time of implantation to appearance of first tumour in each tumour bearing rat.
† A.P.T. = Average of Progression Times in days from appearance of first tumour until death of each tumour bearing rat.
‡ Mes. T. = Mesenchymal Tumour—undifferentiated sarcoma of uncertain classification.
§ Others = Most frequently reticulum cell sarcomas (spontaneous mammary tumours were not included here).

observed a rhabdomyosarcoma in a cat following intramuscular implantation with Ni_3S_2, although the frequency with which this occurs has not yet been established.



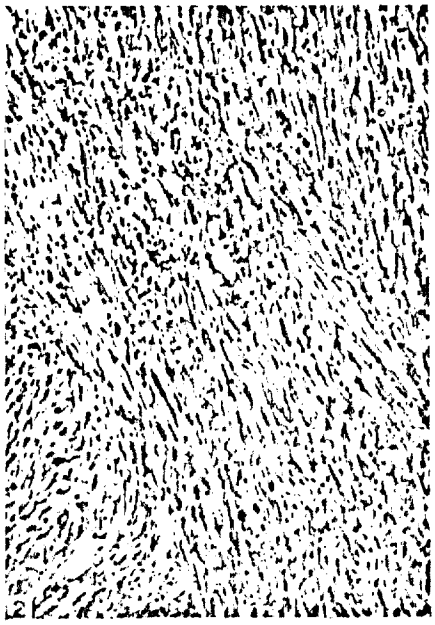


FIG. 1. Mesenchymal tumour of uncertain classification in mouse, induced by 5 mg intramuscular implantation of NiO.

FIG. 2. Rhabdomyosarcoma in mouse, induced by 5 mg intramuscular implantation of NiO.

Induction of Striated Muscle Tumours with Ni_3S_2

Ni_3S_2 being the most active metallurgical carcinogen for the rat regardless of strain[7, 27] has been widely used in our investigations on muscle tumourigenesis. In our experience, over 85 per cent of the primary tumours induced by intramuscular implantation of this compound are of muscle cell origin. The predominating tumour is a pleomorphic rhabdomyosarcoma, although immature and anaplastic types are fairly frequently encountered (Figs. 3, 4 and 5). Alveolar rhabdomyosarcomas have not been observed, with the possible exception of certain primary tumours of the omentum and messenteries.

Rhabdomyosarcoma incidence in Fischer rats increases in a linear fashion in response to graded single intramuscular implantations of powdered N_3S_2 of from 0.25 to 10 mg per site (Fig. A). At doses in excess of 10 mg per site the response curve levels off at approximately 80 per cent incidence. Latent period

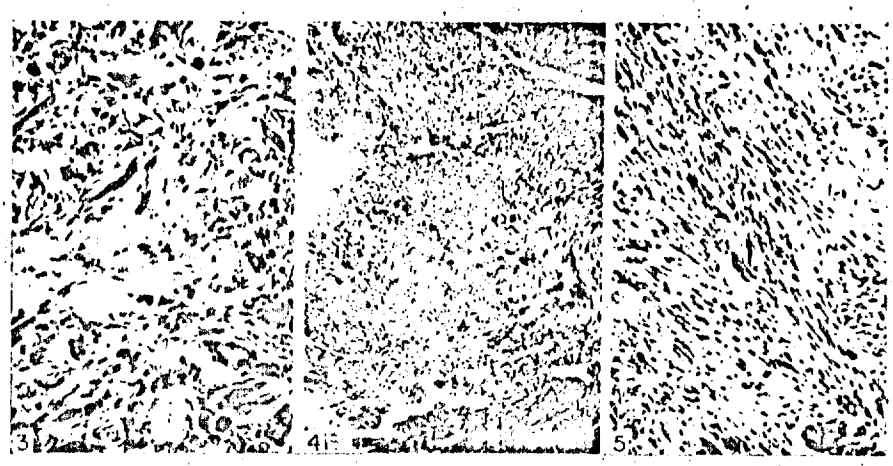


FIG. 3. Pleomorphic rhabdomyosarcoma in rat, showing numerous plasmodia and large strap cells with faint cross striations. Induced by 20 mg subcutaneous implant of Ni_3S_2.

FIG. 4. Immature rhabdomyosarcoma with preponderance of spindle-like cells. Induced by 10 mg intramuscular implant of Ni_3S_2.

FIG. 5. Area from tumour illustrated in Fig. 4 showing differentiation of more obvious myoblastic derivatives.

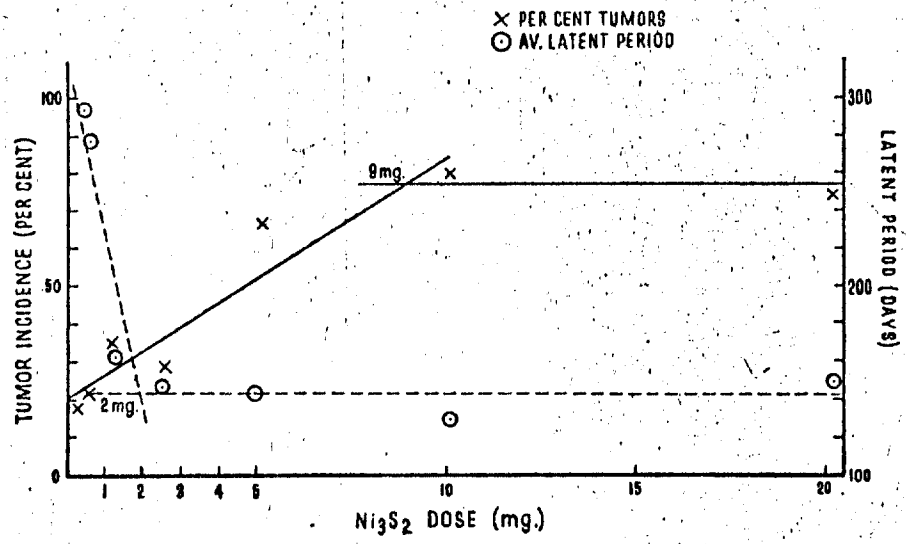


FIG. A. Effect of dose on tumour incidence and latent period. Intramuscular implantation of powdered nickel sulphide (Ni_3S_2) in Fischer rats.

varies inversely with the size of the implant dose; with minimum latency being achieved at about the 2 mg level, in contrast to the 8–10 mg dose needed for maximum incidence. Payne[27] has suggested that carcinogenicity appears to be a reflection of the solubility of the nickel compound used and that rat connective tissue is capable of distinguishing carcinogenic members of related compounds. Our own findings support the generality that the more soluble

TABLE 3. *Relative Solubility and Tumourigenic Activity of Several Compounds of Nickel*

Nickel compounds	Tumourigenic activity		
	Fischer rats (6 mo.+ exposure)		Bethesda B1 (22 mos.)
Relative order of solubility	No. tumours / Sites	Per cent tumour bearing rats	Tumour induct. per cent (Payne)*
Ni sulphate	0/54	0	0
fluoride	3/36	17	—
monosulphide	0/28	0	—
acetate	—	—	7
carbonate	—	—	40
hydroxide	19/40	75	—
oxide	2/40	10	8
sulphide	22/40	85	74

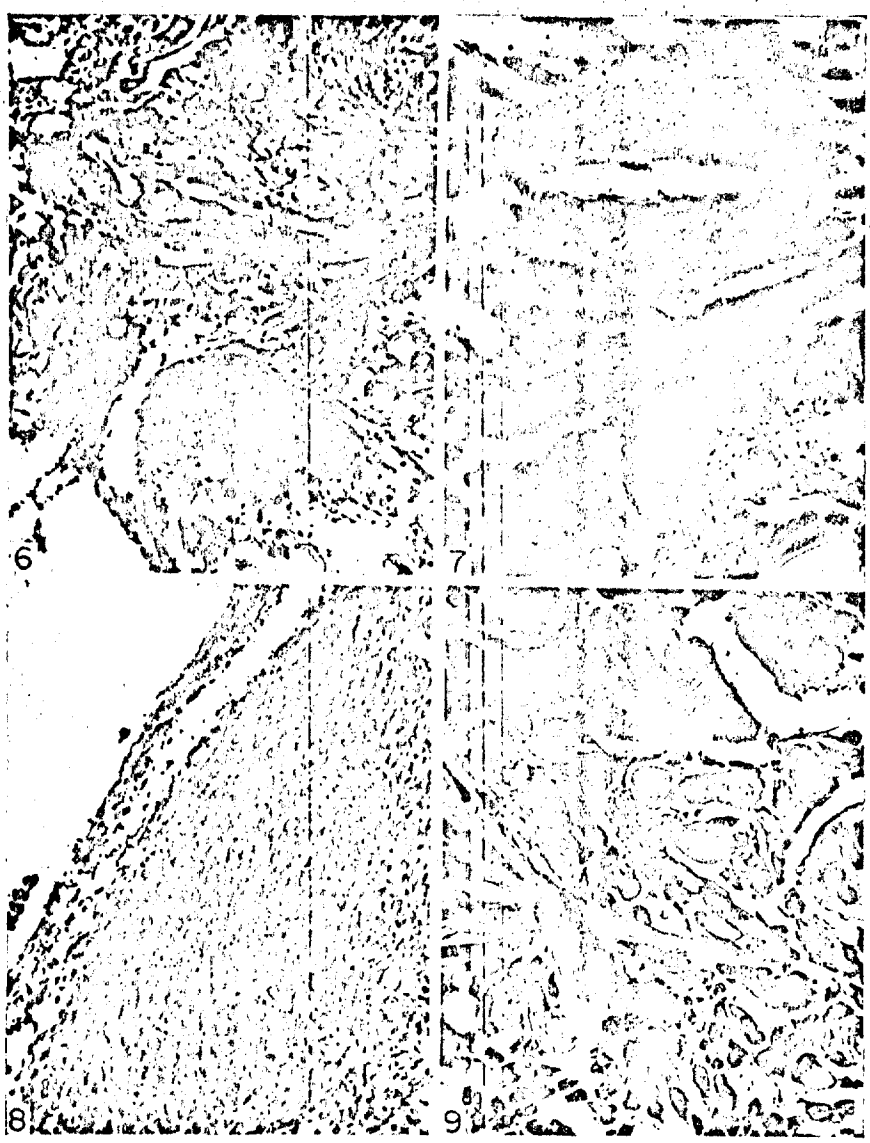
* W. W. Payne, 1964. *Proceedings 55th Ann. Meeting Amer. Assoc. Cancer Res.* (Abstr. No. 197).

W. W. Payne—personal communication.

the nickel compound the greater its toxicity and the less its carcinogenicity amongst the compounds of nickel examined (Table 3). It would seem probable that the metallic nickel in itself is the active carcinogenic agent[22] and that *in vitro* solubility of a particular nickel compound is only one of several indices of its availability *in vivo* for reaction with adjacent tissues and thus, possibly, of its tumour inducing capability.

Subcutaneous implantations of nickel sulphide have given rise to a preponderance of rhabdomyosarcomas, as have the limited number of implantations carried out at intra-abdominal sites (Table 4). Intraperitoneal implantations of powdered nickel sulphide may cause foreign body granulomas up to several months after injection as also may intrasplenic implants.

Nickel induced rhabdomyosarcomas metastasize freely via both the lymphatic and blood streams. Metastases, particularly those involving the lung, frequently contain tumour muscle cells in a more advanced stage of redifferentiation than in the primary tumour from which they originated (Figs. 6, 7, 8, 9).



FIGS. 6, 7. Low and high power views of a lung metastasis of a Ni_3S_2 induced primary rhabdomyosarcoma in a rat. Note high degree of redifferentiation that has occurred in the large muscle straps.

FIGS. 8, 9. Low and high power views of a typical lymph node metastasis of a Ni_3S_2-induced muscle tumour in a rat.

Behaviour of Muscle Tumour Cells on Transplantation and *in vitro*

Induced rhabdomyosarcomas are readily transplantable, cell suspensions in excess of 2000 cells being necessary for successful take. The one such transplant muscle tumour being maintained in this labbratory at present is in its 62nd transfer generation and presents a relatively uniform and anaplastic cell population composed of a majority of uni-nucleated myoblasts. These still exhibit a sufficient tendency towards syncytial and myotube formation to be recognizable as striated muscle derivatives. The transplant tumour is rapid growing, killing the host in approximately 3 weeks and its behaviour and morphology have undergone relatively little change since its early transplant generations. Polyploid nuclei are extremely numerous and the chromosome mode has remained relatively stable at between 54 and 56 since the 11th generation.* Many of the nuclei contain a large metacentric marker chromosome (Figs. 10, 11, 12, 13).

TABLE 4. *Effect of Implantation Site of Ni_3S_2 on Tumour Class and Incidence*

Implant site	No. tum. / Sites	Per cent T.	Tumour classification: Rhabdo.	Mes. T.	Fibro.	Other	No histol.
Intramuscular	32/40	80	25	2	2	0	3
Subcutaneous	28/64	44	16	4	3	1	4
Intraperitoneal	9/37[a]	24	8	1	0	0	0
Intrasplenic	4/20[b]	20	1	0	1	1	1

[a] 4 of the 9 animals developed muscle tumour involving abdominal wall and/or diaphragm.
[b] Experiment still in progress.

Cells from primary and transplant rhabdomyosarcomas have frequently been cultured in our laboratory for *in vitro* studies on the process of neoplastic transformation of mature striated muscle cells. Two such cell lines have been described following prolonged observation:[3] one became and remained predominantly diploid and uniformly fibroblastlike, exhibiting a constant directional growth pattern and a loss of malignancy. In contrast, the other muscle tumour cell line remained heteroploid, pleomorphic and of gradually increasing malignancy through serial subculture. Cells of the diploid strain tended to fuse, becoming multinucleated and fibrilar in old cultures (Fig. 14)—changes indicative of muscle cell redifferentiation *in vitro*.[4, 20]

Studies at present underway in this laboratory† indicate that myoblasts

* Unpublished data: P. K. Basrur, Ontario Veterinary College, Guelph, Canada.
† Unpublished data: L. B. Corbeil, Ontario Veterinary College, Guelph, Canada.

grown *in vitro* from nickel induced rhabdomyosarcomas, while showing definite differentiation, consistently fail to achieve either well developed cross striations or definite contractility. Comparisons between tumour culture myoblasts and those from normal neonatal rat tissues also show the latter to differentiate (apparently in large part through fusion) into multinucleated myotubes much more rapidly than the former. Orderly peripheral migration of nuclei fails to occur in the differentiating tumour muscle cells, which may also tend to remain

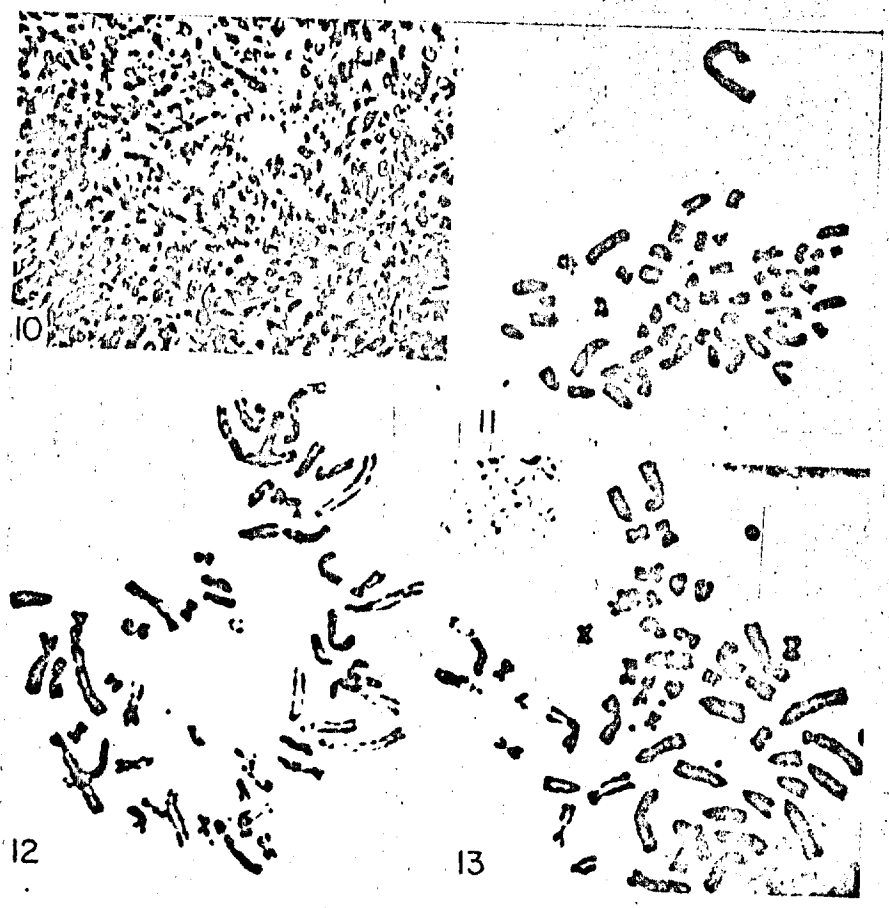


FIG. 10. Transplanted rat rhabdomyosarcoma, fifty-seventh generation. Note numerous heteroploid nuclei and preponderance of uninucleated myoblasts.

FIGS. 11, 12, 13. Nucleus from eleventh, thirty-second, and fifty-seventh transplant generations respectively, showing the modal chromosome complement of 54 through 56. Note presence of large metacentric marker chromosome in Figs. 11 and 12. (Courtesy of Dr. P. K. Basrur.)

FIG. 14. Composite illustration of three aged cultures from a diploid cell line (ALA), derived from a Ni_3S_2-induced rhabdomyosarcoma in a rat. Note the uniform-rowed nuclei, the indications of cross striations, and the lateral fusion of cells indicative of muscle differentiation *in vitro*.

smaller and irregular in outline. Mitotic activity is most frequent amongst the fibroblast-like uninucleate cells but also occurs in both normal and tumour multinucleated myotubes (Figs. 15, 16).

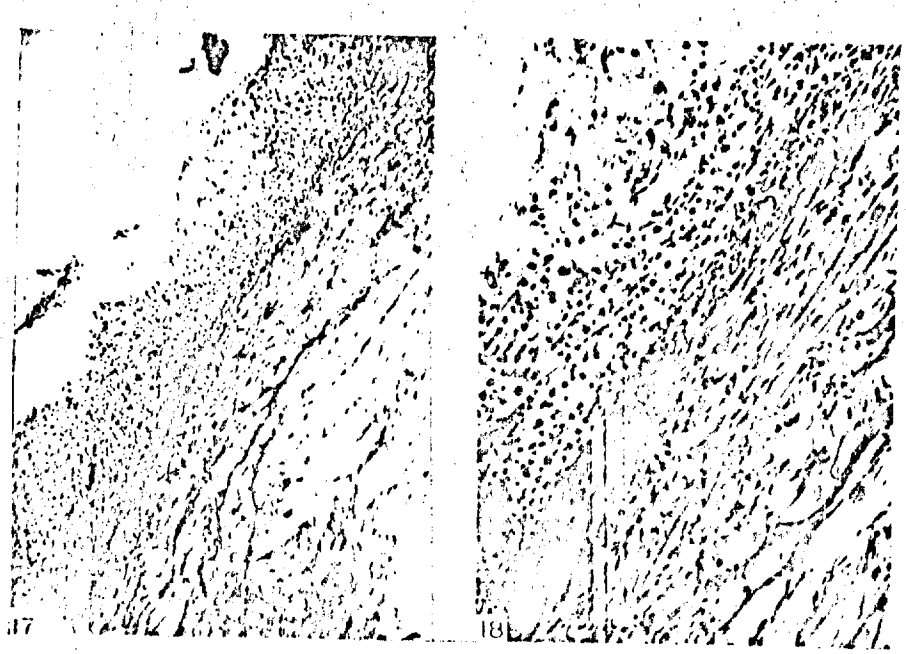
FIG. 15. Multinucleated myotubes from normal neonatal rat culture, showing mitosis. (Courtesy of Dr. L. B. Corbeil.)

FIG. 16. Multinucleated plasmodial-type cell from rhabdomyosarcoma culture, and myoblast, showing mitosis. (Courtesy of Dr. L. B. Corbeil.)

Histological and Cytological Changes During Muscle Tumourigenesis

That clones of viable tumour myoblasts may become established by the 9th week in exposed Fischer rat tissues has been shown by both nickel disc removal and transplantation experiments.[9, 19] Comparisons have been made between tissue changes adjacent to implanted discs of nickel sulphide and those resulting from similar implants of carcinogenically inactive Fe_2O_3.[18] The sequence of gross changes observed in these experiments have consistently shown the nickel compound to induce by far the more drastic degenerative and regenerative changes. The extensive and rapidly developed zone of necrosis, separating the degenerating muscle from the Ni implant, remains fairly constant throughout the induction period. Initial cell damage and eventual proliferation of

the surviving variants that have become resistant to the toxic effect of the chemical or physical carcinogen have been suggested as important factors in the process of neoplastic transformation.[33] In the induction of rhabdomyosarcomas with Ni_3S_2 malignancy is apparently associated with the acquisition



FIGS. 17, 18. Low and high power view of region adjacent to Ni_3S_2 disc-cavity, showing narrow zone of dedifferentiating muscle, extensive necrosis, and relative absence of viable-looking myoblasts.

of resistance by myoblasts derived from dedifferentiating muscle to the cytotoxic effects of Ni. New and mitotically active myoblasts become established at the expense of the necrotic zone and form the focus from which the tumour develops. Prior to the establishment of this "presarcomatous proliferation (approximately 8 weeks) the reaction zone consists of a narrow band of rather loosely arranged muscle fibres in varying stages of degeneration and dedifferentiation. Mitoses are essentially absent at this stage as also are the presence of newly formed viable-looking myoblasts (Figs. 17, 18). From approximately th 8th week on, the uni- and binucleated myoblast population increases steadily, although at first mitoses are infrequent. Subsequent increasing mitotic

FIGS. 19, 20. Low and high power view of a region adjacent to Ni_3S_2 disc-cavity showing "presarcomatous proliferation."

TABLE 5. *Effect of CaEDTA on Nickel Tumourigenesis*

	No. tum. Sites	(%)	ALP (days)	Survivors (23 mos.)
Ni_3S_2 discs (11 mm)	7/8	88	132	0
Ni + CaEDTA (1:9)	4/8	50	309	2
Ni + CaEDTA (1:19)	2/8	25	373	2
CaEDTA (control)	0/8	—	—	3

*Effect of Zinc on Cadmium Tumourigenesis**

	No. tum. Sites	(%)	Interstit. Cell T.	(%)
Cadmium	9/22	41	21/24	88*
Cadmium + Zinc	2/17	12	3/17	18*

* Gunn, S. H., *et al.* 1964. *Proc. Soc. Exptl. Biol. Med.*, **115**, p. 653.

activity is associated with the syncytial formation and attempted redifferentiation characteristic of the early tumour (Figs. 19, 20).

The mechanism involved in tumour induction by metal compounds and their apparent preference for muscle in the rat is not known. It is possible that in the case of nickel tumourigenesis, the mechanism may be similar to that postulated for cadmium, involving the substitution of that metal for zinc in one or more growth regulating enzyme systems.[11] It has been demonstrated that both cobalt and cadmium inhibit the enzymatic oxidation of α ketoglutaric acid[15] and that the keto acids are generally elevated in neoplastic tissues.[10]

Our preliminary findings on the effect of CaEDTA on nickel sulphide tumourigenesis indicate that blocking the availability of the metal by chelation considerably inhibited tumourigenesis (Table 5). Gunn and co-workers[11, 12] have shown that in the rat, systemic flooding with zinc has a similar inhibitary effect both on local and remote tumourigenesis by cadmium.

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J. Wildlife Manage
32(3) 483-8 (1968)

RELATIVE TOXICITY OF LEAD AND FIVE PROPOSED SUBSTITUTE SHOT TYPES TO PEN-REARED MALLARDS

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Abstract: A 30-day toxicity test was made to determine the relative toxicity of lead, a tin–lead alloy, zinc, nickel, teflon-coated steel, and tin, all in shot form, to pen-reared mallard drakes. All of the 15 ducks dosed with lead died. Twenty-seven percent of 15 dosed with alloy, and 20 percent of 15 dosed with zinc also died. Ten of the remaining zinc-dosed ducks showed signs of distress, including losses of muscular control and body weight. There were no deaths among 15 ducks dosed with nickel, 15 dosed with teflon-coated steel, and 15 dosed with tin. Seventy-three percent of those dosed with nickel shot eliminated all shot before the end of the 30-day period. Acid-fast intranuclear inclusion bodies were present in the kidneys of mallards dosed with commercial lead shot, or with tin–lead alloy shot, but not in the kidneys of birds given nickel, tin, or teflon-coated steel shot. Atypical, pale, acid-fast bodies were found in kidneys of 1 of 15 birds dosed with zinc. An iron-containing pigment, which stained positive with the Prussian blue technique, was present in variable amounts in almost all livers. Zinc-dosed ducks that died or were killed while still showing signs of zinc intoxication had higher iron levels in the liver than ducks that had recovered from zinc intoxication.

Mortality of waterfowl due to lead poisoning has been recognized for many years. Despite several research studies (Green and Dowdell 1936, Dowdell and Green 1937, and Jordan and Bellrose 1950) no satisfactory substitute for the presently used commercial lead shot has been developed. In 1965 the Patuxent Wildlife Research Center began a research project aimed at the development of a non-toxic shot. In 1966 the Sporting Arms and Ammunition Manufacturers' Institute and the Patuxent Wildlife Research Center undertook this study as a cooperative research project.

Metals or alloys proposed as possible substitutes for lead are first tested for their acute toxicity to pen-raised mallards. Candidate materials found to be non-toxic are then tested for possible effects on reproduction, for ballistical properties, and for killing power.

The results of the first group of candidate materials tested were reported by Irby et al. 1967, and Locke et al. 1967.

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This paper reports the results of the acute toxicity tests for another group of candidate materials, and is part of a series of studies designed to develop a non-toxic shotgun pellet.

We acknowledge the assistance of John L. Sincock, Ralph Andrews, Larry T. Young, and Robert G. Heath, all of the Patuxent Wildlife Research Center.

MATERIALS AND METHODS

The birds tested were 18-month-old mallard drakes (*Anas platyrhynchos*) reared at the Patuxent Wildlife Research Center. One hundred and five birds were included in the experiment. For 9 months prior to the study they were kept together in a single pen and fed a mixture of commercial duck pellets and mixed grains. During the study they were kept in groups of five in 21 pens 30 × 15 × 6 ft high and offered cracked corn, quartz, and oystershell grit ad libitum.

Each pen contained a feeding trough and a watering trough at ground level, with flowing water continually maintained at a depth of 10 inches.

The following shot types were tested:

Shot Type	*Source*
Commercial lead shot	Winchester-Western
Alloy (60% tin, 40% lead)	National Lead Co.
Zinc	National Lead Co.
92% nickel, 5.5% silicon, 1.7% iron, 0.8% trace elements	Canadian Wildlife Service
Teflon-coated steel	Wheelabrator Corp.
Pure tin	National Lead Co.

Ducks were arbitrarily separated into 21 groups of five ducks each. Each duck was weighed, and then each one, except those in the control groups, was dosed with eight number 6 shot through a flexible plastic tube forced down the gullet to the proventriculus. Three groups of five ducks, 15 birds, were on each test.

Birds dosed with commercial lead shot or zinc shot were weighed and fluoroscoped after 1 week. The controls were also weighed after 1 week, but were not fluoroscoped at that time. These and all other test ducks were weighed and fluoroscoped 2 weeks after the initiation of the study. The experiment was terminated 30 days after dosage and all survivors were killed at that time.

Analysis of variance, in conjunction with Duncan's new multiple range test (Duncan 1955), was used to compare the percentages of mortality associated with the different treatments. The weight changes of the dosed ducks were compared with those of the control birds by use of the t test. Throughout the experiment differences were considered to be real at the 0.05 level of significance.

All test birds were necropsied and selected tissues were retained for chemical analysis and histopathological examination. Tissues selected for microscopic examination were fixed in 4 percent formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, Ziehl-Neelsen acid-fast, or Prussian blue. Unfixed tissues selected for chemical analysis were ashed in a furnace at 550 C. The ash was then dissolved in nitric acid, heated on a hot plate to near dryness, then diluted to an appropriate volume with dilute hydrochloric acid. The Perkin-Elmer atomic absorption spectrophotometer, Model 303, was used for all determinations. Instrument settings were essentially those recommended in the Analytical Methods for Atomic Absorption Spectrophotometry (Perkin-Elmer Corp. 1966).

RESULTS

Gross Effects of Various Shot Types

An analysis of variance, in conjunction with Duncan's new multiple range test, showed that the ducks dosed with commercial lead shot suffered significantly greater mortality than those dosed with any of the five proposed substitutes (Table 1). The ducks dosed with the tin–lead alloy, and those dosed with zinc, were found to have suffered significantly greater mortality than the ducks dosed with the tin, 92 percent nickel, or with teflon-coated steel shot. The difference between the mortality of the ducks dosed with zinc and that of the ducks dosed with the tin–lead alloy was not significant.

Lead.—All 15 of the mallards dosed with commercial lead died within 15 days (Table 1). The time between dosage and death averaged 8 days. These ducks had lost an average of 22 percent of their body weight at death and had retained 5 to 8 shot (average 7.2).

Alloy.—Four of the 15 dosed with tin–lead alloy (60 percent tin, 40 percent lead) died within 30 days. These four lived an average of 21 days after dosage and lost an average of 36 percent of their body weight before death. The 11 survivors lost an average of 14 percent. Weight loss differences be-

Table 1. Mortality and weight losses among mallard drakes during 30 days after force-feeding six types of shot.*

Shot Type	Mortality Among Three 5-Bird Replicates			Percentage Mortality	Post-Dosage Days to Death		Percentages of Weight Loss			
							Dead Birds		Survivors	
					Avg.	Range	Avg.	SD	Avg.	SD
Commercial lead	5	5	5	100	8	5–15	22‡	6.97	—	—
Alloy (60% tin—40% lead)	2	1	1	27†	21	8–30	36‡	14.26	14	9.22
Zinc	2	1	0	20	20	14–28	33‡	11.80	22‡	12.23
92% nickel, 5.5% silicon, 1.7% iron, 0.8% trace elements	0	0	0	0	—	—	—	—	20‡	11.85
Teflon-coated steel	0	0	0	0	—	—	—	—	16	9.10
Pure tin	0	0	0	0	—	—	—	—	11	8.33
Control	0	0	0	0	—	—	—	—	8	11.85

* Fifteen birds on each treatment; each bird given 8 shot of type specified.
† Vertical lines include sets of averages not separable at the 0.05 level of significance.
‡ Significantly different from the controls ($P < 0.05$).

tween survivors and control birds were not statistically significant.

The four that died had retained 1 to 7 shot, an average of 5.2. The one bird that died on the 30th day of the study contained one shot that was worn to a minute fragment. Seven of the survivors had passed all shot before the end of 2 weeks, and three had passed all but one. One survivor retained four shot throughout the study.

Zinc.—Three of 15 mallards dosed with zinc shot died within 30 days. The average length of time from dosage to death was 20 days, and the average weight loss was 33 percent. The average weight loss among survivors was 22 percent, an amount significantly greater than that of the controls.

Two of the 3 mallards that died and 10 of the 12 survivors developed signs of zinc intoxication before the end of the 30-day test period.

Signs of zinc intoxication appeared in the zinc-dosed birds in the following chronological order:

1. Stumbling while running.
2. Stumbling while walking and an inability to run.
3. Loss of the ability to walk without falling; however, retention of the ability to swim.
4. Virtually complete loss of muscular control of the legs, including loss of the ability to swim. Also, loss of the ability to move the wings normally.
5. Total loss of muscular control of legs and presence of spasmodic wing movements. Lack of the ability to fold the wings at the sides. Mouth held open as if panting.

Only three mallards dosed with zinc retained shot until the end of the study. One of these retained five shot but had shown none of the signs of zinc intoxication. The second retained two shot at the end of the study and still showed signs of intoxication. The third duck retained one shot. This bird showed signs of zinc intoxication for 5 days during the study, but was fully recovered at the end of the study.

One of the three that died during the study retained seven shot at death but showed no signs of zinc intoxication. This bird died 17 days after dosage. Each of the other two that died retained six shot at death and both exhibited signs of zinc

Table 2. Concentration of iron (ppm wet weight) in livers of ducks treated with eight shot of various types.*

Commercial Lead†	Tin-Lead Alloy	Tin	Nickel	Teflon-Coated Steel	Control
4,319	1,088	1,740	1,703	1,131	954
3,325	1,432	2,780	1,976	1,036	950
2,607	1,064	762	1,472	1,586	1,153
3,771	1,306	1,494	3,050	1,460	2,921
3,215	1,228	2,414	2,054	1,615	1,210

* Results of iron concentrations in zinc-dosed ducks in Table 3.
† All lead-treated ducks were dead at end of test; all others were alive.

intoxication. One died at 14 days after dosage and the other at 28 days.

Nickel.—None of the birds dosed with nickel shot died during the study although they lost an average of 20 percent of their body weight. Weight losses of these ducks were significantly greater than those of control birds. Four of 15 retained 4 to 7 shot (average 5.7) until the conclusion of the study.

Teflon-coated Steel.—There was no mortality among mallards dosed with teflon-coated steel, but they lost an average of 16 percent of their body weight. This was not significantly different from the 8 percent loss of the control birds. Three ducks retained shot (one to eight) until necropsy. In the bird containing eight shot, all pellets were quite small and granular in appearance.

Tin.—Although mallards dosed with tin shot lost an average of 11 percent of their body weight, there was no mortality among them. This weight loss did not differ significantly from that of the control birds. Fluoroscopy showed that three birds on this dosage retained one to two shot at the end of the first 2 weeks, but no shot were present at necropsy.

Control.—None of the 15 control birds died during the course of this study; however, they lost an average of 8 percent of their body weight. This loss was less than that associated with any treatment.

Results of Histopathologic Examination

Acid-fast intranuclear inclusion bodies (Locke et al. 1966) were present in kidney tubular cells of all mallard drakes dosed with commercial lead shot or tin–lead alloy shot, but none was found in the kidneys of mallards dosed with nickel, teflon-coated steel, or tin, or in those of the control birds.

One of 15 mallards dosed with zinc had small atypical pink (weakly acid-fast) inclusion bodies in the nuclei of five renal tubular cells. In 100 consecutive oil immersion fields examined, these five cells were the only ones so affected. No acid-fast inclusion bodies were found in the kidneys of the other 14 mallards dosed with zinc.

Hemosiderosis of the liver was present in almost every mallard examined. In the control birds, the amount of hemosiderin ranged from accumulations of pigment within the Kupffer cells lining the sinusoids to a light pericanalicular sprinkling of granules within the hepatic cells. There was a marked deposit of hemosiderin within the hepatic cells of one control bird. Hemosiderosis was more pronounced in dosed birds than in control ducks.

Following the microscopic examination, five randomly selected livers from each treatment were analyzed for iron content (Table 2). Because of the wide range of iron levels in the livers of the zinc-dosed ducks, the livers of the 10 remaining zinc-dosed ducks were also analyzed for iron

Table 3. Concentration of iron (ppm wet weight) in the liver of each zinc-dosed duck.

DUCK NUMBER	CONDITION*†	IRON
9	N	813
10	N	1,168
4	N	1,460
3	R	1,653
12	R	203
13	R	1,269
14	R	1,823
8	R	1,381
7	R	2,817
6	R	1,929
5	S	7,514
2	S	7,860
1	D (28)	4,824
11	D (17)	5,821
15	D (14)	3,643

* R = Lived to end of study and recovered at that time.
D = Dead before end of study.
S = Survived to end of study but still showed signs of zinc intoxication.
N = Lived to end of study and never showed signs of zinc intoxication.
† Number of days to death in parentheses.

content. The results of the iron determinations for all 15 zinc-dosed ducks are summarized in Table 3.

DISCUSSION

Retention of Shot

All mallards that died during the experiment retained at least one shot until the time of death. At least one survivor dosed with tin–lead alloy, zinc, nickel, or teflon-coated steel contained shot. No birds dosed with tin contained shot at necropsy.

Nickel shot retained until the end of the study was in almost perfect condition. Therefore, it was concluded that all nickel shot not present at the end of 30 days must have been passed rather than eroded or dissolved. If this conclusion is correct, 73 percent of the birds that received nickel shot passed all their shot.

In contrast, teflon-coated steel shot retained throughout the study showed a considerable decrease in size. This decrease suggests that any teflon-coated steel shot not present could have been eroded, dissolved, or passed. Tin or tin–lead alloy also could have been eroded or dissolved because both are soft and chemically reactive.

Lead vs. Other Treatments

Tin–lead alloy shot was less acutely toxic than commercial lead shot. The assumption that a significant reduction in the quantity of lead contained in a shot should reduce mortality was substantiated because only 4 (27 percent) mallards dosed with tin–lead alloy shot died, whereas 15 (100 percent) dosed with commercial lead shot died.

Pure tin was non-toxic. In contrast, pure lead has been shown to be highly toxic (Jordan and Bellrose 1950:162). The amount of lead contained in eight tin–lead alloy shot (40 percent) is approximately equivalent to that contained in three pure lead shot. Jordan and Bellrose (1950:160) found that this amount of pure lead shot was fatal to only 1 of 10 adult male mallards captured and held for about a year prior to the initiation of their study. Since wild waterfowl containing shot in their gizzards usually contain three or fewer shot (Bellrose 1959:267), further testing of the tin–lead alloy at lower dosage levels might be justified.

Twelve of 15 ducks dosed with zinc showed signs of zinc intoxication. The muscular weakness associated with zinc intoxication would probably make the ducks highly susceptible to predation. Zinc has also been noted as highy toxic to aquatic animals (Skidmore 1964). Therefore, we feel that further consideration of zinc as a substitute for lead shot should be abandoned.

Dosages with 92 percent nickel, teflon-coated steel, and tin shot resulted in no mortality or adverse signs.

Histopathologic Examination

The rather constant occurrence of hepatic hemosiderosis in birds analyzed is at variance with the results of earlier toxicity studies, in which only mallards fed various types of lead or iron shot developed a marked hepatic hemosiderosis. In Locke et al. (1967) hemosiderosis was associated with the production of anemia in lead-poisoned mallards and with iron overloading of the liver in mallards dosed with iron shot.

The mechanism for the production of hepatic hemosiderosis in this study is not understood, but the presence of some degree of hemosiderosis in the livers of control mallards as well as in those of experimentally treated birds suggests that it was due to nutritional or seasonal factors.

Determinations of the iron level in the livers of zinc-dosed ducks indicated that those ducks that died or still showed signs of zinc intoxication at the end of the study had much higher iron concentrations than the control ducks. Iron concentrations in the livers of zinc-dosed ducks that had recovered were similar to the liver iron concentrations of controls. Underwood (1962:181) noted that rats dying of zinc poisoning had depressed iron levels in the liver. Our data on mallards differ from those obtained with rats, and indicate the need for further study of the mechanism of zinc toxicity to mallards.

Analysis of the zinc pellets showed that they were not, in fact, pure zinc, but contained 92 percent zinc, 0.16 percent lead, a trace of iron, and an additional 7 percent not determined. Perhaps the low trace of lead was responsible for the production of the atypical acid-fast intranuclear inclusions seen in the kidneys of 1 of 15 zinc-dosed mallards.

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Received for publication November 10, 1967.

Compt. Rend. Soc. Biol. 151;282-284, 1957

Siderotic Deposits During Chronic Poisoning of Rats from Cobalt and Nickel Salts

Work of G. Guillet, presented by M. Prenant

Since 1951, van Campenhout (1*) and his co-workers as well as Morelle (2*) have published their results concerning the elective toxic effects of cobalt and nickel salts on the pancreas of the guinea pig.

Besides these works, the effect of cobalt salts was especially of interest from the hematological viewpoint, these salts having been used in the treatment of certain anemias in man. Hopps, Stanley and Shideler (3*) studied the polycythemic effect of cobalt on the spleen and bone marrow of the rat.

Also, it seemed opportune to us to relate the facts relative to siderosis observed in various organs of the rat, observations made for a general histophysiologic study of poisoning from cobalt and nickel salts. This accumulation of iron deposits in the organism of poisoned animals does not seem to have been the object of a systematic study. Voigt (4*) and his co-workers in 1954 summarily pointed this out and without insisting on the mobilization of iron in the organism during acute intoxication from cobalt.

The experiments were done on adult albino rats, Wistar strain. Each animal received 2 mg daily of cobalt or nickel chloride by the intraperitoneal

(1*) E. van Campenhout, G. Cornelis and Th. Deulin, Ann. Endocrinol., 1954, 15:p. 89.

(2*) V. Morelle, C.R. Soc. Biol., 1953, 147, p. 1649.

(3*) H.C. Hopps, A.J. Stanley, A.M. Shideler, Amer. J. Clin. Path., 1954, t. 24, p. 1374.

(4*) G.E. Voigt, R. Bog et I. Wolf, Zbl. Allg. Path., 1974, t. 92, p.1.

route. The effects of the treatment are especially evident in animals subjected to a treatment of long duration; 60 to 100 injections.

While the spleens of normal animals contained only a fairly small quantity of ferric compounds localized in the red matter, in the poisoned animals, all the splenic parenchyma was the site of especially great excesses of iron, particularly in the red matter. In this area, the hemosiderin regions appeared in the form of voluminous and very abundant granulations in the cytoplasm of the macrophages of Billroth's strands. These cells are particularly abundant in the vicinity of Malpighian corpuscles. The white matter, which did not contain histochemically detectable iron in the normal animals, was generally lacking in this in the rats subjected to treatment of short duration: 10 to 20 injections. Analysis for iron became positive in animals treated for more than 60 days. In the white matter, the hemosiderin appeared in the form of fine granulations in the cytoplasm of the cells of the reticulum. These elements were especially widespread in the periphery of the germinal center as well as in the borders of Flemming's reactional zones and the corona of lymphocytes and prolymphocytes which surround the germinal center.

The lymph nodes showed, from the point of view of excess deposit of iron, a great similarity to the spleen. In the central lymphatic sinuses as well as the periphery of the follicles, a great number of macrophages overloaded with hemosiderin were observed. In the marginal sinuses as well as in the peripheries of the germinal centers, we found numerous cells of the reticulum filled with very fine granulations, colorable by Tirmann-Schmeltzer's method.

The liver was also the site of an abnormal accumulation of iron deposits. The hemosiderin was especially localized in the Kuppfer cells. In the animals

subjected to a prolonged treatment -- 80 to 100 injections -- certain hepatic cells also contained ferric material.

In rats poisoned by nickel, after long-term treatment, the accumulation of iron deposits in the level of the cortical zone in the kidney is particularly great and recognizable in examination of a section under low magnification.

The excess of iron affected most of the convoluted tubules. The cells of the pars convoluta were largely filled with hemosiderin. We also observed ferric contents in some cells of the connecting intertubular network. In rats treated with cobalt, siderosis was much less severe. Some convoluted tubules gave a positive reaction from the Tirmann-Schmeltzer method.

Testing for iron in other organs was also positive. Certain thyroid vesicles contained ferric material; these were generally inactive peripheral vesicles. The hemosiderin was localized, in part, in the form of voluminous and abundant regions in the cytoplasm of the intervesicular colloidophages, and in part, in the form of fine granulations in the cells of the thyroid epithelium.

Some localizations of iron deposits reported above were common; actually, the spleen, the lymph nodes and to a lesser degree, Kuppfer's cells of the liver were evident in all experimental siderosis. It should be pointed out that the abundance of the iron deposit in the spleen of the normal rat varied with strain and diet. In the control animals studied during the course of the experiments reported above, the splenic matter was poor in iron deposits. Besides the usual localizations, we had reason to diagnosis renal siderosis which is a rare occurrence in mammals. Certainly, iron accumulations in the urinary tubes have been noted in the course of excretion of hemoglobin in the kidney of Batraciens Urodel and Anoura. But in mammals, intrarenal iron

deposits of spontaneous and experimental siderosis are scarce and localized in the intertubular histiocytic elements. On the other hand, chronic experimental poisoning from nickel involved ferric granulations localized in the convoluted tubules.

Concerning the mechanism of this experimental siderosis, hemotological studies would be necessary in order to make a definitive interpretation. It could be involved with an increase of the hemolysis process, and the fact of an erythroblastic reaction in certain animals represents an index in favor of this viewpoint.

Conclusion — Chronic poisoning from cobalt and nickels salts in the rat caused great increase in the amount of iron deposits in the spleen, the lymph nodes, the liver and, in particular, the kidney.

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Compt. Rend. Soc. Biol
151: 282-284 (1957)

Il est évidemment paradoxal de voir augmenter le taux de ces lipides. Le seul phénomène qui paraisse pouvoir être invoqué est une rupture des cénapses au cours du moussage, les lipides devenant alors extractibles à l'éther et entraînables dans les mousses. On est donc conduit à penser que la présence des bulles, et la formation des surfaces correspondantes, entraînent une modification moléculaire telle que les cénapses perdent leur constitution primitive. Ce phénomène ne se retrouve pas avec les lipides du plasma (fig. 2).

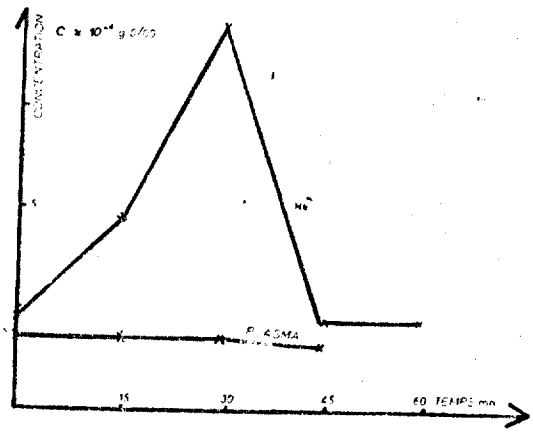

Fig. 2. — Passage comparatif des lipides sur de l'Hb et du plasma moussé.

Il est intéressant de constater que, si l'on peut extraire d'une façon pratiquement totale les protides étrangers, il n'en est pas de même des lipides. La méthode précédente permet d'ailleurs de constater qu'on en trouve les mêmes taux dans les préparations d'hémoglobine aussi purifiée que possible par les méthodes chimiques usuelles.

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Sidéroses au cours de l'intoxication chronique du Rat par les sels de cobalt et de nickel.

Note de G. Guillet, présentée par M. Prenant.

Depuis 1951 van Campenhout (1*) et ses collaborateurs ainsi que Morelle (2*) ont publié leurs résultats concernant l'action toxique élective des sels de cobalt et de nickel sur le pancréas endocrine du Cobaye.

(1*) E. van Campenhout, G. Cornelis et Th. Deulin, *Ann. Endocrinol.*, 1954, t. *15*, p. 89.
(2*) V. Morelle, *C. R. Soc. Biol.*, 1953, t. *147*, p. 1649.

En dehors de ces travaux l'action des sels de cobalt a surtout été envisagée du point de vue hématologique, ces sels ayant été utilisés en traitement de certaines anémies chez l'Homme. Hopps, Stanley et Shideler (3*) ont étudié l'action polycythémique du cobalt sur la rate et la moelle osseuse du Rat.

Aussi nous paraît-il opportun de rapporter des constatations relatives à la sidérose observée dans divers organes du Rat, observations faites dans le cadre d'une étude histophysiologique générale de l'intoxication par les sels de cobalt et de nickel. Cette accumulation de fer figuré dans l'organisme des animaux intoxiqués ne semble pas avoir été l'objet d'une étude systématique. Voigt (4*) et ses collaborateurs en 1954 signalent sommairement et sans insister une mobilisation de fer dans l'organisme au cours de l'intoxication aiguë par le cobalt.

L'expérimentation a porté sur des rats albinos adultes de souche Wistar. Chaque animal a reçu quotidiennement 2 mg de chlorure de cobalt ou de nickel par voie intrapéritonéale. Les effets du traitement sont surtout sensibles chez les animaux ayant subi un traitement de longue durée : soixante à cent injections.

Alors que la rate des animaux normaux ne contient qu'une quantité assez faible de composés ferriques localisés dans la pulpe rouge, chez les animaux intoxiqués tout le parenchyme splénique est le siège d'une surcharge en fer figuré particulièrement intense, surtout en ce qui concerne la pulpe rouge. Dans cette région les enclaves d'hémosidérine apparaissent sous forme de granulations volumineuses et très abondantes dans le cytoplasme des macrophages des cordons de Billroth. Ces cellules sont particulièrement abondantes au voisinage des corpuscules de Malpighi. La pulpe blanche, qui ne contient pas de fer histochimiquement décelable chez les animaux normaux, en est généralement dépourvue chez les rats ayant subi un traitement de courte durée : dix à vingt injections. La recherche du fer devient positive chez les animaux traités depuis plus de soixante jours. Dans la pulpe blanche l'hémosidérine apparaît sous forme de fines granulations dans le cytoplasme des cellules du réticulum. Ces éléments sont surtout répartis à la périphérie du centre germinatif ainsi qu'à la limite de la zone réactionnelle de Flemming et de la couronne de lymphocytes et de prolymphocytes qui entoure le centre germinatif.

Les ganglions lymphatiques présentent, du point de vue de la surcharge en fer figuré, une grande analogie avec la rate. Dans les sinus lymphatiques centraux ainsi qu'à la périphérie des follicules, on observe une grande abondance de macrophages surchargés d'inclusions d'hémosidérine. Dans les sinus marginaux ainsi qu'à la périphérie des centres germinatifs on trouve de nombreuses cellules du réticulum abondamment pourvues de très fines granulations colorables par la méthode de Tirmann-Schmeltzer.

Le foie est également le siège d'une accumulation anormale de fer figuré. L'hémosidérine est surtout localisée dans les cellules de Kupffer. Chez les animaux ayant subi un traitement prolongé — quatre-

(3*) H. C. Hopps, A. J. Stanley, A. M. Shideler, *Amer. J. Clin. path.*, 1954, t. 24, p. 1374.

(4*) G. E. Voigt, R. Bog et I. Wolf, *Zbl. Allg. Path.*, 1954, t. 92, p. 1.

vingt à cent injections — certaines cellules hépatiques contiennent également des inclusions ferriques.

Chez les rats intoxiqués par le nickel, après un traitement de longue durée, l'accumulation du fer figuré au niveau de la zone corticale du rein est particulièrement intense et reconnaissable dès l'examen des coupes au faible grossissement. La surcharge en fer affecte la majeure partie des tubes contournés. Les cellules de la *pars convoluta* sont abondamment pourvues d'inclusions d'hémosidérine. On observe également des inclusions ferrifères dans quelques cellules du réseau conjonctif intertubulaire. Chez les rats traitées par le cobalt, la sidérose est beaucoup moins intense. Quelques tubes contournés donnent une réaction positive par la méthode de Tirmann-Schmeltzer.

La recherche du fer dans d'autres organes est également positive. C'est ainsi que certaines vésicules thyroïdiennes comportent des inclusions ferriques : ce sont en général des vésicules périphériques peu actives. L'hémosidérine est localisée d'une part sous forme d'enclaves volumineuses et abondantes dans le cytoplasme de colloïdophages intravésiculaires, d'autre part sous forme de fines granulations dans les cellules de l'épithélium thyroïdien.

Certaines localisations de fer figuré rapportées ci-dessus sont banales ; en effet la rate, les ganglions lymphatiques et, dans une mesure moindre, les cellules de Kuppfer du foie participent à toutes les sidéroses expérimentales. Il y a lieu de signaler que l'abondance du fer figuré dans la rate du Rat normal est variable avec la souche et le régime alimentaire. Chez les animaux témoins étudiés au cours des expériences rapportées ci-dessus la pulpe splénique est pauvre en fer figuré. A côté de ces localisations habituelles, il y a lieu d'insister sur la sidérose rénale qui représente une éventualité rare chez un Mammifère. Certes une accumulation de fer dans les tubes urinaires a été signalée au cours de l'excrétion de l'hémoglobine dans le rein des Batraciens Urodèles et Anoures. Mais chez les Mammifères le fer figuré intrarénal des sidéroses spontanées et expérimentales est peu abondant et localisé dans les éléments histiocytaires intertubulaires. Dans l'intoxication expérimentale chronique par le nickel, au contraire, il s'agit de granulations ferrifères localisées dans les tubes contournés.

En ce qui concerne le mécanisme de cette sidérose expérimentale l'étude hématologique serait nécessaire pour donner une interprétation définitive. Il pourrait s'agir d'un accroissement des processus d'hémolyse, et la constatation d'une réaction érythroblastique chez certains de ces animaux représente un indice en faveur de cette manière de voir.

Conclusion. — L'intoxication chronique par les sels de cobalt et de nickel détermine chez le Rat une forte augmentation de la teneur en fer figuré de la rate, des ganglions lymphatiques, du foie et, en particulier, du rein.

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Arch Environ Health
19(3) 337-343 (1969)

Nickel Carbonyl

Effects Upon the Ultrastructure of Hepatic Parenchymal Cells

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The acute pathologic reactions of hepatic parenchymal cells following exposure to nickel carbonyl ($Ni[CO]_4$) were investigated by electron microscopy. Nickel carbonyl was administered intravenously to rats in LD_{50} and LD_{100} dosages. Diffuse dilatation of rough endoplasmic reticulum was the most prominent and most consistent ultrastructural abnormality. The mitochondria and other cytoplasmic organelles were essentially unaffected by the injection of $Ni(CO)_4$. Nucleolar alterations were present in hepatocytes during the period from 2 to 24 hours after injection. The affected nucleoli appeared to be smaller than normal, with eccentric localization of dense osmiophilic regions.

A SERIES of investigations in our laboratory has been concerned with the mechanisms of nickel carbonyl ($Ni[CO]_4$) toxicity.[1-4] The acute pathological reactions to $Ni(CO)_4$ have been shown to occur primarily in the lungs, and to a lesser degree in the liver.[5] The ultrastructural alterations which develop in the pulmonary alveoli of rats after administration of $Ni(CO)_4$ were described in a prevous paper.[6] As a continuation of these studies, we have investigated the ultrastructural reactions of the rat hepatocyte following injection of $Ni(CO)_4$.

Materials and Methods

The experimental animals were male rats of the Sprague-Dawley strain, weighing 120 to 150 gm. The rats were maintained on laboratory chow, and were fasted for 16 hours before the administration of $Ni(CO)_4$. Nickel carbonyl was injected into a tail vein by means of a microsyringe, as previously described.[5] Two dosage levels of $Ni(CO)_4$ were tested: (*a*) LD_{50} dosage of 5μl $Ni(CO)_4$/100 gm body weight (equivalent to 2.2 mg Ni/100 gm), and (*b*) LD_{100} dosage of 15μl $Ni(CO)_4$/100 gm body weight (equivalent to 6.6 mg Ni/100 gm). Control rats received sham injections.

Groups of two or three rats were killed at the following intervals after injection: 0, 0.5, 1, 2, and 4 hours, and 1, 2, 3, 4, and 6 days. The livers were excised and weighed. Portions of each liver were fixed in 10% buffered formaldehyde solution, imbedded in paraffin, and examined by light microscopy after staining with hematoxylin and eosin. Additional blocks of tissue (1 cu mm) were fixed in osmium tetroxide (2% solution in phosphate buffer, pH 7.4), dehydrated in ethanol, and embedded in epoxy plastic (Epon 812). Sections (2μ) were examined by phase microscopy without staining. Ultra-thin sections ($< 0.05\mu$) were stained with lead citrate and uranyl acetate and were examined by electron microscopy.

Results

The rats developed severe respiratory symptoms, as previously described.[1-5] At the

Submitted for publication Jan 31, 1969; accepted Feb 14.

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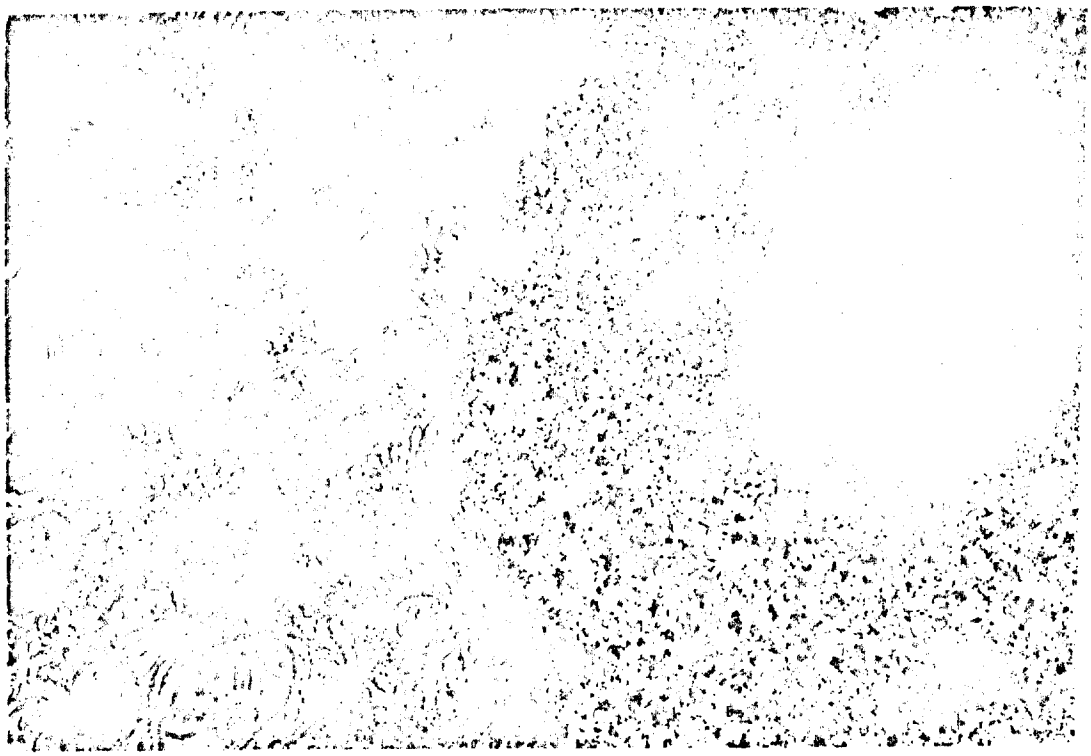
LD_{100} dosage level, all of the rats died by the end of the third day. At the LD_{50} dosage level, deaths usually occurred on the third to fifth days. As previously reported,[5] gross and histologic examinations of the livers revealed mild central congestion on the second to fourth days after injection of $Ni(CO)_4$.

At the LD_{100} dosage level, ultrastructural changes became evident in some hepatocytes as early as two hours and were present in nearly all of the hepatocytes by 24 hours. The most striking alterations developed in the endoplasmic reticulum. The normal endoplasmic reticulum (Fig 1) became transformed into dilated, vesicular structures (Fig 2 to 5). The dilatation of the cisternae of the endoplasmic reticulum was most severe at three days. The dilated sacs of endoplasmic reticulum usually had attached ribosomes; proliferation of smooth endoplasmic reticulum was not a prominent feature.

Complex, whorled, osmiophilic structures were occasionally present in the cytoplasm at 24 hours, and were common by the third day (Fig 5). Such inclusion bodies were usually surrounded by a single membrane. Infrequently, annular membranes were found around mitochondria (Fig 3), but the overwhelming majority of mitochondria appeared normal at all time intervals. No pathologic alterations were found in the other cytoplasmic organelles.

Nucleolar alterations were noted in a minor proportion of hepatocytes during the period from 2 to 24 hours after injection of $Ni(CO)_4$. The nucleoli in these cells were smaller than usual and demonstrated eccentric localization of densely staining regions without clear differentiation into granular and fibrillary components (Fig 6). Careful search failed to disclose the characteristic pattern of nucleolar segregation. In one cell, a nuclear body was seen which was com-

Fig 1.—Hepatocyte from rat killed one hour after $Ni(CO)_4$, LD_{100} dosage. Structures all essentially normal. No lesions detected in nucleus (N), nucleolus (Nu), endoplasmic reticulum (ER), or mitochondria (M) (slightly reduced from × 21,500).



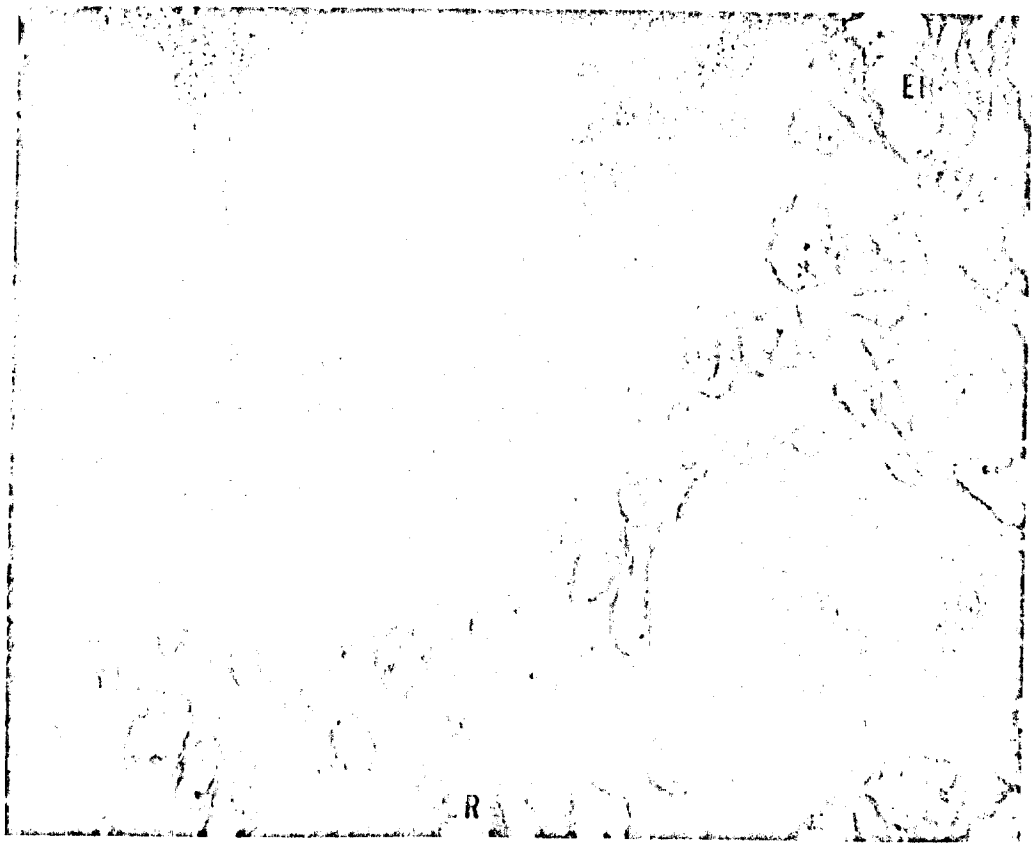
posed of a granular core surrounded by a halo, and limited by a fibrillary and vesicular border (Fig 7). (Similar nuclear inclusion bodies have been described by Krishan et al.[7])

The ultrastructural alterations which were present at the LD_{100} dosage level were also noted at the LD_{50} dosage level, and the changes developed more slowly and involved fewer hepatocytes. By the sixth day, the hepatic ultrastructure had returned to normal in rats which survived an LD_{50} injection of $Ni(CO)_4$.

Comment

The present study has demonstrated that diffuse dilatation of endoplasmic reticulum is the most prominent and most consistent ultrastructural abnormality which occurs in hepatocytes following exposure of rats to $Ni(CO)_4$. Membranous inclusion bodies develop subsequently, presumably derived from the damaged and distended cisternae of the endoplasmic reticulum. Dilatation of the endoplasmic reticulum constitutes a nonspecific response of the hepatocyte to diverse toxic agents, including γ-irradiation,[8] hepatitis virus,[9] and certain chemicals,[10,11] such as white phosphorus,[12] methanol,[11] carbon tetrachloride,[11,13,14] *N*-nitrosodimethylamine (dimethylnitrosamine),[15,16] lasiocarpine,[17] and puromycin aminonucleoside.[18] The most notable feature of the ultrastructural response of the endoplasmic reticulum to $Ni(CO)_4$ is the paucity of

Fig 2.—Hepatocyte from rat killed 24 hours after $Ni(CO)_4$, LD_{100} dosage. Endoplasmic reticulum (ER) severely dilated. Arrow indicates membrane-bound structure containing dense multivesicular body. Nucleus (N), nucleolus (Nu), and mitochondria appear normal (slightly reduced from × 21,500).

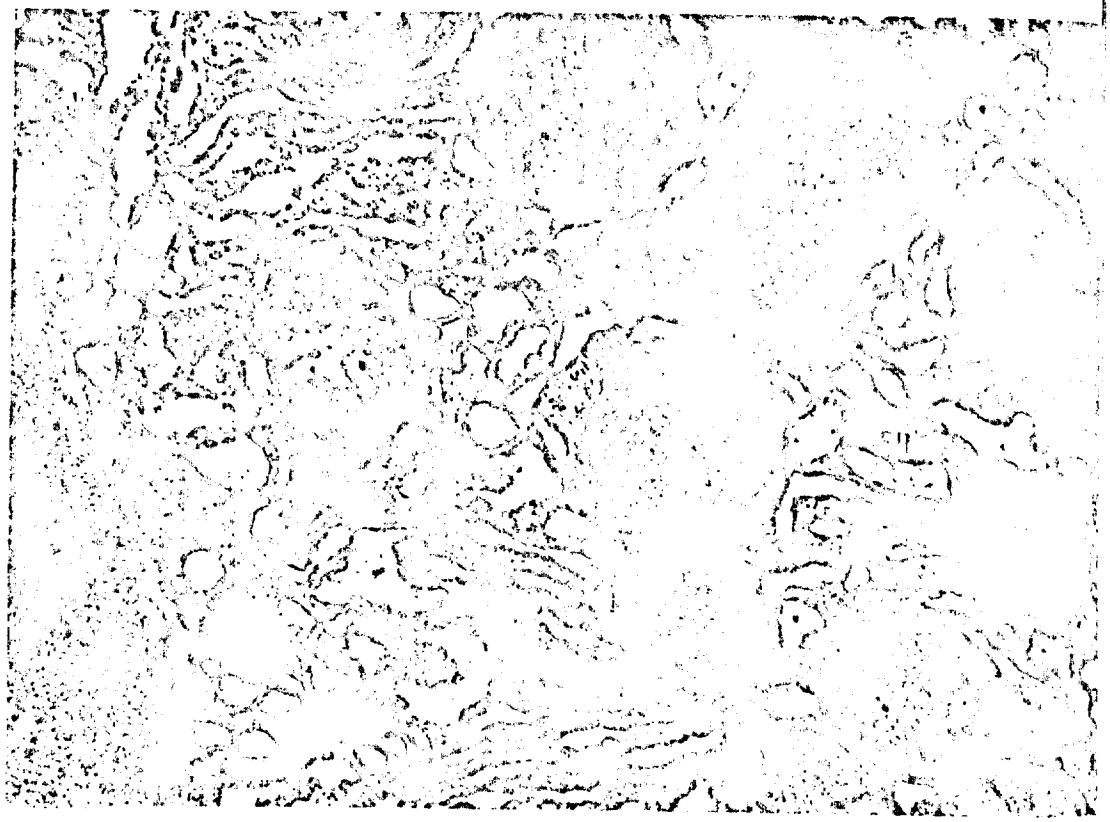


associated lesions in the mitochondria and other cytoplasmic structures. In situations where there is toxic dilatation of the endoplasmic reticulum, lesions usually develop concomitantly in other cytoplasmic organelles. For example, severe dilatation of endoplasmic reticulum occurs in renal tubular cells following injection of mercuric chloride, but the dilatation is associated with diffuse damage to the mitochondria and ground substance.[19]

Structural alterations of the endoplasmic reticulum may be partially responsible for the inhibitory effect of $Ni(CO)_4$ upon induction of hepatic enzymes.[20-23] It may be speculated that the dilated endoplasmic reticulum cannot respond normally to the inducing stimuli. Measurements of the effect of $Ni(CO)_4$ upon leucine tagged with carbon 14 incorporation into microsomal protein are currently being undertaken in our laboratory, in an attempt to correlate ultrastructural alteration of endoplasmic reticulum with functional impairment of hepatic protein synthesis. Such a correlation has previously been documented in studies of carbon tetrachloride[14] and *N*-nitrosodimethylamine[16] toxicity.

Since $Ni(CO)_4$ has been shown to inhibit ribonucleic acid polymerase activity in hepatic nuclei[24] and to impair incorporation of orotic acid tagged with carbon 14 into hepatic RNA,[25] special attention was directed to identifying nucleolar changes. Although atypical nucleolar lesions were seen, we were unable to demonstrate clear-cut nucleolar segregation, such as is produced by several other inhibitors of RNA polymerase activity.[26] This disparity may reflect two different mechanisms of inhibition of RNA polymerase. Nickel carbonyl probably inhibits RNA polymerase activity directly.[27] On the other hand, compounds such as dactinomycin (actinomycin D),

Fig 3.—Hepatocyte from rat killed 24 hours after $Ni(CO)_4$, LD_{50} dosage. Moderate dilatation of endoplasmic reticulum (ER). One mitochondria (arrow) surrounded by dense membranes (slightly reduced from × 21,500).



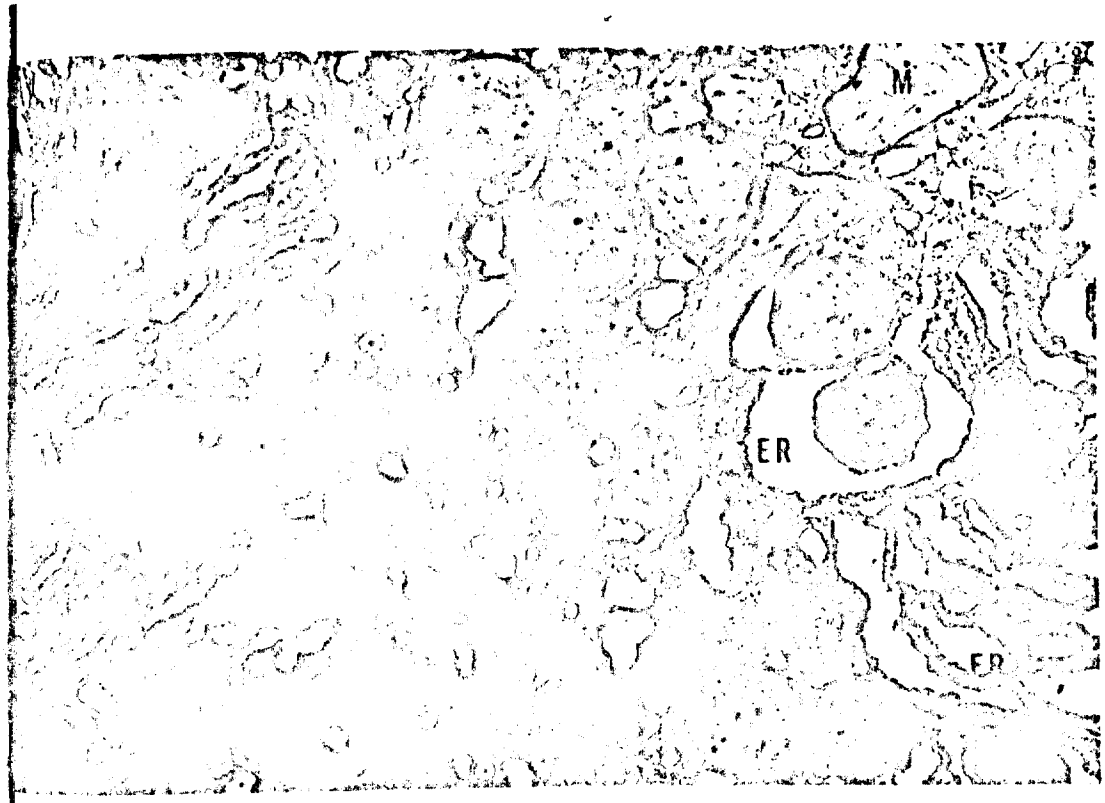
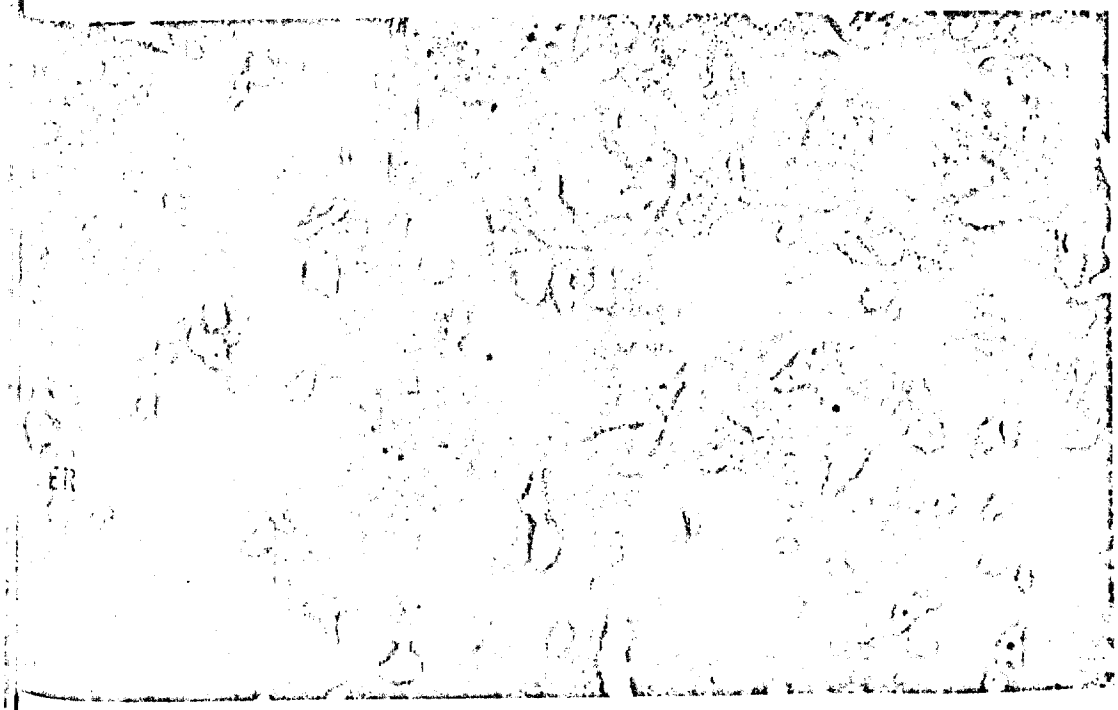


Fig 4.—Portions of cytoplasm of two hepatocytes from rat killed three days after $Ni(CO)_4$, LD_{10} dosage. Dilatation of endoplasmic reticulum (ER) in cell at right more pronounced than in cell on left. Cell junction (arrow) appears normal (slightly reduced from × 15,600).

Fig 5.—Cytoplasm of hepatocyte from rat killed three days after $Ni(CO)_4$, LD_{10} dosage. Moderate dilatation of endoplasmic reticulum (ER). Several irregular structures (arrows) evident, some containing whorled membranes (slightly reduced from × 21,500).



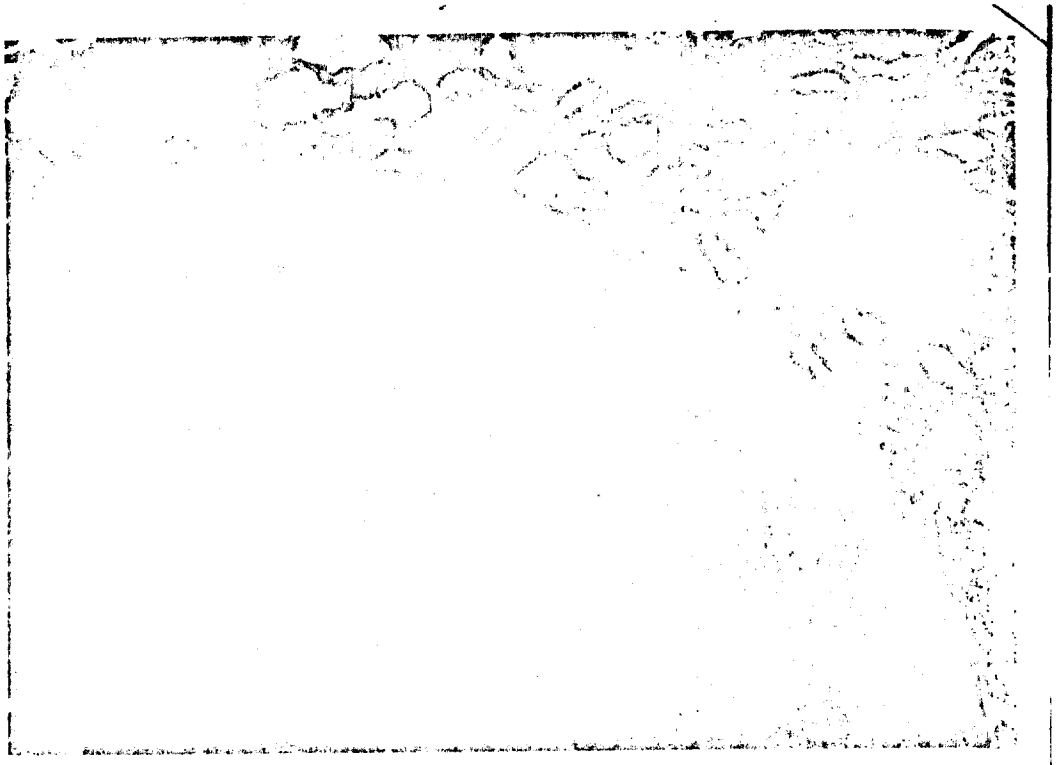


Fig 6.—Hepatocyte from rat killed 24 hours after $Ni(CO)_4$, LD_{10} dosage. Nucleolus (Nu) distorted, with eccentric localization of densely staining material (slightly reduced from × 21,500).

Fig 7.—Hepatocyte from rat killed 24 hours after $Ni(CO)_4$, LD_{10} dosage. Nucleus containing nuclear body (arrow) composed of granular core and peripheral halo, surrounded by filamentous and vesicular border (slightly reduced from × 15,600).

aflatoxin B_1, mitomycin C, nogalamycin, acridine orange, proflavine, and nitroquinoline-N-oxide bind to deoxyribonucleic acid, and are believed to inhibit RNA polymerase activity indirectly by impairing template efficiency for DNA-directed RNA synthesis.[28]

This study was supported by US Atomic Energy Commission grant AT(30-1)-4051; by American Cancer Society grant No. E-374C; and by US Public Health Service research grant (National Cancer Institute) CA-11250-01.

Mrs. Sandra Cullifer and Mr. Norris O. Roszel provided technical assistance.

Generic and Trade Names of Drug

Dactinomycin—*Cosmegen.*

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107 A NEW NICKEL CARCINOGEN. Richard T. Haro and Arthur Furst, Inst. of Chemical Biology Univ. of San Francisco, San Francisco, Cal. and William W. Payne and Hans Falk, National Cancer Institute, Bethesda, Maryland

Nickel has been implicated as a carcinogen. Nickel sulfide discs implanted in the muscle or nickel powder placed in the marrow cavity of rat femurs produced sarcomas. We have evaluated the new pi-complex of nickel dicyclopentadiene, nickelocene, for carcinogenic properties in Fischer-344 rats. Nickelous acetate and nickel powder served as controls. The vehicle was trioctanoin.

Nickelocene and nickel acetate were injected intramuscularly monthly at the I.P. LD_{50} level (50mg/Kg and 35mg/Kg) and nickel powder at 50 mg/rat.

Fibrosarcomas developed at site at the following times: nickel powder 4-6 months; nickelocene and nickel acetate 10-12 months. The tumor incidence was as follows: nickel powder 66%; nickelocene 36%; nickelous acetate 22%. Metastases to the lungs were noted in those animals held for survival (50-60 days after initial tumor appearance). Control animals receiving trioctanoin failed to develop malignant tumors at the injection site.

The nickel powder and nickelocene induced tumors were 100% transplantable. Tumors were palpable 12-15 days after injection. It is concluded that these forms of nickel are carcinogenic under these experimental conditions. (Supported by USPHS, NCI contract # PH 43-64-886).

Mikrochimica Acta [Wien] 1968, 712-718

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Semiquantitative Determination of Micro Amounts of Iron, Copper, Cobalt, Nickel, and Zinc in Blood and Tissue by Circular Thin-layer Chromatography

By

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(Received July 28, 1967)

Introduction

Semiquantitative analytical procedures which are rapid, simple and economical find applications in industrial and clinical laboratories for either obtaining quick information before applying more elaborate analysis or in cases where no more precise results are needed.

The sensitivity and specificity of spot reactions have been utilized in semiquantitative analysis. Comparison of the color intensity of a spot with that of a standard scale allows the concentration of the unknown solution to be determined, and, if the volume is known, the amount of the substance can be estimated. When these reactions are carried out on the spot plate or filter paper, the technique is called "spot colorimetry"[1,2] or "confined spot test"[3]. The ring oven technique[4] allows the collection of substances in the form of rings which can be regarded as "circular spots". The method has been termed "ring colorimetry"[5].

In the present paper, a semiquantitative method for the determination of iron, copper, cobalt, nickel and zinc is developed by making "circular spots" or rings on chromatoplates using the circular thin-layer chromatographic apparatus[6,7]. The error has been minimized to about $\pm 5\%$ and the procedure is rapid, simple and economical. The interference of some 40 metal ions is removed by solvent extraction. This method has been tested for the determination of trace elements in blood and tissue.

Experimental

Reagents

All reagents were of analytical grade or comparable purity.

For determination of iron, copper, cobalt and nickel, aluminium oxide DS-5 (Camag, with $CaSO_4$ binder) and for zinc, cellulose powder CC 41 (Whatman) were used without any further treatment for the preparation of thin layers.

Iron, copper, cobalt, nickel and zinc solutions were prepared by weight by dissolving their nitrate salts in water.

In order to check the interference, known amounts of iron, copper, cobalt, nickel and zinc were mixed with a large excess of the salts of following metals and solutions were prepared[7]: Te^{4+}, U^{6+}, Zr^{4+}, Cd^{2+}, Bi^{3+}, Pb^{2+}, Hg^{2+}, La^{3+}, K^{+}, Li^{+}, Cs^{+}, Rb^{+}, Ba^{2+}, Sr^{2+}, Ca^{2+}, Mg^{2+}, Ce^{3+}, Th^{4+}, Se^{4+}, Al^{3+}, Mn^{2+}, Pd^{2+}, Pt^{4+}, Ru^{3+}, Ge^{4+}, Au^{3+}, Sb^{5+}, Sn^{4+}, Ga^{3+}, As^{3+}, Cr^{3+}, Ti^{4+}, V^{5+}, Mo^{6+}, Rh^{3+}.

The tissue and blood samples were separately ashed for about 12 hours in a muffle furnace at 700° using china crucible[8]. Tissues were dried before ashing.

Apparatus

Circular thin-layer chromatographic apparatus[6] was used. Special care was taken to saturate the developing chamber before running the chromatoplates.

Thin layers of uniform thickness (400 μ) were prepared by mixing 5 g of adsorbent with 12 ml of water using the Camag applicator (Camag, Muttenz, Switzerland). The plates were dried overnight at room temperature (30–32°) and were placed in a Shandon storage cabinet.

Procedure

To a known volume of the test solution (0.5 to 5 ml) containing about 10 to 500 μg each of Fe^{3+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and other metal ions (*cf.* Reagents), sufficient amount (2 to 5 ml) of concentrated hydrochloric acid is added so that the concentration of HCl in the final solution was about 7 *M*. Fe^{3+} is extracted[9] with 5 ml mixture of isobutyl methyl ketone and amyl acetate (2 : 1). The organic layer is removed after washings with 50% HCl in 5 portions of 2 ml each, and analyzed for Fe^{3+}. The aqueous layer along with the washings is transferred to a separatory funnel and was neutralized with $NaHCO_3$. From the neutral aqueous layer, Cu^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} are extracted with 0.5 ml pyridine, 1 ml of 50% ammonium thiocyanate and 10 ml chloroform[9]. The resulting solution is vigorously shaken and the chloroform layer is removed for the analysis of Cu^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}.

A very fine hole is made with a pin in the prepared surface of the chromatoplate. A known volume (1–10 μl) of different concentrations of solution containing iron, copper, cobalt, nickel and zinc is applied to the plate with either "Agla" micrometer syringe (Burroughs Wellcome and Co., London, England) or with a micro pipette (2512: Shandon Scientific Co. Ltd., London, England) taking care that the solution does not spread in the film, thus forming a large spot. Since the Shandon micro pipette has a flat mouth and cannot be used as such, a very fine capillary, having a suitable diameter, is affixed to its mouth. The chromatogram is developed with the circular thin-layer chromatographic apparatus[6] for 1 to 2 minutes using a mixture of acetone, hydrochloric acid (4 *M*) and acetylacetone (45 : 3 : 2) as solvent so that the *Rf* (i. e., the diameter of the "circular spot") should remain the same for each cation. After drying, the chromatoplate is sprayed with different spray reagents in order to determine the sensitivity of each metal ion with the respective spray reagents, and a standard "sensitivity scale" of each metal ion with various spray reagents is prepared.

From the unknown solution, a known volume (1–10 μl) of the solution, after appropriate dilutions, is spotted on chromatoplate. For each dilution a separate chromatogram is run until the dilution limit is reached which contains the "sensitivity" amount of the metal ion with the particular spray reagent. Since the volume of the original test solution, dilutions made, and the sensitivity is known, the concentration of the metal ions can be calculated.

Results and Discussion

The method is dependent on the sensitivity and specificity of color reactions of cations with different spray reagents. The various spray

Table I. Spray Reagents for Sensitivity Standard of Metal Ions

Reagent	Solution
Tannic acid (TA)	10% in water
8-Hydroxyquinoline (HQ)	3% in alcohol
Potassium ferrocyanide (PF)	2% in water
2,2′-Diquinolyl (DQ)	Saturated in alcohol followed by spray with 5% aqueous solution of hydroxylamine hydrochloride
Benzoin oxime (BO)	5% in alcohol
Ammonium thiocyanate (AT)	5% in water
Rubeanic acid (RA)	0.05% in alcohol
α-Nitroso-β-naphthol (NN)	0.2% in alcohol
Dimethylglyoxime (DG)	2% in alcohol
Dimercaptothiodiazol (DT)	1% in alcohol
Dithizone (DZ)	0.2% in carbon tetrachloride
Potassium ferricyanide (PFi)	1% in water

reagents and their method of preparation is given in Table I. Almost all known spray reagents in literature[4,7,10] for these cations were tried but only a few given in Table I were found suitable because this method requires a quick color reaction and, after spraying, a clear background so that the "sensitivity" comparison of the "circular spot" could be made without any difficulty.

The sensitivity standard of iron, copper, cobalt, nickel and zinc with different spray reagents using a mixture of acetone, hydrochloric acid and acetylacetone as solvent is given in Table II. The *Rf* (diameter of the "circular spot") will vary with the change of solvent which will affect the sensitivity standard. The sensitivity standard will also vary with the adsorbent. Hence, the sensitivity standard must be prepared with each solvent and adsorbent, and unknown solutions must be analyzed under identical conditions.

Table II. Sensitivity Standard of Metal Ions

Cation[a]	Color and Spray reagent[b]	Sensitivity[c] (μg)	*Rf*[d]
Fe^{3+}	light violet (TA)	0.04	1.00
	yellow (HQ)	0.06	
	blue (PF)	0.02	
Cu^{2+}	pink (DQ)	0.01	0.98
	light green (BO)	0.40	
Co^{2+}	blue (AT)	0.06	0.41
	brown (RA)	0.04	
	brown (NN)	0.03	
Ni^{2+}	blue (RA)	0.03	0.38
	pink (DG)	0.04	
	light brown (DT)	0.08	
Zn^{2+}	pink (DZ)	0.02	0.98
	light violet (PFi)	0.90	

[a] Alumina, DS-5 (Camag) for Fe^{3+}, Cu^{2+}, Co^{2+} and Ni^{2+}; Cellulose powder CC 41 (Whatman) for Zn^{2+}.

[b] Cf. Table I.

[c] Represents the minimum amount of the metal ion in the volume of the liquid spotted which is visible on the chromatoplate with the corresponding spray reagent provided the diameter of the "Circular spot" i. e., *Rf* value remains the same. By this method, the amount of metal ions as low as "sensitivity" limit can be determined.

[d] Mixture of acetone, hydrochloric acid (4 *M*) and acetylacetone (45 : 3 : 2) was used as solvent.

The results for the semiquantitative determination of iron, copper, cobalt, nickel and zinc are given in Table III which indicate that these

cations can be determined to ± 5%. This seems to be satisfactory considering that no optical apparatus is used and that the sample for the analysis is extremely small. The interference due to other metal ions is removed by solvent extraction. From a mixture of about 40 metal ions (*cf.* Experimental), Fe^{3+} is extracted by a mixture of isobutyl methyl ketone and amyl acetate (2 : 1). Cu^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} are extracted with pyridine, 50% solution of ammonium thiocyanate and chloroform. In the second extraction, Cd^{2+} is extracted partially but does not interfere with the determination of copper, cobalt, nickel and zinc.

Table III. Semiquantitative Determination of Metal Ions by Circular Thin-layer Chromatographic Technique*

Cation	Reagent	Amount added mg/100 ml	Amount found mg/100 ml	Error %
Fe^{3+}	(a) Tannic acid	102.500	100.000	2.44
	(b) 8-Hydroxyquinoline		95.000	7.46
	(c) Potassium ferrocyanide		98.000	4.39
				Mean... 4.76
Cu^{2+}	(a) 2,2′-Diquinolyl	12.576	12.200	2.98
	(b) Benzoin oxime		12.000	4.57
				Mean... 3.77
Co^{2+}	(a) Ammonium thiocyanate	217.325	213.000	1.99
	(b) Rubeanic acid		208.000	4.28
	(c) α-Nitroso-β-naphthol		210.000	3.37
				Mean... 3.21
Ni^{2+}	(a) Rubeanic acid	504.700	480.000	4.89
	(b) Dimethylglyoxime		500.000	0.93
	(c) Dimercaptothiodiazole		475.000	5.88
				Mean... 3.90
Zn^{2+}	(a) Dithizone	123.680	118.000	4.51
	(b) Potassium ferricyanide		116.000	6.21
				Mean... 5.36

* The determination of each cation was made with different spray reagents separately and then the "mean" error was calculated. The analysis of the same cation with different spray reagents provides a useful check on the results. The method was checked by preparing various solutions having different strengths of Fe^{3+}, Cu^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}. Typical results of only one such solution are given in the Table.

After extraction, copper, cobalt, nickel and zinc are in the same solution for analysis. Since copper, cobalt and nickel have different *Rf* values they do not interfere with one other even when they are visible on chromatoplate with the same spray reagent (e. g., Co^{2+} and Ni^{2+} with rubeanic acid). Zinc is determined on the cellulose powder plate in a

separate chromatogram because it cannot be determined on alumina plate though its *Rf* value on alumina plate is 0.40.

Iron, copper, cobalt, nickel and zinc in human blood and human tissues were determined by the present procedure. The results are comparable with the values in the literature[11]. In order to check the accuracy of these results, a known amount of iron, copper, cobalt, nickel and zinc were added in the blood and tissue samples and their recovery was recorded. The results are given in Table IV and V.

Table IV. Determination of Iron, Copper, Cobalt, Nickel and Zinc in Human Blood[a]

Cation	Amount found mg%	Amount added mg%	Total amount mg%	Amount found mg%	Error, %
Fe^{3+}	50.271	15.082	65.353	64.856	0.76
Cu^{2+}	0.108	7.541	7.649	7.738	1.16
Co^{2+}	0.045	7.541	7.586	7.579	0.09
Ni^{2+}	0.013	7.541	7.554	7.557	0.04
Zn^{2+}	1.006	7.541	8.547	8.375	2.01

[a] In actual experiment, 7 g (6.63 ml) of blood was taken.

Table V. Determination of Iron, Copper, Cobalt, Nickel and Zinc in Human Tissue[a]

Cation	Amount found in 100 g muscle (mg)	Amount added (mg/100 g)	Total amount (mg/100 g)	Amount found (mg/100 g)	Error, %
Fe^{3+}	24.860	31.075	55.935	55.313	1.11
Cu^{2+}	0.888	15.538	16.426	16.656	1.40
Co^{2+}	0.149	15.538	15.687	16.034	2.21
Ni^{2+}	nil	15.538	15.538	15.200	2.17
Zn^{2+}	22.374	15.538	37.912	38.067	0.41

[a] In actual experiment, 4.200 g of human muscle (3.218 g after drying) was taken. The results are expressed per 100 g of muscle after drying.

The method holds an excellent promise for the study of airborne particulates[12] and for the determination of trace elements in blood and tissues because of its being reliable, sensitive, fast, simple and economical.

Summary

A semiquantitative method for the determination of iron, copper, cobalt, nickel and zinc is described which is based on circular thin-layer chromatographic technique. The error is minimized to ± 5% and interference of about 40 metal ions is removed by solvent extraction. The

application of the method has been demonstrated by the determination of these metal ions in human blood and tissue and the method is suggested for the study of airborne particulates.

Zusammenfassung

Ein halbquantitatives Verfahren zur Bestimmung von Eisen, Kupfer, Kobalt, Nickel und Zink auf dünnschicht-ringchromatographischem Wege wurde beschrieben. Der Fehler wurde auf ± 5% eingeschränkt und die störende Wirkung von etwa 40 Metallionen durch Lösungsmittelextraktion beseitigt. Die Anwendbarkeit des Verfahrens für die Bestimmung in menschlichem Blut und Gewebe wurde gezeigt bzw. für die Untersuchung atmosphärischer Verunreinigungen empfohlen.

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Compt. Rend. soc. biol. 152: 1093-4 (1958)

Cancer Produced by Nickel and Lesions of Nerve Endings by Fixation of Histamine

by S. Hatem, presented by C. Champy

We have previously observed that organic carcinogenic substances blocked histamine (1, 2, 3). The study extended to mineral compounds. This second part is not finished, but already some results have been obtained.

Thallium forms a compound with histamine (4), and sometimes the addition of thallium salts causes the destruction of nerve endings sometimes bringing about cancers (5, 6).

Silver also combines with amines (7) and further cancers appeared to be numerous when the metal is inserted into the abdominal lining of rats (8).

Nickel has been the object of very extensive studies. In one series of notable experiments (12), the author demonstrated that this metal administered by intravenous injections and dispersed throughout the organism is not carcinogenic while prolonged applications of metallic nickel produce cancer at the site of application. The metal was enclosed in gelatin, the gelatin itself being in an isotonic solution of sodium chloride. The vehicle alone was shown to have no effect on the application site and the author further used another vehicle. This effect of nickel was evident in rats and rabbits, but not in mice; this proves the author declared, that there is certainly cancer from nickel with specific characteristics and not from hypothetical irritation from no matter what metal.

Further, numerous cancers were developed in Norway and Canada caused by the metallic dusts in workers who purified the metal (13). From 1923 to 1948, 47 cases of cancer of the nose and 82 cases of cancer of the lung were observed.

Amine was then put into contact with various nickel salts. The examination of visible spectrums and u.v. enables us to foresee the histamine-nickel meeting, a meeting of the entire molecule by applying to luminous absorption the method of continuous variations of P. Job. The presence of several soluble compounds can be observed. A similar result was found by P. Job (14) in the study of salts of nickel-ammonia compounds. The study has been done for three different salts: nickel chloride, nitrate and sulfate, and the results are consistent.

Thus, histamine is captured by the metal and this shows that the disorderly increase which the nickel causes is of the same degree as that caused by organic carcinogens.

We are far from considering, however, that all minerals likely to combine with amines invariably lead to the start of cancer, given the different characteristics of solubility diffusibility and stability of the systems in contact. The above-named authors have demonstrated that the carcinogenic metals only manifest their effects under exactly determined conditions.

It is also interesting to note that lead observed under the same conditions as nickel doesn't form a soluble compound with histamine (15 to 18). Lead revealed itself as completely inactive as a carcinogen to numerous researchers who administered it in various ways and in particular, by applying it to the skin.

This study is being pursued today, and other metals will be examined as much on the physical-chemical plane as on the biological one.

The verification <u>in vivo</u> was done by the preceding indicated methods (19) and enables us to conclude the fixation of nerve histamine by nickel.

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Compt. rend. soc. biol.
152: 1093-4 (1958)

Cancer du nickel et lésions des terminaisons nerveuses par fixation de l'histamine.

Note de S. HATEM, présentée par C. CHAMPY.

Nous avons observé précédemment que les substances cancérigènes organiques bloquaient l'histamine (1, 2, 3). L'étude s'étend aux composés minéraux. Cette deuxième partie n'est pas terminée mais, déjà, quelques résultats sont acquis.

Le thallium forme un complexe avec l'histamine (4) et, par ailleurs, les applications de sels de thallium provoquent la destruction des terminaisons nerveuses entraînant parfois des cancers (5, 6)

L'argent s'associe également à l'amine (7) et, d'autre part, des cancers apparaissent nombreux lorsque le métal est inséré dans la paroi abdominale de rats (8).

Le nickel a fait l'objet d'études très poussées (9 à 12). Dans une série d'expériences remarquables (12), l'auteur démontre que le métal administré par injections intraveineuses et dispersé dans tout l'organisme n'est pas cancérigène tandis que des applications prolongées de nickel métallique produisent le cancer au lieu même du dépôt. Le métal était enrobé de gélatine, la gélatine étant elle-même dans une solution isotonique de chlorure de sodium. Le véhicule seul s'est révélé sans action au lieu même de l'application et l'auteur a, de plus, utilisé un autre véhicule. Cette activité du nickel se manifeste chez les rats, chez les lapins et non chez les souris ; ceci prouve, déclare l'auteur, qu'il y a bien là un cancer du nickel avec un certain caractère de spécificité et non l'hypothétique irritation d'un métal quelconque.

De plus, les cancers se sont développés nombreux en Norvège et au Canada, provoqués par la poussière métallique chez des ouvriers qui purifient le métal (13). De 1923 à 1948, on a observé 47 cas de cancers du nez et 82 cas de cancers du poumon.

L'amine a donc été mise en contact avec différents sels de nickel. L'examen des spectres visibles et U.V. laisse prévoir la rencontre his-

(1) S. Hatem, *C. R. Acad. Sc.*, 1957, t. *244*, p. 2113.
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tamine-nickel, rencontre par molécule entière en appliquant à l'absorption lumineuse la méthode des variations continues de P. Job. On peut même conclure à la présence de plusieurs complexes solubles. Un résultat analogue avait été trouvé par P. Job (14) dans l'étude des complexes sels de nickel-ammoniac. L'étude a été faite pour trois sels différents : chlorure, nitrate et sulfate de nickel et les résultats sont concordants.

Ainsi, l'histamine est captée par le métal et ceci montre que le désordre de croissance que fait apparaître le nickel est de même ordre que celui provoqué par les cancérigènes organiques.

Nous sommes loin de considérer, cependant, que tout minéral susceptible de s'associer à l'amine précède invariablement la naissance du cancer étant donné les différents caractères de solubilité, de diffusibilité et de stabilité des systèmes en contact. Les auteurs précités ont démontré que les métaux cancérigènes ne manifestaient leur activité que dans certaines conditions parfaitement déterminées.

Il n'en est pas moins intéressant de relever que le plomb ne donne pas, observé dans les mêmes conditions que le nickel, de complexe soluble avec l'histamine (15 à 18). Or, le plomb s'est révélé parfaitement inactif comme cancérigène à de nombreux expérimentateurs qui l'ont administré de façons multiples et, en particulier, par application sur la peau.

Cette étude se poursuit aujourd'hui et d'autres métaux seront examinés tant sur le plan physicochimique que sur celui de la Biologie.

La vérification *in vivo* a été faite par les méthodes précédemment indiquées (19), et permet de conclure à la fixation de l'histamine des nerfs par le nickel.

Action des sels potassiques et magnésiens de l'acide DL-aspartique sur le CO_2 plasmatique total dans l'hypercapnie provoquée chez le Chien.

par H. Laborit, P. Niaussat, J. M. Jouany, B. Weber, J. Martin et C. Baron.

Au cours de l'étude que nous poursuivons de certains sels de l'acide DL-aspartique, certaines constatations expérimentales nous ont amenés à envisager la possibilité d'une action de cet acide aminé sur l'élimination du CO_2 par une autre voie que la voie pulmonaire. L'expérimentation que nous rapportons aujourd'hui permet, semble-t-il, d'apporter quelques précisions à ce sujet.

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C.R. 250:2962-2964, 1960

Oncology--Inhibition of Carcinogenesis of Nickel and Liberation of Bound Histamine

By: Simone Hatem
Presented by: Mr. Christian Champy

The monocalcium disodium salt of ethylenediametetraacetic acid used to combat carcinogenesis caused by nickel liberates the histamine captured by the metal. It seems that there was between the inhibition of tumorogenesis and the liberation of histamine a relationship already apparent in a different form in the case of carcinogenic carbides.

The complexing of nickel (Ni) by histamine (Hi) already glimpsed in aqueous solutions by an electrochemical study (1), (2), (3) was recovered and confirmed by the method of continuous variations applied to luminous absorption by Paul Job. Various nickel salts put in contact with Hi in the aqueous phase enabled the formation of several complex bases in certain cases (4). In concentrated solutions, the crystals appeared in 24 hours. The following complexes are thus characterized:

$(NO_3)_2$ [Ni Hi_2]n H_2O, blue crystals, biaxial, having a very good cleavage plane and intensely pleochromatic (Fig. 1).

Figure 1

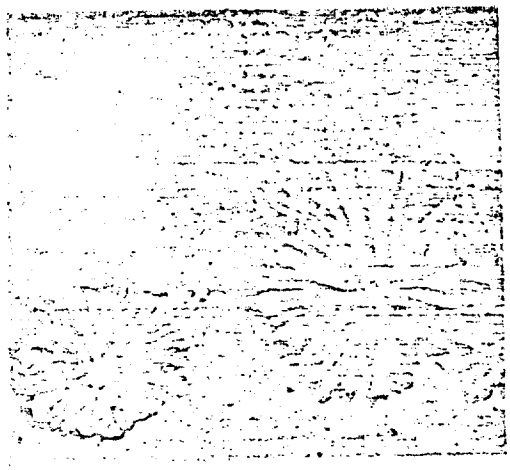

Figure 2

Fig. 1--Crystallization of $(NO_3)_2$ $(NiHi_2)$ n H_2O.
Fig. 2--Crystallization of $(NO_3)_2$ (NiH_3) n H_2O.

$(NO_3)_2$ [Ni Hi_3]n H_2O, violet crystals, negative, single axis, hexagonal, very birefringent (Fig. 2).

Cl_2 [Ni Hi_3] n H_2O, violet crystals, positive single axis, hexagonal, slightly birefringent (Fig. 3).

SO_4 [Ni Hi_3] n H_2O, violet crystals, negative, biaxial, not pleochromatic. Its cleavage is good, slightly birefringent (Fig. 4).

Further, it is known that Ni in the state of metallic powder, causes the formation of cancers in situ (in the respiratory tract of industrial workers) and that the monocalcium, disodium salt of ethylenediaminetetraacetic acid (E.D.T.A.) hinders carcinogenesis provoked in this manner, the nickel is substituted for the calcium in the chelate which is eliminated.

Given the considerable affinity of Ni for Hi, it is interesting to see what the choice of the metal will be once in the presence of the E.D.T.A. salt and the combined Hi. It should be put into consideration that if the Hi is infinitely soluble, the E.D.T.A. is slightly soluble in water.

Figure 3 Figure 4

Fig. 3--Crystallization of Cl_2 (NiH_3) n H_2O.
Fig. 4--Crystallization of SO_4 (NiH_3) n H_2O.

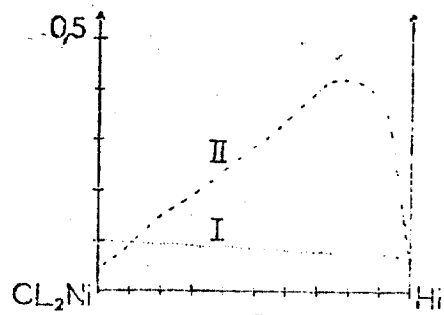

Figure 5

Fig. 5--Continous variations. Optic density of mixtures of equimolecular aqueous solutions of Cl_2 Ni-Hi after three weeks of contact. Concentration c = 0.01 M; λ = 2.500 Å.

Curve I: Compound abolished by the E.D.T.A. salt, e = 0.05 cm.

Curve II: Normal compound, e = 1 cm.

Nickel chloride seems to be strongly indicated for this study. The (Ni, H_3) compound which forms only in the crystallized state appears also in solution (Fig. 5).

Two identical series of mixtures of equimolecular solutions of Ni, Hi are prepared according to the method of continuous variations. Each series comprises concentrations ranging from 0.01 to 0.1 M. In one of the series (Series II), the powder of the E.D.T.A. salt is dispersed.

Although it is slightly soluble in water, the salt is slowly dissolved by the milieu where the Ni, Hi compound pre-exists, and the Ni, Hi reaction takes place. Actually, examined under ultraviolet light three weeks later, the two series are clearly expressed. The density spanned for the spectral examination is chosen so as to situate the simple Ni, Hi constituents at the limit of absorption in a determined area and for a given concentration. A very definite maximum is observed independently of the region and the concentration, in series I, a very definite maximum: the histamine is bound; in series II, a neighboring curve to the right of additivity, a higher pH; the histamine is free (Fig. 5). It is further seen that the slow evaporation of solutions gives rich and abundant crystals, visible to the naked eye in this case, when only a microscope enables detection of weak particles in the other case.

It appears once again that when a substance fights the appearance of a chemical cancer, it also fights, reducing it at its birth, the blockage of histamine.

C. R. 250: 2962-2964, (1960)

CANCÉROLOGIE. — *Inhibition de la cancerogenèse du nickel et libération de l'histamine engagée.* Note (*) de Mlle **Simone Hatem**, présentée par M. Christian Champy.

Le sel monocalcique disodique d'éthylène diamine tétraacétique utilisé pour combattre la cancérogenèse provoquée par le nickel libère l'histamine captée par le métal. Il semble qu'il y ait entre l'inhibition de la tumorogenèse et la libération de l'histamine une parenté déjà apparue sous une forme différente dans le cas de carbures cancérigènes.

La complexion du nickel (Ni) par l'histamine (Hi) déjà entrevue aux solutions aqueuses diluées par une étude électrochimique (1), (2), (3) a été reprise et confirmée par la méthode des variations continues appliquée à l'absorption lumineuse selon Paul Job. Divers sels de Ni mis au contact de Hi en phase aqueuse laissaient prévoir la formation de plusieurs bases complexes dans certains cas (4). Aux solutions concentrées, des cristaux apparaissent en 24 h. Les complexes suivants sont ainsi caractérisés :

$(NO_3)_2\,[NiHi_2]\,nH_2O$ cristal bleu, biaxe, ayant un très bon plan de clivage et un pléochroïsme intense (*fig.* 1).

Fig. 1. Fig. 2.

Fig. 1. — Cristallisation de $(NO_3)_2\,(NiHi_2)\,n\,H_2O$.

Fig. 2. — Cristallisation de $(NO_3)_2\,(NiHi_3)\,n\,H_2O$.

$(NO_3)_2\,[NiHi_3]\,nH_2O$ cristal violet uniaxe négatif, hexagonal, très biréfringent (*fig.* 2).

$Cl_2\,[NiHi_3]\,nH_2O$ cristal violet, uniaxe positif, hexagonal, peu biréfringent (*fig.* 3).

$SO_4\,[NiHi_3]\,nH_2O$ cristal violet, biaxe négatif, n'ayant pas de pléochroïsme.

Son clivage est bon, sa biréfringence est faible (*fig.* 4).

D'autre part, on sait que Ni, à l'état de poussière métallique, provoque la naissance de cancers *in situ* (voies respiratoires chez les ouvriers de l'industrie) [3] et que le sel monocalcique, disodique d'éthylène diamine tétraacétique (E. D. T. A.) entrave la cancérogenèse ainsi provoquée, le nickel se substituant au calcium dans le chélate qui s'élimine.

Étant donné l'affinité considérable de Ni pour Hi, il est intéressant de

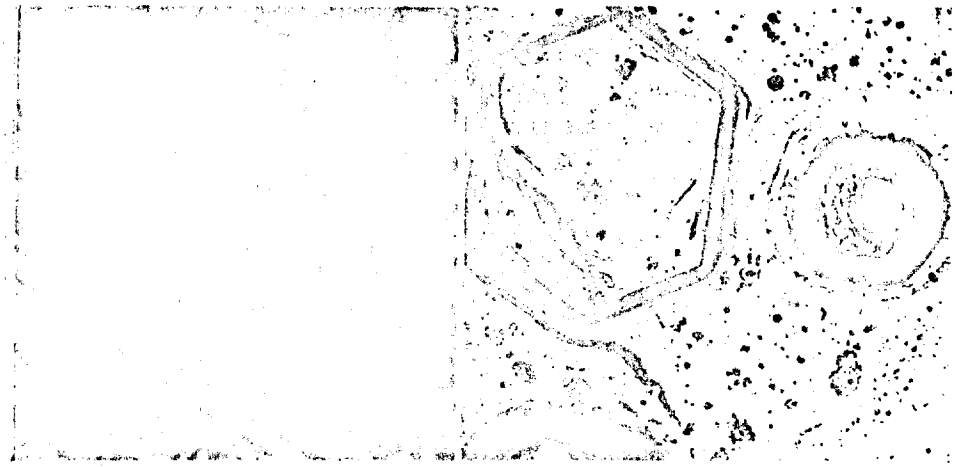

Fig. 3. Fig. 4.

Fig. 3. — Cristallisation de Cl_2 (Ni Hi$_3$) n H_2O.

Fig. 4. — Cristallisation de SO_4 (Ni Hi$_3$) n H_2O.

voir quel sera le choix du métal, celui-ci une fois mis en présence du sel E. D. T. A. et de Hi réunis. Il y a lieu de considérer que si Hi est infiniment soluble, le sel E.D.T.A. est peu soluble dans l'eau.

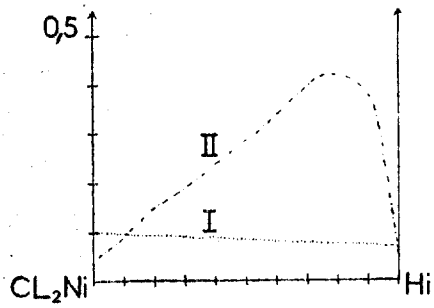

Fig. 5. — Variations continues. Densité optique des mélanges de solutions aqueuses équimoléculaires Cl_2 Ni-Hi après trois semaines de contact. Concentration c = 0,01 M; λ = 2 500 Å.

Courbe I : Complexion annulée par le sel E. D. T. A., e = 0,05 cm.

Courbe II : Complexion normale, e = 1 cm.

Le chlorure de nickel semble tout indiqué pour cette étude. Le complexe (Ni, Hi$_3$) qui se forme seul à l'état cristallisé apparaît aussi en solution (*fig.* 5).

Deux séries identiques de mélanges de solutions équimoléculaires Ni, Hi sont préparées selon la méthode des variations continues. Chaque série comporte des concentrations s'échelonnant de 0,01 à 0,1 M. Dans l'une des séries (*série II*) la poudre du sel E.D.T.A. est dispersée.

Bien qu'il soit peu soluble dans l'eau, le sel est lentement solubilisé par le milieu où le complexe Ni, Hi préexiste, et *la réaction* Ni, Hi *régresse tandis que la réaction* Ni, E.D.T.A. *s'accomplit*. En effet, examinées dans l'ultraviolet, trois semaines après, les deux séries sont éloquentes. L'épaisseur traversée pour l'examen spectral est choisie de façon à situer les constituants simples Ni, Hi à la limite d'absorption dans une région déterminée et pour une concentration donnée. On observe, indépendamment de la région et de la concentration, *dans la série I*, un maximum très accusé : l'histamine est engagée; *dans la série II*, une courbe voisine de la droite d'additivité, un pH plus élevé : l'histamine est libre (*fig.* 5). On vérifie de plus que l'évaporation lente des solutions donne des cristaux abondants, riches et visibles à l'œil nu dans un cas quand seul le microscope permet de déceler des germes chétifs dans l'autre cas.

Il apparaît une fois encore que lorsqu'une substance combat la naissance d'un cancer chimique elle combat aussi, le réduisant à néant, le bloquage de l'histamine.

(*) Séance du 20 avril 1960.

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La séance est levée à 15 h. 20 m.

L. B.

Brit. J. Cancer 18(2): 261-264

THE PRODUCTION OF MALIGNANT TUMOURS BY NICKEL IN THE RAT

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Received for publication March 6, 1964

In May 1932 a Parliamentary question drew attention to a number of cases of nasal cancer which had occurred among workers in a nickel refinery at Clydach, South Wales. The report of the Chief Inspector of Factories for 1931 (published in July 1932) listed several cases, and Stephens (1933), in discussing cases of industrial epitheliomata, suggested that nickel was one of the agents responsible. It was already known that nickel compounds, including gaseous nickel carbonyl, were toxic, but they were not suspected of being carcinogens. Subsequent statistical investigations by Doll (1958) and Gwynne Morgan (1958), and pathological studies by Løken (1950) and Jones Williams (1958) have confirmed the relatively high incidence of respiratory cancer in nickel workers.

Animal experiments by Hueper (1958) on the effect of inhalation of metallic nickel powder by rats and guinea-pigs, and by Sunderman, Donnelly, West and Kincaid (1959) on the effect of inhalation of nickel carbonyl by rats, confirmed that nickel could produce neoplastic changes in the respiratory tract, leading sometimes to frankly malignant tumours (Sunderman *et al.*, 1959). Hueper (1952) had previously shown that powdered nickel, injected into rats by various routes, produced malignant tumours in at least 8 out of 70 animals. He also showed (Hueper, 1955) that the same agent, introduced by five different routes into rats, mice, and rabbits, produced malignant tumours of various types in 27 out of 100 rats, one out of six rabbits, and none of 125 mice. It is of interest that he draws attention to a possible species-specificity.

Recently, Gilman (1962) has shown that nickel sulphide and nickel oxide, injected intramuscularly into rats and mice, produce tumours at the injection site. The tumours he obtained in the rats were mainly rhabdomyosarcomata; in the mice, although many of the tumours had the characteristics of fibrosarcomata, their cellularity, numerous oval nuclei and lack of collagen suggested to him that they contained myomatous elements. He has observed (personal communication) a strain-specificity of the carcinogenic action of nickel in rats.

We are investigating the mechanisms of metal carcinogenesis, and thought it necessary to look for the possible carcinogenic effects of pure nickel powder under the same conditions as those in which cobalt (Heath, 1954 and 1956) and cadmium (Heath, Daniel, Dingle and Webb, 1962; Heath and Daniel, 1964) are carcinogenic.

MATERIALS AND METHODS

Ten female rats of the hooded strain, aged 2–3 months, were used. 0·0283 g. of spectrographically pure nickel metal powder (Johnson Matthey) was shaken into suspension with 0·4 ml. of fowl serum and injected into the muscle of the right thigh of each animal from the medial aspect, approximately parallel with the

femur and directed towards the hip. On microscopical examination the metal powder was found to consist mainly of aggregates ranging from 3 $\mu \times 3\ \mu$ to 117 $\mu \times 87\ \mu$, composed of small, mostly spherical, particles of 0·5–0·8 μ diameter. There were a very few small single particles of about 1·7 $\mu \times 2{\cdot}5\ \mu$; simple trituration of the larger aggregates with a glass rod in a little water did not easily sub-divide them further. The characteristics of this nickel sample were similar to those of the one obtained by Hueper (1955) from the International Nickel Co.

Control injections with fowl serum alone were not made, since in previous work (Heath, 1956), serum produced no reaction or tumours.

RESULTS

There was no clinical evidence of an immediate response to the injection, either local or systemic.

All the animals developed tumours at the injection site. The earliest tumour was noticed 17 weeks after injection and was removed for histological examination at $18\frac{2}{7}$ weeks; most of the others appeared within the next 5 weeks and the last was taken for histological examination at $40\frac{3}{7}$ weeks.

Three animals showed metastases, all of which were in the prevertebral lymph nodes.

Gross appearance of tumours

The major dimensions of the tumours ranged from $3 \times 2\frac{1}{2} \times 2\frac{1}{2}$ cm. to $5 \times 4 \times 4$ cm. Most of the tumours were pinkish-white, and the consistency varied from soft to firm; they showed very little necrosis, but three contained cystic regions filled with blood-stained fluid or thrombus.

Histological appearance of tumours

Primary tumours.—All of the tumours had clearly originated in striated muscle tissue; seven were well differentiated, and in the others differentiation ranged from poor to moderate. In the best differentiated regions, the tissue consisted of interwoven bundles of striated muscle fibres (Fig. 1), the disorientation of which resembled that seen in sections of muscle regenerating after crush injury (Le Gros Clark, 1946). We propose to use the term "rhabdomyoma" for tumours with this appearance, without thereby implying that they are benign. A tumour having this rhabdomyomatous appearance in most regions, together with small interspersed anaplastic components (Fig. 2), was used successfully for serial transplantation; histological examination of the first transplant, which took

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EXPLANATION OF PLATES

All stained with Azan. ×450

FIG. 1.—Area of tumour having an appearance defined as rhabdomyoma.
FIG. 2.—Anaplastic region of a similar tumour.
FIG. 3.—Invasion of tendon by fairly well differentiated rhabdomysarcoma.
FIG. 4.—"Muscle tube", similar to those found in regenerating muscle, lying between two normal muscle fibres.
FIG. 5.—Muscle fibre in which the centre is occupied by discrete cells.
FIG. 6.—Lymph node almost replaced by anaplastic tumour tissue.
FIG. 7.—Lymph node almost replaced by well differentiated rhabdomysarcoma.
FIG. 8.—Cross-sections of rhabdomyomatous fibres showing varying degree of sarcoplasmic vacuolation.

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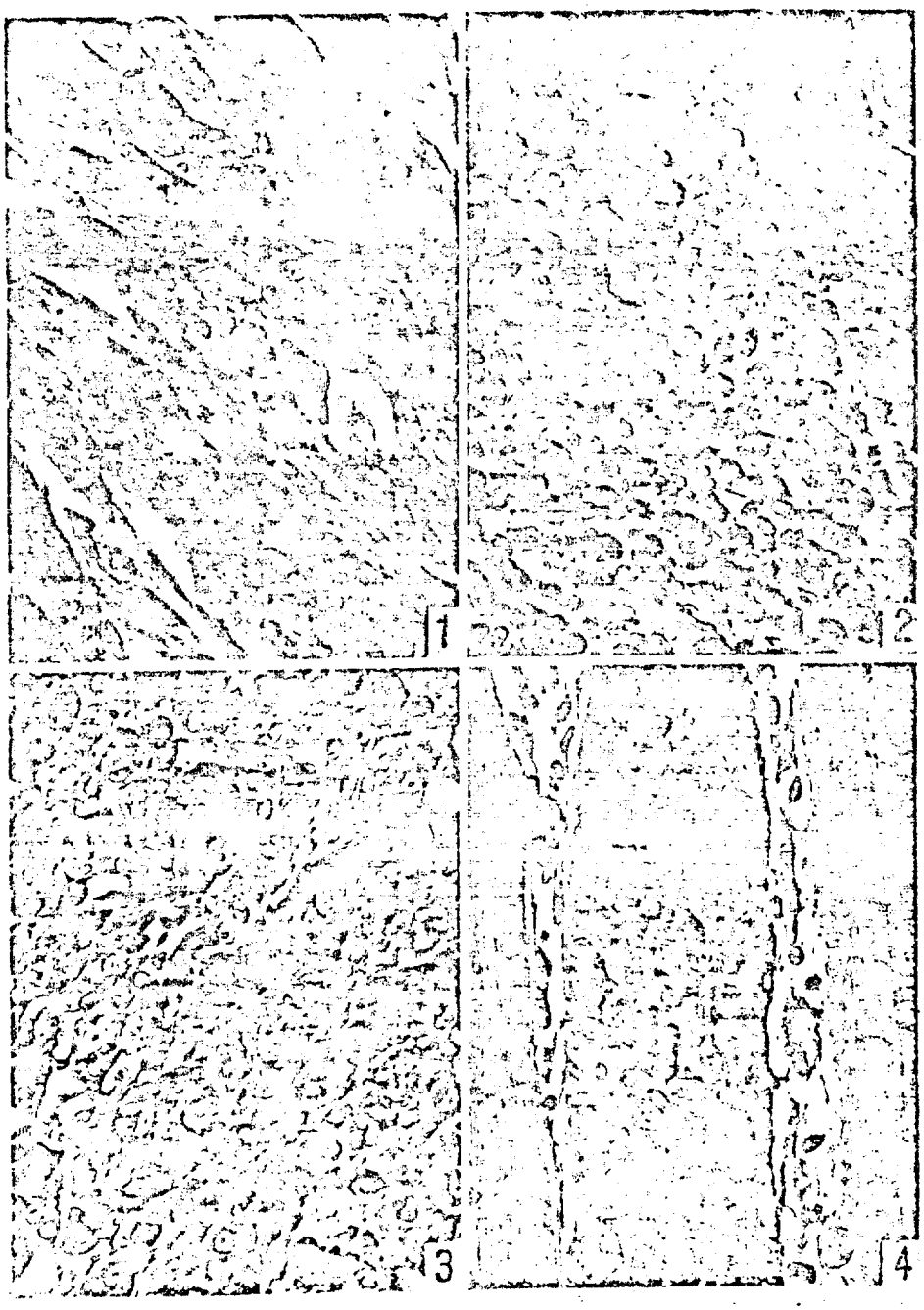

Heath and Daniel.

two months to mature, showed that the proportions of well-differentiated and anaplastic components were reversed. Subsequent transplants grew very much more rapidly (ca. 11 days), indicating that the anaplastic component was of high malignancy and outgrew the well-differentiated, less malignant element.

All the primary tumours were invasive (Fig. 3), and sections that included normal muscle fibres showed invasion of the normal tissue by one or both malignant components. In some areas the histological picture was difficult to interpret, because normal muscle fibres surrounded abnormal fibres identical (cf. Fig. 1) with those seen in the rhabdomyomata; the appearance of these fibres varied, some being almost normal and others obviously abnormal, but manifestly regenerating muscle such as that occasionally seen where the growing edge of a tumour confronts normal muscle was not present. In such regions there was clearly a transformation of normal muscle fibres to abnormal forms unlike anything we have seen in muscle exposed to cobalt or cadmium. It is not yet known whether these altered fibres at the periphery of the tumours were neoplastic, although similar fibres may form massive tumours and did occur in the primary transplant.

Another interesting feature of some tumours was the presence of muscle fibres in which the central sarcoplasm was replaced by a mass of cells, some round, some spindle-shaped and some in mitosis (Fig. 4). Such fibres resemble the "Muskelzellenzehläuche" (muscle cell tubes) described by Waldeyer (1865) in regenerating muscle, and shown clearly by Godman (1957), who gives a full list of references. Some of the cell-tubes seen in the tumours differed from the picture given by Godman, however, in that the two ends and the periphery of the fibre were composed of fully differentiated muscle tissue enclosing a conglomeration of cells (Fig. 5); they were very similar, however, to those observed by Adams, Denny-Brown and Pearson (1953) in muscle regenerating after coagulation by heat.

Metastases.—Of the metastases found in three animals, two were of the anaplastic type (Fig. 6) and the third a rhabdomyosarcoma showing a moderate degree of differentiation (Fig. 7).

DISCUSSION

Of the 26 tumours produced in rats by Hueper (1955) by the intrafemoral injection of nickel metal powder in gelatin suspension, all arose at the injection site; one was derived from bone and the others arose by seepage of the metal into the surrounding tissue. Of the latter, 16 were formed from the periosteal connective tissue and 4 appeared to develop from muscle. The myoblastic sarcoma illustrated in his paper (plate 3, fig. 2) shows a moderate degree of differentiation, and one of the malignant fibres displays clear central regions of a type often seen in our material (Fig. 8). There were also myoblastic elements in some tumours from his intravenous series. 14/16 of his tumours metastasized.

Of 36 tumours produced at the injection sites in Gilman's (1962) series of rats, most and possibly all of those induced by nickel sulphide, and most of those induced by nickel oxide, originated from striated muscle. Those tumours of which he gives illustrations appear to be of moderate to good differentiation. Gilman found metastases in 20/21 rats in which nickel sulphide was implanted and in 7/20 of those implanted with nickel oxide.

In our series of 10 tumours in 10 rats, all were clearly derived from striated muscle; the degree of differentiation was mostly very good, although some

tumours had anaplastic regions. There were metastases in three animals only, and of these only one was well differentiated: it is likely that the low incidence of metastasis was due to the very high degree of differentiation of the primary tumours.

It is worth noting that in our experiments the degree of differentiation of tumours produced by three metallic carcinogens—cobalt, cadmium and nickel—increases in this order. The high degree of differentiation seen in many of the nickel-induced tumours, coupled with the presence of new or altered muscle fibres which in some respects resemble those of normal regenerating muscle, suggests that the muscle tissue injured by nickel is not forced so far along the abnormal pathways of regeneration as that injured by cobalt (Heath, 1960) and cadmium (Heath and Daniel, 1964). A comparative histogenetic study of nickel-induced carcinogenesis must be made to elucidate some of these problems. Gilman and Basrur (1963) have published a preliminary note on the histogenesis of nickel sulphide-induced rhabdomyosarcomata in the rat; in general, the changes they observed in the tissue were broadly similar to those reported previously for cobalt (Heath, 1960).

SUMMARY

Powdered metallic nickel when injected intramuscularly into rats produced tumours of striated muscle origin, most of which were very well differentiated.

We wish to thank Professor Dame Honor Fell, F.R.S. for her continued interest in this work. We are grateful to Miss Angela Orledge for her skilful histological preparations and to Mr. W. G. Stebbings for his care of the animals.

This work was financed by grants from the British Empire Cancer Campaign.

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Brit. J. Cancer
21: 768-79 (1967)

CONTENT AND INTRACELLULAR DISTRIBUTION OF THE INDUCING METAL IN THE PRIMARY RHABDOMYOSARCOMATA INDUCED IN THE RAT BY COBALT, NICKEL AND CADMIUM

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Received for publication July 12, 1967

PREVIOUS studies in this laboratory have shown that cobalt, nickel and cadmium, when injected as the powdered metal into the thigh muscle of rats of the hooded strain, produce a high incidence of rhabdomyosarcomata, whereas a number of other metals, including iron, copper, zinc, manganese, beryllium and tungsten are not carcinogenic under these conditions (Heath, 1956, 1960; Heath and Daniel, 1964a, b; Heath, Daniel, Dingle and Webb, 1962). Tumours, which arise at any time from about 3 months after implantation of the active metals, and which metastasize readily in the primary animal, are transplantable into rats of the same strain. A cobalt-induced tumour, for example, has been maintained by transplantation for 13 years and is now in its 137th passage. Corresponding values for a nickel-induced and cadmium-induced tumour are $3\frac{1}{2}$ years and 54th transplant, and 6 years and 75th transplant respectively. All of these transplants preserve the characteristics of the primary rhabdomyosarcomata; in general the cobalt primary tumours are the least and the nickel tumours the most differentiated.

Since a metallic implant disappears from the site of injection, it is probable that the metal in the dissolved state is one of the main factors in the induction of malignancy. Ions of many of the above mentioned metals, whether carcinogenic or not, are inhibitory to tissue metabolism (e.g. Dingle, Heath, Webb and Daniel, 1962) and are toxic to chick and mammalian cells in culture (Heath, 1953; Daniel, 1961, 1964); no common specific biochemical activity of Co^{2+}, Ni^{2+} and Cd^{2+} has yet been demonstrated.

The work that is reported in this paper is designed to answer the following questions. Firstly, whether the tumours contain high local concentrations of the corresponding metallic ions; secondly, whether these ions are bound firmly and are retained after the dissolution of the metal implants, and, thirdly, whether the carcinogenic cations have a common pattern of distribution, and are associated primarily with a particular intracellular component or organelle.

MATERIAL AND METHODS

Biological methods.—Primary tumours were induced by the injection of a suspension of the finely powdered metal (28 mg.; Johnson, Matthey & Co. Ltd., Hatton Garden, London, E.C.1.) in horse serum (0·4 ml.) into the thigh muscle of 2–3 month old rats of the hooded strain as described by Heath (1956) and Heath and Daniel (1964a, b). Tumours for biochemical work were taken usually between 4 and 5 months after the initial implantation. At this stage of development they were free from any significant central necrosis.

Transplants were maintained by serial passage of tissue fragments (about 1·5 mm.^{3}) from the edge of an actively growing tumour into fresh rats at intervals of about 3–4 weeks. Insertion of the tumour tissue into the thigh muscle was made with a large-bore hypodermic needle.

All experimental animals were fed *ad libitum* on Diet No. 86 (North-Eastern Agricultural Co-operative Society Ltd.) rat cubes, and had free access to water.

Isolation of Cellular Components.—All operations were done in a cold room at 4° C. The tissue was homogenized for 2 min. with 9 vol. 0·25 M sucrose in a modification (J. T. Dingle and C. Mallows, unpublished data) of the motor-driven homogenizer described by Aldridge, Emery and Street (1960). The homogenate was strained through nylon gauze (200 mesh) and fractionated by differential centrifugation, essentially by the method of Schneider and Hogeboom (1950). The nuclear and mitochondrial fractions were sedimented by centrifugation for 10 min. at 700 g and 10,000 g (Serval SS-3 centrifuge). Each fraction was washed by resuspension in 0·25 M sucrose followed by recentrifugation under the above conditions, the washings being combined with the supernatant material before the next step. The microsomes were recovered by centrifugation for 1 hr. at 105,000 g (Spinco Model L Centrifuge), the residual supernatant solution being termed the soluble fraction.

Additional preparations of cell nuclei only were made by the lissapol method of Gilbert and Radley (1964).

Isolation of nucleic acids and deoxyribonucleohistone (DNP).—DNA was isolated by the methods of Marko and Butler (1951) and Kirby (1957), and RNA by the methods of Kay and Dounce (1953) and Kirby (1956). Any modifications that were introduced into these procedures are mentioned in the text. DNP was isolated by extraction with 1 M NaCl, essentially as described by Mirsky and Pollister (1946).

Determination of metal contents.—Glassware was cleaned as described previously (Webb, 1949). Water was re-distilled in a pyrex glass still.

Portions (about 1–2 g. wet weight) of the tumour, or other tissue were digested in a mixture of conc. HNO_3 (17 ml.) and water (3 ml.) on a sand-bath at about 150°. The solutions were evaporated until almost dry residues were obtained. These were dissolved in, and made up to a suitable volume (usually 5 or 10 ml.) with 1 N HCl (prepared from the re-distilled, constant-boiling acid) and the solutions filtered before analysis. Little or no material remained insoluble in HCl after digestion with HNO_3, in contrast to either a HNO_3–$HClO_4$ mixture (Buell, 1950) or H_2SO_4.

In the analysis of subcellular components, most of which were isolated in 0·25 M sucrose, extraction of the metal ions was used whenever possible in preference to digestion. In this procedure, a suitable volume of each fraction was made 5% (w/v) with respect to trichloroacetic acid (TCA) and kept in a glass stoppered flask at 60° C. for several hours, usually overnight. The denatured protein was removed and washed with 5% (w/v) TCA, the combined extract and washings being adjusted to volume with TCA and 5 N HCl to give final concentrations of these acids of 5% and 1 N respectively. In control experiments in which portions of homogenates of primary cadmium, nickel and cobalt tumours were extracted with TCA and also digested with HNO_3, the results obtained by the two procedures were identical.

The concentrations of the metallic ions in digests and extracts were determined

by atomic absorption with a Perkin–Elmer Model 303 Spectrophotometer. Excellent recovery of Cd^{2+}, Ni^{2+} and Co^{2+} was obtained when known amounts of these cations were added to homogenates of both rat liver and of the transplanted tumours before and after digestion, and there was no interference due to the high Na^{+} contents of the solutions. TCA affected the relationships between absorption and concentration of the above cations to different extents and the standard reference solutions that were used in the analysis of extracts were prepared in 5% (w/v) TCA and 1 N HCl.

Most analyses were made either by direct reading, or by scale expansion in conjunction with the recorder-readout accessory. When concentration was necessary, the aqueous solution was adjusted to pH 3·0 in the presence of 0·2 vol. 0·1 M glycine-HCl buffer of this pH, and 0·2 vol. of an aqueous 1% (w/v) solution of ammonium pyrrolidine dithio-carbamate (K. & K. Laboratories Inc., Plainview, New York) were then added. The use of bromo-phenol blue as an internal indicator simplified the pH control and did not interfere with the subsequent analysis. After dilution as necessary to a volume twice that of the initial solution, the chelated cation was extracted into an appropriately smaller volume of methyl isobutyl-ketone. As observed by others (e.g. Slavin, 1964; Sprague and Slavin, 1964a, b; Joyner and Finley, 1966) increased analytical sensitivity was obtained by the use of the organic solvent, and with a 4-fold concentration on extraction, it was possible to extend the lower limit of detection to one tenth of the normal value for Co^{2+}, Ni^{2+} and Cd^{2+}. This procedure was particularly valuable in the recovery of cations from extracts that in addition to TCA contained variable concentrations of sucrose. In aqueous solutions the absorption due to a fixed concentration of any one of the above cations was decreased by increasing amounts of the sugar and analysis of each extract had to be made by the method of additions.

Nucleic acid and protein determinations.—DNA was determined by Burton's (1956) modification of the diphenylamine procedure, and RNA by the orcinol reaction (Mejbaum, 1939). Commercial preparations of salmon sperm DNA (California Corporation for Biochemical Research, Los Angeles, U.S.A.) and yeast RNA (British Drug Houses Ltd., Poole, Dorset) were used as standards. Protein was estimated by the colorimetric method of Lowry, Rosebrough, Farr and Randall (1951) with bovine serum albumin (Fraction V. Armour Pharmaceutical Co. Ltd., Eastbourne) as standard.

Dry weights.—These were determined on tissue samples dried to constant weight at 105° C.

RESULTS

Contents of inducing metal cations in the primary tumours

Analyses were made on tissues from a total of 50 primary tumours (22 induced by nickel, 16 by cobalt and 12 by cadmium). Most of the tumours, which ranged in weight from 4 g. to 25 g., were removed 16–20 weeks after implantation of the powdered metal. A few older tumours (39, 45, 49 and 53 weeks) also were included in the survey. Dry weight : wet weight ratios for the healthy tissue of the tumours varied from 0·147 to 0·183 (mean value 0·162) and there were no significant differences in this ratio for tumours induced by any one of the three metals.

The presence of the cation of the inducing metal was detected in peripheral tissue (i.e. the growing edge) of all tumours except a slow-growing nickel tumour that was analysed 53 weeks after the initial implantation. Ni^{2+} was detected also

in a lymph node tumour that developed in an animal in which nickel powder had been administered intramuscularly. This tumour was probably a metastasis that developed secondarily to the rhabdomyosarcoma, although primary tumours have been observed to arise in the lymph nodes in response to the deposition of nickel sulphide, transported from the site of implantation by macrophages (Daniel, 1966).

In general, the content of ions of the inducing metal decreased from the centre to the periphery, and also with the age of the primary tumour. In both primary nickel and cadmium tumours of similar age, the contents of the cations in the peripheral tissue were very variable (e.g. 0·4–22·4 μg. Ni^{2+}/g. wet wt.; 0·5–56·3 μg. Cd^{2+}/g. wet wt.), whereas in the primary cobalt tumours the Co^{2+} concentration was somewhat more constant (0·35–3·8 μg. Co^{2+}/g. wet wt.). In some animals in which primary tumours were well developed at 16–20 weeks, significant amounts of the cation of the inducing metal (i.e. 0·5–2·0 μg./g. wet wt.) were found in muscular tissue from the opposite leg. The contents of the appropriate cation were also high in the liver, kidney and spleen, and in the limited number of analyses made, were always greater than in the peripheral tissue of the primary tumour. With cadmium implants in particular, very high amounts of Cd^{2+} were found in these organs (Table I); for example, in one series of analyses, the Cd^{2+} content of

TABLE I.—*Cd^{2+} Contents of Peripheral Tissue from Primary Tumours Induced by the Intramuscular Implantation of Metallic Cadmium into Rats and of the Livers, Kidneys and Spleens of the Host Animals*

	Rat number			
	9761	9746	9523	9518
	Cd^{2+} content (μg./g. wet weight)			
Tissue				
Tumour (peripheral tissue)	9·40	56·3	13·0	3·54
Liver	348·0	123·0	137·1	404·0
Kidney	142·5	—	—	178·8
Spleen	18·6	—	—	43·8

the liver was above 400 μg./g. wet wt., and was approximately double that of the kidney (179 μg./g. wet wt.), 9 × that of the spleen (43·8 μg./g. wet wt.) and more than 100 × that of the tissue from the primary tumour in the thigh (3·54 μg./g. wet wt.).

Under the conditions of the present analytical measurements, cations of the inducing metal were not detected in transplants of the cobalt and cadmium tumours, but Ni^{2+} (0·45–0·55 μg./g. wet wt.) was found in some transplants of the nickel tumour.

Intracellular distributions of cations of the inducing metal in primary tumours

Although, in these studies, the tumours were dissected before homogenization, and any visible remnants of the original metallic implant were discarded, the presence of residual metal granules remained a potential source of error. Excessively high values for the cation contents of the initial homogenates, therefore, were considered to indicate contamination, and the results of analyses on the fractions of these preparations were rejected. In the representative results shown in

Table II the contents of Co^{2+}, Ni^{2+} or Cd^{2+} in the homogenates, with the exception of one preparation from pooled cobalt tumours, were within the limits of the analyses summarized in the preceding section. These results show a common pattern of distribution of ions of the inducing metal in all of the three types of primary tumour. Thus, only 15% or less of the total cation content was associated with the soluble fraction, and the remainder was bound by the sedimentable cellular components. The major part was recovered in the nuclear fraction, a smaller amount being found in the mitochondria, and little or none in the microsomes. The latter finding was unexpected, as it was thought that the foreign ions might replace part of the normal complement of Mg^{2+}, or other bivalent cation of the ribosome. Both Zn^{2+} and Fe^{2+} occur regularly in the ribosomes of liver and other tissues (e.g. Petermann, 1964), and were present in appreciable amounts (0·2 and 0·26 μg./mg. protein respectively) in the microsomal fraction of the primary cobalt tumour (Table II).

In each experiment of Table II the subcellular components were isolated from the same homogenate in order that the distribution of the cation of the inducing metal amongst the different fractions of the tumour tissue could be determined. With the exception of one series of Cd^{2+} analyses, in which the recovery was low (88%) there was satisfactory agreement between the cation contents of the initial homogenates and the recovery in the subcellular fractions. It is well known, however, that these subcellular fractions, as isolated in 0·25 M sucrose, are not homogeneous, and the limitations of this method of fractionation have been summarized by Schneider (1961). In particular, the nuclear fraction is liable to contamination by intact cells, mitochondria and connective tissue fibres, and the disintegrated cell membranes also are known to sediment from sucrose homogenates with the nuclear fraction (e.g. Takenchi and Terayama, 1965). The results of Table II, however, show that contamination by unbroken cells and mitochondria cannot explain the high cation content of the nuclear fractions from the primary tumours. Furthermore, the close agreement that was obtained between the results of the four or more replicates of the nuclear suspensions that were analysed, is evidence for the absence of contamination of these fractions by either residual granules of the inducing metals, or by fibres of connective tissue that contained excessive amounts of bound cations. Significant redistribution of cations through adsorption on to the swollen agglutinated nuclei during the fractionation also seems unlikely from the results of a series of control experiments.

Thus, when low concentrations of Co^{2+} and Ni^{2+} were added respectively to homogenates of liver and transplants of the nickel tumour, before fractionation by differential centrifugation, both cations were bound by the sedimentable components (including the microsomes), but about 50% of each remained in the soluble fraction, and binding by the nuclei was not disproportionately great (Table III). An essentially similar distribution of Co^{2+} was found in the subcellular components that were isolated from the livers of animals that had been injected with $CoCl_2$ (Table IV).

In other controls approximately equal weights of primary and transplanted cobalt and nickel tumours were homogenized and fractionated separately. The nuclear fractions from the transplants were then combined with the soluble fractions of the corresponding primary tumours, and portions of these suspensions analysed for the appropriate cations before and after removal of the nuclei by centrifugation. Binding of the cation by the nuclei under these conditions was

TABLE II.—*Intracellular Distribution of Cations of the Inducing Metals in Primary Cobalt, Nickel and Cadmium Tumours*

Inducing metal	Content of inducing metal in initial homogenate: μg./g. wet weight original tissue	Content of inducing metal in initial homogenate: Total content (μg.)	Component	Fraction of homogenate: Nuclear	Fraction of homogenate: Mitochondrial	Fraction of homogenate: Microsomal	Fraction of homogenate: Soluble	Recovery of inducing metal in all fractions (% of total content of homogenate)
Nickel (Rat No. 8630, 8662, 8882, 8888	2·48	162·5	Protein (mg.)	—	212	111	—	—
			Ni^{2+} (μg.)	—	25·7	2·2	—	—
Nickel (Rat No. 9213–9219)	22·05	1102·5	Protein (mg.)	1240	184	100		
			Ni^{2+} (μg.)	924·4	116·7	9·3	45·7	99·4
Nickel (Rat No. 9514–9517)	21·7	710·0	Protein (mg.)	1037	186	945†		
			Ni^{2+} (μg.)	662·0	9·8	32·6†		99·2
Cobalt (Rat No. 8316, 8323, 8650, 8652, 8871, 8876)*	3·84	566·0	Protein (mg.)	5340	1087	585	4044	
			Co^{2+} (μg.)	420·5	36·8	6·0	89·2	97·6
Cobalt (Rat No. 9190, 9191)	30·0	1245·0	Protein (mg.)	1440	403	161	1228	
			Co^{2+} (μg.)	1167·2	71·1	0·0	51·1	103·5
			Zn^{2+} (μg.)	—	67·1	68·3		
			Fe^{2+} (μg.)	—	46·1	41·6		
Cadmium (Rat No. 9224–9226)	12·49	624·5	Protein (mg.)	1756	258	150	987	
			Cd^{2+} (μg.)	438·6	16·3	0·0	95·5	88·4
Cadmium (Rat No. 9752, 9760)	3·01	162·5	Protein (mg.)	1832	364	1582†		
			Cd^{2+} (μg.)	106·0	14·8	29·8†		92·6

* Random samples of the nuclear, mitochondrial and microsomal fractions of the homogenate of these tumours were examined by Dr. T. Hall of the Cavendish Laboratory, Cambridge, with the electron probe microscope. No contamination of any fraction with residual granules of the inducing metal was detected.

† In these analyses the microsomal and soluble fractions were not separated.

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TABLE III.—*Distribution of Co^{2+} and Ni^{2+} amongst the Subcellular Components of Liver and the Transplanted Nickel Tumour after the Addition of these Cations to the Tissue Homogenates Before Fractionation*

Homogenate	Cation added to homogenate	Cation concentration (μg./g. wet weight original tissue)	Cellular fraction	Total protein (mg.)	Total content of added cation (μg.)	Cation concentration (μg./g. protein)	Recovery % of added cation
Liver	Co^{2+} (120 μg.)	3	Nuclear	1420	21·0	14·8	91
			Mitochondrial	1763	33·2	18·8	
			Microsomal	224	7·1	31·7	
			Soluble	2704	48·1	17·8	
Transplanted nickel tumour (Rat No. 9391, 9393)	Ni^{2+} (675 μg.)	15	Nuclear	1520	208·7	137·5	86
			Mitochondrial	650	63·4	97·6	
			Microsomal	242	46·0	190·0	
			Soluble	1927	345·8	179·6	

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low, and accounted for 13% of the Ni^{2+} (3·08 μg.) and 17% of the Co^{2+} (8·1 μg.) in the soluble fractions.

TABLE IV.—*Distribution of Co^{2+} Amongst the Cellular Components of Rat Liver After the Subcutaneous Injection of $CoCl_2$*

Cellular fraction	Experiment I: Protein content mg.	Experiment I: Co^{2+} content μg.	Experiment II: Protein content mg.	Experiment II: Co^{2+} content μg.
Whole homogenate	—	—	—	356·2
Nuclear	1550	57·8	1346	105·5
Mitochondrial	1622	35·7	1406	44·6
Microsomal	368	5·0	624	25·2
Soluble	2572	180·6	2424	158·4

In these experiments male rats (about 250 g. body weight) were injected subcutaneously with 0·5 ml. (≡1·25 mg. Co^{2+}) of an isotonic solution of $CoCl_2$ $6H_2O$ (42·5 mM) in aqueous NaCl (94·5 mM). After being fasted for 17 hr. the rats were killed and the subcellular fractions prepared from homogenates of the appropriately pooled livers.

To eliminate the possibility that metal-rich fragments of cell membranes contaminated the nuclear fraction and contributed to its high cation content, nuclei were isolated from each of the primary tumours by the method of Gilbert and Radley (1964), the purification of the preparations being followed by phase contrast microscopy. The use of the lissapol solution in this procedure seemed less liable to cause artifacts through displacement of bound cations than other aqueous media that contained either Ca^{2+} (Schneider and Petermann, 1950; Wilbur and Anderson, 1951) or Mg^{2+} (Widnell and Tata, 1964). Before analysis, the nuclei, which contained 17·5–21·0% DNA, were precipitated with ethanol (5 vol.) at 0° C. to remove the detergent, and the residue was then dried with ethanol and ether. The results of these determinations (Table V) confirmed that cations of the inducing metal were concentrated in the nuclei from each of the three primary tumours.

TABLE V.—*Contents of Cations of the Inducing Metals in the Isolated Nuclei of Primary Nickel, Cobalt and Cadmium Tumours*

Primary tumour induced by	Cation content of tumour tissue: μg./g. wet weight tissue*	Cation content of tumour tissue: μg./g. dry weight†	Cation content of isolated nuclei μg./g. dry weight
Nickel	27·0	166·7	1086·0
Cadmium	1·18	7·3	49·8
Cobalt	3·82	23·6	176·7

* These analyses were made on randomly selected fragments of the tumour tissue before homogenization.

† Calculated from the average dry weight : wet weight ratio of 0·162 (see text).

Association of cations of the inducing metals with the nucleic acids of the primary tumours

Both Cd^{2+} (21–260 μg./g. DNP) and Ni^{2+} (20–120 μg./g. DNP) were found in the deoxyribonucleohistone from primary cadmium and nickel tumours respec-

tively. In similar preparations from three batches of the primary cobalt tumour, however, the Co^{2+}-content was too small to be determined accurately. Also this cation was not detected in the DNP from transplanted cobalt tumours that were removed 16–17 hr. after the subcutaneous injection of $CoCl_2$ into the hosts.

Most initial preparations of DNA were made by Kirby's (1957) method, with the exception that ethanol was used for precipitation. Thus, although treatment with RNAase was included in the purification of the DNA, the RNA-contents of the preparations were high (30–40%). The contents of the cation of the inducing metal in these mixed nucleic acids from primary nickel, cadmium and cobalt tumours ranged from 52–480, 50–260 and 0–100 μg./g. nucleic acid respectively. When ethoxyethanol and methoxyethanol were used in place of ethanol in the isolation procedure as described by Kirby (1957) the purity of the DNA preparations was increased to 92–95% and a lower, but consistent yield of 2·5–2·8 mg. DNA/g. wet weight tumour tissue was obtained. Only single preparations of DNA that were isolated in this way from the primary cobalt, cadmium and nickel tumours were analysed; these were found to contain 38 μg. Co^{2+}, 31 μg. Ni^{2+} and 52 μg. Cd^{2+}/g. DNA.

One sample of crude DNA (327 μg. Ni^{2+}/g.; 66·5% DNA) that was isolated from a primary nickel tumour with ethanol as the precipitant, was refractionated with ethoxy- and methoxy-ethanol to yield a preparation that was 94·5% pure and contained only 7·8 μg. Ni^{2+}/g. The combined supernatant fractions were dialysed to remove the organic solvents and frozen dried to recover the RNA. This contained 1016 μg. Ni^{2+}/g. (98% of the Ni^{2+} in the initial preparation) in contrast to the bulk RNA (yield 27 mg./g. wet weight tissue) that was isolated from a portion of the initial tumour and which contained 123 μg. Ni^{2+}/g.

These results indicate, therefore, that a significant portion of the total content of inducing cation that is associated with the nuclei of a given primary tumour is bound by the nucleic acid components, and, at least in the nickel tumour, binding by nuclear RNA is much greater than by DNA. Wacker and Vallee (1959), amongst others, however, have shown that the total heavy metal content of RNA is greater than that of DNA. Presumably, many of these cations are contaminants that are introduced during the isolation procedure, since the nucleic acids have a great capacity for base exchange (Kirby, 1957). The content of Zn^{2+} in a number of the initial DNA preparations, for example, was found to vary widely (41–788 μg./g. nucleic acid) and was invariably less (41–73 μg. Zn^{2+}/g. nucleic acid) in those samples that were isolated by the detergent method. Thus, the method of isolation may lead to the redistribution of cations, particularly with the primary tumours on the denaturation of protein with phenol.

DISCUSSION

It is known that intramuscular implants of cadmium, nickel and cobalt dissolve slowly in the tissue fluids. It is not unexpected, therefore, that in the tumours that are induced by these metals there is a decreasing concentration gradient of the corresponding cation from the centre to the periphery. The cation content also decreases with the age of the tumour as the metal is eliminated from the site of implantation. Transplants of these tumours, which develop in the absence of the inducing metals, neither concentrate nor require the corresponding cation in excess of any normal level for growth and survival.

Elimination of the implanted metals leads to the accumulation of the corresponding cations in the liver, kidney and spleen of the host. With cadmium implants the concentration of Cd^{2+} in these organs is very high, and a study of the intracellular distribution of this cation in the liver for example, might be of interest. Although the histology of the liver and other metal-containing organs has not been investigated, macroscopically the tissues appear normal and thus an excessive content of ions of the carcinogenic metals alone seem insufficient to induce the malignant change. As suggested by Heath (1960) it is probable that the induction of tumours at the sites of implantation is due to the combined effects of mechanical and chemical damage by the abrasive particles of metal and the attempted regeneration in the presence of the toxic cation.

In each of the primary tumours most of the total cellular content of the inducing cation appears to be associated with the nuclear fraction. Smaller amounts are found in the mitochondria, 15% or less in the soluble fraction, and little or none in the microsomes. *In vitro*, free ribosomes are known to be precipitated by various bivalent cations which in lower concentrations, may inhibit amino acid incorporation into protein by the particles (Petermann, 1964). Possibly, the low uptake of Co^{2+}, Cd^{2+} and Ni^{2+} by the microsomes *in vivo* is related in some way to their high content of Mg^{2+}. The latter is known to antagonize the uptake of Ni^{2+} and Co^{2+} by both bacteria and mitochondria (M. Webb, unpublished results).

In most of the analyses that are summarized in Table II, the contents of Co^{2+}, Cd^{2+} and Ni^{2+} in the mitochondria of the primary tumours range from about 50–150 mμg. cation/mg. protein. By tracer methods Dingle *et al.* (1962) found that isolated rat liver mitochondria bound about 2 μg. Co^{2+}/mg. protein N when incubated in the presence of 0·5 mM Co^{2+}, a concentration that gave 75% inhibition of pyruvate oxidation. In published experiments, slightly higher values (2·6 μg. Co^{2+} and 2·7 μg. Ni^{2+}/mg. protein N) have been obtained by atomic absorption analysis for the uptake of Co^{2+} and Ni^{2+} by rat liver mitochondria under the same conditions. Keto acid oxidation by mitochondria is extremely susceptible to inhibition by a number of bivalent cations: inhibition by Co^{2+} for example is apparent at a concentration of 50 μM. Although figures are not available for Co^{2+}-binding by the mitochondria at this low concentration, it is probably of the same order as the values (33·8 and 176 mμg. Co^{2+}/mg. protein; i.e. about 200 mμg. and 1035 mμg. Co^{2+}/mg. protein N) that may be derived from the data of Table II. Thus, it is to be expected that the presence of the cations of the inducing metals in the tumour mitochondria will affect their oxidative metabolism. These considerations led to a separate study of the activities of the mitochondria from the primary and transplanted tumours, the results of which are summarized in the following paper (Daniel, Heath and Webb, 1967).

Both DNA and RNA from the primary rhabdomyosarcomata contain the cations of the inducing metals and, subject to the limitations that are mentioned in the Results section, it seems that at least part of the high cation content of the nuclei of each of these tumours is associated with the nucleic acid components. Recently, Menke and Sarif-Sarban (1966) have reported that $^{60}Co^{2+}$ is incorporated into the nucleic acids of the chick embryo, and that the binding of the cation by DNA reaches a saturation point at a dose of 10 μg. Co^{2+}/egg. For the four-times precipitated DNA the Co^{2+} content (4 mμg./g. DNA) at this limit corresponds to average distribution of one cobalt atom in 46×10^6 nucleotides. In the DNA preparations from the primary metal-induced tumours, the isolation of which

involves dialysis and several precipitation steps, the cation contents are much larger than the value given by Menke and Sarif-Sarban (1966), but nevertheless are small when expressed as atoms/mole nucleotide. Studies on the properties of these nucleic acids are now in progress.

The results of Tables II and III show that the distribution of the inducing metal amongst sub-cellular fractions of the primary tumours differs quantitatively from that which occurs when the corresponding cations are added before fractionation to homogenates of transplanted tumours or liver. In particular, in the primary rhabdomyosarcomata 70–90% of the cellular content of the inducing metal is bound by the nuclei whereas in the latter systems binding by the nuclear fraction is much less (20–30%). Although these findings, coupled with those on the isolated nucleic acids, suggest that the carcinogenic metals interact with, and thus alter the genetic apparatus, it should be stressed that only well-developed tumours have been analysed, i.e. in which the malignant change had taken place many cell generations previously. Both Zn^{2+} and Co^{2+} are concentrated by spontaneous mammary tumours in C3H mice when the host animals are injected with the chlorides of these metals, and are found in preparations of DNP from these tumours (Heath and Liquier-Milward, 1950; 1951). It is possible, therefore, that ions of heavy metals interact more readily with the nucleic acids and nucleoproteins of any growing and dividing cells, malignant or normal.

SUMMARY

Primary rhabdomyosarcomata that are induced in rats by the intramuscular implantation of powdered metallic cobalt, cadmium or nickel develop in the presence of excessive local concentrations of the dissolved metals. In each tumour the content of incorporated metal decreases from the centre to the periphery and also, as the implant dissolves and is eliminated from the site of implantation, with the age of the tumour. Transplants of these tumours do not either concentrate or need the corresponding cations in excess of any normal requirements for growth and survival.

In the different primary tumours the distribution of the three inducing metal ions is similar, but differs quantitatively from that found when these cations are added before fractionation to homogenates of either liver or the transplanted tumours. Most of the inducing metal that is incorporated intracellularly by the primary tumours is bound by the nuclear fraction. Smaller amounts are found in the mitochondrial and soluble fractions, and little or none in the microsomes. At least part of the high cation content of the nuclei is due to binding by the nucleic acids.

This work was done with the support of the British Empire Cancer Campaign for Research (J. C. Heath) and of the Medical Research Council (M. Webb).

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Cancer 26(5): 1134-1141 (1970)

ARGINASE ACTIVITY IN NICKEL SULFIDE-INDUCED RAT TUMORS

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The arginase activity of a group of nickel sulfide-induced rat tumors was compared with that of normal rat tissues with and without prior treatment with nickel sulfide. Arginase levels were strikingly greater in tumor tissues than those noted in normal muscle tissue from adult rat and rat embryos. The level of arginase activity in muscle tumors did not approach that noted in the liver tissues either of normal rats or of those bearing nickel sulfide-induced rhabdomyosarcomas. A wide range was observed in the arginase activity of nickel sulfide-induced muscle tumors, an elevation generally paralleling the histopathologic feature and the age of the tumors. The increase in arginase activity in "older" tumors is interpreted to be a reflection of the selection of arginase-rich cells in the environment altered by nickel sulfide.

NICKEL SULFIDE (Ni_3S_2) IS A POTENT CARCINOGEN.[3, 4, 6] Single intramuscular injection with powdered Ni_3S_2 induces tumors in 85 to 90% of Fischer rats within 5 to 6 months.[3] The action of Ni_3S_2 in the induction of tumors is currently under study in our laboratory; enzymes which are activated or inhibited by metallic cations are particular interest in this investigation, as such enzymes would be expected to be altered in a nickel environment. Arginase (EC. 3.5.3.1) has been known to be activated by metal ions including Mn^{++}[9] and Ni^{++}.[7, 9] The present investigation is designed to compare the arginase activity in Ni_3S_2-induced muscle tumors with that of normal muscle tissue and to determine whether short-term in vivo exposure of muscle to nickel enhances arginase activity similar to that reported by Hellerman and Perkins[7] in in vitro studies.

MATERIAL AND METHODS

Tumor induction with Ni_3S_2 has been described elsewhere.[2-4] The tumors employed in this experiment were selected at random from a group of Fischer rats receiving single intramuscular injections of 10 mg of nickel sulfide. The powdered Ni_3S_2 was made into a slurry with ayercillin and was injected into each rear thigh muscle of 3-month-old male Fischer rats, and the routine procedure for housing and maintenance was followed till palpable tumors were detected.

The tumor induction period was recorded as the interval between the time of injection and the appearance of palpable tumors. The age of the tumor was calculated as the period between the first appearance of palpable tumor and the time the rat was sacrificed. The approximate size of each tumor was estimated from the diameter of the tumor obtained using a vernier caliper.

Rats were sacrificed by a blow to the head, and approximately 1.0 g of the tumor was removed from each tumor sample for enzyme assay. A representative portion of each tumor was fixed in neutral buffered formalin for histologic characterization. The tumor tissues for enzyme assay were blotted on filter paper, placed in cold tarred petri plates and their weight determined prior to mascerating and homogenizing in 3.5 volumes of cold distilled water using a hand grind-glass homogenizer. The homogenate was then diluted with an equal volume of carbonate-bicarbonate buffer (pH 9.5) containing 60 μmoles of $MnCl_2$ per ml, and was preincubated for 20 minutes at 55 C to ac-

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Supported by a grant-in-aid of research from the National Cancer Institute of Canada.

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The authors gratefully acknowledge the technical assistance of Mrs. Dorothy G. Friedman.

Received for publication November 10, 1969.

tivate the enzyme.[8] The preincubation solution was slightly turbid due to precipitation of $Mn(OH)_2$ at this *p*H. One-tenth ml of each homogenate was added to 0.4 ml amount of 0.1M arginine in 0.05M carbonate-bicarbonate buffer. Each enzyme reaction mixture (*p*H 9.7) was incubated at 25 C for 45 minutes, and the reaction was then stopped by the addition of 1.0 ml of 0.5M percloric acid. The homogenates were precipitated by centrifugation in a clinical centrifuge at 2900 rpm for 10 minutes and the amount of urea in the supernatant was estimated by color development with α-isonitrosopropiophenone in acid solution.[1] One-half ml of the supernatant was added to tubes containing 5.0 ml of a mixture of H_3PO_4:H_2SO_4:H_2O (27:9:64 by volume) and 0.5 ml of α-isonitrosopropiophenone in alcohol. The tubes were placed in boiling water for one hour prior to cooling and standing at room temperature for 15 minutes. The transmittance of the solutions were recorded at 540 mμ using a Bausch and Lomb "Spectronic 20." The tubes were protected from light during color development since the colored complex is photolabile. The enzyme activity was expressed as μmoles urea produced per mg wet weight of tissue, in 45 minutes at 25 C.

Urea standards and enzyme blanks were determined with each experiment. Enzyme blanks consisted of solutions in which the arginine solution was replaced by buffer and of solutions in which the perchloric acid was added to the enzyme reaction tubes before the addition of the homogenate.

Liver and muscle tissues from normal rats, tumor-bearing rats, and rat embryos of 14- to 17-day age were used as controls for arginase assay. In order to determine the effect of short-term exposure of normal tissues to nickel, liver and muscle from normal adults were assayed for arginase activity, 24 hours after intramuscular injection with Ni_3S_2. A batch of homogenates from each of these samples was incubated with buffer without $MnCl_2$ in order to test the effect of Mn^{++} on arginase levels.

RESULTS

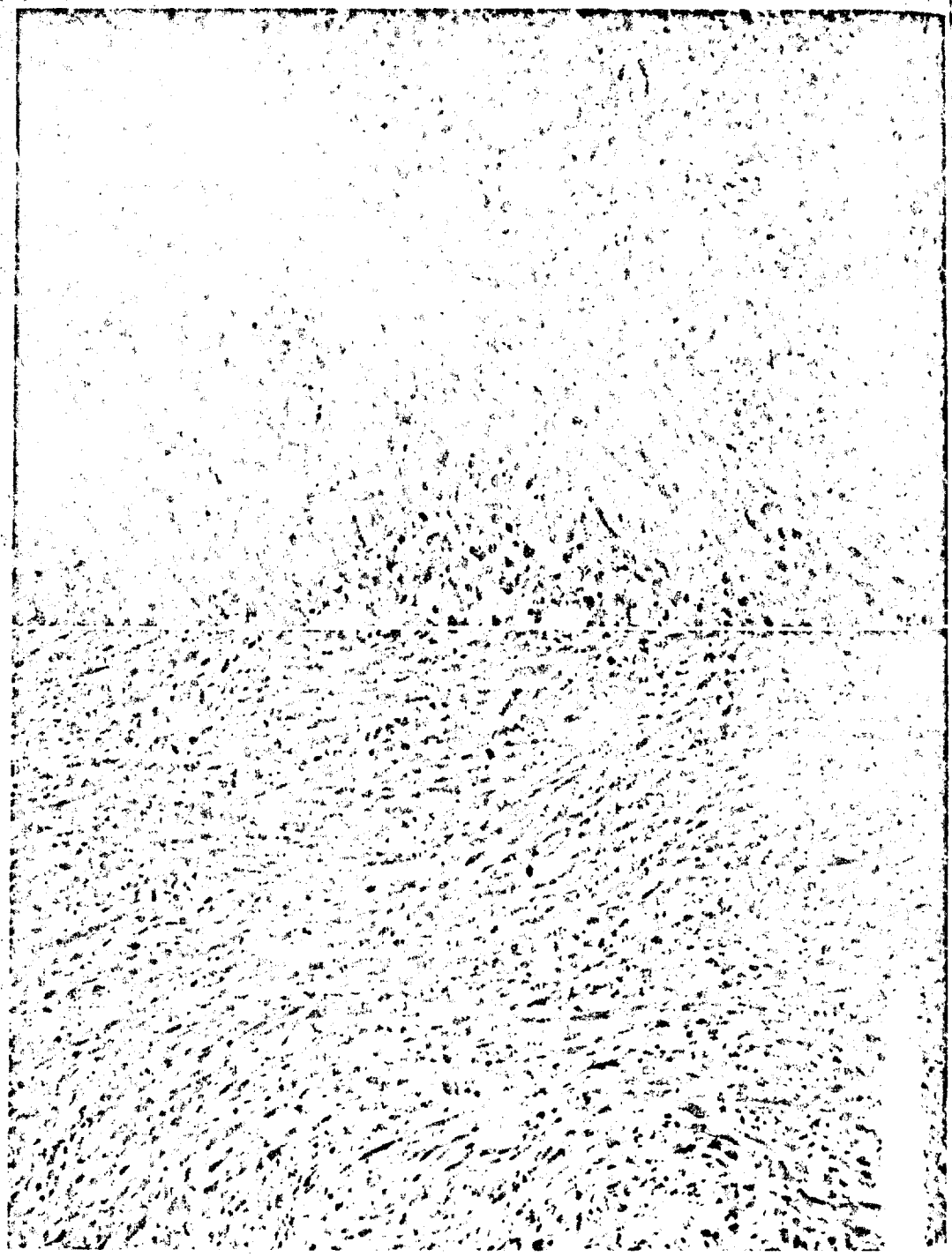
Of the 12 rats injected with Ni_3S_2, 9 developed tumors on both legs while the remaining 3 had a tumor on one leg only. Altogether, 11 tumors were included in this study for arginase assay and histologic characterization.

The histopathologic feature of the tumors indicated that 9 of 11 tumors were of striated muscle origin exhibiting varying degrees of muscle differentiation and mitotic activity (Figs. 1–4). Seven of these were pleomorphic rhabdomyosarcomas, while 2 were classified as rhabdomyoblastomas (Table 1). Of the remaining 2 tumors of nonmuscle origin, one was a "sclerosing hemangioma" exhibiting vascular lesions enclosed by spindle cells which were, at places, arranged in curlicues (Fig. 5). The other tumor which exhibited a high mitotic activity and cells with cytoplasmic processes (Fig. 4) was a reticulum cell sarcoma as confirmed by special stain for reticulum fibers (Fig. 6).

The arginase activity of these tumors varied from 0.009 μmoles to 0.122 μmoles of urea produced per mg wet weight (Table 1). The lowest arginase activity was detected in the tumor classified as sclerosing hemangioma (Table 1). The other tumor of nonskeletal muscle origin also exhibited a relatively low level of arginase although 2 of the skeletal muscle tumors, B2 and D6, also showed relatively low levels of this enzyme. In a majority of tumors of skeletal muscle origin, the arginase values were noted to be within the range of 0.049–0.079. In one rhabdomyosarcoma (Figs. 7, 8) with a large population of multinucleated strap cells, the arginase value was noted to be 0.122 which was the highest value detected in these tumors, while the 2 tumors with high mitotic activity, small patches of necrotic areas, and relatively few strap cells (Figs. 3, 4) exhibited low levels of arginase activity.

It is interesting to note that in 2 rats (F and C) whose tumors from both legs (C3-C4 and F8-F9) were used in this study, the arginase activity varied considerably between tumors (Table 1).

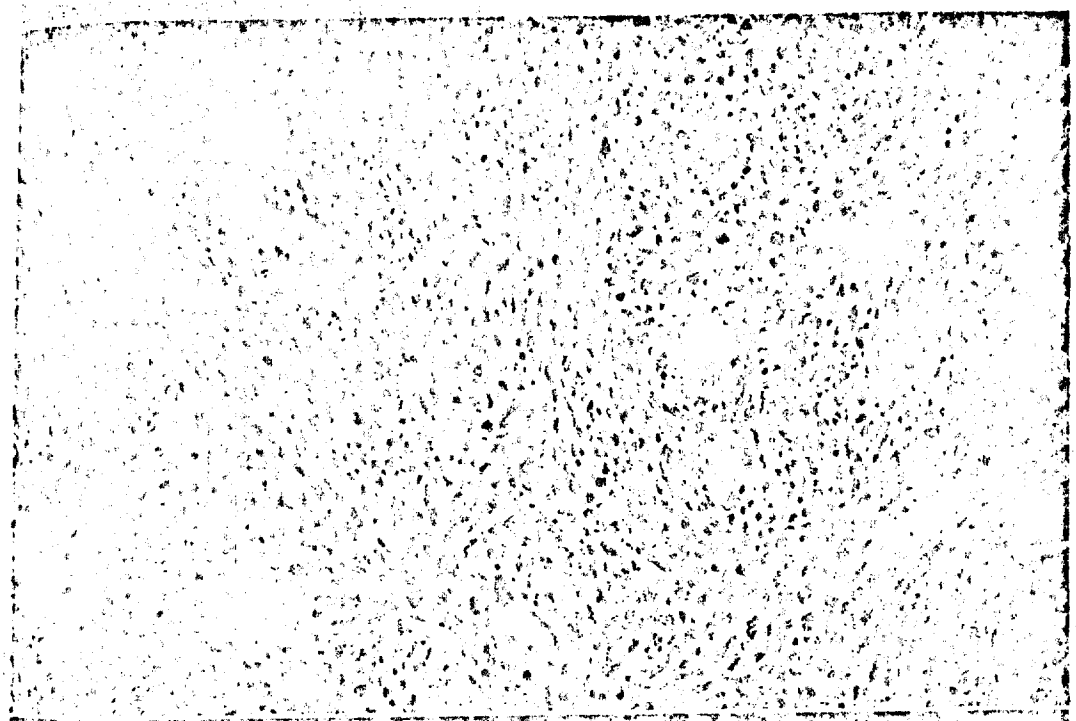
The induction time of the rhabdomyosarcomas varied from 113 to 189 days. Tumor-induction time, or the growth rate as characterized by the approximate size of the tumor at harvest, did not appear to have any apparent relation to the level of arginase activity (Table 1). The age of tumor and arginase activity seemed to show a correlation in that among the tumors classified as rhabdomyosarcomas the highest value for arginase was noted in the oldest tumor (F8) which was sacrificed 55 days after detection.



FIGS. 1–4. Histologic sections of nickel sulfide-induced tumors of skeletal muscle origin (H and E).

FIG. 1 *(top)*. A pleomorphic rhabdomyosarcoma (G10) exhibiting a large number of "redifferentiating" muscle cells and relatively low mitotic activity (×200).

FIG. 2 *(bottom)*. An active rhabdomyosarcoma (A1) with numerous thin strap cells and abundant mitotic activity (×200).



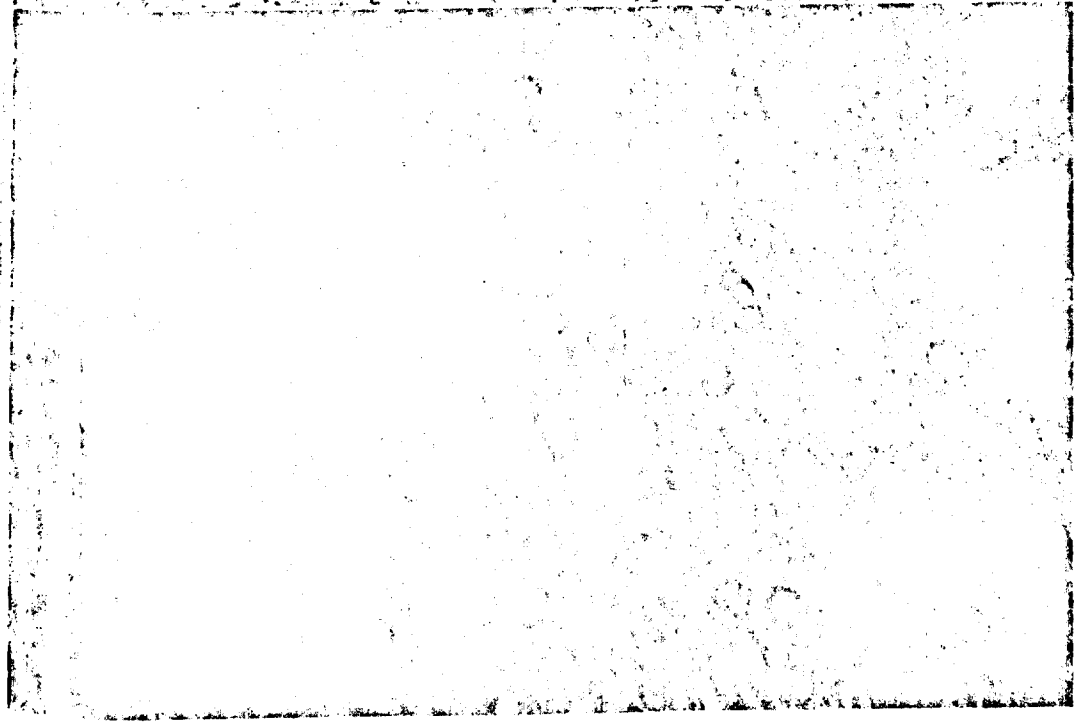


FIG. 3 *(top)*. A rhabdomyoblastoma (D6) showing high mitotic rate and a large number of multinucleate muscle elements (×200).

FIG. 4 *(bottom)*. A higher magnification of a rhabdomyoblastoma (B2). Note the tripolar mitosis in center and the muscle element with peripherally placed nuclei (×400).

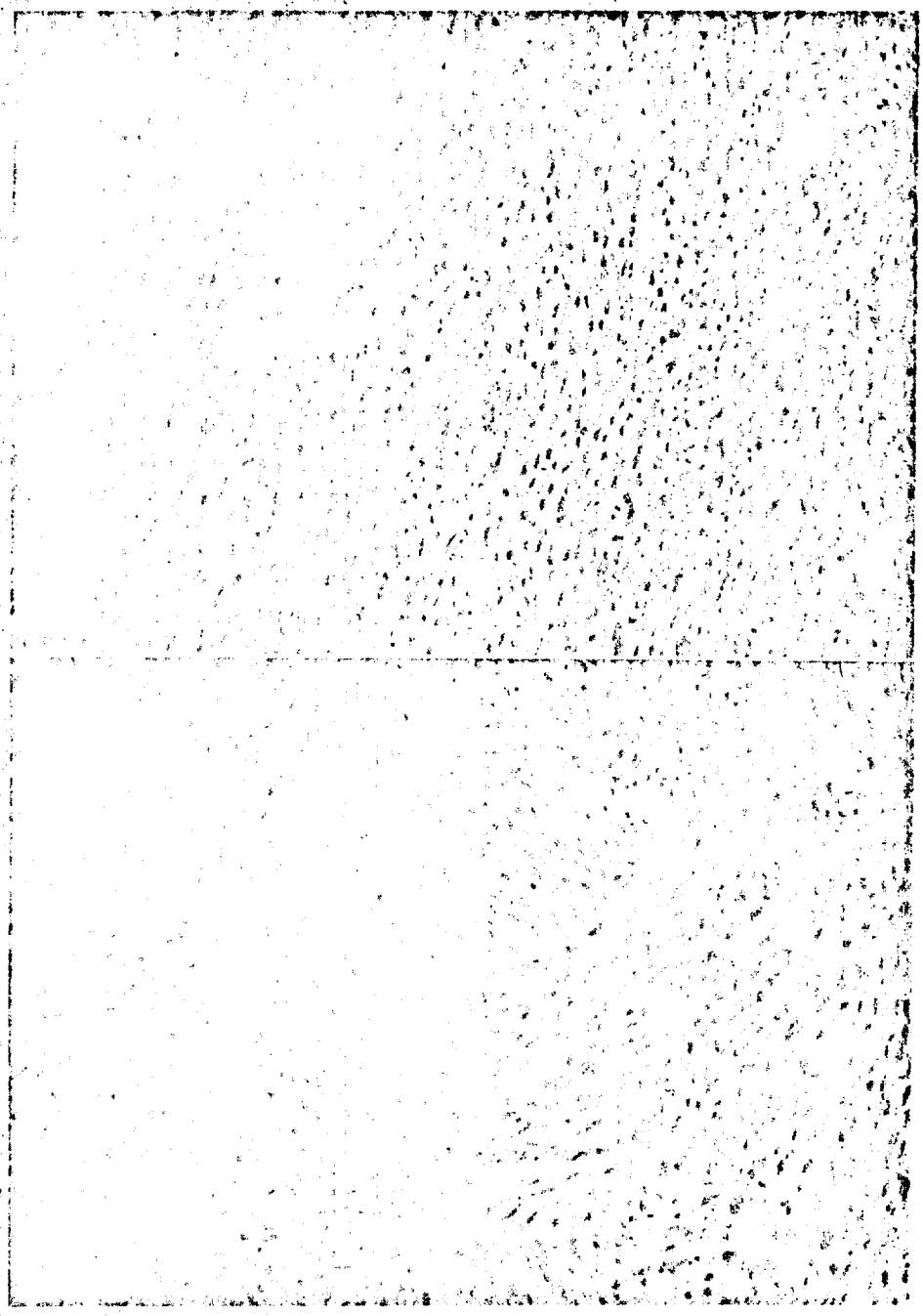
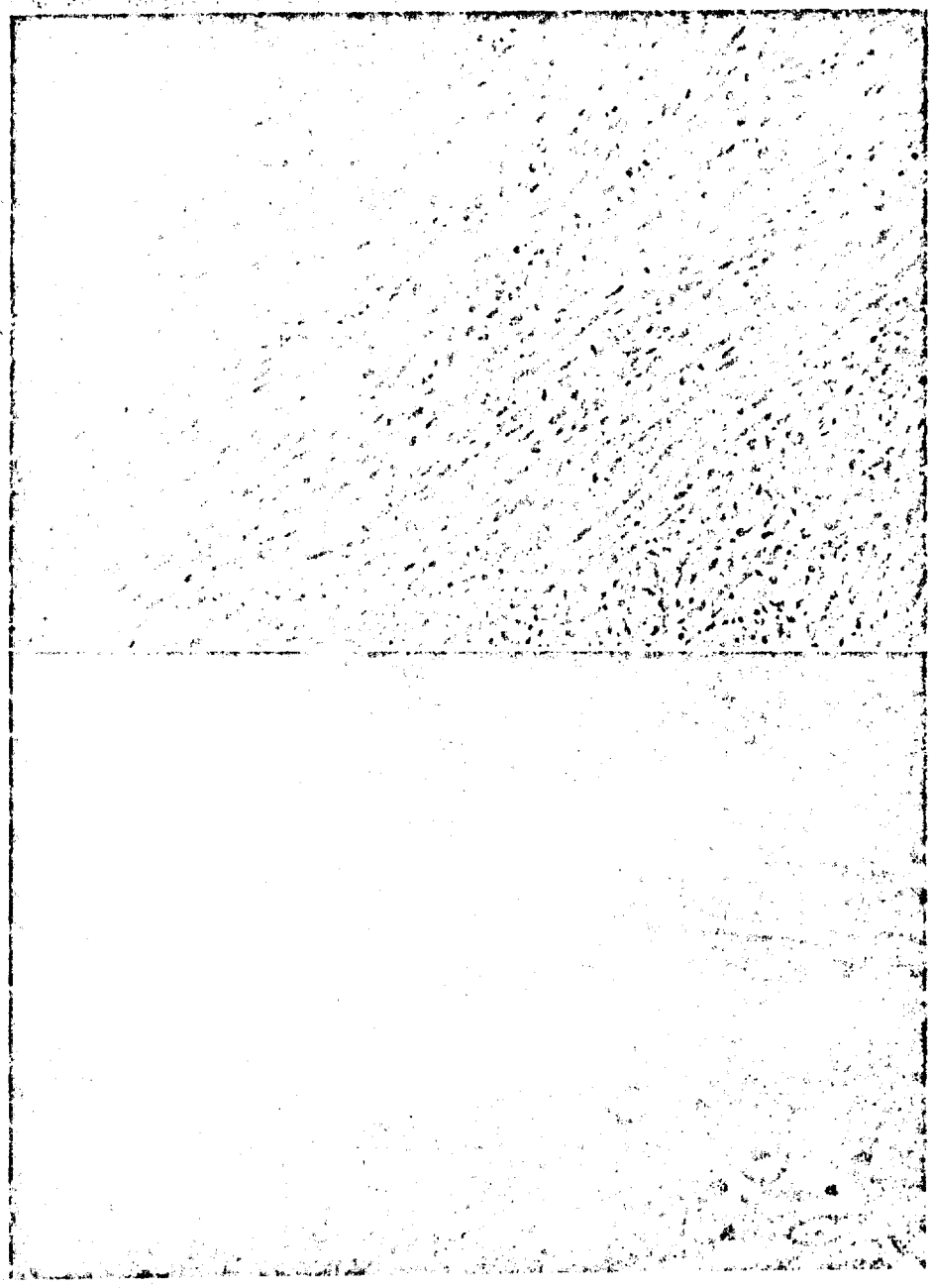


FIG. 5 *(top)*. A sclerosing hemangioma showing vascular lesions surrounded by spindle cells.
FIG. 6 *(bottom)*. A reticulum cell sarcoma (D5).

There was no detectable arginase activity in normal adult muscle and embryonic muscle since the values obtained for these samples were within the range of that noted for the enzyme blanks (Table 2). The arginase levels in "normal" thigh muscle of tumor-bearing rats, and in skeletal muscle exposed to Ni_3S_2 for 24 hours did not exhibit any striking difference, whereas those of liver tissues were relatively high (Table 2).

The arginase activity in the liver homogenates incubated without Mn^{++} was consist-



FIGS. 7 and 8. Histologic sections of the rhabdomyosarcoma (F8) with high arginase activity.
FIG. 7 *(top)*. A typical area of F8 showing a large proportion of redifferentiating muscle elements (×200).
FIG. 8 *(bottom)*. A higher magnification of F8. Note that the mitotic activity in this tumor is relatively low.

ently lower than in the corresponding samples incubated with Mn^{++}. There was no appreciable difference in the values obtained for muscle homogenates from adult and embryos when Mn^{++} was omitted.

DISCUSSION

There was a definite enhancement of arginase activity in the tumor tissues as compared to that of normal muscle. The argi-

TABLE 1. Histopathologic Features, Growth Characteristics, and Arginase Activity of Ni_3S_2-Induced Rat Tumor.

Tumor number	Histopathology	Induction time (days)	Age (days)	Size (4/3 r^3)	Arginase activity* (μmoles of urea/mg wet wt.)
A1	rhabdomyosarcoma	113	12	0.70	0.049
C4	rhabdomyosarcoma	154	20	14.16	0.051
E7	rhabdomyosarcoma	175	12	0.70	0.058
F8	rhabdomyosarcoma	140	55	14.16	0.122
F9	rhabdomyosarcoma	168	27	0.70	0.069
G10	rhabdomyosarcoma	161	47	4.19	0.079
G11	rhabdomyosarcoma	189	19	9.43	0.070
B2	rhabdomyoblastoma	120	17	1.77	0.013
D6	rhabdomyoblastoma	179	8	0.07	0.017
C3	sclerosing hemangioma	133	14	14.16	0.009
D5	reticulum cell sarcoma	168	19	0.70	0.014

* Arginase activity expressed as μmoles of urea produced in 45 minutes per mg wet weight of tissue.

nase activity of tumors did not, however, approach the level of activity found in liver tissues. These findings agree with those reported by Greenstein[5] that the enzyme activity in tumors of rats and mice lies between or below the range noted for normal tissues. The arginase activity in the liver of tumor-bearing animals was lower than that in normal rats. This may be due to a different response of liver cells to Ni^{++} which is known to enter the blood stream following intramuscular injection of Ni_3S_2 or due to systemic effects of the tumor on the liver.

The effect of Ni_3S_2 could be of an indirect type in that it facilitates the selection of a malignant cell (or cells) which has acquired a general complement of enzymes facilitating uncontrolled cell division rather than a direct enhancement of arginase activity in muscle elements by the nickel cation. This conclusion is supported by the fact that a 24-hour exposure to Ni_3S_2 in vivo did not enhance arginase activity of muscle or of liver (Table 2). Hellerman and Perkins[7] found that Ni^{++} activates arginase activity in vitro; however, in the present study, no increase in arginase activity was noted as a result of in vivo Ni_3S_2 treatment. One might suppose that the effect of nickel on arginase activity is being masked by Mn^{++} in the enzyme assay solution. However, this does not appear to be the case, as shown by the data in Table 2. Although the omission of Mn^{++} in the enzyme assay solution reduced the enzyme levels, the relative value of arginase activity in adult and embryonic muscle remained within the range

TABLE 2. Arginase Activity in Liver and Skeletal Muscle of Fischer Rats, with and without Mangane (Mn^{++}) Added In Vitro

Tissues	Source	Arginase activity: With Mn^{++}	Arginase activity: Without Mn^{++}
Liver	Normal adult rat	2.360	1.810
Liver*	Tumor-bearing rat	1.940	1.740
Liver	Ni_3S_2-treated rat+	1.930	1.470
Liver	Rat embryo	0.810	0.290
Muscle	Normal adult rat	0.001	0.002
Muscle*	Tumor-bearing rat	0.006	0.006
Muscle	Ni_3S_2-treated rat+	0.007	0.006
Muscle	Rat embryo	0.002	0.001
	Enzyme blanks	0.004 ± 0.003	

* Histologically unaffected liver and thigh muscle from rats with Ni_3S_2-induced tumor on one leg.
\+ 24 hours after intramuscular injection.

noted for the blanks. It is conceivable that the arginase in vivo is maintained at an optimal level by cations present in the tissue so that addition of nickel would not activate it further. The decrease in arginase activity in the liver tissue of tumor-bearing rats and rats receiving intramuscular injection with Ni_3S_2 may be a reflection of a systemic effect on the liver.

The fact that the embryonic muscle exhibited no arginase activity indicates that the increased arginase activity of the tumors is not merely a reflection of higher mitotic activity coupled with poor differentiation generally noted in tumor tissues. However, a tendency for higher arginase activity was evident in tumors of skeletal muscle origin exhibiting lower mitotic rates and higher degree of muscle differentiation, as that noted in F8. This would indicate that the higher levels of arginase activity are compatible with redifferentiation of tumor cells whereas the lower levels are indicative of increased anaplasia. The increase in arginase activity with the age of rhabdomyosarcomas is perhaps a reflection of the selection of specific cell types capable of redifferentiation in an environment altered by nickel sulfide.

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Effect of Duration of Exposure on Nickel Sulphide Tumorigenesis

NICKEL and nickel compounds, notably nickel sulphide, are potent local acting carcinogens when administered parenterally to laboratory rats[1,2]. The tumour arising from exposure of muscle tissue to powdered nickel sulphide is almost invariably a pleomorphic rhabdomyosarcoma; a fact suggesting a high tissue specificity for this metal[2]. Disks of nickel sulphide implanted in the hip muscles of laboratory rats had essentially the same tumorigenic effect as did the implantation of chips or powder[3]. Comparable results were also obtained when 5-μ particles of nickel sulphide were implanted in 'Millipore' diffusion chambers of 0·45μ porosity[3]. This communication is concerned with establishing the approximate exposure time necessary for nickel sulphide to bring about malignant change in muscle tissue.

Solid disks of pressed nickel sulphide powder measuring 8 mm × 1 mm and weighing approximately 250 mg were utilized for implantation, in order to facilitate the total removal of the carcinogen from the implantation site at exposure times of 2, 4, 8, 16, 32, 64, 128 and 256 days. This geometrical progression of exposure periods was chosen so that histological examination, particularly of the early, acute stages, of the lesion might be undertaken[4]. Fischer rats of our own inbred sub-line were used in this experiment, and an implant of nickel sulphide was inserted into the right gluteal region of each animal. An equal-sized disk of ferric oxide—non-carcinogenic under similar experimental conditions[3]—was placed into the opposite hip as a control. Groups of 15 rats were assigned to each exposure time. At the end of each period, both implants were removed from 10 randomly selected animals of the particular group of 15; these animals were kept under prolonged observation for tumour development. The remaining 5 animals of each group were killed at the end of their period of exposure for histopathological examination[4].

The tumour incidence observed, following the various periods of exposure to the nickel sulphide disks, are summarized in Table 1. No tumours developed at any of the ferric oxide implantation sites.

Table 1. EFFECT OF DURATION OF EXPOSURE TO NICKEL SULPHIDE DISKS ON TUMOUR INCIDENCE

	Days to removal of implants				
	2; 4; 8; 16	32	64	128	256
No. of effective animals*	10 10	9	10	10	10
Palpable tumours	0	0	4	7	10†
Average latent periods (days)	—	—	191	205	230
Range of latent periods (days)	—	—	171–242	160–265	183–256

* Animals surviving longer than the shortest latent period encountered.
† Not randomly selected (see text).

Table 1 shows that no tumour resulted from exposure times of less than 64 days. It is evident that in both the 64- and 128-day groups all tumours arose long after the time of removal of their respective implants. However, the 256-day exposure period exceeded the average tumour latent period for this group and 10 of the 15 animals had already developed palpable tumours at the time of implant removal. The remaining 5 animals, being without palpable tumours, were killed for the histological study. Thus the occurrence of 100 per cent tumour-bearing rats, shown in Table 1 for the 256-day group, does not represent a random incidence sample and the average tumour incidence after this length of exposure was actually 66 per cent.

The results establish the existence of a critical period of carcinogenic action necessary to malignant change in muscle tissue and occurring some time after 32 days' exposure to nickel sulphide. This change had already occurred in 40 per cent of the animals in which the carcinogen was removed at 64 days and in 70 per cent by 128 days.

Fig. 1. Micro-tumour developing in dedifferentiating muscle tissue at 128-day exposure to nickel sulphide. Normal muscle top right. Necrotic zone and implanted nickel disks not shown (below lower border) (hematoxylin and eosin)

plastic films have been reported to require a minimum of six months *in situ* after subcutaneous implantation in order to produce malignant tumours. Removal of plastic implants subsequent to this critical exposure period in no way affected the production of sarcomas, the latent periods of which ranged between 1 and 2 years[5]. Hepatic cancer produced in rats with a diet containing 2-acetylaminofluorine or 4-dimethylaminoazobenzene is also believed to require a specific length of exposure with the critical period occurring in a large proportion of animals at 50–75 days[6].

In addition to the palpable tumours occurring in those animals listed in Table 1, histological changes of an early cancerous nature were observed in 4 of the 5 animals killed for histology at 128 days' exposure and in one of the corresponding 5 animals of the 256-day group. Similar lesions were not noted in tissues from the 64-day group. Such microscopic lesions occurred in the zones of dedifferentiating muscle surrounding the implant and were considered to be actual micro-tumours. A typical trans-section of such a micro-tumour observed in the 128-day group is illustrated in Fig. 1. The tendency of the myoblasts to coarse fibrillation, pleomorphism and syncytial formation along with the reappearance of mitoses are prominent features characterizing this as an early rhabdomyosarcoma.

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Analyst 87, 650-2 (1962)

Spectrophotometric Determination of Nickel in Wheat Fractions

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A method is described for determining nickel in bran, feed and flour; solvent extraction is avoided, and the method is suitable for multiple determinations. Middleton and Stuckey's digestion procedure is included, and the selectivity of dimethylglyoxime for nickel is utilised. A compact apparatus for filtration and the stability of the final colour contribute to the ease and reliability of the method.

Destructive rust epidemics in grain-growing areas have occasioned interest in chemical means of control. New rust-resistant varieties of wheat are often attacked by new races of rust within a few years of their development. Wheat can be protected against infection from the seedling stage to maturity by frequent applications of such compounds as zineb (zinc ethylenebisdithiocarbamate) or sulphur, but this involves a commitment of considerable time and expense without proof of real necessity in a given crop year. Obvious advantage would be offered by a rust-eradicative treatment to be used only when rust is noticeable on the plants. In greenhouse and field tests, low concentrations of nickel salts displayed fungicidal activity against cereal rusts through both protective and eradicative action.[1,2,3,4] Application of these salts to wheat increased acreage yields, and calculations showed that growers would receive greater net returns after deducting the cost of spraying.[5] These findings have stimulated the interest of both plant pathologists and chemical companies engaged in the manufacture and sale of fungicidal chemicals. However, accurate information in terms of the accumulation and distribution of nickel in the grain was required in order to afford a sound basis for judging any possible toxic hazard. It was foreseen that a considerable number of samples would be involved in such a survey; further, this number would be trebled by the necessity for milling the wheat into bran, feed and flour fractions for distribution studies.

Of the analytical procedures available it was considered that either Forster's method[6] or that described by Alexander, Godar and Linde[7] might be adapted for our purpose. These procedures involve multiple solvent extraction of the nickel complex, and it was soon evident that the great amount of time spent in manipulating separating funnels would be a serious drawback to either method. However, preliminary work showed that Alexander, Godar and Linde's method gave reliable results, whereas instability of the colour made Forster's method unsatisfactory for determining nickel in wheat fractions.

Other features detracting from the suitability of these methods were the potential danger introduced by the use of perchloric acid and the constant and close attention required during the preliminary wet digestion.

A method described by Middleton and Stuckey[8] for destroying organic matter had important advantages over other methods. The main reagent used was nitric acid, and only relatively small amounts were needed; the only attention required was the intermittent addition of fresh acid, and there were no critical stages in the procedure. It seemed likely that this method of preparing the sample could be extended to the determination of nickel.

The procedure developed includes this more efficient digestion, retains the selectivity of dimethylglyoxime for nickel and offers the convenience of a stable spectrophotometric end-point.

Method

Apparatus—

Filtration apparatus—The assembly shown in Fig. 1 was devised to facilitate the handling of multiple samples. The manifold at the back consists of a series of T-joints connected by tubing.

Spectrophotometer—A Beckman DU instrument equipped with matched 1-cm silica cells was used.

Glass spatula—A glass handle was attached to a 0·5-inch section taken from the wall of glass tubing that just fitted inside a 15-ml sinter funnel.

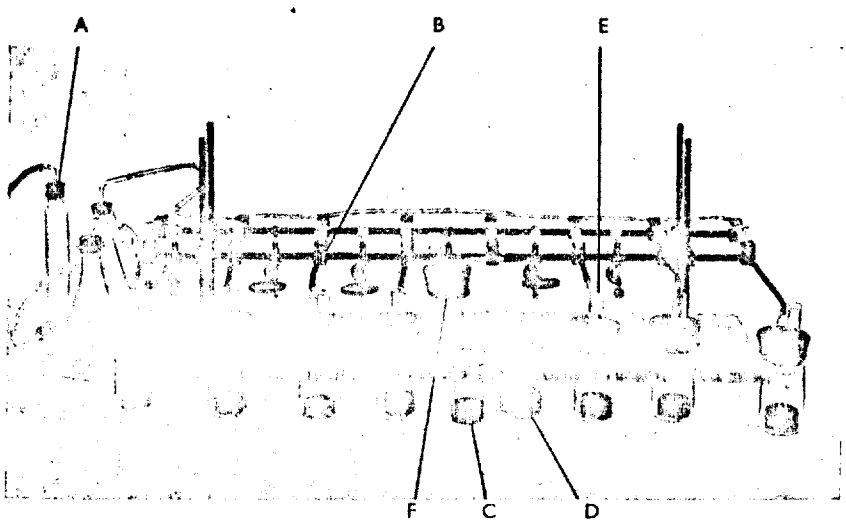

Fig. 1. Filtration assembly: A, scrubbing system (suction flask containing concentrated solution of sodium hydroxide, empty suction flask as spray trap and tower filled with calcium chloride); B, 3-way stopcock; C, inner 200-ml Berzelius beaker, with polythene cover; D, outer 500-ml Berzelius beaker (lipless); E, 15-ml funnel with fine sinter; F, No. 14 rubber stopper

REAGENTS—

Nitric - sulphuric acid mixture—Mix 95 ml of nitric acid, sp.gr. 1·42, with 5 ml of sulphuric acid, sp.gr. 1·84.

Nitric acid, fuming, sp.gr. 1·50.

Sodium tartrate dihydrate solution, 20 per cent. w/v, aqueous.

Dimethylglyoxime solution 1 per cent. w/v, in ethanol.

Ammonia solution, sp.gr. 0·90.

Bromine water, saturated.

Potassium persulphate solution, saturated, aqueous.

Standard nickel solution, 1 mg per ml—Dissolve 6·7301 g of ammonium nickel sulphate, $(NH_4)_2SO_4 \cdot NiSO_4 . 6H_2O$, in water, and dilute to 1 litre.

TREATMENT OF SAMPLES—

Wheat kernels (var. Thatcher) were milled by the procedure described for the Ottawa micro flour mill,[9] and the weights of bran, feed and flour were recorded; the yields were approximately 65 per cent. of flour, 27 per cent. of bran and 8 per cent. of feed.

PROCEDURE—

Place a weighed portion of the sample in a lipless 1-litre Berzelius beaker, moisten with 10 to 15 ml of water, add 10 ml of nitric - sulphuric acid mixture, and evaporate gently to dryness. Allow the beaker and charred contents to cool, add sufficient nitric acid, sp.gr. 1·42, to moisten the residue, cover with a clock-glass, and place on a hot-plate at about 350° C (as registered by a surface thermometer). Evaporate to dryness, and continue heating for about 20 minutes. Remove from the hot-plate, allow to cool, and repeat the addition of nitric acid and subsequent heating until white patches appear in the residue. Substitute fuming nitric acid for the nitric acid, sp.gr. 1·42, when incandescence will not occur in the sample, and repeat the operation until a white residue is obtained. Remove the clock-glass, and continue heating until oxides of nitrogen have been expelled. Cool, add 15 ml of dilute sulphuric acid (1 + 4), cover the beaker, and heat at 350° C until fumes are evolved. Cool, rinse the sides of the beaker with 15 to 20 ml of water, and heat uncovered until fumes are evolved; do not evaporate to dryness. Repeat the rinsing and heating, then transfer the acid solution to a 200-ml Berzelius beaker; repeatedly rinse the 1-litre beaker, with water, adding the rinsings to the contents of the 200-ml beaker, until the final volume is about 100 ml.

Evaporate to about 20 ml, allow to cool somewhat, add 5·0 ml of sodium tartrate solution, and heat to 80° C. Then add 4·0 ml of dimethylglyoxime solution, 2 drops of bromothymol blue indicator solution and ammonia solution, sp.gr. 0·90, dropwise, with constant stirring, until the solution becomes purplish in colour; the pH, which should be 8 to 9, can be checked externally by means of an indicator paper. Cover the beaker, and set aside, preferably overnight.

With use of a glass rod having a rubber-covered tip and repeated washings with small portions of water, transfer all the precipitate to a fine-porosity 15-ml sinter funnel, and retain the beaker for the next step (see C, Fig. 1). Add 1·0 ml of saturated bromine water to the contents of the funnel, and bring the liquid into contact with the entire wall area by means of the glass spatula. After 5 minutes, apply suction, and collect the filtrate in the beaker retained previously. Continue to apply suction, and wash the sinter with hot water. (The inner beaker is covered with a polythene disc during this operation to exclude any rubber "crumbs" from the stopper—see C, Fig. 1; the stem of the sinter funnel passes through a hole bored in the disc.) Add a second 1-ml portion of bromine water as before, and wash well with hot water.

Boil the filtrate for at least 15 minutes to expel bromine, keeping the volume constant by making occasional additions of water. (If desired, this is a convenient stage at which to interrupt the procedure.) Place the beaker and solution in cold water, and add, with stirring after each addition, 1 ml of ethanol, 3 ml of sodium tartrate solution, 5 ml of potassium persulphate solution, 5 ml of ammonia solution and 2 ml of dimethylglyoxime solution (in that order). Transfer the solution to a 50-ml calibrated flask, dilute to the mark with washings from the beaker, mix well, and measure the optical density at 465 mμ. If a white precipitate forms, pass a portion of the solution through a sinter funnel before measuring the optical density. The final red-brown colour is stable for at least 3 hours.

STANDARD GRAPH—

Place suitable portions of diluted standard nickel solution in a series of separate beakers, add 1 ml of dimethylglyoxime solution to each, and shake. Add 2 ml of saturated bromine water, boil as described above for the samples, and continue as above. A graph of optical density against weight of nickel present is linear (slope 0·0045) and passes through the origin.

TEST FOR NICKEL IN FILTRATES—

Place the filtrate in a separating funnel, and extract three times with 5-ml portions of chloroform. Combine the chloroform solutions, and extract with a 10-ml and then a 5-ml portion of 0·33 N hydrochloric acid. Place the combined acid extracts in a beaker, add 2 ml of saturated bromine water, and boil until chloroform has been completely expelled. Place the beaker and contents in cold water, and continue as described above for the sample.

DISCUSSION OF THE METHOD

Table I shows results for the recovery of nickel added to untreated wheat fractions, the weights taken being the same as those of the samples. The satisfactory results and the absence of nickel in any of the filtrates tested confirmed (*a*) that recovery of the nickel was complete and (*b*) that Middleton and Stuckey's method for destroying organic matter can be used in determining nickel.

TABLE I

RECOVERIES OF NICKEL FROM WHEAT FRACTIONS

Fraction	Weight of sample, g	Nickel added, μg	Nickel recovered, μg
Bran ..	1·0000	10·0	7·4
		30·0	26·8
		60·0	56·0
Feed ..	1·0000	10·0	9·7
		30·0	28·7
		60·0	57·1
Flour ..	10·0000	10·0	10·2
		30·0	32·3
		30·0	31·6
		60·0	60·0
		60·0	59·0

The proposed procedure is comparatively rapid and extremely convenient for determining the nickel contents of a large number of wheat samples. An analyst's daily average output of twelve to fifteen determinations can be increased by using additional sets of apparatus. Detailed information on the various field treatments and the resulting distribution and accumulation of nickel in the wheat fractions is given elsewhere.[10]

I thank Mr. J. W. Kemp for technical assistance.

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Received *February 19th*, 1963

Cancer Research
11:257-8 (1951)

EXPERIMENTAL STUDIES IN METAL CARCINOGENESIS. I. NICKEL SARCOMAS IN RATS. W. C. HUEPER. (National Cancer Institute, Bethesda, Md.)

The rapidly increasing importance of metals and metal compounds as carcinogenic agents in man and experimental animals has provided the basis for an extensive series of experiments on metal carcinogenesis. Of the various metals and metal compounds (chromium, chromite ore, nickel, beryllium, arsenic, uranium, asbestos) used, only parenterally injected nickel powder has produced so far a significant number of cancerous reactions at the sites of deposition (femoral cavity, pleural cavity, nasal sinuses). Of the different species employed, rats alone have shown such responses. (None have been observed up to the present time in dogs, rabbits, and guinea pigs.) Twenty-five rats received injections in the femoral cavity of 0.05 cc. of a 25 per cent suspension by volume in lanolin. Another set of 25 rats received five injections of 0.05 cc. of the same suspension in the right pleural cavity, while 20 rats were given two injections of 0.1 cc. of a 12.5 per cent suspension by volume in lanolin in the nasal sinuses. Of the total number of 70 rats, 15 died without neoplastic reactions within the first 6 months of the experiment. Of the 24 rats that died within 7–14 months after the start of the experiment, 10 showed sarcomatous growths of spindle-cell, round-cell, and giant-cell structure at the site of injection, usually originating from a markedly hyperplastic periosteal tissue. There were metastases present in several cases.

Texas Repts. Biol. & Med.
10(1) 167-186 (1952)

EXPERIMENTAL STUDIES IN METAL CANCERIGENESIS*

I. Nickel Cancers in Rats

W. C. Hueper

For more than a century arsenicals were the only metallic chemicals that exhibited upon occupational, environmental or medicinal exposure, causal relations to cancers in man. Observations made during the last two decades in the field of occupational cancerigenesis have more or less definitely incriminated several additional metals or metallic compounds, such as chromates, nickel carbonyl, and asbestos. Experimental investigations moreover have shown that beryllium and selenium are cancerigenic for certain species. To this list of cancerigenic metals there may be added the various radioactive metals of natural or synthetic origin that have produced cancers in man and/or experimental animals.

In view of the growing scientific and practical importance of metal cancerigenesis and the inadequacy or the complete lack of experimental investigations of this rather new facet of the cancer problem, a series of experimental studies were initiated dealing with the following metals or their compounds: arsenic, chromium, chromite ore, nickel, uranium, beryllium, and asbestos. In the selection of the technical approaches the following principles and considerations were applied:

The primary aim of the experiments was the demonstration of cancer producing properties of elementary metals. No attempts therefore were made to imitate in the experimental conditions (type of metal compound, intensity and kind of exposure) those met in human experience. The animals for this reason received, wherever necessary, maximal tolerated or injectable doses of powdered metals at sites where their slow resorption seemed to be assured (marrow cavity of femur, pleural cavity, and nasal sinus).

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*From the National Cancer Institute, Bethesda, Maryland. Received for publication October 9, 1951. Presented at the Fifth Annual Symposium on Fundamental Cancer Research at The University of Texas M. D. Anderson Hospital for Cancer Research, in Houston, Texas, on April 20, 1951.

Since human and experimental experience have shown that cancers develop at sites where cancerigens act over long periods of time even when present in only minute quantities, it was thought that the relatively low solubility of the metals in water and fat, would favor through a depot action their gradual release in concentrations that would not cause severe local metallo-toxic necroses, but rather a specific metallo-biologic action (metal catalysis, enzyme metabolism, metallic protein complexes, etc.).

For each substance tested several species (dog, rabbit, guinea pig, rat) were used so as to overcome, as much as possible, any species specific lack of susceptibility to the potential cancerigens.

In the selection of the particular species employed, care was taken to exclude species or strains possessing a marked genetic tendency toward the "spontaneous" development of cancers in organs or tissues in which experimental cancerous responses to the metals tested might be expected. Through this arrangement it was thought that any results obtained might be unequivocal, despite a smaller number of test and control animals used than that required when animals belonging to "cancer strains" are employed.

The present communication reports the experimental procedure used and results obtained in rats to which pure metallic nickel powder suspended in lanolin was administered by three different parenteral routes.

Experimental Procedures

Female albino rats of the Osborne Mendel strain were used. There were 25 animals 6 months old in the two series that received injections of a suspension of pure metallic nickel powder in lanolin into the right femur or into the right pleural cavity, while there were 20 rats 4 months old in the series which were given injections of such a suspension into the right nasal sinuses.

Intrafemoral Administration: The suspension of nickel powder in lanolin used contained 12.5% of nickel by volume and 61% of nickel by weight. Approximately 0.05 cc. of this suspension containing about 50 mgms. of nickel was injected into

the narrow cavity of the right femur. The suspension was warmed upon a water bath until it could be withdrawn through a 19 gauge needle into a 1 cc. tuberculin syringe. The needle was then inserted through a hole drilled into the distal end of the femur with the aid of a #1 dental drill activated by an electric motor with a flexible driving shaft (hobby drill). A second hole was drilled into the proximal part of the femoral shaft. The injection was continued until the grey black soft buttery material exuded from the hole in the proximal end. There was always some nickel suspension in the perifemoral tissue. The wound was then closed with sutures. The operation was performed under ether anesthesia.

Intrapleural Administration: Through the supraclavicular fossa 0.05 cc. of the same nickel suspension were injected into the right pleural cavity. These administrations were made in monthly intervals for a total of 5 injections.

Intranasal Sinus Injections: A nickel powder suspension in lanolin containing 12.5% of nickel by volume and 43.8% by weight was used. Through a 19 gauge needle 0.1 cc. of the suspension was introduced into the right nasal sinuses. The needle was placed for this purpose on the floor of the nasal cavity and its tip slightly raised so as to avoid the puncture of the palate. The needle was then gently pushed forward until a moderate bony resistance was felt. Under slight pressure this bony membrane was perforated and the needle further advanced until a firm bony resistance was experienced. The needle was then withdrawn for a very short distance and the suspension was delivered into the sinus cavities. As a rule very little bleeding from the nostril was met during or after the injection. Three such injections were made to each rat in intervals of 2 months between the individual injections.

Experimental Observations

Intrafemoral Injection Series: The surgical incisions healed well in all animals except one, in which the femur fractured and a fistulous duct was formed. Two rats died during the first 6 months of the experiment without showing gross evidence of tumor formation or any other symptoms referable to a metallotoxic or biologic effect. After about 8 months a

slight swelling of the right thigh was noted in one rat. During the following 8 months similar developments appeared in 3 additional rats. These tumors were firmly attached to the deep tissues but did not seem to involve the skin. They were firm in some parts and soft in others and grew rather rapidly. They extended in most instances into the pubic region and into the abdominal cavity. One became fixed to the spine and pelvic bones. They varied in size from that of a golf ball to that of a tennis ball. In one rat, with the fistula, however, there was a navy bean sized firm node beneath the fistulous opening and attached to the bone. In a fifth rat the right thigh was normal but the abdomen became increasingly distended by a tumor mass. These animals died or were sacrificed during the period of 7 to 16 months after the start of the experiment. During the same time 12 additional animals died without showing signs of neoplastic responses. There are six rats still alive. It appears thus that among the 17 rats that survived the minimal latent period of 7 months and died within 16 months after the injection 5 developed neoplastic responses, 4 involving the site of injection (30 per cent tumor incidence).

The post-mortem examinations of the rats showed that the tumors located in the right femoral region were rather well encapsulated toward the cutaneous surface, but at the same time were firmly attached to the femur. The involvement of the bone extended in one rat well into the pelvic ring and the spine. The tumor tissue on section exhibited usually a large central necrotic area, while the marginal parts were of homogeneous, white bacon-like texture. Pea sized to bean sized nodes of similar appearance and consistency were found in the abdominal lymph nodes and in the lung. In the rat with the abdominal tumor mass numerous smaller nodes were found in the mesentery and the retroperitoneal tissue. The rat with the fistula and the tumor surrounding this duct exhibited a second pea sized medullary growth located on the snout and forming there a projecting mass that was slightly adherent to the bone underneath.

In the rats which died without any neoplastic reactions a blackish material could be demonstrated in and around the right femur. One of them revealed a bean sized cyst filled

with debris and located in the muscular tissue beneath the original incision. The main pathology in these animals consisted of purulent pleurisy and pneumonia or enteritis.

The histologic examination of the tumors and tissues of the rats gave the following findings:

The sections taken from parts of the three tumors topographically closely related to the right femur and firmly attached to it show masses of irregular spindle shaped cells containing round, oval, elongated, hyperchromatic nuclei and originating from the periosteal tissue which contains a layer of newly formed osteoid and cancellous bony tissue (figs. 1, 2 and 3). The tumor tissue is found in close proximity to black

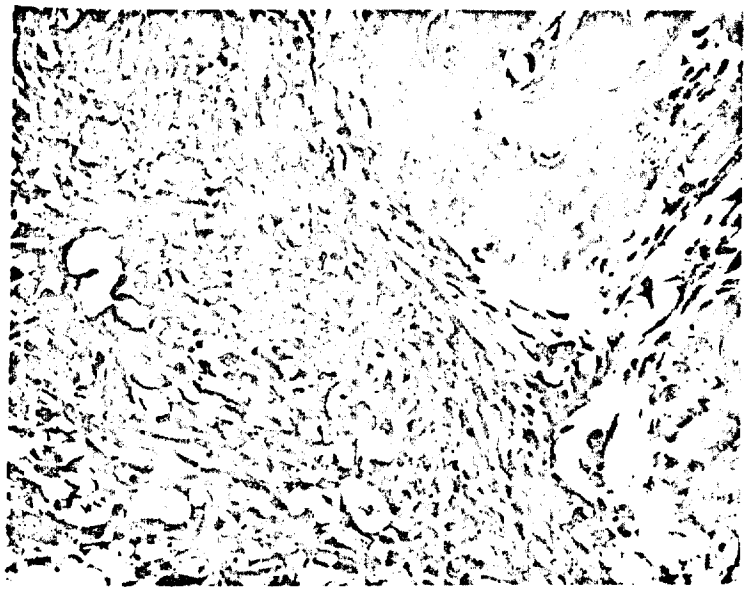

FIG. 1. R-435, Osteogenic spindle cell sarcoma of right femur.

nickel deposits. In distal portions of the primary tumors as well as in metatases the structural characteristics differ a great deal. In some parts the sarcomatous tissue is composed of irregularly arranged bundles of hyperchromatic spindle cells

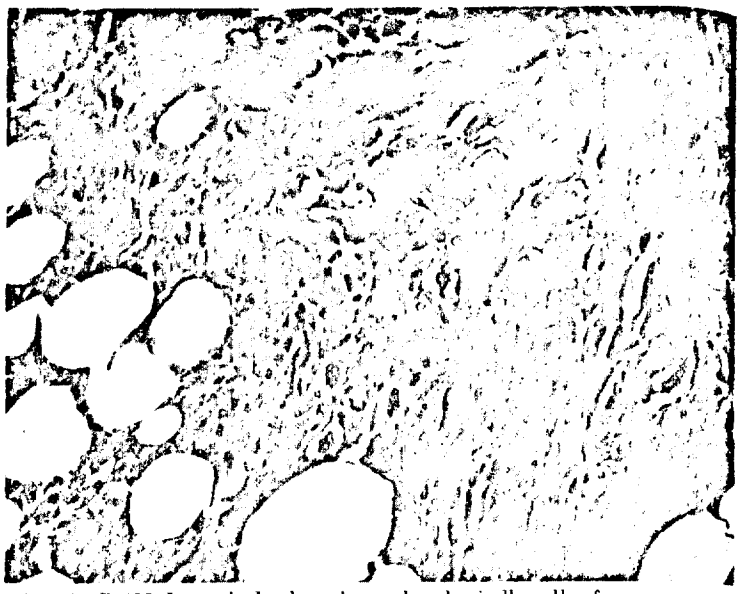

FIG. 2. R-432, Irregularly shaped round and spindle cells of a sarcomatous tumor originating from the femoral periost invading subcutaneous fat tissue.

FIG. 3. R-422, Femoral cortex with periosteal spongy bone and spindle cell and round cell sarcomatous tissue.

having a moderate amount of fibrillar intercellular substance. Giant cells are scattered in this cellular matrix (fig. 4). The

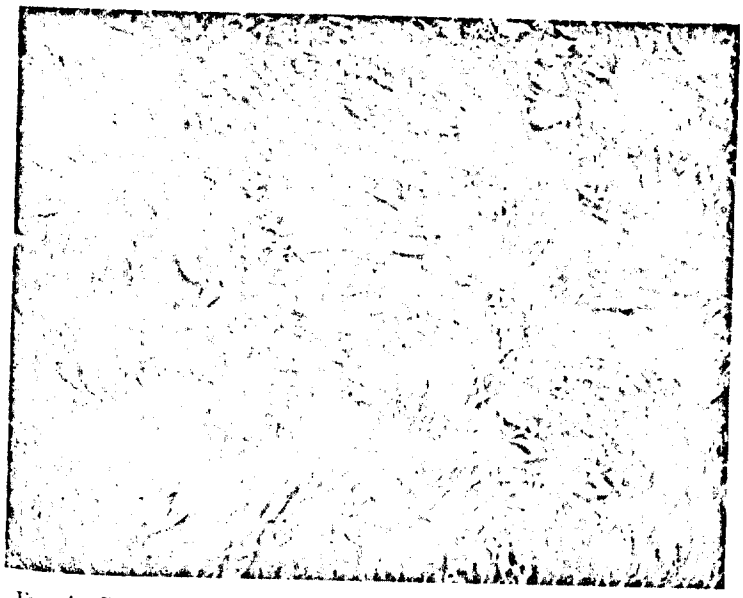

FIG. 4. R-422, Spindle cell and fibrosarcomatous areas with scattered giant cells.

nuclei are occasionally arranged in palissade formation. In other areas the tumor cells are irregularly round and show a dense scattering of huge cytoplasmic complexes containing small, hyperchromatic, nuclear bodies, resembling osteoclastic syncytial formations (fig. 5). In two cases parts of the primary and metastic tumors display large solid masses of indistinctly round cells arranged in small groups of ill defined, solid alveoli (fig. 6). Adjacent normal tissues, such as muscle tissue and fat tissue as well as mesentery, kidney, spleen, lung, pancreas and abdominal and mediastinal lymph nodes are diffusely invaded by tumor cells (fig. 7).

The tumor that seemed to originate in the abdominal cavity of one rat consists of masses of small round cells embedded in a scanty loose connective tissue. In some areas the tumor cells

FIG. 5. R-422, Irregularly round shaped sarcoma cells with an admixture of large syncytial masses containing many small hyperchromatic nuclear bodies.

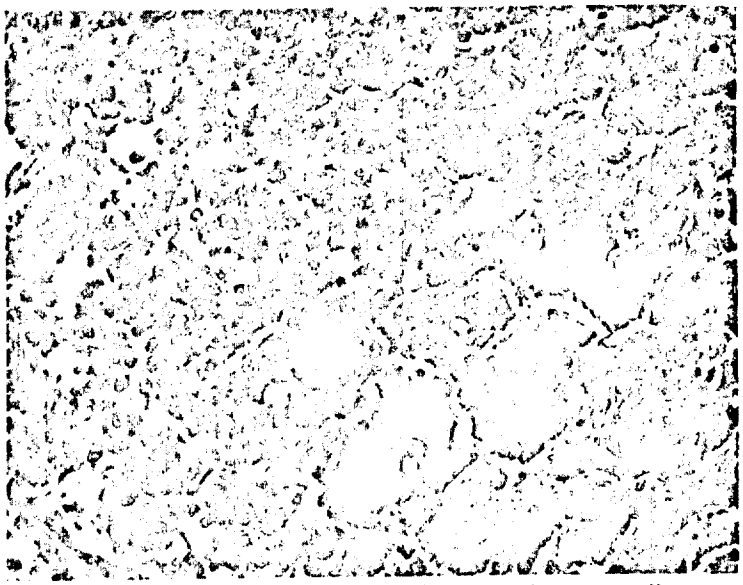

FIG. 6. R-435, Alveolar arrangement of large round sarcoma cells.

FIG. 7. R-422, Round and oval shaped sarcoma cells invading the muscle tissue of the thigh.

FIG. 8. R-434, Small round cell sarcoma apparently originating from abdominal lymph nodes.

display an abortive follicular arrangement. The femur cavity contains black amorphous material surrounded by a fibrous marrow and necrotic bone trabecles. The bone marrow located more distally from the nickel deposits consists of dense myeloid tissue, while the bone trabecles are hyperplastic.

Sections taken from the tissue surrounding the fistulous duct connecting the skin of the thigh with the femur, found in one rat, reveal irregular nests of small epidermal cells in a fibrous stroma. They extend from an atypical epithelial coating of the fistula. Periosteal proliferations of spongy bone at the base of the duct are invaded by these carcinomatous nests (fig. 9). The surface epidermis is normal. Deep seated

FIG. 9. R-429, Newly formed periosteal spongy bone surrounded by nests of epidermal carcinoma cells, extending from a fistulous duct.

carcinomatous cell nests are present in the subcutaneous tissue of sections of the small tumor node removed from the snout. The surface epithelium of the skin is normal.

The histological examination of the femur and muscle tissue of the right thigh of non-tumor bearing animals shows as a

rule some periosteal thickening and often a proliferation of osteoid and osseous tissue. The bone marrow is in general a dense myeloid tissue, that only in the immediate proximity of nickel particles exhibits necrotizing, atrophic and fibrosing changes. In a few instances osteoid tissue is present within the muscle tissue. This is usualy without any pathologic changes. The examination of all other organs (lung, heart, aorta, liver, spleen, pancreas, adrenal, kidney, sternum, stomach, ovary, salivary gland, bladder) shows occasionally abnormal alterations especially acute inflammatory reactions of the lungs and pleura as well as acute circulatory responses, all of which, however, do not bear any direct relation to the administration of nickel into the femur.

The administration of metallic nickel powder into the femur cavity of rats thus resulted within 7 to 16 months afterwards in the development of three osteogenic spindle cell sarcomas, one squamous cell carcinoma of a fistulous duct and, possibly, one round cell sarcoma originating in the abdominal cavity.

Intrapleural Injection Series: The injections were in general well tolerated. In two rats, however, sudden death occurred when the injection was given accidentally into the lungs. Following the fifth month there developed in the region of the right chest of three rats rapidly growing tumors which either extended downward into the sternum and abdominal wall or upward and backward involving then the spine. The tumors were rather firm and well fixed to the thoracical wall. The tumors varied in size from that of a large walnut to that of an oversized golf ball.

Of the 18 animals that died during the 16 months of the experiment post-morten examinations were performed on 12 rats. Six rats were destroyed by cannibalism. The autopsies performed on the three tumor rats show that the tumors are firmly attached to the bony structures of the thoracic wall (ribs, sternum, spine). The tumors are well encapsulated toward the skin. The cut surface reveals a homogeneous bacon-like texture. Black areas representing injected nickel are present within the tumor tissue of one case. Bloody serous fluid is found in the pleural cavity. In one rat white-yellow nodules are attached to the pleural surface of the lungs. The

cervical and axillary lymph nodes are enlarger, white, firm, and homogeneous in texture. The other organs are essentially normal.

The histological examination of the tumors and organs shows that the tumors are mainly composed of irregularly round and spindle shaped cells which are not infrequently of large size, have hyperchromatic round, oval or elongated nuclei and sometimes are intermingled with huge syncytial masses containing numerous small nuclei or larger chromatic masses (figs. 10, 11, and 12). There is evidence that at least in one case the tumor

FIG. 10. R-445, Anaplastic sarcoma cells with a dense scattering of large giant cells having irregularly shaped, hyperchromatic, nuclei.

appears to originate from periosteal tissue of the rib, since there is a diffuse transition of cartilagenous and osseous tissue into round and spindle cell sarcomatous tissue (fig. 13). Nickel particles are demonstrable in close proximity to sarcomatous tissue. Round cell and spindle cell masses are found in lymph nodes, abdominal wall, lung and sternum. The histologic

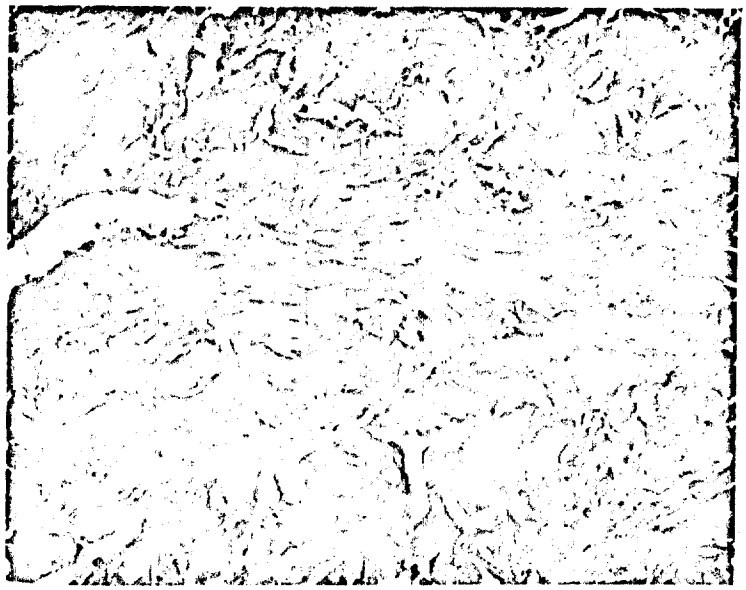

FIG. 11. R-446, Spindle cell sarcomatous tissue containing a moderate number of irregularly shaped large cells with a pink, homogeneous cytoplasm.

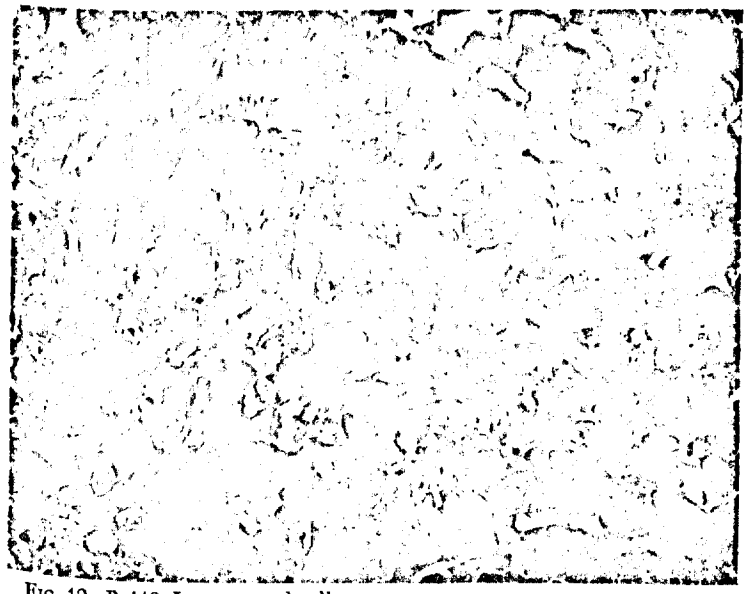

FIG. 12. R-449, Large round cell sarcoma.

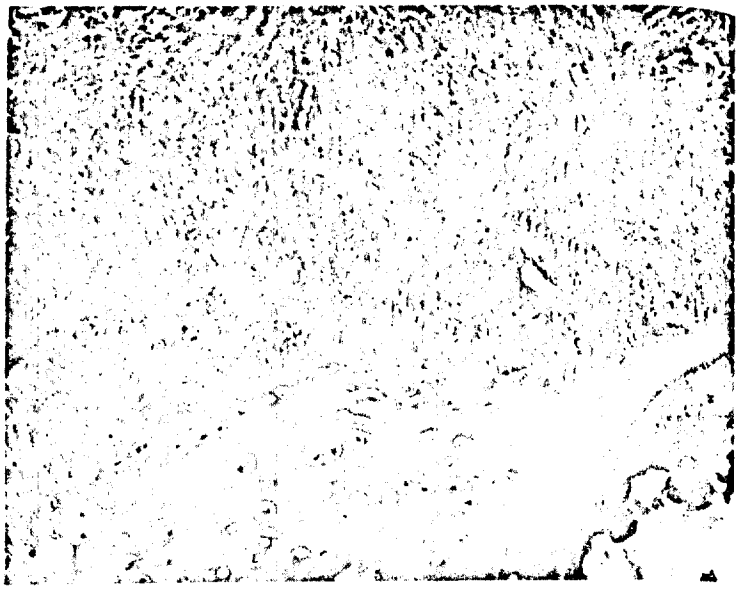

FIG. 13. R-449, Periosteal portion of a large round cell sarcoma originating from a rib.

examination of the axillary tissue of a fourth rat which had not shown grossly any tumor formation reveals around black nickel deposits a proliferation of atypical, hyperchromatic, irregularly round and oval cells which represent apparently early sarcomatous foci elicited in the connective tissue by the presence of the nickel particles (fig. 14).

The findings of the histologic examination of the other organs of this series are without definite relation to the administration of nickel into the pleural cavity with the following exception. Black nickel deposits are present in the thoracical and pulmonary pleura where they are embedded in a highly capillarized granulation tissue forming brush-like excrescences. In two rats nickel particles surrounded by cellular granulomatous tissue are present in many pulmonary vessels (fig. 15). In two other rats, which died during the injection, nickel particles without perivascular inflammatory reactions fill many pulmonary capillaries.

FIG. 14. R-455, Black nickel particles surrounded by hyperchromatic, irregularly round and oval cells suggesting an early stage of a sarcomatous transformation of mesenchymatous cells adjacent to nickel.

FIG. 15. R-429, Pulmonary tissue with intracapillary nickel particles surrounded by a dense mononuclear and lymphoid granuloma.

There were thus 4 sarcomas in the 10 rats that survived from 7 to 16 months the course of intrapleural injections of nickel powder into the pleural cavity. In all instances the tumors developed at the site of injection and were histologically similar to those observed in the thigh of rats injected intrafemorally with the identical material.

Intranasal Sinus Injection Series: The injections were in general well tolerated. Two rats died after the second injection. Several of the rats developed following the injections marked swelling of the snout, which, however, subsided after several weeks. In one rat this development led to the formation of a cystic pea sized mass on the dorsum of the nose. Of the 6 rats that have died during the first 8 months of the experiment 3 died 7 and 8 months, respectively, after the start of the experiment. One of these rats had a distended abdomen, while the second had the swelling of the nose.

The autopsy of the last mentioned rat shows a navy bean sized black nodule that is firmly attached to the skin and which reveals upon sectioning a necrotic cystic mass. The other organs including the nasal sinuses are grossly normal. In the second rat that died 7 months after the first injection the lymph nodes of the mediastinum are enlarged and hemorrhagic, the intestines are fused into a large mass including the mesentery and the greatly enlarged abdominal lymph nodes. These are dark red. The spleen is of normal size. The autopsy findings in the other rats show often purulent pleurisy and pneumonia. The nasal sinuses appear to be grossly normal. There are occasionally some small hemorrhagic spots in the brain.

Upon the histologic examination of the organs of this series the nasal sinuses exhibit often chronic inflammatory reactions. The cystic mass present on the nose of one rat consists of necrotic debris surrounded by an inflammatory wall. The enlarged mediastinal and abdominal lymph nodes found in a second rat are composed of solid masses of large round cells having a rather ample amount of cytoplasm (fig. 16). There is no neoplastic involvement of any other organ.

FIG. 16. R-641, Large round cell sarcoma originating from mediastinal or abdominal lymph nodes.

The intranasal sinus injection of nickel powder was followed in one out of 2 rats that died 7 to 8 months after the first injection by the development of a round cell sarcoma involving thoracical and abdominal lymph nodes. The neoplastic responses observed so far among the 70 rats which received by various routes injections of nickel powder are summarized in table 1.

TABLE I

Neoplastic Responses in Rats after Nickel Injections

Route	No. Rats	Deaths 0–6 months	Deaths 7–16 months	Tumor at Site of Inj.	Tumors remote from site
Femur	25	2	17	4	1
Pleura	25	8	10	4	0
Nasal Sinus	20	6	3	0	1

It appears from this listing that there were 10 malignant tumors among the 30 rats that died between 7 to 16 months

after the first parenteral administration of nickel powder suspended in lanolin. In 8 of these cancers the site of the cancer was closely related to the nickel depot produced. In several instances histologic examination revealed the proximity between nickel deposits and malignant growths. Of special significance concerning causal relations between the nicked deposition and the subsequently appearing cancer is the fact that one of the neoplastic reactions is of carcinomatous nature.

Comments

The scientific and practical importance of these observations lies in the fact that reports from English copper nickel ore refineries made during the past twenty years have shown an excessive frequency of cancer of the lung, nasal cavity and nasal sinuses among workers engaged in operations in which exposure to nickel carbonyl exists (Cooper; Amor; Bridge; Stephens). According to a recent report of Merewether there occurred between 1923 and 1948, 47 cancers of the nares and nasal sinuses and 82 cases of cancer of the lung among members of this occupational group. No similar observations so far have been recorded from Canada and Germany where the Mond Process of nickel ore refining is employed (Goldblatt and Wagstaff). While in previous years some investigators favored the view that exposure to arsenic containing sulfuric acid fumes generated during the refining process was responsible for these respiratory cancers, this view has been abandoned as untenable by practically all investigators in more recent years. Circumstantial evidence incriminates exposure to nickel carbonyl vapors, since inhaled nickel carbonyl is rapidly decomposed in the lungs into carbon monoxide and very finely dispersed metallic nickel which is precipitated on the respiratory surfaces, as well as nickel dust.

Experimental proof for this concept has been lacking up till now. Campbell exposed mice to the inhalation of nickel dust 6 hours a day, 5 days a week for one year and found increase of lung tumors of doubtful statistical significance. The experimental observations reported here appear to be of much more substantial nature as to the existence of a causal relation between an exposure to finely dispersed metallic nickel and the

development of cancer in the tissues where this metallic matter is deposited.

Although the total number of cancers observed among the rats treated with nickel powder is relatively small, the absence of similar tumors among 70 rats injected by the various routes noted with lanolin and among another series of 70 rats identically treated with injections of asbestos powder suspended in lanolin as well as among some 150 rats injected intramuscularly into the thigh with suspensions of various mineral oils and other organic chemicals incorporated into lanolin supports the view that the cancers observed in rats at the sites of injection of metallic nickel powder represent specific manifestations of experimental metal cancers. Some doubt in this respect, however, may be entertained in regard to the two round cell sarcomas that apparently originated from mediastinal or abdominal lymph nodes.

Conclusions

Three series of rats injected with a suspension of nickel powder in lanolin into the femur, pleural cavity and nasal sinuses exhibited cancerous reactions in 30 per cent of the animals that died from 7 to 16 months after the start of the experiment.

Of the total number of cancers observed 6 were apparently osteogenic sarcomas, 1 originated from connective tissue at the site of injection, 1 from the epithelial lining of an osteomyelitic fistula, while 2 seemed to have developed from abdominal lymph nodes.

The evidence obtained strongly suggests that finely dispersed metallic nickel may elicit in tissues of contact cancerous reactions.

The minimal latent period of the tumors observed is about 6 months.

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NOTE: When the experiment was terminated 24 months after its start there were 6 rats with cancers at the site of injection, and 2 rats with cancers in the abdominal cavity in the intrafemoral series, while 8 cancers of the site of injection had been observed in the intrapleural series. Two intra-abdominal cancers were found in the series given injection into the nasal sinuses. There was thus a total of 14 cancers at the site of injection; 42 per cent of rats surviving more than 6 months.

In new experiments with nickel obtained by the courtesy of the International Nickel Co., Inc., injected into femurs of rats in a gelatine base, sarcomas were formed at the site of injection within 4 months.

J. Nat. Cancer Inst.
16: 55-73 (1955)

Experimental Studies in Metal Cancerigenesis. IV. Cancer Produced by Parenterally Introduced Metallic Nickel[1]

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The rapidly expanding production and use of metallic nickel and its compounds in the chemical, metallurgical, aviation, and other industries (paint, ink, linoleum, oil, etc.), as well as the increasing introduction of some of their products into the home in the form of kitchen utensils, household goods, paints, and other consumer goods, provide extensive contacts of large population groups with this metal. The significance of metallic nickel as a carcinogen so far has been restricted to the occurrence of cancers of the nasal cavity, paranasal sinuses, and lung among English, Norwegian, and Canadian nickel refinery workers (*1*) and to the experimental production of cancers in rats at the sites of implanted nickel powder (*2*).

Because of the limited scope of the initial experimental studies, a reinvestigation of the problem on an enlarged and modified scale was undertaken. In the original experiment previously reported, the following were used: rats of the Osborne-Mendel strain, nickel obtained from the Chicago Apparatus Company, and lanolin as the dispersing vehicle. In the present study nickel provided through the courtesy of the International Nickel Company was employed. In addition to rats of the Osborne-Mendel strain, animals of the Wistar strain were used so as to eliminate, or at least mitigate, any special strain-specific influence upon the type of reactions elicited. For overcoming any possible effects of species-specific factors, other animals, *viz.*, mice, guinea pigs, and rabbits, were given nickel by various routes (intrafemoral, intramuscular, intravenous, intraperitoneal, intrapleural). A highly viscous gelatin solution was substituted for lanolin in most of these experiments in view of the alleged anticarcinogenic action of this material, reported in connection with the testing of carcinogenic polycyclic hydrocarbons (*3–5*).

Experimental Procedures

The powdered metallic nickel purchased from the Chicago Apparatus Company consisted of irregularly shaped, ragged particles ranging in

[1] Received for publication December 15, 1954.
[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

diameter from 2 to 50 microns with the mean between 10 to 30 microns. The powdered nickel obtained from the International Nickel Company, on the other hand, was prepared by the disintegration of nickel carbonyl and was composed of minute round particles usually clumped together in small chains or in loose, larger clumps. The individual particles as a rule measured less than 1 micron in diameter, although the average-sized clump or branched chain of beads was usually 2 to 20 microns in length, with some clumps as large as 100 microns. While the powdered metal in its original form was employed in all implantation experiments, a fraction of nickel (International Nickel Company) consisting of particles measuring 0 to 4 microns in diameter was prepared through fractionation of the powder in a particle analyzer (American Instrument Company) for intravenous injection.

I. Intrafemoral Implantation of Nickel

Rats, rabbits, and dogs were used for implanting a suspension of powdered nickel in the marrow cavity of the femur. Since the dogs that were operated on more than 5 years ago have so far not shown any gross evidence of tumor formation and since they will be kept under observation for an additional 2 to 3 years, no further reference will be made to this aspect of the experiment.

a) Two series of 50 rats each of the Wistar strain, approximately 3 months old and equally divided as to sex, were used for implanting about 50 mg. of powdered nickel obtained from the International Nickel Company (INCO) and the Chicago Apparatus Company (CAC), respectively. The rats were anesthetized for this purpose. A hole was drilled through the cortex of the right femur into the marrow cavity with an electric hobby drill having a flexible shaft and provided with a #1 dental burr. Then by use of a tuberculin syringe and a 20-gauge needle, about 0.1 cc. of a 5-percent nickel suspension in a 20-percent gelatin solution prepared with physiologic saline solution was injected through this hole into the marrow cavity.

Through back pressure there was usually some seepage of the suspension from the marrow cavity around the needle into the periosteal tissue. The great majority of the rats tolerated the surgical intervention well. A few rats later developed abscesses and osteomyelitic complications, sometimes associated with fistula formation. Differential leukocyte counts were made before the injection of nickel and again about 12 months later. No significant changes were noted except for a rise in eosinophilic leukocytes from an average of 2 percent before the injection to an average of 4.1 percent in the rats after implantation with INCO nickel, and a rise from 2.3 percent to 9.1 percent in rats given CAC nickel.

Following an observation period of 18 months, during which time 9 tumors of the right thigh were found among the rats of the CAC series, the 20 surviving animals were reinjected in the left femur with the same dose of nickel previously used for the right thigh. This experiment was performed for testing whether the first deposition of nickel and a possible

systemic immunochemical reaction to it might influence the relative tendency toward tumor formation in a positive or negative direction in the as yet untreated left thigh. During the subsequent 10 months of the total survival period of 28 months (at the end of which time the last surviving rats were sacrificed), one tumor developed in the left thigh, while three appeared in the right thigh (table 1).

The first tumors at the site of nickel implantation appeared after a minimal induction period of 4 to 5 months. A total of 27 cancers appeared among the 100 rats composing the two groups. The relative incidence rate of tumor formation for the two types of nickel used was practically identical. The tumors varied in size from 1 cm. to 6 cm. in diameter. They were usually of firm consistency, attached to the femur, sometimes extending to and involving the pelvic girdle, and they were of medullary whitish-pink appearance and fibrillar texture on the cut surface. The centers of the larger tumors as a rule were soft, dirty grayish-red, and

TABLE 1.—*Survival periods and tumor formation in thighs of rats after intrafemoral deposition of nickel*

Months	1–3	4–6	7–9	10–12	13–15	16–18	19–24	25–28	Total
Deaths INCO	1	9	2	3	3	12	11	9	50
Deaths CAC	.....	5	7	5	3	10	10	10	50
Tumors INCO	.....	5	.....	2	.....	1	5	1	14
Tumors CAC	.....	1	5	1	.....	2	3	1	13

necrotic. In several tumors the neoplastic tissue surrounded black deposits. Sixteen of these neoplasms were present in female rats and 11 in males.

The histologic examination of these cancers showed them to be of different histogenetic origin. The majority (16) consisted of large, round, oval, spindle, or elongated cells with intercellular fibril formation indicating their origin from periosteal connective tissue. However, 4 of these cancers had histologic characteristics of muscular derivation; 5 displayed cellular whirl formation and nuclear garland together with a palisade arrangement suggesting a neurogenic origin; 1 exhibited an angiosarcomatous structure; and 1 had histologic features suggesting its reticulum-cell derivation from bone-marrow cells (figs. 1 and 2). Metastases were noted in 14 of these tumor rats (9 INCO, 5 CAC). They involved regional and remote lymph nodes (abdominal and mediastinal), kidney, spleen, pancreas, liver, lung, and heart (fig. 3).

In addition to these malignant tumors at the site of nickel deposition, a considerable number of additional malignant and benign neoplasms were observed among the rats of both series. Some of them occurred in rats with nickel sarcomas; others were found in animals without such cancerous reactions in the thigh. Several animals had multiple primary tumors. Two rats had round-cell sarcomas of the ileocecal lymph nodes extending into the retroperitoneal and mesenteric lymph nodes. Four rats had single or multiple adenofibromas of the breast, while a fifth

showed an adenocarcinoma of the mammary gland. In three of these rats with breast tumors, there was also present a hemangioma of the adrenal medulla, and in one rat there was a basophilic adenoma of the anterior lobe of the hypophysis. Adrenal hemangiomas were noted in 2 additional rats. Basophilic adenomas of the pituitary gland occurred in four additional rats. One rat had a myeloid leukemia; another, a cystadenoma of the ovary. A pancreatic insuloma measuring 2 mm. in diameter was discovered in one rat during the histologic examination of the sections (fig. 4). Altogether there were 20 additional tumors present in the animals of both series, making a total of 47 malignant and benign neoplasms among the 100 rats used.

Control rats intrafemorally injected with gelatin.—Twenty-three female rats of the Wistar strain, 7 months old, were injected through a drill hole into the marrow cavity of the right femur with 0.1 cc. of a 20-percent gelatin solution prepared with physiologic saline. The surgical procedure and the injection of gelatin were tolerated without untoward effects. Table 2 gives the survival periods of these rats.

TABLE 2.—*Survival periods of rats intrafemorally injected with gelatin solution*

Months	4–6	7–9	10–12	13–15	16–18	19–21	22–24
Deaths	2	3	1	0	2	5	10

None of the rats developed a tumor of the right thigh. There were, however, 10 benign or malignant neoplasms among these rats. Multiple tumors of the same type were present in two different rats and multiple tumors of different types were present in 4 rats. Two rats had round-cell sarcomas of the ileocecal lymph nodes forming round masses measuring up to 10 cm. in diameter. A third rat had a nodular, soft, multicystic, fused mass in this region, which upon histologic examination revealed a lymphangiectatic, edematous structure with solid cellular areas composed of small and large mononuclear cells, suggesting a presarcomatous condition. A fourth rat displayed at necropsy a solitary, firm node measuring 3 cm. in diameter and located in the ileocecal region with several smaller ones at the mesenteric root. The cut surface of the large node was medullary and white and showed a cheesy, yellowish center. The wall of the large node was composed of a cellular connective tissue which formed the wall of a cyst lined by squamous-cell carcinomatous masses. These masses infiltrated the wall in trabeculae composed of polygonal and spindle-shaped epithelial cells with hyperchromatic nuclei. Five rats had adenomas or adenofibromas of the breast which were multiple in two of them and which measured up to 10 cm. in diameter. In one rat the breast tumor was associated with a basophilic pituitary adenoma. There were three additional rats with basophilic pituitary adenomas and one rat with an adrenal hemangioma. The largest of both types of these tumors measured 1 cm. in diameter and were dark red in

color. One rat had an extensive, and in part, nodular lymphoid leukemia (involving the liver, spleen, kidneys, adrenals, heart, lung, and brain) causing a considerable enlargement of the liver and spleen which measured 8 × 2.5 × 1 cm. There were thus 14 tumors present in the 23 control rats.

Comment.—From the observations reported, it may be concluded that the 27 sarcomas of different histogenetic origin found at the site of nickel deposition are causally related to the metal and thus represent true metal cancers. These findings on the cancerigenic action of nickel in rats confirm those previously reported, when 6 sarcomas of different types and 1 squamous-cell carcinoma were found in 25 rats intrafemorally injected with a similar amount of nickel (CAC) suspended in lanolin. In both experiments the total nickel cancer yield was about 28 percent of the total number of rats injected (125). Regional and distant metastases were found in 17 of the 34 cancers. The total absence of cancers of the thigh in the rats injected with gelatin only indicates the specificity of the nickel effect. The wide variation in histogenetic types of cancer observed in these rats, ranging from osteogenic sarcoma through rhabdomyosarcoma, neurogenic sarcoma, angiosarcoma, and reticulum-cell sarcoma to squamous-cell carcinoma, indicates that no tissue specificity exists in relation to the cancerigenic action of nickel.

The presence of a considerable number and variety of benign and malignant tumors remote from the site of nickel deposition (25 tumors among 125 rats) raises the question as to whether these may be attributable to a systemic action of nickel producing a spectrum of different tumors. However, a comparison of these neoplastic reactions with those noted among the rats comprising the two control groups (gelatin and lanolin) (*6*), which exhibited a total of 17 different tumors among 48 rats, and with those found in rats intrafemorally injected with beryllium, arsenic, and asbestos does not support such a view, especially as these neoplasms were similar in histologic type and topographic distribution to those seen in the test series. The conclusion seems to be justified that the tumors which developed at a site remote from the site of nickel implantation were not causally related to nickel.

There is no evidence that the creation of a nickel depot influenced in any distinct way the subsequent tumor related to the production of a second depot made at another site some 18 months later. Although the only sarcoma which developed in the left thigh appeared as early as 4 months after the second nickel implant was placed and exhibited an explosive growth, no other tumor development in the left thigh followed. Moreover, once the tumor had become established, an extraordinarily high proliferative rate was observed with many other nickel cancers.

b) Six 3-month-old Dutch rabbits weighing between 1,750 and 2,000 gm. were injected into the right femoral cavity with about 0.25 cc. of powdered nickel (CAC) suspended in lanolin (12.5-percent nickel by volume, 43.8-percent by weight). A second injection was given 27 months later, when twice the original dose was administered. The

rabbits recovered well from these procedures and gained weight, reaching a maximal weight between 3,000 and 4,000 gm. Blood studies made at varying intervals up to 24 months after the initial injection of nickel did not reveal any significant changes in the number of erythrocytes and leukocytes, in the differential count and in the amount of hemoglobin.

Two additional rabbits were injected with lanolin only.

The necropsy and histologic observations made on 4 of the 6 rabbits injected with nickel revealed the presence of a completely hyalinized spleen with highly atrophic lymph follicles and a hyalinosis of the renal glomeruli in one of them. In a second rabbit there was a nodular mass measuring 4 cm. in diameter located at the junction of the uterine horns. Upon dissection, a soft, reddish mass of homogeneous texture was found projecting into the distended uterine lumen which contained a moderate amount of serous fluid. The node consisted of closely packed, papillary glandular formations often forming cysts lined by a papillary columnar epithelium. Since there was no invasion of the myometrium by these glandular structures, the node represented an endometrial adenoma.

The sixth rabbit, which died 43 months after the implantation of nickel, showed at necropsy a mass of well encapsulated, firm nodules measuring 3 × 3 × 3 cm. to 5 × 3 × 3 cm. in diameter located in the right thigh. The centers of these nodes were hemorrhagic, while the marginal portions were grayish-pink in color and homogeneous in texture. Numerous similar medullary nodules were present in both kidneys, spleen, right ovary, parametrium, liver, cervical lymph nodes, and lungs. The largest of these nodules measured 4 cm. in diameter. Survival periods for these rabbits are presented in table 3.

TABLE 3.—*Survival periods of rabbits injected intrafemorally with nickel (CAC)*

Months	13–18	31–36	37–42	43–48
Deaths	1	1	3	1

The histologic examination showed the nodular masses to be composed of irregularly arranged bundles of slender spindle cells surrounded by a moderate amount of fibrillar connective tissue diffusely invading the surrounding normal tissues. The examination of the right femur revealed the presence of similar tumor growth within the marrow cavity (figs. 5 and 6). In view of the location of the tumor at the site of injection of the nickel, it may be concluded that the fibrosarcoma represents a cancerous response to the nickel. The observation indicates that the cancerigenic action of nickel is not restricted to rats but extends to other species.

II. Intravenous Injection of Nickel

Mice, rats, and rabbits were injected with a suspension of powdered nickel (INCO) in gelatin solution prepared with physiologic saline.

a) Twenty-five strain C57BL male mice, 6 weeks of age, were injected in the tail vein with 0.05 cc. of an 0.005-percent suspension of nickel powder in a 2.5-percent gelatin solution. The injections were repeated after a 2-week interval. Four mice died immediately after the second injection.

There were no pertinent gross and histologic observations, except for circulatory disturbances in some of these animals. Table 4 gives the survival periods of the mice of this group.

TABLE 4.—*Survival periods of mice after intravenous injections of nickel*

Months	0–1	5–15	13–14	15–16
Deaths	4	2	13	6

b) Twenty-five 3-month-old rats of the Wistar strain were injected in the vena saphena with an 0.5-percent nickel suspension prepared with physiologic saline. The nickel powder used was composed of particles measuring 0 to 4 microns in diameter. The individual dose given was 0.5 cc. per kg. of body weight (0.1–0.18 cc. per rat ranging in weight from 100–184 gm.). A total of six injections was given at one-week intervals.

Four rats died during the injection. Differential leukocyte counts made before the first injection and on two occasions during the last 8 months following injection did not reveal any significant changes. The last surviving rats were sacrificed at the end of an observation period of 28 months (table 5).

TABLE 5.—*Survival periods of rats after repeated intravenous injections of nickel*

Months	0–3	4–6	7–9	10–12	13–18	19–24	25–28
Deaths	5	3	10	1	0	3	3
Tumors	0	0	7	0	0	0	0

Seven rats developed tumors in the region of the groin and in the proximal portion of the thigh, where nickel had escaped through leakage from the vena saphena into the subcutaneous tissue. They appeared about 7 to 8 months after the first injection. The tumors were bilateral in one rat and measured from 1 to 5 cm. in diameter. They were firm and encapsulated toward the skin but adherent to the pelvic girdle or femur. Metastases were formed by only one of these tumors which produced a fused, nodular retroperitoneal mass measuring 2 × 4 × 0.5 cm., and several hemorrhagic nodules in the lungs measuring up to 1 cm. in diameter.

Upon histologic examination, these tumors were found to consist of masses of large spindle cells, which in two of them were apparently of myoblastic character, while in one they were associated with trabecular bone incorporated into the spindle-cell matrix. Masses of multinucleated, huge giant cells representing apparently regenerating muscle cells were present within the tumor masses of several rats. In another rat, the

histologic examination of the tissue of the groin revealed a highly cellular, fibroblastic tissue which, through its cellular irregularity, suggested a presarcomatous condition (figs. 7 and 8).

In addition to these nickel-induced tumors there were other neoplasms remote from the site of nickel deposition in some of the rats (1 fibroadenoma of the breast, 2 adenomas of the breast with atypical glandular, papillary and solid epithelial formations, 3 basophilic pituitary adenomas measuring up to 1 cm. in diameter, and 3 hemangiomas of the adrenal medulla, 2 of which were associated with the hypophyseal tumors).

The lungs of 2 rats showed emboli of black, amorphous material which was evidently nickel powder. Similar deposits were not observed in the reticuloendothelial cells of the various organs studied.

c) Ten albino rabbits, about 3 months old, weighing from 2,000 to 3,000 gm., were given at weekly intervals six injections of a 1-percent powdered nickel suspension (0.5 cc. per kg. of body weight) in 25-percent gelatin solution. Five control rabbits received by vein similar amounts of the 25-percent gelatin solution.

The injections were not well tolerated. The rabbits had convulsive reactions developing during the injection of the dispersion. Some of the nickel leaked from the vein into the surrounding tissue where it gave rise to black induration, necroses, and abscesses. Several of the rabbits gained some weight during the period of the injections; others lost a few hundred grams. Two rabbits died with paresis of the hind legs 4 and 12 months, respectively, after the start of the experiment. The others died or were sacrificed at various times during the experiment. The control rabbits were healthy 40 months after the start of the experiment (table 6).

TABLE 6.—*Survival periods of rabbits after repeated intravenous nickel injections*

Months	0–12	13–18	19–24	25–32	33–36	37–44
Deaths	4	1	1	3	1	Test series

Erythrocyte, leukocyte and differential counts, determinations of the coagulation and bleeding times, and of the hemoglobin content were done at varying intervals and the urine was examined for albumin, sugar, acetone, blood, bile, and the presence of casts and cells in the sediment.

There were no significant changes in these elements apart from a 50- to 100-percent increase in bleeding time after the sixth injection. Since this phenomenon was common to both the test and the control series, it is apparently elicited by the introduction of the macromolecular gelatin molecules into the blood (7).

The necropsy findings in all rabbits were negative for tumors. In the pulmonary capillaries of two rabbits, black amorphous clumps were present which were surrounded by multinucleated endothelial syncytial formations. One rabbit had a completely hyaline pulp with atrophic lymph follicles, as well as a marked interstitial fibrosis and parenchymal atrophy of the pancreas.

Comment: Repeated intravenous injections of nickel suspensions into mice, rats, and rabbits did not cause the development of tumors in any organ or tissue to which nickel particles might have been brought by the blood stream. The only cancers which were found originated in the subcutaneous tissue of rats into which nickel had seeped from leakage of the injected vein.

III. Intramuscular Injection of Nickel

Fifty strain C57BL mice, 6 weeks old, were injected in the muscle tissue of the right thigh with approximately 0.02 cc. of an 0.05-percent suspension of powdered nickel (INCO) in a 2.5-percent gelatin solution. Nine died during the first 9 months, 15 during the next 3 months, and 26 died or were sacrificed during the 13th to 18th month of the experiment. There were no local reactions at the site of deposition of the nickel. A few of the mice showed hyaline lesions in the spleen and liver. None developed a tumor at the site of injection. This negative experience in mice contrasts sharply with the positive observations made in rats, in which the seepage of nickel suspension from the femoral cavity into the surrounding connective tissue and muscle caused the development of fibrosarcomas and myosarcomas.

IV. Intrapleural Injection of Nickel

Fifty strain C57BL male mice, 6 weeks old, were injected in the right pleural cavity with 0.02 cc. of an 0.06-percent suspension of powdered nickel (INCO) in a 2.5-percent gelatin-saline solution. Table 7 gives the survival periods for these mice.

TABLE 7.—*Survival periods of mice intrapleurally injected with nickel*

Months	0–6	7–12	13–18	19–24
Deaths	8	5	33	4

The post-mortem examination and a histologic study of the organs of these mice showed enlarged spleens, kidneys, and livers with mild to marked hyaline lesions in some of the mice. There were no tumors.

The negative outcome of this experiment on mice is in agreement with the negative results obtained in the other studies in which this species was used. It contrasts, on the other hand, with the positive results previously obtained in rats, in which the intrapleural introduction of powdered nickel (CAC) suspended in lanolin was followed by the development of spindle-cell sarcomas of the thoracic wall near or around nickel deposits in 8 of 25 Osborne-Mendel rats.

Comments

The production of sarcomas at the sites of deposition of finely powdered metallic nickel (thigh and groin) in 28 percent of rats injected in the femoral cavity and/or saphenous vein with this metal suspended in gelatin

solution confirms previously reported observations concerning the development of cancers in the thigh and thoracic wall of rats following an introduction of nickel suspended in lanolin. Since tumor formation was noted in two different strains of rats (Osborne-Mendel and Wistar), with two different types of nickel (International Nickel Company and Chicago Apparatus Company) and with two different types of vehicle (lanolin and gelatin), there can be no doubt that nickel, when present in rather concentrated amounts in the tissue of rats, is capable of eliciting malignant tumors. The lack of a systemic cancerigenic action of nickel when generally dispersed through the blood after its intravenous administration suggests that either a minimal amount of this metal is needed at any one point for insuring a cancerigenic effect, or the body is able to dispose effectively of minute amounts of this metal when it is present in the form of very small particles.

Furthermore, the observations made indicate that the cancerigenic action is to a certain extent species-specific, since cancers were not obtained in mice intramuscularly, intrapleurally, or intravenously injected with nickel suspended in gelatin solution. The occurrence of a fibrosarcoma in the femur of a rabbit 43 months after an intrafemoral introduction of nickel in lanolin, on the other hand, demonstrates that rabbits apparently are susceptible to a cancerigenic action of nickel. The absence of similar reactions in rabbits intravenously injected with nickel may be explained by the fact that they did not survive sufficiently long to cover the induction period of metal cancers in rabbits, which seems to be in the neighborhood of 2 to 5 years (*8*).

Moreover, these positive observations made in two species provide additional evidence in support of the contention that the carcinogenic action in rats is a specific one and not an expression of some hypothetical nonspecific irritative effect of the metal seen only in rats (*6*).

The appreciable number of benign and malignant tumors found in rats remote from the deposition of nickel raises the question of a possible systemic spectrum effect of this metal apparently found in the form of lung cancers in rabbits following an intrafemoral implantation of chromium and cobalt (*8*), or resembling that effect noted with several carcinogenic amino compounds (4-aminodiphenyl, *p*-dimethylaminoazobenzene, and 2-acetylaminofluorene) in rats and dogs by several investigators (*9–11*). However, when comparing the number, types, and distribution of the various tumors found remote from the site of nickel introduction with those observed in rats belonging to the control series, it becomes most doubtful that such an action of nickel was present.

Special consideration in this respect, however, may be in order concerning the insuloma seen in one of the rats following an intrafemoral nickel implantation, since in another experiment three identical pancreatic tumors were found among 25 rats intraperitoneally or intrafemorally injected with another cancerigenic metal, namely, chromium. Because of the great rarity of these tumors not only in man but also especially in experimental animals (*12, 13*), the suspicion remains that the genesis of

these neoplasms may somehow be related to the administration of the two cancerigenic metals, although no information exists as to any special metabolic or toxic action of these metals upon the pancreatic islets similar to that exerted by alloxan, suggesting thereby a specific affinity of the metals for the cellular constituents of these endocrinic formations. There was also no anatomic evidence in the rat carrying the insuloma of an islet growth-stimulating action of estrogen or progesterone, recently reported for rats by Haist (*14*). Since there was no evidence of a glycogenesis of the liver in this particular rat, it is highly doubtful whether the tumor produced insulin.

According to the experimental evidence available, cancer formation has so far been observed after the implantation, intravenous injection, or inhalation of the following metals:

a) Nickel implanted intrafemorally, intrapleurally, and subcutaneously into rats and rabbits causing local tumors (*2*, *6*).

b) Chromium implanted intrafemorally into rabbits causing local and remote lung tumors (*8*).

c) Cobalt implanted intrafemorally into rabbits causing local and remote lung tumors (*8*) and implanted intramuscularly into rats causing local tumors (*15*).

d) Beryllium injected intravenously into rabbits eliciting sarcomas of bones [Gardner (*16*); Barnes (*17*); Barnes, Denz, and Sissons (*18*); Dutra, Largent, and Roth (*19*)]; and inhaled by rats producing bronchiogenic carcinomas (*20*).

e) Uranium implanted intrafemorally and intrapleurally into rats eliciting local cancers (*21*).

While some information on the biologic activity of chromium and cobalt exists, the data available on the biologic activity of nickel are very scanty. Nickel apparently forms complexes with proteins since cutaneous contact with this metal is not infrequently the cause of an allergenic dermatitis. It is prone to form chelates with organic compounds, such as dimethyl glyoxime, and may combine with carbamates and fatty acids. Chemical and biologic evidence indicates that it is an oxidation antagonist and a hydrogenation catalyst and may become soluble in tissue fluids. Recent spectrographic studies of Araki and Mure (*22*) have demonstrated its presence in benign and malignant tumors of man and experimental animals. The significance of nickel in tumors is not known. Its distribution in the various tissues resembles that of zinc. Since some students of nickel have suggested that it has a relation to insulin, Araki and Mure proposed that nickel may have an inhibitory action on tumor growth. It is evident from the enumeration of the various data available on the possible and actual biologic action mechanisms of nickel that its carcinogenic effect may take various biologic pathways, none of which so far has been explored.

It may be noteworthy in this connection that Wase, Goss, and Boyd (*23*) working with radioactive nickel, found that nickel apparently has a high value for the complex formation constant of nickel with lung protein.

The reasons for this affinity of nickel for lung protein, however, are as yet not known.

Conclusions

1) The intrafemoral and subcutaneous introduction of powdered nickel into rats resulted in the production of sarcomas of different histogenesis in about 28 percent of the implanted animals.

2) Of six rabbits that survived a similar procedure for over 3 years, one also developed a metastasizing endosteal fibrosarcoma of the femur. No tumors, on the other hand, were elicited in mice given nickel intravenously, intraperitoneally, or intramuscularly.

3) Although many benign and malignant tumors were found in rats remote from the site of nickel deposition, there is no reliable evidence indicating a carcinogenic spectrum effect of nickel similar to that reported by Schinz and Uehlinger for chromium and cobalt.

4) The occurrence of an insuloma in the pancreas of a rat intrafemorally implanted with nickel is reported.

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PLATE 3

FIGURE 1.—Neurogenic sarcoma in the thigh of a rat with intrafemorally implanted nickel showing palisade and garland arrangement of nuclei. × 340

FIGURE 2.—Myoblastic sarcoma in the thigh of a rat with intrafemorally implanted nickel showing multinucleated myoblastic cells. × 340

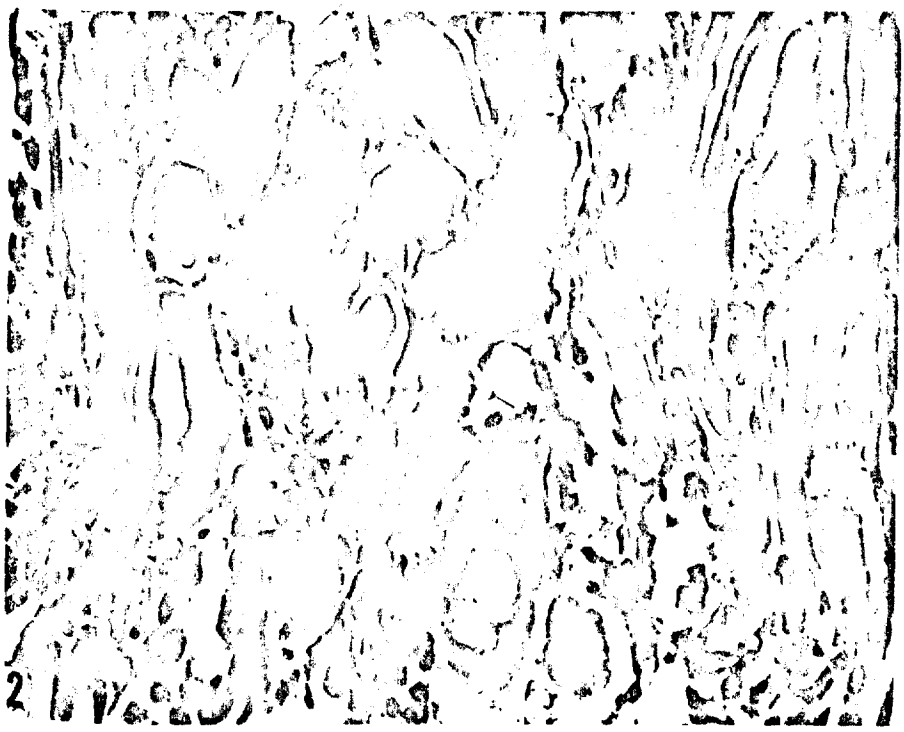

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PLATE 4

FIGURE 3.—Spindle-cell sarcoma in the heart of a rat following intrafemoral implantation of nickel. × 340

FIGURE 4.—Endosteal fibrosarcoma of the narrow cavity of the femur of a rabbit following intrafemoral implantation of nickel. × 340

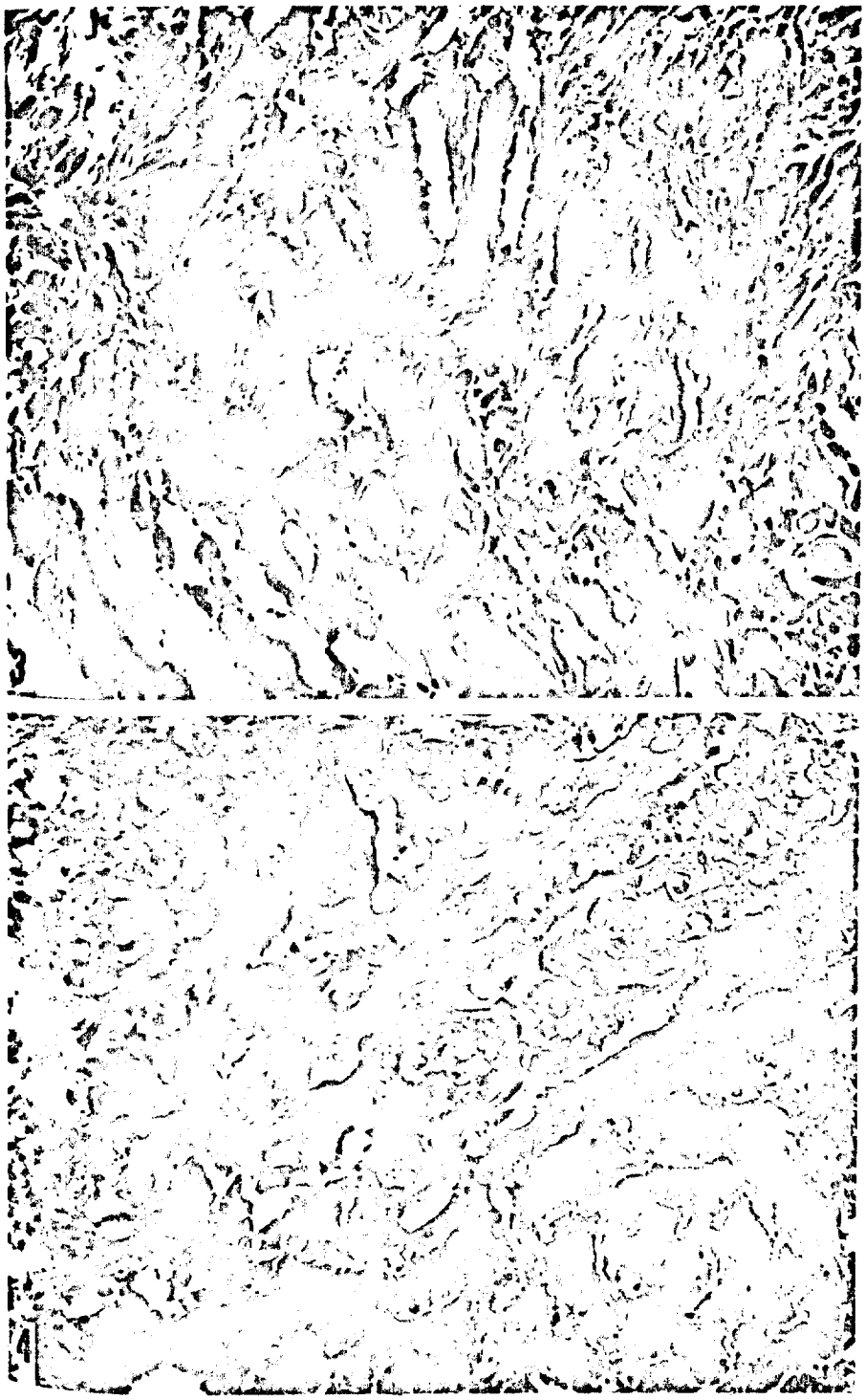

PLATE 5

FIGURE 5.—Metastatic fibrosarcoma in the lung of a rabbit from an endosteal primary cancer. × 340

FIGURE 6.—Insuloma in the pancreas of a rat intrafemorally injected with nickel. × 43

FIGURE 7.—Metastatic oval-cell sarcoma in the kidney of a rat originating from a primary sarcoma of the groin following injection of nickel into the vena saphena. × 340

FIGURE 8.—Metastatic spindle-cell sarcoma in the lung of a rat following intravenous injection of nickel into the vena saphena and the formation of a sarcoma in the region of the groin. × 340

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Arch Environ Health
5: 445-62 (1962)

Experimental Studies in Metal Carcinogenesis

Chromium, Nickel, Iron, Arsenic

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In a series of epidemiologic and experimental studies published during the last decade, various etiological and epidemiologic aspects of metal carcinogenesis in man and animals were explored.[1-22] The inorganic chemicals investigated were chromium, nickel, beryllium, uranium, arsenic, and asbestos. The present concluding report on this important and challenging subject presents the results of several experiments conducted with chromium compounds, metallic nickel, metallic iron, and arsenious oxide.

I. Studies on Hexavalent and Trivalent Chromium Compounds

The experiments performed with chromium compounds were undertaken for the following purposes:

1. *Intramuscular and Intrapleural Injection of Sodium Dichromate.*—In previous experiments on the carcinogenicity of chromium compounds the methodologic procedure used consisted of the intrapleural and intramuscular implantation of hexavalent chromium compounds with low or medium solubility in water. Such compounds were only slowly mobilized from their depots and exerted a prolonged local effect.

While some of the compounds previously tested are produced during the smelting process and contaminate the plant atmosphere, a major respiratory exposure in such establishments is related to highly water-soluble compounds like sodium and potassium monochromate or dichromate. Although it may be anticipated that a small part of such chromium compounds, when in contact with tissue proteins, is retained transitorily in the form of chromium-protein complexes, the bulk is readily absorbed into the blood and lymph and excreted in the urine. Despite the essentially fleeting effect of these highly water-soluble and corrosive chromium compounds, they may exert or contribute to a carcinogenic effect upon the tissues of the respiratory tract, if such relatively mild exposures are repeated and pro-

Submitted for publication Jan. 2, 1962.
National Cancer Institute.

TABLE 1.—*Distribution of Deaths of Rats Intramuscularly and Intrapleurally Injected with Sodium Dichromate Solution*

	Months After Start							Tumors		Cancers At Site
	0-6	7-9	10-12	13-15	16-18	19-21	22-24	Benign	Malignant	
Intrapleural	10	2	1	8	5	2	11		3	1
Intramuscular	2	3	2	10	5	4	13	4	2	0
Untreated	1	4	3	8	6	10	28	4	12	0

longed. This type of exposure prevails for workers employed at certain operations in chromate-producing and using plants.

To investigate this premise, experimental conditions were devised which to some degree imitated the type of exposure sustained by chromate workers. Two sets of 20 male and 19 female 3-month-old rats of the Bethesda black strain received injections into the muscle tissue of the thigh and the right pleural cavity, respectively, with 2 mg. of sodium dichromate dissolved in 0.05 cc. of a 10% gelatin solution administered at monthly intervals. A total of 16 injections was given. Survivors were killed at the end of a 24-month observation period. The results are summarized in Table 1.

The only cancer situated at the site of injection was an adenocarcinoma of the right lung extending into the myocardium (Fig. 1). The cancers located in other organs were 2 reticulum cell sarcomas of the liver, 1 round cell sarcoma of the ileocecal lymph nodes, and 1 squamous cell carcinoma of the uterine mucosa. Since such cancers occur on a "spontaneous" basis in animals of this strain at a frequency similar to that observed in this experiment, they are evidently unrelated to the treatment given. The benign tumors noted were 1 adenofibroma of the breast, 1 cystadenoma of the ovary, 1 adenoma of the renal cortex, and 1 cholangioma.

From the evidence obtained, it may be concluded that sodium dichromate is capable of eliciting cancers of the lung under proper conditions of exposure. Although there was only one cancerous response of this type elicited, this conclusion is justified because similar carcinomatous reactions were observed in the lungs of rats following an intrapleural administration of other car-

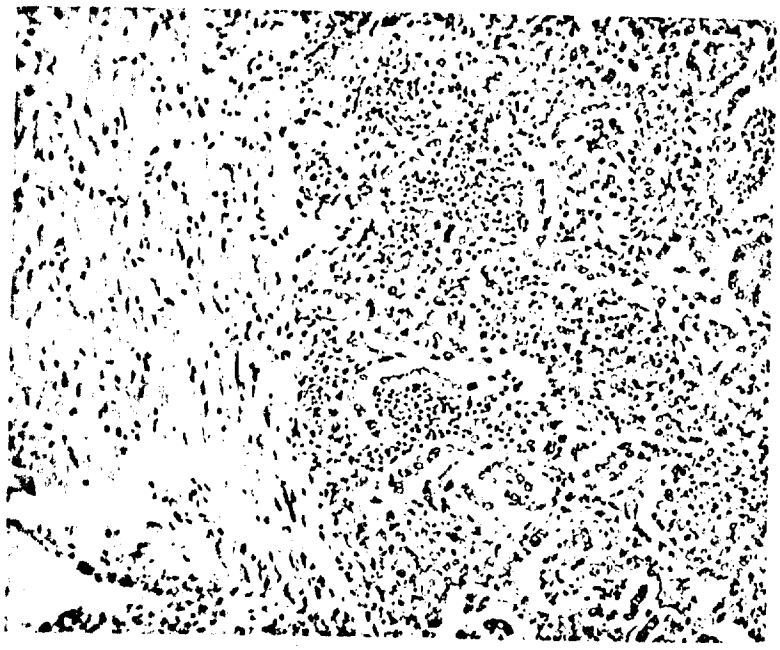

Fig. 1.—Adenocarcinoma of the lung invading myocardium of a rat intrapleurally injected with a solution of sodium dichromate.

cinogenically more potent chromium compounds.[1-12]

2. *Intratracheal Injection of Calcium Chromate, Strontium Chromate, and Zinc Chromate.*—In view of the marked sarcomatogenic and carcinogenic action of calcium chromate, strontium chromate, and zinc chromate when introduced into the pleural cavity of rats, demonstrated in previous experiments,[10-12] it seemed pertinent to investigate the effects of these chromates upon the bronchial mucosa. For this purpose, a PE 10 polyethylene tubing was inserted into a 17-gauge spinal type needle. The needle was then introduced into the trachea, and the polyethylene tubing was pushed down until it hit the bifurcation. The needle was then withdrawn leaving the tube in place. The end of the tube was now connected with a tuberculin syringe filled with a suspension of 200 mg. of a chromate (calcium chromate, strontium chromate, zinc chromate) in a 10% gelatin solution. The individual dose given was 0.05 cc. (2 mg. of the salt). The rats were anesthetized with sodium pentobarbital (Nembutal) for this procedure and were suspended for 5 minutes on a string from their upper front teeth after injection so as to favor the settling of the chromate particles in the lower portions of the bronchial tree. This treatment was repeated every 2 months until a maximum of 5 instillations containing up to 10 mg. of chromate had been given. While the rats usually survived the immediate effects of this operation, many died from pulmonary complications within the first 4 weeks after each application.

Equal amounts of a 10% gelatin solution were administered intratracheally to a control group. In a second control group, 12.5 mg. of calcium chromate was implanted in a gelatin capsule into the right pleural cavity and into the muscle tissue of the thigh of 2 sets of rats. The animals were observed for a maximal period of 2 years when survivors were killed for histopathologic study. The results obtained are summarized in Table 2.

The intrathoracic tumors found in 3 out of 218 rats injected intratracheally with calcium chromate and strontium chromate, respectively, were fibrosarcomas (Fig. 2). Bronchiectases lined with metaplastic squamous cell epithelium, peribronchial bronchiolar adenomatosis, and cystic adenosis in chronic inflammatory, fibrotic tissue were often found in the lungs of these rats, but only one animal had definite carcinomatous changes. Cancers at nonpulmonary sites in these rats were, in 3 cases, round cell sarcomas of the ileocecal lymph nodes and, in 1 rat, a fibrosarcoma of the mesentery. One rat had an adenofibroma of the breast. This rather meagre yield of malignant pulmonary neoplasma after an intratracheal injection of up to 10 mg. of carcinogenically potent chromates is in strong contrast to a tumor yield of 50% or better obtained when 12.5 mg. of calcium chromate placed in a gelatin capsule was introduced into the pleural cavity or the muscle tissue of rats. Gelatin alone did not elicit any abnormal neoplastic response in these tissues. Gelatin solution instilled intratracheally did not produce pulmonary tumors.[15,16] Among animals of this series there occurred 1 mesothelioma of the

TABLE 2.—*Death Distribution and Tumors in Rats Intratracheally Injected with Calcium Chromate, Strontium Chromate, and Zinc Chromate*

	Months							Total No.	Tumors		Cancers at Site
	0-6	7-9	10-12	13-15	16-18	19-21	22-24		Benign	Malign.	
Calcium chromate	78	4	3	—	—	—	—	85	—	3	2
Strontium chromate	40	2	6	2	4	—	6	60	1	2	1
Zinc chromate	51	11	—	3	3	1	4	73	0	1	0
Gelatin	7	2	2	1	8	7	8	35	0	4	0
Calcium chromate I.P.	2	4	2	1	1	—	4	14	0	8	8
Calcium chromate I.M.	1	1	—	1	3	1	1	8	0	6	4

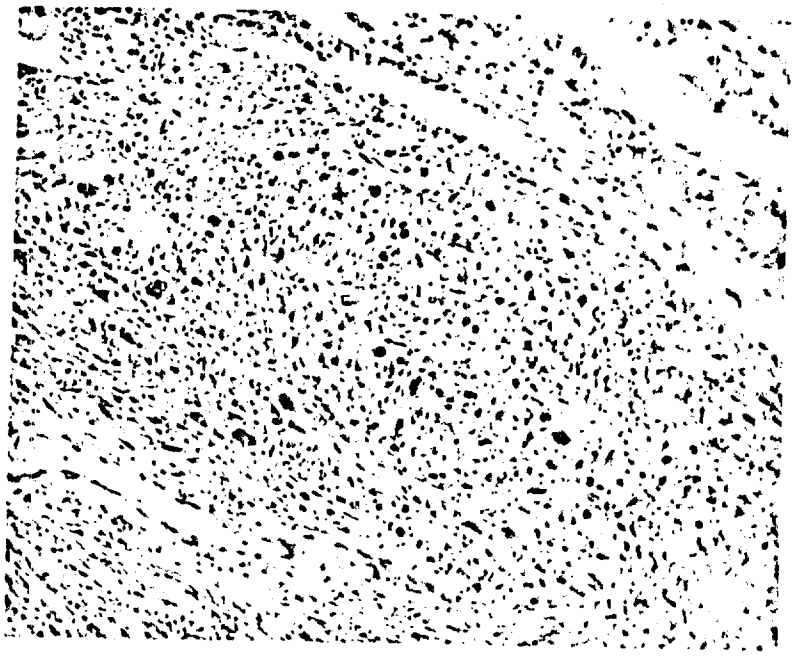

Fig. 2.—Fibrosarcoma of the lung in a rat intratracheally injected with suspensions of strontium chromate.

pleura, 1 reticulum cell sarcoma of the liver, and 1 round cell sarcoma of the ileocecal lymph nodes, i.e., cancers which appear without any intervention in rats of this strain with about the same frequency.

A plausible explanation for the obvious failure of this experiment in producing carcinomas of the lung following an induced direct and an apparently adequate bronchial and bronchiolar exposure to proven carcinogenically potent chromates is not readily available.

3. *Intrapleural and Intramuscular Implantation of Chromic Acetate.*—The claim that occupational exposures to trivalent chromates do not cause cancerous reactions has repeatedly been advanced,[21,22] because chromium in this valence form was said to be chemically and biologically inactive. Since Grogan had shown that finely dispersed trivalent chromium is converted by the action of oxygen into hexavalent chromium and reconverted apparently by reduction in the cell into a trivalent depot chromium, the validity of this thesis needed to be tested.

Two sets of Bethesda black rats, consisting of 42 animals receiving implantations in the right pleural cavity and of 35 with implantations into the right thigh muscle of 25 mg. of chromic acetate (Mallinckrodt Chem., black crystals) placed into a gelatin capsule, were used. A second implantation of an identical amount of chromic acetate was made one month later. Six additional implants were made thereafter at 2-month intervals, making a total of 8 implantations. The observation period extended over 24 months, at the end of which all surviving animals were killed and subjected, like all animals which died earlier, to autopsy. Greenish encapsulated material was found in the muscle tissue of rats which received intramuscular implants.

TABLE 3.—*Death Distribution and Tumors in Rats Intrapleurally and Intramuscularly Implanted with Chromic Acetate*

	Months							Tumors		
	0-6	7-9	10-12	13-15	16-18	19-21	22-24	Benign	Malign.	Cancers at Site
Intrapleural	2	3	12	5	5	6	9	1	5	0
Intramuscular	1	1	5	5	7	6	10	1	6	1

The death distribution and the tumor yield are summarized in Table 3.

Only one of the intramuscularly injected rats developed a neoplasm at the site of implantation. This was an anaplastic spindle cell sarcoma with giant cells invading the surrounding muscle tissue. None of the other cancers occurring in either series seemed to have any connection with the implanted chromic acetate. One was a fibromyxosarcoma of the subcutaneous tissue of the back, 3 were mesotheliomas of the pleura, 3 were round cell sarcomas of the ileocecal lymph nodes, and 3 others were carcinomas of the uterine endometrium. Of the 2 benign tumors, one was a multifocal papilloma of the urinary bladder and the second one was an adenofibroma of the breast.

In contrast to the marked carcinogenic effect elicited by some of the parenterally administered hexavalent chromates reported on in previous publications, chromic acetate exerted at best a weak carcinogenic influence upon muscular tissue. Whether this phenomenon is common to all trivalent compounds, however, remains to be investigated by testing less water-soluble trivalent chromium compounds in bioassays.

4. *Immunochemical Studies with Chromium-Protein Complexes.*—A number of years ago (1936) the concept was advanced that the development of various occupational cancers might be related to chemoallergic reactions resulting from an interaction of chemical carcinogens, such as certain metals and aromatic amino- and azo-compounds, with proteins or nucleoproteins of the host organism.[23-28] This theory received considerable support in intervening years from the demonstration of the in vitro and in vivo formation of protein complexes with various carcinogens (3,4-benzpyrene,[30] *p*-dimethylaminoazobenzene,[31] 1,2-dibenzanthracene,[32] 1,2,5,6-dibenzanthracene,[33-35] beryllium[36]). Clinical observations with various carcinogenic metals, such as chromium and nickel, had moreover shown that they act frequently as allergic sensitizers to the human skin. Several investigators[37-42] had obtained chemospecific antibody reactions and anaphylactic phenomena in guinea pigs and rabbits sensitized to chromium by parenteral injections and skin applications of chromium compounds dissolved in several types of protein solutions.

In previously published observations[18,19] preparatory to the present studies, it had been ascertained that chromium salts under suitable experimental conditions form complexes with egg albumin and plasma proteins. An attempt was then made at demonstrating the antigenic property of such complexes in immunochemical experiments on animals injected with such agents. The blood plasma of workers employed in the production of chromates, some of whom had developed cancer of the lung was examined for the presence of immune bodies of chromium-specific nature (1955).[26] The possibility of such immune reactions was likely, since Clark[43] in 1959 showed that blood plasma albumin is denatured by both trivalent and hexavalent chromium compounds at pH <5.4.

Experimental Procedures

1. *Preparation of Haptens and Antigens.*—Haptens: The following chromium reagents were prepared and standardized for use in introducing Cr^{III} and Cr^{VI} containing haptenic groups into the various proteins to be employed as antigens in precipitin tests, and for the sensitization of animals for the formation of antisera.

Potassium Chromate. A total of 38.840 gm. of the dried reagent neutral salt in one liter of double-distilled water contained 10.388±0.006 mg. C^{VI} per milliliter as determined by iodometric comparison with a dichromate primary standard.

Chromic Acetate. Chromic acetate+aq., Mallinckrodt reagent grade, 49.432 gm. per liter in double-distilled water yielded 0.097% brownish-red insoluble material. Iodometric analysis of the filtered solution gave 12.123±0.006 mg. Cr^{III} per milliliter. These 2 solutions served as stock solutions from which appropriate dilutions were made in using Cr^{III} and Cr^{VI} as haptens.

Chromite Ore and Chromite Ore Roast Residue: Commercial powdered chromite ore* was micropulverized and particles in the range 0μ-4μ separated out in a Roller Particle Size Analyzer and used in these experiments. The residue still contains some 3%-4% chromium.

* Supplied by the Mutual Chemical Company of America, Baltimore, Md.

Antigens: Egg Albumin. A single lot of egg albumin scales was used throughout. Sterile stock solutions containing from 60-100 mg. protein per milliliter were prepared by dissolving the protein in phosphate buffer pH 7.35 (adjustment of pH with 0.1 N NaOH when necessary), filtration through a Seitz bacteriological filter, and storage of 3-5 ml. aliquots in sealed ampoules at 3-5 C.

Egg Albumin-Cr Complexes. Sufficient Cr^{III} or Cr^{VI} (stock solution diluted so as not to cause precipitation of the protein) was added to sterile egg albumin solutions to provide ratios of Cr (micrograms) to protein (milligrams) from 1-5.

Human Plasma and Sera. Heparinized human blood was obtained from individual donors. After the removal of the formed elements by centrifugation, the plasma was stored under sterile conditions in sealed ampoules. Sera obtained by permitting spontaneous clotting of blood were also stored under sterile conditions.

Human Plasma- or Sera-Cr Complexes: These were formed by adding Cr^{III} or Cr^{VI} to the protein solutions to provide ratios of Cr (micrograms) to protein (milligrams) from 1-5.

Human Plasma-Chromite Ore. Chromite ore, 0μ-4μ particle size, was added to human plasma in the range 5-10 mg. per milliliter plasma, mixed on a shaking machine, centrifuged, and the supernatant protein solutions used as antigen.

Human Plasma-Chromite Ore Roast Residue. Pulverized roast residue was added to human plasma in the range 1.25-2.50 mg. per milliliter plasma, mixed on a shaking machine, centrifuged, and the supernatant protein solutions used as antigen. The roast residue contains Cr^{VI}, readily seen by the yellowish coloration of the aqueous supernatant fluid of an extract of the pulverized residue.

Animal Plasma. In addition to the plasma of human beings, plasma was obtained from rats, rabbits, sheep, goats, and guinea pigs. These were stored under sterile conditions.

Animal Plasma-Cr Complexes. Cr^{III} and Cr^{VI} solutions were added to each of the animal plasmas listed above so as to give Cr-protein ratios equivalent to those employed with egg proteins and human plasma.

Roast Residue. A total of 125 mg. of roast residue was added to boiled double-distilled water in a closed system, permitted to stand for 24-48 hours, centrifuged, and the supernatant extract employed.

Chromite Ore. An aqueous extract of chromite ore was prepared in a similar manner.

2. *Preparation of Antisera Against Chromium-Protein Complex.*—Sets of 2 virgin young rabbits were injected intraperitoneally once a week for 6 successive weeks, with 1 cc. of solutions of the following antigens for producing specific antisera.

A. Egg albumin solution in phosphate buffer at pH 6.4. Each cubic centimeter of the sterile solution contained approximately 100 mg. of egg albumin.
B. Egg albumin-potassium chromate solution.
C. Egg albumin-potassium chromate solution.
D. Human serum-chromium acetate solution.
E. Human serum-potassium chromate solution.
F. Rat serum-chromium acetate solution.
G. Rat serum-potassium chromate solution.

This regimen was repeated after an injection-free interval of one month.

One week after the last injection the animals were bled from the carotid artery under sterile conditions. The blood corpuscles were separated from the serum by centrifugation, and the supernatant fluid was filled into sterile glass ampoules which were sealed and stored in the refrigerator for future use. The antisera were then tested for their precipitin-antibody titers against solutions of the protein component of the antigen. In these tests 0.1 cc. of the various protein antigens diluted with isotonic saline at a ratio of 1:10 was overlaid on 0.5 cc. of increasing dilutions of antiserum (1; 1:10; 1:100; 1:1,000; 1:10,000; 1:20,000; 1:50,000). The test was considered positive if a ring-shaped white precipitate (P) or an opalescent ring (O) appeared at the surface of contact of the 2 solutions. Each of these 2 types of reactions was graded into 4 arbitrary grades depending upon their relative degree of intensity. Sera used in subsequent tests had a maximal antibody titer of up to 1:10,000 to 1:20,000 against the specific protein and a zero titer against other proteins used in antigen production.

The antisera were then tested for the presence of chromium-specific antibodies against antigens containing a chromium-protein complex. Their protein component differed from that employed in the production of the antiserum. Before the reactions were read, the test tubes were incubated for one hour at 37 C and then refrigerated overnight.

Table 4 presents the results. The successful production of chromium-specific antibodies is demonstrated.

The evidence obtained through these and similar tests strongly suggested that chromium-specific antibodies were formed by rabbits receiving repeated parenteral injections of trivalent and hexavalent chromium compounds dissolved in protein solutions of various species (chicken, man, rat). With this evidence available an attempt was made to demonstrate the presence of chromium-specific antibodies and antigens in the blood serum of 12 workers engaged for varying periods in the production of chromates who had developed occupational cancer of the lung. Sera of 10 members of the Environmental Cancer Section, National Cancer Institute, engaged in research work with chromium compounds were similarly tested. The human sera were tested for chromium-specific antibodies against antigens prepared from trivalent

TABLE 4.—*Chromium-Specific Precipitin Reactions Obtained with Various Antisera and Antigens Prepared with Trivalent and Hexavalent Chromium*

Antigen Dilutions	Rabbit Antiserum Prepared with Trivalent & Hexavalent Chromium in Rat Plasma						Saline
	Undiluted	1:1	1:10	1:25	1:100	1:1,000	
Egg albumin Cr[3]							
1:10	Px	Px	Px	Oxx	Oxx	Oxx	—
1:100	Pxx	Px	Px	Oxx	Oxx	Oxx	—
1:1,000	Pxx	Pxx	Ox	Ox	—	—	—
1:10,000	—	—	—	—	—	—	—
Saline	—	—	—	—	—	—	—
Egg albumin Cr[6]							
1:10	Px	Oxxx	Ox	—	—	—	—
1:100	—	—	—	—	—	—	—
Rabbit plasma Cr[3]							
1:10	Ox	Ox	Ox	—	—	—	—
1:100	Ox	—	—	—	—	—	—
Rabbit plasma Cr[6]							
1:10	Oxxx	Oxx	Ox	—	—	—	—
1:100	Oxx	Ox	—	—	—	—	—
1:1,000	Ox	Ox	—	—	—	—	—
1:10,000	Ox	Ox	—	—	—	—	—
Human plasma Cr[3]							
1:10	Pxxx	Px	—	—	—	—	—
1:100	Pxx	Px	Px	—	—	—	—
1:1,000	Pxx	Px	—	—	—	—	—
1:10,000	Px	Px	—	—	—	—	—
Human plasma Cr[6]							
1:10	Pxxx	Oxxx	—	—	—	—	—
1:100	Pxx	Px	—	—	—	—	—
1:1,000	Px	Px	—	—	—	—	—
1:10,000	Px	Px	—	—	—	—	—
Rat plasma Cr[3]							
1:10	Pxx	Pxx	Px	Px	Px	Px	—
1:100	Px	Px	Px	Px	Px	Oxxx	—
1:1,000	Px	Px	Px	Oxxx	Oxxx	Ox	—
1:10,000	Px	Px	Px	Px	Oxx	—	—
Rat plasma Cr[6]							
1:10	Pxxx	Pxx	Px	Oxxx	Ox	Ox	—
1:100	Pxx	Pxx	Px	Px	Pxx	Ox	—
1:1,000	Px	Px	Px	Px	Oxxx	Oxx	—
1:10,000	Px	Px	Px	Px	Oxx	Ox	—
	Pxx	Pxx	Px	Px	Px	Px	—

P indicates precipitin ring; O, opalescence.

and hexavalent chromium compounds, chromite ore, and chromite roast residue added to human plasma, rabbit plasma, and rat plasma. Some of the long-term workers seemed to have chromium-specific antibodies in their sera, since their sera elicited definite precipitin reactions in undiluted form and at a 1:10 dilution when brought in contact with the various antigens, particularly those prepared with trivalent chromium. Since the results were not always consistent and were demonstrable only when rather high concentrations of antigen were used, they must be considered equivocal at present. The observations seem sufficiently promising to justify a continuation of investigations on immunochemical reactions to chemical carcinogens in man and animals.

II. Studies in Metallic Nickel

The distinct carcinogenicity of metallic nickel when introduced into the pleural cavity, muscle tissue, and subcutaneous connective tissue has been established.[2,5] The results obtained by inhalation of powdered metallic nickel are not entirely satisfactory. Since Sundermann, Donnelly, West, and Kincaid[44] reported the occurrence of squamous cell carcinomas in the lungs of rats following repeated inhalations of nickel carbonyl, it seemed desirable to reinvestigate this area by modified experimental methods.

1. *Intrapulmonary Deposition of Powdered Metallic Nickel.*—Although it had been demonstrated that carcinomas of the lung develop in rats after an intrapleural implantation of chromite ore roast,[2,5] the identical technical approach using powdered metallic nickel had yielded only sarcomas. It

was thought that the positive results obtained with chromite ore roast might be attributable to the fact that this material is corrosive and might establish direct contact with lung tissue through the mechanism of chemical trauma, while powdered metallic nickel might not readily penetrate into the lung tissue. Therefore, sarcomas originated from mediastinal connective tissue into which the metal particles were transported by phagocytic action. It was, therefore, decided to inject powdered metallic nickel suspended in a 10% gelatin solution into the lungs of rats to insure direct contact with pulmonary tissue.

Finally powdered metallic nickel obtained by the disintegration of nickel carbonyl † was used in these experiments. By thoracotomy 34 rats of the Bethesda black strain (20 females, 14 males), 3 months old, received injections into the right lung of approximately 0.02 cc. of a suspension of nickel powder in gelatin solution. The solution contained 2 gm. of nickel per 10 cc. of 10% gelatin. While most rats tolerated this procedure quite well, about 25% died within the subsequent 72 hours. The procedure was repeated on the surviving rats once 12 months later. The maximal observation period was 24 months. The results of this experiment and the death distribution of the rats are presented in Table 5.

The cancer involving the right lung of 1 rat was a spindle cell sarcoma. The other 2 cancers found in rats of this series were a squamous cell carcinoma of the uterine endometrium and a squamous cell carcinoma of the skin of the cheek. Local fibrosis, in some animals containing brown-black pigment and circumscribed areas of bronchiolar adenomatosis, was present in many of the injected lungs. While the occurrence of a spindle cell sarcoma in one injected lung confirmed the carcinogenic property of metallic nickel, the experiment failed in its principal purpose, i.e., the production of bronchiogenic carcinomas.

2. *Inhalation of a Combination of Powdered Metallic Nickel, Powdered Limestone, and Sulfur Dioxide by Rats and Hamsters.*—The failure of most investigators to obtain bronchiogenic carcinomas in experimental animals by the inhalation of various carcinogenic agents only (exceptions: nickel, beryllium, and radioactive chemicals)[45] suggested that in the experimental animal additional factors acting upon the bronchial mucosa together with the carcinogen might be essential for eliciting a carcinogenic response. The success reported in this respect by Sundermann et al.[44] when using nickel carbonyl per se seemed to support this view, since nickel carbonyl disintegrates readily into finely dispersed metallic nickel and carbon monoxide and is extremely toxic to the lung tissue. The likelihood, therefore, existed that the production of bronchiogenic carcinomas in rats by this procedure might be attributable to a combined action of chemical trauma impairing normal defensive mechanisms of the bronchial mucosa and permitting a direct and prolonged action of a carcinogen simultaneously introduced upon these tissues.

Among the various irritative chemicals which occur together with specific carcinogens in the general atmosphere as well as in the air of plants with pulmonary cancer hazards and which exert an injurious effect upon the bronchial mucosa, sulfur dioxide is one of

† Courtesy of Prof. H. Oettel, Badische Anilin & Soda Fabrik.

TABLE 5.—*Death Distribution and Tumor Yield in Rats Following an Intrapulmonary Deposition of Metallic Nickel*

	Months							Tumors		
	0-6	7-9	10-12	13-15	16-18	19-21	22-24	Benign	Malignant	Cancers at Site
Rats	8	2	1	4	5	4	10	0	3	1

† Courtesy of Prof. H. Oettel, Badische Anilin & Soda Fabrik.

the most prominent and widely distributed air pollutants of this type. It was conceivable, therefore, that this chemical when inhaled together with carcinogenic agents might act via chemical trauma to the bronchial mucosa as a cocarcinogen. Applying these considerations to experimental design, rats and hamsters were exposed to the inhalation of sulfur dioxide and mixture of powdered nickel and powdered limestone. The mineral mixture, composed of 3 to 4 parts of nickel and 1 part of limestone for the hamsters and of 1 part of nickel and 1 part of limestone for the rats, was discharged into the exposure chamber by a Wright's dust feed apparatus by means of compressed air containing 20 to 35 ppm of sulfur dioxide. The limestone was mainly added to the nickel to prevent the nickel particles from forming conglomerates and to enable a continuous operation of the dust feed apparatus over 5-6 hours per day without delivering toxic amounts of nickel into the exposure chambers. Between 50 and 65 gm. of this mixture was discharged daily into each of the 2 chambers used. The majority of the nickel particles measured from 1μ to 3μ in diameter. The nickel powder ‡ (98.95% nickel, 0.2% iron, 0.12% cobalt, 0.6% copper, and various other inorganic components in minute amounts) had been ground in a ball mill to obtain a suitable particle size.

The death distribution and tumor yield obtained in animals of both species are presented in Table 6.

None of the tumors involved the lungs in either rats or hamsters. While the lungs of many rats exhibited chronic inflammatory, fibrosing changes with bronchiectases, squamous cell metaplasia of the epithelial lining, and peribronchial adenomatosis, none of these lesions was definitely malignant. The lungs of the hamsters, in contrast, showed scarcely any chronic effects ascribable to the exposure. The predominant reaction consisted in more or less diffuse hemorrhages into alveolar lumina.

The essentially negative outcome of this experiment duplicates several similar experiences and demonstrates the technical shortcomings evidently existing in the production of lung cancers in experimental animals by carcinogens of established carcinogenicity for the human lung.

III. Studies on the Effects of Parenterally Introduced Metallic Iron

Isolated reports on the occurrence of lung cancer in individuals occupationally exposed to the inhalation of iron oxide [1,25,26] who had siderosis of the lung suggested that iron in some form at this particular site might be somewhat carcinogenic. This suspicion was enhanced by observations of Faulds and Stewart [46,47] and of McLaughlin and Harding [48,49] of an excessive frequency of lung cancer among English hematite miners and iron foundry workers suffering from occupational siderosis of the lung. Similar observations were recently reported for iron ore miners in Lorraine.[50,51] The experimental production of sarcomas in mice and rats following repeated subcutaneous injections of large amounts of an iron-dextran complex was also attributed to a carcinogenic role of the iron in this antianemic preparation.[52,53] It is noteworthy that pulmonary carcinomas and pleural and peritoneal mesotheliomas seemed to be especially frequent in persons suffering from asbestosis after having been exposed to dust of blue Cape asbestos which

‡ Purchased from the Fisher Scientific Company.

TABLE 6.—*Death Distribution and Tumor Yield in Rats and Hamsters Inhaling a Combination of Powdered Metallic Nickel, Limestone, and Sulfur Dioxide*

	Months							Tumors		Cancers at Site	No. & Sex of Animals
	0-6	7-9	10-12	13-15	16-18	19-21	22-24	Benign	Malign.		
Rats	27	4	9	16	18	16	30	6	7	0	120 60m, 60f
Hamsters	14	—	6	8	6	9	57	6	8	0	100m

TABLE 7.—*Death Distribution and Tumor Yield in Rats Intrapleurally and Intramuscularly Implanted with Powdered Metallic Iron*

	Months							Tumors		Cancers at Site	Site of Implant.
	0-6	7-9	10-12	13-15	16-18	19-21	22-24	Benign	Malignant		
Rats	2			2	6	5	20	0	4	0	Intrapleural
	1	2	4	6	5	6	11	0	3	0	Intramuscular

contains about 44% iron.[54] The fact that asbestos bodies found in asbestotic lungs, including pulmonary cancer tissue, are coated with a sheath composed of an iron-protein complex has stimulated the speculation that iron present in the coating or the iron-protein complex may be involved in cancers in asbestotic lungs.

Large worker groups are exposed to metallic iron and iron oxide dust. Iron pentacarbonyl exerts a distinct pathogenic action on the lung similar to that of nickel carbonyl and has been increasingly used industrially.[55] Thus, there seems to be a need for an experimental study of the alleged carcinogenicity of iron.

Finely powdered metallic iron produced by degradation of iron carbonyl § of an average particle size of 2.45μ was used in the study. Pellets of 25 mg. of iron suspended in 50 mg. wool fat were placed surgically in the right pleural cavities of 35 Bethesda black rats (20 males, 15 females), 3-months old. An equal number was implanted with pellets of identical composition in the muscle tissue of the right thigh. A second implant was placed at these sites 12 months later. Table 7 presents the death distribution and tumor yield of both series.

None of the cancers observed in rats of both series involved the implantation site. The powdered iron implanted into the pleural cavity elicited in some rats the development of nodular foreign-body granulomas (sideromas) consisting of masses of phagocytic and foreign-body giant cells attached to the pleura, often extending into the adjacent pulmonary parenchyma and containing masses of powdery brownish-black intracellular and extracellular pigment which, at places, formed solid deep black-stained blotches. In other rats the iron-powder-containing pleural de-

§ Supplied by Prof. H. Oettel, Badische Anilin & Soda-Fabrik.

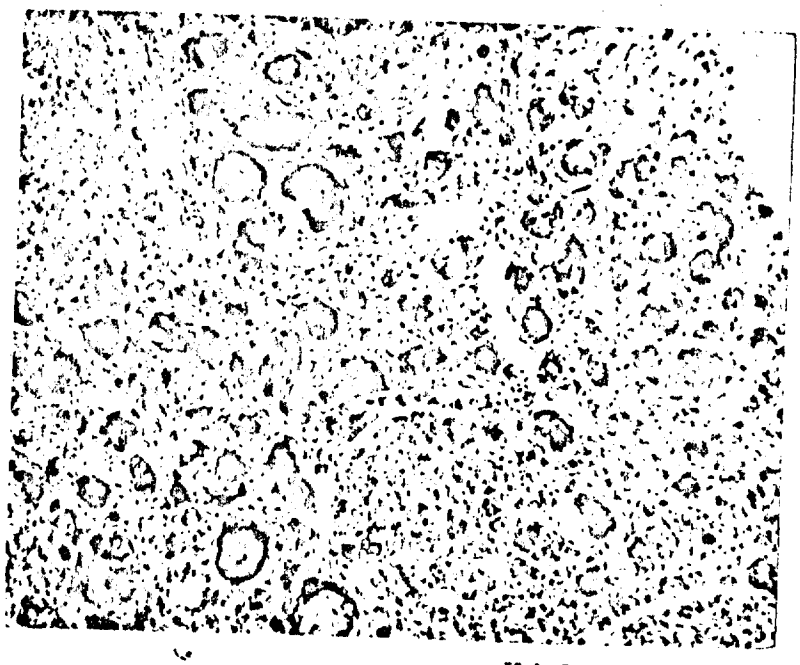

Fig. 3.—Pleural sideroma in a rat with phagocytic cells and giant cells containing large amounts of finely powdered and conglomerated carbonyl iron.

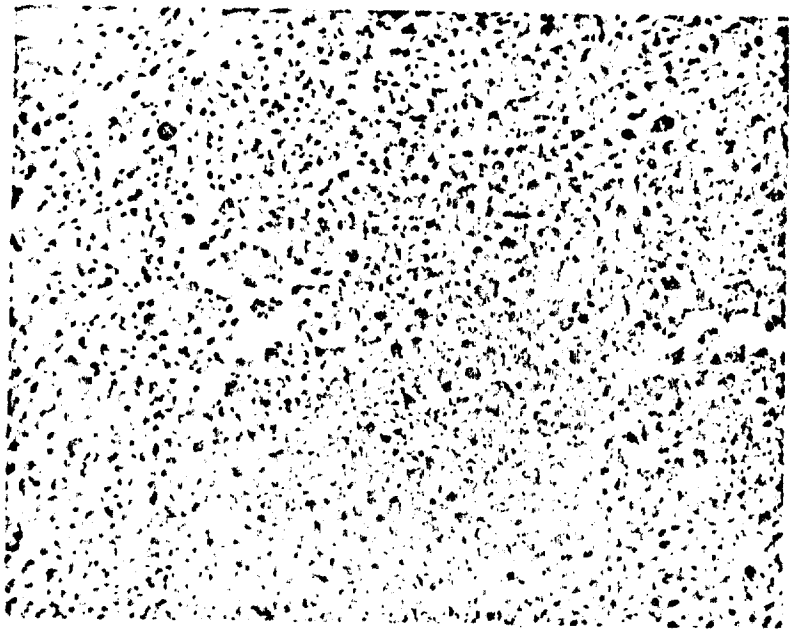

Fig. 4.—Massive accumulation of phagocytes, some containing iron, in a sideroma formed in the muscle tissue of the thigh of a rat.

posits formed a thick coating covering a larger area of the pleura and displaying similar cellular reactions. The matrix of both formations exhibited at times a remarkable amount of hyaline and fibrotic material (Fig. 3). Histologically similar sideromatous nodular lesions were found in the thigh muscle of rats with intramuscular iron deposits (Fig. 4). The absence of any distinct inflammatory reaction around such formations was noteworthy.

The failure to produce any type of cancerous response around intramuscular and intrapleural depots of finely powdered iron does not support the concept that iron in general possesses carcinogenic properties. Negative experimental observations recorded by Müller and Ehrhardt [56] also support this conclusion.

IV. Prolonged Oral Administration of Arsenious Oxide in a 12% Alcoholic Solution to Rats

Recent observations reported from Germany on the frequent occurrence of chronic arsenicosis, multiple cancers of the skin, and cancers of the lung, larynx, nasal sinuses, and liver among vineyard workers exposed in previous years to arsenical pesticides and to wine heavily contaminated with arsenic provided a stimulus for another attempt to produce experimental arsenic cancers.[57,58] The urgent need for such evidence had become especially apparent because of doubts concerning the validity of available medical evidence on this matter.[59,60,61]

In the new impressive German observations on the carcinogenic action of arsenicals in man, the arsenicals were introduced mainly in an alcoholic solution. In view of the negative or equivocal results obtained in experimental animals exposed to arsenicals in other forms by several previous investigators,[62,25,63] the use of an alcoholic solution of arsenicals orally administered to animals (rats, mice) seemed worth a trial, especially in view of the fact that the sites of arsenic cancers among German vineyard workers had included, in addition to the skin and the liver, various parts of the respiratory tract.

Male and female Bethesda black rats, 3 months old, and C57 black mice, 2 months old, were given arsenious oxide (As_2O_3) in the drinking water. Four series of 50 animals each were used. The first set of animals received 4 mg. of arsenious oxide in 1 liter of a 12% aqueous ethyl alcohol solution. The arsenic content of this medium was increased by 2 mg. per liter each month until a maximum of 34 mg. of arsenious oxide per liter

TABLE 8.—*Death Distribution and Tumor Yield in Rats and Mice Given Arsenious Oxide and Ethyl Alcohol in Their Drinking Water*

Treatment	Species	Months							Sites & No. of Cancers				
		0-6	7-9	10-12	13-15	16-18	19-21	22-24	Pleura	Liver	Lymph Node	Uterus	Skin
Arsenic + alcohol Set I	Rats	1	3	3	5	10	12	16	3	2	4	2	2
	Mice	25	25	—	—	—	—	—	—	—	—	—	—
Arsenic + water Set II	Rats	4	2	2	2	5	2	32	2	1	2	—	—
	Mice	5	2	12	16	6	3	3	—	—	—	—	—
Alcohol Set III	Rats	6	1	4	4	7	13	15	3	2	1	—	—
	Mice	13	33	4	—	—	—	—	—	—	—	—	—
Water Set IV	Rats	0	0	2	1	9	10	28	1	5	2	—	1
	Mice	7	10	11	11	9	2	—	—	—	—	—	—

was reached after 15 months. The arsenic level was stabilized at this point for the 24-month duration of the experiment. Since rats consumed between 20 and 25 cc. of this fluid per day, the daily intake of arsenious oxide per rat rose from approximately 0.1 mg. at the start to about 0.8 mg. at the end. The second set of animals was kept on an aqueous solution of arsenious oxide of the same concentrations as those employed in the first set. The third set of animals was fed 12% ethyl alcohol, and the fourth set received ordinary tap water.

Fluid intake and weight were checked at frequent intervals. While the rats tolerated the drinking water containing arsenic and/or alcohol well and gained weight, the mice reacted rather badly to drinking water spiked with arsenic. They showed little gain in weight and died rather early. An intestinal infection resulting in liver abscesses in the mice of all groups complicated the experiment and influenced its results. Table 8 presents the death distribution of the rats and mice of the 4 sets as well as the tumor yield.

With the exception of a leukemia present in a mouse in Set IV on plain water, cancers were absent among the mice of all sets. While the total number of cancers was highest among rats on an alcoholic solution of arsenious oxide (13 cancers), they did not differ in type from those "spontaneously" developing in animals of this strain. In fact, the control rats of Set IV had more reticulum cell sarcomas of the liver than those of Set 1 and had 8 cancers, one of which was a squamous cell carcinoma involving the cheek. It was identical in site and type to the 2 cancers of the skin found in rats of the principal test Set 1. None of the rats revealed any lung cancers.

The purpose of this experiment was not achieved, i.e., conclusive experimental evidence concerning the carcinogenic property of arsenicals was not obtained.

Comment

The experimental observations reported here and in previous publications on the carcinogenicity of metallic chromium and its trivalent and hexavalent compounds indicate that the relative carcinogenicity of these agents for rats and mice is determined by their relative biologic availability, i.e., mainly their relative solubility in water, especially when administered parenterally in a single dose exerting depot action. This depends in its intensity and duration on the dose as well as on its relative solubility in aqueous biologic fluids. From the total experimental evidence at hand, it is possible to construct a scale of carcinogenic responses with a peak when chromium salts moderately soluble in water are used and its lowest levels when highly water-soluble compounds (chromium acetate, sodium dichromate) which are rapidly removed from the site of introduction are employed or when the chromium-containing agent is insoluble or little soluble in water (metallic chromium, chromite ore, barium chromate) (Table 9). The valence of a particular chromium compound does not seem

TABLE 9.—*Cancerous Responses at the Sites of Implantation of Several Chromium Compounds in the Thigh Muscle and Pleural Cavity of Rats*

Compound	Route 35 Rats	No. Cancers at Site	% Cancer Yield	Min. Latent Period
Chromic chromate	IM	30	86	7
	IPI	34	97	7
Calcium chromate	IM	10	29	7
	IPI	28	80	7
Sintered calcium chromate	IM	13	37	6
	IPI	21	60	6
Strontium chromate	IM	16	46	6
	IPI	17	50	6
Barium chromate	IM	0	0	—
	IPI	2	6	14
Lead chromate	IM	3	9	—
	IPI	3	9	16
Sodium dichromate	IM	0	0	—
	IPI	1	3	10
Chromite roast residue	IM	1	3	19
	IPI	8	23	12
Zinc yellow	IM	16	45	5
	IPI	22	65	6
Chromium acetate	IM	1	3	16
	IPI	0	0	—
Chromite ore roast	IM	3	8	10
	IPI (25)	2	8	19
Chromium metal	IM	0	0	0
	IPI	0	0	0
Chromite ore	IM	0	0	0
	IPI	0	0	0

to play any important or essential role. The present experiments indicate that often repeated exposures to small and noncorrosive amounts of highly water-soluble chromium compounds may also be effective in producing malignant reactions in the tissues of contact.

In view of these experimental findings, it may be assumed that occupational lung cancer hazards are not limited to chromate makers and producers and to packers of chrome pigments but probably extend to members of all those occupations in which there is respiratory contact with the various chromium compounds found to be carcinogenic in animals. Such occupations are spray painters applying chrome-pigment-containing paints to airplanes, railroad cars, oil and gas pipes, ships, bridges and similar steel structures, also welders, metal cutters, and scrap metal smelter workers handling steel parts coated with such paints; moreover, aniline dye producers using chromates as oxidants, chrome platers, and printers inhaling chrome-pigment-containing inks discharged into the atmosphere of workrooms from rotary presses. It is, therefore, likely that the general scope of lung cancer hazards associated with an inhalation of chromium compounds is distinctly greater than that usually assumed.

The extremely meagre yield of intrathoracic tumors observed in rats receiving repeated intratracheal instillations of suspension of several chromates with demonstrated high carcinogenicity for lung and mesenchymatous tissues lacks a satisfactory explanation, especially since this approach gave good results in hamsters injected intratracheally with suspensions of 1,2,5,6-dibenzanthracene [64] and in rats receiving instillations of radioactive chemicals by this route.[65,66] It may be possible either that the severe corrosive action of the chromates used on the bronchial mucosa antagonized the development of a cancerous response or that the instilled material became so generally distributed within the bronchial tree that a locally effective carcinogenic dose was not obtained within the observation period of the experiment.

Since there is currently a shift of emphasis in cancer research from cure to prevention,[67] studies on immunochemical aspects of occu-

pational and environmental carcinogenesis assume significance in human cancer control.[68,27,28] Tests of this type, if available, should prove valuable for etiologic, diagnostic, and prophylactic reasons. They might be applied in preemployment examinations for the selection of workers most suitable for work in carcinogenic industrial operations [68] and may be used to provide a biologic method for checking on the effectiveness of technologic and sanitary preventive measures.

The immunochemical investigations described are merely exploratory and remain fragmentary for reasons beyond our control. These studies are reported mainly to point out an area of much needed research in the occupational and environmental cancer problem. It is likely that future investigations may be more successful, since those recorded here were done when the carcinogenic properties of several specific chromates had not yet been detected.

The negative outcome of the experiment in which rats and hamsters inhaled powdered metallic nickel and sulfur dioxide was not a surprise, because simultaneously conducted experiments on rats exposed to inhalation of fumes generated by the application of heat from carcinogenic coal tar and petroleum asphalt containing irritative constituents, such as acridines, had not resulted in lung cancers. The reasons for this failure are obscure. These results are in disagreement with clinical and statistical observations made on the positive role which chronic bronchitis of carcinogenically specific or nonspecific origin has played in the development of human bronchogenic carcinoma.[69]

The low tumor yield obtained by an intrapulmonary introduction of powdered metallic nickel into rats was not anticipated because of the considerable carcinogenicity which this agent had previously displayed when deposited intrapleurally. These disappointing observations with intrapulmonary implants of nickel contrast with the definite carcinogenic responses obtained in mice and rats following an intrapulmonary introduction of other carcinogens, such as beads of radioactive strontium [70] and textile strands impregnated with a carcinogenic polycyclic aromatic hydrocarbon (1,2,5,6-dibenzanthracene).[71] The reasons for this discrepancy of pulmonary response to different carcinogens directly introduced as depots into the parenchyma of the lung are not immediately apparent and deserve further study.

A carcinogenic role of iron, either alone or in complexed form, recently favored by Haddow and Horning,[53] was postulated years ago by Warren and Drake [72] in primary carcinoma not infrequently found in hematochromatotic livers. Haddow and Horning [53] proposed several mechanisms by which iron might exert a carcinogenic effect: through an imbalance and maldistribution of iron in cells interfering with cellular function, or by a disturbance of the action of metal enzymes, mainly of the respiratory type, either directly upon such enzymes or through an oxidative effect upon SH groups essential for proper enzyme activation.[73] Haddow considers the formation of metal-protein or -nucleoprotein chelates as a possible factor in the carcinogenic effects of certain metals.

The negative results obtained in the experiments described as well as the doubtful role of siderosis among South African Bantus in the production of cancer of the liver, excessively frequent in these Africans, indicate that any carcinogenic action of iron depends upon the presence of iron in a specific form, if iron is actually a primary carcinogenic agent involved in the production of cancers in organs affected by siderosis.[74-78] Since iron present in pulmonary siderosis may occur in various forms, i.e., elementary iron, ferrous oxide, ferric oxide, or a salt, such as iron carbonate, or as a chemically complex iron ore,[79,80] a survey of worker groups exposed to the inhalation of iron in its various forms is needed to obtain reliable data on whether the risk of cancer of the lung is uniformly elevated in persons with siderosis of all types or occurs only in individuals with one or several specific types of siderosis. Future experimental investigations might include studies on the respiratory, oral, and parenteral introduction of natural and synthetic carbon- and silicon polymer-iron

complexes, to study the role of macromolecular components of such complexes in carcinogenic responses of various organs and tissues.

The negative outcome of our experimental approach to the iron cancer problem does not invalidate existing epidemiologic evidence obtained in man, nor does it negate medicolegal implications of such epidemiologic evidence.

A similar statement is in order concerning the carcinogenic action of arsenicals in man. It is regrettable that the experiments reported did not provide conclusive experimental evidence on the carcinogenic effect of arsenicals in animals. However, the existence of a symptom complex characterizing chronic arsenic poisoning and the development of cancer on the basis of chronic arsenical lesions of the skin provide reliable and significant medical evidence on this point. More recently obtained epidemiologic data on the occurrence of endemic arsenicosis associated with multiple skin cancers in populations consuming drinking water or wine contaminated with arsenicals [57,58] are of particular importance. The most recent German observations indicate that arsenic may become a powerful carcinogen to man under proper conditions of exposure, since cancers among vineyard workers were not only very frequent but affected, in addition to the skin, also the lung, nasal sinuses, larynx, liver, and esophagus.[57] Such cancers not infrequently appeared in several organs of the same person. Finally, past and present failures in producing cancer in animals exposed to various arsenicals in different vehicles via several routes do not prove that arsenicals are not carcinogenic to animals but are merely part of the obstinate pattern of experimental research in occupational carcinogenesis encountered in the past with most occupational carcinogens. With perserverance and some luck arsenicals one day may be shown to cause cancers in animals.

Conclusions

1. Experimental evidence indicates that hexavalent and trivalent chromium possesses carcinogenic properties.

2. Highly water-soluble trivalent and hexavalent chromium compounds when brought in contact with tissue at frequent intervals and in low concentrations to avoid significant corrosive effects are capable of producing cancers.

3. The tumor yield obtained by repeated, intratracheal instillation of suspensions of 3 carcinogenically highly potent chromates, calcium chromate, zinc chromate, and strontium chromate, was remarkably low and limited to the formation of sarcomas in the lung.

4. Immunochemical investigations with chromium-protein complexes showed that such agents are capable of eliciting chromium-specific antibodies. Tests for the presence of immune bodies in the serum of chromate producers gave equivocal results.

5. While powdered nickel implanted intrapleurally into rats elicited cancers in about one-third of the animals, intrapulmonary deposited nickel powder produced intrapulmonary sarcoma in only one of 34 rats.

6. The inhalation of powdered metallic nickel in combination with sulfur dioxide by rats and hamsters did not give rise to lung tumors in any of 2 species studied suggesting that the combined action of a purely irritative chemical, such as sulfur dioxide, with a carcinogen does not necessarily induce a carcinogenic response by the lung.

7. Rats implanted intrapleurally with finely powdered chemically pure iron or fed arsenic dissolved in water or a 12% alcoholic solution did not develop any cancers in the target organs, i.e., the lung or mediastinum or the skin, lung, and liver, respectively.

The technical assistance of Miss J. Zuefle, Mrs. R. Walker, Mrs. M. Johnson, Mr. H. Auerbach, and Dr. C. Grogan in various phases of the experimental work is herewith gratefully acknowledged.

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BRIT. J. Cancer
17(4)681-686(1963)

EFFECTS OF METHANDROSTENOLONE ON MUSCLE CARCINOGENESIS INDUCED IN RATS BY NICKEL SULPHIDE

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Received for publication August 10, 1963

EXPERIMENTAL production of malignant tumours by nickel and its compounds has been previously reported in different animal species (Hueper, 1952, 1955, 1958). It was recently demonstrated that a single instramuscular injection of nickel sulphide is capable of producing a high incidence of rhabdomyosarcomas in rats (Gilman, 1962). The carcinogenicity of this metallic salt seems rather selective for striated muscles, but not specific. Malignant muscle tumours have also been observed in animals injected with cobalt and chromium compounds (Gilman and Ruckerbauer, 1962; Heath, 1956, 1960; Hueper and Payne, 1959).

That rhabdomyosarcoma can be induced in laboratory animals is of paramount interest, because of its high malignancy and ability to produce extensive metastases, a finding uncommon in experimental carcinogenesis with chemical agents. Animal studies also provide some additional criteria for diagnosis of this undifferentiated tumour in man. The present paper deals with experiments on the effect of methandrostenolone, an anabolic and myotrophic steroid, on the induction of rhabdomyosarcomas by nickel sulphide in rats and the growth of such tumours when transplanted into animals of the same strain.

MATERIALS AND METHODS

The two experimental series were carried out in young female Sprague Dawley rats weighing 100–115 g., divided into groups and treated as indicated in Tables I, II and III. The animals were maintained on Purina Laboratory Chow and tap water, *ad libitum*.

Experiment No. 1.—In this first series, both groups of rats were injected on the first day with 0·1 ml. of a 10 per cent aqueous suspension of nickel sulphide (kindly supplied by Doctor J. P. W. Gilman from the Ontario Veterinary College, Canada) to which 2000 units of penicillin G had been added; injections were given in the right gastrocnemius. To ascertain that the nickel powder was always administered at the same site, a 20 gauge $\frac{3}{4}$ in. long needle attached to a tuberculin syringe was introduced at the junction of the Achilles tendon and the muscle, and the injection substance was expelled $\frac{3}{4}$ in. from the point of entry; treatment with methandrostenolone (kindly supplied by Ciba Company, Montreal) began on the same day. The steroid was injected subcutaneously as a microcrystal suspension at the daily dose of 0·5 mg. in 0·2 ml. of saline. Animals were under observation for a period of 217 days. Each week, the legs were examined by palpation in order to detect development of the tumour in the early stages and to follow its progression.

At autopsy, a careful dissection was made in order to weigh the primary tumour and to evaluate the importance and extent of metastases. All animals were thoroughly examined in this manner whether they died spontaneously or were killed with chloroform upon completion of the experiment. Tissues were fixed in Susa solution for subsequent staining with haemalum-phloxine-saffron and Masson's trichrome.

Experiment No. 2.—Using one of the tumours previously induced in the control animals, transplantation was carried out after two successive passages in rats of the same strain. On subcutaneous passage in tissue slices, the tumour was found to grow sufficiently rapidly for further transplantation in less than 15 days, keeping its original histological characters but without formation of necrotic tissue. Transplantation was made using a cellular suspension prepared by crushing 2 g. of fresh tissue in 10 ml. of physiological saline. The integrity of the cells was found to be well preserved when examined under the microscope. The suspension was injected under sterile conditions in a volume of 0·3 ml. underneath the skin of the central lumbar region. Methandrostenolone, as in the previous experiment, was injected in areas remote from the site of implantation at a daily dose of 0·5 mg. The animals were killed with chloroform on the 45th day and the tumours were dissected and fixed in Susa solution for subsequent weighing and histological studies.

RESULTS

Observations arising from the first experiment are summarized in Tables I and II. There was little or no local reaction at the site of injection until the 137th

TABLE I.—*Action of Methandrostenolone on Tumour Development at Site of Injection of Nickel Sulphide*

Treatment	Number of rats	Number of rats with tumours	Time of appearance of 1st tumour (days)	Overall average time of appearance (days)	Average progression time* (days)	Survivors after 217 days observation
Nickel sulphide	15	5	149	$176 \cdot 4 \pm 10 \cdot 0$ ($P = 0 \cdot 05$)	$29 \cdot 4 \pm 6 \cdot 2$ ($P < 0 \cdot 05$)	13
Nickel sulphide + methandrostenolone	10	10	137	$157 \pm 4 \cdot 1$	$46 \cdot 2 \pm 3 \cdot 7$	5

* Average time between appearance of 1st tumour and death.

day, when the first tumour became apparent in a methandrostenolone-treated rat. Twelve days later, 1 animal from the control group also exhibited a palpable tumour. As the experiment progressed, the difference between the two groups became more obvious, especially with regard to the frequency of neoplasm. By the end of the experiment, only 5 out of 15 control animals had histologically demonstrable tumours, while none of the steroid-treated rats escaped cancerization by nickel sulphide. In the untreated controls, in addition to the lower frequency of the tumours the average time of appearance was 19 days later than in the treated animals. This delay shortened the observation period of progression and the tumours being, therefore, relatively smaller in volume and weight by the end of the experiment caused less mortality than in the treated animals.

TABLE II.—*Action of Methandrostenolone Upon Weight of Rhabdomyosarcomas and Distribution of Metastases*

Treatment	Number of rats	Number of rats with tumours	Average weight of tumours (g.)	Incidence and distribution of metastases						
				Lymphatics of pelvic organs	Lymphatics of abdominal wall	Aortic lymph glands	Lungs	Spleen	Heart	Kidney
Nickel sulphide	15	5	$17 \cdot 28 \pm 4 \cdot 2$ ($P < 0 \cdot 1$)	0	0	2	1	0	0	0
Nickel sulphide + methandrostenolone	10	10	$29 \cdot 9 \pm 7 \cdot 4$	2	2	10	8	1	1	1

When separated from the surrounding skin and adherent-muscle layers, the tumours were seen to be of firm consistency and greyish in colour; their size varied between 1 and 5 cm. The larger ones usually invaded the entire thickness of the abdominal muscle, propagating in the direction of the inguinal region and often penetrating into the pelvis. Upon sectioning, nickel deposits were apparent within fibrous tissue, being centrally located (Fig. 2); this area was surrounded by a more greyish tissue that appeared either as a uniform layer or as separate nodules, some of which contained necrotic tissue. As a rule, metastases spread along the aortic lymph glands, which were markedly hypertrophied and whitish in colour. Only 2 out of the 5 tumour-bearing animals in the control group had metastases: one of these animals showed a metastatic nodule in the lung parenchyma (Table II). In contrast, 8 of the steroid-treated rats had metastases that extended to the lungs in every case; 2 animals, in addition, showed involvement of abdominal organs, such as the kidney and spleen (Fig. 1), and of the myocardium.

Histological examination of tumours at the site of origin disclosed a cellular pattern that varied from the centre to the periphery. The centrally-located nickel deposits appeared as black dust-like granules surrounded by reticular giant cells. Necrotic foci were more or less numerous depending upon the rate of tumour growth; they were formed by acidophilic granular coagulated material, pyknotic cells, cellular debris, and phagocytes. Very few muscle fibres were recognizable in these areas, except for some occasional enucleated hyaline fibre remnants. A common feature was the presence of reticular cells containing a ferric pigment. The necrotic areas were eventually replaced by a combination of fibrous tissue and regenerated muscle cells; these were polynucleated and appeared as elongated or as typical plasmodes. Malignant cells were usually identified in outer layers exhibiting large atypical nuclei containing several nucleoli. Some were elongated like spindle cells, but the majority appeared as giant multinucleated cells with syncytial cytoplasm. There were a few mitotic figures except in areas rich in connective tissue cells; in areas where the neoplasm invaded normal muscle fibres the interstitial cellular reaction was almost absent. Metastases, whether in the aortic lymph nodes, abdominal organs, or lungs, usually consisted in polynucleated giant cells as previously described, and a number of more differentiated rhabdomyoblasts that occasionally showed double striations (Fig. 3).

Table III summarizes results of the second experiment. It was found that methandrostenolone reduced both the take and the development of the transplanted rhabdomyosarcomas. In addition, the tumour nodules in the steroid-

EXPLANATION OF PLATE

FIG. 1.—Gross appearance of Rhabdomyosarcoma of leg and of its metastases in different organs. *Left*, tumour nodule in the spleen. *Upper centre*, neoplastic infiltration of great omentum. *Lower centre*, primary tumour in gastrocnemius. *Right*, large tumour nodule in left lung.

FIG. 2.—Mid-section of Rhabdomyosarcoma at site of induction. Note black nickel deposits embedded in fibrotic tissue, which differs from upper neoplastic tissue in being whiter in colour.

FIG. 3.—Histological aspect of a metastatic nodule in lung. Note typical rhabdomyoblasts, some of which are elongated and others appear as plasmoidal cells. ×340.

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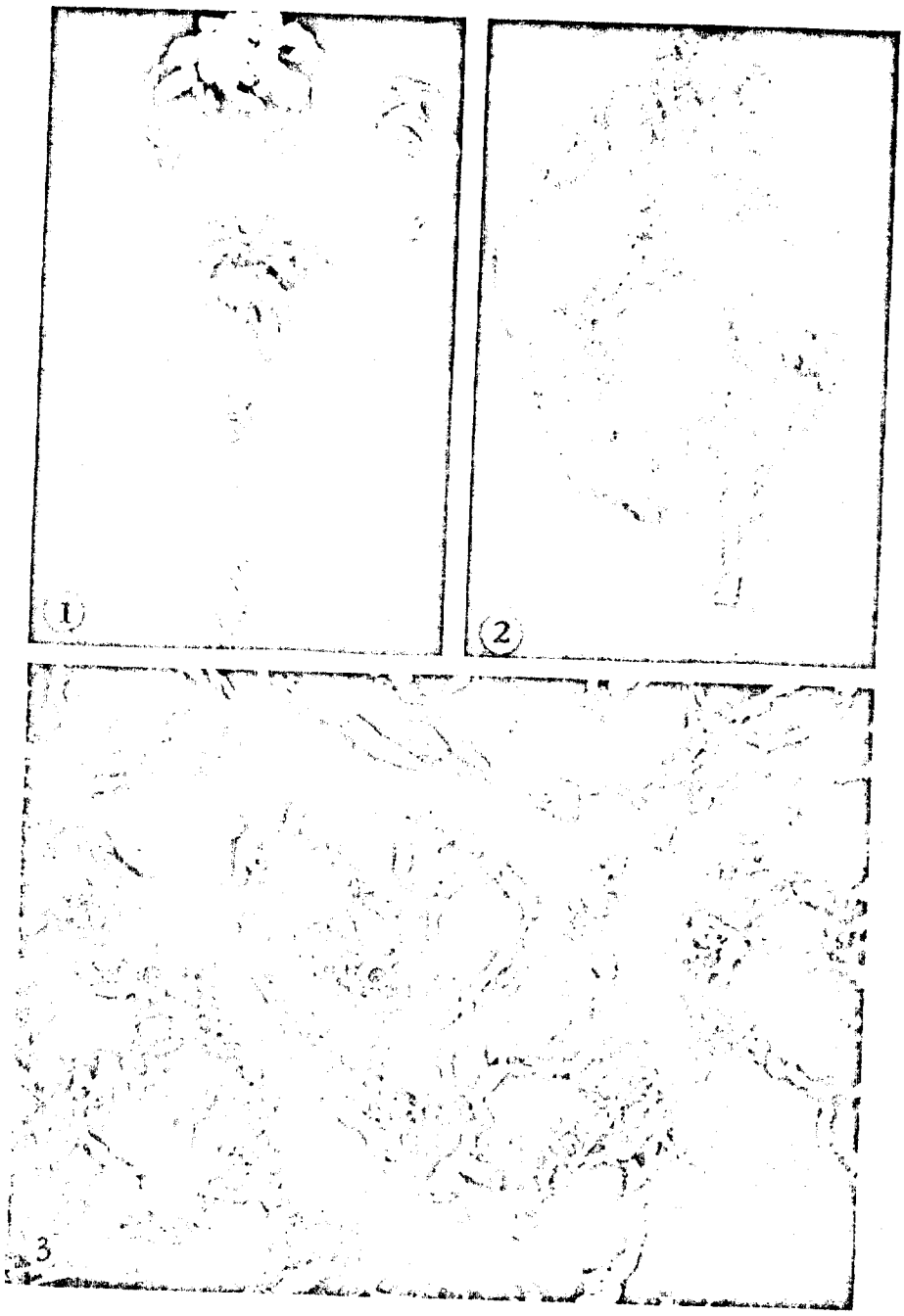

Jasmin.

TABLE III.—*Action of Methandrostenolone Upon Development of a Transplanted Rhabdomyosarcoma in the Rat*

Treatment	Number of rats	Body weight gain (g.)	Overall average time of appearance (days)	Average weight of tumours (g.)
--	12	10	$28 \cdot 5 \pm 2 \cdot 1$ ($P < 0 \cdot 3$)	$4 \cdot 26 \pm 2 \cdot 1$ ($P < 0 \cdot 2$)
Methandrostenolone	12	6	$32 \cdot 8 \pm 3 \cdot 4$	$2 \cdot 66 \pm 0 \cdot 46$

treated animals were more indurated upon sectioning; histological examination revealed them to be partly fibrotic. The tumours did not spread in either group, but were adherent to the underlying tissues.

DISCUSSION

There have been several reports pertaining to modification in activity of a carcinogen by hormone treatment (Bielschowsky, 1961; Bielschowsky and Horning, 1958; Huggins, Briziarelli and Sutton, 1959; Mühlbock and Van Nie, 1960). This phenomenon is particularly noteworthy at the level of target organs influenced by endocrine secretions (Mühlbock, 1960). It is also well known that such tissues as the mammary glands may be genetically conditioned to become neoplastic, if stimulated by a trophic hormone. As far as rhabdomyosarcoma is concerned, we were unable to find any data indicating that the development of this tumour is hormone dependent. In surveying 114 cases of human rhabdomyosarcomas, Stout (1946) noticed a slight preponderance of tumours in males. On the other hand, a detailed study of spontaneous muscle tumours in several species of animals did not provide any evidence of sex difference upon the incidence of such tumours (Blachser, 1961). Experiments recently carried out in our laboratory and reported elsewhere (Jasmin, Bajusz and Mongeau, 1963), revealed that this is also the case in a rat subjected to nickel sulphide.

In the present study, the myotrophic steroid significantly promotes muscle tumorigenesis as evidenced by a higher incidence of tumours, a reduction of the latent period, and lengthened progression time. In fact, after 217 days, the experiment was terminated because all surviving animals of the second group exhibited respiratory disturbances because of lung metastases. Only one animal in the control group showed metastases in the lungs.

Methandrostenolone, on the other hand, exerted no significant effect upon the development of transplanted rhabdomyosarcomas. The steroid even manifested some retarding action as judged by incidence, overall time of appearance, and weight of the tumours. Thus, it appears that this tumour when it becomes autonomous and transplantable is no longer responsive to hormonal treatment. A possible explanation for the reverse action of methandrostenolone is that the steroid causes an adverse systemic effect upon tumour growth, as previously reported (Jasmin, Bois and Mongeau, 1960). Consequently, the myotrophic steroid would act mainly by increasing the susceptibility of muscle tissue at a critical stage during nickel tumorigenesis, an action that most probably results from an increase in the metabolic rate of the muscle fibres.

SUMMARY

Methandrostenolone, an anabolic and myotrophic steroid, was found to accelerate the carcinogenic activity of a single injection of nickel sulphide into the gastrocnemius of rats: the incidence of rhabdomyosarcoma was 100 per cent in steroid-treated rats, in comparison with 33 per cent in untreated controls. A reverse action was observed when methandrostenolone was administered to animals transplanted with a previously-induced rhabdomyal tumour.

The action of methandrostenolone consisted mainly in shortening the latent period of induction, resulting in a relatively higher incidence of tumoutrs and more widespread metastases, for a given period of observation.

This investigation has been supported by a grant from the National Cancer Institute of Canada.

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NATURE, VOL. 219, SEPTEMBER 28, 1968

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Effect of Inhibition of Adenosine Triphosphatase Activity on the Fine Structural Organization of the Brain Capillaries

According to Torack and Barrnett[1] the activity of 5-nucleoside phosphatase in the brain capillaries is localized in the basement membrane of the capillary wall and in the surface membranes of the glial end feet surrounding the capillaries. Thus with respect to the fine structural localization of the activity of adenosine triphosphatase (ATPase) the capillaries of the brain differ markedly from those of other tissues in which ATPase activity is located in the pinocytotic vesicles of the endothelial cytoplasm. By means of a sensitive method based on the fluorescence of trypan blue it has previously been shown[2] that when ATPase is inhibited the permeability of the haematoencephalic barrier is enhanced. Thus it seems that the activity of ATPase in the brain capillaries does not indicate the site of accelerated transport—as Barrnett suggested[3]. The activity of this enzyme is, however, in close correlation with the specific functions of the blood-brain barrier.

I have examined the fine structure of the brain capillaries by electron microscopy after inhibiting the activity of ATPase using nickel chloride.

Kaye has stated[4] that the inhibition of ATPase by 10^{-3} M nickel chloride is to a large extent specific. I first attempted to establish whether ATPase is also inhibited in the vessels of the brain after intraperitoneal administration of nickel chloride. Ten rats were injected intraperitoneally with nickel chloride (0·25 g/kg dissolved in physiological saline), and were killed after 30–60 min or 3, 6, 12 and 24 h. For histochemical demonstration of ATPase activity Padykula's method[5] was used. Between 30 and 60 min after the administration of nickel chloride 20–50μ areas of capillary walls showed no ATPase activity. Between 3 and 6 h after nickel chloride has been given, the vessels of the brain can no longer be demonstrated by this histochemical ATPase technique. After 12 h, enzyme activity can be detected in parts of the capillary wall and after 24 h ATPase activity in the capillaries of the rats given nickel chloride is practically the same as that of the control group. The activity of non-specific alkaline phosphatase[5] did not show any alterations; thus nickel chloride seems to inhibit specifically only ATPase.

Electron microscopic investigations revealed the changes in the fine structure of brain vessels after the inhibition of ATPase by nickel chloride. The enzyme was inhibited as described. Samples were fixed, by Millonig's method[6], in buffered osmic acid and embedded in 'Durcupan' (Fluka) after gradual dehydration with alcohol. Thin sections were stained by Reynolds's[7] procedure, and for inspection and photography of the sections a Tesla 242 D table type electron microscope was used.

Thirty minutes after administration of the enzyme inhibitor I found a change in the basement membrane of the capillary wall. This membrane which covers the endothelial cells with a continuous single layer[8] lost its

Fig. 1. Section of capillary wall of rat cerebellum 30 min after administration of 0·25 g/kg of nickel chloride. On the site marked with an arrow the basement membrane forms glove-finger-like processes. L, lumen of the capillary; M, mitochondria in the cytoplasms of the glial end feet; BM, basement membrane. (×45,000.)

characteristic features and formed fingerlike processes 0·5–2μ long. These labyrinth-like formations were most pronounced on sites where basement membrane was in contact with glial cytoplasm (Fig. 1). In material examined 12 h after the administration of the enzyme inhibitor the labyrinth-like formations gradually disappeared (Fig. 2) and the basement membrane became thick and looser in structure. This looseness in the fine structure of the basement membrane could be a result of exudation. Twenty-four hours after nickel chloride had been given (Fig. 3) the basement membrane was still thick (0·25–1·2μ on average), but the looseness had disappeared, so that the membrane formed a thick homogenous wall around the capillaries.

According to Torack and Barrnett[1] and our earlier observations[9,10], the activity of ATPase and cholinesterase in the brain capillaries is important in the regulation of the permeability of the barrier between blood and brain. After inhibition of ATPase activity with nickel chloride the permeability of the haematoencephalic barrier has been found to be enhanced[2]. I have now found profound changes in the fine structure of the capillaries. The labyrinth-like structure of the basement membrane made the brain capillaries resemble capillaries of the brain areas outside the blood–brain barrier[11]. In that the widening of the basement membrane is associated with an enhanced permeability our data agree with observations that when the permeability of the glomerular filter is enhanced the glomerular basement membrane of the kidney thickens[12,13].

It seems therefore that normal ATPase activity is important not only in the regulation of capillary permeability in the brain, but also in the maintenance of the normal submicroscopic organization of the basement membrane. Thus the thickening of the basement membrane of the capillaries[14] in the brains of humans suffering from different neurological diseases can be explained as a result of the inhibition of ATPase. Of course, it is still possible that the inhibition of ATPase actually affects the glial cells, and fingerlike hypertrophy of the basement membrane could be simply a consequence of dilated pericapillary spaces, resulting from the retraction of glial end feet.

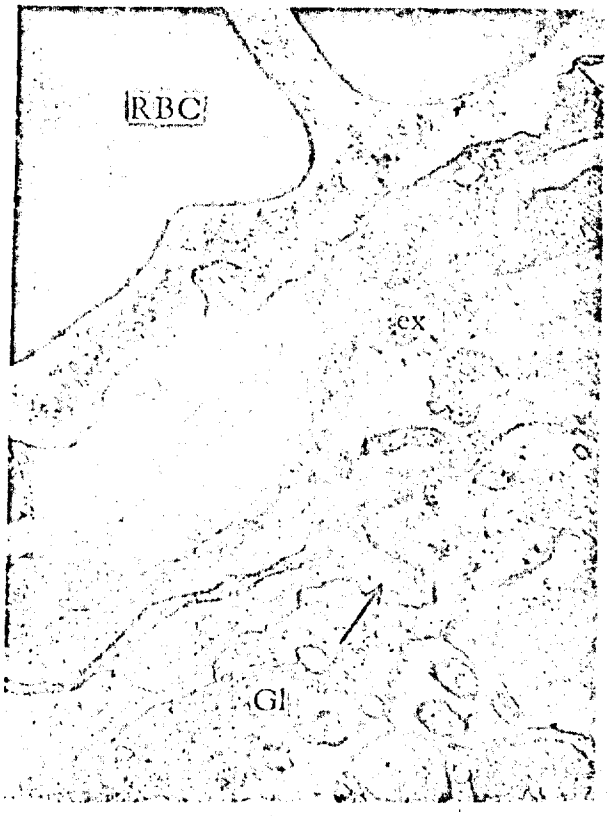

Fig. 2. Twelve hours after inhibition of ATPase looseness is visible in the structure of the basement membrane. The arrow points to an extrusion of the external part of the basement membrane. RBC, red blood cells; ex, exudate; Gl, glial cytoplasm. (×45,000.)

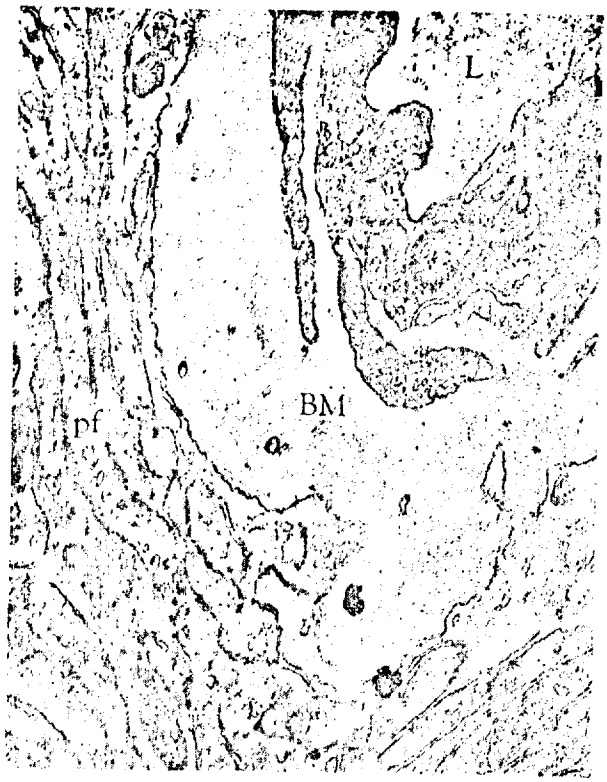

Fig. 3. Twenty-four hours after administration of nickel chloride the thickened basement membrane surrounded the endothelial cytoplasm. L, Lumen of the capillary; BM, basement membrane; pf, parallel fibres. (×45,000.)

I thank Dr T. Várkonyi for help and technical assistance.

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Received May 23; revised July 18, 1968.

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Hyperglycemia and Islet Cell Damage Caused by Nickelous Chloride

By Ichiro Kadota and Mitsutaro Kurita

THE RECENT report[1] that cobaltous chloride causes both transitory hyperglycemia and alpha cell damage in the pancreatic islet of guinea pigs was confirmed in rabbits by Goldner, Volk and Lazarus.[2, 3] While we were investigating the effect of zinc, cobalt and nickel on the experimental production of diabetes caused by organic reagents, we found that nickelous chloride caused an effect similar to that of cobaltous chloride. Its administration produced in the rabbit a transitory hyperglycemia and lesions of the islet cells, particularly the alpha cell. We also could confirm the previously reported effect of cobaltous chloride.[1-3] Recently, Creutzfeldt and Schmidt[4] also confirmed the effect of cobaltous chloride on blood sugar and islet cells in various animals.

Materials and Methods

Adult rabbits were used as experimental animals. The blood sugar determinations were carried out by Hagedorn-Jensen's method.[5] We obtained biopsies of pancreatic tissue one to four hours after injection of the agents and fixed the tissue in Bouin's solution. For histologic examination, Gomori's chromium-hematoxylin phloxine method[6] was employed. A second injection of nickelous chloride was given to a few rabbits after 48 hours, and the change in blood sugar was observed.

Results

Toxicity of Nickelous Chloride

Some rabbits receiving 20 mg. per kilogram of body weight died immediately or within one hour. Doses of 10 to 15 mg. per kilogram seemed to give optimal results.

Changes in Blood Sugar Level

The changes in the blood sugar in 22 rabbits after administration of nickelous chloride are shown in table 1. With doses of 10 mg. per kilogram, 7 out of 11 rabbits showed transitory hyperglycemia after one to four hours. All rabbits given injections of 15 mg. per kilogram showed considerable hyperglycemia, from 150 to 300 mg. per cent, which returned to normal after 24 hours (fig. 1). A second injection of 10 mg. per kilogram caused a new transitory hyperglycemia.

In alloxan diabetic rabbits the administration of nickelous chloride caused augmentation of hyperglycemia as shown in table 2.

From the Research Laboratories of the Osaka Red Cross Hospital, Osaka, Japan.
Received for publication October 29, 1954.

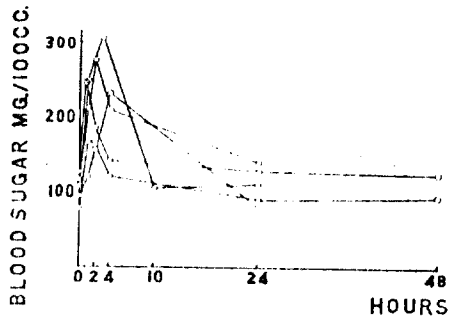

FIG. 1. Changes in blood sugar level in rabbits after intravenous administration of 15 to 20 mg. per kilogram of nickelous chloride.

TABLE 1. *Changes in Blood Sugar After Intravenous Injection of 0.5% Nickelous Chloride Aqueous Solution*

Rabbit	Body wt. (gm.)	Injected doses (mg./kg.)	Blood sugar; mg./100 ml. of Blood								
			Before injection	Hrs. after injection							
				1	2	3	4	10	24	48
94	2,120	10	79	217	191		140		76	82
95	2,100	10	74	79	98		79		98	95
104	2,180	10	105	163	147		88		123	
102	1,980	10	107	247		307		105	110	
103	2,320	10	77	95	95		89		72	75
101	2,000	10	93	172	132		86		109	112
105	2,080	10	89	111	84		89		79	
106	3,220	10	77	84	93		82		84	
107	1,960	10	96	125	93		115		100	
108	2,200	10	109	158	113		107		100	
109	2,000	10	95	165	127		104		127	
96	2,100	15	116	245	185	141			127	125
125	1,980	15	127	214	273		210		139	
126	2,000	15	79	168	163		121		91	95
127	2,100	15	86	153						
129	2,000	15	76	135	107				80	
130	2,000	15	90	152	137		183			
131	2,100	15	114	236	167		220			
128	2,250	20	77	163						
155	2,000	20	84	153		237			93	
156	2,000	20	73	117		150			85	
157	2,000	20	91	121	156		230		80	
Second injection after 48 hrs.										
102	2,020	10	123	186	167		121		116	
103	2,320	10	88	97	107		74		75	

TABLE 2.—*Changes in Blood Sugar After Intravenous Injection of Nickelous Chloride in Alloxan Diabetic Rabbits*

Rabbit	Alloxan doses (mg./kg.)	Duration of hyperglycemia (days)	Body wt. (gm.)	Injected NiCl doses (mg./kg.)	Blood sugar, mg./100 ml. of blood						
					Before injection	Hrs. after injection					
						1	2	3	4	24	48
154	200	30	2,260	15	151	174	168		160	155	157
158	200	23	1,900	15	152	212	207	222		254	311
158	200	30	1,600	15	194	270		260 (after 1 week)		316	240
159	200	15	2,060	20	164	286					
160	200	21	1,300	30	186	154	176		150		

Histologic Change of Islet Cells

Biopsies of the pancreas were performed in four rabbits one hour after the administration of nickelous chloride. The islets were, in general, small; slight congestion of the capillaries and irregularity of the cell cords were seen. The alpha cells were reduced in number, or had disappeared, leaving some cellular debris. Their cytoplasmic granules disappeared mostly. The appearance of nuclei was compact and pyknotic. Only a few alpha cells remained visible at the periphery.

Most islets appeared to be composed chiefly of beta cells, which, however, had lost most of their cytoplasmic granules. The cells were swollen and contained many vacuoles. Most of the nuclei were pyknotic, although we also observed some large and faintly stained ones. A few nuclei were left without protoplasma as evidence of cellular degeneration.

Four hours after the injection the changes of the islets were almost the same as those after one hour. However, after 24 hours, the cellular damage was more marked. The grade of beta cell damage also was increased, showing empty spaces with cellular debris (figs. 2–5).

DISCUSSION

A close relation between the trace metals and the pancreas, particularly the function of the islets, is suggested by the histochemical demonstration of zinc in the islets and the analytical detection of several of these metals in the pancreas. We can report now that the administration of nickelous chloride causes a transitory hyperglycemia and islet cell damage similar to the effect of cobaltous chloride.

A marked hyperglycemia, from 150 to 300 mg. per cent was observed between one and four hours after the administration of 15 to 20 mg. per kilogram of nickelous chloride, which is close to the lethal dose for rabbits. This hyperglycemia was somewhat more marked than that caused by cobaltous chloride. With cobaltous chloride we obtained values almost identical to those of Goldner, Volk and Lazarus.[2, 3] With zinc acetate we did not observe any changes in blood sugar.

The histological islet changes caused by nickelous chloride were mainly marked alpha cell destruction and, to a lesser degree, degranulation and destruction of the beta cells. We could not find vacuolization of alpha cells as observed in islets of guinea pigs treated with cobaltous chloride.[1]

FIG. 2. Pancreatic islet of rabbit; biopsy one hour after intravenous injection of 20 mg. per kilogram of nickelous chloride. Only a few alpha cells remain at the periphery of the islet, showing degranulated cytoplasm and pyknotic nuclei. Beta cells are also considerably destroyed.

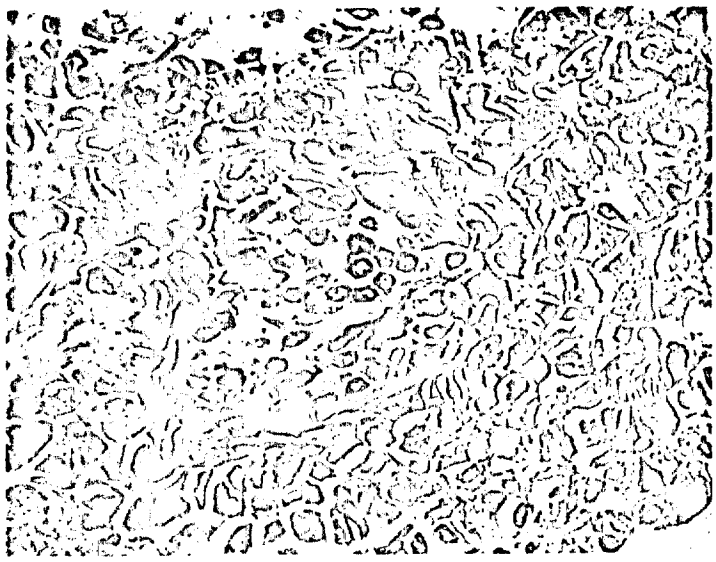

FIG. 3. Pancreatic islet of rabbit; biopsy one hour after intravenous injection of 20 mg. per kilogram of nickelous chloride. Degenerated alpha cells and degranulated beta cells are visible in the islet.

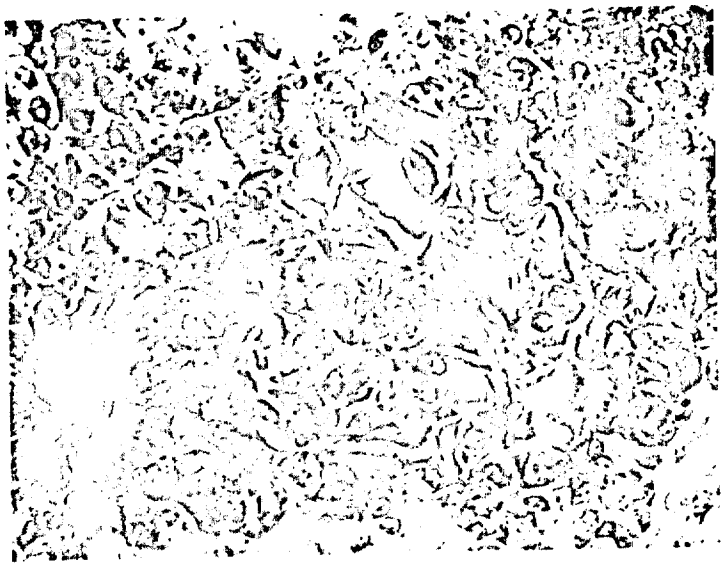

FIG. 4.—Pancreatic islet of rabbit; biopsy four hours after intravenous injection of 15 mg. per kilogram of nickelous chloride. Degenerated alpha cells are located at the periphery. Most of the beta cells are degranulated.

FIG. 5. Pancreatic islet of rabbit, biopsy one hour after intravenous injection of 15 mg. per kilogram of cobaltous chloride. The normal cell arrangement is lost. Alpha cells remain only at the periphery of the islet with considerable degeneration. Beta cells also show disintegration.

These findings are similar to the necrosis of alpha cells caused by diethyldithiocarbamate, as previously reported by us,[7] and also are similar to those reported by Goldner, Volk and Lazarus[2,3] in the rabbit treated with cobaltous chloride. The islet cell damage induced by cobaltous chloride was in our experiments more marked than that by nickelous chloride, although the degree of hyperglycemia was somewhat greater in the latter instance. We could also find lesions of the beta cells caused by cobaltous chloride.

Although Creutzfeldt and Schmidt[4] reported hyperglycemic action of nickelous chloride in rabbits, they observed no effect on the blood sugar in rat and guinea pig. The fluctuation of the blood sugar may be presumed to be a result caused by the direct action of nickel and cobalt on the islet cells, although an extrapancreatic action must be considered. In the case of diethyldithiocarbamate[7] we observed an initial hypoglycemia, and during the phase of hypoglycemia there was conspicuous alpha cell damage. Therefore, we presumed that the beta cell damage was secondary to the alpha cell lesion. Nickel and cobalt, on the other hand, seem to damage alpha and beta cells simultaneously. The role of nickel and cobalt for the function of the pancreas and its islets is not yet clear. It is very interesting, nevertheless, that experiments by us and others demonstrate a close relation between these metals and the function of islets of Langerhans. These observations may give a clue toward elucidation of the mechanism of insulin production and of the origin of the hyperglycemic-glycogenolytic factor.

Summary

After the administration of 10 mg. per kilogram of nickelous chloride in rabbits, we observed a transitory hyperglycemia in about half the animals. All rabbits administered 15 to 20 mg. per kilogram of nickelous chloride showed hyperglycemia, reaching maximal levels in one to two hours and returning to normal after 24 hours. In the islets of these rabbits we found destruction of alpha cells, and to a lesser degree, beta cell damage with degranulation. These results are similar to those produced with cobaltous chloride; however, the cobalt hyperglycemia was less marked.

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[GANN, 51, 377~382; December, 1960]

A STUDY ON THE RELATION OF SOME METALS IN MELANOTIC TUMOR FORMATION IN *DROSOPHILA* FLIES*

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(Biological Laboratory, Kobe University, Kobe)

Genetic and physiological studies have shown that "melanotic tumor" *in Drosophila* is intimately related to tryptophan metabolic pathway connected with the appearance of brown pigment in the eye, with a suggestion that the mechanism of the formation of the character must be considered through the tyrosine-melanin approach (Kanehisa 1956 a, b, 1957 a, b). The present investigation deals with an attempt to learn if there is any relation between metal accumulation and melanotic tumor formation. The author is greatly indebted to Professor D.F. Poulson, Yale University, Professor Hideo Kikkawa, Osaka University, Dr. V.T. Bowen, Yale University and Professor Sajiro Makino, Hokkaido University, for their keen interest, and various sorts of help in the carrying out the present work and their valuable criticisms. Thanks are also given to Dr. J.E. Sang, Poultory Research Center, Edinburgh, for his advice on metal analysis and synthetic media.

MATERIALS AND METHOD

Fifty sterized, normal shaped egges were collected within 4 hours after laying, in each strain of Oregon R, *bw*, *v*, *st*, *tu*, *bw tu*, *tu* and *tu st* strains which were based on genetical matings (Kanehisa 1956 a, b). Each lot was placed in a "creamer" containing 5 ml synthetic media. Table 1 shows the components aed quantity of the synthetic medium used here which is a slight modification of Sang's formula (Sang 1956). To maintain a constant humidity in a creamer, the cups were completely sealed with celophane tape. They were kept at 25±1°C in a refrigerator. The viability and melanotic tumor-incidence of flies were examined by flies which emerged in each creamer. On the other hand, the dry metarials of pupae from synthetic media were ashed by "wet ashing" method and analyzed by a modification of Fisher's method (Fisher 1934, 1937): the amouns of hydrochloric acid, sulfuric acid, perchloric acid and water for wet ashing were regulated to a constant in each step of heating, and the samples were completely ashed in an electric oven under a constant temperature (160°C in the surface of oven and 200°C in the micro-Kijeldahl flask) for a certain number of hours to avoid she risk of evaporation of metals by means of heating in acid-solution (some data regarding such a risk will be published in another paper). One tenth of the volume of the ashing solution was used for analysis of iron with 0.5% α-α dipiridile solution after addition of 10% ammonium citrate, at pH 3.5 with the use of 2M sodium acetate. To prevent the formation of precipitates of iron, aluminum and

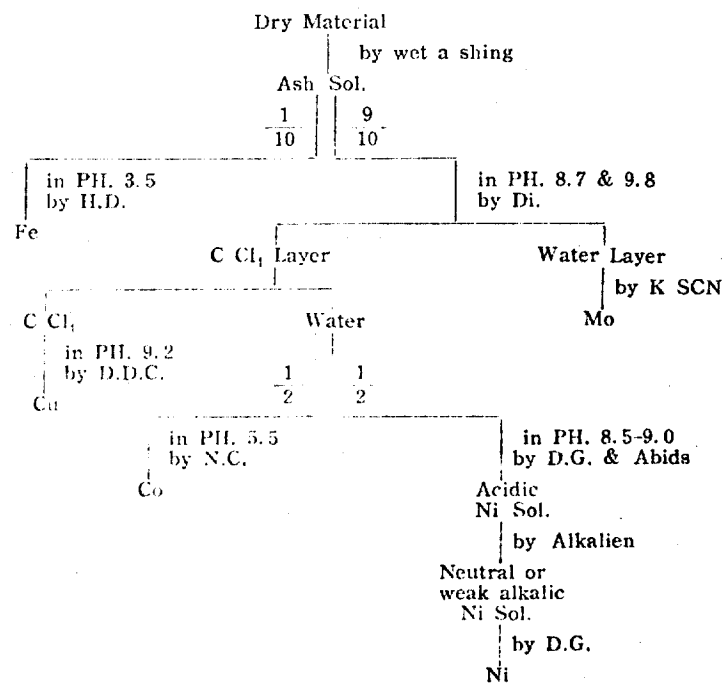
* The present investigation was carried out at the Osborn Zoological Laboratory, Yale University with financial support from the National Science Foundation for 1956 1957, through the courtesy of Dr. D.F. Poulson. The essential results were reported before the Xth International Congress of Genetics held in Montreal, Canada in 1958.

Table 1. Medium used for experiments.

Components	Quantity	Components	Quantity
Ager (Oxoid, Kobe no. 1)	2	Folic acid	0.00006
Casein (vitamin free)	5	Choline	0.001
Fructose	0.75	$NaHCO_3$	0.126
Cholesterol	0.015	$FeSO_4 \cdot 7H_2O$	0.002
Lecithin	0.20	$CaCl_2$	0.002
R.N.A. (stayc)	0.40	$MgSO_4$	0.002
Thiamine	0.0002	$MnSO_4 \cdot 4H_2O$	0.020
Riboflavine	0.0002	NaCl	0.002
Nicotinic acid	0.0001	KH_2PO_4	0.183
Ca-pantothenate	0.0003	Na_2HPO_4	0.189
Pyridoxine	0.00004	Water to	100 ml
Biotin	0.000006		

other metals, 20% ammonium citrate was used for ashing saples. Method of analysis of copper, molydenum and nickel of this solution was almost the same as that of Kikkawa and Ogita (1956). The general procedure of the analysis used is given in Scheme 1. A Beckman-spectrophotometer was used for quantitative analysis of metals.

Scheme 1. Analytical Method



H.D : Hydroquinone & dipyridin. D.D.C : Sodium-diethyl-dithiocarvamate. Di: Dithizone. N.C : Nitroso-cresol. D.G. : Dimethyl-glyoxime

Results and Discussion

Viability of flies and melanotic tumor-incidence: The results of observations of viability of flies and melanotic tumor-incidence are shown in Table 2. From the table, it is evident that each melanotic tumor incidence of the tumor strains was approximately the same as the one shown from the regular medium, that the melanotic

Table 2. Viability and "melanotic tumor" -incidence of tumor and non-tumor flies in *D. melanogaster* from a synthetic medium.

Viability (%)		"mel. tu." -incidence (%)
tu	52 (416/800)	51
bw tu	54 (432/800)	56
v tu	65 (435/750)	72
tu st	67 (536/800)	93
+	79 (603/800)	
bw	82 (656/800)	*tu*, *bw tu* < try. *tu**
v	71 (520/800)	
st	73 (560/800)	(with 95% Confidence Interval)
non-*tu tu* ,*bw* try. *tu*, *bw tu* try. *tu* } Viab.		Decreased viability ratio
try. non. *tu*→try. *tu*<non. try. non. *tu*→non. try. *tu*		

* means tumor strains with genes related to tryptophan metabolism concerning brown pigment formation.

tumor-incidence of the tumor strains with genes related to tryptophan metabolism in brown pigment formation of eye (*v tu*, *tu st*) is relatively higher than that of the tumor strains having no such genes (*tu*, *bw tu*), further that viabilities of the tumor strains are relatively lower than those of non-tumor strains, and that viabilities of *v tu* and *tu st* flies are higher than those of *tu* and *bw tu* flies because the viabilities of non-tumor strains with the gene related to the tryptophan metabolism (*v*, *st*) are lower than those of non-tumor flies (wild, *bw*). Taking up the ratio of decreased viability between non-tumor and tumor strains, the decreased ratio of non-tumor strains with *v* or *st* genes to tumor strains with *v* or *st* is lower than that of non-tumor strains without *v* or *st* to tumor strains without *v* or *st* genes. It seems therefore that tumor formation itself is related to "lethality" of the flies, and that the tryptophan metabolism is related to increaseing viability through melanization. These results support those of some previous observations reported by Glass *et al* (1957) and Kanehisa (1957a).

Metal analysis: Table 3 shows the metal content in the medium (5 ml). The metal contents in tumor and non-tumor flies are shown in Table 4, (I) and (II). As clearly shown in these data, copper contents in flies of the melanotic tumor strains

are approximately parallel to the order of their tumor-incidence; namely, the higher the melanotic tumor-incidence, the greater the copper content. This evidence should be considered as a secondary result following melanization, rather than following tumor formation itself. To see the relation of metal incorporation between non-tumor flies and tumor flies, the ratio of metal contents was measured in both types of flies

Table 3. Metal contents in the medium (5ml.)

Fe	188.0 r
Cu	28.0
Co	11.8
Mo	4.1
Ni	3.7

Table 4. Metal contents in tumor and non-tumor flies (I).

	+	*bw*	*v*	*st*	*tu*	*bw tu*	*v tu*	*tu st*
Fe	1290*	550	555	301	321	230	84	129
Cu	302	248	240	265	201	220	235	247
Mo	24	121	235	177	109	149	101	115
Ni	88	170	145	160	195	154	100	114
								(II)
Fe	1072	574	536	220	510	219	52	96
Cu	265	232	225	231	182	197	210	228
Mo	25	106	199	157	118	172	132	144
Ni	70	201	189	139	231	159	111	106

* r gr.w.

(cf. Table 5, (I)′ and (II)′). It is significantly clear from the table that copper, molybdenum or nickel incorporation is relatively higher in comparison with the amounts of iron in melanotic tumor formation. As to relative amount of molybdenum to copper, there is an opposite relation between the change of wild, or *bw* into *tu* or *bw tu* and that of *v*, or *st* to *v tu*, or *tu st*. Namely in the former, the accumulation of molybdenum is higher than that of copper, while an opposite relation is seen in the latter. Further, the quantity of nickel accumulation shows a coupling relation to that of molybdenum. It is strongly suggested from these results that

Table 5. The uptake relation between each metal into "mel. tu." (I′)

	+ *tu*	*bw*/*bw tu*	*v*/*v tu*	*st*/*tu st*
Cu Fe	0.3*	0.5	0.1	0.5
Cu Ni	3.4	1.4	0.7	0.7
Cu Mo	7.9	1.3	0.4	0.6
Ni Fe	0.1	0.4	0.3	0.6
Mo Fe	0.06	0.3	0.3	0.7
Mo Ni	0.5	0.7	1.6	1.2

(II')

Cu/Fe	0.7	0.4	0.1	0.4
Cu/Ni	4.8	0.9	0.6	0.7
Cu/Mo	7.1	2.0	0.7	0.8
Ni/Fe	0.2	0.6	0.2	0.6
Mo/Fe	0.1	0.3	0.2	0.5
Mo/Ni	0.8	0.5	0.9	0.9

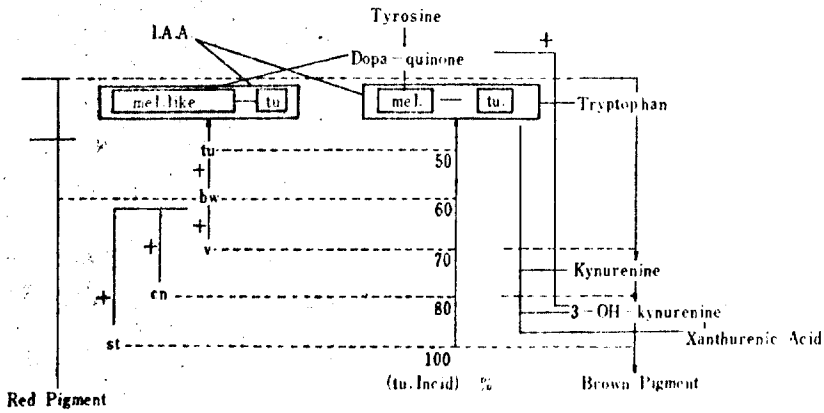
* $\frac{\text{Cu/Fe (+)}}{\text{Cu/Fe (}tu\text{)}}$

non-tumor ⟶ tumor Fe↖↗Cu, Mo, Ni

\+ ⟶ *tu*
bw ⟶ *bw tu* Cu↖↗Mo↗Ni

try. ⟶ try. *tu* Cu↖↗Mo↗Ni

molybdenum or nickel also has an intimate relation to tumor formation itself in some way. It was reported that melanotic tumor-incidence was increased by addition of tryptophan to the culture medium (Mittler 1952, Hinton *et al* 1951, Hartung 1951, Friedman *et al* 1951, Plaine and Glass 1955, Kanehisa 1956, and others), and that tryptophan metabolites like 3-hydroxy-kynurenine xanthurenic acid and others which are related to brown pigment formation of the eye are intimately related to melanotic tumor formation (Kanehisa 1957a). Glass (1957) suggested that 3-hydroxy-kynurenine is intimately related to melanotic tumor formation. Further, Glassman (1957) reported that melanin-like pigment was produced by the combination of aromatic amine compounds like kynurenine and 3-hydroxy-kynurenine with quinones *in vitro*. On the other hand, Hartung (1955) showed that "melanotic tumor" was induced in wild flies of *D. melanogaster* and non-melanotic tumor strain in *D. virilis* (Maruyama)



I.A.A.: indole-acetic acid. mel. like-tu.: melanotic tumor-like abnormal masses.

Scheme 2. Possible relations in tryptophane metabolic way, tyrosine-melanin metabolism and tumor formation in *Drosophila*

through the treatment with indole-acetic aceid. The auther (1955) also found tumor-like melanotic mass in *D. virilis* flies in Maruyama strain after the same treatment. Such abnormal masses were also found to occur in *v bw tu*, *cn bw tu* and *bw tu st* flies based on genetical matings (Kanehisa 1955). From the data obtained from the present investigation and some other tumor studies, it is apparent that, in the event where brown pigment can not be formed, both melanotic tumor-incidence and viability of flies are higher and the absorption rate of molybdenum or nickel is lower, than in the case were brown pigment can be formed. Then, it seems probable that the increase in volume of tryptophan or tryptophan derivatives related to brown pigment formation causes higher tumor-incidence in parallel to the accelation of melanization. A possible illustration to show the above relationship is presented in Scheme 2.

SUMMARY

1. Quantitative measurements of some metals (Cu, Fe, Mo and Ni) were made in pupae of tumor and non-tumor strains (Oregon R, *bw*, *v*, *st*, *tu*, *bw tu*, *v tu* and *tu st*) of *Drosophila melanogaster* with the use of synthetic medium through the metal analysis with the acid-ashing treatment. Viability of flies and their melanotic tumor-incidence were also examined.

2. It is shown that tumor formation itself is related to "lethality" of flies and that the tryptophan metabolism in relation to brown pigment formation in the eye is associated with increasing viability through melanization.

3. From the results of metal analysis it is strongly suggested that Mo or Ni has an intimate relation to tumor formation itself in some way.

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Direct Determination of Cadmium, Nickel, and Zinc in Rat Lungs

By Atomic Absorption Spectrophotometry

Archives of Environmental Health
27(6) 387(1973)

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An atomic absorption method for the direct determination of metals in mammalian tissues has been developed. The procedure, which involves digestion of the sample with a quaternary ammonium hydroxide, allows one to measure metals normally present in low concentrations, such as cadmium and nickel, with a great degree of sensitivity and a minimal amount of sample handling.

At present atomic absorption is the most commonly used method for the determination of cadmium.[1] Sensitivities are high if a nonbiological matrix is present,[2,3] if the biological matrix is sufficiently dilute to prevent chemical-physical interferences, or if a special apparatus is used.[1,4] Since in animal tissues cadmium is present in much lower concentrations than zinc or copper, the common practice has been to increase the sample size or concentrate the sample.

A procedure has been developed in this laboratory for the specific analysis of cadmium and nickel in a variety of tissues. The method utilizes a quaternary ammonium hydroxide or tetramethylammonium hydroxide for the dissolution of the tissue; these solubilizers originally were developed for use in scintillation counting.[5] More recently their usage has been extended to gas chromatographic examination of mammalian tissues[6] and as a medium for sample preparation prior to zinc analyses by atomic absorption spectroscopy.[7] The recognized advantage of minimizing sample handling is supplemented by the increased sensitivity with which certain metals are detected. This method can be extended to the analysis of those trace metals which do not form insoluble hydroxides under basic conditions.

Materials and Methods

White, two-month-old male rats were used for all experiments.

Reagents.—All chemicals were reagent grade unless otherwise specified. All water used was triply distilled in a glass still. All glassware was boiled one hour in aqua regia, rinsed five times with distilled water, immersed in 1% (W/V) edetic acid overnight, and rinsed again five to ten times. Buffer solutions were prepared daily. The buffer used was sucrose (0.17 M), mannitol (0.15 M), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) (0.005 M), pH 7.4.[8]

Standard Solutions of Metals.—A stock solution of $CrCl_3 \cdot 6\,H_2O$ (5.128 gm), $NiCl_2$ (4.05 gm), zinc metal (0.1000 gm), and $CdCl_2$ (0.163 gm) was prepared in 100 ml of 10% (W/V) nitric acid. Copper (0.5 gm) was dissolved separately in 10% (W/V) nitric acid (1 liter). Both solutions were stored at 4 C in acid washed polyethylene bottles. Both solutions were diluted with water at the time of use and aliquots taken for analysis. The final concentrations of metal ions ranged from 5×10^{-5} to 5×10^{-6} gm/ml. Stock solutions are prepared monthly.

Preparation of Lung Tissue.—The rats were anesthetized with pentobarbital sodium, the rib cage opened, and the lungs perfused with buffer through the right ventricle of the heart until the lung was visibly cleared of blood. Lungs were then washed four successive times with 10-ml aliquots of buffer according to standard procedures,[9] separated from the trachea and bronchi, and pressed through a tissue press, pore diameter 1.5 mm. The tissue remaining in the press was analyzed separately. The remainder of the lung was divided into approximately equal portions on a wet weight basis, lyophilized overnight, weighed again, and analyzed for metal content.

Procedure.—Dried lung samples weighing 200 to 300 mg wet weight, (50 to 100 mg dry weight) were digested with an aliquot of the organic base in toluene (46% weight by volume), in a ratio of 2 ml base for every 100 mg dried tissue, at 60 C for 24 hours in a shaking incubator. After digestion, the sample was diluted 2½ times its volume with toluene and analyzed directly. Reagent blanks and standards were run concurrently and both inter-

Submitted for publication April 19, 1973; accepted June 11.

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Table 1.—Standard Curve for Cadmium and Nickel in the Presence of Lung Tissue*

Concentration, μg/ml	Average Tissue Wet Weight ± SE, gm	Mean Absorbance ± SD (Log)		Coefficient of Variation, %	
		Cadmium	Nickel	Cadmium	Nickel
0	0.25469 ± 0.00039	0.0292 ± 0	0.0223 ± 0	0	0
0.1 Cadmium, 1.0 Nickel	0.25421 ± 0.00119	0.0696 ± .002	0.0622 ± 0.003	2.8	4.8
0.2 Cadmium, 2.0 Nickel	0.25600 ± 0.00210	0.1211 ± 0.004	0.0969 ± 0.003	3.3	3.1
0.3 Cadmium, 3.0 Nickel	0.24964 ± 0.00894	0.1675 ± 0	0.1378 ± 0.017	0	12.3
0.4 Cadmium, 4.0 Nickel	0.25707 ± 0.00043	0.2077 ± 0.01	0.1612 ± 0	4.8	0
0.5 Cadmium, 5.0 Nickel	0.25521 ± 0.00316	0.2441 ± 0	0.1838 ± 0.005	0	6.0

*Average of three determinations.

Table 2.—Cadmium, Zinc, and Nickel Concentrations in Whole Lung as a Function of Exposure to Cadmium Oxide Aerosol*

Exposure Time, hr	Concentration		
	Cadmium	Zinc	Nickel
Control	$2.98 \times 10^{-7} \pm 0.4$†	$1.47 \times 10^{-5} \pm 0.23$	< Limit
54	$3.05 \times 10^{-6} \pm 0.06$	$1.76 \times 10^{-5} \pm 0.04$	$8.01 \times 10^{-6} \pm 0.02$
58	$3.14 \times 10^{-6} \pm 0.44$	$1.62 \times 10^{-5} \pm 0.14$	$1.13 \times 10^{-5} \pm 0.10$
66	$5.79 \times 10^{-6} \pm 0.14$	$1.53 \times 10^{-5} \pm 0.14$	$9.42 \times 10^{-6} \pm 2.10$
72	$6.02 \times 10^{-6} \pm 1.56$	$1.84 \times 10^{-5} \pm 0.11$	$1.14 \times 10^{-5} \pm 0.11$

*Cadmium concentration in aerosol varied from 45μg/cu m to 92μg/cu m. Concentration values given are mean and standard deviations of five analyses, expressed as grams of metal per grams of sample.

†Average of three determinations.

nal and external standards were used. All metal additions were made prior to the digestion procedure. In the case of cadmium and nickel, the standard curve was not significantly altered by the presence of tissue (the matrix effect is minimal for 200 mg quantities of tissue), an observation leading ultimately to the use of external standards. The tissue metal concentrations reported were by the method of interpolation. All samples were diluted with toluene for zinc and copper analysis in a ratio varying from 1:2 to 1:20, depending on the weight of the tissue used. The dilution represents a source of error in the final evaluation of concentrations for these two metals. Every metal concentration was run in quadruplet or quintuplet, with and without pressed whole lung. Where internal standards are recorded, they consist of at least three whole lungs, pressed and divided into approximately equal portions, to which the metals were added. The weight of lung present in the standard is listed in the relevant table.

Analyses were performed on an atomic absorption spectrophotometer, equipped with a Boling burner and a strip chart recorder, using standard methods.[2,3]

Inhalation Experiments.—Cadmium oxide aerosols were generated according to a method established by Horstman et al[10] and passed into the inhalation chambers. White, male, two-month-old rats were exposed daily for eight-hour periods. The total period is listed for each experiment.

Results and Comment

Table 1 shows data for the working curve of internal standards covering the range 0.1 to 0.5×10^{-6} and 1.0 to 5.0×10^{-6} gm/ml for cadmium and nickel, respectively. The means, standard deviations, and coefficients of variations are listed for each metal. The coefficient of variation is somewhat larger for nickel, for which this method is less sensitive. Although the slope of the standard curves varies slightly from day to day, the coefficients of variation for triplicate analyses performed on one day are generally less than 3%. If one uses external standards, the coefficient of variation is closer to 2%, which is very close to the coefficient of variation for the apparatus in measurement of the same sample ten times (1.8%). For cadmium analyses the average slope with use of standard acid wet ashing procedures,[11] is about 0.357, as a measurement of percentage of absorption against microgram of metal.

Table 3.—Cadmium Retention by the Lung as a Function of Exposure Times to Cadmium Oxide Aerosol*

Exposure Period, hr	Total Cadmium Exposure, gm†	Total Metal Recovered, gm		Cadmium Retention, %	Calculated‡ Inhaled Cadmium in Lungs, gm	Calculated Retention of Inhaled Cadmium, %
		Cadmium	Zinc			
54	6.071×10^{-3}	3.68×10^{-6}	2.32×10^{-5}	0.061	56.98×10^{-6}	6.5
58	7.268×10^{-3}	4.29×10^{-6}	2.37×10^{-5}	0.059	33.02×10^{-6}	12.9
66	7.927×10^{-3}	7.68×10^{-6}	2.48×10^{-5}	0.097	39.21×10^{-6}	19.5
72	8.523×10^{-3}	9.67×10^{-6}	3.16×10^{-5}	0.113	44.87×10^{-6}	21.5
Control	0	4.27×10^{-7}	2.30×10^{-5}	. . .	0	. . .

*Concentration of cadmium in aerosols varied from 47 to 90×10^{-6} gm/cu m. Particle mass median diameter is equal to 0.15μ, with 97% being less than 0.94μ.[4]

†Total amount of cadmium to which animals were exposed was calculated from concentration of cadmium per cubic meter times number of cubic meters in exposure period.

‡Figures based on inhalation rate of 85.5 per minute and lung volume of 2 ml.

Use of the present method produces an average slope around 1. For nickel, the slope changes from 0.222 in acid, to 0.666 in this organic base.

Since copper and zinc concentrations are determined after dilution, and therefore subject to a certain amount of dilution error, a National Bureau of Standards sample (No. 1577) of bovine liver was subjected to the same procedure and analyzed. The copper recovery was 113% ± 5.0%, and zinc, 112% ± 6% in three separate analyses.

The precision of the method in repeated analyses of control and exposed lung tissue is presented in Table 2. All lungs were divided into approximately five equal portions and each portion analyzed for cadmium, nickel, and zinc. The standard deviations for each lung varied from a low of 0.02 ppm to 1.56 ppm, depending somewhat on the exposure to the inhaled metal. Within measured ranges of cadmium and nickel concentrations, 200 to 300 mg of wet tissue from an exposed animal are necessary for precision. If the animal is unexposed, 300 to 400 mg are required for the cadmium analysis. Although one would not expect any difference in the nickel concentration before or after exposure to cadmium, the nickel concentration was below the level of detection in the control animals. Sample weights can be reduced by as much as 90% to 95% for the analysis of zinc and copper.[7]

Using the data given in Table 3, the total amount of cadmium recovered from the lung was calculated and compared with the total amount of cadmium present in the inhalation chamber during that period, as well as with the estimated amount of cadmium inhaled by the rats. It can be seen that the percent retained (6% to 20%) of that "inhaled," is larger by a factor of 50 than the amount retained from the total exposure. These figures are somewhat comparable to those reported in the literature,[12] which for short acute exposures varied from 6% to 13% for rats, and under chronic conditions, lasting eight months, are estimated at 2% for rabbits. Both of these studies were performed at higher exposure levels than those in the present experiments. Under the conditions of the present study, all animals are exposed to a relatively small particle size, (89% are less than 0.53μm in diameter)[10] which for cadmium oxide would be relatively soluble. The animals were killed 16 hours after removal from the chamber, and the total exposures were not large. Experiments performed in these laboratories after five- to six-week exposure periods, to be reported in the future, do not show much increase in lung retention. Under the conditions of these experiments then, it would appear that of the cadmium oxide aerosol absorbed, a substantial portion remains in the lung.

In summary, data have been presented to illustrate a fast and relatively precise method for the analysis of cadmium and nickel in animal tissues.[13] The technique has been used to measure cadmium concentrations in the rat lung after exposure to cadmium oxide aerosols.

This investigation was supported by the National Institute of Occupational Safety and Health grant 5-R01-OH-00347-02.

Emil Pfitzer, PhD, assisted in the construction of inhalation experiments.

William Barkley ran the inhalation chambers. Tony Horstman, PhD, performed the particle sizing.

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Wallerstein Lab. Commun.
18(63) 285-289 (1955)

Determination of Traces of Nickel in Malt Beverages†

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A SENSITIVE and precise colorimetric method for the determination of traces of nickel in worts, beers, and brewing materials was developed for investigations on the effect of this metal on fermentation and wildness. This is a continuation in part of the studies conducted in this laboratory on the effects of trace metals in the brewing process.

The presence of traces of nickel in worts and beers is assuming greater importance with the increasing use of nickel or nickel-containing equipment in breweries. The contamination of worts with traces of nickel of the order of a few parts per million may lead to toxic effects on the yeast and slowing of the fermentation. Traces of nickel in the finished beer tend to form gas-evolving nuclei producing the condition of wildness, or gushing beer. This paper will present the details of the technique and data relating to the determination of nickel. The results of tests on the effects of traces of nickel on fermentation and the production of wildness will be presented elsewhere (1).

Hagues (2) in 1931, in a paper on the contamination of beer by copper and nickel, proposed a method which involved dry ashing the sample and finally visually matching the color produced by strongly alkaline dimethylglyoxime with standards in Nessler tubes after standing overnight. The method lacks sensitivity, as 0.6 part per million appears to be the lowest detectable limit. In a paper in 1945, Essery (3) notes the many difficulties encountered in the determination of trace metals in wort. After an elaborate dry ashing and solubilization of the ash, he determined nickel by visual comparison of the color produced by oxidation with bromine and treatment with ammonia and dimethylglyoxime. Citric acid was used to prevent interference of phosphates, but it is stated that if matching is delayed for a short time, precipitation ensues which interferes with the color comparison. The author states he obtained a recovery of 93 percent. The presence of 3 to 4 parts per million of iron produced no interference, but the effect of the presence of traces of copper was not mentioned. After the present method was in use, the paper of Andrews and Harrison (4) appeared in 1954. Their method utilizes a wet digestion to destroy organic matter, and the nickel is determined colorimetrically

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with *alpha*-furildioxime in chloroform. The interference of copper is eliminated by extraction of the chloroform solution with dilute sulfuric acid. Very good recoveries are reported. The levels of nickel found in the beers and brewing materials are in line with those found by us.

The method reported here is a refinement of the reaction involved in the method of Essery. The reaction was first reported by Feigl (5) who found that nickel and dimethylglyoxime, when oxidized by lead peroxide, produced a red-colored compound in alkaline solution. Rollet (6) substituted bromine water as the oxidizing agent and used the reaction to determine nickel quantitatively in steel and various organic compounds. He achieved an accuracy of within 5 percent and reported copper to interfere. Sandell (7) recommended the separation of nickel from copper by chloroform extraction of the nickelous dimethylglyoxime. Babco (8) investigated the reaction of nickel with dimethylglyoxime in the presence of bromine water and concluded that the mechanism involved oxidation of the dimethylglyoxime and also found that the order of mixing reagents is important.

The direct determination of nickel in beer, without ashing, was tried, but it is not satisfactory in the range of desired sensitivity. Colored materials are produced from the beer constituents during the test, and the colored nickel complex is not readily extractable by solvents from the dark reaction mixture. The method, as here presented, involves dry ashing of the beer or wort. The ash is taken up in dilute hydrochloric acid and extracted with a carbon tetrachloride solution of dithizone to remove the interference of copper and iron. The aqueous layer is treated with dimethylglyoxime and bromine and then made alkaline, developing the brown-to-red nickel color. The intensity of the color, which is directly related to the concentration in the range 0.0 to 0.5 part per million, is then read in a photometer. After the ashing step the reactions are conducted in a stoppered 15-ml graduated centrifuge tube and then transferred to a 10-ml volumetric flask for color development.

Reagents

Hydrochloric acid, 1 to 1 and 1 to 9

Dithizone (diphenyl thiocarbazone) —0.05% in carbon tetrachloride. Keep refrigerated in brown bottle.

Dimethylglyoxime — 1% in 95% ethyl alcohol

Bromine water — a saturated solution of bromine in distilled water

Ammonium hydroxide, concentrated (28% ammonia)

Standard nickel solution, nickel ammonium sulfate, $NiSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$, 6.730 grams per liter, 1 ml = 1 mg nickel

Calibration of Photometer

To graduated 15-ml centrifuge tubes, add 1 ml 1 to 9 hydrochloric acid and aliquots of freshly prepared, appropriately diluted standard nickel solution, keeping total volume under 6 ml. Cover the range from 0.0 to 0.5 mg nickel in suitable increments. Dilute to the 6-ml mark with distilled water, add 4 ml dithizone solution, and continue as described below.

The intensity of color developed is proportional to the concentration of nickel in the above range, so that if the response of the colorimeter or photometer employed is linear, a straight line calibration plot should be obtained, and an average calibration factor converting scale readings to parts per million may be calculated. If the response of the photometer is non-linear, a graphic method with a calibration curve may be used.

Method

Evaporate 100 ml of wort or "degassed" beer to dryness in a silica dish previously cleaned by boiling with 1 to 1 hydrochloric acid. Char with a Bunsen flame, then ash in a muffle furnace at 500 to 550°C until ash is white. (In the case of dry materials such as malt, weigh out a suitable charge, such as 10 grams, and char and ash as above.) To the ash add 2 ml of 1 to 1 hydrochloric acid and 2 ml of distilled water,

taking care to wash down the sides of the dish. Evaporate to dryness on a boiling water bath, and while dish is still hot, take up residue with 1 ml of 1 to 9 hydrochloric acid and 2 ml of distilled water. Carefully transfer the acid solution to a graduated 15-ml centrifuge tube (graduated in 0.1-ml divisions). Wash out the silica dish with small increments of water, transferring to tube until the 6-ml mark is reached.

Add 4 ml of dithizone solution, cork tube tightly, and shake vigorously for 30 seconds and allow layers to separate. Repeat shaking four times. If color of dithizone turns brownish, draw off spent dithizone and add a fresh 4-ml portion. Repeat shake-outs and dithizone additions until no change in original dithizone color occurs. Using a 5-ml volumetric pipet, carefully remove 5 ml of the aqueous (top) layer and transfer to a 10-ml volumetric flask. Add 0.5 ml dimethylglyoxime solution, mix, and then add 1.0 ml saturated bromine water. Mix and let stand 10 minutes. (The solution should be brownish due to the excess bromine. If not, add more bromine water.) Then add 0.5 ml ammonium hydroxide, mix thoroughly, and dilute to the 10-ml mark with distilled water. Transfer to centrifuge tube and centrifuge for 3 minutes at approximately 2,500 rpm. Read the supernatant liquid (within 15 minutes) in a photoelectric colorimeter, using a green filter and a suitable cell. Set the "zero" reading of the instrument with water. The authors have used a 13-mm cell and a filter photometer with a composite glass filter having a maximum transmission at 500 millimicrons.

A reagent blank is run by mixing 1 ml of 1 to 9 hydrochloric acid and 5 ml distilled water in a graduated centrifuge tube and proceeding from the step where dithizone is added.

The results are calculated in the usual manner for a colorimetric procedure. If the calibration data is a linear function, multiply the factor (converting photometer readings to parts per million nickel) by the difference obtained by subtracting the blank photometer reading from the sample photometer reading. If a curvilinear function is obtained for the calibration, then pick off the values for the sample and blank from a curve constructed on graph paper relating photometer readings to parts per million nickel. Subtract the blank results from the sample value to obtain the parts per million of nickel to be reported.

For beers containing nickel in excess of 0.5 part per million, reduce the charge of sample used for ashing. Select a charge such that the color intensity of the solution read in the photometer falls within the value of the calibration curve and gives readings of acceptable precision.

Calibration Data

A plot of the calibration data obtained on the Klett-Summerson photoelectric colorimeter gives a straight line, indicating that Beer's Law is obeyed in this range of concentrations.

Recovery and Interference Data

To determine the precision and reliability of the method, a series of tests measuring the recovery of added nickel was carried out. Table I gives the results of tests for the determination of the recovery of added nickel in a series of beers. Recovery tests were also conducted on beers to which

Table I: Recovery of added nickel

Sample	ppm added metal Copper	Iron	Nickel	Nickel found ppm	Added nickel recovered ppm
Beer A	—	—	—	0.02	—
	—	—	0.025	0.044	0.024
	—	—	0.05	0.07	0.05
	—	—	0.10	0.13	0.11
	—	—	0.40	0.43	0.41
Beer B	—	—	—	0.01	—
	2.0	5.0	—	0.01	—
	—	—	0.025	0.031	0.021
	—	—	0.05	0.05	0.04
	2.0	5.0	0.05	0.06	0.05
	—	—	0.10	0.12	0.11
	2.0	5.0	0.10	0.12	0.11
	—	—	0.40	0.41	0.40
	2.0	5.0	0.40	0.41	0.40

2 parts per million of copper and 5 parts per million of iron were added to see whether the presence of these levels of copper and iron interfere. Two parts per million of copper and 5 parts per million of iron are much above the level of pickup generally found in beer. Inspection of the data shows that excellent recovery of added nickel can be expected from this method and that there is no interference from copper and iron at levels much beyond those ordinarily encountered.

Replication

Very good replication of results is indicated. Table II contains results of tests conducted in triplicate on a series of beers. Replication is good both at levels of nickel generally encountered for normal beers (Beers A to D) and at levels indicating nickel pickup (Beer E).

Table II: Replication of results

Nickel-parts per million

	1	2	3
Beer A	0.01	0.01	0.01
Beer B	0.02	0.02	0.02
Beer C	0.02	0.02	0.01
Beer D	0.04	0.04	0.04
Beer E	0.85	0.84	0.84

Summary

A precise colorimetric method for the determination of traces of nickel in beers and ales is presented. The technique involves ashing the samples, taking up the ash with dilute acid, and shaking out with dithizone in carbon tetrachloride. The soluble colored nickel complex is formed in the aqueous layer by treatment with dimethylglyoxime, bromine water, and ammonia. The color formed is proportional to the quantity of nickel present. Recovery of added nickel is good, and copper and iron do not interfere.

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[*Résumé*]

Le dosage de traces de nickel dans les boissons de malt.

Une méthode colorimétrique précise pour le dosage de traces de nickel dans les bières et les autres boissons à base de malt est présentée. La technique comprend la calcination de l'échantillon, puis les cendres sont dissoutes par de l'acide dilué et l'on agite la solution acide avec du dithizone (diphenyl thiocarbazone) dans du tetrachlorure de carbone pour supprimer l'interférence du cuivre et du fer. Le complexe de nickel, soluble et coloré, se forme dans la couche aqueuse en traitant par du diméthyle glyoxime, de l'eau de brome et de l'ammoniaque. La coloration qui prend naissance est proportionnelle à la quantité de nickel présent. Les ajoutes de nickel se retrouvent bien et le cuivre et le fer ne gênent pas la réaction.

[*Resumen*]

Determinación de huellas de níquel en bebidas a base de malta

Se da a conocer un método colorimétrico para la determinación de huellas de níquel en cerveza o en otras bebidas a base de malta. La técnica comprende: calcinar una porción de la cerveza, disolver las cenizas en ácido clorhídrico diluido, y agitar la solución ácida con ditiazona (difeniltiocarbazona) disuelta en tetracloruro de carbono. A una alícuota de la capa acuosa se agrega dimetilglioxima, agua de bromo, y amoniaco, los cuales con el níquel forman un compuesto complejo que queda disuelto y colora la solución. La intensidad del color es proporcional a la concentración del níquel presente.

El método es preciso como se ha podido demostrar en ensayos en los cuales se determinaron los incrementos de níquel agregados. La presencia de cobre y hierro en la cerveza no afectó los resultados.

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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

APR 23 1974

OFFICE OF
AIR AND WATER PROGRAMS

Dr. Renee Ford
Tracor Jitco Inc.
1300 E. Gude Drive
Rockville, Md. 20851

Dear Dr. Ford:

Enclosed is the information you requested of Mr. Roger Lee on nickel in drinking water.

The numbers in the first column are the location of where the sample was collected and the numbers in the second column are the values for nickel as analyzed by the laboratory in Cincinnati, Ohio.

The numbers in the first column, location identification, are as follows:

Digits 1 & 2 are EPA Region (01-10)

Digits 3 & 4 are the States (01-54)

Digits 5 - 9 are the cities within States (00001-99999)

Digits 10 - 12 are the counties within States (001-999)

Digit 13 is used as an incremental number.

A listing of the Regions and States is attached.

There is a total of 9169 values stored in the computer file, 4182 have a value of zero (meaning an analysis was run on the sample but no detectable amount was recorded) and 4977 have a value of from .001 to 1.3.

Also enclosed is a sample copy of a frequency table giving the number of samples in ranges from zero to 3 times the drinking water standards limit. This can be run for nickel, using 0.005 mg/liter, by State with a national total, if it is desired.

In the tables S=surface source and G=ground source, there are eight tables per page and it would take 7 pages to develop tables for the 50 states and a national total.

These tables would be better for your monogram, rather than the 168 pages of individual values.

If the Division can be of further assistance, please let me know.

Sincerely yours,

George C. Kent

George C. Kent, Chief
Basic Data Unit
Water Supply Division

426-8877

Enclosures - 3

Analyses 1969-1974 of water supply systems in USA
Surface water systems
Ground water systems
Combined systems

NICKEL(0.0050)

	<0	0	0.00050	0.00100	0.00150	0.00200	0.00250	0.00300	0.00350	0.00400	0.00450	0.00500	0.01000	0.01500	>0.01500
ALABAMA															
S	0	34	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	17	0	0	0	0	0	0	0	0	0	0	0	0	1
C	0	107	0	0	0	0	0	0	0	0	0	0	0	0	0
ALASKA															
S	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
C	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
ARIZONA															
S	0	14	0	0	0	0	0	0	0	0	0	0	0	0	1
G	0	32	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ARKANSAS															
S	0	4	0	1	0	0	0	0	0	0	0	0	0	0	0
G	0	4	0	1	0	0	0	0	0	0	0	0	0	0	0
C	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
CALIFORNIA															
S	0	166	0	5	0	0	0	0	0	0	0	0	0	0	1
G	0	350	0	42	0	0	0	0	0	0	0	0	0	0	15
C	0	17	0	4	0	0	0	0	0	0	0	0	0	0	0
COLORADO															
S	0	49	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	57	0	1	0	0	0	0	0	0	0	0	0	0	13
C	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
CONNECTICUT															
S	0	192	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	174	0	0	0	0	0	0	0	0	0	0	0	0	2
C	0	18	0	0	0	0	0	0	0	0	0	0	0	0	0

NICKEL(0.0050)

	<0	0	0.00050	0.00100	0.00150	0.00200	0.00250	0.00300	0.00350	0.00400	0.00450	0.00500	0.01000	0.01500	>0.01500
DELAWARE															
S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
WASHINGTON D.C.															
S	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FLORIDA															
S	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	113	0	0	0	0	0	0	0	0	0	0	0	0	6
C	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0
GEORGIA															
S	0	12	0	1	0	0	0	0	0	0	0	0	0	0	0
G	0	294	0	31	0	0	0	0	0	0	0	0	0	0	6
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HAWAII															
S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
IDAHO															
S	0	21	8	0	0	0	0	0	0	0	0	0	0	0	0
G	0	106	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
ILLINOIS															
S	0	141	0	0	0	0	0	0	0	0	0	0	0	0	6
G	0	58	0	1	0	0	0	0	0	0	0	0	0	0	1
C	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0

NICKEL(0.0050)

	<0	0	0.00050	0.00100	0.00150	0.00200	0.00250	0.00300	0.00350	0.00400	0.00450	0.00500	0.01000	0.01500	>0.01500
INDIANA															
S	0	15	0	0	0	0	0	0	0	0	0	0	0	0	1
G	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
IOWA															
S	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	5	0	0	0	0	0	0	0	0	0	0	0	0	2
C	0	2	0	0	0	0	0	0	0	0	0	0	0	0	1
KANSAS															
S	0	26	0	1	0	0	0	0	0	0	0	0	0	0	3
G	0	162	0	0	0	0	0	0	0	0	0	0	0	0	10
C	0	48	0	1	0	0	0	0	0	0	0	0	0	0	3
KENTUCKY															
S	0	169	0	4	0	0	0	0	0	0	0	0	0	0	3
G	0	211	0	3	0	0	0	0	0	0	0	0	0	0	20
C	0	13	0	1	0	0	0	0	0	0	0	0	0	0	0
LOUISIANA															
S	0	78	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	43	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MAINE															
S	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	783	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MARYLAND															
S	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	24	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

NICKEL(0.0050)

	<0	0	0.00050	0.00100	0.00150	0.00200	0.00250	0.00300	0.00350	0.00400	0.00450	0.00500	0.01000	0.01500	>0.01500
MASSACHUSETTS															
S	0	897	0	0	0	0	0	0	0	0	0	0	0	0	4
G	0	46	0	1	0	0	0	0	0	0	0	0	0	0	0
C	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0
MICHIGAN															
S	0	26	0	1	0	0	0	0	0	0	0	0	0	0	0
G	0	3	0	0	0	0	0	0	0	0	0	0	0	0	1
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MINNESOTA															
S	0	38	0	1	0	0	0	0	0	0	0	0	0	0	1
G	0	52	0	1	0	0	0	0	0	0	0	0	0	0	0
C	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
MISSISSIPPI															
S	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	26	0	1	0	0	0	0	0	0	0	0	0	0	15
C	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
MISSOURI															
S	0	343	0	4	0	0	0	0	0	0	0	0	0	0	1
G	0	75	0	2	0	0	0	0	0	0	0	0	0	0	5
C	0	3	0	2	0	0	0	0	0	0	0	0	0	0	0
MONTANA															
S	0	11	0	1	0	0	0	0	0	0	0	0	0	0	2
G	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	2	0	0	0	0	0	0	0	0	0	0	0	0	3
NEBRASKA															
S	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	10	0	0	0	0	0	0	0	0	0	0	0	0	5
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

NICKEL(0.0050)

	<0	0	0.00050	0.00100	0.00150	0.00200	0.00250	0.00300	0.00350	0.00400	0.00450	0.00500	0.01000	0.01500	>0.01500
NEVADA															
S	0	8	0	0	0	0	0	0	0	0	0	0	0	0	2
G	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NEW HAMPSHIRE															
S	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
NEW JERSEY															
S	0	53	0	0	0	0	0	0	0	0	0	0	0	0	2
G	0	83	0	1	0	0	0	0	0	0	0	0	0	0	6
C	0	17	0	0	0	0	0	0	0	0	0	0	0	0	0
NEW MEXICO															
S	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	61	0	3	0	0	0	0	0	0	0	0	0	0	1
C	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
NEW YORK															
S	0	110	0	16	0	0	0	0	0	0	0	0	0	0	4
G	0	278	0	10	0	0	0	0	0	0	0	0	0	0	29
C	0	11	0	1	0	0	0	0	0	0	0	0	0	0	0
NORTH CAROLINA															
S	0	94	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	31	0	1	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NORTH DAKOTA															
S	0	55	0	0	0	0	0	0	0	0	0	0	0	0	1
G	0	45	0	3	0	0	0	0	0	0	0	0	0	0	0
C	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0

NICKEL(0.0050)

	<0	0	0.00050	0.00100	0.00150	0.00200	0.00250	0.00300	0.00350	0.00400	0.00450	0.00500	0.01000	0.01500	>0.01500
OHIO															
S	0	158	0	3	0	0	0	0	0	0	0	0	0	0	7
G	0	60	0	3	0	0	0	0	0	0	0	0	0	0	5
C	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0
OKLAHOMA															
S	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OREGON															
S	0	26	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	345	0	1	0	0	0	0	0	0	0	0	0	0	5
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PENNSYLVANIA															
S	0	16	0	1	0	0	0	0	0	0	0	0	0	0	3
G	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RHODE ISLAND															
S	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SOUTH CAROLINA															
S	0	13	0	3	0	0	0	0	0	0	0	0	0	0	0
G	0	30	0	0	0	0	0	0	0	0	0	0	0	0	1
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SOUTH DAKOTA															
S	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

NICKEL(0.0050)

	<0	0	0.00050	0.00100	0.00150	0.00200	0.00250	0.00300	0.00350	0.00400	0.00450	0.00500	0.01000	0.01500	>0.01500
TENNESSEE															
S	0	79	0	2	0	0	0	0	0	0	0	0	0	0	1
G	0	316	0	3	0	0	0	0	0	0	0	0	0	0	24
C	0	7	0	0	0	0	0	0	0	0	0	0	0	0	1
TEXAS															
S	0	9	0	0	0	0	0	0	0	0	0	0	0	0	3
G	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
UTAH															
S	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	2	0	0	0	0	0	0	0	0	0	0	0	0	1
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
VERMONT															
S	0	126	0	1	0	0	0	0	0	0	0	0	0	0	5
G	0	169	0	3	0	0	0	0	0	0	0	0	0	0	26
C	0	37	0	0	0	0	0	0	0	0	0	0	0	0	1
VIRGINIA															
S	0	17	0	2	0	0	0	0	0	0	0	0	0	0	1
G	0	128	0	1	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
WASHINGTON															
S	0	61	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	21	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
WEST VIRGINIA															
S	0	40	0	4	0	0	0	0	0	0	0	0	0	0	0
G	0	32	0	2	0	0	0	0	0	0	0	0	0	0	2
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

F NCY TABLES
F L SUPPLIES
05/31/74

NICKEL(0.0050)

	<0	0	0.00050	0.00100	0.00150	0.00200	0.00250	0.00300	0.00350	0.00400	0.00450	0.00500	0.01000	0.01500	>0.01500
WISCONSIN															
S	0	18	0	0	0	0	0	0	0	0	0	0	0	0	5
G	0	19	0	0	0	0	0	0	0	0	0	0	0	0	3
C	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
WYOMING															
S	0	47	0	5	0	0	0	0	0	0	0	0	0	0	0
G	0	257	0	0	0	0	0	0	0	0	0	0	0	0	8
C	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0
PUERTO RICO															
S	0	14	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	2	0	0	0	0	0	0	0	0	0	0	0	0	1
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
VIRGIN ISLANDS															
S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	4	0	0	0	0	0	0	0	0	0	0	0	0	1
C	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
NATIONAL TOTAL															
S	0	3229	8	56	0	0	0	0	0	0	0	0	0	0	57
G	0	[illegible]	0	116	0	0	0	0	0	0	0	0	0	0	215
C	0	345	0	10	0	0	0	0	0	0	0	0	0	0	10

BIOCHIMICA ET BIOPHYSICA ACTA VOL. 9 (1952) 170-179

TYROSINASE AND POLYPHENOLOXIDASE. THE ROLE OF METALLIC IONS IN MELANOGENESIS

by

DENIS KERTÉSZ

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The mechanism by which tyrosinase acts on monohydric phenols has been under discussion for a long time[1-5]. ONSLOW AND ROBINSON[6] were the first to propose that the monophenolase action is not due to the enzyme molecule itself but to the *o*-quinones produced by the enzymic oxidation of *o*-dihydric phenols. After the adverse criticism of PUGH[7] the same view was reaffirmed by KEILIN AND MANN[8] and by CALIFANO AND KERTÉSZ[9]. More recently WARBURG[10] states also that probably in the case of monohydric phenol oxidation *o*-diphenols are first formed by a slow reaction, and these then act catalytically by virtue of the change *o*-diphenol $\rightleftharpoons$ *o*-quinone. This is the same catalytic change which, in presence of traces of catechol, enables the enzyme to oxidize hydroquinone, ascorbic acid and reduced coenzymes I and II[10].

However, most authors, following NELSON AND DAWSON[5], believe that the oxidation of the monohydric phenols is a function of the enzyme molecule itself and that the enzyme is primed whilst oxidizing an dihydric phenol and so acquires the capacity to act on monohydric phenols. MALLETTE AND DAWSON[11], to explain the loss of monophenolase activity during the purification process, suppose that different centres of the same protein molecule are responsible for the monohydric phenol and dihydric phenol oxidation. The diminution of the activity on monohydric phenols (in their terminology the cresolase activity of tyrosinase) and the augmentation of the activity on *o*-dihydric phenols (in their terminology the catecholase activity of tyrosinase) would be the result of the greater fragility and loss of part of the molecule responsible for the cresolase activity, or of the unmasking of new active centres responsible for the catecholase activity during the purification process.

Evidence reported in this paper not only fails to support the above views, but shows that the cresolase or monophenolase activity of the enzyme belongs to free, and not protein-bound, metallic ions which hasten the spontaneous reaction[9] between *o*-quinones and monohydric phenols. In agreement between earlier theoretical[12] and experimental[13,14] work the oxidation of the monohydric phenols appears to be represented correctly by the following reactions

(I) *o*-dihydric phenol $\rightarrow$ *o*-quinone $\rightarrow$ ulterior products $\rightarrow$ melanin

(II) *o*-quinone + monohydric phenol + H_2O = 2 *o*-dihydric phenol

of which only the first of (I) is enzymatic.

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METHODS

Enzyme preparation. Different preparations of polyphenoloxidase apo-enzyme, from potato peelings extracted with HCN, were described earlier[16,18]. In this work a stable powder was used which is similar to one described recently[19], but of a more simple preparation. 1300 g of potato peelings are extracted with 700 ml of extraction liquid (0.05 molar KCN neutralized and buffered to pH 7). The extract is centrifuged for a few minutes to eliminate the starch and then brought up to 30% concentration in acetone and centrifuged again. The precipitate is discarded. The supernatant is brought up to 60% concentration in acetone and centrifuged. The precipitate is resuspended in 180 ml of extraction liquid. The trouble suspension obtained is brought up again to 60% concentration in acetone, filtered on a Buchner funnel and the precipitate is washed 5 times with acetone. It is dessicated first in an air current, later in a dessicator under vacuum. It can be pulverized after 24 hours; the powder is stable (at least 12 months at + 2° C) and it is not hygroscopic. During the whole procedure the extraction liquid is maintained at 0°, acetone at —20° C; all operations are made in a cold room at + 2° C (for further details see ref. 16). Immediately before use, the powder is suspended at a ratio of 50 mg/ml in Pyrex-redistilled water and the liquid is sharply centrifuged for 30 minutes to eliminate the abundant insoluble matters. The clear solution obtained is practically inactive without added copper on Dopa; its content in non-dialysable substances is 16 mg/ml and in copper 0.02%. 1 ml of the solution contains, if copper is added, 60 enzyme units (Fig. 1) as defined by LERNER *et al.*[17].

Activity determination. The activity of the enzyme was determined manometrically by measuring the oxygen uptake in the Warburg apparatus at 38° C. Substrates (in amounts shown in the legends of the figures) were added from the side arms after 15 minutes of temperature equilibration, at 0 time. The reaction mixtures still contained 0.25 ml of the enzyme solution, 1.25 ml of 0.1 molar phosphate or phosphate-citrate buffer at pH 6.8, the amount marked on the figures respectively of copper and of other metals and Pyrex-redistilled water to make the total volume up to 2.5 ml. The central well of the flasks contained 0.1 ml of NaOH 15%. All solutions were prepared with Pyrex-redistilled water; reagents were chosen and glass-ware washed metal-free. The importance of these precautions was emphasised by BARRON *et al.*[18], by WARBURG[10], and recently by the author[16].

Copper determination. Copper was determined with sodium diethyldithiocarbamate according to KEFFIN AND MANN[8]. Colorimetric readings were made with a Coleman spectrophotometer, at 440 mμ.

RESULTS

Influence of metals on Dopa oxidation. Influence of copper is reported in Fig. 1. It is evident that the enzyme is already fully active in presence of 0.00005 millimols (0.08%) Cu and that presence or absence of excess copper is indifferent. The insignificantly lower activities of the enzyme measured with the highest amounts of copper are artefact and due to the fact that the enzyme is slightly and slowly inactivated in presence of a great excess of copper. This is shown by the two lower curves (A and B) of the Fig. 1, where the Warburg flasks containing 0.0001 millimols and 0.0016 millimols of Cu were reopened, the side arm cavities washed (with the aid of a pipette) 7 times with Pyrex-redistilled water and then filled with the same amount of Dopa solution as previously. The new substrate is added to the old test solution after 7 minutes of temperature equilibration. As the whole procedure did not take more than 15 minutes, the potato enzyme was held 120 minutes at 38° C in

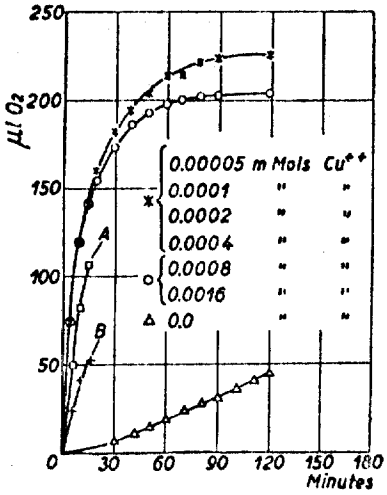

Fig. 1. The effect of varying amounts of copper on the enzymic oxidation of 1 mg Dopa. For curves *A* and *B* see text. All flasks contain the same amount of apo-enzyme corresponding (in presence of copper) to 15 enzyme units.

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contact with Dopa and Cu with almost continuous agitation before the second 0 time. The enzyme which acted in presence of 0.0001 millimol Cu is (calculating the greater dilution) only 12% inactivated; the enzyme which acted in presence of 0.0016 millimols of Cu is about 55% inactivated during this time.

As it was found by KUBOWITZ[10], copper is strictly specific and cannot be replaced by any other metal. Tests were made on: cobalt (as cobaltous chloride), vanadium (as divanadyl tetrachloride and vanadyl sulphate), nickel (as sulphate), zinc (as chloride), iron (as ferric chloride), chromium (as potassium chromic sulphate), magnesium (as sulphate) and manganese (as manganous sulphate). Cobalt in Vitamin B_{12} is also completely inactive, as is iron in Cytochrome C (experiments not reported).

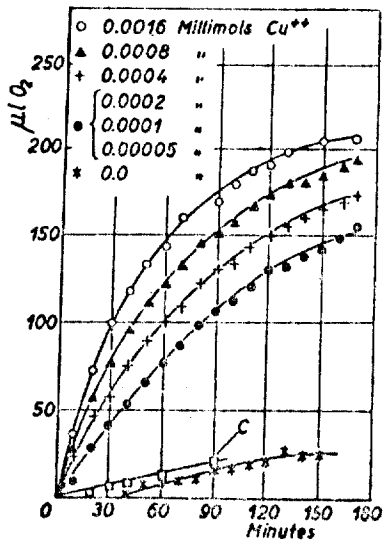

Fig. 2. The effect of varying amounts of copper on the enzymic oxidation of 0.90 mg tyrosine + 0.1 mg Dopa. Curve *C* represents maximum autoxidation, in presence of 0.0016 millimol copper and in absence of apo-enzyme. All other flasks contain the same amount of apo-enzyme as in Fig. 1.

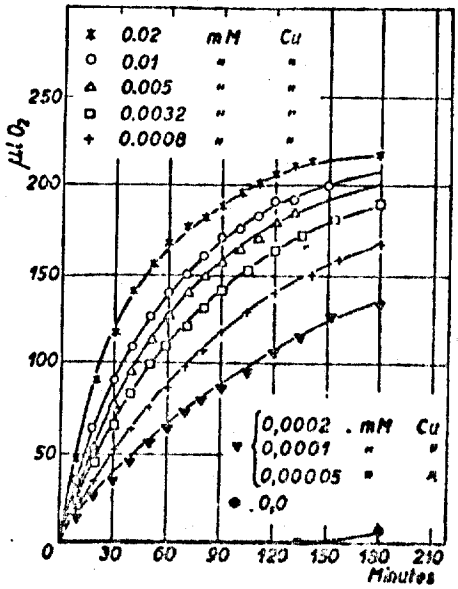

Fig. 3. The effect of varying amounts of copper on the enzymic oxidation of 0.90 mg tyrosine + 0.05 mg Dopa in 0.05 molar citrate-phosphate buffer. All flasks contain the same amount of apo-enzyme as in Fig. 1.

Influence of copper on tyrosine oxidation. Completely different is the action of copper on tyrosine oxidation. The enzyme has the same activity in presence of 0.00005, 0.0001 and 0.0002 millimols of copper, however (Fig. 2), further augmentation of this amount of copper determines further augmentation of the activity on tyrosine. Using citrate-phosphate buffer of MCILWAIN instead of SÖRENSEN's phosphates permits a greater variation of copper concentration and thus an enlargement of the previous findings (Fig. 3), but, as was found by MASON AND WRIGHT[10] for Dopa oxidation at pH 6.6, the enzyme is slightly less active in presence of citrate. The lower activity of the enzyme in this experiment is partly due also to the lower concentration of Dopa.

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Influence of other metals on tyrosine oxidation. The non-specificity of copper is shown in Fig. 4. Here, after having added 0.00005 millimols of copper, equimolecular amounts of cobalt, vanadium, nickel, zinc, iron, chromium, magnesium and manganese (as before) were added. Cobalt, vanadium and nickel accelerate significantly the activity, but copper is about 2.5 times as active as the other three. Presence or absence of zinc, iron, chromium, magnesium and manganese is indifferent. Cobalt in Vitamin B_{12} is inactive or at least is not more active than inorganic cobalt; Cytochrome C is slightly inhibitory (experiments not reported).

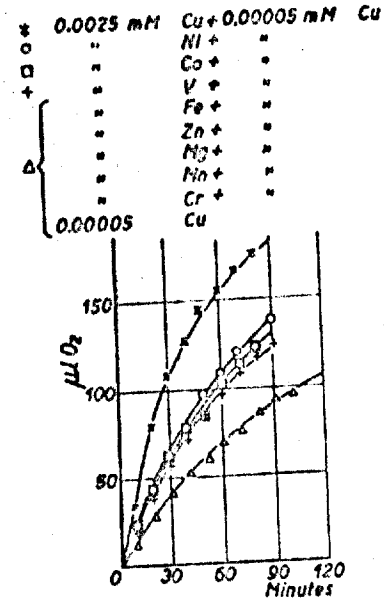

Fig. 4. The effect of equimolecular amounts of copper, nickel, cobalt, vanadium, iron, zinc, magnesium, manganese and chromium on the enzymic oxidation of 0.9 mg tyrosine + 0.05 mg Dopa. All flasks contain the same amount of apo-enzyme as in Fig. 1.

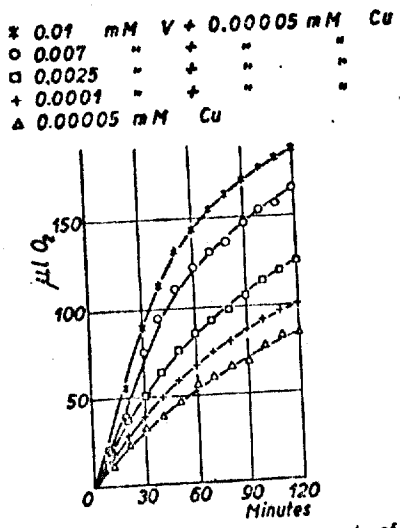

Fig. 5. The effect of varying amounts of vanadium on the enzymic oxidation of 0.9 mg tyrosine + 0.05 mg Dopa. All flasks contain the same amount of apo-enzyme as in Fig. 1.

Fig. 5 shows that an augmentation of vanadium concentration determines a corresponding augmentation of activity on tyrosine.

Demonstration that copper accelerating tyrosine oxidation is free and non protein-bound. In the lowest of the curves reported in the Fig. 2 and 3 it was shown that the apo-enzyme has the same activity on tyrosine in presence of 0.00005 millimols, 0.0001 and 0.0002 millimols of copper and that the activity begins to increase only when this amount is surpassed. It is highly improbable that in all the following trials, especially with the highest concentration used, copper should remain always bound to protein. However, it can be easily demonstrated, by two different methods, that even the lowest amount of copper, which gives a measurable increase of the activity on tyrosine, is already in excess and is not protein-bound.

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One of the demonstrations is based on the fact, found by KUBOWITZ[19], that copper bound to polyphenoloxidase protein does not dialyse (if not against HCN). In Fig. 6 it is shown how dialysis against Pyrex-redistilled water influences the activity of the enzyme to which 0.0008 millimols copper were added. Dialysis does not modify the activity on Dopa, where only the protein-bound specific copper is acting, but reduces the activity on tyrosine at the same level as it would have without excess of copper.

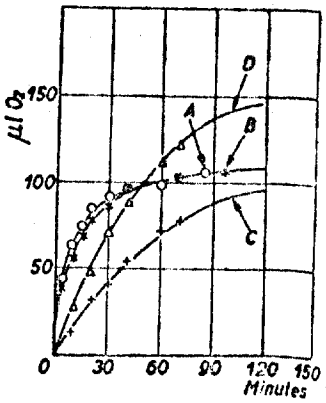

Fig. 6. The effect of dialysis on the enzymic oxidation of 0.5 mg Dopa (Curves *A* and *B*) and on the enzymic oxidation of 0.9 mg tyrosine + 0.05 mg Dopa (Curves *C* and *D*). After having added 0.0008 millimols of copper, *A* and *C* are dialysed (see text), *B* and *D* not. All flasks contain the same amount of apo-enzyme as in Fig. 1.

Another, yet more categoric proof can be presented by virtue of the complex interrelations existing between ascorbic acid, catechol, copper and the apo-enzyme. KUBOWITZ[10] and KEILIN AND MANN[8] have discovered that copper bound to catalytically active positions of the polyphenoloxidase protein becomes strictly specific for *o*-dihydric phenols and it loses its original capacity to oxidize directly ascorbic acid[18]; it can do so only in the presence of a catalytic amount of catechol, by virtue of the change *o*-dihydric phenol ⇌ *o*-quinone. It was recently shown[16] that copper bound to proteins of the enzyme solution is incapable of the direct catalysis of ascorbic acid oxidation also when it occupies positions which are catalytically inactive, and that copper has a certain preference for the catalytically active positions of the apo-enzyme molecule. If copper is not in excess and it is all protein-bound, in presence of traces of catechol, an augmentation of the amount of apo-enzyme determines a proportional augmentation of the ascorbic acid oxidation, which follows exclusively the indirect mechanism of KUBOWITZ[19]. If, however, copper is in excess over the protein, then indirect and direct oxidation proceeds contemporaneously, and an augmentation of the amount of apo-enzyme not only does not determine an augmentation, but actually reduces the rate of ascorbic acid oxidation, because it reduces the quantity of free copper ions present[16]. In the experiment reported in Fig. 7, in presence of 0.0001 millimols of copper and of 0.0001 millimols of catechol a fourfold augmentation of the amount of apo-enzyme determines a proportional augmentation of the oxidation; on the contrary, in presence of 0.0004 millimols

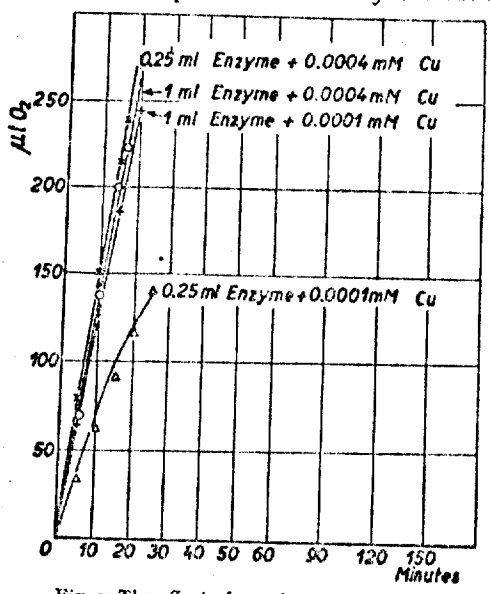

Fig. 7. The effect of varying apo-enzyme concentrations in presence of an excess (0.0004 millimols) and in presence of an amount not in excess (0.0001 millimols) of copper on the enzymic oxidation of 3.51 mg ascorbic acid + 0.01 mg catechol. 1 ml enzyme = 60 enzyme units.

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of copper and 0.0001 millimols of catechol, the originally high rate of ascorbic acid oxidation is in fact reduced by a fourfold augmentation of the apo-enzyme concentration.

DISCUSSION

ONSLOW AND ROBINSON[6] suggested that *o*-quinones react directly with monohydric phenols because they found that by repeated adsorptions and elutions they could reduce the activity of the potato-enzyme on tyrosine but not on catechol, and that the lost activity on tyrosine could be restored by adding traces of catechol. We arrived at the same conclusion[8] because we found that the enzymic oxidation of tyrosine, after the induction period, proceeds at a constant linear rate which is independent of the quantity of the *o*-dihydric phenol added to shorten the induction period, and that in the absence of enzyme *o*-benzoquinone can oxidise tyrosine to an *o*-dihydric phenol. The constant rate of the monohydric phenol oxidation was also found by BEHM AND NELSON[21]; and LERNER *et al.*[17] in the first of a series of valuable papers have not only rediscovered the same particularities of the monohydroxyphenol oxidation, but have also shown that the induction period is proportional to the negative logarithm of the amount of *o*-dihydroxyphenol added, as it was predicted theoretically[22].

In criticising the direct *o*-quinone theory, NELSON AND DAWSON[5] state: "Undoubtedly, the strongest argument against the view that products of catechol oxidation are alone responsible for the initial oxidation of monohydric phenols is the fact that the ratio of catecholase activity to monophenol action of the enzyme can be widely varied during the preparation of tyrosinase". As PUGH[7] rejected ONSLOW AND ROBINSON's views principally because she could *not* vary the relative activity of the enzyme on catechol and on *p*-cresol, it is disconcerting to see the same argument used as the strongest one in an exactly contrary sense. Whatever is the value of this argument, the fact remains that the relative activity of the enzyme could be changed previously only in the sense of a loss of the monophenolase activity; a change more reasonable to explain on the basis of the *o*-quinone theory[8,10] than admitting a specific monophenolase activity of the enzyme itself, which leads one necessarily to attribute quite new and unprecedented properties to the tyrosinase molecule[11].

However, a real difficulty which indeed can not be explained by the simple *o*-quinone theory is represented by the different behaviour of enzymes of animal and vegetal origin. *Sepia*[9] and melanoma[17] enzymes oxidise Dopa very rapidly and tyrosine, if less rapidly, still at a considerable speed, whilst enzymes of vegetal origin[8,19] oxidise catechol and Dopa very rapidly, but their action on tyrosine is relatively moderate. So animal enzymes appear to function more as a tyrosinase and vegetal enzymes more as a catecholase or polyphenoloxidase; in the terminology of NELSON AND DAWSON[5] the first present a low catecholase/cresolase ratio, the second a high one. This different specificity of animal enzymes makes it necessary to postulate the presence of an additional factor or factors, which can accelerate the action of vegetal enzymes on monohydric phenols, especially on tyrosine.

It is shown in this paper that at least four metals, copper, cobalt, vanadium and nickel can accelerate the action of the potato enzyme on a monohydric phenol and can raise it to a higher level than it was in the original extract. Iron, zinc, magnesium, manganese and chromium were tried and found completely inactive, but an eventual

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activity of some other metal, owing to the little specificity of the reaction, cannot be excluded *a priori*.

The action of the four active metals is completely different in Dopa and in tyrosine oxidation. Copper is highly specific in Dopa, as it is in *o*-dihydric phenol oxidation in general[8,19] and cannot be replaced by any other metal as the prosthetic group of the enzyme; if the enzyme is once saturated by the relatively small amount of copper required, it is fully active and this activity cannot be augmented by an excess of the same or of any other metal. As the active copper is completely protein-bound and all other metal is inactive, dialysis against Pyrex-redistilled water does not have any influence on the activity on Dopa (Fig. 6).

Presence of copper in the small quantity sufficient to saturate the active positions of the enzyme molecule is also indispensable in tyrosine oxidation, but with this amount of copper alone the activity is only moderate. In agreement with the fact discovered by KUBOWITZ[19] that polyphenoloxidase protein can bind more copper than it uses as prosthetic group and that the surplus copper is fixed on catalytically inactive positions ("falsche Stellungen") of the molecule, the activity on tyrosine remains moderate until the inactive places also of the enzyme molecule and of the impurities (which of course have only inactive places) are occupied (Fig. 2 and 3). The activity begins to increase only when this amount of copper is once surpassed and where there are free and non-protein-bound copper ions in solution. As excess copper dialyses easily, activity on tyrosine is reduced to the minimum level by dialysis against Pyrex-redistilled water (Fig. 6); moreover, it catalyses directly ascorbic acid oxidation (Fig. 7), which can not be done by actively- or inactively-bound copper[16]. A definite relationship exists between copper concentration and rate of tyrosine oxidation; if the time required for the oxidation of 50% tyrosine is plotted against the logarithm of the amount of copper added, a linear curve is obtained which presents a sharp break at the value where there are no free ions in solution, that is where all copper is bound. Below this value, as stated before, the time of half oxidation remains constant (Fig. 8).

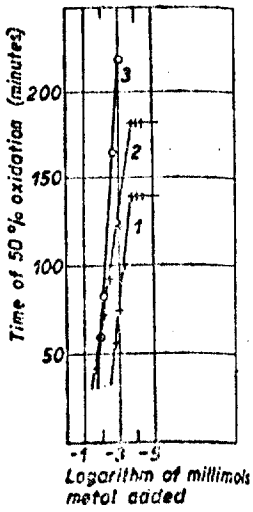

Fig. 8. Relationship between amount of metall added and enzymic tyrosine oxidation. Curve 1: Copper in phosphate buffer (from the experiment reported in Fig. 2). Curve 2: Copper in citrate-phosphate buffer (from the experiment reported in Fig. 3). Curve 3: Vanadium (from the experiment reported in Fig. 5).

The specificity of excess copper in tyrosine oxidation is limited to the fact that it is more active than cobalt, vanadium or nickel (Fig. 4). That the same quantitative relationship holds also for the other active metals as for copper is shown by curve 3 of Fig. 8, where half oxidation time is plotted against the logarithm of vanadium concentration.

The point and mechanism of intervention of metals results clearly from a comparison of their action on Dopa and on tyrosine. This comparison cannot be made on catechol and *p*-cresol; the difficulties of using catechol as substrate are well known[5,18,16] and *p*-cresol, besides being highly unphysiological, is too autoxidable to serve as a useful substrate to distinguish between monohydric phenol and *o*-dihydric phenol oxidation. Although the ulterior products of Dopa and of tyrosine oxidation (exactly as those of catechol and of *p*-cresol oxidation[23,24,25]) are unknown, it was shown by RÜPER's fun-

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damental researches that the first product of the tyrosine oxidation is Dopa and the second is its *o*-quinone[1,26,27] and there can be no doubt that in the two over-all reactions:

(I) Tyrosine → Dopa → *o*-quinone → ulterior products → melanin
(II) Dopa → *o*-quinone → ulterior products → melanin

all the ulterior products, the nature and importance of which is yet under discussion[15,28], are identical. As the over-all tyrosine oxidation (I) is much slower than over-all Dopa oxidation (II), the reaction tyrosine → Dopa is rate-determining in tyrosine oxidation and, the two reactions being in all other respects identical, all agents which accelerate tyrosine but not Dopa oxidation necessarily intervene in the reaction tyrosine → Dopa.

The so-called tyrosinase appears as a very complicated system, composed of: 1. an enzyme with copper as prosthetic group, specific for *o*-dihydric phenols; 2. an *o*-dihydric phenol; 3. certain free metallic ions. The complexity of this system is increased by the interdependence of its components inasmuch as the quantity of *o*-dihydric phenol (or respectively of *o*-quinone) present depends on the amount of the enzyme[18] and on both the quality and quantity of the metallic ions present. Yet more complications arise from the fact that only free metallic ions increase the originally low rate of tyrosine oxidation and in consequence all constituents which can bind metal ions, especially proteins inert on *o*-dihydric phenol oxidation, will influence the activity on monohydric phenols, as will the metallic ions always present in common laboratory distilled water. In this context it is useful to point out the decisive importance of the use of Pyrex-redistilled water: if the enzyme were prepared in common distilled water and dialysed afterwards against the same, its activity on tyrosine would not change and it would appear to belong to the protein molecule. The error introduced by the use of common distilled water is greater in the case of pure or of dilute enzyme solutions[16]; moreover it is constant in a given Laboratory and so escapes detection.

Although names are not of very great importance, the name "tyrosinase" is probably the most inappropriate among the numerous designations used for the enzyme studied in this paper. Admitting the direct action of this enzyme on monohydric phenols and calling it tyrosinase, we already possessed a most particular enzyme, which

1. if dilute, could act on other substances but not on its own substrate[29,9];
2. had a much greater action on a number of other substances than on tyrosine[5,8,10];
3. could oxidise other substances immediately, but tyrosine only after an induction period[1-5];
4. could act at distance, without immediate contact with its substrate[13,14];
5. ought to possess quite unique properties for an enzyme molecule[11].

The results reported in this paper are in agreement with the views that the oxidation of tyrosine is a non-enzymatic reaction between *o*-quinone and tyrosine and render the name tyrosinase still more inappropriate: this enzyme corresponds to the system polyphenoloxidase + *o*-dihydric phenol (or *o*-quinone) + metallic ions, which latter function probably as electron transmitters and hasten the non-enzymic reaction between tyrosine and *o*-quinone.

The author cannot see any justification for the conservation of the term "tyrosinase" except to keep the name under which the discovery and the fundamental researches of B. BERTRAND and of H. S. RAPER were made.

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SUMMARY

A study was made to explain the different behaviour of animal and of vegetal polyphenol-oxidases on monohydric- and dihydric-phenols.

1. It was found that the first phase of the tyrosine oxidation (the transformation of tyrosine in Dopa) is considerably accelerated by excess of copper, by cobalt, by vanadium and by nickel, whilst iron, zinc, chromium, magnesium and manganese are inactive.

2. Excess of copper, cobalt, vanadium and nickel have no action on the second, enzymic phase of tyrosine oxidation (the transformation of Dopa in its *o*-quinone) where only copper bound to polyphenoloxidase protein is active.

3. It was demonstrated, by two independent methods, that the acceleration of tyrosine oxidation is determined by free and non-protein-bound metal ions.

4. and that there is a quantitative relationship between metal concentration and velocity of tyrosine oxidation.

5. Tyrosinase is identified with a complex system composed of an enzyme specific for *o*-dihydric phenols, of an *o*-dihydric phenol (or *o*-quinone) and of free metallic ions. The role of the latter is to accelerate the spontaneous reaction between *o*-quinone and tyrosine.

6. The appropriateness of the name "tyrosinase" is discussed.

RÉSUMÉ

La présente étude a été entreprise afin d'essayer d'expliquer le comportement différent des polyphénoloxydases animales et végétales vis-à-vis des phénols mono- et divalents.

1. Nous avons trouvé que la première phase de l'oxydation de la tyrosine (transformation de la tyrosine en dopa) est accélérée considérablement par un excès de cuivre, par le cobalt, le vanadium et le nickel, tandis que le fer, le zinc, le chrome, le magnésium et le manganèse sont inactifs.

2. Un excès de cuivre, de cobalt, de vanadium ou de nickel n'exerce aucune action sur la seconde phase enzymatique de l'oxydation de la tyrosine (transformation de dopa en son *o*-quinone) où seul le cuivre lié à une protéine polyphenoloxydasique est actif.

3. Nous avons démontré par deux méthodes indépendantes que l'accélération de l'oxydation de la tyrosine est déterminée par des ions metalliques libres et non liés à une protéine et

4. qu'il existe une relation quantitative entre la concentration du métal et la vitesse de l'oxydation de la tyrosine.

5. La tyrosinase a été identifiée comme étant un système complexe composé d'un enzyme spécifique pour les phénols contenant 2-OH en position *o*-, d'un tel phénol divalent (ou d'une *o*-quinone) et d'ions métalliques libres. Le rôle de ces derniers consiste à accélérer la réaction spontanée entre l'*o*-quinone et la tyrosine.

6. La justesse du nom "tyrosinase" est discutée.

ZUSAMMENFASSUNG

Es wurde ein Versuch gemacht, um das verschiedene Verhalten von tierischen und pflanzlichen Polyphenol-Oxydasen gegen einwertige und zweiwertige Phenole zu erklären.

1. Es wurde festgestellt, dass die erste Phase der Oxydation von Tyrosin (die Verwandlung von Tyrosin in Dopa) durch einen Überschuss von Kupfer, durch Kobalt, durch Vanadium und durch Nickel sehr beschleunigt wird, während Eisen, Zink, Chrom, Magnesium und Mangan inaktiv sind.

2. Ein Überschuss von Kupfer, Kobalt, Vanadium und Nickel übt keine Wirkung auf die zweite, enzymatische Phase der Tyrosinoxydation (Verwandlung von Dopa in sein *o*-Chinon) aus; hier ist nur das an Polyphenol-Oxydase-Protein gebundenes Kupfer wirksam.

3. Es wurde mit Hilfe zweier unabhängiger Methoden bewiesen, dass die Beschleunigung der Tyrosin-Oxydation durch freie und nicht proteingebundene Metallionen bewirkt wird, und

4. dass zwischen der Metallkonzentration und der Geschwindigkeit der Tyrosin-Oxydation ein quantitatives Verhältnis besteht.

5. Tyrosinase wurde als ein komplexes System, bestehend aus einem für Phenole mit 2 OH-Gruppen in *o*-Stellung spezifischen Enzym, aus einem solchen zweiwertigen Phenol (oder *o*-Chinon) und aus freien Metallionen, identifiziert. Die Rolle der letzteren besteht darin, dass sie die spontane Reaktion zwischen *o*-Chinon und Tyrosin beschleunigen.

6. Die Angemesseuheit des Namens "Tyrosinase" wird diskutiert.

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Received November 12th, 1951