analyses revealed no significant difference in the incidence of carcinomas among the three groups at the 5% level.

Discussion

The procedure used for evaluating the carcinogenicity of AA and $KAsO_2$ is unique (*4*, *13*) and requires some comment. Because the tumors attributed to arsenic are almost exclusively of the skin of man (*9*, *11*), and because the skin of man is exposed to many known carcinogenic stimuli, including both ionizing and ultraviolet radiations, as well as many chemical carcinogens, such as coal tar and petroleum products, it is of great interest to test arsenicals in conjunction with an incomplete carcinogenic stimulus.

The tests were based on the fact that the formation of skin tumors in mice may be divided into two stages called initiation and promotion, and that substances exist with potency to accomplish either one or the other stage (*1*, *2*). If $KAsO_2$ were found to possess predominantly either initiating or promoting power, exposure to $KAsO_2$ alone would not ordinarily result in tumor formation. Thus the contradictory nature of reports on the carcinogenicity of $KAsO_2$ might be resolved.

Second, the arsenicals were fed in conjunction with exposure of the animals to substances able to cause either the initiation or promotion of skin tumors. The existence of subthreshold levels of each of these agents is readily demonstrable as well as the additive nature of quantities of either initiators or promoters insufficient to elicit maximal tumor response (*2*). Therefore, it is likely that even if the arsenical possessed only a subthreshold initiating or promoting action, that property would be revealed by addition to a submaximal stimulus of the same kind provided by either DMBA or croton oil. The data in Figure 1 show that the initiating dose of 5 μg. of DMBA was submaximal while a comparison of the results shown in Figures 4, 5, and 7 with those in 9, 10, and 11 shows that the lower level of croton oil employed in the fourth experiment did not cause a maximal response (experiments 3 and 4 were run simultaneously with the mice obtained from a common pool). The higher incidence of carcinomas among the mice of experiment 3 compared to experiment 4 was significant at the 1% level.

Finally, although the procedure that was used is unique for carcinogenicity testing, its use in this respect has adequate precedent in research on dissection of the carcinogenic process (*1*, *2*, *13*) to justify application to problems of carcinogenicity testing where appropriate. These techniques utilizing mice selectively bred for susceptibility to skin tumor formation make a relatively rapid and highly sensitive test.

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FEED ADDITIVES

Metabolism of Selenium in the Mammalian Organism

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THE World Health Organization Expert Committee Report of 1961 (*35*) on "Evaluation of the Carcinogenic Hazards of Food Additives" makes the following statement regarding selenium: "It seems clear that too little selenium is harmful and gives rise to a deficiency state. However, too much selenium may cause toxic effects and may perhaps constitute a carcinogenic risk." Despite the proposal that selenium be included among the "essential" dietary trace elements (*26*, *28*, *29*), there are many unanswered questions about selenium metabolism in the mammalian organism. The function of selenium is not clear. The purpose of this paper is to review in part results from laboratory studies which may add to the general knowledge of the metabolism of selenium and assist in an eventual evaluation of dietary selenium.

Nearly all of the experiments employed the radioactive tracer technique which made possible the detection of submicro amounts of this element. The amount of Se^{75} and selenium in the single dose administered to the experimental animals was kept within trace amounts; thus, both radiation and toxic effects from selenium were avoided, and the metabolism under study was considered normal.

Retention of Selenium in Lactating Dog

One of the pathways by which selenium may be eliminated from the animal body other than through the kidney, gastrointestinal tract, and lung is through the mammary glands, into the milk of lactating animals (*1*). A lactating dog, having five 1-day-old pups, was injected subcutaneously with 0.64 mc. of $Se^{75}O_3^{-2}$ which contained 7 μg. of selenium. Milk samples were collected at various time intervals from 4 hours (*17*) throughout a period of 7 weeks and were separated into skim milk, casein, and milk serum. All the fractions including whole milk

In an evaluation of selenium as either a dietary essential or a carcinogenic risk, it is important to note that administered selenium is retained by the animal for extended periods of time. Selenium passes the placental barrier as well as the mammary glands and into the milk proteins. In specific cells, as the erythrocytes, selenium once incorporated remains there throughout the life span of the cell. It appears that selenium has an effect on growth and is readily incorporated into rapidly growing and embryonic tissues. Selenium is incorporated into tissue proteins which include specific proteins such as hemoproteins, enzymes, and nucleoproteins. It is present in the various intracellular fractions of liver.

were assayed for Se^{75} activity. The five pups were nourished on the mother's milk and sacrificed at different time intervals over a 2-month period. The tissues were assayed for Se^{75} activity. Expressed as c.p.m. per organ, the greatest concentration of Se^{75} appeared in the total muscle, liver, small intestines, and kidney. Se^{75} was present in the milk proteins until lactation ceased. When the Se^{75}-treated dog was bred again without further administration of the isotope, the second litter delivered by Caesarean section produced only two pups, one of which was abnormal. The exact reason for the reduced number in the second litter and the presence of an abnormal pup must await further investigation. This pup had a normal body except for the head, which was abnormal. There was practically no brain tissue, and there were no eyes. The second pup was normal as far as could be detected. Delivery of the pups by Caesarean section demonstrated that Se^{75} passes the placental barrier, since radioactivity was present in the various tissues of the pups. Again, the greatest concentration of activity was found in the muscle, liver, small intestines, and kidney. Nearly all the Se^{75} was present in the proteins of the milk. About 90% of whole milk and skim milk activity was protein-bound. In both pregnancies, more than 50% of the total activity present in the milk was found in the casein, and 45 to 48% in the milk serum. At the time of a second pregnancy, 236 days after the initial injection of Se^{75}, the isotope was still present in the mother dog. It is important to note that Se^{75} could be detected in the milk 278 days after injection of 0.64 mc. of selenious acid containing 7 μg. of selenium.

Retention of Selenium in Blood

One of the interesting phases of selenium metabolism is the apparent incorporation of selenium in blood proteins (*30, 34*). In earlier studies in the rat, 24 hours after injection of Se^{75} the whole blood activity was in favor of the red cells, which was 80%, while in the plasma it was 20% (*9*). Later, experiments with dogs were conducted to study the rates of change of Se^{75} in the various components of blood over a long period of time (*15*). Normal adult dogs were injected subcutaneously with subtoxic amounts of inorganic selenium. At frequent intervals over a period of nearly a year, samples of whole blood plasma and washed red blood cells were obtained, and Se^{75} activities were recorded and plotted against time. Since these data represent a biological function, the curves were assumed to be exponential of the form $y = ae^{-kt}$. The values for the a and k parameters were determined by use of the method of least squares. With the k parameter known, half-time values were determined. The rate of disappearance of Se^{75} from whole blood, plasma, and red blood cells can be described as a multiple-component rate function. Of importance is the fact that the greatest rate of disappearance of selenium from the red blood cells is at 100 to 120 days after the initial injection. These results are interpreted to mean that selenium, once it becomes incorporated in the RBC of the dog, remains there throughout the life span of the cell. As in the milk protein studies, after administration of subtoxic amounts of Se^{75} it is possible to detect selenium in various blood proteins as long as 310 days after injection.

Serum proteins incorporate Se^{75}. Sixty per cent of the total serum radioactivity was equally distributed between the alpha-2 and the beta-1 globulins (*19*). In other studies, it was found that selenium is incorporated into leucocytes (*7*).

Retention of Selenium in the Developing Egg

Congenital abnormalities (monstrosities) are known to occur in embryonic chicks or in chicks hatched from eggs laid by hens fed selenium compounds or grains containing these compounds (*32*). Experiments were undertaken to gain insight into the effects of selenium on the embryonic development of chicks. As others have reported, it was found that monstrosities could be produced by injection of toxic amounts of stable selenium salts into hen's eggs. After injection of subtoxic amounts of $Se^{75}Cl_4$ into the air sack of eggs, followed by the assay of Se^{75} in the various tissues of the hatched chicks, a wide but varied distribution of selenium was found in the tissues examined (*21*). On the basis of the percentage of the original dose, the highest concentration (55%) appeared in the viscera, including the egg yolk. The greatest concentration appeared in the viscera, carcass, outer shell membrane, shell, gizzard, and liver; lesser amounts in the feet, muscle, brain, feathers, blood, bone, and eyes; and traces in the kidney, beak, lung, heart, and bile. Important is the fact that nearly all the tissue Se^{75} is protein-bound. During the early embryo stages, there is relatively rapid and profound developmental activity, at which time the plans of many organ systems appear. It is conceivable that excessive amounts of selenium at this stage of development may cause irreversible damage. Selenium appears to have an effect on growth, and in a not completely explained fashion appears to interfere with sulfur metabolism. These experiments demonstrate that fast developing embryonic tissues incorporate selenium.

Chemistry (Form or Forms) of Tissue Selenium

One of the greatest challenges today in the selenium field is the determination of the form or forms of selenium in the animal tissues. Evidence from the author's laboratory has been reported in support of the contention that part of the protein-bound selenium in mammalian tissues exists as selenocystine, the selenium analog of cystine. Dog liver proteins tagged in vivo with Se^{75} were purified and hydrolyzed, and the constituent amino acids thus obtained were separated by techniques of paper chromatography (*20*). The greatest activity appeared in the cystine-selenocystine area, with less in the methionine and selenomethionine area, and in the leucine area. Interesting is the fact that these compounds behave like amino acids in ion exchange and precipitation studies, and are isolated along with the amino acids found normally in liver protein by paper chromatography. In another approach to the problem, experiments were conducted in which dogs given injections of Se^{75} were fed bromobenzene, and the mercapturic acid fraction of the urine

was isolated and found to contain Se^{75} (*12*). Assuming that sulfur and selenium have similar biological properties because they are related chemically, as in sulfur metabolism, it is believed that tissue-bound rather than free amino acid selenium is the source for selenomercapturic acid synthesis. Further, it appears that there exist isomorphic forms of MA-SeMA. Other such forms existing in biological systems are cystine-selenocystine, and cystathionine-selenocystathionine. The evidence indicates that in the dog the selenium analog of mercapturic acid can be formed. These results, therefore, provide further evidence that trace amounts of administered selenium appear as selenocystine in the tissues of the dog.

Hair, which is high in cystine, has proved to be of practical value in extending the studies on the fixation of selenium in tissue proteins (*11*). The affinity of selenium for sulfhydryl compounds has led investigators to assume that selenium enters into a chemical combination with keratin. Mixed disulfides of radioactive keratin were prepared from hair of dogs injected with single doses of $H_2Se^{75}O_3$. The protein *S*-sulfokerateine was prepared and was found to contain 16% of the original Se^{75} hair activity. In separate experiments, after several months the concentration of Se^{75} in hair on a dry-weight basis was higher than in most tissues on a wet-weight basis, and cystine isolated from hair by conventional methods contained Se^{75}.

One important phase of selenium metabolism in the mammalian organism is its incorporation into tissue proteins. Incorporation of selenium into specific proteins regardless of form or manner of administration includes hemoproteins [hemoglobin (*9*), cytochrome-c (*10*), myoglobin (*6*)], muscle enzymes [myosin (*16*) and aldolase (*6*)], and nucleoproteins (*6*). Whether the extent to which selenium alters enzyme action is proportional to the degree to which it combines with specific tissue proteins is unknown. There is no question that excessive amounts of selenium are toxic and cause retardation of growth. However, Poley *et al.* in 1941 (*27*) demonstrated growth stimulation in chickens when the ration was supplemented with subtoxic levels of selenium. Nesheim and Scott (*23*) more recently have shown that 0.4 to 1.0 mg. of Se per kg. of a Torula yeast diet has growth-promoting effects over similar diets without selenium. Some selenium compounds are capable of functioning in place of their sulfur analogs; occasionally they act as antagonists. For instance, selenomethionine can replace methionine in an enzyme preparation of *Escherichia coli* (*24*), which activates amino acids prior to their incorporation into proteins; also, it can be converted to functional selenoadenosyl methionine by an enzyme of yeast (*22*). Selenopantethine can replace pantethine as a growth factor for *Lactobacillus helveticus* (*4*), but it acts as an antagonist of pantethine in a pigeon liver system (*5*). Selenocystine interferes with the uptake of cystine by leukemic leukocytes (*33*). Since seleno-75-cystine can be made by neutron activation of selenocystine, a comprehensive study of this new radioactive amino acid was made (*14*). After injection of trace amounts of seleno-75-cystine into the rat, over a 48-hour period Se^{75} was present in all tissues and in all intracellular liver fractions, including the nucleoproteins derived from these fractions (*8*). The greatest concentration of Se^{75} occurred in the liver, muscle (total), and kidney. Se^{75} activity in the different liver intracellular fractions expressed as a percentage of the total homogenate showed 40% in the soluble fraction, 35% in the mitochondrial fraction, 16% in the microsomes, and 11% in the nuclei. In the nucleoproteins derived from the various intracellular fractions, about 70% of the Se^{75} was found in the protein fraction. Extension of the author's previous studies covered the incorporation of Se^{75} into growing tissues, especially rat uterus, after administration of trace amounts of seleno-75-cystine with and without estradiol. Animals treated with estradiol seleno-75-cystine incorporated 1.5 times more Se^{75} expressed as c.p.m. per gram, or five times more Se^{75} as c.p.m. per whole uterus, than their untreated litter mates. This demonstrated that there is an increased incorporation of Se^{75} after administration of selenocystine into normal uterine tissue which had been induced to grow rapidly by estradiol treatment.

Selenium has been observed to be distributed widely not only in various tissue proteins, but also specifically in the intracellular liver fractions of the rat (*18*). From the results of experiments, the following possible intracellular metabolic pathway for selenium is suggested. The initial total specific activity in the soluble fraction and the high percentage of nonprotein selenium indicate that this particular fraction may be the site of entry of the inorganic selenium into the rat liver cell. The soluble fraction appears to be the metabolic pool or space initially containing nonprotein selenium subsequently utilized for protein incorporation. The microsomes appear to be the initial site for incorporating selenium into protein as indicated by the high, protein-bound, selenium content and the rapid incorporation of Se^{75} into protein in the first phase of the time-distribution curve. From the microsomal fraction, the protein-bound selenium is probably transferred in this quantitative order:—soluble fraction, mitochondria, and nuclei. In principle, this concept of selenium protein synthesis is essentially in agreement with the generally accepted role of microsomes in protein synthesis.

The rat can incorporate selenium into cytochrome-c and does so at a relatively rapid rate in the young adult female rat (*10*). The fact that selenium can substitute for vitamin E in preventing some of the symptoms in animals deficient in vitamin E suggests a metabolic function for this trace element. Recently reported experiments implicate selenium as well as vitamin E in the in vivo synthesis of ubiquinone (*2*).

There has been considerable interest lately in selenium as related to the physiology and biochemistry of muscle. There is substantial evidence to support the view that selenium prevents certain muscle diseases in animals. Since earlier distribution studies showed that about 35% of the total body Se^{75} content appeared in the body musculature, it was of interest to establish whether or not selenium could be incorporated into two muscle enzymes, aldolase and myosin (*16*). Selenium does incorporate into the muscle protein myosin, where about a third of the muscle radioactivity was calculated to be present. ATPase activity of isolated normal myosin from animals not exposed to selenium was unaffected by concentrations of SeO_3^{-2} from 10^{-2} to $10^{-6}M$. Aldolase, the enzyme present in muscle which catalyzes the breakdown of a hexose into two trioses in glycolysis, like myosin incorporates selenium, and its enzyme activity is not affected by 10^{-2} to $10^{-6}M$ SeO_3^{-2}. Apparently, myosin and aldolase are not the enzymes directly involved in selenium deficiency muscle diseases.

Selenium is as effective as vitamin E in preventing dietary liver necrosis in the rat; multiple necrotic degeneration in the mouse; and exudative diathesis in the chick (*25*). Since 74% of the serum vitamin E is carried by the serum lipoproteins (*3*), the selenium content of lipoproteins was determined. In the dog and rat, both the alpha and beta lipoproteins incorporate Se^{75}, with the greater percentage in the *alpha* lipoproteins (*13*). Even though selenium is present in lipoproteins in small amounts, there is a question as to the significance of this trace element in serum lipoproteins. Studies indicate that vitamin E and selenium have related antioxidant properties (*31*). In vitamin E deficiency, peroxidation is widespread and is particularly damaging to the mitochondria and microsomes of the cells where lipide free-radical intermediates react at random with structural and functional proteins, lipides, and other compounds (*31*). It is conceivable that selenium may act as an antioxidant in serum lipoproteins as it probably does in the tissues.

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FEED ADDITIVES

Implications of Selenium in Large Animal Nutrition

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White muscle disease has been produced in lambs by feeding their dams prenatally and through lactation on an alfalfa hay–oats diet containing less than 0.02 p.p.m. Se, and has been prevented by raising the dietary Se level to 0.06 p.p.m. or more. Diagnoses of muscle damage were made by histological examination and by demonstration of elevated, though extremely variable, serum glutamate–oxalacetate transaminase levels. Provision of Se in a single dose to protect throughout pregnancy was effective via the parenteral route when a slow-absorption vehicle was used, but less effective when a similar amount was given orally, as sodium selenite, in aqueous solution. Levels of Se in the blood of lambs closely approximated that of their dams in the respective treatment groups. Whole blood levels of 0.11 p.p.m. Se in the ewes and 0.12 p.p.m. Se in the lambs were found compatible with WMD prevention in this study.

For many years, selenium was viewed by nutritionists solely as a toxic material from the point of view of potential hazards to livestock (*32*). It is, therefore, particularly interesting that recent work has attributed beneficial functions to this element. For example, minute amounts of selenium added to the diet will, under varying circumstances, prevent necrosis of the liver of rats (*23*), exudative diathesis of chicks (*25*), multiple necrotic degeneration in mice (*5*), liver necrosis in pigs (*8*), and white muscle disease in lambs (*15, 20*). In addition, there has been evidence presented indicating a positive growth response in lambs to selenium supplementation of the ewe (*6, 14, 18*), although other data show that such responses are not consistently obtained (*12, 26, 34*). Also, in Canadian experiments, administration of subtoxic levels of selenium to sheep produced an increase in wool fiber thickness and total fleece weight (*29*). Reviews on the subject of selenium in nutrition have been prepared by Hashem (*11*), Sharman (*27*), Schwarz (*22*), Cousins and Cairney (*4*), and Scott (*24*), among others.

Experiments in this general area of interest carried out at Oregon State University have been oriented toward the control of white muscle disease (WMD) in young ruminants, and have involved diets composed of natural feedstuffs. The procedures followed have been described in some detail by Schubert *et al.* (*21*). While it has been tacitly accepted by the investigators that the disease is of dietary origin, the precise nature of the nutrient interrelationships has not been described, due partly to the complexity of dealing with diets composed of crude feedstuffs, and more particularly to what Schwarz has termed "(the) elusive quality about the element" which has made selenium analysis most difficult (*22*). This paper describes an experiment designed to demonstrate relationships between selenium content of forage