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THE METABOLISM OF ^{35}S-(1,2-DICHLOROVINYL)-L-CYSTEINE IN THE CALF*

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Abstract—^{35}S-(1,2-dichlorovinyl)-L-cysteine (DCVC) was injected intravenously into calves to study the rate and nature of its metabolism and the possible preferential accumulation of the tracer in hematopoietic tissues. The DCVC molecule is attacked very rapidly as shown by the fact that, although it disappeared from the blood plasma and lymph in less than 80 min, none could be found in the urine in spite of a very high rate of excretion of the tracer. Evidence was obtained for the presence of at least nine radioactive compounds in the urine, among which only inorganic sulfate has been identified. Among the radioactive products in the urine the proportion of inorganic sulfate increased rapidly while that of others decreased. Inorganic sulfate and ^{35}S associated with the protein and lipid fractions of blood plasma and lymph fluid reached the highest concentrations in about 40 min. The bile collected between 20 and 40 min after injection of ^{35}S-DCVC contained extremely high levels of radioactivity in the form of inorganic sulfate and three unidentified components, but not DCVC.

The highest concentration of radioactivity was found in the kidney; the liver contained one-fourth to one-sixth as much, other organs and tissues less. In liver and kidney, as in subcellular fractions prepared therefrom, over half the radioactivity was associated with the protein fraction and one-sixth to one-fourth in the lipid fraction. Very little radioactivity was found in bone marrow cells removed by biopsy or in red bone marrow collected at slaughter.

The metabolism of DCVC in the calf is strikingly different from that in the rat, in keeping with the different biological response of these two species to DCVC.

APLASTIC anemia in calves[1] can be induced by the daily intravenous injection of as little as 0·015 mg of *S*-1,2-(dichlorovinyl)-L-cysteine[1,2] for several months‖ or by a single dose of as little as 2 mg per kg body weight. In the latter case there is no apparent effect until about 2 weeks have elapsed, when a severe blood dyscrasia develops rapidly which terminates in death 1 to 2 weeks later. If smaller daily doses of DCVC, of the order of 0·1 to 0·2 mg per kg, are injected intravenously for 10 days,[3] the calves develop

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a severe hematologic crisis after about 3 weeks, from which some, but not all animals recover.

In contrast to the calf, the rat is much more resistant to the toxic effects of DCVC[3] and does not develop aplastic anemia. When DCVC is administered to the rat, some of it is excreted unchanged in the urine, some as the N-acetyl derivative, and some is metabolized to inorganic sulfate and at least one other compound.[4] It became of interest, therefore, to ascertain whether the metabolism of DCVC in the calf follows a similar pattern and whether DCVC or products of its metabolism are localized in the hematopoietic tissues or other organs. This paper summarizes some of our observations on the metabolism of DCVC in the calf, as revealed by ^{35}S-labeled DCVC, previously synthesized for this purpose.[5]

EXPERIMENTAL PROCEDURES

Animals. Female Holstein calves weighing about 45 kg were purchased in the open market, housed in pens provided with clean wood shavings, and fed whole cows' milk, a grain mixture, and good quality alfalfa hay. They had access to water and a mineralized salt block. They were observed for clinical evidence of abnormalities and conditioned for a period of about 2 weeks while complete blood counts were made frequently. Two calves were selected for the desired preliminary surgical procedures.

Surgical procedures. Calf A was fitted with a polyethylene cannula which connected the thoracic duct to the humeral branch of the left cephalic vein. The cannula was secured to each vessel by ligatures, exteriorized, and cut in the middle; the two cut ends were joined by a polyethylene sleeve. This preparation permitted collection of lymph uncontaminated with blood, and it also afforded, while lymph was not being collected, its normal discharge into the venous flow.

Calf B, in addition to the above, was fitted with a polyethylene catheter which was threaded through an incision in the gall bladder via the cystic duct into the hepatic duct, where it was fastened by ligatures; the bile duct was ligated near the duodenum. The catheter was exteriorized through the surgical incision. Through the same incision the pancreatic duct was reached and ligated near its point of entry into the duodenum. The flared end of a polyethylene catheter† was inserted, fastened to the duct with ligatures, and exteriorized through a puncture in the abdominal wall. All incisions were closed by sutures.

These surgical procedures were done under anesthesia induced by sodium pentobarbital, while 0·9% sterile NaCl solution was allowed to run continuously into the right jugular vein at a rate of about 200 ml/hr. The trachea was intubated during the operation. Before surgery all solid food was withheld for 36 hr and liquids for 12 hr. Methylatropine nitrate (5 mg) and procaine penicillin (200,000 units) were injected just before induction of anesthesia.

Recovery from the operations was prompt; calf A was used for the tracer experiment 48 hr, calf B, 24 hr after surgery. At that time the calves appeared to be clinically normal. They were suspended in a canvas sling which was fastened to a stanchion.‡

* Unpublished observations by the authors.

† Made from polyethylene tubing (PE 280, Clay Adams, I.D. 0·085 "X OD 0·128")

‡ We are indebted to Drs. A. F. Sellers and C. M. Stowe, Dept. of Veterinary Physiology, for use of the facilities in the large animal laboratory.

The height of the sling was adjusted so that the calf could rest in it or stand on the floor. The calves had access to water and hay during the tracer experiment,* and they were fed milk at intervals of about 8 hr.

DCVC was dissolved in aqueous, sterile 0·9% NaCl (2 mg per ml); a 0·02-ml aliquot was removed for determination of the radioactivity, and the remainder was injected through a deep indwelling catheter into the left external jugular vein, the syringe and catheter being rinsed with sterile 0·9% NaCl solution. The amounts of DCVC and radioactivities thus administered were: to calf A 1·44 mg/kg, $5{\cdot}10 \times 10^9$ disintegrations per minute (DPM); to calf B 1·57 mg/kg, $2{\cdot}56 \times 10^9$ DPM.

For quantitative collection of urine, as voided, a rubber conduit was fitted over the vagina of calf A and fastened with rubber cement in a tight seal. After each discharge this conduit was rinsed by injection of a known volume of distilled water. To permit continuous collection of urine as it reached the bladder, calf B was fitted with an indwelling, retaining, urethral catheter through which fluid injected into the bladder could be quantitatively recovered. The catheter drained through fixed polyethylene tubing into test tubes or polyethylene bottles. In this manner 19 specimens of urine were collected from calf B during the first 2 hr when the greatest changes in radioactivity were anticipated; during the remaining 22 hr, 28 specimens were collected. The urine was stored at 4°.

Feces were collected on plastic sheets as voided, transferred into plastic bags, and kept at −15° until sampled. For this purpose they were dried, first in a ventilated, steam-heated cabinet, and finally in a vacuum oven at 52°. The dried material was weighed, crushed in plastic bags, and representative specimens burned in Schöniger flasks and analyzed as described in the preceding paper.[4] The feces from calf B were of a light gray color, a fatty consistency, and appeared to contain some undigested curds of milk.

From calf B bile and pancreatic juice were collected from the respective cannulas through exteriorized polyethylene tubing into test tubes. For the first 3 hr there was a continuous flow of bile at a rate of 0·4 to 0·6 ml/min. Thereafter the flow of bile became erratic and intermittent, and it ceased after 12·5 hr. Evidence of jaundice developed in calf B after 18 hr which caused us to terminate the experiment after 24 hr. Post-mortem examination revealed a small thrombus which obstructed the cannula in the hepatic duct.

The flow of pancreatic secretion continued at a rate of 0·3 to 0·4 ml/min throughout the 24 hr of the experiment with calf B. Intermittently, after some vigorous motion by the calf, the colorless secretion became tinged with traces of bile. Post-mortem examination showed a small duct which led from the proximal end of the bile duct to the pancreatic duct; about half the time this anastomosis was not functional. Because the radioactivity of the bile was much higher than that of the colorless pancreatic secretion, contaminated specimens of the latter were not considered in the analysis of the results.

Lymph (10 ml) was collected intermittently† and at the same time as venous blood, from the disconnected slip-joint of the exteriorized anastomosis between the thoracic duct and the cephalic vein. The lymph, in heparinized tubes, was centrifuged for

* We acknowledge the assistance of R. O. Wollan, Health Physicist of the University, who monitored for escape of radioactivity while the experiment was in progress.

† See first footnote page 478.

separate analysis of the fluid and the cells. Blood was withdrawn at intervals* with a heparinized syringe through an indwelling polyethylene catheter from the right external jugular vein. Bone marrow was collected by puncture of the crest of the ileum and ischium four times from calf A and three times from calf B during the course of the experiment, and immediately after slaughter from ribs and sternebrae. Measured volumes of blood, lymph, and bone marrow were centrifuged in the cold, the cells were separated from the fluid and washed once with cold homologous blood plasma (collected on the day before injection of ^{35}S-DCVC), and twice with 0·9% NaCl. They were dried *in vacuo* and weighed.

Calf A was slaughtered after 72 hr, calf B after 24 hr. Specimens of many organs were removed at that time, great care being taken to avoid contamination. The tissues were dried, ground, and oxidized by the procedures used previously for rat tissues.[4]

Analysis of radioactivity. Aliquots of blood plasma, lymph, and bile, diluted with known volumes of water when necessary, were plated on weighed planchets, dried under an infrared lamp, and counted with a thin-window Geiger-Muller counter; appropriate corrections were made for background activity and self-absorption, where necessary. Eluates from the ion-exchange column were counted by the same procedure.

Aliquots of the dried hemic cells or tissues (10 mg) were mixed with 20 mg sucrose and burned in a 2-L Schöniger flask[6] containing 5 ml 0·01 M NaOH and 5 ml bromine water. After combustion and absorption of the vapors the solutions were evaporated and made to volume, for counting of aliquots.

Radioactivity of the solutions obtained from combustion of specimens of calf B, and of 1 : 10 aqueous dilutions of urine, blood plasma, lymph fluid, and pancreatic secretion from the same animal were counted with a scintillation counter,† the vials containing up to 0·60 ml of the aqueous sample, 1·0 ml of Hyamine‡ hydroxide 10× (1 M in methanol), 5·0 ml of absolute ethanol, and 10 ml of toluene containing per liter 5·0 g 2,5-diphenyloxazole§ and 0·3 g p-[2-(5-phenyloxazolyl)]-benzene,§ adapted from the system used by Bartlett and Shelata.[7] The counting efficiency of this system for ^{35}S was 58·6%. The results obtained with the different counting systems are expressed in absolute terms, as disintegrations per minute, using a certified sulfate standard† for reference. All results were corrected for decay of ^{35}S to the day of administration of the tracer.

Fractionation of tissues and fluids. Kidney and liver homogenates in 0·25 M sucrose were separated into subcellular fractions by centrifugation.[8] For chemical fractionation, 2 ml of homogenate, plasma, or lymph fluid was deproteinized with 4 ml of cold 0·6 M $HClO_4$. The precipitate was washed four times with 2 ml of 0·4 M $HClO_4$. The supernatants of the acid extracts and washings were combined, neutralized with 12 M KOH, freed in the cold of $KClO_4$, and lyophilized. The dried residues were dissolved in 2 ml of water, used for assay of radioactivity, paper chromatography,‖ and determination of nitrogen[9] and sulfate,[10], where pertinent.

* With calf B, blood and lymph were collected at the following time intervals after injection of ^{35}S-DCVC: 10, 20, 40, 80, 120 min; 4, 8, 12, 24 hr; blood also at 2 min. Bone marrow was collected 4, 8, 12, 24 hr after injection. With calf A the corresponding time intervals were: for blood and lymph 20, 40, 80, 120 min; 4, 8, 12 24 48, 72 hr; for bone marrow 4, 12, 24, 48, 72 hr.

† Nuclear Chicago Corporation, model 703.

‡ Packard Instrument Company, LaGrange, Ill.

§ Pilot Chemicals, Inc., Watertown, Mass.

‖ Unpublished work by the authors.

The lipid fraction of the homogenates or fluids was obtained by four successive extractions of the $HClO_4$-insoluble precipitates at 50° with ethanol : ether (3 : 1 vol/vol) followed by two extractions with CH_3Cl : methanol : HCl (200 : 100 : 1 vol/vol). The combined extracts were flash-evaporated, and the residue was mixed with a known weight of cellulose powder prior to combustion in a Schöniger flask. The extracted precipitates were dried *in vacuo* and weighed, and aliquots were mixed with sucrose for combustion. Radioactive components of urine were separated by paper chromatography or ion- exchange separation as in the case of rat urine.[4] The paper chromatograms were cut into 1-cm segments for counting, or scanned,* and the tracings were evaluated with the aid of a planimeter. The identity of sulfate was established by its chromatographic and electrophoretic movements, and by treatment with $BaCl_2$ or benzidine which, in the case of blood plasma extracts, precipitated 99·8% and 99·1%, respectively, of the radioactivity.

Subcellular localization of ^{35}S. Radioautograms were made of smears of blood, lymph, and bone marrow by applying a liquid emulsion† to the smear. Paraffin sections of formalin-fixed tissues were made and covered with Kodak AR-10 stripping film. Both types of preparations were exposed for 100 days at 4°, then treated with Kodak DK 19B developer, rinsed, and fixed with Kodak acid fixer. The hemic smears were stained with Leishman-Giemsa stain and the tissue sections with hematoxylin and eosin.

RESULT AND DISCUSSION

The results obtained in the 24-hr experiment with calf B are in many respects most informative. They will be presented in some detail with confirmative or supplementary data obtained with calf A.

^{35}S in blood and lymph. As shown in Fig. 1, after i.v. injection of ^{35}S-DCVC there was a rapid, essentially exponential disappearance of ^{35}S from blood plasma. The transfer of ^{35}S into the mixed blood cells was slow, and there was no evidence of equilibration between plasma and cells. Throughout the experiment the radioactivity per volume of the lymph fluid was slightly below that of the blood plasma. In contrast to the cells of the whole blood, the lymph cells acquired only traces of radioactivity. With calf A essentially the same relationships were found. Between hours 24 and 72 there was a steady, parallel decrease in the radio-activity of blood plasma and lymph. Ion exchange separation[11] of the acid-soluble components in a pooled specimen from the first four samples of blood plasma from calf B revealed only two major components, corresponding to inorganic sulfate and DCVC respectively.

Fractionation of blood plasma and lymph, followed by paper chromatography—procedures which afforded on the average 87% recovery of the total radioactivity—revealed a very rapid disappearance of DCVC, as shown in Fig. 2; in the 80-min specimens none could be detected. Simultaneously there occurred a rapid increase of the "sulfate" fraction which consisted mainly, although not necessarily wholly, of inorganic sulfate (see below under *urine*). After disappearance of DCVC, the sulfate fraction of plasma accounted for more than 90% of the acid-soluble radioactivity; in the lymph, through the first 12 hr, this proportion was distinctly less. As DCVC

* Vanguard model 800 auto scanner.

† Kodak NTB, Eastman Kodak Co.

disappeared from the body fluids there occurred also a rapid increase in the radioactivity in the lipid and protein fractions, which reached a maximum in 40 to 80 min and then gradually decreased. In the blood plasma the protein-bound radioactivity was distinctly higher than in the lymph. When the results were calculated in terms of specific activity (per mg total S), the highest values in the plasma and lymph were

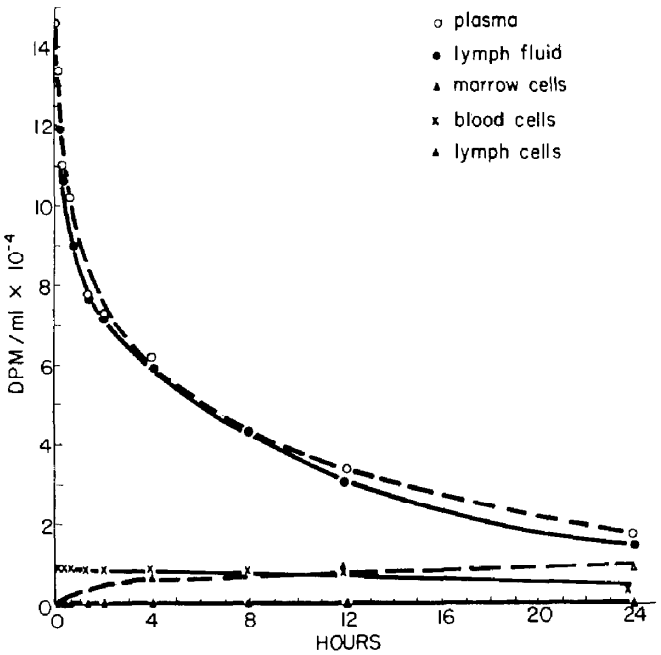

FIG. 1. Radioactivity in blood plasma, lymph fluid, blood cells, lymph cells, and bone marrow cells from calf B after i.v. injection of ^{35}S-DCVC at 0 hr.

obtained in the specimens collected 20 and 40 min after injection of DCVC. In the blood plasma of calf A, nondialyzable^{35}S was observed within 10 min; this fraction reached a maximum of 20% in 20 min and then remained fairly constant at about 12% of the total radioactivity of the blood plasma. As was the case with rats[4] a small amount of radioactivity became also firmly associated with crystalline hemoglobin prepared[12] from the 72-hr specimen of blood of calf A. This radioactivity could not be removed by dialysis against water, 0·9% NaCl, or by extraction with trichloroacetic acid before or after treatment with sodium sulfite or 2-mercaptoethanol. The radioactive component was bound to the hemoglobin, presumably through covalent bonds other than disulfide.

The rapid disappearance of DCVC from the blood plasma and the concurrent appearance of radioactivity in the sulfate, protein, and lipid fractions emphasize the speed with which DCVC is metabolized in the calf. This is also in accord with the quick appearance of several radioactive components, including inorganic sulfate in the urine.

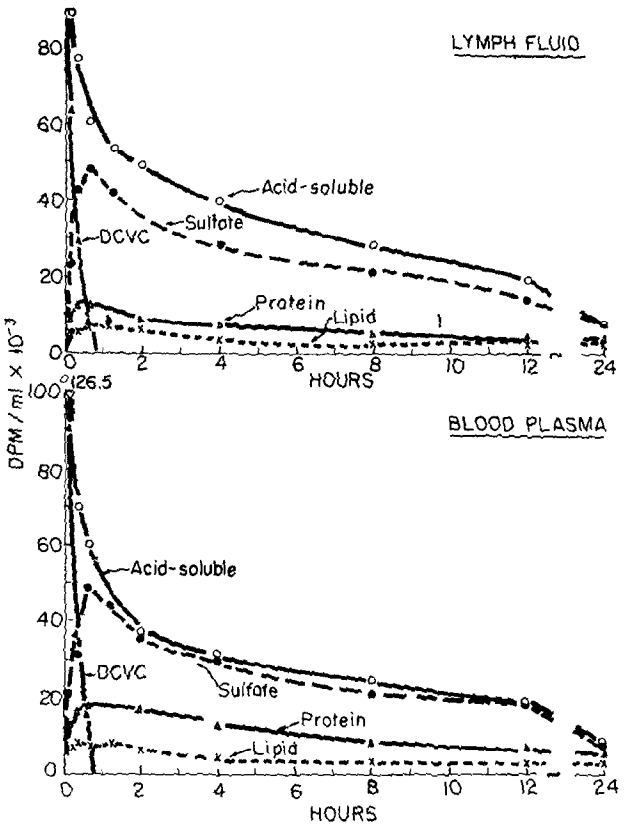

FIG. 2. Distribution of ^{35}S in various fractions of blood plasma and lymph fluid from calf B after injection of ^{35}S-DCVC at 0 hr.

TABLE 1. EXCRETION OF ^{35}S IN BILE

Specimen	Time interval (min)*	Flow rate (ml/min)	Radioactivity (DPM/ml)
1	0– 10	0·48	860
2	10– 20	0·65	$1·80 \times 10^5$
3	20– 40	0·51	$6·80 \times 10^6$
4	40– 80	0·43	$4·28 \times 10^6$
5	80–120	0·40	$1·29 \times 10^6$
6	120–175	0·41	$5·00 \times 10^5$

* After injection of DCVC, while specimen was being collected.

*^{35}S in bile.** The flow of bile remained steady during the first 3 hr while six specimens were collected, and during this time apparently the most important changes occurred (Table 1). The specimen collected between 20 and 40 min after injection of DCVC contained by far the highest radioactivity (per ml), exceeding that of urine and blood plasma during the same periods, about 4·5- and 80-fold respectively. Separation of

* We acknowledge with thanks the assistance of H. Hof with the study of the radioactive components of bile.

this bile on Dowex-50 ion-exchange resin by the procedure of Moore and Stein[11] revealed the presence of at least three radioactive components (corresponding to urine fractions A = 46·5%, C = 6·4%, and E = 47·1% shown in Fig. 4) among which the "inorganic sulfate" fraction A contained almost one-half of the total activity. Paper chromatography of lyophylized, water-soluble solids of this specimen of bile revealed a fourth radioactive component of high mobility (Rf 0·95) which may have been formed from the interaction of ^{35}S-DCVC with components of normal, nonradioactive bile as shown by control experiments. By means of paper chromatography, no evidence was found for the presence in the bile of ^{35}S-labeled taurine, taurocholic acid,[14] or cysteic acid, DCVC or N-acetyl DCVC.[4] Only the presence of inorganic radioactive sulfate could be verified in bile, which is in accord with its rapid appearance in blood plasma, lymph, and urine. The high radioactivity of the bile collected within 40 min after injection of ^{35}S-DCVC suggests that DCVC undergoes rapid metabolic changes in the liver.

Pancreatic secretion. The radioactivity of this secretion was much lower than that of any other fluid examined. The highest radioactivity, 7,000 DPM/ml, was found in a specimen uncontaminated with bile, collected between 40 and 80 min after injection of DCVC. The specimen collected between 0 and 10 min contained only 120 DPM/ml, all of it acid-soluble (0·2 M $HClO_4$), while the last specimen, collected during hour 24 of the experiment, contained 970 DPM/ml, 82·4% of which was acid-soluble. Among ten specimens which were uncontaminated with bile, an average of 75·2% of the radioactivity was acid-soluble and there was no evidence that a greater proportion of the radioactivity became protein bound with longer elapsed time since injection of DCV.

^{35}S in feces. During the 72-hr experiment calf A excreted in the feces 17·8% of the administered radioactivity, not including the material remaining in intestine at slaughter. The radioactivity rose from 466,000 DPM/g dry weight in the first specimen, (collected after 7·5 hr) to 25·6 million/g in the third specimen (collected after 9·75 hr) and then gradually decreased to 1 million DPM/g after 67 hr. In calf B, where the entry of bile into the intestine was prevented, the radioactivity was much lower, the maximum being 250,000 DPM/g dry weight in a specimen collected after 15·5 hr, and there was no distinct period of maximum excretion of ^{35}S. Evidently most of the radioactivity entered the contents of the intestinal tract of calf A via the bile. Chromatography on Dowex-50[12] of an aqueous extract of the most radioactive fecal specimen from calf A revealed the presence of at least three radioactive compounds which correspond to components A, B, and C in Fig. 4. The component corresponding to component E in Fig. 4, which was prominent in the bile of calf B, was not observed in the fecal extract from calf A.

^{35}S in tissues. For ease of comparison the data in Table 2 are expressed in relative terms, using as point of reference the kidney, which contained by far the highest radioactivity. If one considers the difference in time elapsed between injection of DCVC and slaughter of the two calves there is, with few exceptions, a remarkable correspondence in the relative radioactivity of the various organs and tissues. Aside from the relatively high radioactivity of kidney and liver, the high activity of the vitreous humor in both calves and of the aqueous humor and the salivary glands in calf B are noteworthy. The presence of sulfated mucopolysaccharides in these tissues, as well as in cartilage, which was more radioactive in calf A, may explain their relatively

high radioactivity. The relatively low radioactivity of the bone marrow, particularly in calf B, is of special interest inasmuch as in the calf the manifestations of DCVC toxicity suggest that it elicits a block in the normal hematopoietic activity. If the bone marrow is a target organ for DCVC, the amounts of this compound involved would be extremely small. It is quite possible, however, that DCVC contributes to the formation of a metabolic antagonist which, although formed elsewhere, eventually

TABLE 2. RADIOACTIVITY OF CALF TISSUES

Tissue	Relative radioactivity per gram dry tissue	
	Calf B (24 hr)	Calf A (72 hr)
Kidney	100 ($1{\cdot}97 \times 10^6$ DPM)	100 ($3{\cdot}69 \times 10^6$ DPM)
Liver	26·9	15·3
Aqueous humor	11·7	1·2
Vitreous humor	11·8	15·7
Salivary gland, submaxillary	10·0	5·7
Lymph node, retropharyngeal	9·78	6·8
Lymph node, prefemoral	9·00	5·7
Lymph node, mesenteric	9·00	5·5
Pancreas	8·16	6·9
Abomasum	7·71	5·6
Lymph node, prescapular	6·81	6·1
Small intestine (duodenum)	6·62	3·8
Ovaries	6·55	5·4
Spleen	6·55	4·5
Auriculoventricular valves	6·10	7·9
Large intestine	5·92	3·5
Lung	5·41	5·8
Auricle, left	5·41	4·3
Adrenals	4·61	4·3
Thyroid	4·41	
Pituitary, whole	4·40	4·3
Brain, cortex	4·16	3·3
Ventricle, left	3·52	4·4
Bladder	3·47	
Thymus	3·45	3·4
Cartilage, costochondral	3·07	15·1
Aorta	2·81	6·0
Cornea	2·04	2·7
Skeletal muscle	1·12	
Bone marrow	0·79	6·6
Lenses	0·59	
Tibial bone	0·48	1·1

finds its way into the bone marrow and its hematopoietic loci. The higher relative radioactivity of the bone marrow of calf A, and its slow increase in the specimens withdrawn by biopsy during the progress of both experiments are in accord with the suggestion made above.

The analyses summarized in Table 3 were made to obtain some information about the chemical homogeneity of ^{35}S-labeled compounds in tissues and on their possible localization in subcellular fractions of kidney and liver. In these tissues from calf B over half the total radioactivity was associated with the protein fraction of the tissues, about 20% was acid-soluble and most of the remainder was lipid soluble. The appearance of radioactivity in the lipid and protein fractions has also been observed when ^{35}S-DCVC was incubated for 30 min with bovine kidney homogenates.* In the

* Unpublished work by the authors.

mitochondrial and the microsomal fractions of both liver and kidney the lipid-soluble radioactivity was present in higher relative amounts than the acid-soluble ^{35}S. As might be expected, only a relatively small proportion of the total radioactivity was found in the nucleic acid fraction, but more appeared in the liver than in the kidney. In calf A, slaughtered after 72 hr, the relative radioactivity of the nucleic acid fraction of liver and kidney was 4·6% and 1·9% of the total, much higher than in calf B which was slaughtered after 24 hr.

TABLE 3. DISTRIBUTION OF ^{35}S IN LIVER AND KIDNEY, CALF B

Fraction Organ	Nuclear	Mito- chondrial	Micro- somal	Super- natant	Total
	DPM as per cent of homogenate activity				
Acid-soluble					
Kidney	1·5	3·2	0·68	16·8	22·2
Liver	1·1	1·8	0·93	16·5	20·3
Lipid-soluble					
Kidney	1·8	6·1	2·4	5·2	15·4
Liver	3·3	9·4	4·6	9·0	26·3
Nucleic acid					
Kidney	0·02	0·06	0·02	0·25	0·36
Liver	0·25	0·42	0·16	0·43	1·26
Protein					
Kidney	6·3	24·2	8·4	23·3	62·1
Liver	11·1	15·8	10·0	15·3	52·2

Further evidence of extensive association of radioactivity with the proteins of liver and kidney was obtained by subjecting these fractions to exhaustive dialysis against water, at 4°, before and after digestion with Cotazyme,* a treatment through which about 60% of the "protein-bound" radioactivity became dialyzable.

The mitochondrial and microsomal fractions of liver and kidney had the highest radioactivity when calculated in terms of total nitrogen (Kjeldahl) content; calculation in terms of sulfate (after combustion of the tissue) failed to disclose a distinct pattern of highest concentration of radioactivity.

The radioautographs revealed high concentrations of radioactivity in the parenchymal cells of liver, in the tips of the villi of the small intestine, and in the kidney, in which the glomerular epithelium showed a low, but the tubular epithelium a high, accumulation of radioactivity.

^{35}S in urine. The procedure used with calf B permitted analysis of specimens collected, as excreted, during short intervals. The data in Fig. 3 show that after the first 7 min there was an extremely rapid increase in the amount and rate of excretion of ^{35}S. During the next three intervals of 3, 4, and 5 min, respectively, the excretion was from 14 to 16 million DPM, a rate which thereafter dropped to 50% or less of this value. Due to marked fluctuations in the flow rate of urine (which may be a reflection of kidney damage caused by DCVC), the concentration of ^{35}S in the urine showed much greater variations than the absolute rate of excretion. At the beginning of the hour 5 the rate of excretion had decreased to 1 million DPM which was never exceeded

* A mixture of proteolytic enzymes from pancreas, manufactured by N. V. Organon, Oss, Holland.

thereafter. If one considers the small amounts of DCVC administered (1·5 mg/kg) it is remarkable that calf B excreted during 1, 2, and 24 hr 14·9%, 22·6% and 55·7% (cumulative) of the administered radioactivity. In calf A the rate of excretion, although also high, was somewhat slower: 7·0%, 16·3%, 40%, and 50·4% of the administered dose having been voided in 44 and 142 min, and 23·5 and 72 hr respectively.

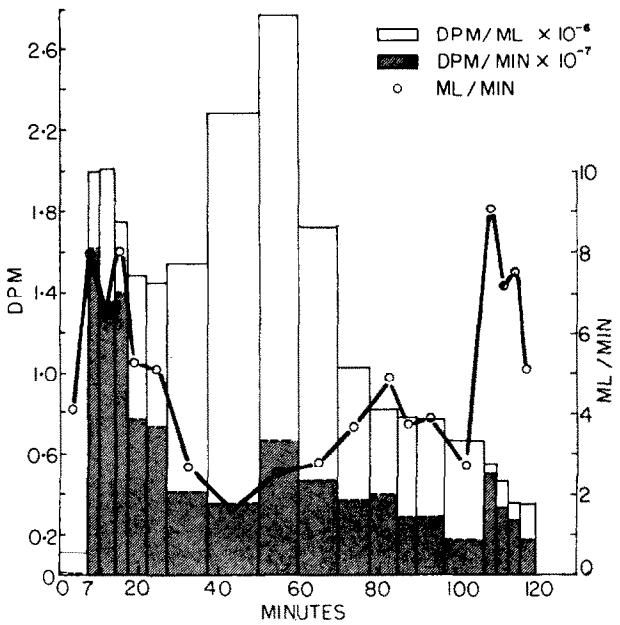

FIG. 3. Rate of urine flow ○——○. Rate of excretion of ^{35}S, shaded bars. Concentration of ^{35}S in urine, open bars. After i.v. injection of ^{35}S-DCVC at 0 min.

Nature of radioactive components in urine. Although the resolution of components through paper chromatography by methods which were satisfactory for rat urine[4] was not so sharp with calf urine, it became evident that the first few specimens of urine collected from calf B contained at least six radioactive components, five of which had virtually disappeared in specimens collected 2 hr after administration of ^{35}S-DCVC. Ion-exchange separation[11] on Dowex 50-X5 resin was used with the urine from both calves. The elution patterns of two specimens collected from calf B for 3-min intervals between 7 and 10 and 110 and 113 min after injection of ^{35}S-DCVC are shown in Fig. 4. With increased elapsed time after injection of ^{35}S-DCVC, the excretion pattern shifted largely to a predominance of component A which, in the second hour, was mainly inorganic sulfate. This is further emphasized by the data in Table 4 which also shows for comparison the excretion pattern observed with rat urine.[4] In rat urine the components A, C-1, and F referred to in Table 4, were identified[4] (conclusively) as inorganic sulfate, N-acetyl-DCVC, and DCVC respectively. In calf urine only inorganic sulfate could be identified in component A; but electrophoresis of the second specimen of urine from calf B, on Whatman 3MM paper, in veronal–acetate buffer, pH 8·6, ionic strength 0·1, revealed that the "sulfate fraction" A (Fig. 4) was not homogeneous. Quantitative analysis demonstrated that of the total

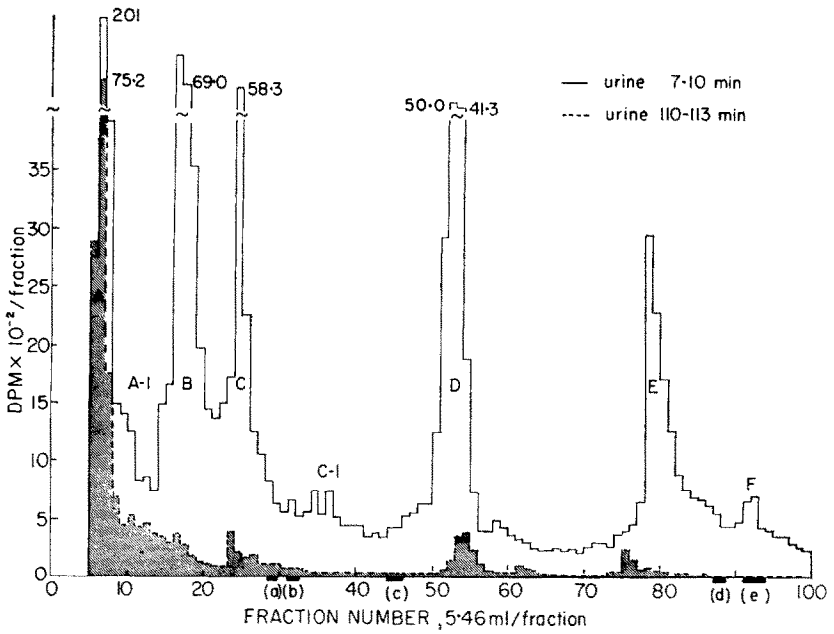

FIG. 4. Elution pattern of urine from calf B, from Dowex 50-X5; ——, pattern from 5 ml urine collected 7 to 10 min after injection of ^{35}S-DCVC and containing $2{\cdot}02 \times 10^6$ DPM/ml; – – –, pattern from 5 ml urine collected between 110 and 113 min after injection of ^{35}S-DCVC and containing $0{\cdot}46 \times 10^6$ DPM/ml. Bars below abscissa show areas of elution of authentic specimens of (a) *S*-carboxymethyl cysteine; (b) N-acetyl DCVC and DCVC-sulfoxide; (c) *S*-(1,2-dichlorovinyl)-mercaptoacetic acid; (d) *S*-(1,2-dichlorovinyl)-mercaptopropionic acid; (e) DCVC. *S*-(1,2-dichlorovinyl)-mercaptoethylamine was not eluted with 546 ml of eluent.

TABLE 4. DISTRIBUTION OF RADIOACTIVITY IN COMPONENTS OF URINE

Component* from Dowex-50	Specimen and time interval (min)†					
	Calf A 0–44	Calf B 7–10	Calf B 10–14	Calf B 60–70	Calf B 110–113	Rat‡
	Per cent of total radioactivity in eluates					
A§	22·1	32·5	50·0	71·3	82·9	24·7
A-1	3·6					2·6
B	16·5	25·7	16·3	12·6	7·8	2·1
C	15·3	14·7	11·8	6·1	3·4	2·4
C-1	4·4					59·5
D	25·2	16·2	16·2	10·1	4·4	
E	11·6	10·3	5·6		1·6	0·9
F	1·3	0·6				8·1
Recovery of ^{35}S applied to column (%)	50	51	58	61	74	88

* Components emerging with the same volumes of eluent, as shown in Fig. 4.

† Minutes after administration of ^{35}-DCVC.

‡ A mixture of urine from two rats, 65% from a specimen voided 5 hr, 35% from a specimen voided 12 hr after i.p. injection of ^{35}S-DCVC.

§ Component A was not homogeneous when examined by paper electrophoresis or by differential precipitation with benzidine before and after oxidation with bromine.

radioactivity in component A (which had been separated from specimens collected from calf B in the time intervals of 7 to 10, 10 to 14, 60 to 70, and 110 to 113 min after injection of ^{35}S-DCVC), 30%, 46%, 84%, and 85%, respectively, could be precipitated as benzidine sulfate. After oxidation with bromine* the proportion of radioactivity which could thus be precipitated from these specimens increased to 78%, 86%, 98%, and 92% respectively. Application of the same oxidative procedure to components B and C, which had been separated from the urine collected between 10 and 13 min, afforded radioactive benzidine sulfate only after bromine oxidation when the yields were 11% and 46% of the total radioactivity of components B and C. It is evident, therefore, that the procedure of ion-exchange separation used was not effective in resolving the complex mixture of radioactive compounds which appear in the urine of the calf shortly after administration of ^{35}S-DCVC. The poor recovery of the total radioactivity applied to the exchange column emphasizes this point (Table 4). Using the methods,[4] among others, which led to the successful identification of DCVC and its N-acetyl derivative in rat urine, a diligent search, including dilution analysis, was made for the presence of these compounds in the urine of both calves. The elution pattern shown in Fig. 4 suggested that at least small amounts of these compounds might be present in the first few urine specimens collected; the rapid clearance of DCVC from the blood gave further impetus to a search for it in the urine. In no case could any decisive evidence be obtained for the presence of DCVC or N-acetyl DCVC in the urine of the calves. If they were present, the amounts were very small, considering the total radioactivity administered and excreted. None of the other radioactive components (other than inorganic sulfate) could be identified. The location in the elution pattern of several compounds which are structurally related to DCVC is shown in Fig. 4. No evidence was found for the presence of radioactive cysteine, cystine, taurine, or cysteic acid in the urine of these calves.

These studies show that the DCVC molecule is attacked rapidly in the calf and that its sulfur moiety gives rise to many different compounds, including inorganic sulfate. One or more of these sulfur compounds becomes rapidly associated with the protein fraction of tissues, or with lipid-soluble components, or both (or becomes soluble in lipid solvents). If water-soluble compounds with similar functional groups can enter analogous reactions with the sulfur-containing products formed *in vivo* from DCVC, this would account for the many different radioactive compounds that were found in bovine urine. The sulfur-containing fragment which is so rapidly formed in bovine tissues from DCVC is apparently an extremely reactive entity. The possibility that it may function as a biological alkylating agent has been considered in the context of our studies with bovine tissues *in vitro*.† Various aspects of this problem are now under investigation in these laboratories.

* The appropriate fraction eluted from the Dowex-50 column was diluted with 5 volumes of water, treated with half the total diluted volume of bromine water, and boiled for 1 hr.

† Unpublished work by the authors.

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