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Radioisotopic Studies on Percutaneous Absorption. II.[1] Comparison of the Rate of Absorption through Mouse Skin of Several Water-soluble Substances from Emulsion-type Ointments

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In a comparison of the percutaneous absorption of $^{22}Na^{+}$ with that of $^{36}Cl^{-}$, zinc-EDTA-^{65}Zn, ferric-EDTA-^{59}Fe, ferric-tiron-^{59}Fe or ferric-chromotropic acid-^{59}Fe by the "double isotopic method," it was demonstrated that water-soluble substances added simultaneously to absorption or hydrophilic ointment were absorbed at widely different rates through hair-clipped mouse skin. These results suggest that water-soluble substances added to an emulsion-type ointment may be absorbed through the skin into the body not as emulsified particles themselves but as solution or particles ultimately free of ointment base.

It was also demonstrated that percutaneous absorbability of ferric-chromotropic acid-^{59}Fe was significantly lower than that of ferric-tiron-^{59}Fe when a comparison of the rates of absorption of these two radioactive compounds was made indirectly by using the absorption rate of $^{22}Na^{+}$ added simutlaneously with each ^{59}Fe-chelate compound to the absorption ointment as a standard. Thus, it was inferred that, when other conditions are similar, the percutaneous absorbability of water-soluble substances from emulsion-type ointment through mouse skin may be decreased with an increase in molecular size.

Notwithstanding the wide acceptance of the "lipid theory"[3] to explain the absorption of medicaments through the skin, many articles in the medical and pharmaceutical literature have indicated the percutaneous absorbability of various water-soluble substances, *e.g.*, heavy water,[4] sodium iodide-^{131}I,[5] sodium phosphate-^{32}p,[6] sodium chloride-^{22}Na,[7] mercuric chloride-^{203}Hg,[7] chromic chloride-^{51}Cr,[8] sodium chromate-^{51}Cr,[8] L-methionine-^{35}S,[9] and cyanocobalamin-^{57}Co.[10]

In a previous paper,[1] we have demonstrated that several predominantly water-soluble substances were absorbed with considerable rapidity from emulsion-type ointment through hair-clipped mouse skin as measured by the disappearance of radioactivity from the skin after topical application of either hydrophilic or absorption ointment containing sodium iodide-^{131}I, ferric-EDTA-^{59}Fe, ferric-tiron-^{59}Fe or sodium chloride-^{22}Na.

It was also observed in the previous paper[1] that ferric-EDTA-^{59}Fe had a tendency to be absorbed faster than ferric-tiron-^{59}Fe from these ointment bases. However, an exact comparison could not be made since the rate of absorption of a substance might be influenced by various factors, among which is the viscosity of each applied ointment.

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The present work was undertaken to determine whether there are distinct differences in the rates of absorption of several water-soluble substances from emulsion-type ointments through hair-clipped mouse skin using the "double isotopic method."

Material and Method

Ointment Bases——Ointment bases used in this study were absorption ointment (W/O type) and hydrophilic ointment (O/W type), the compositions of which have been described in a previous paper.[1] These ointment bases were kindly given by Mr. K. Shiizu of the Hospital Pharmacy, Chiba University.

Radioisotopes——^{22}NaCl (specific activity: 51 mCi/mmole) was purchased from the Radiochemical Center, Amersham. H^{36}Cl (specific activity: 12 μCi/mmole), $^{59}FeCl_3$ (specific activity: 48 mCi/mmole) were obtained from the Oak Ridge National Laboratory, Oak Ridge. Na^{36}Cl was prepared by neutralizing the above H^{36}Cl with sodium hydroxide solution.

Chelating Agents and Other Chemicals——EDTA, tiron (4,5-dihydroxy-*m*-benzenedisulfonic acid disodium salt), chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulfonic acid), and cupferron (ammonium salt of N-nitrosophenylhydroxylamine) were purchased from Wako Pure Chemicals Industries, Ltd., Tokyo. Other chemicals used were of reagent grade from commercial sources.

Preparation and Application of Radioactive Ointment——Two kinds of labeled water-soluble compounds (either Na^{36}Cl or a labeled chelate compound added to ^{22}NaCl) were mixed with an ointment base on a watch glass as specified in the legend of each table. About 50 mg of each radioactive ointment were weighed exactly and applied to a 5 cm^2 area of hair-clipped mouse skin (dd strain, male, weighing 20 to 25 g) in an animal room at 23° and 60—70 per cent humidity. During the application and the different holding periods for absorption, the mice were fixed on their backs in the same animal room.

Determination of Per Cent Absorption of Radioisotopes——After the mice were sacrificed with ether, the area of skin where radioactive ointment had been applied was removed with scissors and the amount of the two radioisotopes contained in each skin sample was measured by the method described in the succeeding sections. To calculate the per cent absorption of radioisotopes, standards were prepared by applying about 50 mg of each radioactive ointment (weighed exactly) to a 5 cm^2-piece of hair-clipped skin removed from a normal mouse. These control skin samples were treated the same as the experimental skin samples.

Measurement of ^{22}Na and ^{36}Cl in a Skin Sample Containing $^{22}Na^+$ and $^{36}Cl^-$——The excised skin was dissolved in a solution of 2 ml of 5% NaOH and 2 ml of 0.5% NaCl by gently boiling the preparation for 20 min in a Kjeldahl-flask. After the solution was cooled to room temperature, it was acidified with conc. HNO_3 using phenolphthalein as the indicator. An excess of one or two drops of acid was added. Immediately, 1.5 ml of 10% $AgNO_3$ were added to precipitate AgCl. The whole suspension was heated with gentle boiling for one hour after the further addition of 3 ml of conc. HNO_3. After this step, all operations were carried out under a tungsten lamp. The precipitate was collected by centrifugation at 2000 × ***g*** and washed with 2 ml of water. The above supernatant fluid and washings were combined and diluted to 10 ml with water. A 5-ml aliquot of this solution was transferred to a test tube and the radioactivity of ^{22}Na was assayed with a well-type scintillation counter.

The above AgCl precipitate was washed successively with water, acetone, and ether on a suction funnel, and finally spread uniformly on a filter paper disc having a diameter of 2 cm (Toyo-roshi No. 5C). The paper disc was placed in a stainless steel planchet and the radioactivity of the ^{36}Cl was assayed with an end-window Geiger-Mueller counter. Recoveries of ^{22}Na and of ^{36}Cl from ^{22}NaCl-skin and Na^{36}Cl-skin control samples were more than 98 and 97%, respectively, when corrected for sample volume and self-absorption.

Measurement of ^{22}Na and ^{65}Zn in a Skin Sample Containing $^{22}Na^+$ and Zinc-EDTA-^{65}Zn——The excised skin was dissolved in a solution of 3.5 ml of conc. HNO_3, 0.15 ml of 40% NaOH, 0.15 ml of 0.01M $ZnCl_2$, and 2 ml of water by gentle boiling in a Kjeldahl-flask. Subsequently, 2 ml of water were added and the preparation was evaporated. After this evaporation process was repeated three more times, the preparation was adjusted to a volume of 5 ml with water and cooled to room temperature. The solution was made weakly alkaline with the addition of 40% NaOH and again acidified with 50% acetic acid to a one-drop excess addition. At this stage, the pH was between 5 and 6. This solution was diluted to 10 ml with water and was extracted with 7 ml of 0.3% dithizone in xylol. 5-ml aliquots of the water layer and of the xylol layer were assayed in a well-type scintillation counter to measure the amounts of ^{22}Na and ^{65}Zn, respectively. Recoveries of ^{22}Na and ^{65}Zn from ^{22}NaCl-skin and zinc-EDTA-^{65}Zn-skin control samples were more than 98 and 97%, respectively, when corrected for sample volume.

Measurement of ^{22}Na and ^{59}Fe in a Skin Sample Containing Ferric-EDTA-^{59}Fe, Ferric-tiron-^{59}Fe or Ferric-chromotropic acid-^{59}Fe in Addition to $^{22}Na^+$——The excised skin was dissolved in a solution of 3 ml of conc. HNO_3, 0.25 ml of 20% NaCl, and 0.1 ml of 0.1M $FeCl_3$ by gentle boiling in a Kjeldahl-flask. After addition of 2 ml of conc. HNO_3, the mixture was evaporated to about 2 ml. The addition of conc. HNO_3 and the evaporation were repeated four more times. Subsequently, 2 ml of water were added and the preparation was again evaporated. After this evaporation process was repeated three more times, the preparation was

adjusted to a volume of about 5 ml with water and cooled to room temperature. The solution was neutralized with 40% NaOH and again acidified by the addition of 0.5 ml of 50% acetic acid. At this stage, the pH was between 3 and 3.5.

This solution was adjusted to 10 ml with water and then mixed with 6 ml of chloroform and 1 ml of 5% cupferron. The mixture was shaken vigorously for one minute and then allowed to separate over night at room temperature. The upper layer was transferred to another test tube, mixed with 0.05 ml of 0.1M $FeCl_3$ and shaken vigorously for one minute after the addition of 4 ml of chloroform and 1 ml of 5% cupferron. After separation of the two layers, a 5-ml aliquot was withdrawn from the upper layer and the amount of ^{22}Na was assayed in a well-type scintillation counter. Meanwhile, the two chloroform layers were combined, the volume was adjusted to 10 ml with fresh chloroform, a 5-ml aliquot was withdrawn to measure the amount of ^{59}Fe and this sample was assayed in the same counter. Recovery in the control skin samples of ^{22}Na from $^{22}NaCl$-skin and of ^{59}Fe from ferric-EDTA-^{59}Fe-, ferric-tiron-^{59}Fe-, and ferric-chromotopic acid-^{59}Fe-skin was more than 97%, when corrected for the sample volume.

Measurement of ^{59}Fe and ^{65}Zn in a Skin Sample Containing Ferric-EDTA-^{59}Fe and Zinc EDTA-^{65}Zn——The excised skin was dissolved in a solution of 3.5 ml of conc. HNO_3, 0.15 ml of 0.01M $FeCl_3$, 0.05 ml of 0.01M $ZnCl_2$, 0.15 ml of 40% NaOH, and 2 ml of water by gentle boiling in a Kjeldahl-flask. Subsequently, 2 ml of water were added and the solution was evaporated. After this evaporation process was repeated three more times, the preparation was adjusted to a volume of about 5 ml with water and cooled to room temperature. The solution was transferred to a graduated test tube, neutralized with 40% NaOH and again acidified by the addition of 0.5 ml of 50% acetic acid. At this stage, the pH was between 3 and 3.5. After addition of 0.15 ml of 0.1M tiron, the preparation was adjusted to 10 ml with water and extracted with 10 ml of 0.2% dithizon in xylol. The amount of ^{59}Fe in 5 ml of the water layer and of ^{65}Zn in 5 ml of the xylol layer was assayed in a well-type scintillation counter. Recovery in the control experiments of ^{59}Fe from ferric-EDTA-^{59}Fe-skin and of ^{65}Zn from zinc-EDTA-^{65}Zn-skin was more than 97%, when corrected for the sample volume.

Result and Discussion

Comparison of $^{22}Na^+$ and $^{36}Cl^-$ Percutaneous Absorption

In order to determine whether there are distinct differences in the rates of absorption of various water-soluble substances from emulsion-type ointments through hair-clipped mouse skin, we first compared the per cent absorption of $^{22}Na^+$ and $^{36}Cl^-$ which are both absolutely water-soluble simple monovalent ions. The sodium chloride added to each oitment was doubly labeled with ^{22}Na and ^{36}Cl. After application of the radioactive ointment to the animals, the remaining amount of ^{22}Na and of ^{36}Cl on each skin sample was measured as described in "Material and Method." As shown in Table I, the degree of absorption of chloride ions was slightly greater than that of sodium ions in both ointment bases. The difference was small but statistically significant on paired observations except in the case of the absorption ointment of 20 hr-duration in which both radioisotopes were almost completely absorbed through the skin.

As for the subcutaneous absorption of Na^+ and Cl^- in mice, Secher-Hausen[11] reported that radioactive sodium ions were cleared significantly more slowly than radioactive chloride ions after the subcutaneous injection of labeled isotonic saline. In a comparison of the percutaneous absorption of several inorganic electrolytes from aqueous solutions through young (3—6 days old) rat skin, Vrignaud, *et al.*[12] reported that anions appeared to penetrate more easily than cations.

Comparison of $^{22}Na^+$, Zinc-EDTA-^{65}Zn, and Ferric-EDTA-^{59}Fe Percutaneous Absorption

As shown in Table II, the degree of absorption of zinc-EDTA-^{65}Zn was very low in comparison to that of $^{22}Na^+$ under all conditions tested. These data show that water-soluble substances simultaneously added to an emulsion-type ointment could be absorbed at widely different rates. Therefore, it may be inferred that water-soluble substances added to each

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Table I. Percutaneous Absorption of ^{22}Na and ^{36}Cl after Application to Mouse Skin of Emulsion-type Ointments Containing ^{22}Na ^{36}Cl

Ointment base	Time of duration of application (hr)	Amount of ointment applied to each mouse (mg)	Per cent absorption of radioisotopes		$p^{a)}$
			^{22}Na	^{36}Cl	
Absorption ointment	1	51.6	68.6	75.9	<0.005
		54.2	66.1	71.6	
		54.6	56.1	62.8	
		54.1	63.8	68.4	
	2	52.0	74.2	81.3	<0.025
		52.3	81.7	85.2	
		53.1	77.9	84.9	
		53.1	76.9	88.7	
	5	49.4	94.6	95.8	<0.1
		53.7	81.7	85.8	
		52.2	96.9	97.3	
		52.3	90.7	92.7	
	20	43.4	98.6	98.5	>0.1
		44.9	97.5	97.7	
		44.2	98.5	97.9	
		51.1	97.9	98.2	
Hydrophilic ointment	7	53.6	46.0	52.7	<0.05
		52.1	29.0	36.8	
		55.2	48.5	62.3	
		53.7	46.1	68.6	
	20	51.6	54.1	80.3	<0.005
		53.5	41.7	65.7	
		53.6	52.4	82.6	
		53.5	41.0	66.7	

An aqueous solution (0.15 ml) containing 0.05 μmole of $^{22}NaCl$ and 5 μmoles of $Na^{36}Cl$ was mixed with 1.0 g of each ointment base. In the case of the hydrophilic ointment of 7 hr duration, 0.15 ml more water was added to the above solution before mixing with the ointment base.

a) The level of significance of difference between per cent absorption of ^{22}Na and that of ^{36}Cl calculated by *t*-test (paired observations).

emulsion-type ointment are absorbed through the skin into the body not as emulsified particles but rather as solution or particles ultimately free of ointment base. The larger absorption of the water-soluble substances from absorption oitment (W/O type) than from hydrophilic ointment (O/W type) (Table I and II) might be due to the greater affinity for the skin of the former ointment than that of the latter ointment. The smaller absorption of $^{22}Na^+$ in experiments of Table II than in the experiments of Table I might be a reflection of the differences in the viscosity of the ointments.

The data expressed in Table II also suggest the possible relationship between the molecular size and the percutaneous absorbability of water-soluble substances from emulsion-type ointments. In order to gain some insight into this problem, we compared the percutaneous absorption of $^{22}Na^+$ and of ferric-EDTA-^{59}Fe by a similar method. As shown in Table III, a large difference in the degree of absorption of the two radioactive compounds was observed again. Table IV summarizes the results of an experiment in which percutaneous absorbability of ferric-EDTA-^{59}Fe and of zinc-EDTA-^{65}Zn was compared. Since no significant difference was observed (Table IV), it appears that the slow absorption rate of metal-EDTA chelates as compared to the absorption rate of sodium ions is not due to the specificity of the metals but mainly due to the molecular size of the chelate compounds.

Comparison of Ferric-tiron-^{59}Fe and Ferric-chromotropic Acid-^{59}Fe Percutaneous Absorption

For the purpose of gaining further insight into the relationship between molecular size and percutaneous absorbability of water-soluble substances from emulsion-type ointments,

a comparison of the absorption of ferric-tiron-^{59}Fe and of ferric-chromotropic acid-^{59}Fe was performed. These compounds are similar in chemical properties but differ considerably in molecular size. Since it was impossible to compare the percutaneous absorption of these two radioactive compounds directly on the same ointment, an indirect method was employed as expressed in Table V. In this method, percutaneous absorption of $^{22}Na^{+}$ and of each ^{59}Fe-chelate compound was measured by the "double isotopic method." Those animals in which percutaneous absorption of 65 to 75% of the applied ^{22}Na occured were selected for comparison of absorption of the two different ^{59}Fe complexes. The relative absorption of ^{59}Fe to ^{22}Na was calculated on each of these animals, and these values in each group were compared.

Table II. Percutaneous Absorption of ^{22}Na and ^{65}Zn after Application to Mouse Skin of Emulsion-type Ointments Containing both ^{22}NaCl and Zinc-EDTA-^{65}Zn

Ointment base	Time of duration of application (hr)	Amount of ointment applied to each mouse (mg)	Per cent absorption of radioisotopes		$p^{a)}$
			^{22}Na	^{65}Zn	
Absorption ointment	5	51.0	48.8	19.1	<0.005
		50.3	46.3	19.5	
		50.0	34.2	12.5	
		52.7	50.6	19.2	
	20	52.2	86.8	40.1	<0.005
		50.6	92.3	56.2	
		50.0	85.9	40.8	
		51.4	94.6	64.3	
Hydrophilic ointment	5	51.8	17.1	15.2	<0.025
		51.4	17.3	11.7	
		50.3	13.9	8.7	
		52.0	12.2	5.8	
	20	51.8	39.5	16.4	<0.005
		53.5	35.4	19.5	
		54.6	36.0	17.8	
		54.4	28.2	16.3	

An aqueous solution (0.3 ml) containing 5 μmoles of ^{22}NaCl, 5 μmoles of $^{65}ZnCl_2$, and 10 μmoles of EDTA was adjusted to a pH between 6 and 7 by spraying NH_3-containing air. This solution was mixed with 2.0 g of each ointment base.
a) The level of significance of difference between per cent absorption of ^{22}Na and that of ^{65}Zn calculated by *t*-test (paired observations).

Table III. Percutaneous Absorption of ^{22}Na and ^{59}Fe after Application to Mouse Skin of Absorption Ointment Containing both ^{22}NaCl and ferric-EDTA- ^{59}Fe

Time of duration of application (hr)	Amount of ointment applied to each mouse (mg)	Per cent absorption of radioisotopes		$p^{a)}$
		^{22}Na	^{59}Fe	
5	50.2	67.4	36.0	<0.005
	52.2	44.2	14.8	
	52.8	65.4	26.8	
	52.1	62.1	32.4	
20	51.8	97.9	84.2	<0.005
	51.0	97.6	75.1	
	50.1	94.5	62.5	
	51.7	91.9	49.2	

An aqueous solution (0.3 ml) containing 5 μmoles of ^{22}NaCl, 5 μmoles of $^{59}FeCl_3$, and 10 μmoles of EDTA was adjusted to a pH between 6 and 7 by spraying NH_3-containing air. This solution was mixed with 2.0 g of absorption ointment.
a) The level of significance of difference between per cent absorption of ^{22}Na and that of ^{59}Fe calculated by *t*-test (paired observations).

Table IV. Percutaneous Absorption of ^{59}Fe and ^{65}Zn after Application to Mouse Skin of Absorption Ointment Containing both Ferric-EDTA-^{59}Fe and Zinc-EDTA-^{65}Zn

Time of duration of application (hr)	Amount of ointment applied to each mouse (mg)	Per cent absorption of radioisotopes		$p^{a)}$
		^{59}Fe	^{65}Zn	
5	53.8	21.1	20.1	>0.1
	54.5	44.4	42.0	
	52.7	16.5	17.9	
	52.4	38.1	37.2	
	53.8	38.1	37.0	

An aqueous solution (0.3 ml) containing 0.5 μmole of $^{59}FeCl_3$, 0.5 μmole of $^{65}ZnCl_2$ and 5 μmoles of EDTA was adjusted to a pH between 6 and 7 by spraying NH_3-containing air. This solution was mixed with 2.0 g of absorption ointment.
a) The level of significance of difference between per cent absorption of ^{59}Fe and that of ^{65}Zn calculated by *t*-test (paired observations).

As seen in Table V, ferric-chromotropic acid-^{59}Fe was absorbed from absorption ointment through hair-clipped mouse skin significantly more slowly than ferric-tiron-^{59}Fe. Since the chromotropic complex is larger, it may be inferred that percutaneous absorbability of water-soluble substances from emulsion-type ointments through mouse skin is decreased with an increase in molecular size when other conditions are similar.

In this connection, it is of interest that Marzulli, *et al.*[13] demonstrated a decrease in penetrating capacity of trialkylphosphates through isolated human skin as the carbon chain length was increased. However, Wahlberg[14] observed no difference in absorption through excised human and guinea pig skin between $^{51}Cr^{3+}$ and $^{51}CrO_4^{2-}$. Further studies are necessary to elucidate the factors determining the percutaneous absorbability of water-soluble substances from emulsion-type ointments.

Table V. Comparison between Ferric-tiron-^{59}Fe and Ferric-chromotropic acid-^{59}Fe in the Rate of Absorption from Absorption Ointment through Mouse Skin

Form of ^{59}Fe	Time of duration of application (hr)	Amount of ointment applied to each mouse (mg)	Per cent absorption of radioisotopes		Relative absorption of ^{59}Fe to $^{22}Na^{a)}$
			^{22}Na	^{59}Fe	
Ferric-tiron	4	53.6	65.9	10.6	0.161
		52.9	73.8	9.6	0.130
		51.0	72.8	10.7	0.147
		53.2	71.9	10.0	0.141
			mean 71.1[b)]		mean 0.145[c)]
Ferric-chromotropic acid	4	53.8	71.6	4.2	0.059
		54.3	70.8	4.7	0.066
		54.9	65.8	5.7	0.087
		55.1	65.5	3.9	0.060
			mean 68.4[b)]		mean 0.068[c)]

An aqueous solution (0.3 ml) containing 5 μmoles of $^{22}NaCl$, 0.5 μmole of $^{59}FeCl_3$, and 2 μmoles of tiron (or chromotropic acid) was adjusted to a pH between 6 and 7 by spraying NH_3-containing air. This solution was mixed with 2.0 g of absorption ointment.
a) Ratio of per cent absorption of ^{59}Fe to that of ^{22}Na.
b) No significant difference between the two means ($p>1$; F-test).
c) Significant difference between the two means ($p<0.005$; F-test).

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