A CHEMICAL AND PHYSIOLOGICAL STUDY OF COBALT METABOLISM IN A MONOGASTRIC, HERBIVOROUS, PSEUDO-RUMINATING ANIMAL

by

ELLIOTT LEE HIX

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INTRODUCTION

Many chemical elements are recognized today to be essential to both plants and animals for the continuance of their normal physiological functions. These chemical elements are required in various quantities by the animal body and those elements required in very small amounts are known as "trace elements". Many of these trace elements occur in such minute quantities in both plant and animal tissues that until relatively recent it was not possible to quantitatively analyze for them by the methods of routine chemical analysis. However, today, improved analytical methods, instruments and techniques have been developed so that accurate determination of the trace elements is possible.

The trace elements, in which category cobalt is classified, are also referred to as "minor elements", "rare elements", and "micro-nutrients". These terms, however, are somewhat ambiguous considering, in the absolute sense, the true intention of the term "trace element". The chemist classifies the rare elements as those elements occurring in nature in minute amounts without regard to their influence on, or function in, the animal organism. Micro-nutrients, quite to the contrary, designates those elements used by the plant or animal organism in minute quantities and serving in the capacity of a nutrient.

An arbitrary classification of rare and minor elements may logically include, to mention a few, iron, copper, cobalt, zinc, boron, fluorine, iodine, barium, and strontium. Of these men-

tioned, iron, copper, cobalt, iodine, and fluorine are considered essential for optimum nutrition in ruminating animals, and many vital physiological functions have been attributed to these trace elements. Zinc, boron, barium, and strontium have not been shown to be essential nor to possess physiological activity. Experimental observations, however, have indicated that these elements may possibly play an important role in the physiology of our domestic animals.

Spectrographic analysis has demonstrated the presence of copper, cobalt, nickel, manganese, lithium, lead, silver, cadnium, rubidium, and fluorine in animal tissues. Practically every element from uranium to calcium has been demonstrated qualitatively in animal tissues. However, the presence of these elements does not indicate their necessity in the physiological phenomena peculiar to the animal specie, but they are most likely present in the animal incidental to their presence in the foods consumed by the animal.

While broken bones, depraved appetite, and other gross physical symptoms first called our attention to the needs of animals for minerals, bone and blood studies and chemical and physical balance techniques have been the means by which quantitative needs for all-round optimum nutrition have been established.

Recent discoveries resulting from the vast researches regarding the trace elements should teach many things. In the first place, one cannot rest on the assumption that, aside from salt, calcium, phosphorus, and iodine in special cases, commonly

fed rations will always meet the mineral needs of livestock. Numerous cases have revealed that this is not true, and it is commonly accepted that the same feed may vary widely in mineral content. More important perhaps, the recently discovered cobalt deficient areas in the United States were uncovered where the cobalt shortage was severe enough to develop acute physical symptoms which demanded attention. Undoubtedly there are many more widespread cases of deficiencies too mild to manifest symptoms and thus be detected by the gross observations of the veterinarian. The vitamin chemists and physiologists have shown conclusively that the subacute deficiencies recognizable only by chemical and physiological tests are the more numerous and the more important (1). Undoubtedly the same principle applies not only to such trace elements as cobalt, but to all the inorganic elements in general.

With the recent emphasis on vitamin research, the physiology of the trace elements, particularly the micro-nutrients, has been, by comparison, neglected. Many investigators feel that they need the same detailed attention (2). It may be that the animal nutritionist will come back to the present viewpoint that the trace elements are generally unimportant in practice because of the contention that they are always supplied in the usual rations.

Since the recognition of the first cobalt deficiency in ruminating animals in 1937 by Neal and Ahmann of the Florida Experiment Station (3), cobalt has been under continuous research from the standpoint of its physiological function, the nutritional

requirements of the ruminant and the toxic levels in practical salt licks commonly used in Florida. An interesting survey by Briggs (4) reveals that mineral studies, of which trace elements were more important, were the major ones under investigation in 1940 by all the departments of animal husbandry in the United States.

It has been suggested by several investigators (5, 6) that cobalt, which is known to be required by ruminating but not by nonruminating animals, functions primarily through some unknown mechanism in the rumen, probably related to the rumen microflora. This theory enjoys some but not conclusive experimental support. The discovery by Rickes et al. (7), simultaneously with that of Smith (8), that vitamin B_{12} is a cobalt complex pointed to the possibility that this vitamin is an intermediary in the metabolism of cobalt in the animal species requiring the element.

It appears that the end of the already long list of B-complex vitamins is not yet in sight. Until recently the list appeared to be approaching completion. The vitamin B_{12} molecule is of particular interest to chemists and physiologists because it contains 4.5 per cent cobalt. This trace element has long been recognized as a stimulator of erythropoiesis, but this is the first time that a trace element has been found as an integral part of a vitamin. The biochemists, physiologists, and nutritionists are at present in the midst of a most interesting phase of research in this field.

With this new line of attack, a dearth of new and valuable

knowledge has been published in the last year regarding cobalt metabolism in general (5, 6, 9, 10) and the physiological relationship between cobalt and vitamin B_{12} (11-14). Such a fundamental discovery as the need of cobalt by the ruminating animal has had wide application, not only in economical animal production in areas deficient in cobalt, but in both animal and human physiology, biochemistry, and veterinary and human medicine.

The role of nutrition in animal health, and conversely in disease and impaired reproduction, is an important one. The successful veterinarian is today acquiring information on animal nutrition to complete his knowledge of prevention and treatment of animal disease by means of improved nutrition. Vitamins and the micro-nutrients are exceedingly important.

In the light of the discoveries already made, the physiologist is led to wonder to what extent conditions in animals referred to under the general term, "unthriftiness", are due to an undiscovered need of a specific nutrient for a specific physiological function. To understand this unthriftiness in cobalt deficient animals, the physiological function (s) of cobalt must be known. Aside from the function of cobalt in the animal body, the animal organs, tissues, and processes in general associated with cobalt metabolism must be known before a specific physiological function can be attributed to cobalt.

The present investigation was designed to study the metabolism of cobalt in a common laboratory animal closely related to the ruminating animal in its physiology. The nature of such an

investigation required the techniques of the physiologist and biochemist. Realizing that little could be gained in such an investigation without first producing a state of cobalt deficiency in the experimental animals, an experimental period of five months was felt sufficient to induce a cobalt deficiency. The chemical analysis of cobalt in feeds, animal organs, and blood and feces required especial consideration and is given somewhat more emphasis than merely a technique of experimentation. The chemical analysis of cobalt is indeed worthy of thorough investigation in itself.

REVIEW OF LITERATURE

Cobalt

Historical. The first incidence of a recognized trace element deficiency occurring in livestock under natural conditions in the United States appears to have been a copper deficiency in Florida, reported in 1921 by Neal, Becker, and Shealy (15). Previous to this time economical production of livestock was generally not possible even though Florida abounded in abundant and apparently healthy grasslands. Early investigations revealed deficiencies of iron and copper and other minerals in the soil, and salt licks were heavily fortified with these minerals. Even then, however, there were evidently other deficiencies existing as substantiated by the general overall emaciation and stunted growth in grazing animals.

Since about 1907 in certain areas of South Land, New Zealand, sheep production had suffered severely from a disease known as "Morton Mains" disease. In midsummer the sheep would lose weight, the wool became coarse and 30 per cent or more would die. The remainder would make a poor recovery. Serious efforts in the control of the disease were started in 1932, and the malady was attributed to a cobalt deficiency (16). Investigations from 1935 through 1937 by Denham (17) conclusively established the disease in New Zealand as a cobalt deficiency, and complete recovery of affected animals could be obtained by providing cobalt-containing salt licks.

In 1937, Neal and Ahmann of the Florida Experiment Station (3), encountered a condition of malnutrition in controlled feeding trials with calves on a ration consisting of locally grown feeds. Having previously eliminated every possibility of a trace element deficiency, with the exception of cobalt, spectrographic analysis of the feeds revealed the total absence of cobalt. The feeding of a cobalt salt resulted in immediate and complete recovery. By 1939 a cobalt deficiency was conclusively established in Florida, and it was shown to be widespread over the state (18).

For over a hundred years a nutritional anemia in sheep had prevailed in New Hampshire, the cause of which was unknown until 1944 when Keener et al. (19) observed a cobalt deficiency in cattle.

Since 1937, cobalt deficiency diseases, characterized by anemia, have been acknowledged and reported in Florida (3), North

Carolina (20), New Hampshire (19), Michigan (21), Wisconsin (22), Canada (23), New Zealand (16), Australia (24), England (25), Scotland (26), Germany (27), and Tasmania (28).

The cobalt deficiency disease in Florida is known as "salt sick", "burton-ail" in New Hampshire, "bushsickness" in New Zealand, "enzootic marsumas" in Australia, "pining" in England and Scotland, and "coastiness" in Tasmania.

Etiology. In general the cobalt deficient areas in the United States are located along the Atlantic coast line and Great Lakes region. Geologically, the deficient areas in the South and those in Australia are similar in soil type, consisting principally of sands and soils badly leached. Even though soil type appears to be a contributing factor, Grimmett (29) found that certain soil types, on which cobalt deficiencies were observed, contained 10 ppm of total cobalt, and Askew (30) found that the pH of the soil played a major role in the relative solubility and, consequently, relative availability of cobalt to plants. Plants would not be able to assimilate the cobalt in its insoluble and unavailable form and it is likely that cobalt deficiencies could occur in livestock where the soil contains normal concentrations of cobalt. Gallup et al. (31) have shown that grazing animals consume considerable amounts of soil in comparison to dry-lot fed animals. This perhaps explains why many cobalt deficiencies in sheep and cattle were not manifested until isolated to rigidly controlled feeding experiments.

Cobalt deficient pastures, according to Askew and Maunsell

(32) and McNaught (33), contained 0.01 to 0.07 ppm of cobalt whereas "healthy" pastures generally afforded 0.07 to 0.30 ppm. Underwood and Harvey (34) found deficient soils to average 0.04 ppm while normal soils averaged 0.13 ppm. The analyses of Baltzer (21) and Becker et al. (35) are in substantial agreement with the aforementioned.

The clinical symptoms of cobalt deficiency in cattle and sheep are similar to those of general malnutrition. Many of these symptoms, however, are also manifested in cases of copper deficiencies (15) and manganese and iron deficiencies (36), therefore, only a response to cobalt feeding in the case of the affected animal is clear evidence that a lack of cobalt is the cause of the symptoms observed. Many cobalt deficiencies may overlap a nutritional anemia area that responds to copper and iron supplement (3).

Fertilizing deficient soils with cobalt chloride at a rate of two pounds per acre definitely increased the cobalt content of the herbage grown on these soils to such an extent that cobalt deficient sheep recovered rapidly (37). Nelson and Rigg (38) found an application of one pound of cobalt sulfate per acre was markedly evident two years after treatment. Four ounces per acre was effective for almost two seasons in maintaining sheep in good condition, as reported by Anon (39).

From these investigations it is quite evident that the requirements of ruminants for cobalt are indeed minute although the actual minimum requirements are not known. It is generally re-

garded, however, that sheep require 0.1 mg of cobalt daily while cattle require 1.0 mg (40). Such cobalt levels cannot be considered the requirements, but more aptly are sufficient to prevent cobalt deficiencies. The practical aspects of the cobalt problem have been solved, however, very little is known at present with regard to the action of cobalt in the animal body.

Dr. McCliermont, of Australia, recently related to the author that cobalt deficiencies in sheep in southern Australia are continuously being uncovered. Administration of cobalt salts frequently fails to restore the animals to health indicating perhaps the existence of a conditioned deficiency. The Australian workers at present are unable to ascertain the physiological action of cobalt. This phase of the problem is at present under critical investigation.

Through private communication, G. K. Davis of the Florida station, C. F. Huffman of the Michigan station and P. H. Phillips of the Wisconsin station, have related that cobalt deficiencies in those areas are not evident today due to fertilization practices. Cobalt-containing salt licks are used extensively by animal husbandmen in those areas and livestock production is economically sound as in other progressive districts.

Physiological Functions. Even though many investigations have been designed to study the physiological action of cobalt in the ruminant and simple gastric animals, little has been gained in a direct manner. The problems which handicap such physiological studies, such as the apparent differences in function in the

simple and polygastric animals, the minute quantities of cobalt that must be dealt with, the inadequacy of chemical analysis and many more, tends to broaden one's perspective of the difficulties that must be overcome in even the most elementary studies.

In cobalt deficient areas it has been shown that copper quite frequently is also somewhat deficient in the soil and upon the administration of cobalt recovery was not always the rule (3). Copper administration alone to cobalt deficient animals only aggravated the condition, indicating that perhaps cobalt and copper function in conjunction with one another in hematopoietic respects. Provided such is the case, iron and manganese must also be involved (36, 41). The physiological action of cobalt in producing hematological responses in simple gastric animals appears to be the result of a combination of metals and not cobalt alone (42).

Dorrance (43) found that cobalt in small doses induced erythropoietic activity in the liver and spleen and increased erythropoiesis in the bone marrow of rats. Significant increases in hemoglobin values also were observed. Frost et al. (44) observed the same effects in dogs, and the investigation of Kato and Iob (45) indicates that cobalt aids in the utilization of iron in promoting hamatopoiesis.

Marston (24) found that the livers of sheep suffering from a cobalt deficiency contained levels of iron above the normal and a copper content appreciably below the normal, but the blood copper level was unchanged. This is in good agreement with Kato

and Iob's findings, and it appears well grounded that cobalt or some cobalt complex aids in the utilization of iron for hemoglobin formation. In the absence of cobalt, iron is stored in the liver in an inactive form. Such a contention is supported by the findings of Baltzer (21).

The chemical analyses of Bowstead et al. (46), Askew and Watson (47), Grimmett (29), and Underwood and Harvey (34) reveal that the blood cobalt remains quite constant while the liver cobalt content remains only fairly constant in sheep under conditions of cobalt feeding. In cobalt deficient animals the blood cobalt level does not vary at all comparable to the liver content, indicating that the liver cobalt content determines largely the degree of unthriftiness and that the blood change is probably secondary.

Ray et al. (5) have found that orally administered cobalt salts at a rate of 1 mg daily to cobalt deficient sheep brought about a rapid increase in appetite and hemoglobin values. Injection of cobalt salts gave a much slower response. Irrespective of the method of cobalt administration, the most common response was an increase in the hemoglobin content of the blood. Injected cobalt was unable to produce a significant response in hemoglobin regeneration. In view of the effect of cobalt on the production of polycythemia in simple gastric, nonruminating animals, as demonstrated by Kato and Iob (45) and others (43, 44, 48), the inability of injected cobalt salts to bring about a noticeable response in hemoglobin regeneration in the ruminant is rather

strange.

Following up the apparent beneficial effect of cobalt on appetite, Gall et al. (49) observed a 45 per cent reduction in rumen microflora count in cobalt deficient sheep. Injected cobalt gave no response in rumen microflora count, while oral administration of cobalt produced excellent results evidenced by an increase in number and vigor of the microorganisms in the rumen contents. Digestion trials with cobalt deficient lambs indicate that the cellulose-splitting microorganisms of the rumen are not affected (10). Hale et al. (11), using chickens as assay animals, assayed the rumen contents of both cobalt deficient and cobalt supplemented sheep and found invariably that some growth factor (s) was not present in the rumen contents of cobalt deficient sheep. In some instances the cobalt deficient rumen contents gave less growth than the basal ration, and the addition of cobalt sulfate without rumen contents was ineffective in correcting the retarded growth in the assay animals. The investigations of Ray et al. (12) and Ray et al. (5) suggest that in cobalt deficiency the production of B-vitamins in the rumen of the deficient animal is disturbed and the symptoms of the deficiency may be due to avitaminosis B-complex.

Ruminating animals normally do not manifest symptoms of vitamin B-complex deficiencies due to the biosynthesis of these vitamins by the rumen microflora (50-52). Since vitamin B_{12} has been shown to contain cobalt, the possibility exists that a cobalt deficiency in sheep and cattle may be due to a lack of vitamin B_{12}

synthesis in the rumen. Experimental evidence indicates that cobalt is apparently not involved in vitamin B_{12} synthesis in simple gastric animals as cobalt acts directly (53, 54). However, the simple gastric animals apparently require vitamin B_{12} which is normally supplied in their diet (13, 14, 55, 56).

These investigations indicate that the simple gastric, nonruminating animals, such as the rat, rabbit, dog, and pig respond favorably to vitamin B_{12} and polycythemia is marked by the administration of small quantities of cobalt. It appears that these animals require vitamin B_{12} and that biosynthesis of this vitamin by the microflora of the gut is not the case. Good (57) found this to be true in young rabbits. Evidence indicates that the dog might require elemental cobalt for normal hematopoiesis, but the experimental evidence is not conclusive (54).

That cobalt promotes bacterial activity in the rumen of ruminants, and that a reduction in the number of active rumen microorganisms is associated with cobalt deficiency, appears to be well established. Consequently, cobalt promotes vitamin synthesis, and Rickes et al. (58) have isolated a microorganism capable of synthesizing vitamin B_{12} which provides more evidence that the physiological function of cobalt in ruminants is perhaps indirect.

Requirements. Two outstanding features of cobalt seem to lie in its secondary mode of action in the ruminant, as it appears, and the very small amounts required. The analyses of Becker et al. (35) show normal soils to contain an average of 0.2 ppm of

cobalt. Since the relationship of soil to plant is generally regarded to be direct with respect to cobalt content, a grazing animal would theoretically have a daily intake of about 1 mg of elemental cobalt.

The requirements of animals for cobalt are indeed small, and it is believed that only ruminating animals require cobalt. Efforts to induce a cobalt deficiency in nonruminants have failed indicating that the requirements of simple gastric animals for cobalt are unusually minute (59). However, with ruminating animals, even though the requirements are small, these requirements must be met.

Comar et al. (60), using radioactive cobalt in metabolism studies with rats and cattle, found that in the rat, only 0.25 per cent of the administered cobalt was retained. Only the liver showed a small accumulation of cobalt and this was not retained.

The quantitative requirements for cobalt in the case of sheep and cattle can be approximated on the basis of analytical determinations of cobalt contained in the herbage in both "healthy" and "sick" areas. On this basis it appears that 0.1 mg of cobalt for sheep and 2.0 mg for mature cattle daily is sufficient. Normally, cattle grazing on "healthy" pasture may be considered to have a daily intake of approximately 5 mg of elemental cobalt and sheep about 1.5 mg daily.

A salt lick containing 1 ounce of cobalt sulfate per 100 pounds of salt is considered adequate, and in areas where sheep and cattle suffer from a cobalt deficiency, such a salt lick is

commonly used (18, 22). A cobalt supplement is not indicated in areas free from cobalt deficiencies in the vegetation.

Chemical Analysis of Cobalt

In studies of the role of cobalt in animal physiology it is important to be able to determine small amounts of cobalt in the animal tissues. This is particularly true when laboratory animals such as the rabbit or guinea pig are used, since not only is the concentration of cobalt low, but the amount of tissue available for analysis is small.

Cobalt is, in occurrence, associated with iron, copper, sulfur, and nickel. The usual samples are alloys and minerals, and the majority of the methods of analysis are concerned with such samples. Biological occurrence, however, is important today, and many methods of analysis have been designed in the past decade to deal with minute concentrations of cobalt. A blue color with thiocynate and colloidal dispersions with nitrosonaphthols are the classical methods of determination (61). Many of the more recent analytical methods have sacrificed accuracy and precision of analysis in gaining greater sensitivity. The minute concentrations of cobalt in biological samples demands sensitivity of analysis, however, accuracy of analysis is equally demanding.

Cobalt may be determined volumetrically by titration with cyanide, potentiometrically, colorimetrically, or spectrophotometrically. These methods, however, with the exception of spectrophotometric analysis, are not applicable to determinations of

cobalt in biological materials. In certain cases cobalt may be determined spectrographically or polarographically. Young (62) has described the analytical procedures in some detail. Recent analytical methods utilizing complex organic reagents are important so that currently there appears to be no one outstanding method.

The first attempt at cobalt analysis in biological materials by colorimetric means appears to be that of Stare and Elvehjem (63), using Van Klooster's nitroso-R-salt reagent. Their method was sensitive to 0.01 mg of cobalt, but they were unable to demonstrate the presence of this element in the bodies of normal rats and pigs. The nitroso-R-salt reagent is, however, much more sensitive than the work of Stare and Elvehjem indicated. A modification of their method by McNaught (64) by further refinement resulted in a procedure sensitive to 0.00005 mg of cobalt. Recoveries of cobalt from liver samples ranged from 90 to 105 per cent. A later modification by McNaught (65) resulted in no significant increase in accuracy or sensitivity.

Cobalt reacts with nitroso-R-salt (Sodium 1-nitroso-2-hydroxynaphthalene-3,6-disulfonate) to give a soluble red colored complex salt which is stable in hydrochloric or nitric acids. The determination is based on the fact that the colored complexes formed by most of the common elements with nitroso-R-salt are destroyed by these acids. With the exception of the Nitrosocresol reaction proposed by Ellis and Thompson (66), the nitroso-R-salt

reaction furnishes the most sensitive method for the determination of traces of cobalt such as occur in soils, plants and animal organs (67). The cobalt complex is ordinarily formed in hot acetate medium at a pH of 5.5. After the cobalt complex has been thus formed, mineral acid is added to decompose the complexes of most of the other heavy metals. Iron and copper interfere if present in more than small amounts. Both copper and iron are removed in the last stages of the analysis by adding hydrated sodium acetate and boiling briefly. Alternatively, the iron in the ferric state may be precipitated by an excess of ammonium hydroxide, however, coprecipitation of cobalt may occur (68). Copper may be precipitated as the sulfide by passing hydrogen sulfide through the sample solution as used by McNaught (64). The ferric iron may be removed by acidifying the sample solution with hydrochloric acid and extracting ferric chloride with ether (61). Bashir and McCollum (69) elect to disregard copper and iron interference in the analysis of plant materials.

Another means of eliminating copper and iron interference in the cobalt analysis is to extract the cobalt with dithizone, forming a cobalt-dithizonate complex (70-73). Also for this purpose, Bayliss and Pickering (74) have used ammonium thiocynate, and Moeller (75) has demonstrated the use of 8-hydroxyquinoline. Ellis and Thompson (66), using dithizone to extract cobalt at pH 8.5, obtained excellent results with as little as 0.0004 mg.

The use of dithizone in this capacity appears to have resulted from the observations of Fisher who found dithizone

(Diphenylthiocarbazone) to give a series of bright colored complexes with cobalt, copper, zinc, nickel, stannous, and others (61). The relation of the metals when converted to dithizonates is well brought out by the pH-extraction curves of Wichmann (76). Snell and Snell (61) have recently published the most commonly used methods of cobalt analysis in minerals, soils, plants, and biological materials.

In spite of the obvious refinements in the methods of analysis for cobalt, much remains to be desired. Through private communication, C. F. Huffman, research professor of dairying at Michigan State College and G. K. Davis of the University of Florida, both of whom have had considerable experience in the field, have related to the author that they regard the chemical analysis of cobalt a poor criterion of the cobalt content of feed. Spectrographic analysis of cobalt has not been successful in our laboratory, even in solutions containing known but very minute quantities of cobalt. The difficulty has not been ascertained as yet, but probably lies or arises in the activation of the cobalt atom.

MATERIALS AND METHODS

Experimental Design

A single factorial design was used in which a total of 20 rabbits were grouped into two subgroups of 8 and 12 animals each. The group of 8 animals was again subdivided into two groups of 4

animals each. The group of 12 animals, consisting of young inbred rabbits, was used in a parallel study which has recently been reported by Good (57) of this department. The present study considers this group of animals only from the standpoint of determining the adequacy of the experimental diet in promoting growth.

The two groups of 4 animals each were placed on a low cobalt semipurified diet on October 22, 1949 for an experimental period of 5 months. Normal hematological values were established for the lot, and one animal from each group was exsanguinated and the normal cobalt content of liver, blood, and feces determined. Throughout the experimental period, Group 1 (control) received a daily allowance of 0.04 mg of elemental cobalt administered weekly as a component of the basal mineral supplement. Group 2 (experimental) received the basal mineral supplement free of cobalt. The animals had access to the experimental diet and water ad libitum. Each week, throughout the experimental period, body weights were recorded and the blood erythrocyte count, hemoglobin and color index was determined. Animals that died during the experiment were autopsied, the livers and fecal samples taken and analyzed for cobalt. At the termination of the experiment, the 3 remaining animals were euthanized and the livers and feces analyzed for cobalt.

Experimental Animals. As mature and aged animals are considered more apt to react to dietary deficiencies, and with due consideration for the inherent advantages in dealing with a more or less static physiology, mature rabbits of mixed breed and sex

were chosen as experimental animals. Cobalt has been shown to be of importance only in ruminant nutrition, therefore, the rabbit, being herbivorous, was considered a satisfactory substitute for the herbivorous ruminating animals. Of more importance in selecting the rabbit as the experimental animal is the peculiar act of coprophagy or pseudorumination characteristic of this animal specie.

In performing this type of pseudorumination, the animal catches its feces and ingests them. It is believed that the rabbit benefits materially from such action (77). The great length of the small intestine of the rabbit is generally believed to be a provision for dealing with bulky foodstuffs and for enabling it to utilize fiber (78). Thus the nutritional and physiological peculiarities of the rabbit are closely allied to those of the ruminant.

Experimental Diet and Mineral Mixture. The preparation of the experimental diet, low in cobalt, was based on the chemical analyses of cobalt in foods by Grimmett (79), Bashir and McCollum (69) and Hurwitz and Beeson (80). The purified diet has limitations which prevented its use, especially in an experiment using herbivorous animals in which the roughage factor presents a problem. The ingredients of even a purified diet cannot be considered pure and free from mineral matter. In view of this and other considerations, the semipurified experimental diet consisted of the following:

Ground yellow corn	70 pounds
Whole milk powder	15 pounds
Purified casein	10 pounds
Corn starch	5 pounds

This diet consists of purified sources of the various nutrients such as protein supplied by casein, carbohydrate and energy supplied by corn starch, and fat supplied by whole milk powder. Yellow corn was added to give the diet the necessary bulk, to improve palatability, to supply vitamin A and others, and to improve the overall physical balance of the diet. Casein counteracts the deficiency of the amino acids lysine and tryptophan in the corn. The diet contains 19 per cent protein, 6.6 per cent fat, 59 per cent carbohydrate, and 1.7 per cent mineral matter.

The experimental diet was supplemented with two mineral mixtures consisting of C. P. chemical salts. One differed from the other only with regard to cobalt. These mineral mixtures supplied the following daily allowances of the various mineral components:

Iron, as ferric ammonium citrate	2.00 mg
Manganese, as manganous sulfate monohydrate	2.00 mg
Copper, as cuprous sulfate pentahydrate	0.20 mg
Cobalt, as cobaltous sulfate heptahydrate	0.04 mg
Potassium iodide	0.01 mg
Sodium chloride	200.00 mg

The components of the diet were mixed in hundred pound lots and stored in covered containers free from cobalt contamination.

Every precaution was taken to prevent contamination by cobalt during the preparation and handling of the diet. The corn was ground fine in a hammer mill to facilitate complete utilization of the nutrients by the experimental animals.

Blood Analysis. Blood analyses were made weekly. Erythrocyte counts were performed by conventional methods, two counts being made on the same blood sample and the average of the two taken as representative. Blood samples were taken from the marginal ear vein for both the erythrocyte count and hemoglobin determination.

The hemoglobin was determined by the method of Cohen and Smith (81). The technique consisted of taking 0.05 cc of blood in a micropipette, wiping the tip clean, and transferring the blood to exactly 10 cc of 0.1 N hydrochloric acid. After standing for one hour at room temperature, the brown colored "acid hematin" was measured spectrophotometrically in a Coleman Model 14 Spectrophotometer at 520 mu wave length. Calculation of hemoglobin was made as follows:

$$\frac{2-\log G_{520}}{\text{Density std.}} \times 0.075 \times \frac{100}{0.05} \times \frac{10}{100} = \text{gms Hb per 100 cc}$$

In standardizing the spectrophotometer for the hemoglobin analyses, total iron in rabbit blood was determined by the method of Wong (82) as modified by Ponder (83). A spectral transmittance curve determined on the reddish potassium ferricyanide revealed maximum light absorption at 490 mu wavelength. All iron deter-

minations were made at this wavelength. Beer's Law is effective over the entire concentration range, and the iron analysis was reproducible with an accuracy of ± 1.1 per cent.

Method of Chemical Analysis

The chemical analysis of cobalt occurring in normal concentrations in biological materials is exceedingly difficult. Preliminary analyses using the method of McNaught (64) as modified by Sandell (67), in which ferric iron is extracted with ether and copper is precipitated as the sulfide, proved quite unsatisfactory. A method was desired which would be adaptable to systematic analysis, prove accurate in the desirable range with little sacrifice in sensitivity, require a small sample, eliminate long and laborous procedures and adapted to conditions existing in the laboratory. In order to achieve this end, the author has combined the most desirable procedures of many published methods, principally that of Ellis and Thompson (66) and McNaught (65). The principal changes made were designed to eliminate the interference of iron and copper in the analysis, to make the method applicable to different apparatus and to eliminate sources of contamination during the analysis. The author's analytical scheme and methods used are described in the following section without comment. A discussion of the developments, details, and theoretical aspects of the scheme is given later.

Preparation of Ash Solution. In the ashing of samples, proper care was exercised from the standpoint of adventitious

contamination with cobalt as well as possible losses of cobalt in the form of volatile salts when the ashing was completed at high temperature.

Fresh livers were air dried and allowed to come into equilibrium with the moisture content of the air. Weights were taken and the analysis expressed on the air-dry basis. Feces were oven dried at 100-110 degrees C to constant weight and the analysis expressed on the dry basis. Twenty-five cc of blood was taken for analysis, an equal volume of concentrated nitric acid was added to the blood in a pyrex beaker and then evaporated to near dryness. With the use of small portions of acid, quantitative transfer was made to a silica ashing dish, evaporated to dryness, and heated further until all volatile matter was driven off. Dry ashing was completed in an electric muffle at 500 degrees C. Feeds were dry ashed per se in the electric muffle at 550-600 degrees C. With most of the animal tissues ashing was not complete the first time. In this event 10 cc of 1:1 redistilled nitric acid and 1 cc of purified potassium nitrate was added, evaporated to dryness, and ashed again at 600-650 degrees C. The white ash was then dissolved in 10-15 cc of redistilled 1:1 hydrochloric acid and transferred quantitatively to a 250 cc pyrex separatory funnel.

Analytical Procedure. After transferring the ash solution to a separatory funnel, the ferric chloride was extracted three times with 20 cc portions of diethyl ether. The ether was removed completely and 1 cc of ammonium citrate solution was added for each

gram of dry tissue. The pH was adjusted to 8.5 with redistilled 1:1 ammonium hydroxide using phenolphthalein as an internal indicator. The formation of a precipitate was prevented by adding more ammonium citrate. To the buffered solution, 10-15 cc of purified dithizone in carbon tetrachloride was added and shaken vigorously for 30 seconds. The red colored solvent phase was drawn off and the dithizone extractions repeated until the carbon tetrachloride phase, as it separated out, retained its pure green color. The cobalt was then all in the dithizone extract. The extractions were combined and evaporated to dryness in a pyrex beaker on a hot plate.

The residue remaining after evaporating the carbon tetrachloride of the dithizone was oxidized with 3 cc of perchloric acid ($HClO_4$) under gentle reflux until colorless. The perchloric acid was then evaporated to dryness, the snow-white salts were taken up in 10 cc of dilute redistilled aqua regia, and the solution boiled for one minute to dissolve any solid material.

After cooling to room temperature, 2 cc of 0.1 per cent nitroso-R-salt and 2 grams ($\pm$ 0.1 gm) of hydrated sodium acetate was added and the pH checked for 5.5 with Bromcresol green. The resulting green solution was then boiled gently for one minute to produce the cobalt-nitroso-R-salt reaction. After removing from the hot plate, 1.5 cc of concentrated hydrochloric acid was added and the solution boiled further for one minute to break down any heavy metal complexes formed.

The resultant red to light-orange colored cobalt complex was

cooled and made up to volume for spectrophotometric measurement of light transmittance at 430 mu wave length. When the concentration of cobalt was exceptionally low, the colored solution was concentrated by boiling to 5 cc. The standard series method of comparison was used and as little as 0.00006 mg of cobalt could be determined using a minimum volume of 5 cc with absorption cells 1 cm in diameter. A blank was carried through all the stages of the analysis and used in the final spectrophotometric measurements.

For reference purposes a standard cobalt solution was prepared by dissolving 0.4936 gm of cobalt nitrate hexahydrate ($Co(NO_3)_2 \cdot 6H_2O$) in double distilled water and made up to a volume of 1 liter. This solution contained 0.1 mg of elemental cobalt per cc and was further diluted as necessary in the preparation of the standard cobalt solution containing 0.1 ug of cobalt per cc. The calculations were made as follows:

$$\frac{2\text{-log } G_{430}}{\text{Density std.}} \times \frac{1.0}{\text{Sample wt.}} = \text{ug cobalt per gm}$$

where G_{430} is the galvanometer reading in per cent transmittancy at 430 mu wavelength. The calculations were simplified by using a standard cobalt solution containing 1 ug of elemental cobalt in 10 cc total volume, and the above formula holds only under such conditions.

RESULTS

The results of the chemical analysis of cobalt in the various components of the experimental diet are given in Table 1. The term "mixed feed" refers to the experimental diet after being mixed and stored. On the basis of the analyses of the individual feeds, the mixed feed should not contain more than 0.25 ug of cobalt per gram. However, an average of 0.49 ug of cobalt for the mixed feed is shown, the increase being presumably due to contamination during the mixing.

In Tables 2 and 3, the group averages of body weight, erythrocyte count, hemoglobin and blood color index of the experimental animals, over a period of 20 weeks, are shown to indicate no possibility of a cobalt deficiency in the experimental group (Group 2) on the basis of the hematological data. The animals that died during the experiment all manifested slight and severe anemias; an anemia characterized by a normal color index. Statistical treatment of the data in Tables 2 and 3 is given in Table 4.

Table 1. Cobalt content of the various components of the semi-purified diet and the diet as a whole after being mixed.

Sample	Sample wt. grams	Cobalt ug/sample	Cobalt ug/gm	Average
Milk Powder I	16.9092	4.820	0.285	0.287
Milk Powder II	12.3700	3.590	0.290	
Casein I	4.6547	0.520	0.112	0.119
Casein II	5.4655	0.698	0.127	
Corn Starch I	7.5982	0.254	0.034	0.033
Corn Starch II	7.8051	0.255	0.032	
Yellow Corn I	11.4700	2.420	0.211	0.226
Yellow Corn II	12.0198	2.920	0.242	
Mixed Feed I	19.5807	9.900	0.505	0.494
Mixed Feed II	8.2599	4.010	0.483	

Table 2. Hematological data and body weight averages of Group 1 (receiving 0.04 mg of elemental cobalt daily) over an experimental period of 20 weeks. Average of three animals.

Week	Body wt. pounds	Erythrocytes mill/cu mm	Hemoglobin gms/100 cc	Color index
1	7.25	6.12	13.6	0.94
2	7.32	6.66	14.0	0.88
3	7.62	6.43	13.2	0.87
4*	7.40	6.76	11.8	0.73
5	7.33	6.85	12.3	0.76
6	7.02	5.93	10.8	0.77
7	6.96	5.08	10.6	0.87
8	7.05	6.06	11.6	0.80
9	7.23	4.52	10.1	0.94
10	7.61	4.56	10.8	1.00
11	7.39	5.00	10.9	0.92
12	6.99	5.42	11.6	0.90
13**	7.06	5.56	11.8	0.89
14	6.46	5.47	11.6	0.89
15	6.90	4.30	9.6	0.94
16	6.87	4.95	9.9	0.84
17	6.23	4.22	9.7	0.97
18	6.57	4.22	8.2	0.82
19	6.34	3.76	8.0	0.91
20	6.41	4.27	9.3	0.92

* One animal died, leaving two in the group.
** One animal taken off the experiment; died one week later.

Table 3. Hematological data and body weight averages of Group 2 (receiving no cobalt supplement) over an experimental period of 20 weeks. Average of three animals.

Week	Body wt. pounds	Erythrocytes mill/cu mm	Hemoglobin gms/100 cc	Color index
1	6.44	5.92	13.5	0.96
2	6.36	6.68	13.9	0.88
3	6.66	6.48	13.8	0.90
4	6.71	5.88	12.9	0.93
5	6.56	6.70	14.1	0.88
6	6.79	5.93	13.2	0.94
7	6.82	5.62	12.4	0.93
8	6.86	5.55	12.0	0.91
9	6.67	5.65	12.7	0.95
10	6.97	5.67	12.9	0.96
11	6.80	5.37	11.8	0.92
12	6.65	5.70	12.3	0.91
13	6.56	5.34	12.2	0.96
14*	6.25	5.51	12.5	0.95
15	7.22	4.68	10.5	0.91
16	7.14	5.23	11.8	0.95
17	7.28	5.30	11.4	0.91
18	7.08	5.77	12.1	0.88
19	7.20	4.57	11.0	1.01
20	6.92	5.64	13.1	0.97

* One animal died due to causes unknown, leaving two animals in the group.

Table 4. Statistical analysis of the hematological data in Tables 2 and 3 illustrating the overall effect of cobalt supplementation and no cobalt on the gains in weight and changes in the blood picture of the experimental animals.

Group		Initial value	Mean value	Terminal mean	Gain	Per cent mean gain
1	BW	7.25	7.00	6.77	-0.48	- 6.62
	RC	6.12	5.31	4.43	-1.69	-27.61
	Hb	13.60	11.00	8.10	-5.50	-40.44
2	BW	6.44	6.80	7.20	0.76	11.80
	RC	5.92	5.67	5.38	-0.54	- 9.12
	Hb	13.50	12.50	11.40	-2.10	-15.55

The results of the cobalt analyses of livers and feces are given in Table 5, and a summary is given in Table 6. These data indicate the changes in cobalt content of livers and feces occurring during the 20 weeks experimental period compared to normal cobalt levels in rabbit livers and feces.

Table 5. Cobalt analyses of liver and feces of the experimental animals over a period of 20 weeks.

Week	Animal	Liver cobalt ug/gm	Fecal cobalt ug/gm
2	2*	0.103	0.207
1	4	0.174	0.128
1	5	0.155	0.134
12	1*	0.198	0.210
12	8	0.095	0.101
20	3*	1.138	0.654
20	6	0.040	0.180
20	7	0.067	0.199

* Animals receiving a daily allowance of 0.04 mg of cobalt.

Table 6. Summary of the cobalt content of livers and feces of the experimental animals on a low cobalt semipurified diet for 20 weeks. Illustrating the changes in cobalt content. Cobalt content expressed as micrograms per gram of liver on the air-dry basis and feces on the dry basis.

	Normal	Cobalt fed	No cobalt
Liver	0.164	0.480	0.067
Feces	0.131	0.357	0.160

DISCUSSION

In the chemical analysis of cobalt, particular care must be taken to prevent contamination during the analysis. This is especially true during the dry-ashing in the electric muffle. Dry-ashing was chosen because reagents added in the digestion and ashing of the sample are apt to introduce cobalt and impurities which might interfere in the subsequent cobalt analysis. However, Drabkin (84) has used an oxidation mixture satisfactorily, and Parks et al. (85) have preferred a wet-ashing procedure.

In the analysis of plant materials and the components of the experimental diet, preliminary extraction of ferric iron was not necessary. The ammonium citrate, about 1 cc per gram of sample, sufficiently buffers the iron during the alkaline dithizone extraction of cobalt, leaving the ferric iron quantitatively in the aqueous phase. With samples of liver and feces, however, the citrate buffer did not eliminate iron interference and ether extraction became necessary. Ether extraction of iron also partially extracts phosphorous (86). Three ether extractions of the acid ash solution completely removes the iron as shown by a negative qualitative test. Interference due to iron may also be eliminated by precipitating the ferric iron with potassium fluoride and filtering (87).

The dithizone (diphenylthiocarbazone) was purified by the method of Parks et al. (85). The reagent used had a concentration of 0.5 gram per liter of carbon tetrachloride which is several

times more concentrated than is ordinarily used. Such a concentration was used in order to keep the number of dithizone-carbon tetrachloride extractions at a minimum. The use of a less concentrated dithizone reagent resulted in many laborious extractions and exceptionally large volumes to be later evaporated. The modified analytical procedure, using dithizone extraction, gave 96.2 per cent recovery of as little as 0.1 ug of added cobalt, which is comparable to the claims of authors using dithizone extraction (66, 85) and those using other methods (64, 65, 87).

In determining the most desirable spectral wavelength at which the red to light-orange colored cobalt-nitroso-R-salt complex was to be measured spectrophotometrically, the spectral characteristics of this cobalt complex and the nitroso-R-salt reagent was determined, the results of which are shown in Fig. 1. The concentration of the nitroso-R-salt reagent was 0.02 per cent which constituted the spectrophotometric blank used throughout the analysis. As shown, the wavelength at which maximum absorption occurs is in the near ultraviolet for the reagent and at 430 mu in the case of the cobalt complex.

The cobalt-nitroso-R-salt complex showed excellent agreement with the Lambert-Beer Law (86) over the range of 0.06 to 1.5 ug of cobalt per cc concentration. The spectrophotometer was standardized for this concentration range. Consequently, in the analysis, the sample or its aliquot should contain not more than 15 ug of cobalt nor less than 0.06 ug. The standard series method of comparison was used, and the method is sensitive to 0.065 ug

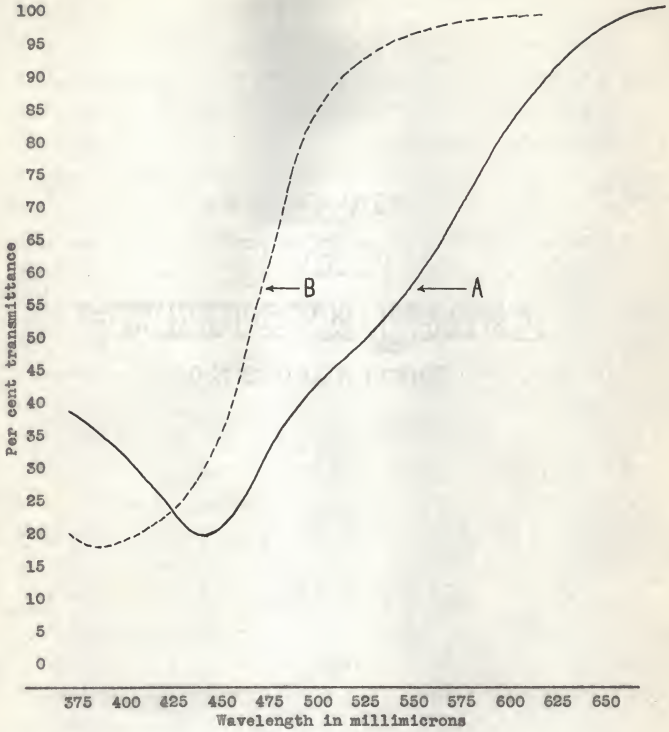


Fig. 2. Spectral transmittance curve for the cobalt-nitroso-R-salt complex (a) equivalent to 1 ug of cobalt per cc and for the nitroso-R-salt reagent equivalent to a concentration of 0.02 per cent.

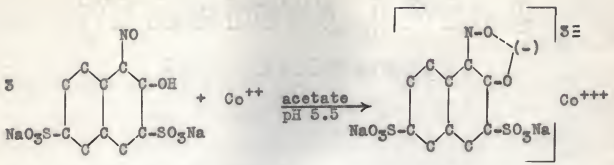
of cobalt using absorption cells 1 cm in diameter and a volume of 10 cc. In the extreme limits of the sensitivity of this analysis, that is, a sample containing 0.065 ug of cobalt prepared by the standard series method, 90.2 per cent of the incident light was transmitted. Then,

$$D = -\log T = (2-\log G_{430}) = 2-\log 90.2 = 2-1.9552 = 0.0448$$

$$\frac{0.0448}{0.688} \times 1.0 = 0.065 \text{ ug of cobalt,}$$

which is in excellent agreement with the standard cobalt solution containing 1 ug. This also demonstrates the validity of the Lambert-Beer Law.

The electro-valence of cobalt is 2 or 3. However, the trivalent salts of cobalt are difficult to synthesize, and they break down readily under atmospheric conditions (62). Malyuga, cited by Snell and Snell (61), has shown that cobalt combines with 3 mols of the nitroso-R-salt reagent, and Mellan (89) identifies the reactive grouping as $-\underset{NO}{C} = \underset{OH}{C}-$. Logically then, the divalent cobalt salts, such as the cobaltous chlorides ($CoCl_2$) and nitrates ($Co(NO_3)_2$) formed on the addition of dilute agua regia to the salts of cobalt, copper, nickel, zinc, etc., in the final stages of the analysis, react with nitroso-R-salt in the following manner:



Sodium 1-nitroso-2-hydroxy-naphthalene-3,6-disulfonate

Cobalt-nitroso-R-salt

The reaction being endothermic is accomplished in hot sodium acetate trihydrate at pH 5.5. The reaction does not go to stoichiometric completion, and consequently full color development, unless the pH is rigidly controlled, the quantity of nitroso-R-salt is slightly in excess and the period of boiling is just sufficient and not extended for a period longer than one minute. Loss of color intensity has been attributed to prolonged boiling and to the addition of excess quantities of nitric or hydrochloric acids in breaking down the heavy metal complexes (90). The yellow color of the excess reagent may be completely removed by the addition of bromine (74). A blank was run in the present scheme with appropriate corrections made which served to eliminate errors due to contamination during the analysis. On the addition of 2 grams ($\pm$ 0.1 gm) of sodium acetate the pH of the solution was found invariably to be very close to 5.5.

The analysis of the various components of the experimental diet gave results comparable to those of other investigators (91), however, the milk powder and casein show a much higher cobalt

content than is to be expected. Theoretically, the diet as a mixture should not have a cobalt content greater than that of the corn which shows an average of 0.23 ug per gram. The diet as a whole, however, shows 0.49 ug of cobalt per gram, and this is indeed strange for such a large degree of contamination seems unlikely. The agreement between analyses on samples of the mixed diet, varying in weight by as much as 10 grams, is excellent and therefore eliminates the possibility of extraneous contamination during the analysis. Again, however, the fecal determinations on the experimental animals do not give support to the relatively high cobalt content of the diet, but is in fair agreement with the cobalt content of the corn which made up 70 per cent of the diet. The corn, having been ground in a feed mill, did not show any appreciable contamination; however, Hood et al. (92) have found that some cobalt was contributed to feed in the course of grinding in a ball mill.

As the experimental animals were placed on the semipurified diet, normal blood erythrocyte count and hemoglobin was established on the basis of the average of all animals. The normal erythrocyte count was 5.93 million per cu mm and the hemoglobin was 14.0 grams per 100 cc of blood. Table 2 demonstrates an anemia in the control group after four weeks on the diet, extending over a period of three weeks. As this anemia, characterized by a steady decline in hemoglobin level and a low color index, was first recognized, it was thought that perhaps a vitamin deficiency was slowly establishing itself. A slight diarrhea was also mani-

fested. At the first recognition of these symptoms, 1 mg of pteroylglutamic acid and 2 mg of pyridoxine were administered orally, and a remarkable response was observed. The appetite of the anemic animals increased and the diarrhea rapidly disappeared. This treatment was repeated once again at a week's interval with no noticeable response. The blood picture fluctuated profusely for several weeks with or without vitamin treatment. However, the diarrhea did not recur. In spite of the conclusions of Simpson et al. (93) and Passmore (94) regarding vitamin synthesis in rabbits on purified diets, a definite avitaminosis complex was undoubtedly manifested by the experimental animals on this particular diet. This is readily explained on the grounds of diet change.

The animals were reluctant in eating the ration which had the appearance of good physical substance, but very hygroscopic. After a day or so of coaxing, the animals took readily to the feed, consuming an average of 60 grams daily. This diet change was undoubtedly not favorable or conducive to microorganismal biosynthetic activity in the gut. The compacting of the food in the lower gut and the following diarrhea produced an unfavorable microflora, or inhibited the normal flora to such an extent that the various substances produced by this flora, essential to their host, were critically deficient. After a period of adaptation, the microflora was again stabilized and these essential factors once again became available to their host. This explanation is supported by the fact that recovery from the anemia was spon-

taneous irrespective of vitamin administration.

The adequacy of the experimental diet for growth of young rabbits, as demonstrated in Fig. 2, seems to be apparent.

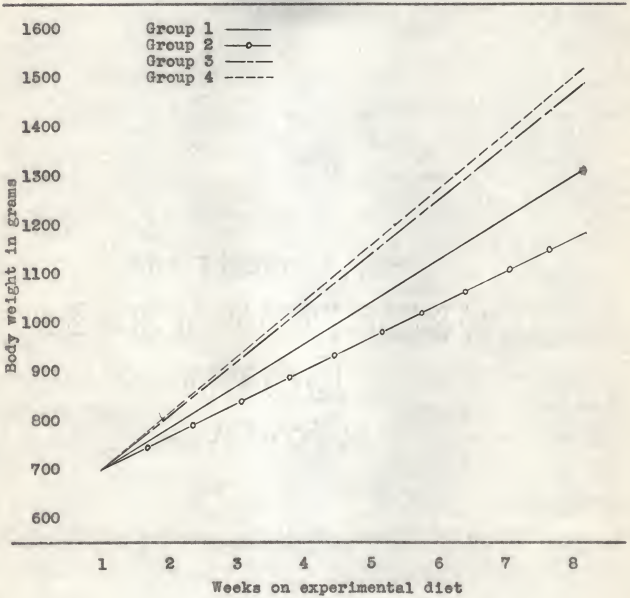


Fig. 2. Statistical treatment of body weight gains of 12 young rabbits on the semipurified diet for a period of 8 weeks.

Table 4 also shows the diet to be adequate for maintenance of mature and aged rabbits with respect to body weight. After a five months period on the diet, the control group showed a 7 per cent loss in weight while the experimental group showed a 12 per cent gain. The data of Table 4 also indicates a toxic action of cobalt in the control group. A possible toxicity is substantiated by the tremendous amount of cobalt stored in the liver of animal 3. It is significant also that for a period of 8 weeks, animal 3 comprised the control group, thus effecting the group data considerably in favor of cobalt toxicity.

Considering the metabolism of cobalt, it is apparent that the dietary to fecal cobalt ratio will not be the same regardless of the quantity of feed eaten. The fecal cobalt would, however, be controlled by many variable physiological factors such as the overall efficiency complex, the relative solubility of the cobalt salts, the renal threshold of cobalt concentration, the degree of cobalt storage in the liver and other organs, and the influence of the pseudoruminating act in which the feces are taken directly from the anal orifice.

The average daily intake of cobalt, based on the diet analysis and daily feed consumption, was about 30 ug for the experimental group while the control group had the same intake plus a 40 ug daily supplement, giving this group an intake of 70 ug. Table 6 shows that normal livers and feces contain an average cobalt content of 0.164 and 0.131 ug per gm, respectively. Animals having a daily intake of 70 ug of cobalt show 0.48 ug per gm of

liver and 0.357 ug per gm of feces, while those with a daily intake of 30 ug show 0.067 ug per gm of liver and 0.160 ug per gm of feces at the end of five months. As Eden (95) has found that the rabbit eliminated 96 per cent of the copper ingested through the feces, it is assumed that this is the major channel of elimination. The ingestion of large amounts of cobalt in an insoluble form would enormously increase the fecal output without materially altering the liver or perhaps the urinary cobalt level. In an analysis of the data in an endeavor to arrive at some conclusion regarding the metabolism of cobalt, it is significant that the act of coprophagy in this specie renders sound and logical interpretation difficult. Even though the pseudoruminating act was not observed during the entire experimental period, it may nevertheless be assumed that coprophagy also occurred to a normal extent in this experiment.

The question now is, in what measure did the pseudoruminating act cause such lagging in the evacuation of feces that direct and gross errors have arisen. Quite obviously, this cannot be ascertained, but observations during the experimental period support the viewpoint that coprophagy is a natural and normal physiological action essential to this animal specie (77, 96).

The portion of the cobalt that was absorbed in the gut therefore, cannot be separated from that portion which originally failed of absorption. Since this is true and since the re-excreted portion may be a large as well as a variable part of the whole, it is impossible to arrive at a figure of any value for

the metabolism of cobalt. The problem is made even more difficult by the small quantities of cobalt available in a balance study of this nature. The apparent metabolism of mineral matter in general may be regarded as of no certain significance, since the fecal mineral matter may include relatively large amounts of mineral substances excreted into the gut from the blood.

During the last week of the experiment, animal 3 consumed very little feed and the feces were scarce, an entire week being required to collect enough for analysis. On autopsy the gut was relatively empty, the urinary bladder distended with urine and the stomach was filled completely with its contents heavily infiltrated with hair, indicating profound coprophagy. As this animal was progressively becoming anemic, it is not known whether this was an expression of a depraved appetite or a desire for some unknown nutritional factor. The blood picture of this animal and the high cobalt content of the liver (1.138 ug per gm) indicate a toxic effect of cobalt in the amount ingested.

Normal sheep livers contain an average of 0.22 ug, rat livers 0.14 ug, and rabbit livers 0.14 ug of cobalt per gram according to McNaught (64). On the basis of normal rabbit livers (0.164 ug per gm), the cobalt content of the liver of animal 3 was increased seven fold and the feces content six fold, by a daily intake of 70 ug of cobalt. After five months, the two remaining animals in the experimental group receiving no cobalt showed an average liver cobalt content of 0.067 ug and a fecal cobalt content of 0.160 ug per gram. The liver cobalt of one animal in this group showed

only 0.04 ug per gram and the blood picture showed a conflict between normality and anemia. However, animal 7 showed 0.095 ug of cobalt per gram of liver after five months on the low cobalt diet and there was not once an indication of a developing anemia.

Sheep suffering from a cobalt deficiency show an average liver cobalt content of 0.06 ug per gram (64), and McNaught (33) found the average cobalt content of healthy pasture to be 0.64 ug with a range of 0.34 to 0.94 ug. The present diet analyzing 0.49 ug is then seen not to be critically low in cobalt. In view of this, it is indeed strange that the animals on this diet showed better than a 100 per cent reduction in liver cobalt content. The only logical explanation then would lie in a low degree of intestional absorption. A 25 cc sample of blood did not reveal cobalt in quantitative amounts, and this fact gives more significance to the low coefficient of absorption.

Comar et al. (60) have found that only 0.25 per cent of orally administered radioactive cobalt was retained by rats and that the feces was the major route of elimination. Considering this to be the case with rabbits, and the data certainly indicates a low absorption coefficient for cobalt, the animal on the experimental diet would absorb only 0.075 ug of cobalt daily (30 ug x 0.0025 = 0.075). The livers of these animals showed only 0.067 ug of cobalt, thus indicating an extremely low coefficient of absorption.

An interesting aspect of this analysis is that the cobalt fed animals showed an increase of 0.31 ug of cobalt per gram of

liver while the animals receiving roughly one-third the amount of cobalt of the aforementioned showed a decrease of 0.10 ug. This relationship is interesting not only from the standpoint of absorption, but from the standpoint of cobalt disposition by the liver. This indicates that perhaps the normal cobalt content of liver is not normal at all, but lies within the limits normally revealed by analysis only as a consequence of the cobalt content of feeds and the apparently low absorption coefficient. The liver then stores cobalt even in amounts detrimental to its normal functioning due presumably to the lack of a physiological mechanism capable of dealing with cobalt. The storage of cobalt in the liver in toxic amounts is not peculiar to the rabbit (97, 98, 99); however, the act of coprophagy would appear to increase the absorption of cobalt by its many passages through the alimentary tract.

It thus appears that the rabbit is not a satisfactory animal for use in cobalt metabolism studies because of the peculiar act of coprophagy it engages in. The obvious similarity in physiology between the rabbit and the ruminant does not extend to cobalt metabolism, and Crampton et al. (100) and others (78) regard the rabbit as an unsuccessful substitute for the ruminant in digestion studies. The obviously minute requirements for cobalt, if any, adds another disadvantage in using the rabbit as an experimental animal.

There are a number of aspects of the role of cobalt in animal nutrition about which further information would be desirable. Co-

balt reserves of animals and cobalt content of pastures where deficiency diseases have been cured by cobalt are recorded in only very few instances. Mild cobalt deficiencies are undoubtedly much more widespread in forms characterized by mild or borderline clinical symptoms. The diagnosis of borderline deficiencies by blood analysis is not a dependable means of diagnosis. Furthermore, clarification of the role of cobalt in the dual deficiency of copper and cobalt would be helpful. Then too, the role of cobalt in metabolism is not known. It is to be hoped that future studies similar to those of Comar and associates (60, 101) on cobalt metabolism, using radioactive cobalt as a tracer, will throw light on the role of this element in the animal organism.

SUMMARY AND CONCLUSIONS

A chemical and physiological balance study was carried out in an endeavor to learn something about cobalt metabolism in a common laboratory animal closely allied to the ruminant in its physiology. Eight rabbits were maintained on a semipurified diet, low in cobalt, for an experimental period of five months. Four of these animals received a daily allowance of 0.04 mg of cobalt in addition to a mineral supplement, while the remaining four animals received a mineral supplement free of cobalt. Chemical and physiological balance was studied throughout the experimental period.

A modification of the Nitroso-R-salt-Nitrosocresol methods of chemical analysis of cobalt is described for cobalt analysis

of feeds and biological materials. This modified procedure, using dithizone extraction of cobalt, is sensitive to 0.000065 mg of cobalt when determined spectrophotometrically and using absorption cells 1 cm in diameter. This extraction procedure gives 96.2 per cent recovery of cobalt in samples containing as little as 0.0001 mg of cobalt.

Attempts at producing a cobalt deficiency in the rabbit were unsuccessful, and the requirements of this specie, if any, must be very low. The cobalt content of rabbit livers was reduced to a level at which sheep normally manifest symptoms of a cobalt deficiency.

Normal rabbit livers contain 0.164 ug of cobalt per gram on the air-dry basis while normal feces contain 0.131 ug on the dry basis. Rabbits having a daily intake of 0.07 mg of elemental cobalt after a 20 weeks period stored only 0.00032 mg per gram of liver. A daily intake of 0.03 mg resulted in a decrease of 0.0001 mg of cobalt per gram of liver from the normal over the same period.

The experimental data leads to an hypothesis of a constant absorption coefficient for cobalt. The cobalt content of normal rabbit livers appears to be a consequence of the cobalt content of the animals' feed and the absorption coefficient. The major source of cobalt elimination is through the feces. The constant cobalt absorption coefficient hypothesis is based on liver and fecal cobalt analyses under varying cobalt intakes.

It appears that a daily intake of 0.07 mg of elemental cobalt

over a period of five months is toxic to mature and aged rabbits. The agreement between the level of cobalt intake, liver storage of cobalt, and elimination of cobalt through the feces is very good, giving somewhat more significance to the hypothesis of a constant absorption coefficient.

The blood erythrocyte count, hemoglobin and color index is not a reliable criterion for the diagnosis of anemias in the rabbit unless critically performed and without exciting the animal. The erythrocyte count and hemoglobin content of blood is controlled by many variable factors such as the time of day the determination is made, temperature to which the animal has been exposed, and the age and plane of nutrition.

The experimental animals, having a daily cobalt intake less than one-third that amount required by sheep, failed to manifest symptoms of a cobalt deficiency. Consequently, the rabbit is not a good substitute for the ruminant in studies of this nature.

The conclusions from this study have been drawn with considerable reservation as the number of experimental animals employed in the experimental design is not sufficient for sound and safe deduction.

The chemical analysis of cobalt remains to be a limiting factor in cobalt metabolism studies. Perhaps if some chemical spot test for blood cobalt, sensitive enough to indicate sub-clinical levels of cobalt in sheep and cow's blood, could be devised, the veterinarian could undoubtedly uncover many more cases of mild cobalt deficiencies. Such mild cases are of considerable economic importance to the animal husbandman.

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