Methyl 9- and 10-ethylthiostearates: bp 193—195° (3 mmHg), *Anal.* Calcd. for $C_{21}H_{42}O_2S$: C, 70.33; H, 11.80; S, 8.94. Found: C, 70.61; H, 11.87; S, 8.95. IR ν_{max} cm^{-1}: 2920, 2850, 1740, 1465, 1435, 1370, 1250, 1205, 1175, 720. NMR (CCl$_4$) τ: 6.38 (s, COOCH$_3$), 7.35—7.85(m), 8.00—8.90(m), 9.10(t).

Methyl 9- and 10-*n*-propylthiostearates: Yield 94.4%, bp 202° (3 mmHg), *Anal.* Calcd. for $C_{22}H_{44}O_2S$: C, 70.90; H, 11.90; S, 8.60. Found: C, 71.01; H, 11.92; S, 8.45. Mass Spectrum *m/e* (relative intensity, %): 372 (98.0, M$^+$), 329 (100, M$^+$—C$_3$H$_7$), 259 (66.0, [C$_3$H$_7$SCH(CH$_2$)$_8$COOCH$_3$]$^+$), 245 (66.7, [C$_3$H$_7$SCH(CH$_2$)$_7$-COOCH$_3$]$^+$), 215 (98.6, [CH$_3$(CH$_2$)$_8$CHSC$_3$H$_7$]$^+$), 201 (99.0, [CH$_3$(CH$_2$)$_7$CHSC$_3$H$_7$]$^+$).

Methyl 9- and 10-isopropylthiostearates: Yield 93.9%, bp 196° (3 mmHg), *Anal.* Calcd. for $C_{22}H_{44}O_2S$: C, 70.90; H, 11.90; S, 8.60. Found: C, 70.62; H, 11.99; S, 8.75. Mass Spectrum *m/e*: 372 (48.1, M$^+$), 329 (100, M$^+$-C$_3$H$_7$), 259 (43.5, [C$_3$H$_7$SCH(CH$_2$)$_8$COOCH$_3$]$^+$), 245 (43.2, [C$_3$H$_7$SCH(CH$_2$)$_7$COOCH$_3$]$^+$), 215 (60.5, [CH$_3$(CH$_2$)$_8$CHSC$_3$H$_7$]$^+$), 201 (59.5, [CH$_3$(CH$_2$)$_7$CHSC$_3$H$_7$]$^+$).

Methyl 9- and 10-*n*-butylthiostearates: Yield 95.1%, bp 213° (3 mmHg), *Anal.* Calcd. for $C_{23}H_{46}O_2S$: C, 71.44; H, 11.99; S, 8.29. Found: C, 71.37; H, 11.84; S, 8.15. Mass Spectrum *m/e*: 386 (100, M$^+$), 329 (100, M$^+$—C$_4$H$_9$), 273 (64.0, [C$_4$H$_9$SCH(CH$_2$)$_8$COOCH$_3$]$^+$), 259 (64.7, [C$_4$H$_9$SCH(CH$_2$)$_7$COOCH$_3$]$^+$), 229 (87.2, [CH$_3$(CH$_2$)$_8$CHSC$_4$H$_9$]$^+$), 215 (88.1, [CH$_3$(CH$_2$)$_7$CHSC$_4$H$_9$]$^+$).

Binding of Calcium by Soluble Fraction from Normal Rat Liver

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The calcium-binding activity in the soluble fraction of a normal rat liver was studied. Calcium binding in the supernatant depends on the competition between a cation-exchange resin and a soluble calcium-binding substance for added calcium. The calcium concentration found in the supernatant of the test system was approximately 12-fold of that of calcium contained in the soluble fraction of a normal rat liver. The binding of calcium in the supernatant of the test system using the heat-treated soluble fraction increased linearly up to 0.75 mM calcium and was saturated 1.0 mM calcium. When the radiocalcium-binding activity was expressed as S/R net, the radiocalcium-binding activity existed in soluble fraction, and it increased approximately 4-fold by the heat treatment. These results suggest that the calcium-binding factor exists in the soluble fraction of a normal rat liver.

A calcium-binding protein (CaBP) was first reported to be present in the duodenal mucosa of vitamin D-treated rachitic chicks by Wasserman and Taylor.[2] A similar protein has been found in the small intestine of a number of mammals including the rat, dog, pig, cow, monkey, and human.[3] Vitamin D-dependent CaBP has also been identified in the kidney of which

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there is a large flux of calcium.[4] Recently, CaBP was isolated from porcine parathyroid glands which the rate of biosynthesis and secretion of parathyroid hormone was regulated by the concentration of calcium in the extracellular fluid.[5] Thus, substantial evidence has been obtained to suggest an involvement of CaBP in the calcium transport system of respective tissues.

More recently, we found that the concentration of calcium in rat liver is markedly increased by thyrocalcitonin which has a hypocalcemic effect.[6] On the basis of this fact, the possibility that CaBP may be involved in the transport of calcium in the liver cells led to the present investigation. We observed that, although CaBP reportedly has not been detected in the liver of animals,[5] soluble fraction from normal rat liver exhibited a calcium-binding activity. This paper describes the presence of calcium-binding activity in the soluble fraction from a normal rat liver.

Materials and Methods

Preparation of Soluble Fraction——Male Wistar strain rats, each weighing approximately 120 g, were used in these experiments. They were kept at room temperature of $25° \pm 1$ and fed Purina chow and tap water freely. The animals were killed by decapitation and the livers were perfused with Tris-HCl buffer (pH 7.4) (containing 0.1M Tris, 0.12M NaCl, 0.004M KCl, cooled to 4°). The liver was removed, cut into small pieces, suspended 1: 4 in Tris-HCl buffer (pH 7.4) and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at $1800 \times g$ in a refrigerated centrifuge for 10 min and the supernatants was spun at $105000 \times g$ for 60 min. The supernatants from the latter was collected and heated at 60° for 10 min: The solution was then cooled and recentrifuged at $38000 \times g$ for 20 min.

Calcium-binding Assay——The calcium-binding activity in the supernatant fractions was determined by the method as modified by Wasserman and Taylor.[2] The procedure depends on the competition between a cation-exchange resin and a soluble calcium-binding substance for added calcium. The resin, Chelex-100, was first washed with Tris-HCl buffer and then diluted with the same buffer to a concentration of 0.1 ml resin per 0.2 ml of the suspension. While the resin was maintained in suspension by a magnetic stirrer, 0.2 ml was pipeted into a small test tube containing 1 ml of either supernatant fraction or Tris-HCl buffer alone. This was followed by 1 ml of $CaCl_2$ (1.0 mM). The tube content was then mixed thoroughly for 1 min and centrifuged at $1800 \times g$ for 10 min. An aliquot of 1.0 ml of the supernatant solution was then transferred to the test tube to determine the bound calcium. Calcium remaining in the supernatant of the test system was calculated by substracting the amount of calcium present in Tris-HCl buffer. Calcium was determined by the atomic absorption spectrophotometry after chloric acid digestion.[7]

In the experiments using radiocalcium, 0.1 ml of $^{45}CaCl_2$ solution (0.5 μCi ^{45}Ca and 4.0 μg cold calcium) was placed in a small test tube containing 1 ml of either supernatant fraction or Tris-HCl buffer alone. The tube content was then mixed vigorously for 20 sec and centrifuged. An aliquot of 0.2 ml of the supernatant solution was then transferred to a liquid scintillation counting vial, 5 ml of Bray's solution was added to each vial, and ^{45}Ca radioactivity was counted by a liquid scintillation counter.[8] Channel ratio values indicated that quench corrections were not required. The concentration of protein was determined by the method of Lowry, et al.[9]

Results and Discussion

The concentration of calcium in soluble fraction of normal rat liver was determined. The results summarized in Table I indicate that calcium was present in a small amount in the soluble fraction. Table II summarizes the calcium-binding data and protein concentration in soluble fraction of a normal rat liver. There was some calcium remaining in the supernatant solution of the test system when calcium was added to the test system. When the data are

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expressed as nmol of calcium remaining per g of protein in the supernatant solution of the test system, the concentration of calcium in the supernatant solution of the test system was approximately 12-fold that of calcium contained in the soluble fraction of a normal rat liver. Although heat treatment markedly decreased the concentration of protein in the soluble fractions, the concentration of calcium remaining in the supernatant of the test system was not significantly decreased by heating. It was found that calcium present was completely stable to heating. The effect of the added calcium concentration on calcium remaining in the supernatant of the test system from the soluble fraction of a rat liver was examined and its representative experiment is shown in Fig. 1. The remaining of calcium in the supernatant of the test system

TABLE I.   Calcium Concentration in Soluble Fraction
of Normal Rat Liver

Soluble fraction	Calcium value[a]
μg/g wet tissue	0.32±0.04
nmol/g protein	15.70±3.12

a)   mean ± S.E of an experiment with five animals

TABLE II.   Binding of Calcium by Soluble Fraction from Normal Rat Liver

Values	Soluble fraction	
	None	Heat-treated
Protein concentration (mg/ml)	158.0 ± 8.5[a]	45.7 ± 2.0
Calcium in supernatant (μg/ml)[b]	1.23± 0.09	1.13± 0.13
Calcium in supernatant nmol/g protein	180.6 ±11.2	577.2 ±57.5

a)   mean ± S E of an experiment with five animals
b)   Calcium binding determined by competition between Chelex-100 resin and soluble calcium-
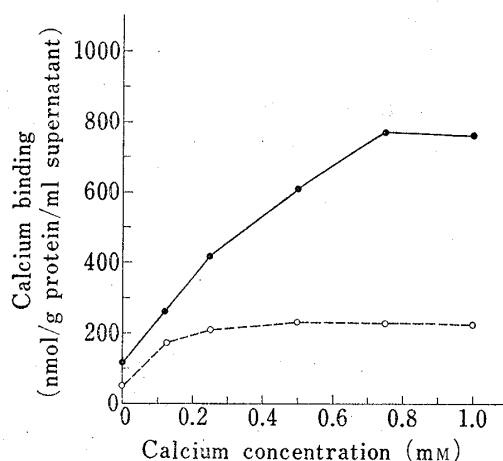binding substance for added calcium.

Fig. 1.   Effect of added Calcium Concentration on Calcium Remaining in the Supernatant of the Test System of Soluble Fraction from Normal Rat Liver

Calcium binding determined by Chelex-100 resin assay.   A duplicate experiment gave the same relative data.
---○---: soluble fraction,
—●—: heated soluble fraction

was saturated with 0.1 mM calcium, while calcium remaining in the supernatant of the test system using the heated soluble fraction increased linearly until 0.75 mM calcium and was saturated with 1.0 mM calcium. These results indicate that the test system would be suitable for the assay of calcium binding.

Results of these experiments suggested that the calcium-binding activity existed in the soluble fraction from a normal rat liver. To determine this possibility, calcium-binding activity in the soluble fraction was determined by the use of a method similar to that of Wasserman and Taylor.[2] When the calcium-binding activity was expressed as S/R net (Table III), the data indicated that the calcium-binding activity existed in the soluble fraction from a normal rat liver, and that it was increased approximately 4-fold by heat treatment. Rationality for expressing the calcium-binding activity as S/R net has been described by Wasserman and Taylor.[10]

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Table III. Binding of Radiocalcium by Soluble Fraction
from Normal Rat Liver

Treatment	Protein (mg/ml supernatant)	Radiocalcium binding activity[a]	
		S/R net (ml supernatant)	S/R net/mg protein (ml supernatant)
None	1.05	0.097[b]	0.092
Heat-treated	0.28	0.091	0.325

a) Calcium binding determined by Chelex-100 resin assay, and calcium binding activity
was expressed as

$$S/R \text{ net} = \frac{^{45}\text{Ca in test supernatant (\%)}}{^{45}\text{Ca in test resin (\%)}} - \frac{^{45}\text{Ca in blank supernatant (\%)}}{^{45}\text{Ca in blank resin (\%)}}$$

b) A duplicate experiment gave the same relative data.

The present results clearly demonstrate that the calcium-binding activity exists in the soluble fraction of a rat liver. Gel filtration studies with Sephadex G-75 further suggested that the activity is associated with a protein.[11] Presumably, calcium-binding protein in the soluble fraction of a rat liver is involved in the transport of calcium in the liver cells.

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Benzodiazepines. XI.[1] Further Examination of the Chromic Acid Oxidation of 2-Aminomethylindoles to 2,3-Dihydro-2H-1,4-benzodiazepin-2-ones

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In the chromic acid oxidation of 2-aminomethyl-5-chloro-1-methyl-3-phenylindole (1) to 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (2), 1 reacts rapidly with chromic acid to form an oxidation intermediate A, which on acetylation with acetic anhydride gives 2-acetamido-2'-benzoyl-4'-chloro-N-methylacetanilide (3). The rate for formation of 2 from A has been determined by following the change of the quantities of 2 and 3 obtained by periodical sampling followed by quenching with acetic anhydride. The rate is first order with respect to the concentration of A.

In the preceding papers of this series the oxidative ring enlargement of 2-aminomethyl-indoles to 1,3-dihydro-2H-1,4-benzodiazepin-2-ones have been reported.[3] The present study was undertaken in order to consider more closely the mode of formation of 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (2) by the chromic acid oxidation of 2-aminomethyl-5-chloro-1-methyl-3-phenylindole (1).

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